Adaptive plant responses to sequential biotic and abiotic stress

- Unraveling the underlying genetics -

Silvia Coolen

Adaptive plant responses to sequential biotic and abiotic stress Unraveling the underlying genetics
PhD thesis, Utrecht University, Plant-Microbe Interactions
Silvia Coolen (2018)

ISBN: 978-94-6332-343-7
Cover & layout: Silvia Coolen
Photo's: Hans van Pelt and Silvia Coolen
Printed by: GVO drukkers & vormgevers

The research described in this thesis was supported by the Netherlands Organization for Scientific Research (NWO) through the Dutch Technology Foundation STW Perspective Program 'Learning from Nature'

Adaptive plant responses to sequential biotic and abiotic stress

- Unraveling the underlying genetics -

Adaptieve plant responsen tijdens opeenvolgende biotische en abiotische stress

- Ontrafeling van de onderliggende genetica -

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
maandag 4 juni 2018 des ochtends te 10.30 uur

door

Silvia Coolen

geboren op 8 november 1985
te Deurne

Promotor: **Prof. dr. ir. C.M.J. Pieterse**

Copromotor: **Dr. S.C.M. van Wees**

To my mother and family

Aan mijn moeder en familie

Contents

Chapter 1

General introduction

Plant self-defense in multi-stress situations

Abstract

In nature plants are often exposed to a multitude of biotic and abiotic stress factors that occur sequentially or simultaneously. To cope with these stresses, plants have evolved response mechanisms that are essential for plant survival and can be of great value for future crop protection and breeding purposes. However, still little is known about how these adaptive mechanisms cooperate to maximize plant survival under multi-stress conditions. Knowledge on plant single stress responses provides insufficient insight in the complexity of plant multi-stress signaling as the plant's responses to multiple-stress environments seem to be distinct from the sum of the individual stress responses. With a rapidly changing climate, plant stresses that never coincided before can be considered possible in the future, making multi-stress research of even greater importance. Many factors influence plant survival during stress encounters, including the timing and severity of the stress, previously encountered stresses, plant stress signal crosstalk, host defense manipulation by pathogens and insects, plant to plant communication, and the genetic background of the plant. Stress signaling, phytohormones and specific transcription factors emerged as central regulators of plant combinatorial stress responses, making them key components to study. Here, we provide an overview of recent advances in our understanding of the complexity and functioning of interacting stress signaling networks.

Introduction

In nature, plants have to cope with a wide variety of biotic and abiotic stress conditions that frequently occur in different combinations, for varying durations and severities, and sequentially or simultaneously. As plant growth is often greatly affected by stress, this especially impacts current agriculture where diverse ecosystems have been replaced with monocultures that are much more vulnerable to changing climatic conditions and evolving biotic stresses (Balmer *et al.,* 2014; Felton *et al.,* 2016). Despite crop protection measures, global losses in agriculture due to pathogens and pests are estimated at 25-40% for the major food and cash crops, representing a value of over €500 billion worldwide. Moreover, crops chronically attain only about 50% of their potential yield due to the negative effects of abiotic stresses, such as drought, heat, cold, water logging, high salinity and toxic compounds. Also the combination of different stresses such as the 2003 drought and heat wave in Europe caused a 30% reduction in crop yield (Ciais *et al.,* 2005). To survive under combinations of different biotic and abiotic stress conditions, wild plants have evolved intricate mechanisms to perceive external signals and translate these into an optimal adaptive response to maximize the chance for survival. Studying naturally evolved plant adaptations to environmental stress can therefore provide great insight in plant multi-stress tolerance and provide tools for future resistance breeding (Thoen *et al.,* 2017).

To obtain more insight in plant responses to different stresses, single stress situations have been extensively studied. However, a growing body of evidence has shown that plant responses to combinations of different stresses are distinct from the sum of the individual stress responses (Atkinson

and Urwin 2012; Rasmussen *et al.,* 2013; Santino *et al.,* 2013; Kissoudis *et al.,* 2014; Coolen *et al.,* 2016; Davila Olivas *et al.,* 2016; Davila Olivas *et al.,* 2017b), suggesting a complexity of antagonistic and synergistic interactions between the stress response pathways involved. Moreover, certain adaptive plant responses may only become apparent under multi-stress conditions, while they remain unexpressed during single stress situations. Furthermore, plant responses to both single and multiple-stresses are influenced by a plethora of signals including the plant's physiological and developmental stage, previously encountered stresses, plant-plant communication, stress perception and signaling, crosstalk of stress induced pathways, plant genetics, transgenerational induced epigenetic changes and high-jacking of plant stress signaling by pathogens and insects. Progress in our knowledge of how plants cope with multi-stress conditions will provide important tools for designing new strategies in the protection of plants against combinatorial stresses. To date, knowledge on the integrative signals and points of convergence in different combinations of partially overlapping stress signaling pathways is still in its infancy. In this review we aim to provide an overview of recent literature on plant adaptive responses in view of plant multi-stress signaling.

Plant abiotic and biotic stress perception, signaling and responses

Plant biotic stress responses start off at the basis of the first layer of protection where structural barriers such as waxy cuticles, pigments, trichomes and preformed antimicrobial metabolites prevent or attenuate invasion and stress induction by potential attackers. Whenever this first layer of protection is breached the second layer of protection comes in action. As a post-invasive line of protection, plants evolved a sophisticated innate immune system by which they recognize non-self-molecules and signals from stressed or injured cells, and respond to that by activating an effective counter response (Jones and Dangl 2006; Howe and Jander 2008).

Plant stress perception. Potential threats can be perceived by the plant via both extracellular and intracellular receptors that bind to substrates such as pathogen derived elicitors called pathogen-associated molecular patterns (PAMPs), plant derived elicitors that are released upon tissue damage called damage-associated molecular patterns (DAMPs), and pathogen or insect effector molecules that interfere with plant stress signaling intracellularly (Davis *et al.,* 1986; Davis and Hahlbrock 1987; Cabanne and Donèche 2002; Jones and Dangl 2006; Ferrari *et al.,* 2007; Miya *et al.,* 2007; Howe and Jander 2008; Coll *et al.,* 2011; Duran-Flores and Heil 2016). Although the receptor substrates for abiotic stresses remain unknown, these stress signals are known to be perceived by different receptors including CALCIUM/CALMODULIN-REGULATED RECEPTOR-LIKE KINASE 1 (CRLK1), RECEPTOR-LIKE PROTEIN KINASE1 (RPK1) and CYSTEINE-RICH REPEAT RECEPTOR-LIKE KINASE 5 (CRK5), which are involved in cold stress and drought tolerance and abscisic acid (ABA) signaling, respectively (Osakabe *et al.,* 2010; Yang *et al.,* 2010; Lu *et al.,* 2016). For the perception of biotic stresses several receptors have been identified including PAMP receptors CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1), elongation factor thermo-unstable (EF-TU) receptor (EFR), FLAGELLIN SENSING 2 (FLS2) and DAMP receptors PEPTIDE (PEP1–8) RECEPTOR 1 and 2 (PEPR1 and PEPR2), for the

perception of fungal chitin, bacterial EF-TU, bacterial flagellin and plant peptides, respectively (Zipfel *et al.,* 2006; Chinchilla *et al.,* 2007; Yamaguchi *et al.,* 2010; Couto and Zipfel 2016). Although these receptors recognize distinct molecules, plant receptors are known to form heteromers with shared co-receptors that together activate both general and stress-specific plant signaling pathways. For instance, the plant chitin receptor CERK1 that forms heteromers with LysM-CONTAINING RECEPTOR-LIKE KINASE 5 (LYK5) for recognition of fungal chitin, is also involved in perception of peptidoglycans together with LysM DOMAIN-CONTAINING GPI-ANCHORED PROTEIN 1 and 3 (LYM1 and LYM3; Miya *et al.,* 2007; Willmann *et al.,* 2011; Cao *et al.,* 2014). Another example is the BRASSINOSTEROID INSENSITIVE 1 (BRI1)-ASSOCIATED RECEPTOR KINASE 1 (BAK-1) that was shown to be essential for perception of bacterial flagellin and EF-TU and plant peptides Pep1–8 (Zipfel *et al.,* 2006; Chinchilla *et al.,* 2007; Yamaguchi *et al.,* 2010; Couto and Zipfel 2016). The latter indicates that although receptors perceive and respond to specific molecules, they also share components of the plant's stress signaling network that potentially connects different stresses with each other.

Early plant stress signaling. Abiotic and biotic stress perception subsequently leads to the phosphorylation and activation of receptor kinases resulting in a rapid calcium (Ca^{2+}) influx and phosphorylation of receptor-like cytoplasmic kinases (RLCKs) and calcium-dependent protein kinases (CPKs) that recruit and phosphorylate respiratory burst oxidase homolog D (RbohD; Mersmann *et al.,* 2010; Dubiella *et al.,* 2013; Liu *et al.,* 2013; Kadota *et al.,* 2014). Activation of RbohD results in the production of extracellular reactive oxygen species (ROS) that depolarizes plant cells within minutes after elicitor application (Jeworutzki *et al.,* 2010). Both Ca^{2+} and ROS were shown to act as second messengers and spread throughout the plant, activating plant stress signaling. In FLS2-mediated signal transduction, Ca^{2+} influx and ROS production are both dependent on the BAK1 co-receptor, indicating the importance of receptor heteromerization for stress signal transduction (Chinchilla *et al.,* 2007; Jeworutzki *et al.,* 2010). Ca^{2+} was shown to spread throughout the plant with a speed of up to 2.4 cm min^{-1}, facilitating systemic plant stress signaling (Choi *et al.,* 2014). Similar to Ca^{2+} signaling, ROS was shown to spread throughout the plant via a 'ROS wave' that triggers systemic cell to cell communication with a speed of 8.4 cm min^{-1} (Mittler *et al.,* 2011; Suzuki *et al.,* 2013; Couto and Zipfel 2016). Besides rapid second messengers induced signaling, receptor activation leads to downstream mitogen activated kinase (MAPK) signaling that activates transcription factors (TFs) involved in stress signaling and regulation (Asai *et al.,* 2002; Mittler *et al.,* 2011; Couto and Zipfel 2016). These include TFs from different families such as, ABA-responsive element-binding proteins (AREBs), no apical meristem (NAM) ATAF1 and 2 and cup-shaped cotyledon (CUC) (NAC) TFs, WRKYs, APETALA 2 (AP2)/ethylene-responsive element-binding factors (ERFs; e.g. C-repeat binding factors/dehydration-responsive-element-binding TFs, CBF/DREBs), myeloblastosis (MYB) TFs, myelocytomatosis (MYC) TFs, basic domain-leucine zipper (bZIP) TFs (e.g. TGA binding TFs), and zinc finger proteins (ZFPs; Fujita *et al.,* 2004; Yu *et al.,* 2008; Bi *et al.,* 2010; Li *et al.,* 2010b; Li *et al.,* 2010c; Seo and Park 2010; Zhang *et al.,* 2010a; Zhang *et al.,* 2010b; Zhu *et al.,* 2010; Cheng *et al.,* 2011; Mizoi *et al.,* 2012; Chen *et al.,* 2014; Shi *et al.,* 2014). These transcription factors are known to regulate different stress-driven signaling pathways including the production of phytohormones that amplify stress signals (Figure 1).

Amplification of plant stress signaling. Depending on the nature of the stress, plants make use of phytohormone-driven signaling pathways to amplify stress signaling, including the production and accumulation of ABA, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET; Anderson *et al.,* 2004; Robert-Seilaniantz *et al.,* 2011; Pieterse *et al.,* 2012; Vos *et al.,* 2013b; Broekgaarden *et al.,* 2015; Caarls *et al.,* 2015; Vos *et al.,* 2015). In response to abiotic stress, biotrophic pathogens, herbivory and wounding or necrotrophic pathogens plants make use of ABA, SA, JA and ABA, or JA and ET signaling, respectively (Glazebrook 2005; Qin *et al.,* 2011). Phytohormonal accumulation was shown to be linked to early plant stress signaling involving ROS production, showing the importance of general and early stress responses in stress specific phytohormonal regulation. For instance, ABA, SA, JA and ET production and accumulation were shown to be dependent on RbohD-mediated ROS production and Ca^{2+} signaling including activation of RLCKs and CPKs (Du *et al.,* 2009; Gao *et al.,* 2013; Liu *et al.,* 2013; Suzuki *et al.,* 2013). On the other hand ET and SA were also shown to regulate RbohD-dependent ROS production and JA was shown to increase ROS scavenger activity, indicating the bidirectional regulation of phytohomones and Ca^{2+} and ROS second messengers (Boutrot *et al.,* 2010; Mersmann *et al.,* 2010; Qiu *et al.,* 2014). Furthermore, diverse TFs were shown to be involved in the production and regulation of phytohormones (Figure 1). For instance, production and accumulation of ABA was shown to be mediated by AREBs, DREBs and NAC transcription factors, SA production by WRKY TFs, ET production by WRKYs, ERF and NAC TFs and JA production by ERF and MYC TFs (Dong *et al.,* 2003; Boter *et al.,* 2004; Liu and Zhang 2004; Lorenzo *et al.,* 2004; Delessert *et al.,* 2005; Fujita *et al.,* 2006; Van Verk *et al.,* 2011; Jensen *et al.,* 2013; Chen *et al.,* 2014; Hickman *et al.,* 2017). Aside from an important role in the production and regulation of phytohormone-driven pathways, TFs play an important role in cross-communication between signaling pathways, necessary for fine-tuning complex signaling events when multiple signals collide.

Plant stress signaling crosstalk. To cope with a multitude of different stresses plants rely on a well-balanced and cross-communicating signaling system (Figure 1). Within the set of phytohormones known for their cross-communication abilities, SA and JA are the most extensively studied. SA is well known to repress JA signaling via non-expressor of pathogenesis-related (*PR*)-genes (NPR1), and specific TGA and WRKY TFs (Mao *et al.,* 2007; Gao *et al.,* 2011; Birkenbihl *et al.,* 2012; Caarls *et al.,* 2015; Kloth *et al.,* 2016). Moreover, SA-mediated repression of JA biosynthesis was recently shown to be mediated by ROS H_2O_2 scavenging enzyme CATALASE 2 (CAT2; Yuan *et al.,* 2017). JA induced repression of SA and synergy between both pathways have also been observed, suggesting an evolutionary importance for extensive crosstalk between these pathways (Laurie-Berry *et al.,* 2006; Mur *et al.,* 2006). ET was shown to play an important modulating role in SA-JA crosstalk, allowing activation of both pathways through ET-mediated blockage of SA induced JA repression (Leon-Reyes *et al.,* 2010). However, SA was also shown to act in synergy with ET, promoting induced celldeath in ozone (O_3) treated plants (Rao *et al.,* 2000). Having access to both SA and JA pathways can be of great importance in defense against hemi-biotrophic pathogens such as *Pseudomonas syringae*, which switches from an early biotrophic stage to a necrotrophic stage later during infection. JA signaling itself is known to result in two distinct antagonizing signaling branches

modulated by ET and ABA (Anderson *et al.,* 2004; De Vleesschauwer *et al.,* 2010; Pieterse *et al.,* 2012). The combination of JA and ET, triggered by necrotrophic pathogens, leads to downstream activation of ERF TFs, whereas the combination of JA and ABA results in downstream activation of MYC TFs. Aside from its modulating effect on JA signaling, ABA is also known to suppress SA signaling (Ward *et al.,* 1989; Audenaert *et al.,* 2002; Mohr and Cahill 2007), suggesting a negative interaction when biotrophic pathogens and abiotic stress are combined.

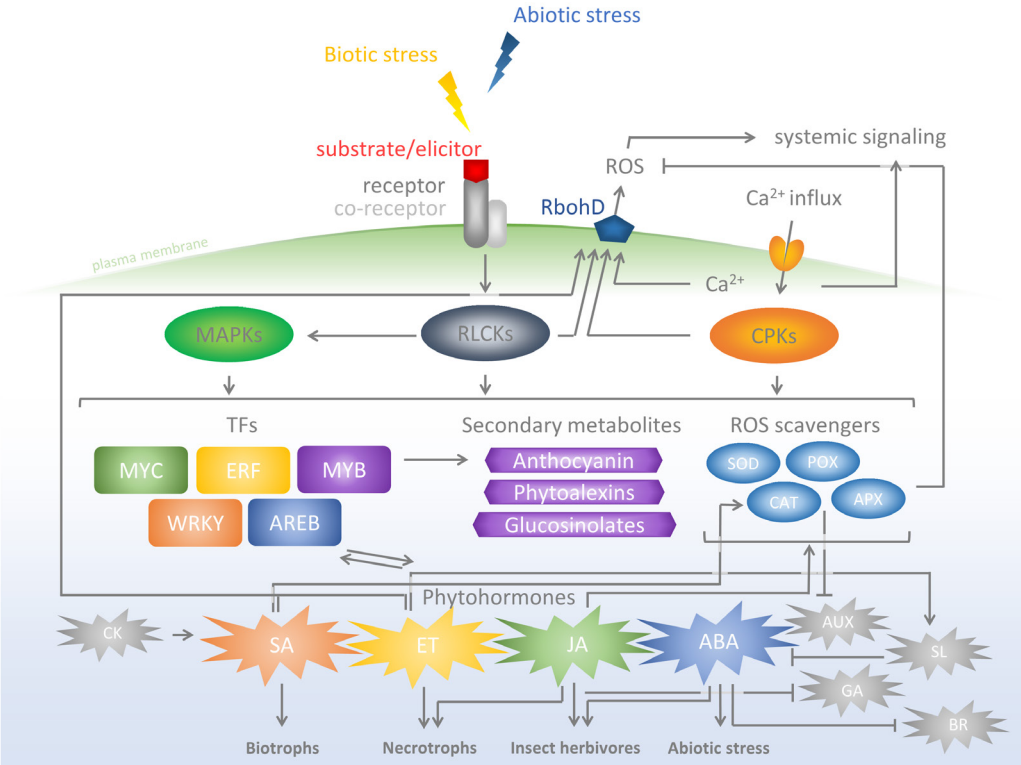


Figure 1. General overview of plant stress signaling. Plant stress perception leads to the activation of RLCKs, MAPKs and Ca^{2+} influx, which in turn results in the activation of CPKs. Ca^{2+}, RLCKs and CPKs are involved in the activation of RbohD, which produces extracellular ROS that together with Ca^{2+} acts as second messenger in systemic signaling throughout the plant. Activation of CPKs, RLCKs and MAPKs leads to downstream stress signaling, involving the activation of TFs (e.g. WRKYs, ERFs, MYCs, AREBs and MYBs) that regulate the production of phytohormones and secondary metabolites (e.g. anthocyanin, phytoalexins and glucosinolates). Each phytohormonal signaling pathway generally induces defenses against specific stress types. SA accumulation triggers defense against biotrophic pathogens, ET/JA accumulation triggers defenses against necrotrophic pathogens, JA/ABA accumulation against herbivores and ABA accumulation triggers tolerance against abiotic stress. SA and ET also promote RbohD-associated ROS production and JA in its turn is known to increase the activity of ROS scavengers that reduce tissue damage as a result of ROS production. Growth and development-associated phytohormones AUX, GA, CKs, BRs and SLs interact with stress signaling, balancing the plants investment in defense/tolerance or plant growth.

Plant stress and growth signaling crosstalk. Besides the cross-communication between different stress-driven plant signaling pathways, stress signaling is also known to communicate with development and growth related phytohormones auxin (AUX), gibberellin (GA), cytokinins (CKs), brassinosteroids (BRs) and strigolactones (SLs; Peleg and Blumwald 2011; Pieterse *et al.,* 2012; Waters *et al.,* 2017).

Plant stress responses often result in growth retardation, suggesting that plant stress signaling is prioritized over growth and development. Increasing evidence shows an important role for AUX not only in plant development and growth but also in plant defense (Kazan and Manners 2009; Hentrich *et al.,* 2013). AUX and SA were shown to act antagonistically as AUX was shown to be repressed by SA and H_2O_2 scavenging enzyme CAT2 that was recently shown to mediate SA-induced repression of indole-3-acetic acid (IAA) accumulation during defense responses (Wang *et al.,* 2007; Yuan *et al.,* 2017). Interestingly CAT2 was also found to be involved in SA-JA crosstalk (Yuan *et al.,* 2017), linking SA, JA and AUX signaling. Also JA and AUX were shown to have both an antagonistic and synergistic relationship (Tiryaki and Staswick 2002; Nagpal *et al.,* 2005; Liu and Wang 2006; Khan and Stone 2007; Hentrich *et al.,* 2013), again indicating bi-directionality of signaling pathways. Stress signaling is often found to negatively regulate plant growth and development. For instance, JA is known to negatively affect GA signaling via MYC2 and growth repressing DELLA-proteins, resulting in growth inhibition (Hong *et al.,* 2012; Yang *et al.,* 2012). Abiotic stress related ABA was demonstrated to negatively regulate plant growth via BR-mediated signaling (Zhang *et al.,* 2009). On the contrary, plant growth related signaling was also shown to negatively regulate stress signaling. For example, CK receptor histidine kinases, that are known to be positive regulators of plant growth, were shown to act as negative regulators of ABA (Phan Tran *et al.,* 2010). CKs were also shown to enhance SA defense signaling (Choi *et al.,* 2010) and together with the ABA-mediated suppression of SA (Ward et al., 1989; Audenaert et al., 2002; Mohr and Cahill 2007), a possible role for CKs in SA-ABA crosstalk may be suggested. The latest addition to the plant phytohormone family are strigolactones, involved in synergistic interactions with mycorrhizal fungi and germination cues for parasitic plants (Al-Babili and Bouwmeester 2015). Strigolactones were shown to originate from carotenoids in a distinct route from ABA biosynthesis. Furthermore, strigolactones were shown to interact with ABA and ET signaling and therefore possibly influence diverse plant stress signaling pathways (Waters *et al.,* 2017). Confirmation of their role in plant stress signaling was obtained with a strigolactone-deficient *CAROTENOID CLEAVAGE DIOXYGENASES 8* (*CCD8*) RNA interference (RNAi) line displaying increased susceptibility to necrotrophic pathogens *Botrytis cinerea* and *Alternaria alternate* (Torres-Vera *et al.,* 2014).

Plant stress adaptive responses. Plant stress signaling eventually leads to a plant adaptive response to counteract potentially harmful situations. Early plant stress H_2O_2 (ROS) production is one of the first plant responses leading to stress resistance, since H_2O_2 is known for its direct toxic effects on pathogens (Lu and Higgins 1999). Furthermore, Ca^{2+}, ROS and MAPKs are known to be involved in the production of protective pigments (e.g. anthocyanin) and toxic secondary metabolites (e.g. glucosinolates and phytoalexins; Figure 1) that protect the plant against light-induced damage, insects and pathogens. For example, Ca^{2+} was shown to be involved in sucrose-mediated anthocyanin accumulation, giving plants protection to light induced damage (Shin *et al.,* 2013). Chitin and oligogalacturonide (OG, DAMP) induced signaling was shown to trigger a rapid RbohD-mediated ROS burst and MAPK signaling leading to activation of WRKY TFs and transcriptional activation of phytoalexin (e.g. camalexin) biosynthesis, known to be involved in resistance to

necrotrophic fungi like *B. cinerea*, aphids (*Myzus persicae)* and *Spodoptera littoralis* caterpillars (Miya *et al.,* 2007; Galletti *et al.,* 2008; Ren *et al.,* 2008; Schlaeppi *et al.,* 2008; Mao *et al.,* 2011; Kettles *et al.,* 2013). The production of secondary cell walls and secondary metabolites like glucosinolates were shown to involve the activation of MYB transcription factors that are induced in several plant stress signaling pathways and protect plants against insects (Bhargava *et al.,* 2013; Burow *et al.,* 2015; Frerigmann *et al.,* 2016; Hickman *et al.,* 2017). Furthermore, physiological adaptations such as stomatal closure to prevent pathogens from entering or stomatal opening for cooling down in case of heat stress are driven by diverse stress signals. For instance, flagellin-induced ET signaling was shown to result in closure of stomata to prevent bacterial invasion into the plant's apoplast. Abiotic stress induced ABA on the other hand was shown to result in stomatal closure independent of ET induced closure, allowing the plant to respond to different stress responses independently (Lozano-Durán *et al.,* 2014).

Eventually stress perception followed by stress specific signaling will determine the plant's adaptive response that will ensure survival. However, in multi-stress situations the plant needs to make a choice about which adaptive response should be prioritized to maximize the chance of survival. Advances in plant stress signaling research revealed that most stress signaling pathways share components with one another, making the plant stress signaling network a complex interconnected web with large potential for regulation.

Additional complexity to plant abiotic and biotic stress responses

Plant multi-stress responses are a paradigm of complex signaling events that occur when plants encounter different stress signals. Besides the fact that stresses trigger different plant signaling pathways simultaneously and may depend on the type and location of the tissue (local or systemic), another dimension of complexity is added when taking into consideration that pathogens and insects are able to high-jack plant stress signaling for their own benefit using effector molecules. Furthermore, plant stress signaling is influenced by plant to plant communication, transgenerational epigenetic changes, stress severity and timing and plant genetics.

High-jacking plant stress signaling. Plant defense signaling and crosstalk play an important role in (re-)directing the plant's defense strategy. However, pathogens and insect herbivores can make use of the plant's cross-communication abilities for their own benefit. Pathogens are known to influence plant responses via excreted effector molecules and the production of phytohormones or phytohormone mimics such as coronatin of *P. syringae*. Coronatin was shown to mimic JA and promote *P. syringae* virulence by repression of plant SA-induced defense signaling via MYC2 (Zheng *et al.,* 2012). *P. syringae* was also shown to repress ET-induced stomatal closure with its HOPM1 effector to ensure easy passage into the plant's apoplast (Lozano-Durán *et al.,* 2014). In addition, *P. syringae* was shown to repress both PAMP (flagellin and EF-Tu) and DAMP (OG)-induced plant stress signaling with the help of its AvrPto effector molecule (Gravino *et al.,* 2017). Also herbivores such as the small cabbage white caterpillar *Pieris rapae* was shown to make use of plant crosstalk

by redirecting the plants defense strategy from a herbivore-induced JA response involving ABA-mediated induction of the JA MYC branch, to a less destructive JA response involving ET and the JA ERF branch. Oral secretions of *P. rapae* were shown to be sufficient to redirect the JA pathway towards the ERF branch which is favorable for the caterpillar (Verhage *et al.*, 2011). Furthermore, herbivory was shown to rapidly trigger AUX and plant genes involved in multidimensional growth, which is likely to be induced by herbivores to ensure sufficient food recourses for their development (Machado *et al.,* 2013; Coolen *et al.,* 2016; Davila Olivas *et al.,* 2016; Machado *et al.,* 2016). Also necrotrophs such as *B. cinerea*, that induce JA signaling, are known to manipulate their hosts. With the production of phytotoxic sesquiterpene botrydial *B. cinerea* induces a hypersensitive (HR) response in plants that is accompanied by programmed cell death, facilitatating the infection. This mechanism was shown to be mediated by SA (Rossi *et al.,* 2011). Thus, plant flexibility through cross-communicating signaling pathways can also be a disadvantage since biotic threats may use them to trick plants into inducing ineffective or even adverse responses.

Neighbor and transgenerational plant communication. To ensure population survival, plants are known to emit volatile organic compound (VOC) alarm signals that generate a primed state of defense against threats in neighboring plants (Heil and Karban 2010; Dicke 2016). The phenomenon of interplant communication was also found for parental plants that leave epigenetic signatures for their offspring in the form of heritable epigenetic modifications (e.g. DNA methylation or RNA interference; Heil and Karban 2010). For example, plants under high salinity stress emit VOCs that induce salt stress tolerance in neighboring plants (Lee and Seo, 2014) and offspring of plants exposed to herbivore-induced wounding, mechanical wounding or JA treatment show enhanced resistance to herbivore attack. The latter mechanism was shown to be dependent on RNA interference and priming of JA-dependent defense responses (Rasmann *et al.,* 2012). Also offspring of plants challenged with *P. syringae* (or its effector Rpt2) were shown to be more resistant to *P. syringae* and oomycete pathogen *Hyaloperonospora arabidopsidis* (Slaughter *et al.*, 2011; Luna *et al.*, 2012), showing that the epigenetic changes not only give protection to the original inducer. DNA methylation was shown to be responsible for *P. syringae*-induced transgenerational resistance to *P. syringae* (Boyko *et al.*, 2010; Bilichak *et al.*, 2012; Dowen *et al.*, 2012). In addition, progeny of plants infected with Tobacco mosaic virus (TMV) were shown to have increased resistance to TMV, *P. syringae* and *Phytophthora nicotianae*, also as a result of altered DNA methylation (Kathiria *et al.,* 2010). Both plant to plant communication via VOCs and heritable epigenetic changes significantly influence plant stress responses similarly to when having encountered a prior stress. However, epigenetic effects may go unnoticed while greatly affecting plant responses.

Plant evolutionary strategies. Under the Influence of selective pressure plants have adapted their defenses to fit their natural environment. Therefore, differences in their responses may be expected. A microarray study identifying the plant response and stress regulatory networks under a combination of cold, heat, high-light, salt, and bacterial flagellin treatment suggested that out of the ten tested *A. thaliana* accessions the extensively used model accession Col-0 behaves as an outlier (Rasmussen

et al., 2013). The latter potentially has great implications for plant stress research as most studies are performed with this accession. Moreover, this indicates that there is natural variation in plant responses to these stresses within the population of naturally occurring *A. thaliana* accessions that can be mined for naturally occurring plant adaptive responses (Atwell *et al.,* 2010; Baxter *et al.,* 2010; Rasmussen *et al.,* 2013; Kloth *et al.,* 2016; Davila Olivas *et al.,* 2017b; Thoen *et al.,* 2017). Furthermore, a recent study on natural genetic variation has shown that the plant's life strategy plays an important role in plant responses as winter annuals were shown to be more resistant to drought, aphids and thrips, while summer annuals are more resistant to *P. rapae* and *Plutella xylostella* caterpillars (Davila Olivas *et al.,* 2017a), showing the importance of evolutionary differences between plants.

Stress timing and severity. Plant stress type, timing and severity can greatly influence the plant's defense strategy. An increasing body of research focusses on transcriptional analyses of plant responses to multiple stresses, which provides a wealth of information about signaling pathways and plant responses. However, applying stresses in a naturally occurring and relevant manner can result in quite some challenges and considerations. For example, a transcriptome study on the response of *A. thaliana* plants subjected to drought and nematode infection showed that the majority of the transcripts regulated by the stress combination are regulated by drought alone. This suggests that plant prioritizes the potentially more damaging abiotic stress over a biotic stress when applied simultaneously (e.g. mild nematode infection with severe drought stress; Atkinson *et al.*, 2013)). However, in a study of plant drought stress and infection with necrotrophic fungus *B. cinerea* the opposite was found (Coolen *et al.,* 2016). The plant's transcriptional responses were greatly influenced by the biotic stress and showed only a signature of the abiotic stress. This difference in stress response prioritization can be the result of the order of application, their severity and timing. In the study of drought and *B. cinerea* for instance a recovery period was included after drought stress that might influence the plant's response.

Plant stress signaling, crosstalk and additional factors (e.g. high-jacking of plant defenses by pathogens and insects, plant to plant communication, transgenerational modifications, natural genetic variation, and severity and timing of stresses) greatly influences the plant's responses to abiotic and biotic stresses. When combining different stresses, stress specific and unique multi-stress responses may occur, showing a glimpse of the complexity of the plant's adaptability during combinations of abiotic and biotic stresses.

Plant adaptive responses to multi-abiotic stresses

Abiotic stresses are important plant stress factors that often occur simultaneously and have great impact on plant health, growth and reproduction (Mittler 2006). Because of increasing extreme environmental changes these naturally occurring conditions are hard to manage, challenging plant adaptabilities. Abiotic stresses are known to alter plant physiology and involve ABA signaling, though other phytohormones are also known to be involved in plant abiotic stress responses.

Drought and heat stress. Amongst the main abiotic factors limiting plant growth and crop productivity are drought and heat. In the field these stress types are rarely present individually, hence plants are often subjected to a combination of them (Ciais *et al.,* 2005; Johnson *et al.,* 2014). Physiologically, the combination of these two stresses seem to be conflicting. On the one hand drought stress induces stomatal closure to retain water, resulting in less evaporation cooling and eventually reducing photosynthesis by limited CO_2 availability and heat buildup (Carmo-Silva *et al.*, 2012). Heat stress also results in less photosynthesis due to photosystem damage, but on the other hand it causes the stomata to open, providing more evaporation cooling, resulting in excessive water loss by the plant. As individual stress, both drought and heat were shown to involve ABA, SA, JA, ET, AUX, CKs, a wide variety of TFs and small non-coding RNAs (Larkindale and Knight 2002; Rizhsky *et al.,* 2004; Larkindale *et al.,* 2005; Lin *et al.,* 2008; Dobra *et al.,* 2010; Rampino *et al.,* 2012; De Ollas *et al.,* 2013; Ding *et al.,* 2013; Du *et al.,* 2013; Hasanuzzaman *et al.,* 2013; Miura *et al.,* 2013; Johnson *et al.,* 2014; Kuromori *et al.,* 2014; Sailaja *et al.,* 2014), suggesting that both responses are quite similar. However, when drought and heat stress occur simultaneously an unique response involving enhanced respiration, suppression of photosynthesis and a complex expression pattern of defense and metabolic transcripts is observed (Rizhsky *et al.*, 2004; Rampino *et al.*, 2012; Johnson *et al.*, 2014). Genes that can be found elevated in the combined stress include receptor-like kinases, small guanosine triphosphate (GTP)-binding proteins, MYB TFs, protein kinases and different membrane channels. Metabolic analysis revealed the accumulation of sucrose and other sugars such as maltose and glucose that act as the major osmoprotectants in the combined drought and heat response, whereas proline was the major osmoprotectant in the drought stress response (Rizhsky *et al.,* 2004; Dobra *et al.,* 2010; Prasch and Sonnewald 2013; Johnson *et al.,* 2014; Sekmen *et al.,* 2014). Besides acting as an osmoprotectant, sucrose was found to be involved in H_2O_2-detoxification during oxidative stress that occurs in both heat and drought stress (Ramel *et al.,* 2009). Plant tolerance to a combination of drought and heat stress was shown to involve ROS scavenging by detoxifying enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidases (POX; Sekmen *et al.,* 2014), showing the importance of early and general stress responses to plant tolerance to combined abiotic stresses. Since JA is known to increase the activity of these enzymes, drought and heat tolerance may also be facilitated by JA (Qiu *et al.,* 2014).

Drought, heat and elevated CO_2. Abiotic factors such as CO_2 availability can have a major influence on other plant abiotic stress responses. For instance, changes of leaf metabolites and transcripts in response to drought stress are delayed or even eliminated by CO_2 enrichment in maize (Sicher and Barnaby 2012). Moreover, elevated CO_2 was shown to protect *A. thaliana* plants, that exhibit C3 photosynthesis, from oxidative membrane damage during drought, heat and a combination of drought and heat stress (Wang *et al.,* 2012; Zinta *et al.,* 2014). This protective mechanism was shown to involve antioxidant defense metabolism and reduced photorespiration that led to a decrease in oxidative stress. The inhibition of photorespiration was also observed in maize (C4-) plants as a result of elevated CO_2 under drought stress conditions, suggesting that the protective mechanism of CO_2 is effective in both C3 and C4 plants.

Salt and heat stress. The combination of salt and heat stress results in physiologically conflicting responses that are also observed during drought and heat stress. Heat stress results in increased respiration and will therefore require more water uptake by the plant to attain turgor pressure. This leads to more salt uptake and will therefore increase salt stress even further (Mittler 2006). Although with each individual stress plants are shown to have the ability to survive, the survival rate of plants that encounter a combination of salt and heat stress is dramatically decreased (Suzuki *et al.,* 2016). Salt stress in *A. thaliana* was shown to increase the plants Na^+ concentration by 200% and K^+ by 60%, whereas the combination of salt and heat resulted in a comparable Na^+ concentration along with an increased NA^+/K^+ ratio (Suzuki *et al.,* 2016). In tomato this ratio was shown to decrease in a combination of salt and heat stress, resulting in a better cell water status and increased photosynthesis as compared with salinity alone (Rivero *et al.,* 2014). This might suggest that tomato plants are better protected against a combination of salt and heat stress, or *A. thaliana* has a different mechanism of protection. Protection against osmotic stress as a result of salt and heat stress was shown to rely on the accumulation of trehalose in tomato, whereas for salt stress individually proline and sucrose were shown to be mainly accumulated and for heat stress sucrose choline and glycine betaine were shown to be the main accumulated sugars (Rivero *et al.,* 2014). The accumulation of trehalose was shown to be linked to the maintenance of a high K^+ concentration and a low Na^+/K^+ ratio.

The plant's transcriptional response to a combination of salt and heat stress was shown to result in an unique profile compared to each stress individually (Suzuki *et al.,* 2016). Each of the stresses and their combination were shown to result in massive transcriptional changes leading to both induction and even more repression of transcripts of which the majority are categorized as ABA-associated. ABA responsive genes were found upregulated in both individual and combined stresses and were shown to be important for tolerance to a combination of salt and heat stress. Furthermore, RbohD was shown to be specifically upregulated by the combination of salinity and heat stress and expression of ROS scavenger CAT2 was found to be reduced, suggesting an increased oxidative stress in the combined stress treatment. Salt stress tolerance was shown to be obtained with exogenous application of JA, resulting in an increased activity of ROS scavengers such as activities of SOD, POX, CAT and APX (Qiu *et al.,* 2014). In accordance to that, *A. thaliana* plants deficient in APX1-mediated ROS scavenging activities were shown to be more sensitive to a combination of drought and heat stress (Miller *et al.,* 2007; Koussevitzky *et al.,* 2008; Sekmen *et al.,* 2014).

Drought and salt stress. Plant responses to drought and salt stress share more than half of the genes that are induced upon stress application (Seki *et al.,* 2002), suggesting that these stresses induce quite similar plant responses. In contrast to the great overlap in shared genes during both individual stresses, the combination of salt and heat stress results in an unique plant response involving plant defenses, signal transduction, transport, photosynthesis and energy production (Li *et al.,* 2011). Besides the suppression of CO_2 assimilation, the combination of drought and salt stress leads to significant metabolic changes compared to the single stresses, including sugars, organic acids, amino acids and nucleotides. In contrast to the accumulation of sugars that were found for the combination of drought and heat stress (sucrose, maltose and glucose), drought and salt stress

results in the accumulation of proline that could also be found in individual drought stress (Rizhsky *et al.,* 2004; Dobra *et al.,* 2010; Rivero *et al.,* 2014; Sun *et al.,* 2015).

Salt stress and light availability. Light intensity has major effects on the plant's ability to cope with salt stress. In a study on wheat it was shown that plants under low light intensity were more sensitive to salt stress than plants grown under high light intensity (Ivanov, 2010). The rate of photosynthesis was shown to rapidly decrease with low light intensity and relatively high salt concentrations. Light stress induced ROS production was shown to result in the accumulation of anthocyanin, involved in tolerance to light stress (Vanderauwera *et al.,* 2005). Both cytosolic ROS scavenger APX and thylakoid APX (tyAPX) are involved in light stress induced ROS accumulation (Vanderauwera *et al.,* 2005; Miller *et al.,* 2007) and APX and tyAPX single and double mutants were shown to be more tolerant to salt stress (Miller *et al.,* 2007).

Drought and cold stress. Although only 10% of drought-inducible genes are cold-inducible, plants under drought stress are known to trigger ABA-dependent freezing tolerance (Mäntylä *et al.,* 1995; Seki *et al.,* 2002; Shinozaki *et al.,* 2003). Soybean (*Glycine max* L.) MYB transcription factor gene *MYBJ1* was shown to be involved in both drought and cold stress. Overexpression of *MYBJ1* was shown to enhance plant tolerance to drought and cold stress (Su *et al.,* 2014). Furthermore soybean DREB1B was shown to target ABA receptor PYL21 and enhances ABRE-mediated gene expression in an ABA-independent manner in drought, salt, heat and cold stress (Kidokoro *et al.,* 2015), indicating the importance of this TF in different abiotic stress responses.

The latter studies suggest that the molecular features signed by the single abiotic stress can be rapidly rewired by additional abiotic stresses. This can sometimes benefit the plant, as in case of CO_2 in combination with drought and heatwaves. Environmentally closely-related stresses like drought, heat and salt stress trigger signaling pathways with shared components. When they occur together, the plant's protective strategy activates an unique signaling network ensuring the best physiological status that fits with the new unfavorable multi-stress environment.

Plant adaptive responses to multi-biotic stresses

Aside from environmental conditions giving rise to abiotic stresses, plants are often exposed to a variety of biotic threats including bacteria, fungi, nematodes, insects and viruses. Depending on the nature of the attacker, plants are capable of inducing different defense strategies in which phytohormones SA, JA, ET and ABA play important regulatory roles (Pieterse *et al.,* 2012).

Multiple insects. Insects are one of the most abundant organisms, comprising an estimated six million species of which fifty percent is herbivorous (Schoonhoven *et al.,* 2005). Recently, multiple studies indicated that attack from various insects affect plant defenses to subsequent attackers

(Stam *et al.,* 2014). For example, infestation of *A. thaliana* with the whitefly *Bemisia tabaci* strongly suppressed genes that were induced by diamondblack moth *Plutella xylostella* caterpillars (Zhang *et al.,* 2013). In another study in which tomato plants were infested with *Macrosiphum euphorbiae* aphids and *Spodoptera exigua* caterpillars it was shown that aphids suppress almost a third of the genes regulated by caterpillars, whereas caterpillars suppress over two third of the genes regulated by aphids (Rodriguez-Saona *et al.,* 2010). This indicates that the order of insect infestation influences the plant response and that the insect's feeding strategy along with its damaging potential may be part of the plant's evolutionary strategy.

Multiple pathogens. Simultaneous infection with multiple pathogens can have different outcomes depending on the lifestyle of the pathogen and the plants response. Exogenous application of SA to mimic (hemi-)biotrophic induced SA accumulation in plants renders plants less susceptible to hemi-biotroph *Fusarium oxysporum* (Mandal *et al.,* 2009). As a result of plant defenses against hemi-biotrophic pathogen *P. syringae,* avirulence (avr) protein-induced HR-mediated programmed cell death was not only shown to limit bacterial spreading but also benefits the necrotrophic fungus *B. cinerea* (Govrin and Levine 2000). However, *P. syringae* avr proteins are not essential for the increased susceptibility to necrotrophic pathogens in *P. syringae* infected plants. As shown by Spoel *et al.* (2007), *P. syringae* increased susceptibility to necrotrophic pathogen *Alternaria brassicicola* irrespective of avr proteins. SA-mediated suppression of the JA signaling was shown to be responsible for the increased plant susceptibility, showing the great impact that *P. syringae* can have on subsequent plant infections with necrotrophs. Furthermore, SA production was shown to be locally important for penetration resistance to *B. cinerea* (Ferrari *et al.,* 2003; Ferrari *et al.,* 2007). The later studies show that a previously encountered stress can greatly influence an encounter with a subsequent stress, rendering the plant more susceptible in both biotroph-necrotroph scenarios.

Insects and pathogens. Simultaneous infection with pathogens and infestation by insect herbivores can have different outcomes like seen in multiple pathogen infections. In case of herbivore-induced plant defenses triggered by *P. rapae* caterpillars, both infection by the hemi-biotrophs *P. syringae* and *Xanthomonas campestris* pv. *armoraciae* can be suppressed in local plant tissue in a SA, JA and ET dependent manner (De Vos *et al.,* 2006b). This effect was shown to be limited to local tissue, indicating the specificity of the *P. rapae* induced suppression of hemibiotrophs. Mechanical or herbivore-induced wounding is also known to induce resistance against certain pathogens in systemic tissue, including necrotrophic fungus *B. cinerea* (Chassot *et al.,* 2008; Benikhlef *et al.,* 2013; Coolen *et al.,* 2016). This indicates that plant defenses may differ greatly in locally affected and systemic tissues, influencing subsequent stress signaling in a tissue specific manner. In contrast to herbivore-induced plant defenses against different pathogens, infection with hemi-biotrophic pathogen *P. syringae* was shown to result in plant susceptibility to the cabbage looper moth *Trichoplusia ni* (Groen *et al.,* 2013). This effect was shown to be dependent on the antagonism between SA and JA signaling. SA was also shown to inhibit JA-induced plant resistance to insect herbivores such as *S. exigua* via the MYC2 transcription factor (Cipollini *et al.,* 2004; Nakata *et al.,*

2013), again showing the importance of SA-JA crosstalk in plant resistance against insect herbivores. However, bacterial-induced plant defenses can also lead to the attraction of insect herbivores as was shown by Mann *et al.* (2012). Infection of citrus plants with plant phloem-limited bacterial pathogen *Candidatus Liberibacter asiaticus* was shown to lead to volatile-mediated (Methyl SA, MeSA) attraction of African citrus psyllid, which not only acts as a pathogen vector for *C. Liberibacter asiaticus* but also feeds on plant sap (Mann *et al.,* 2012). Apparently in this case the plant's cry for help only brings along more problems.

Insects and virusses. In systemic tissue, feeding by JA-inducing *P. rapae* caterpillars represses SA-inducing turnip crinkle virus (TCV; De Vos *et al.,* 2006b). This effect was shown to be specifically induced by *P. rapae* and could not be achieved by mechanical wounding alone. In the SA-JA crosstalk perspective, plants infected with SA inducing tomato spotted wilt virus (TSWV) are more susceptible to JA inducing western flower thrips, *Frankliniella occidentalis*. Moreover, there seems to be a direct benefit for the virus with this crosstalk effect, as the plant is more attractive to thrips that acts as a vectors for these viruses (Abe *et al.,* 2012). Also in potato a negative effect of virus infection on plant resistance to insects was shown as potato virus Y infection was shown to hinder defense to Colorado potato beetle *Leptinotarsa decemlineata* (Petek *et al.,* 2014). Thus, in case of plant-insect-virus interactions evolutionary adaptations of plant defenses benefit virus transportation via repression of plant defenses against insect vectors, which is also seen for bacteria and insects (Mann *et al.,* 2012).

In the simultaneous interaction between biotic agents, usually the most invading one rewires the plant transcriptome in a way that the plant could be favorable to become a niche opportunity. In the sequential stress, the first pathogen can enhance plant susceptibility against the second one, as demonstrated by the effect of *P. syringae* on the cabbage looper moth infected plants. Moreover, the infection by the first pathogen can prime the plant to improve the defense against the second one, as well demonstrated by the effect of *P. rapae* on *X. campestris* or TCV infected plants.

Plant adaptive responses to combined biotic and abiotic stresses

Although the habitat range of pests and pathogens can be seasonal or spatial separated from major abiotic stresses, subsequent encounters can greatly influence the plants adaptability. Together with an increasing global temperature, global climate changes are known to facilitate the spread of plant pests that are predicted to create new opportunities for different stress combinations (Chakraborty and Newton 2011; Nicol *et al.,* 2011). Both abiotic and biotic stresses engage similar hormone-regulated plant signaling pathways, whereas the consequences for the plant can be very different and has a great impact when induced sequentially.

Drought and insects. Drought stress is one of the most common abiotic stresses that can easily co-occur with insect pests. Meta-analysis on the effects of herbivory on drought stressed plants reveals that sap feeder performance is negatively affected by plant drought stress, benefitting the plant (Huberty and Denno 2004). In addition, the extent of the drought stress is also shown to influence phloem feeders. When drought stressed plants are able to recover their turgor pressure, phloem feeders could potentially benefit from the stress-induced increases in plant nitrogen. In soybean drought stress leads to less leaf chewing herbivores, likely to be caused by the lack of plant vigor (Grinnan *et al.,* 2013). Furthermore, herbivore generalist *S. littoralis* prefers to eat from drought stressed plants over well-watered plants, whereas specialist *Pieris brassicae* prefers well-watered plants over drought stressed plants. Surprisingly, *P. brassicae* performance on drought stressed plants was better than on well-watered plants (Gutbrodt *et al.,* 2011). The latter finding suggests that the relation between the stressed plant and *P. brassicae* is more complex than the interaction with *S. littoralis*, as otherwise *P. brassicae* would be more likely to choose for the drought stressed plant which benefits its performance. Explanations for why feeding strategy and performance are not in line can be sought in differences in feeding stimulus (glucosinolates and other secondary metabolites), though it might also be a strategy to avoid parasitoids that are potentially attracted to drought stressed plants and a glucosinolate rich diet that negatively affect parasitoids (Scascighini *et al.,* 2005; Gols and Harvey 2009).

Drought and nematodes. Plant responses to a combination of drought and nematode infection was shown to result in an unique set of transcripts that were dominated by drought-induced responses. These include a *RAPID ALKALINIZATION FACTOR-LIKE 8* (*RALFL8*), *METHIONINE GAMMA LYASE* (*MGL*) and *AZELAIC ACID INDUCED 1* (*AZI1*). Although the overexpression of *RALFL8* enhances the susceptibility of transgenic plants to nematodes and drought individually, MGL enhances resistance to nematodes and *AZI1* conferred susceptibility to drought stress, no candidates affecting the double treatment were found (Atkinson *et al.,* 2013). This suggests that plant responses to either stress follows a distinct route without affecting the other too much. Another explanation can be that drought stress was so severe that plant responses to this stress almost completely overshadowed the prior nematode infection, indicating that the plant reacted to the most threatening stress.

Drought and pathogens. When comparing 20 micro-array studies, both rice and *A. thaliana* responses to drought stress and bacterial infection were shown to share approximately 38.5% of all differentially expressed genes (DEGs), involved in hormonal signaling, oxidative stresses and metabolic processes (Shaik and Ramakrishna 2013). In particular in the *A. thaliana* datasets, meta-analysis showed that genes responsive to ABA, SA, ET, JA as well as those involved in oxidative stresses are induced by both drought and bacterial infection, suggesting synergistic interactions when both stresses occur. In accordance to that exogenous application of ABA results in increased resistance to hemi-biotrophic bacterial pathogen *P. syringae* and increased plant susceptibility to *B. cinerea* (Audenaert *et al.,* 2002; Mohr and Cahill 2007; Curvers *et al.,* 2010). The mechanism of ABA-induced susceptibility to *B. cinerea* was described as being the result of the negative regulation

of ABA on SA. Furthermore, SA that is known to be induced by several pathogens was shown to enhance plant heat tolerance, tolerance to low temperatures and drought stress (Dat *et al.,* 1998; Janda *et al.,* 1999; Saruhan *et al.,* 2012), indicating the importance of SA in abiotic stress responses. When looking at drought stress and plant infection with necrotrophic fungus *B. cinerea*, both individual stresses have been reported to repress genes involved in multidimensional cell growth (Coolen *et al.,* 2016). However, when plants experience drought stress and subsequent infection with *B. cinerea* a mild induction of these genes was observed. It could be that the drought-induced repression of growth is compensated after a drought recovery, potentially affecting plant responses to *B. cinerea*. However, it could also be that the combination of drought and *B. cinerea* leads to an unique response resulting in multidimentional cell growth.

UV light and pathogens. When plant cells are exposed to a combination of flagellin epitope flg22 from bacterial pathogens and UV-B, defense-related compounds such as the phytoalexins camalexin and scopoletin as well as a structural barrier of lignin, to restrict pathogen spread, are significantly induced (Schenke *et al.,* 2011). By contrast, production of UV-protective flavonols, induced by UV-B, are attenuated by the simultaneous application of flg22. It appears that this crosstalk involves antagonistic regulation of two opposing MYB transcription factors, the positive regulator of the flavonol pathway MYB12 and the negative regulator MYB4 and was shown to involve chromatin remodeling (Schenke *et al.,* 2014). UV-C treatment of *A. thaliana* plants was shown to induce resistance against the necrotrophic fungus *B. cinerea* (Stefanato *et al.,* 2009). This effect was also shown in post harvested strawberry and involves SA (Pombo *et al.,* 2011).

Heat stress, drought stress and virus infection. Heat and drought stress were both shown to significantly alter turnip mosaic virus (TuMV) infection and when combined increase susceptibility of *A. thaliana* to infection with TuMV (Prasch and Sonnewald 2013). On the other hand TuMV can inhibit heat-induced stomata opening, potentially affecting the heat responses in *A. thaliana*. Analyzing the plant's transcriptome, virus-treated plants display enhanced expression of defense genes, which is altered in plants additionally subjected to heat and drought stress. An interesting expression pattern for *PR* genes was found in which virus infection leads to an up-regulation of *PR* gene transcripts, including *PR1*, *PR2*, and *PR5*, which are down regulated by additional heat stress. By contrast, *PR* genes are not altered under a combined virus and drought stress situation. The combination of a virus infection together with heat stress increases the levels of hexoses in the plant's apoplast that could lead to enhanced expression of *PR* genes (Herbers *et al.,* 2000; Kocal *et al.,* 2008). However, transcripts of cell wall-bound invertases, that are responsible for the release of hexoses into the plant's apoplast, are not up-regulated when heat or heat and drought are applied together with the TuMV. This might suggest a non-transcriptionally regulated activation of invertase activity.

The studies mentioned above carefully describe the complexity of abiotic and biotic stress plant-interactions. The outcome of the interactions can have multiple directions, and can be highly dependent on very specific and subtle differences. The described findings clearly point out that the

plant's transcriptomic response to two stresses is far from an additive response to the two stresses individually. At the end the plant's defense strategy determines how the interaction will set off. How it will end remains quite unpredictable from single stress knowledge as the highly complex multi-stress interactions are formed by many different components of the plant and its stress factors.

Concluding remarks

As member of a complex biotic community, plants often deal with multiple abiotic and biotic stress factors. Plant multiple stress responses are not simply additive or contrasting, the outcome of the interaction will most probably be dependent on the evolutionary strategy of the plant, stress timing and the strength of the threat. Overall, interactions between plants and multiple stresses are very complex as each contributing stress factor can have multiple ways of interacting with the plant's signaling pathways triggered by other stresses. Understanding how naturally existing plant stress-responsive mechanisms function under multi-stress environments, how plant defense networks are built-up and where key regulators are within the system, is essential for future multi-stress-focused crop breeding. In order to manipulate and steer plant stress responses in favorable directions, discovery of signaling networks and key signaling nodes underlying multiple biotic and abiotic stress will provide new insights for broad spectrum stress tolerance. Ultimately extensive high density time point signaling network analyses will provide more insight into the complexity and architecture of (phytohormonal) signaling dynamics that lead to specific signaling events in plants (Windram *et al.,* 2012; Hickman *et al.,* 2017). Detailed knowledge of plant responses to combined biotic/abiotic stress will open new doors for the development of novel strategies for sustainable crop protection.

In the context of future perspectives, plants and also food producing crops are likely to encounter complex multi-stress conditions more frequently, as our changing climate with extreme weather conditions will support pests and pathogens to spread more easily and even beyond seasons where they normally occur (Grieve *et al.,* 2010; Chakraborty and Newton 2011; Nicol *et al.,* 2011; Bebber *et al.,* 2013; Garrett *et al.,* 2013; Syvertsen and Garcia-Sanchez 2014). Together with our rapidly increasing human population, reaching 9.3 billion people by 2050, serious problems for our food security are predicted (Mittler and Blumwald 2010; Newton *et al.,* 2011; Atkinson and Urwin 2012; Garrett *et al.,* 2013; Teixeira *et al.,* 2013). A dramatic increase in efficient food production is required in order to meet with future food demands (UN 2011; FAO 2012). To reach these demands, pest management is of great importance. Where pesticides were once important tools for pest reduction, the usage of these products is greatly reduced nowadays, because of health risks and resistance buildup (Gilden *et al.,* 2010; Meissle *et al.,* 2010; Gressel 2011). Therefore, using and improving natural adaptive mechanisms of plants with resistance breeding, which already has been used for many centuries, provides a good alternative. Innovative approaches to enhance tolerance for biotic and abiotic stresses are necessary to sustainably increase crop production, without adversely affecting our ecological footprint. Biotechnological approaches and novel non-GMO mutational breeding technology can be used to target sequences of master switch genes in crops. Regulatory genes of multiple stresses could be targeted in marker-assisted breeding programs or by *cis*-genic

approaches to engineer second-generation GM crops. Studies on the interaction between plants and multiple micro- or macro-organisms clearly addresses effects at the levels of gene expression, hormonal cross-talk, metabolic changes, species interaction and community dynamics.

Thesis outline

In nature plants need to cope with a wide variety of biotic and abiotic stresses, often simultaneously or in sequence, thereby forcing the evolution of plant traits that allow plants to quickly adapt to changing environments. Studies on combinatorial plant stresses represent an emerging field of research that will become increasingly important as global climate change more frequently exposes plants to extreme environmental conditions. The research described in this thesis is focused on exploring the natural genetic variation in *Arabidopsis thaliana* related to the ability of plants to cope with a combination of different biotic and abiotic stresses. Because biotic and abiotic stresses are known to induce stress signaling pathways that partly overlap, studying plant responses to combinatorial stresses may unravel key players that allow plants to better cope with multiple stresses simultaneously. A better understanding of plant stress responses under complex environmental conditions improves fundamental knowledge that may support breeders in improving multiple stress resistance in crops. The work described in this thesis was part of the collaborative project "Mining the natural genetic variation of combinatorial stress responses of *A. thaliana* to identify new tolerance pathways for multiple biotic and abiotic stresses", which was part of the STW Perspective program "Learning from Nature to Protect Crops" (STW-LfN). In this project, we worked together with the laboratories of Entomology and Genetics of the Wageningen University, and the industrial partners KeyGene N.V., Genetwister Technologies B.V., and Royal Van Zanten B.V.

In **Chapter 1**, an overview is given of recent advances in our understanding of the complexity and functioning of interacting stress signaling in view of plant multi-stress signaling. Plant stresses are perceived via receptors that bind to stress specific molecules and result in signal transduction leading to the activation of plant phytohormones and transcription factors that are involved in amplifying and regulating plant stress signaling. To carefully fine-tune their stress signaling, plants are known to make use of extensive cross-communicating signaling pathways that allow them to swiftly adapt to changing environmental conditions. However, pathogens and insects have some sophisticated tricks up their sleeves to tackle these well adapted plants. With the help of effector molecules and phytohormone mimics they high-jack plant stress signaling for their own benefit, leaving the plant poorly protected. In turn, plants evolved stress signaling pathways that eventually lead to adaptations that minimize stress-induced damage and ensure survival. These adaptations include physiological changes and the production of anti-microbial and toxic compounds to ward of biotic threats. However, when plants encounter multiple stresses simultaneously or sequentially, the outcome of the interaction is often hard to predict form the response to individual stresses. Therefore, an increasing number of studies is now focusing on unraveling plant responses to multiple stresses. Moreover, with a rapidly changing climate, plant stresses that never coincided before can be considered possible in the future, making multi-stress research of even greater importance.

In **Chapter 2**, we used RNA-seq to monitor and analyze dynamic transcriptome changes in *A. thaliana* accession Col-0 over time in response to *B. cinerea* infection, *P. rapae* infestation, drought stress, or

a combination of these stresses. The main goal of this study was to identify key genes or processes involved in adaptive plant responses to combinations of the applied stresses. Meta-analysis of the overall data sets revealed extensive overlap in genes induced or repressed during the individual and combined stress treatments, indicating that the adaptive plant responses to these stresses at least partially share similar sections of the plant stress signaling network. In addition, the meta-analysis revealed that regardless the nature of the first stress, *A. thaliana* swiftly changes its transcriptome to the last applied stress, suggesting that the previous stress encounter did not majorly affect the transcriptome induced by the subsequent stress. Nevertheless, this approach allowed us to pinpoint specific sectors in the plant stress signaling network that were markedly different in combinations of stresses, relative to the respective single stress treatment. For instance, prior treatment of the plant with a drought period or herbivory, significantly slowed down the *B. cinerea*-induced activation of genes in the ethylene signaling sector, which may be causally related with the impact of the first stress on the level of disease resistance. Overall, the effect of pretreatment was shown to have a significant effect on early plant responses to *B. cinerea*, whereas pretreatment did not have a great impact on drought or *P. rapae*-induced plant responses. This result was supported with phenotypic data. Overall, the results show that there is a first stress signature that can have great influence on the outcome of the interaction although the majority of later responses swiftly change to that of the latest encountered stress. The latter suggests that plants are well capable of adapting its survival strategies to a changing environment, although influenced by previous stresses.

In **Chapter 3**, the phenotypic variation of 346 *A. thaliana* accessions of the HapMap collection was investigated for the level of resistance against the necrotrophic pathogen *Botrytis cinerea* and the effect of prior herbivory by *Pieris rapae* or drought stress on the level of resistance to *B. cinerea*. This study revealed that *A. thaliana* possesses a large natural genetic variation that affects the level of resistance to *B. cinerea* infection. Interestingly, herbivory by *P. rapae* prior to *B. cinerea* infection increased the level of plant resistance to *B. cinerea* infection in the majority of the 346 accessions. Application of drought stress prior to the *B. cinerea* infection also affected the level of plant resistance to *B. cinerea*, resulting in both increased susceptibility and increased resistance depending on the accession. Univariate GWAS analysis pointed to different sets of candidate genes involved in *B. cinerea* resistance or traits involved in multi-stress resistance. To help pinpointing key players in single and multi-stress resistance, quantitative trait loci (QTL) mapping was performed on a multi-parent population of recombinant inbred lines descending from multiple natural accessions present in the HapMap collection of 350 accessions. Finally, fine mapping was performed on fully sequenced genomic regions to generate final candidate gene lists. Future research has to determine their role in plant sequential stress responses.

In **Chapter 4**, the whole STW-LfN consortium joined forces in mining the genome of *A. thaliana* to find genes involved in the plant's ability to cope with different stresses. To this end, a collection of 350 naturally occurring *A. thaliana* accessions of the Haplotype map (HapMap) collection was phenotypically screened for their responses to different abiotic and biotic stresses as well as some of

their combinations. These stresses included biotic stresses, such as pathogen and insect attack, and abiotic stresses, such as drought and salinity. The data from all partners was collected, providing a wealth of information on plant phenotypic traits during different stresses. Using the collective data, a search for genes involved in adaptive responses to the multiple stresses was performed by single trait (univariate) genome-wide association studies (GWASs), highlighting potential candidates. Subsequently, a meta-analysis of all the data was performed including a newly developed multi-trait (multivariate) mixed model analysis. Using this multivariate GWAS analysis, contrasting effects of genes involved in biotic and abiotic stress resistance were found. In addition, the multivariate approach yielded an unique set of shared and trait specific genes involved in one or more biotic or abiotic stresses that were not identified in the univariate GWAS analysis. Interestingly, genes with a small contribution to the univariate analysis were found to gain power in the multi-trait analysis, resulting in the identification of genes that are potentially interesting for further research on multi-stress resistance.

In **Chapter 5**, we explored the natural genetic variation of the *A. thaliana* HapMap collection to identify plant loci that influence the choice of *P. rapae* butterflies for host plants for oviposition. *P. rapae* is a specialist on Brassicacea species, such as *A. thaliana*, and is thought to be relatively insensitive to their defense systems. To investigate if we could identify genes involved in host selection for oviposition by *P. rapae*, 350 *A. thaliana* accessions were screened in an experimental setup in which *P. rapae* butterflies were allowed to choose between all 350 accession for oviposition. We observed that the oviposition behavior of *P. rapae* was influenced by daylight and oviposition was preferred on plants at the edges and corners of our experimental cage setup. In addition, more eggs depositions were found on medium and large plants compared to small plants and spontaneous plant chlorosis and necrosis was found to be an unfavorable trait for host selection by *P. rapae* butterflies. In order to find plant genes involved in host selection by *P. rapae*, an univariate GWAS analysis was performed on the obtained data of 346 accessions, yielding candidate genes that were further investigated with fine mapping. Fine mapping results showed significant associations with a jasmonic acid (JA) biosynthesis gene. Future functional analysis of the JA biosynthesis gene and the other identified candidates will be required to reveal their role in host selection for oviposition by *P. rapae*. Knowledge on the genetic basis of *P. rapae* oviposition preference may be used in breeding strategies that are aimed at reducing the attractiveness of crop plants for insect herbivores.

In **Chapter 6**, the findings of the research presented in this thesis are discussed and future perspectives for the research field of plant multi-stress signaling and directions for breeders are outlined.

Chapter 2

Transcriptome dynamics of Arabidopsis during sequential biotic and abiotic stresses

Silvia Coolen[1]*, Silvia Proietti[1]*, Richard Hickman[1]*, Nelson H. Davila Olivas[2]*, Ping-Ping Huang[3]*, Marcel C. Van Verk[1,4], Johan A. Van Pelt[1], Alexander H.J. Wittenberg[5], Martin De Vos[5], Marcel Prins[5], Joop J.A. Van Loon[2], Mark G.M. Aarts[3], Marcel Dicke[2], Corné M.J. Pieterse[1] and Saskia C.M. Van Wees[1]

* These authors contributed equally to this work

***The Plant Journal* 86**, 249–267 (2016)

1. Plant–Microbe Interactions, Department of Biology, Utrecht University, PO Box 800.56, 3508 TB, Utrecht, the Netherlands

2. Laboratory of Entomology, Wageningen University, PO Box 16, 6700 AA, Wageningen, the Netherlands

3. Laboratory of Genetics, Wageningen University, PO Box 16, 6700 AA, Wageningen, the Netherlands

4. Bioinformatics, Department of Biology, Utrecht University, PO Box 800.56, 3508 TB, Utrecht, the Netherlands

5. Keygene N.V., PO Box 216, 6700 AE, Wageningen, the Netherlands

Summary

In nature, plants have to cope with a wide range of stress conditions that often occur simultaneously or in sequence. To investigate how plants cope with multi-stress conditions, we analyzed the dynamics of whole-transcriptome profiles of *Arabidopsis thaliana* exposed to six sequential double stresses inflicted by combinations of: (i) infection by the necrotrophic fungus *Botrytis cinerea*, (ii) herbivory by chewing larvae of *Pieris rapae*, and (iii) drought stress. Each of these stresses induced specific expression profiles over time, in which one-third of all differentially expressed genes was shared by at least two single stresses. Of these, 394 genes were differentially expressed during all three stress conditions, albeit often in opposite directions. When two stresses were applied in sequence, plants displayed transcriptome profiles that were very similar to the second stress, irrespective of the nature of the first stress. Nevertheless, significant first-stress signatures could be identified in the sequential stress profiles. Bioinformatic analysis of the dynamics of coexpressed gene clusters highlighted specific clusters and biological processes of which the timing of activation or repression was altered by a prior stress. The first-stress signatures in second stress transcriptional profiles were remarkably often related to responses to phytohormones, strengthening the notion that hormones are global modulators of interactions between different types of stress. Because prior stresses can affect the level of tolerance against a subsequent stress (e.g. prior herbivory strongly affected resistance to *B. cinerea*), the first-stress signatures can provide important leads for the identification of molecular players that are decisive in the interactions between stress response pathways.

Introduction

Plants are continuously threatened by a wide range of harmful microbial pathogens and insect herbivores. Besides these biotic stresses, plants are also exposed to extreme abiotic environmental conditions such as drought, heat, cold, water logging, high salinity or toxicity. Adaptive plant responses to single biotic and abiotic stresses have been extensively studied. Both biotic and abiotic stress responses are associated with the action of the phytohormones jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), and salicylic acid (SA), and to a lesser extent with cytokinin, brassinosteroids and auxin (Robert-Seilaniantz *et al.,* 2011; Pieterse *et al.,* 2012; Giron *et al.,* 2013; O'Brien and Benková 2013; Kazan and Lyons 2014; Broekgaarden *et al.,* 2015) JA and ET are generally involved in defense against pathogens with a necrotrophic lifestyle, whereas defenses against biotrophs are commonly controlled by SA (Glazebrook 2005). ABA is associated with plant development and abiotic stresses (Yamaguchi-Shinozaki and Shinozaki 2006), such as drought, but its role in modulating JA-dependent defenses against insect herbivores and SA-dependent defenses against pathogens is becoming increasingly evident (Yasuda *et al.,* 2008; Verhage *et al.,* 2011; Vos *et al.,* 2013b). Antagonistic and synergistic interactions between hormonal signal-transduction pathways are thought to provide the plant with a regulatory potential to adapt to its complex biotic and abiotic environment while utilizing its resources in a cost-efficient manner (Reymond and Farmer 1998; Robert-Seilaniantz *et al.,* 2011; Pieterse *et al.,* 2012; Vos *et al.,* 2013a; Vos *et al.,* 2015).

In natural and agricultural settings, plants often have to cope with multiple stress conditions simultaneously. In the context of climate change, it is highly likely that the frequency and complexity of these multi-stress conditions will increase and further threaten crop yield. Abiotic stresses can significantly affect plant responses to biotic stresses and vice versa, depending on the timing, nature, and severity of the stresses (Atkinson and Urwin 2012; Appel *et al.,* 2014; Rejeb *et al.,* 2014)). How plants regulate and prioritize their adaptive response when exposed to multiple stresses is largely unknown. Several studies have investigated plant responses to different stress factors occurring simultaneously or sequentially (Mohr and Cahill 2003; De Vos *et al.,* 2006b; Van Oosten *et al.,* 2008; Atkinson *et al.,* 2013; Prasch and Sonnewald 2013; Rasmussen *et al.,* 2013; Santino *et al.,* 2013; Kissoudis *et al.,* 2014; Rivero *et al.,* 2014; Sewelam *et al.,* 2014; Stam *et al.,* 2014; Suzuki *et al.,* 2014; Ramegowda and Senthil-Kumar 2015; Sham *et al.,* 2015). From these studies, the picture emerged that different stress signaling pathways are interconnected in a network that is under control of key regulators such as MAP kinases, transcription factors and the above-mentioned stress-related hormones (Fujita *et al.,* 2006; Robert-Seilaniantz *et al.,* 2011; Pieterse *et al.,* 2012; Rejeb *et al.,* 2014; Caarls *et al.,* 2015).

In order to gain insight in the complexity of the plant response to combinatorial stresses, several recent studies investigated changes in the transcriptome of *Arabidopsis thaliana* (hereafter called Arabidopsis) in response to simultaneous exposure to abiotic and biotic stresses (Atkinson *et al.,* 2013; Prasch and Sonnewald 2013; Rasmussen *et al.,* 2013; Suzuki *et al.,* 2014; Ramegowda and Senthil-Kumar 2015; Sham *et al.,* 2015). Generally, the responses to the single stresses were different from those to the double stresses. However, these studies often focused on a single time point, representing only a snapshot of the transcriptional changes that are induced by a single or combinatorial stress. The influence of one stress on the other may primarily have an effect on the timing of the response to the second stress, causing the detection of large transcriptional differences in combinatorial stresses at one time point, while over time these differences may be much smaller or are the result of a shift in the phasing of the expression profiles.

In order to gain detailed insight into how plants cope with multiple stresses, we here investigated how a first stress influences the nature and dynamics of the transcriptional response that is induced by a second stress. We chose to study the response of the model plant species Arabidopsis to two biotic stresses (infection by the necrotrophic fungus *Botrytis cinerea* and herbivory by larvae of *Pieris rapae*) and to one abiotic stress (drought stress by water withhold). These stresses were chosen because in previous studies it was demonstrated that the plant hormones JA, ABA, and/or ET are involved in adaptive plant responses to these respective stresses. We hypothesized that combining these stresses may lead to hormonal signal interactions that potentially affect the outcome of the response to the second stress. Several previous studies have identified thousands of Arabidopsis genes that change in expression in response to the selected single stresses (Reymond *et al.,* 2000; Reymond *et al.,* 2004; De Vos *et al.,* 2005; Ferrari *et al.,* 2007; Huang *et al.,* 2008; Rowe *et al.,* 2010; Birkenbihl *et al.,* 2012; Windram *et al.,* 2012; Rehrig *et al.,* 2014; Clauw *et al.,* 2015), but their dynamic behavior during multi-stress conditions is largely unknown.

Botrytis cinerea is considered to be the second most important plant pathogen (Dean *et al.,* 2012), infecting over 200 cultivated plant species and causing significant economic damage to crops worldwide. Moreover, *B. cinerea* has become an important model for studying interactions between plants and necrotrophic pathogens (Van Kan 2006; Laluk and Mengiste 2010). As a necrotroph, *B. cinerea* kills plant tissue prior to feeding by using different mechanisms that cause plant decay, e.g. enzymatic degradation of the cell walls, generation of toxic reactive oxygen compounds, or secretion of host non-selective toxins. JA and ET participate in the defense response of Arabidopsis against *B. cinerea* (Thomma *et al.,* 1998; Thomma *et al.,* 1999; Diaz *et al.,* 2002; Geraats *et al.,* 2002; Rowe *et al.,* 2010; El Oirdi *et al.,* 2011), while ABA and SA can have a negative effect on *B. cinerea* resistance (El Oirdi *et al.,* 2011; Liu *et al.,* 2015; Vos *et al.,* 2015).

Insect herbivores consume over 15% of the plant biomass produced annually in temperate and tropical ecosystems making insect herbivory a major conduit by which energy flows through food webs (Cyr and Pace 1993; Agrawal 2011; Johnson 2011). The Small Cabbage White butterfly *P. rapae* is one of the most destructive pests of cruciferous plants because it has adapted to the glycoside toxins known as glucosinolates that are produced by crucifers as chemical defenses (Hopkins *et al.,* 2009). Arabidopsis and other plants activate additional defense responses that reduce the performance of leaf-chewing *P. rapae* caterpillars on pre-infested plants (De Vos *et al.,* 2006b). It has been shown that this herbivore- or wound-induced resistance also extends systemically to undamaged plant parts (Howe and Jander 2008; Vos *et al.,* 2013b). JA is an important primary signal in herbivore-induced local and systemic defenses in various plant–herbivore interactions, while ABA has a modulating role in the JA responsiveness (Bodenhausen and Reymond 2007; Howe and Jander 2008; Soler *et al.,* 2013; Vos *et al.,* 2013b). SA is reported to inhibit the JA-dependent defense pathway that is induced by *P. rapae* feeding (Koornneef *et al.,* 2008).

Drought is one of the most frequently experienced abiotic environmental stresses in plants. Low water availability in the rhizosphere leads to a reduction in leaf stomatal conductance and growth (Schachtman and Goodger 2008). Adaptive responses to drought also involve metabolic, osmotic, and structural adjustment, as well as the production of proteins with DNA damage control and repair functions (Ingram and Bartels 1996). ABA accumulation is essential for the adaptation to drought, but also ABA-independent regulatory systems are involved in drought stress-responsive gene expression. In the latter, JA and ET have been implicated as important regulators (Bray 1997; Shinozaki *et al.,* 2003; Riera *et al.,* 2005; Huang *et al.,* 2008).

In this study, we used RNA-Seq to analyze the dynamics of the transcriptome changes that occurred in Arabidopsis over four time points in response to *B. cinerea* infection, *P. rapae* feeding, drought stress, and all six combinations of sequential double stresses. Our results show that irrespective of the first stress, Arabidopsis is capable of swiftly adapting its transcriptome to respond to the second stress. Over time, this second stress-induced transcriptome is highly similar to that of plants that did not receive a first stress, but contains clear first-stress signatures, which may play a role in the phenotypic interaction between consecutive stresses.

Results

Experimental approach for RNA-Seq analysis of single and sequential stress time series

In order to capture a maximal dynamic range of the stress responses, the response to each of the three main stresses was monitored in a different time frame of four time points, depending on how quickly the stress response developed (Figure 1). The transcriptional response to each single and sequential stress was compared at each time point to a non-treated control (for treatments not involving *B. cinerea*) or a mock-treated control (same 100% relative humidity conditions as *B. cinerea* treatments) that was harvested at the same time as the stress treatment. For the study of *B. cinerea* stress, a time span between 6 and 24 h after inoculation with a 5-µl droplet of 1 x 10^5 spores ml^{-1} was chosen, because previous studies showed that the earliest transcriptional changes can be observed around 6 h after application of the inoculum, while at 24 h after inoculation massive changes in gene expression can be detected (Windram *et al.,* 2012; Vos *et al.,* 2015). For the study of *P. rapae* stress, we chose a time span between 3 and 24 h after infestation by larvae of stage L1 because previous studies demonstrated that this would yield a maximal dynamic range of transcriptional responses (Reymond *et al.,* 2000; De Vos *et al.,* 2005; Verhage *et al.,* 2011).

For the induction of drought stress, 4-week-old Arabidopsis plants that had previously been watered with equal amounts of water were subsequently withheld from water for 7 days. At day 5 of water withhold, drought stressed plants were clearly smaller and darker colored than the watered control plants, a phenotype that progressed further on day 6 and 7 when they were at the point of wilting. The transcriptome time series were chosen at 5, 6 and 7 days after water withhold, and at day 8 (7 + 1 day), which was 1 day after re-watering. The recovery response at day 8 was chosen as the fourth time point of the drought time series because this recovery response after drought stress is interesting by itself, and at this time point the sequential treatment with *B. cinerea* and *P. rapae* was executed and thus could function as a reference treatment.

Prior to applying the second stress, further development of the first stress was stopped by changing the 100% relative humidity condition to 70% (first stress *B. cinerea*), by removing the caterpillar (first stress *P. rapae*), or by re-watering the plants (first stress drought). Developmental leaf number 8 was used for applying *B. cinerea* or *P. rapae* as second stress. For all treatments, leaf number 8 was harvested for RNA-Seq analysis. When leaf number 8 was not damaged by *P. rapae*, the next-closest *P. rapae*-damaged leaf was harvested. Three biological replicates per treatment and time point were subjected to RNA-Seq. Each of the three biological replicates consisted of four 'number 8' leaves that were pooled to form one sample. After harvest, leaves were processed and subjected to RNA-Illumina sequencing. On average, 14.6 million reads (range 8.5– 29.8 million) were generated per sample with >90% of sequences aligning to the Arabidopsis genome after quality filtering (Van Verk *et al.,* 2013).

Time series transcriptome profiling following single and sequential stresses

In this study, our aim was to analyze the dynamic transcriptome changes that are triggered by the single stresses and investigate how the nature and dynamics of these transcriptome profiles were affected by pre-exposure to each of the other two stresses. First, a set of differentially expressed genes (DEGs) derived from each single stress time series was selected according to their significance in fold-change expression (false discovery rate (FDR) <0.05) and an additional threshold level of at least two-fold change ($-1 > \log_2 > 1$) in comparison to the respective control (Table S1). The first observation that can be made from the RNA-Seq results is that over time there are clear differences in the number of genes that are significantly activated or repressed during the different single stress conditions (Figure 2). For responses to *B. cinerea* (total 2076 unique DEGs) and *P. rapae* (total 3952 unique DEGs), a strong increase in the number of activated genes is observed over time, while relatively few genes are repressed. Upon exposure to drought stress (total 4032 unique DEGs for the first three time points, plus 482 additional unique DEGs for the 1 day after re-watering time point), relatively more genes become repressed than activated. A prior stress did not dramatically change the number of DEGs relative to the single stresses (Figure 2). Clustering the union of DEGs of the single stress sets (total 7355 unique DEGs), and subsequent gene ontology (GO) analysis (Boyle *et al.*, 2004) of overrepresented biological processes in each cluster highlights the differentially regulated biological processes during the plant response to the single stresses and uncovers similarities and contrasts between the different stress responses (Figure 3 and Table S2).

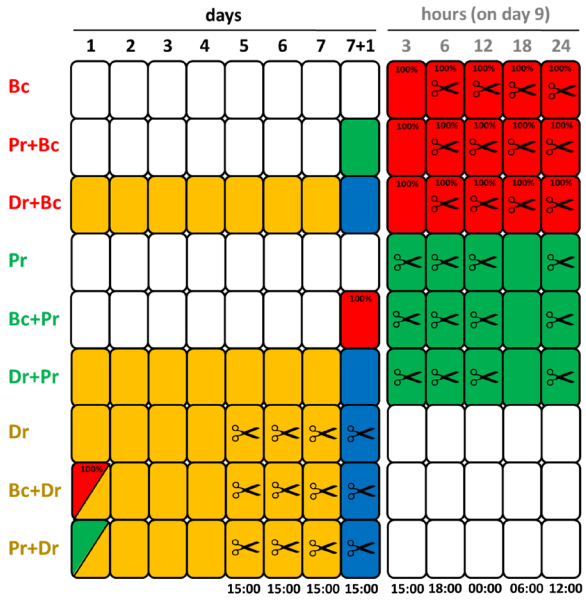


Figure 1. Experimental schedule of treatments and harvests for RNA-Seq time series of single and sequential double stresses. The schedule shows the timing of treatments and time points of harvest for the three main treatments, *B. cinerea* (Bc, red), *P. rapae* (Pr, green) and drought (Dr, yellow), and the respective pre-treatments. Each single and sequential double stress sample had a mock/ control (not visualized) that was harvested at the same time point as the stress treatment. Mock-treated plants were cultivated under the same conditions as their respective *B. cinerea*-treated plants (same periods of 100% RH). Untreated control plants were cultivated under the same conditions as their respective *P. rapae*- and/ or drought-treated plants. First stresses were stopped by either lowering RH from 100 to 70% (after 1 day in case of Bc pre-treatment), removing caterpillars from plants (after 1 day), or re-watering after a 7-day period of drought (7 + 1; blue). In case the second stress was drought, the pre-treatments with *B. cinerea* and *P. rapae* were performed right after the last moment of watering. 100%; period of 100% RH instead of standard 70% RH; timeindications at the bottom indicate time of the day at which plants were harvested.

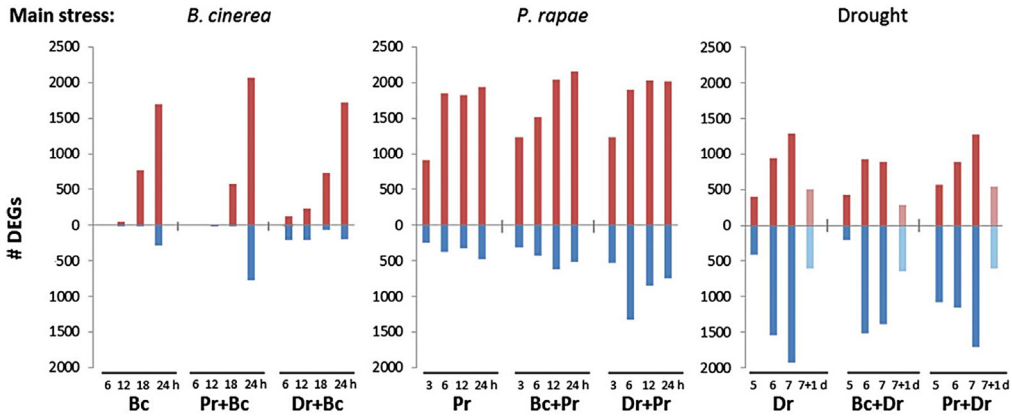


Figure 2. Numbers of DEGs at different time points in single and sequential stress responses. Graphs show the number of activated (red bars) and repressed (blue bars) differentially expressed genes (DEGs) for all single stresses and their corresponding sequential double stresses at different time points after treatment (FDR <0.05; >2-fold). The one day after re-watering time point of the drought treatments is indicated as '7 + 1 day'. Bc, *B. cinerea*; Pr, *P. rapae*; Dr, drought; Pr + Bc, Dr + Bc, Bc + Pr, Dr + Pr, Bc + Dr, and Pr + Dr, respective sequential double stresses.

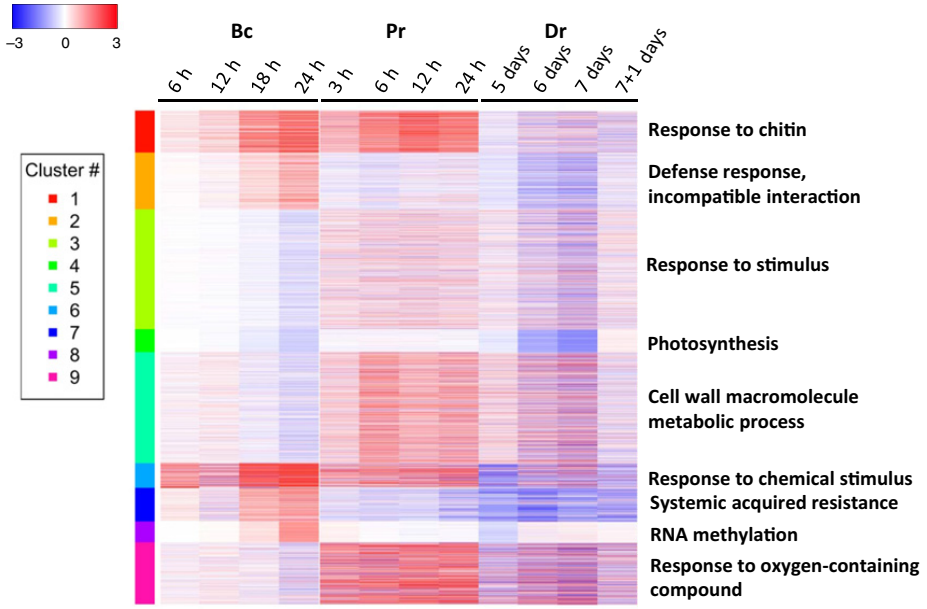


Figure 3. Clustering of the single stress DEGs. Heatmap showing the expression patterns of the union of differentially expressed genes (DEGs) in the three single stresses at different time points after induction (total 7173 unique genes). DEGs were clustered using mclust yielding nine gene clusters (colored bars on the left). On the right side, the most significant GO term for each cluster (full data set in Table S2). Bc, *B. cinerea*; Pr, *P. rapae*; Dr, drought. For drought stress, the time point 1 day after re-watering (7 + 1 day) was included in the cluster analysis. Blue–red color key for change in gene expression level:-3 > $\log_2$ fold change >3.

Core DEGs that are shared between the single stress responses

To investigate to what extent genes and biological processes are shared between the three single stress responses, we compared their DEGs. Figure 4(a) shows that there is a large overlap between the DEGs of the single stress responses, ranging from 1716 genes shared between the drought and *P. rapae* sets, to 788 genes between the drought and *B. cinerea* sets, and 777 genes between the *P. rapae* and *B. cinerea* sets. Of all 7173 DEGs (excluding the 1 day after re-watering time point), 2493 DEGs (35%) are shared with one or both of the other stresses. A core set of 394 DEGs (5%) was differentially expressed in response to all three single stresses, and clustered into 12 co-expressed gene clusters (Figure 4b). Among this core set of shared DEGs are several well-characterized hormone-responsive marker genes, including *LOX2* (At3g45140), *JAZ7* (At2g34600), and *JAZ8* (At1g30135) (JA responsive), *PDF1.2* (At5g44420) and *ORA59* (At1g06160) (JA/ET responsive), *PR4* (At3g04720), *ERF5* (At5g47230), *ERF6* (At4g17490), and *ACS2* (At1g01480) (ET responsive), *Rap2.6L* (At5g13330), and *HAI1* (At5g59220) (ABA responsive), and *PR-1* (At2g14610), *PR-5* (At1g75040), and *FRK1* (At2g19190) (SA responsive) (Table S1 for details on their expression patterns). In Figure 4(c) the expression patterns of well characterized marker genes of the response to *B. cinerea* (*PDF1.2*; At5g44420), *P. rapae* (*LOX2*; At3g45140), and drought stress (*RAD18*; At5g66400) are depicted, confirming that the different stress treatments resulted in the expected response. When looking at the co-expressed gene clusters, only the genes of cluster 1 (top GO terms related to 'response to oxygen-containing compound', 'response to JA' and 'response to wounding'; Table S3) are regulated in the same direction (activated) during all three individual stress conditions (Figure 4b). All other gene clusters behave clearly different in response to the three single stresses and are often regulated in opposite directions (Figure 4b). For example cluster 8 (top GO terms related to 'response to other organism', 'defense response' and 'immune system process') is activated by *B. cinerea*, but repressed by *P. rapae* and drought. Conversely, cluster 10 (top GO terms 'multidimensional cell growth', 'response to light stimulus', and 'cell wall organization') is activated by *P. rapae*, but repressed by *B. cinerea* and drought. The fact that there is an overlap in the expression of genes under all three single stresses, whether in the same or in opposite directions, suggests that these genes or their regulators may act as a point of convergence if plants were to experience these stresses in combination.

***Botrytis cinerea* data set: effect of herbivory and drought stress on dynamics of *B. cinerea*-induced gene expression**

To investigate the effect of *P. rapae* infestation and drought stress on the dynamics of the transcriptome changes that are induced by *B. cinerea* infection, we analyzed the expression patterns over time of all 2076 *B. cinerea*-responsive DEGs. Clustering of this group of genes yielded 10 clusters of co-expressed genes across the *B. cinerea* single and sequential stress data sets. Gene clusters that are activated in response to *B. cinerea* infection are enriched for GO terms such as 'response to chitin' (Figure 5 (cluster 3) and Table S4), reflecting recognition of fungal chitin by the plant immune system (Pel and Pieterse 2013), and 'response to ET stimulus', reflecting the high level of ET emission that is related to plant responses to *B. cinerea* infection (Broekgaarden *et al.,* 2015).

In addition, gene clusters that are repressed in response to *B. cinerea* infection are associated with GO terms such as 'multidimensional cell growth' (Figure 5 (cluster 9) and Table S4), highlighting the antagonistic relationship between plant growth and defense (Wang and Wang 2014).

Interestingly, the expression patterns over time in the sequential double stress treatments appear in general very similar to the ones of the *B. cinerea* treatment alone. This suggests that Arabidopsis swiftly reprograms its transcriptome to the response that is induced by *B. cinerea* infection, thereby overruling effects of the prior stresses herbivory and drought on *B. cinerea*-responsive gene expression. For example, at the moment drought pre-treated plants were inoculated with *B. cinerea* (one day after re-watering of drought-treated plants), more than 1000 genes were still differentially expressed in the analyzed leaf (number 8) due to the prior drought treatment (508 DEGs activated and 610 DEGs repressed at time point 7 + 1 day; Table S1; Figure 3 last lane). At 6 h after *B. cinerea* inoculation this drought-induced effect is still clearly visible in the *B. cinerea*-responsive gene set (compare the 6 h lane in Bc versus Dr + Bc treatment in Figure 5), but at the later time points this effect quickly dampens off and the *B. cinerea*-responsive genes start to follow the same pattern as in the *B. cinerea* single treatment. This effect is also visible in the left panel of Figure 4(c) where the expression patterns of the core set of 394 DEGs are plotted. Also prior exposure to *P. rapae* inflicted clear differences in the expression patterns of the *B. cinerea*-responsive genes during the first two time points (compare the first two time points in the Pr + Bc treatments with those in the Bc treatment), but at later time points the gene expression patterns of the core DEGs become very similar to that of the *B. cinerea* single stress treatment. Nevertheless, during the sequential stress responses some clusters show a first-stress signature, e.g. the genes in clusters 2, 3 and 5 in Figure 5 show a delayed activation when plants experienced herbivory or drought stress prior to *B. cinerea* infection.

In order to identify in greater detail co-regulated genes of which the expression pattern in response to *B. cinerea* infection was affected by either herbivory or drought stress, we used the bioinformatics tool Wigwams (Polanski *et al.,* 2014). The Wigwams algorithm identifies gene modules showing evidence for co-regulation in multiple gene expression time series and identifies signatures of condition- dependent regulatory mechanisms in co-regulated gene sets. Wigwams identified 35 modules of co-regulated genes in the *B. cinerea* data sets (Figure S1). Analysis of these clusters for co-expression revealed gene modules of which the expression patters were clearly affected in one or both of the sequential stress treatments in comparison to the *B. cinerea* treatment alone (examples shown in Figure 6). These gene modules represent signatures of a previous stress in the *B. cinerea*-induced transcriptome profile, and may thus be functionally related to the effect of the first stress on the outcome of the plant response to *B. cinerea* infection. The genes in these Wigwams modules are given in Table S5 along with their GO term analysis.

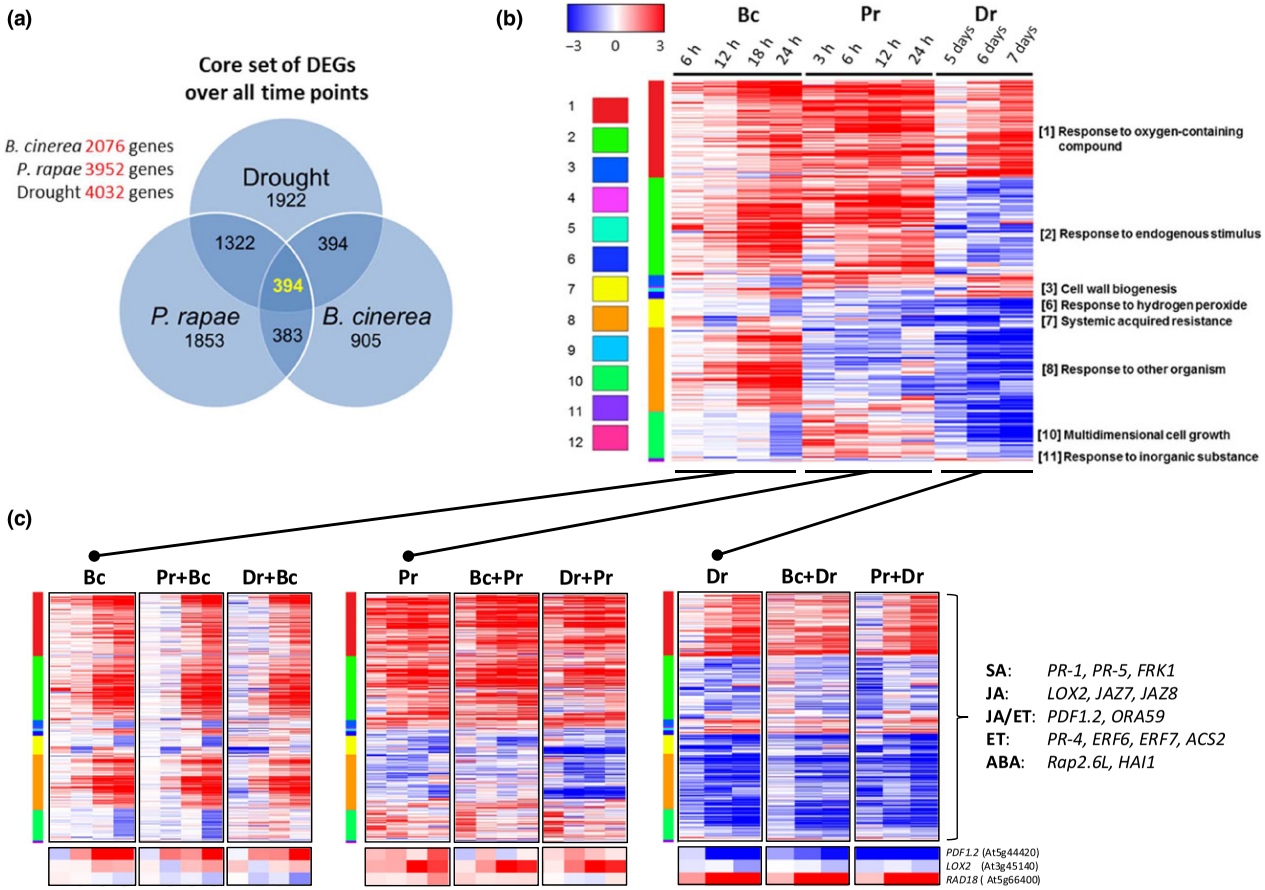


Figure 4. Shared DEGs between the single stress responses. (a) Venn diagram showing the overlap between the DEGs of each of the single stress responses. The total number of unique DEGs per single stress over all time points is shown in red (full data set in Table S1). (b) Hierarchical clustering of the 394 core DEGs that are shared between the three single stresses (Cosine similarity metric; 12 clusters are color coded in the square boxes on the left). On the right side, the most significant GO term for the largest clusters (full data set in Table S3). (c) Comparison of the expression patterns of the 394 core DEGs in response to the single and respective sequential double stresses. Different lanes in (c) reflect the transcription profiles at the time points after treatment as indicated above the lanes in (b). Gene names in the lower right corner represent marker genes of the SA, JA, JA/ET, ET and ABA response pathways that are among the 394 core DEGs. PDF1.2, LOX2, and RAD18 represent known marker genes for the response to B. cinerea, P. rapae, and drought, respectively. Bc, *B. cinerea*; Pr, *P. rapae*; Dr, drought; Pr + Bc, Dr + Bc, Bc + Pr, Dr + Pr, Bc + Dr, and Pr + Dr, respective sequential double stresses. Blue-red color key for change in gene expression level: -3 > $\log_2$ fold change >3.

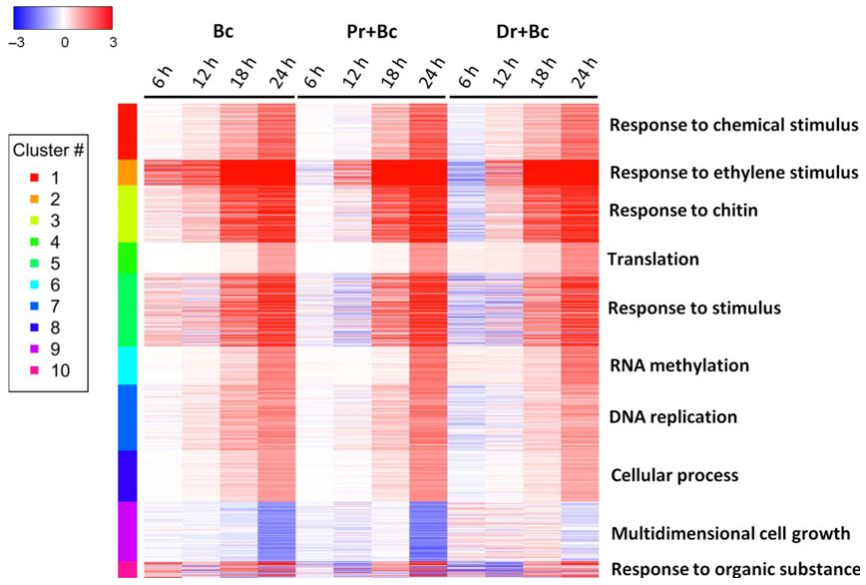


Figure 5. Dynamics of the expression of the *B. cinerea* set of DEGs during single and sequential double stresses. Heatmap showing the expression patterns of the 2076 *B. cinerea*-responsive DEGs during *B. cinerea* infection on mock pre-treated (Bc), *P. rapae* pre-infested (Pr + Bc) or drought pre-treated (Dr + Bc) Arabidopsis plants. The *B. cinerea*-responsive DEGs were clustered using mclust yielding 10 clusters (colored bars on the left). On the right side, the most significant GO term for each cluster (full data set in Table S4). Blue–red color key for change in gene expression level: -3 > $\log_2$ fold change >3.

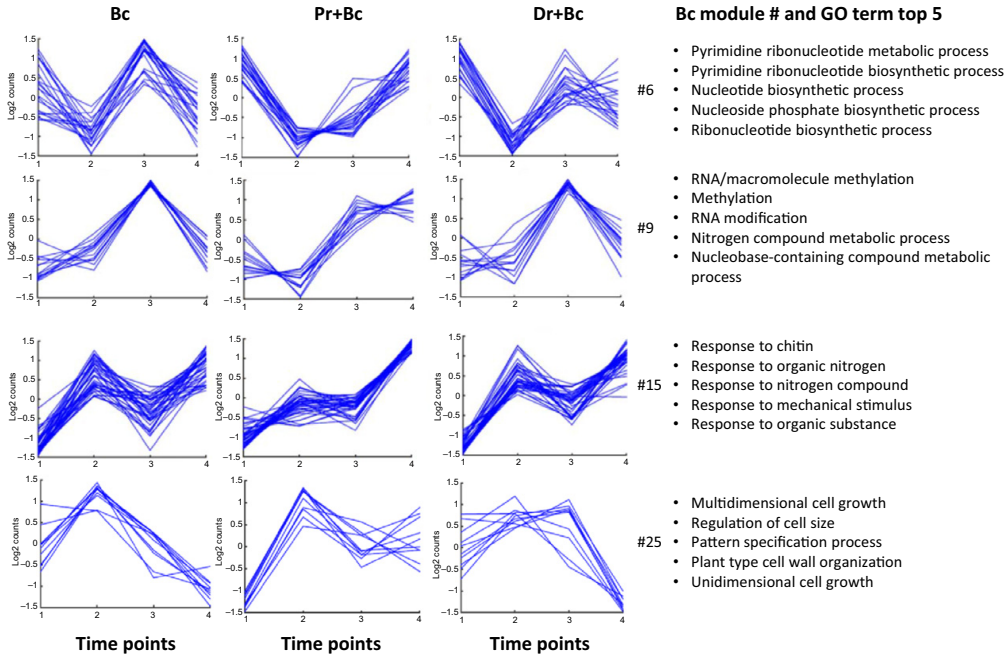


Figure 6. Expression patterns of selected Wigwams modules from the *B. cinerea* set of DEGs during single and sequential stress conditions. A selection of Wigwams modules of co-expressed gene clusters is depicted that show a different pattern in one or both of the sequential stresses *P. rapae*-*B. cinerea* (Pr + Bc) and drought-*B. cinerea* (Dr + Bc) in comparison to the single stress *B. cinerea* (Bc). The modules represent standardized patterns of differential gene expression over time (Log_2 counts). Blue-colored graphs indicate modules of which the genes are significantly co-expressed over time in the given stress condition. Time points 1, 2, 3 and 4 represent 6, 12, 18 and 24 h after *B. cinerea* inoculation. The top five GO terms with highest significance in the respective modules are given (full data set for all Wigwams modules is presented in Table S5).

Among the *B. cinerea*-responsive Wigwams modules of which the co-expression pattern is different when plants were previously exposed to herbivory or drought stress, are gene modules with GO term enrichments for rather general plant processes such as nucleoside biosynthesis and metabolism (modules 6 and 9), and cell growth (module 25), but also modules related to more specific plant processes, such as response to chitin and nitrogen (module 15). Functional analysis of underlying candidate genes should reveal their importance for effects on the outcome of the second stress response.

Further knowledge about the biological processes that are affected when *B. cinerea* infection is preceded by either drought stress or herbivory can be gained by analyzing the phasing of gene expression under the different single and sequential stress conditions. We did this by analyzing at which time point a GO term becomes significantly overrepresented in the *B. cinerea*-related DEG set. For this, we clustered all *B. cinerea*-responsive DEGs according to their time point of first differential expression, divided them over activated and repressed genes, and performed GO term analysis on them. Figure 7 shows the timing and strength of the onset of significant GO term enrichment in the single and sequential double stress conditions. In the *B. cinerea* single stress data set, GO terms related to responses to ET, fungus, chitin, SA, and oxygen-containing compound, or to processes such as systemic acquired resistance, respiratory burst, and defense appear early in the activated gene set, reflecting the importance of these processes in the plant response to this necrotrophic pathogen. Prior infestation with *P. rapae* clearly delayed the appearance of these GO terms (become visible at 18 hai in Figure 7), while pre-treatment with drought stress did not have a dramatic effect on the phasing of the activated genes. For the repressed genes in the *B. cinerea* set of DEGs, pre-infestation with *P. rapae* has clearly only minor effects on the GO term phasing. By contrast, pre-treatment with drought stress noticeably affected the phasing of GO terms related to responses to fungus, JA, SA, ABA, chitin, and oxygen-containing compound, and to auxin metabolic process, defense, systemic acquired resistance, and glucosinolate biosynthetic process. Remarkably, biological processes related to hormone action prevail in the *B. cinerea*-responsive processes that are sensitive to modulation by prior exposure to one of the other stresses.

Effect of herbivory or drought stress on resistance to *B. cinerea*

Both herbivory and drought stress imposed a first-stress-signature in the dynamics of the *B. cinerea*-induced transcriptome profiles. Wigwams analysis gained insight into the identity of candidate genes related to these first-stress signatures (Figure S1 and Table S5), whereas analysis of GO term enrichment provided global insight into the biological processes that were affected by the stress pretreatments (Figure 7). To investigate whether the two prior stresses affected the resistance level to *B. cinerea* infection we performed disease resistance bioassays. Inoculation of 5-week-old Arabidopsis Col-0 plants with *B. cinerea* resulted in the development of spreading lesions in about 60% of the inoculated leaves (Figure 8). Plants that were exposed to drought stress prior to *B. cinerea* inoculation showed a similar percentage of leaves with spreading lesions (approximately 70%). Interestingly, plants that were exposed to herbivory prior to *B. cinerea* inoculation showed a

significantly enhanced level of resistance against *B. cinerea* infection (average approximately 35% spreading lesions). Together these results indicate that a first stress can have strong effects on the outcome of the adaptive stress response to a second stress, depending on the nature of the first stress.

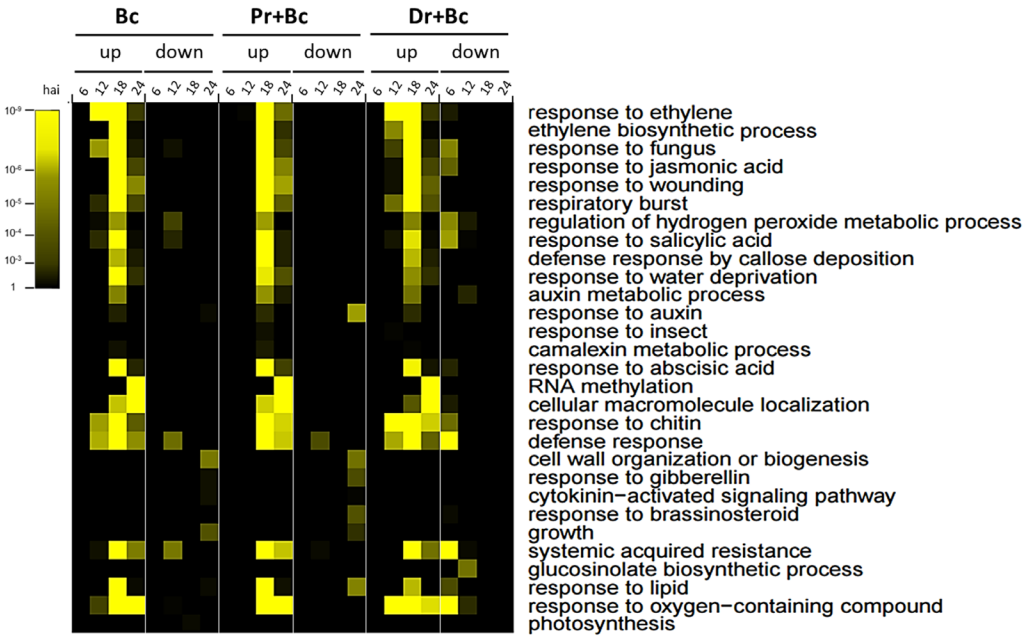


Figure 7. Timing of GO term overrepresentation patterns in *B. cinerea* single and sequential stress data sets. Heatmap represents the strengths of the *P* values of GO term overrepresentation in the *B. cinerea*-responsive DEG sets (corresponding to the gene lists in Table S1) that become significantly activated (up) or repressed (down) for the first time at the given stress conditions and time points. Color index represents level of significance (*P* values). On the right, overrepresented GO terms. Bc, *B. cinerea*; Pr, *P. rapae*; Dr, drought; hai, h after *B. cinerea* infection.

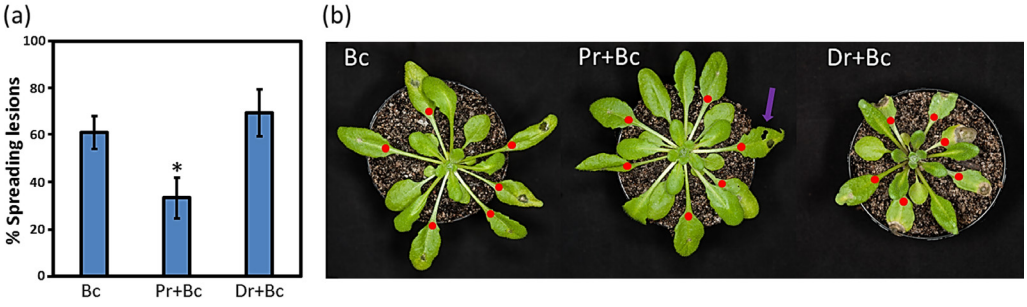


Figure 8. Effect of herbivory and drought stress on resistance of Arabidopsis to *B. cinerea*. (a) Quantification of *B. cinerea* disease symptoms on Arabidopsis accession Col-0 plants (% spreading lesions per plant). On each plant, six leaves were inoculated with one droplet of *B. cinerea* spores. Three days later, the average number of leaves with spreading lesions was determined per plant. Asterisk indicates statistically significant difference from single stress (Bc) treatment ($n = 6$ plants; Student's *t*-test; $P < 0.05$). (b) Photographs of *B. cinerea* disease symptoms 3 days after inoculation. Bc, *B. cinerea*-inoculated plants; Pr + Bc, *B. cinerea*-inoculated plants that prior to inoculation were exposed to herbivory by *P. rapae* larvae for 24 h; Dr + Bc, *B. cinerea*-inoculated plants that prior to inoculation received a drought treatment for 7 days, followed by a re-watering phase of one day. Red dots, *B. cinerea*-inoculated leaves; purple arrow, damage caused by *P. rapae* feeding.

Pieris rapae data set: effect of _B. cinerea_ infection and drought stress on dynamics of _P. rapae_-induced gene expression

The same approach as described above for the *B. cinerea* transcriptome data was taken to investigate the effect of prior *B. cinerea* infection and drought stress on the transcriptional dynamics that are induced by *P. rapae* feeding. Analysis of the global expression profiles of the 3952 *P. rapae*-responsive DEGs yielded nine clusters of co-expressed genes during single and sequential *P. rapae* stress (Figure 9). As expected, *P. rapae* feeding induced many genes related to the GO term 'response to JA stimulus' (Figure 9 (cluster 7) and Table S6), reflecting induced defenses that are triggered by herbivory-inflicted wounding (Wasternack 2015). In addition, *P. rapae* feeding repressed SA-related genes associated with GO terms 'defense response' and 'systemic acquired resistance' (Figure 9 (cluster 3) and Table S6), reflecting the antagonistic relationship between JA- and SA-dependent defenses (Pieterse *et al.,* 2012). In analogy with what we observed in the *B. cinerea* data sets, the general gene expression patterns over time overlapped greatly between the responses to *P. rapae* single and sequential double stress treatments, again suggesting that Arabidopsis is capable of reprogramming its transcriptome to the last stress encountered, thereby overruling the effects of the prior stresses. For instance, while prior drought stress impacted the expression of over 1000 genes in the leaf tissue just before the start of the *P. rapae* treatment (Table S1 and Figure 3 last lane), already from the first time point (3 h) after herbivory this effect was mostly vanished in the *P. rapae*-induced profiles, which readily followed a similar expression pattern as in the *P. rapae* single treatment (Figure 9). A similar pattern is visible in the core set of 394 DEGs (Figure 4c, middle panel). Nevertheless, during the sequential stresses first-stress signatures can be detected, e.g. genes in cluster 3 of Figure 9 and clusters 7 and 8 of Figure 4(c) (middle panel) show a weaker repression in the *B. cinerea* pre-treatment and a stronger repression in the drought pre-treatment. In general, these *P. rapae*-related results confirm previous findings (Davila Olivas *et al.,* 2016).

To pinpoint co-regulated genes whose expression pattern in response to herbivory is affected by prior *B. cinerea* infection or drought stress, the set of *P. rapae*-responsive DEGs was analyzed with the Wigwams algorithm. Wigwams identified 93 modules of co-regulated genes in the *P. rapae* set of DEGs. Analysis of these clusters for co-expression under the single and sequential double stress conditions revealed gene modules of which the expression patterns were clearly affected by one or both of the sequential double stress treatments in comparison to the *P. rapae* treatment alone (examples shown in Figure 10; full set in Figure S2). The identities of the genes in the *P. rapae*-related Wigwams gene modules are given in Table S5 along with their GO term analysis. It is beyond the scope of this paper to discuss the identity of the genes in detail. However, among the *P. rapae*-responsive Wigwams modules of which the co-expression pattern is clearly different when plants were pre-exposed to *B. cinerea* infection or drought stress, are gene modules with GO term enrichments for xylem, phloem and tissue development (module 3), cell wall biosynthesis processes (module 45), and responses to JA and wounding (module 69).

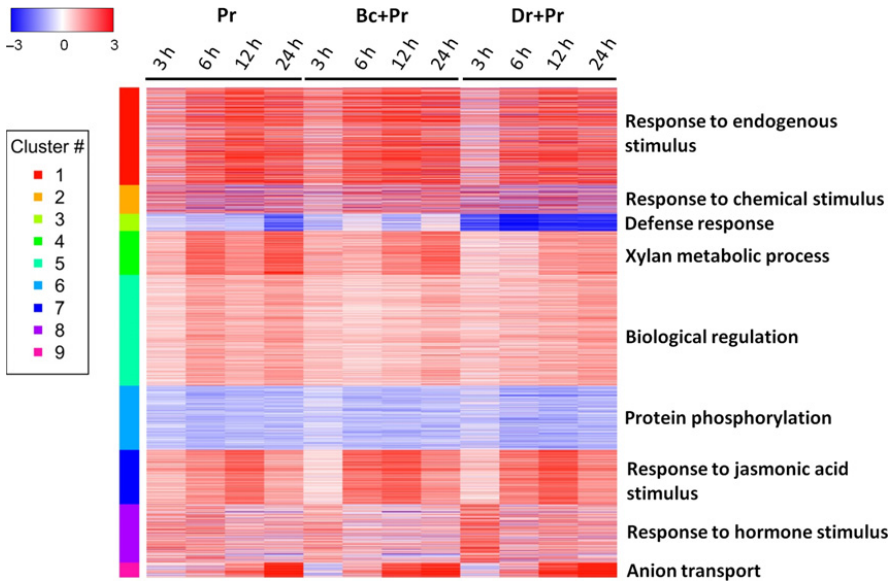


Figure 9. Dynamics of the expression of the *P. rapae* set of DEGs during single and sequential double stresses. Heatmap showing the expression patterns over time of the 3952 *P. rapae*-responsive DEGs during feeding of *P. rapae* on control (Pr), *B. cinerea* pre-infected (Bc + Pr), or drought pre-treated (Dr + Pi) Arabidopsis plants. The *P. rapae*-responsive DEGs were clustered using mclust yielding nine clusters (colored bars on the left). On the right side, the most significant GO term for each cluster (full data set in Table S6). Blue–red color key for change in gene expression level: -3 > $\log_2$ fold change >3.

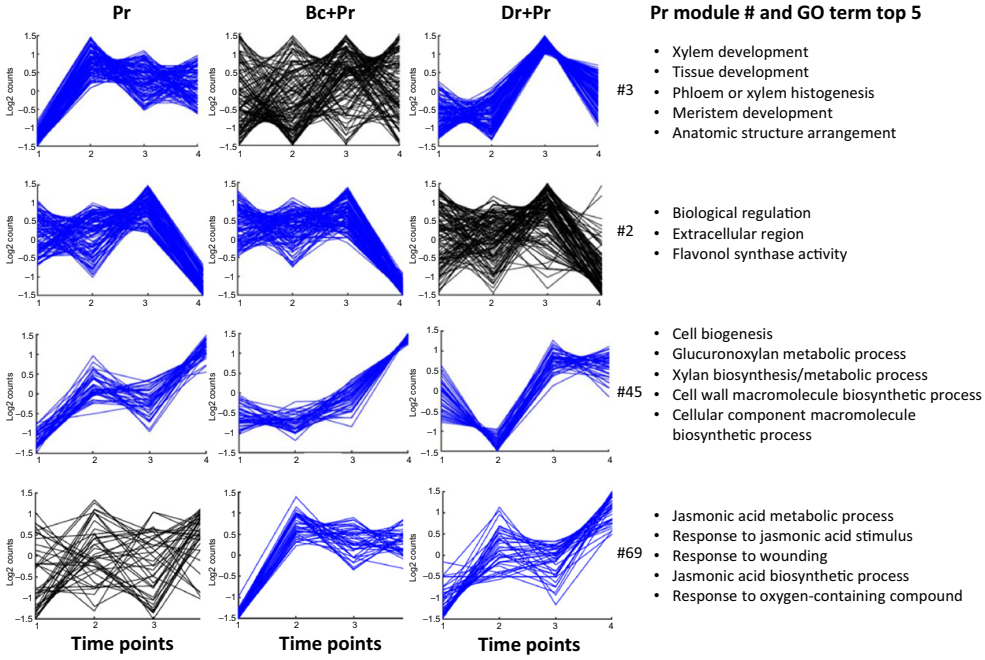


Figure 10. Expression patterns of selected Wigwams modules from the *P. rapae* set of DEGs during single and sequential stress conditions. A selection of Wigwams modules of co-expressed gene clusters is depicted that show a different pattern in one or both of the sequential stresses *B. cinerea*-*P. rapae* (Bc + Pr) and drought-*P. rapae* (Dr + Pr) in comparison to the single stress *P. rapae* (Pr). The modules represent standardized patterns of differential gene expression over time ($\log_2$ counts). Blue-colored graphs indicate modules of which the genes are significantly co-expressed over time in the given stress condition. In the black-colored graphs, the genes in the module are not significantly co-expressed. Time points 1, 2, 3 and 4 represent 3, 6, 12 and 24 h after *P. rapae* infestation. The top 5 GO terms with highest significance in the respective modules are given (full data set for all Wigwams modules is presented in Table S5).

Also for the *P. rapae* data set, phasing of the regulation of biological processes that are associated with herbivory and influenced by prior *B. cinerea* infection or drought stress was determined. Therefore, the level of significance of overrepresentation of the GO terms for the *P. rapae* set of DEGs at their first time of differential expression was assessed. In the *P. rapae* single stress data set, GO terms related to responses to chitin, wounding, JA, ET, SA, auxin, ABA, water deprivation, osmotic stress, and oxygen containing compound are already highly enriched at 3 h after infestation in the activated set of DEGs (Figure 11), reflecting the importance of these processes in the response of Arabidopsis to herbivory. In many cases, pre-infection with *B. cinerea* strengthened the level of overrepresentation of these GO terms at different time points after *P. rapae* infestation, while pre-treatment with drought stress often weakened them. In the repressed set of DEGs, drought stress clearly enhanced the overrepresentation of GO terms related to responses to chitin, JA, fungus, ABA, SA, bacterium, and oxygen-containing compound, and to processes such as systemic acquired resistance, defense response to fungus, and negative regulation of programmed cell death, while *B. cinerea* infection had no major effect on the phasing of these GO terms. Overall, these data indicate that *B. cinerea* infection and drought treatment prior to *P. rapae* infestation affects the timing of several defense-related processes, in particular responses to JA, ABA, SA and ET, corroborating the notion that different stresses interact via the hormone-regulated signaling network.

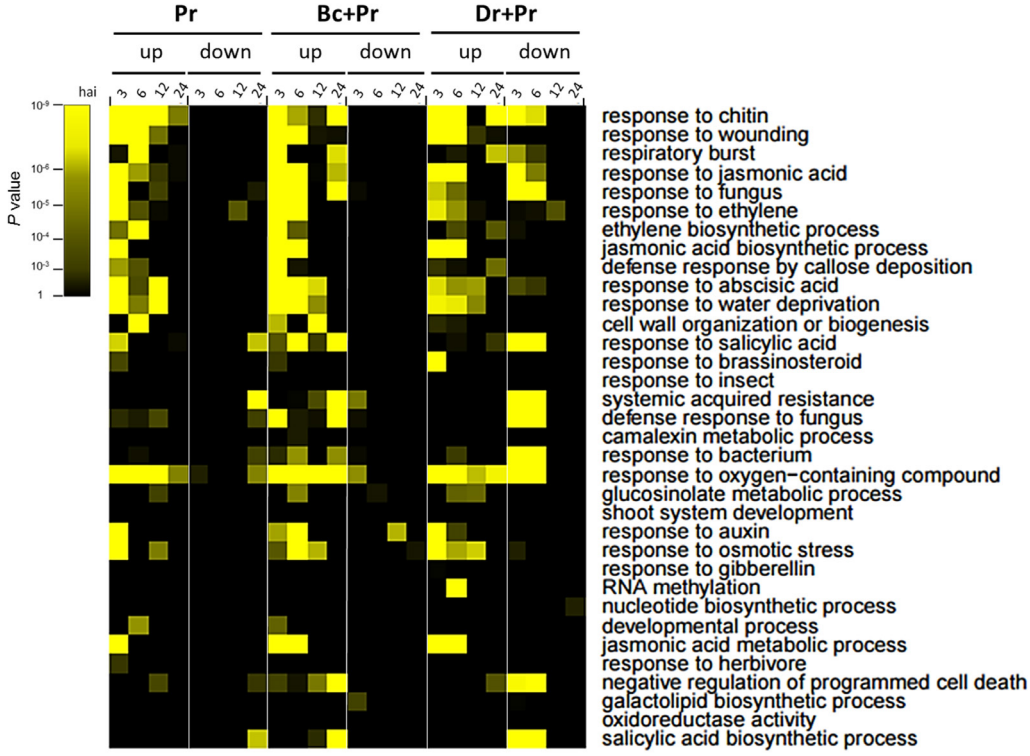


Figure 11. Timing of GO term overrepresentation patterns in *P. rapae* single and sequential double stress data sets. Heatmap represents the strengths of the *P* values of GO term overrepresentation of *P. rapae*-responsive DEG sets (corresponding to the gene lists in Table S1) that become significantly activated (up) or repressed (down) for the first time at the given stress conditions and time points. Color index represents level of significance (*P* values). On the right, overrepresented GO terms. Bc, *B. cinerea*; Pr, *P. rapae*; Dr, drought; hai, h after *P. rapae* infestation.

Drought data set: effect of *B. cinerea* infection and herbivory on dynamics of drought stress-induced gene expression

Also for drought stress we investigated the effect of the other two stresses on the dynamics of the transcriptome changes that are induced by this abiotic stress. We analyzed the dynamics of the global expression patterns of the 4032 drought-responsive DEGs during single and sequential stress with drought as the second stress, which yielded 10 clusters of co-expressed genes (Figure 12). GO term analysis of overrepresented biological processes in each cluster highlights the main differentially regulated biological processes. As expected, drought stress-induced a relatively large number of genes related to GO term 'response to water deprivation' (Figure 12 (cluster 6) and Table S7) and GO terms related to 'response to oxygen-containing compound' (clusters 3 and 10, and cell wall-related processes (cluster 7). Another feature that stands out is the association of drought stress with massive repression of genes, many of which are associated with biological processes such as 'photosynthesis' and 'defense response' (clusters 1, 2, 5 and 8), reflecting the fact that drought-stressed plants shift their strategy from energy-demanding processes related to growth and immunity to adaptation to the abiotic stress condition. Interestingly, after 1 day of re-watering (7 + 1 day columns in Figure 12), the drought-induced transcriptional changes that intensified over the 7- day period of water withhold, were for 77% (3106 of the 4032 DEGs; Table S1) reset towards basal levels within 24 h, demonstrating the plant's ability to swiftly redirect transcriptional programming when drought stress is relieved. Similar to what we observed for the *B. cinerea* and *P. rapae* sequential double stress responses, the gene expression patterns over time in the sequential drought double stress treatments were to a large extent similar to those inflicted by the single drought treatment. In the core set of 394 DEGs it is clear that on the first time point after the start of the drought treatment (5 days) *B. cinerea* and *P. rapae* pre-treatment still had noticeable effects on the drought-induced gene expression profiles (Figure 4c, right panel; compare the left lanes of Dr, Bc + Dr, and Pr + Dr). However, at the later time points (6 and 7 days) these effects dampened off and the expression patterns became more similar to that of the drought single treatment. Nevertheless, prior stress caused by *B. cinerea* infection or *P. rapae* infestation left first-stress signatures in the drought-induced transcriptome.

Wigwams analysis of co-regulated genes in the drought data sets identified 72 co-expressed gene modules under the single and sequential double drought stress conditions (examples in Figure 13; full set in Figure S3). The identities of the genes in these Wigwams gene modules are given in Table S5 along with their GO term analysis. Wigwams modules with clear changes in expression pattern when drought-stressed plants were pre-treated with either *B. cinerea* or *P. rapae* represent genes related to the biological processes such as SA and defense signaling (module 10 and 55), and nucleosome organization (module 11). Future analysis of candidate genes in these modules should reveal their importance for the outcome of the combinatorial stress responses.

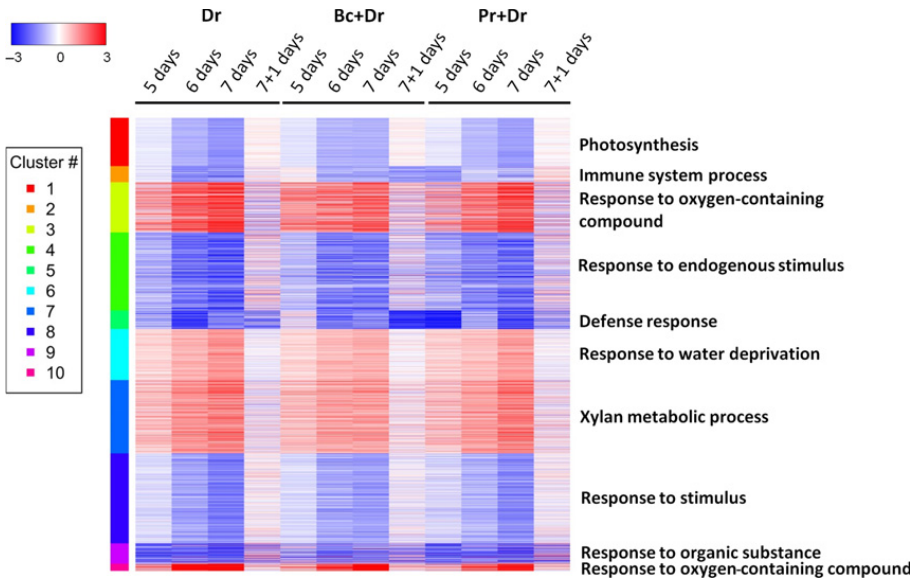


Figure 12. Dynamics of the expression of the drought set of DEGs during single and sequential double stresses. Heatmap showing the expression patterns over time of the 4032 drought-induced DEGs during a 7- day period of water withhold and 1 day after re-watering on control (Dr), *B. cinerea* pre-infected (Bc + Dr), or *P. rapae* pre-infested (Pr + Dr) Arabidopsis plants. The drought-responsive DEGs were clustered using mclust yielding 10 clusters (colored bars on the left). On the right side, the most significant GO term for each cluster (full data set in Table S7). Blue–red color key for change in gene expression level:-3 > $\log_2$ fold change >3.

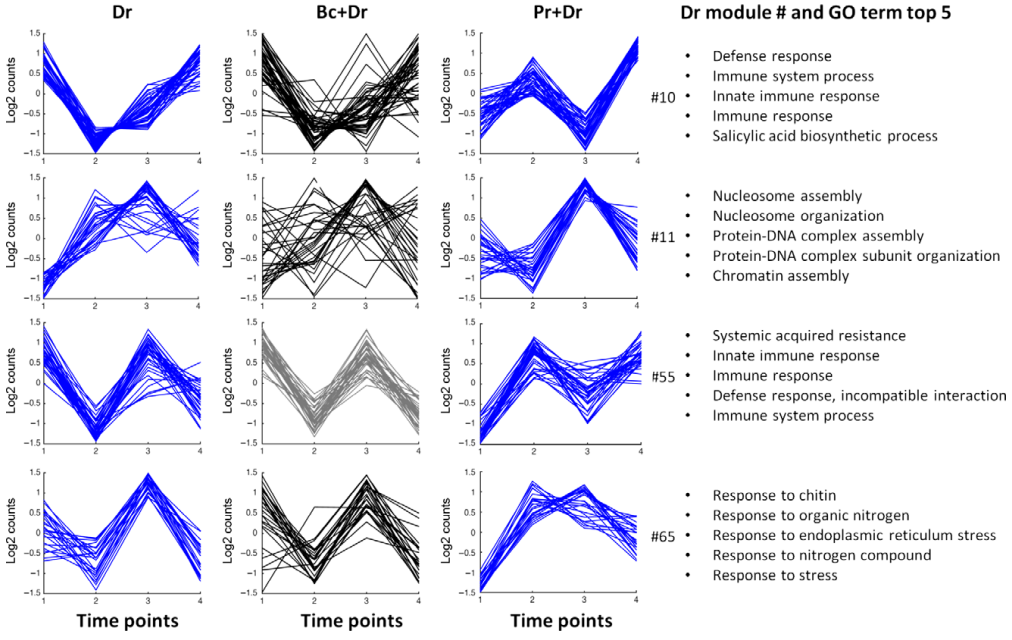


Figure 13. Expression patterns of selected Wigwams modules from the drought set of DEGs during single and sequential stress conditions. A selection of Wigwams modules of co-expressed gene clusters is depicted that show a different pattern in one or both of the sequential stresses *B. cinerea*-drought (Bc + Dr) and *P. rapae*-drought (Pr + Dr) in comparison to the single stress drought (Dr). The modules represent standardized patterns of differential gene expression over time (Log_2 counts). Blue-colored graphs indicate modules of which the genes are significantly co expressed over time in the given stress condition. In the black-colored graphs, the genes in the module are not significantly co-expressed. Time points 1, 2, 3 and 4 represent 5, 6, 7 and 7 + 1 day after onset of water withhold, in which the 7 + 1 day time point represents plants that were re-watered for 1 day after the 7-day drought period. The top 5 GO terms with highest significance in the respective modules are given (full data set for all Wigwams modules is presented in Table S5).

Also for the drought DEGs, we analyzed the timing and level of significance of overrepresentation of all the GO terms in the single and sequential double stress time series (Figure 14). In the drought single stress data set, GO terms related to responses to oxidative stress, water deprivation, osmotic stress, ABA, and oxygen-containing compound, and to processes such as phenylpropanoid biosynthesis, cell wall biogenesis, and lignin metabolism are enriched in the activated gene set at the first day of sampling, while responses to wounding and JA follow somewhat later. In the repressed gene set, GO terms related to responses to JA, SA, chitin, fungus, insect and oxygen-containing compound and to processes such as photosynthesis, shoot system development, systemic acquired resistance, glucosinolate metabolic process, nitrogen compound transport, and respiratory burst are enriched already at the first sampling point. This highlights the biological processes that are engaged or affected during drought stress. Interestingly, pre-infection with *B. cinerea* accelerated the phasing of activated genes associated with biological processes such as responses to chitin, wounding, osmotic stress, ABA, and JA. In the repressed gene set, GO terms related to responses to chitin, fungus, and SA, and to systemic acquired resistance became later enriched than in the single stress data set. When plants were pre-infested with *P. rapae*, the phasing of the drought-responsive genes is also clearly affected. Many GO terms in the drought activated gene set become more prominently enriched at later time points. Moreover, in the repressed gene sets GO terms related to responses to water deprivation, osmotic stress, wounding, JA, ABA and ET are highly overrepresented at the first time point of sampling, while this is not the case in the single stress treatment. Like in the *B. cinerea* and *P. rapae* data sets, biological processes related to hormone action become relatively often differentially enriched in the sequential double treatments in comparison to the single stress treatment.

Effect of stress interactions on plant resistance

For all three main stresses tested, prior treatment with one of the other stresses imposed a first-stress-signature in the dynamics of their transcriptome profiles. Wigwams analysis provided insight into the identity of the co-expressed genes related to these first-stress signatures (Figures 6, 10 and 13), whereas analysis of GO term enrichment at the onset of gene induction provided global insight into the biological processes that were affected during the time course by the prior stress treatment (Figures 7, 11 and 14). As an example for the effect of prior stress on the level of plant resistance to a second stress, we showed that the level of infection by *B. cinerea* can be significantly altered when plants were pre-disposed to herbivory (Figure 8). The bioassays with the other sequential stress treatments showed no strong effects of prior stress treatment on the performance of the specialist herbivore *P. rapae* (Davila Olivas *et al.,* 2016). In future research, we will functionally analyze candidate genes from the first-stress signatures in the second stress profiles to investigate their putative role in a diverse range of multi-stress interactions.

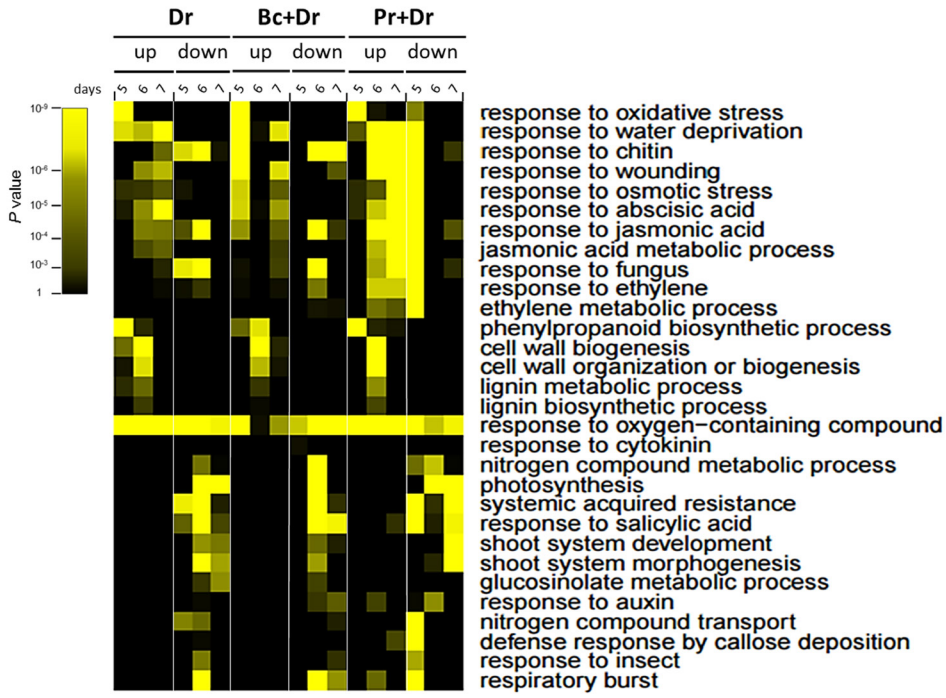


Figure 14. Timing of GO term overrepresentation patterns in drought single and sequential stress data sets. Heatmap represents the *P* values of GO term overrepresentation of drought-responsive DEG sets (corresponding to the gene lists in Table S1) that become significantly activated (up) or repressed (down) for the first time at the given stress conditions and time points. Color index of *P* values represents level of significance. On the right, overrepresented GO terms. Bc, *B. cinerea*; Pr, *P. rapae*; Dr, drought; days, day after water withhold.

Discussion

Plants are often exposed to different abiotic and biotic stresses, which can occur simultaneously or sequentially. How plants selectively adapt their response to this complexity of stresses is largely unknown. In this study, we aimed to gain insight into how plants respond to a biotic or abiotic stress when previously exposed to another stress, using a necrotrophic pathogen, an insect herbivore, and drought as main stress factors. By analyzing the dynamics of the Arabidopsis transcriptome over four consecutive time points we were able to show that: (i) on average 35% of the DEGs in a given single stress is also differentially regulated in one or both of the other two single stresses, albeit often in different directions; (ii) irrespective of the nature of the first and second stress applied, genes responsive to the second stress rapidly follow a similar pattern as that induced by the second stress alone; (iii) the Wigwams algorithm identified first-stress signatures of co-expressed genes that behave differently in the sequential double stress profile in comparison with the single stress treatment; (iv) plant hormone- related biological processes play a dominant role in the interaction between the studied stress-induced transcriptomes supporting previous findings (Rejeb *et al.,* 2014); and (v) a previous stress can affect the outcome of a response to a sequential second stress, resulting in altered plant resistance.

Transcriptome profiles of different single stress responses show significant overlap

The expression profiles induced by the single stresses *B. cinerea* infection, *P. rapae* infestation, and drought are clearly different in timing and numbers of activated and repressed genes (Figure 2). Clustering of the profiles of all DEGs (7355) from the three single stress responses shows that, in general, genes related to stress-related processes are overrepresented in the activated gene sets, while genes related to growth become repressed (Figure 3). This confirms the notion that plants under stress prioritize appropriate adaptive responses over growth (Vos *et al.,* 2013a; Vos *et al.,* 2015). One-third of all DEGs under single stress conditions was also differentially expressed under one or both of the other single stress conditions (Figure 4a). A set of 394 DEGs was differentially expressed in response to all three single stresses and clustering of their expression profiles clearly shows that the expression of the genes in the 12 distinguished clusters is often regulated in opposite directions (activated or repressed), depending on the nature of the stress (Figure 4b). Only one cluster, containing an overrepresentation of genes related to the GO term 'response to oxygen-containing compound' shows a general activation of genes under all three single stress conditions, highlighting that production and responsiveness to oxygen-containing compounds are central to stress responses in general. Overall, these results indicate that a significant proportion of the stress-related transcriptome is engaged by all three stresses tested. The differential stress-type specific activation or repression of genes suggests their positive versus negative function in different adaptive stress responses. When a plant experiences a combination of stresses, this may cause synergistic or antagonistic effects on the level of tolerance to the stresses at hand.

The transcriptome is rewired to the last stress response, but the prior stress leaves first-stress signatures in the second stress profile

Analysis of the dynamics of both the single and sequential double stress transcriptome profiles showed that the transcriptome profiles of all possible double stress combinations were remarkably similar to those of the last encountered stress if applied individually (Figures 4c, 5, 9, and 12). In analogy, 1 day after re-watering, the drought-induced transcriptome was largely reset to the non-stressed condition (Figure 12). Apparently, plants are highly plastic in their capacity to adapt to changes in their biotic and abiotic environment, and swiftly rewire their transcriptome to the latest stress encountered. Nevertheless, it has been demonstrated that prior exposure to biotic or abiotic stresses can have dramatic effects on the outcome of the response to a second stress (Rejeb *et al.,* 2014).

To identify genes and biological processes that could contribute to the interaction between different stress responses, different types of analyses have been carried out. When globally inspecting the transcriptional profiles of the single and sequential double stress profiles, it is difficult to pinpoint obvious effects of a first stress on the dynamics of the transcriptional response to a second stress. Using mclust clustering of the transcriptional profiles of the *B. cinerea*, *P. rapae* and drought sets of DEGs (Figures 5, 9 and 12) different gene clusters were identified that showed distinct behavior over time during the single and sequential double stress responses. However, in this way relatively small

differences between the single and sequential stresses were detected. In order to better pinpoint co-expressed gene clusters that represent first-stress signatures in the second stress transcriptome profiles, we used the bioinformatics tool Wigwams (Polanski *et al.,* 2014). We were able to dissect the transcriptional profiles into modules of co-regulated genes in time across different conditions. This approach highlighted modules whose gene expression patterns differed from the single stress profile in one or both of the sequential double stress profiles (Figures 6, 10, 13, and S1–S3). Future studies should reveal the role of candidate genes in these clusters in shaping the outcome of the adaptive stress responses in the sequential dual stress conditions. Moreover, the Wigwams modules of co-regulated genes could aid in dissecting the regulatory circuitry underlying plant responses to combinatorial stresses, e.g. by analyzing the representation of transcription factor binding motifs in the promoters of the gene modules.

Hormone-related responses prevail in biological processes that are differentially enriched in the double stress transcriptional profiles

Zooming in on the biological processes that are differentially enriched among the transcriptional profiles of the single versus the sequential double stresses, we monitored GO term enrichment at the time points of first differential expression of all genes. Plots of all biological GO terms that become significantly represented in the set of DEGs at the different time points provide a landscape of the timing at which these biological processes significantly change (Figures 7, 11 and 14). Interestingly, among all biological processes that become clearly more enriched in the sequential double stresses over their respective single stresses (either in activated or repressed DEGs) are GO terms related to the response to the stress-related hormones JA, ABA, SA, and ET and occasionally to auxin. This observation suggests that responses to these hormones are likely to play a central role in the interaction between the signaling pathways that regulate the adaptive responses to the sequential double stresses. In the past, JA, ABA, ET, and SA have been demonstrated to be crucial positive or negative regulators of plant resistance against *B. cinerea* (JA, ET, and SA; Thomma *et al.,* 1998; Thomma *et al.,* 1999; El Oirdi *et al.,* 2011; Vos *et al.,* 2015), *P. rapae* (JA and ABA; De Vos *et al.,* 2006b; Bodenhausen and Reymond 2007; Vos *et al.,* 2013b), and drought stress (ABA; Yamaguchi-Shinozaki and Shinozaki 2006). Hence, interactions between the different hormone-controlled signaling pathways may be decisive in the outcome of the adaptive response when two stresses are encountered sequentially.

Effect of prior stress on level of resistance to subsequent stress

Classic examples of interactions between defense pathways are the different forms of induced resistance that are triggered by pathogens, insect herbivores and beneficial microbes as they all change the outcome of the defense response against a subsequent invasion by another pathogen or insect in a positive or negative manner (De Vos *et al.,* 2006b; Howe and Jander 2008; Poelman *et al.,* 2008; Van Oosten *et al.,* 2008; Pieterse *et al.,* 2014; Vos *et al.,* 2015). Also for abiotic stresses effects on the level of resistance against other abiotic and biotic stresses are documented (Fujita

et al., 2006; Rejeb *et al.,* 2014). For the combinations of sequential stresses that were investigated here, we found that prior infestation by *P. rapae* caterpillars changed the level of resistance against *B. cinerea* (Figure 8), even though the global transcriptional profiles induced by *B. cinerea* as single or second stress did not differ dramatically (Figure 5). It can thus be concluded that subtle first-stress signatures in the double stress transcriptional profile may have significant effects on the outcome of the adaptive response to the second stress, although it cannot be excluded that non-transcriptional changes may also contribute to changes in the level of resistance against the second stress. Previously, it was shown that herbivory on Arabidopsis by *P. rapae* results in a systemic increase in the levels of JA, and that this can prime systemic tissues for enhanced JA-dependent anti-herbivory defenses (Vos *et al.,* 2013b). Since JA-regulated defenses play a major role in resistance against the necrotrophic pathogen *B. cinerea* as well, herbivory-induced priming of JA responsiveness may contribute to the enhanced resistance level against *B. cinerea* (Figures 7 and 8).

This study was aimed at analyzing the dynamics of gene expression patterns in response to a set of single and sequential double stresses. Future research will be focused on biological validation of candidate genes in the Wigwams modules with putative major roles in shaping the outcome of sequential double stresses. Knowledge on how plants cope with different stresses simultaneously or in sequence will aid in breeding for multi-stress tolerant crops.

Experimental procedures

Plant cultivation

Seeds of *Arabidopsis thaliana* accession Col-0 were sown in cultivation containers filled with autoclaved river sand. Sand was supplied with half-strength Hoagland solution containing sequestrene as described (Van Wees *et al.,* 2013). In order to attain 100% relative humidity (RH) for germination, cultivation containers were enclosed in a tray with water and covered with a transparent lid. Seeds were stratified for 2 days at 4°C in the dark to ensure a homogeneous germination after which the tray was moved to a growth chamber (t = 0) with an 8-h day/16-h night rhythm, a temperature of 21°C, and a light intensity of 100 µmol m^{-2} sec^{-1}. After 10 days, the lids of the trays were slightly opened and gradually removed over a 4-day period to adjust to the 70% RH present in the growth chamber. Fourteenday-old seedlings were transplanted to individual pots containing a mixture of river sand and potting soil (1:1 (v:v)). Pots were supplied with water from the bottom up three times per week. At an age of 3 weeks the plants were supplied once with half-strength Hoagland solution.

Rearing of *P. rapae* and treatment with *P. rapae* caterpillars

Pieris rapae caterpillars were reared on cabbage plants (*Brassica oleracea* convar. *capitata* var. *alba*) under greenhouse conditions (24°C, with natural daylight). Butterflies were supplied

with flowering plants such as *Lantana camara* for their (nectar) food. When flowers were scarce, additional food (solution of 20% honey and 10% sucrose) was offered to the butterflies. Inbreeding of the population was minimalized by adding wild butterflies and caterpillars from the Dutch Flevopolder to the existing population. After starving for 1 h, first-instar (L1) larvae were placed on Arabidopsis leaves using a fine paint brush as described (Van Wees *et al.,* 2013).

Cultivation of *B. cinerea* and treatment with *B. cinerea* spores

Botrytis cinerea strain B05.10 (Staats and Van Kan 2012) was grown on half-strength Potato Dextrose Agar (PDA; Difco™ BD Diagnostics, Franklin Lakes, NJ, USA) plates containing penicillin (100 µg ml^{-1}) and streptomycin (200 µg ml^{-1}) for 2 weeks at room temperature. Spores were collected, filtered through glass wool, and re-suspended in half-strength Potato Dextrose Broth (PDB; Difco™ BD Diagnostics) to a final density of 1 x 10^5 spores ml^{-1}. After a 3-h incubation period, the spores were used for inoculation by applying 5-µl droplets on Arabidopsis leaves as described (Van Wees *et al.,* 2013). For the RNA-Seq analysis, four droplets were applied on a single leaf, while for disease resistance assays, a single droplet was administered to the leaf.

Single and sequential double stress treatments

Single and sequential double stress treatments were applied according to the schedule shown in Figure 1. Developmental leaf number 8 was treated with the second stress and harvested for RNA-Seq analysis. Individual leaves were numbered from oldest to youngest. For single and sequential double stress treatments in which *B. cinerea* was the second stress, developmental leaf number 8 of 5-week-old plants was inoculated with *B. cinerea* by pipetting four 5-µl droplets of spore suspension (1 x 10^5 spores ml^{-1}) onto the leaf. Plants were kept at 100% RH for the remaining time period. Mock-treated plants received droplets of half-strength PDB and were kept at 100% RH. Pre-treatment with drought was started when plants were 4 weeks old by withholding water for 7 days, after which plants were re-watered and allowed to recover for 1 day before plants were inoculated with *B. cinerea*. *P. rapae* pre-treatment was started 1 day prior to *B. cinerea* inoculation by placing a single *P. rapae* L1 caterpillar on leaf 7 and allowing it to feed for 1 day. Only plants of which leaf number 8 was undamaged were used for inoculation with *B. cinerea* as second stress. Leaf number 8 was harvested at 6, 12, 18, and 24 h after inoculation with *B. cinerea*.

For single and sequential double stress treatments in which *P. rapae* herbivory was the second stress, two *P. rapae* L1 larvae were transferred to developmental leaf number 8 of 5-week-old plants. Pre-treatment with drought was achieved as described above for the *B. cinerea* experimental set-up. *B. cinerea* pre-treatment was performed 1 day prior to introduction of *P. rapae* by inoculating leaves 6 and 7 with one 5-µl droplet of *B. cinerea* spore suspension (1 x 10^5 spores ml^{-1}) per leaf and placing the plants at 100% RH for 1 day. A mock treatment for the *B. cinerea* pretreatment was included by placing droplets with half-strength PDB on the leaves and keeping the plants at 100% RH for 1 day. Leaf number 8 was harvested at 3, 6, 12 and 24 h after the start of *P. rapae* feeding. When leaf

number 8 was not damaged by *P. rapae* (because it had moved to another leaf), the next-closest *P. rapae* damaged leaf was harvested.

For single and sequential double stress treatments in which drought was the second stress, 4-week-old plants were refrained from watering for 7 days. After 7 days of water withhold, plants were re-watered and allowed to recover for 1 day. *B. cinerea* pretreatment was performed at the beginning of day 1 of the drought period by inoculating leaves 6 and 7 with one 5-µl droplet of *B. cinerea* spore suspension (1 x 10^5 spores ml^{-1}) per leaf and placing the plants at 100% RH for 1 day. A mock treatment for the *B. cinerea* pre-treatment was included by placing droplets with half-strength PDB on the leaves and keeping the plants at 100% RH for 1 day. *P. rapae* pre-treatment was performed at the same time as the *B. cinerea* pre-treatment by placing one *P. rapae* caterpillar on leaf 7 and allowing it to feed on the plant for 1 day. Only plants of which leaf number 8 was undamaged were used to harvest leaf 8. Leaf number 8 was harvested at 5, 6, 7, and 7 + 1 days after the onset of water withhold (with 7 + 1 representing the time point of 1 day after re-watering).

For each treatment and time point, three biological replicates were used for RNA-Seq analysis. Each of the three biological replicates consisted of four pooled 'number 8' leaves harvested from four similarly-treated plants. For all treatments in which *B. cinerea* inoculation was used as first or second stress, a mock treatment was performed in which plants were inoculated with droplets of half-strength PDB and placed at 100% RH for 1 day. For all treatments without *B. cinerea*, controls consisted of untreated plants. After harvest, leaf samples were immediately frozen in liquid nitrogen and stored at -80°C.

Experimental design

The experiment was carried out in a fully randomized factorial design with two factors; time and treatment, with time having four levels (four time points analyzed per stress combination) and treatment having five levels (control, mock, two different first stresses per sequential stress, and one single stress). The climate chamber space was divided in three blocks, in which time was randomized. Within every time point, treatments were assigned randomly to the plants. RNA extraction was carried out in batches of approximately 20 randomly chosen samples.

RNA extraction, library preparation, and RNA-Seq alignment

RNA was extracted using the Plant RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. All samples were treated with DNase I on column using the Qiagen RNase-Free DNase Set. Quality of RNA was checked by determining the RNA Integrity Number (RIN) with an Agilent 2100 bioanalyzer and RNA LabChip (Agilent Technologies, Santa Clara, CA, USA). For the library preparation samples with a RIN value ≥6 were used. The samples were processed according to the TruSeq Stranded mRNA HT Sample Prep Kit from Illumina (Illumina Inc., San Diego, CA, USA). This protocol allows the identification of strand-specific transcripts. First, poly-A RNA was isolated from the total RNA using poly-T oligo-attached magnetic beads. Subsequently, poly-A

RNA was fragmented using divalent cations under elevated temperature. First-strand cDNA was synthesized using random primers. Strand specificity was achieved by replacing dTTP with dUTP in the second Strand Marking Mix (SMM), followed by second strand cDNA synthesis using DNA polymerase I and RNase H. Samples were sequenced with an Illumina Hi-seq 2000 sequencer using three sequencing runs. Samples were randomly assigned to seven lanes of the Illumina flow cells within each run.

Processing of raw sequencing data, alignment of the RNA-Seq data to the Arabidopsis genome, and downstream processing was performed as described (Van Verk *et al.,* 2013). RNA-Seq reads were aligned to the Arabidopsis genome (TAIR version 10) using TopHat v2.0.4 (Trapnell *et al.,* 2009) with parameters: 'transcriptome- mismatches 3', 'N 3', 'bowtie1', 'no-novel-juncs', 'genomeread-mismatches 3', 'read-mismatches 3', 'G', 'min-intron-length 40', 'max-intron-length 2000.' Gene expression levels were calculated by counting the number of mapped reads per annotated gene model using HTSeq-count v0.5.3p9 (Anders *et al.,* 2014). For downstream analyses, raw read counts were normalized for between sample differences in sequencing depth (Love *et al.,* 2014). Differential gene expression was calculated using DESeq2 (Love *et al.,* 2014) for all stress treatments and time points relative to the appropriate non-stress-treated control/mock treatment that was cultivated and harvested in exactly the same way as the stress-treated samples. The raw *P. rapae* RNA-Seq data have been used in a previous study (Davila Olivas *et al.,* 2016). In the study of Davila Olivas *et al.,* the raw *P. rapae* RNA-Seq reads were analyzed independently of this study with the specific goal to identify *P. rapae*-responsive genes that are affected by prior drought stress or *B. cinerea* infection and possibly link them to effects on changes in *P. rapae* resistance. In the present study, the raw *P. rapae* RNA-Seq reads were used in the larger framework of analyzing global dynamics of gene expression profiles during multiple combinatorial stress conditions in which also *B. cinerea* and drought stress were analyzed as second stresses. All raw RNA-Seq read data are deposited in the NCBI Short Read Archive (http://www.ncbi.nlm.nih.gov/sra/) under the BioProject accession code PRJNA315516.

Gene ontology analysis

To identify enrichment of GO terms in the different sets of DEGs, 'Go term finder' (Boyle *et al.,* 2004) analysis was performed using an *A. thaliana* gene association file downloaded from ftp.geneontology.org on 2 May 2013. The default background set was used (all 30504 transcripts in the database that have GO annotations). GO term finder tests for overrepresentation of GO categories using the hypergeometric distribution and FDR for multiple testing (*P*-value ≤0.05). Figures showing heatmaps of *P* values were generated using the R package (version 3.2.1; http://www.r-project.org/).

Clustering

Hierarchical clustering of the core set of single stress DEGs was performed on $\log_2$ fold-change expression values using the R function hclust with a cosine similarity metric and average linkage. The cutree function was used with a visually determined cut height to partition the resulting dendrogram into clusters. Clustering of the core set of single stress DEGs and shared main treatment datasets was performed using model-based clustering package mclust version 4 in R (Fraley *et al.,* 2012) with the number of clusters optimized in the range 1 to 10 using the Bayesian information criterion.

Wigwams analysis

To identify modules of co-expressed genes across single and sequential stresses the Wigwams algorithm was applied (Polanski *et al.,* 2014), using $\log_2$ transformed expression values for the DEGs of each single stress across their respective sequential double stresses. For each main treatment, Wigwams was run with default arguments to partition genes into modules that indicate co-expression in subsets of the relevant main treatments.

Acknowledgements

We are thankful to Kim Vermeer, Michel Arts, Elena Kapsomenou, Benno Augustinus, Robert Veldman, and Tom Van den Beuken, who helped with collecting the samples, and Raymond Hulzink and Roy Gorkink (KeyGene) for their skilled execution of the RNA sequencing work and technical discussions. This work was supported by the Netherlands Organization for Scientific Research (NWO) through the Dutch Technology Foundation (STW) STW Perspective Programme 'Learning from Nature' [STW10988], STW VIDI Grant no. 11281 (to SCMvW), STW VENI Grant no. 13682 (to RH), FP7-PEOPLE-2012-IEF no. 327282 (to SP), and ERC Advanced Grant no. 269072 of the European Research Council (to CMJP).

Supporting information

Additional Supporting Information may be found in the online version of this article. http://onlinelibrary.wiley.com/doi/10.1111/tpj.13167/

Figure S1. Expression patterns of all 35 Wigwams modules from the *B. cinerea* set of DEGs during single and sequential stress conditions.

Figure S2. Expression patterns of all 93 Wigwams modules from the *P. rapae* set of DEGs during single and sequential stress conditions.

Figure S3. Expression patterns of all 72 Wigwams modules from the drought set of DEGs during single and sequential stress conditions.

Table S1. Differentially expressed genes (DEGs) of *Arabidopsis thaliana* (AGI numbers of DEGs; FDR

<0.05; >2-fold) in response to *B. cinerea* infection, *P. rapae* infestation, drought stress, and their six sequential combinations at four consecutive time points.

Table S2. Enriched GO terms in gene clusters shown in Figure 3 (Clustering of all DEGs of the single stresses).

Table S3. Enriched GO terms in gene clusters shown in Figure 4(b) (Shared core DEGs between the three single stress responses).

Table S4. Enriched GO terms in gene clusters shown in Figure 5 (Dynamics of the expression of the *B. cinerea* set of DEGs during single and sequential double stresses).

Table S5. Enriched GO terms in Wigwams modules of *B. cinerea*, rapae, and Drought set of DEGs.

Table S6. Enriched GO terms in gene clusters shown in Figure 9 (Dynamics of the expression of the *P. rapae* set of DEGs during single and sequential double stresses).

Table S7. Enriched GO terms in gene clusters shown in Figure 12 (Dynamics of the expression of the drought set of DEGs during single and sequential double stresses).

Chapter 3

Mining the natural genetic variation of adaptive responses of *Arabidopsis thaliana* to *Botrytis cinerea* during sequential biotic and abiotic stresses

Silvia Coolen, Johan A. Van Pelt, Saskia C.M. Van Wees and Corné M.J. Pieterse

Plant-Microbe Interactions, Department of Biology, Utrecht University, PO Box 800.56, 3508 TB, Utrecht, the Netherlands

Abstract

Plants are often part of a complex environment in which they encounter many different biotic and abiotic stresses individually, sequentially or simultaneously. Previous studies mainly focused on plant responses to single stresses, but this does not always provide a good prediction of how plants respond and adapt to the multitude of stresses that plants encounter in agricultural or natural environments. Therefore, we aimed to unravel genetic factors that contribute to the plant's ability to swiftly adapt its responses to different stresses. In this study we focused on the plant's adaptive response to infection by the necrotrophic fungus *Botrytis cinerea*, as individual stress and when preceded by *Pieris rapae* herbivory or drought stress. Using 346 natural *Arabidopsis thaliana* accessions, we found a wealth of variation in the level of resistance against *B. cinerea* when the plants were exposed to *B. cinerea* as a single stress. This variation correlated only partly with that observed when *B. cinerea* inoculation was preceded by either *Pieris rapae* herbivory or drought stress, indicating that the level of *B. cinerea* resistance is influenced by these prior stress factors. To study the genetic factors contributing to the adaptive response of *A. thaliana* to *B. cinerea* infection under multistress conditions, we performed a single nucleotide polymorphism (SNP)-based genome-wide association (GWA) study supported by quantitative trait loci (QTL) mapping and fine mapping with full genome sequences of 164 accessions. We found several genes with known and putative roles in *B. cinerea* defense and additional candidate genes with putative roles in the plant's adaptive response to a combination of herbivory, drought and *B. cinerea* infection.

Introduction

Under natural conditions, plants encounter many different stresses individually, sequentially or simultaneously. To withstand these stresses, plants make use of constitutive and adaptive defenses to ensure survival. Plant constitutive defenses consist of structural and chemical barriers, such as waxy cuticles, trichomes and constitutively produced antimicrobials, such as saponins that hinder potential threats (Osbourn 1996). Adaptive plant responses to stress are generally induced after recognition of a potential threat and are often regulated by phytohormones, such as jasmonic acid (JA), salicylic acid (SA), ethylene (ET) and abscisic acid (ABA) (Robert-Seilaniantz *et al.,* 2011; Pieterse *et al.,* 2012; Broekgaarden *et al.,* 2015). Depending on the type of stress, plants adjust their adaptive response to counteract potential threats, thereby making use of JA and ABA signaling in response to herbivory or wounding, of JA and ET signaling upon attack by necrotrophic pathogens, and SA signaling after infection by biotrophic pathogens . Cross-talk between hormonal signal-transduction pathways is thought to play an important role in the ability of plants to swiftly adapt to a variety of biotic and abiotic environmental conditions and have therefore been in the focus of many studies on plant stress adaptation (Reymond and Farmer 1998; Yamaguchi-Shinozaki and Shinozaki 2006; Verhage *et al.,* 2011; Pieterse *et al.,* 2012; Vos *et al.,* 2013b; Caarls *et al.,* 2015). Plant responses to single stress factors are subject to complex regulatory mechanisms. Environmental conditions and additional stress factors can further increase this complexity, as they can greatly influence the outcome of a plant's response to a single stress (De Vos *et al.,* 2006b; Van Oosten *et al.,* 2008; Kanani

et al., 2010; Verhagen *et al.,* 2010; Atkinson *et al.,* 2013; Prasch and Sonnewald 2013; Rasmussen *et al.,* 2013; Santino *et al.,* 2013; Rivero *et al.,* 2014; Sewelam *et al.,* 2014; Stam *et al.,* 2014; Suzuki *et al.,* 2014; Kissoudis *et al.,* 2015; Coolen *et al.,* 2016; Davila Olivas *et al.,* 2016). In nature, plants co-evolved with a large variety of biotic and abiotic stresses. Hence, genetic variation amongst naturally occurring accessions can provide insight into naturally evolved plant adaptive responses. To study the genetics of these responses, genome-wide association (GWA) mapping has been used in many studies, revealing genes with important functions in diverse processes of plant growth and survival (Atwell *et al.,* 2010; Baxter *et al.,* 2010; Kloth *et al.,* 2016; Davila Olivas *et al.,* 2017b; Thoen *et al.,* 2017).

One of the most notorious necrotrophic plant pathogens is *Botrytis cinerea*, which is responsible for crop losses in a wide range of crop plants (Dean *et al.,* 2012). In a single stress environment, primary necrotic lesion formation by *B. cinerea* is affected by the ability of the fungus to overcome plant constitutive defenses, whereas for the outgrowth and success of *B. cinerea* secondary (or spreading) lesion formation the pathogen needs to overcome inducible plant defenses (Van Kan 2006). To overcome the plant's structural barriers, *B. cinerea* produces a large array of enzymes and toxins that target plant cell walls (Blanco-Ulate *et al.,* 2014). Besides enabling pathogen invasion, break-down of pectin in the plant cell wall results in the release of plant self-molecules (oligogalacturonides, OGs) that act as damage-associated molecular patterns (DAMPs) and trigger the plant's general stress response. Subsequent downstream signaling events lead to the production of reactive oxygen species (ROS), nitric-oxide (NO), callose, glucanases, chitinases, and anti-microbial phytoalexins (e.g. camalexin) that help the plant to limit pathogen ingress (Davis *et al.,* 1986; Davis and Hahlbrock 1987; Cabanne and Donèche 2002; Ferrari *et al.,* 2007; Ferrari *et al.,* 2013; Duran-Flores and Heil 2016). Once entered into the plant apoplast, recognition of *B. cinerea* is facilitated by pattern recognition receptors (PRRs) that recognize so-called pathogen-associated molecular patterns (PAMPs), consisting of molecules that are essential for the pathogen, such as cell wall chitin in the case of *B. cinerea*. Chitin is recognized via the LYSIN MOTIF (LysM) RECEPTOR KINASE 5 (LYK5) and receptor-like kinase (RLK) CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) complex. Association of this complex with the kinases BOTRYTIS-INDUCIBLE KINASE 1 (BIK1) and PBS1-LIKE 27 (PBL27) leads to activation of downstream mitogen activated kinase (MAPK) signaling pathways ultimately resulting in the activation of effective plant defense responses (Liu *et al.,* 2012; Cao *et al.,* 2014; Le *et al.,* 2014; Shinya *et al.,* 2014; Yamada *et al.,* 2016). Activation of *B. cinerea*-induced defense signaling results in the accumulation of JA and ET, which in turn activate defense-related genes such as *PLANT DEFENSIN1.2* (*PDF1.2*; Thomma *et al.,* 1998; Thomma *et al.,* 1999). Plant defenses against *B. cinerea* also involve ABA and SA signaling, which shapes the plant's defense response at different stages of the infection (Audenaert *et al.,* 2002; Ferrari *et al.,* 2003; Liu *et al.,* 2015; Vos *et al.,* 2015). Attempts to unravel the genetics and transcriptional dynamics behind plant responses to necrotrophs, such as *B. cinerea*, revealed many genetic components (Denby *et al.,* 2004; AbuQamar *et al.,* 2006; Rowe and Kliebenstein 2008; Davis *et al.,* 2009; Anuradha *et al.,* 2011; Windram *et al.,* 2012; Coolen *et al.,* 2016). Several genes underlying plant resistance to *B. cinerea* have been described. They involve components of structural barriers, general stress signals such

as reactive oxygen intermediates (ROI), different hormonal biosynthesis and signaling pathways (predominantly JA and ET), glucosinolates and phytoalexins (e.g. camalexin), the plant's nutrient status, and the circadian rhythm (Thomma *et al.,* 1999; Kachroo *et al.,* 2001; Audenaert *et al.,* 2002; Berrocal-Lobo *et al.,* 2002; Alonso *et al.,* 2003; Ferrari *et al.,* 2003; Mengiste *et al.,* 2003; Kliebenstein *et al.,* 2005; Veronese *et al.,* 2006; Bessire *et al.,* 2007; Ferrari *et al.,* 2007; Lionetti *et al.,* 2007; Rowe *et al.,* 2010; Manabe *et al.,* 2011; Birkenbihl *et al.,* 2012; Brauc *et al.,* 2012; Cui *et al.,* 2013; Abro *et al.,* 2014; Hevia *et al.,* 2015; Ingle *et al.,* 2015). In natural and agricultural settings, plants often have to cope with multiple stress conditions simultaneously. Although, studies on plant resistance against *B. cinerea* have revealed many components with roles in defense against this pathogen, still little is known about the regulation and prioritization of such plant adaptive responses when plants are exposed to *B. cinerea* in a multistress environment.

In a previous study we investigated the effect of prior herbivory by *Pieris rapae* and drought stress on the dynamics of the transcriptional response of *Arabidopsis thaliana* to *B. cinerea* infection (Coolen *et al.,* 2016). We showed that when a plant is encountering different stresses in sequence, it is well capable of swiftly adapting it's transcriptional response to the last stress encountered. The transcriptional response to herbivory or drought stress was largely overruled by the plant's response to *B. cinerea* infection, although transcriptional signatures of the first stress were still detectable in the second stress transcriptional profile (Coolen *et al.,* 2016). In the present study we aimed to gain insight into the genetic basis of the plant's ability to adapt to these sequential stresses in naturally occurring *A. thaliana* accessions. To this end we studied the genetic basis of the phenotypic differences that we observed in *B. cinerea* disease severity among 346 *A. thaliana* accessions during single and sequential double stress conditions. The preceding biotic stress factor studied here is herbivory by *Pieris rapae* caterpillars, one of the most destructive plant pests on cruciferous plants. Plant defense against this attacker is regulated by the phytohormones JA and ABA (Vos *et al.,* 2013b). The preceding abiotic stress factor that was used in this study is drought stress, one of the most-encountered abiotic stresses in agriculture. The phytohormone ABA plays an important role in the adaptation to this stress (Yamaguchi-Shinozaki and Shinozaki 2006). The combination of these prior stresses with *B. cinerea* infection are particularly interesting because the plant's adaptive response to these stresses shares components of the JA and ABA hormonal signaling pathways. Hence, this study could potentially reveal important novel players in cross communication between the involved stress signaling pathways (Pieterse *et al.,* 2012; Caarls *et al.,* 2015). To study the genetics behind plant adaptive responses to *B. cinerea* infection under single and multi-stress conditions, we performed GWA mapping on a collection of 346 globally collected *A. thaliana* accessions. These accessions are thought to have evolved under different selection pressures depending on their habitat, potentially leading to genetic variation that can be mined for naturally occurring plant adaptive responses to multiple stresses. To aid in the selection of candidate genes we additionally performed quantitative trait loci (QTL) mapping and fine mapping using available full genome sequences of diverse *A. thaliana* accessions. Our results reveal several candidate genes with potential roles in the plant's adaptive response during sequential double stresses.

Results

Experimental approach to study sequential stress responses

To study the genetic basis of the effect of prior biotic and abiotic stresses on the plant response to *B. cinerea* infection, 346 naturally occurring accessions of the *A. thaliana* haplotype map (HapMap) collection (Baxter *et al.,* 2010; Platt *et al.,* 2010) were screened for susceptibility to infection by *B. cinerea,* either as a single stress, or when preceded by *P. rapae* herbivory or drought stress. These preceding stresses were chosen because the plant response to these stresses shares important signaling components with the defense response to *B. cinerea,* theoretically resulting in antagonistic or synergistic effects on the level of *B. cinerea* resistance during sequential stress conditions. For herbivory by *P. rapae,* a short 24-h period of exposure to herbivory was chosen (Figure 1), because plant defense responses to this attacker are triggered very rapidly (Coolen *et al.,* 2016). First effects of herbivore feeding on defense-related gene expression are typically detectable within 3 h after introduction of the caterpillar onto the leaf. At 12-24 h after infestation, the herbivory- and JA-responsive marker gene *LIPOXYGENASE2* (*LOX2*) peaks in expression (Coolen *et al.,* 2016).

For drought stress, a moderate drought treatment was chosen in which plants were withheld from watering for 7 days after which they were re-watered and allowed to recover for 24 h (Figure 1). The 7-d drought treatment resulted in visible growth retardation, dark coloration of the leaves, and first signs of wilting. By comparing leaf areas from normally-watered and drought-stressed plants it is clear that the 346 tested *A. thaliana* accessions respond differently on water withhold, leading to phenotypic variations that range from highly drought sensitive to highly drought tolerant (Supplementary Figure S1 and Supplementary Table S1). Induced expression of the drought-responsive gene *RAD18* (*AT5g66400*) is typically observed in this setup from day 5 of the water withhold (Coolen *et al.,* 2016).

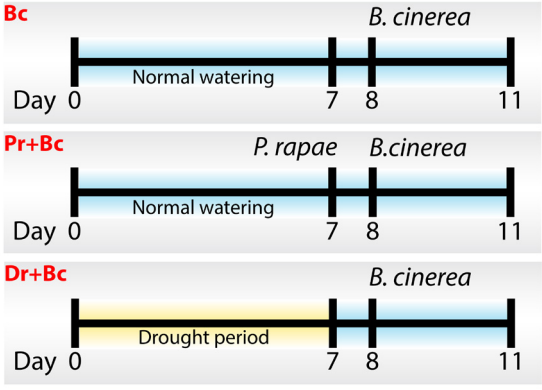


Figure 1. Experimental design of the timing of the single and sequential double stress treatments. Single stress *B. cinerea*-treated plants (Bc) were inoculated on 6 leaves with *B. cinerea* spores when 4 weeks old. In the *P. rapae*/*B. cinerea* sequential double stress (Pr+Bc), a single *P. rapae* larvae was allowed to feed for 24 h on a single leaf per plant (day 7) prior to inoculation of 6 non-damaged leaves with *B. cinerea* (day 8). In the drought/*B. cinerea* sequential double stress, plants were withheld from watering for 7 d (day 0-7), afer which they were re-watered and allowed to recover for 1 d (day 7) prior to inoculation of 6 leaves with *B. cinerea* (day 8). The percentage of leaves with spreading lesions was scored 3 d after inoculation (day 11). The treatments were synchronized so that all treatments were inoculated with *B. cinerea* simultaneously.

Plant responses to *B. cinerea* are detectable at the gene expression level within 6 h after application of the inoculum (Coolen *et al.,* 2016). The infection itself becomes clearly visible at 48 h after inoculation with the start of watery lesions, rapidly followed by expansion of the lesions, and clearly visible cell death 3 d after inoculation (Figure 2). The sequential stress combinations with *B. cinerea* as second stress and herbivory and drought as first stress were chosen because both herbivory and drought stress are expected to impact the JA response pathway that regulates defense against *B. cinerea.* As a readout of the level of *B. cinerea* resistance, we assessed the frequency by which *B. cinerea* inoculations inflict spreading lesions as a measure of the ability of *B. cinerea* to infect plant leaves in single and double stress situations. The experiment was designed in such a way that all stress treatments and measurements were performed simultaneously (Figure 1 see experimental procedures).

Phenotypic variation in plant responses to *B. cinerea* under sequential (a)biotic stress

Screening 346 naturally occurring *A. thaliana* accessions of the HapMap collection revealed that there is a large natural variation in plant susceptibility towards *B. cinerea* infection when the pathogen was applied as a single stress (Figure 2A and Supplementary Table S2). Plant phenotypes ranged from highly susceptible, with all inoculated leaves heavily infected, to highly resistant phenotypes that hardly formed any spreading lesions. Herbivory by *P. rapae* caterpillars, in the 24 h preceding the *B. cinerea* infection, significantly reduced *B. cinerea* disease severity in 101 of the 346 *A. thaliana* accessions, whereas none of the accessions became enhanced susceptible to the disease after herbivory (Student's *t*-test; $p \leq 0.05$). Susceptibility to *B. cinerea* was also affected by drought stress in the 7 d preceding the infection, resulting in both increased susceptibility and increased resistance, depending on the accession. In 75 of the 346 tested accessions, drought stress significantly increased the level of *B. cinerea* resistance, while in 6 accessions the level of *B. cinerea* resistance was significantly reduced (Student's *t*-test; $p \leq 0.05$). Figure 2B shows that the correlation between the single and sequential double stress datasets is moderate to strong in strength (R^2= 0.43-0.61). This suggests that the effects of both herbivory and drought stress on the level of *B. cinerea* resistance are partly dependent on the level of *B. cinerea* resistance observed in the single stress condition.

Genome-wide association mapping of loci related to plant adaptive responses to *B. cinerea* under sequential (a)biotic stress

To analyze the genetic variation of the level of resistance to *B. cinerea* under conditions of single and sequential (a)biotic stress, and to ultimately identify candidate genes that affect the level of *B. cinerea* resistance during multi stress conditions, we performed a GWA study. For the GWA analysis, we used normally-distributed and experimental design-corrected quantitative data of 346 *A. thaliana* accessions, representing the level of disease susceptibility in the single stress condition and the two sequential double stress conditions. Moreover, we calculated residuals for the relative effect of the sequential stress to the *B. cinerea* single stress (Supplemental Table S3). The residual

data were used in the GWA analysis to search for SNPs that are associated with traits that influence the level of *B. cinerea* resistance, but are itself not part of the *B. cinerea* resistance response during the single stress condition. To obtain an estimate of the breeding value for each trait, we calculated the narrow sense heritability (h^2; Kruijer *et al.,* 2015), which reflects the additive genetic effects that contribute to the observed phenotypes. Narrow sense heritability values ranged between 0.17 and 0.40 for the GWA input data, whereas for the residuals it ranged between 0.02 and 0.05 (Table 1). These values suggest that there is a moderate to low selection for the tested traits within the tested set of accessions.

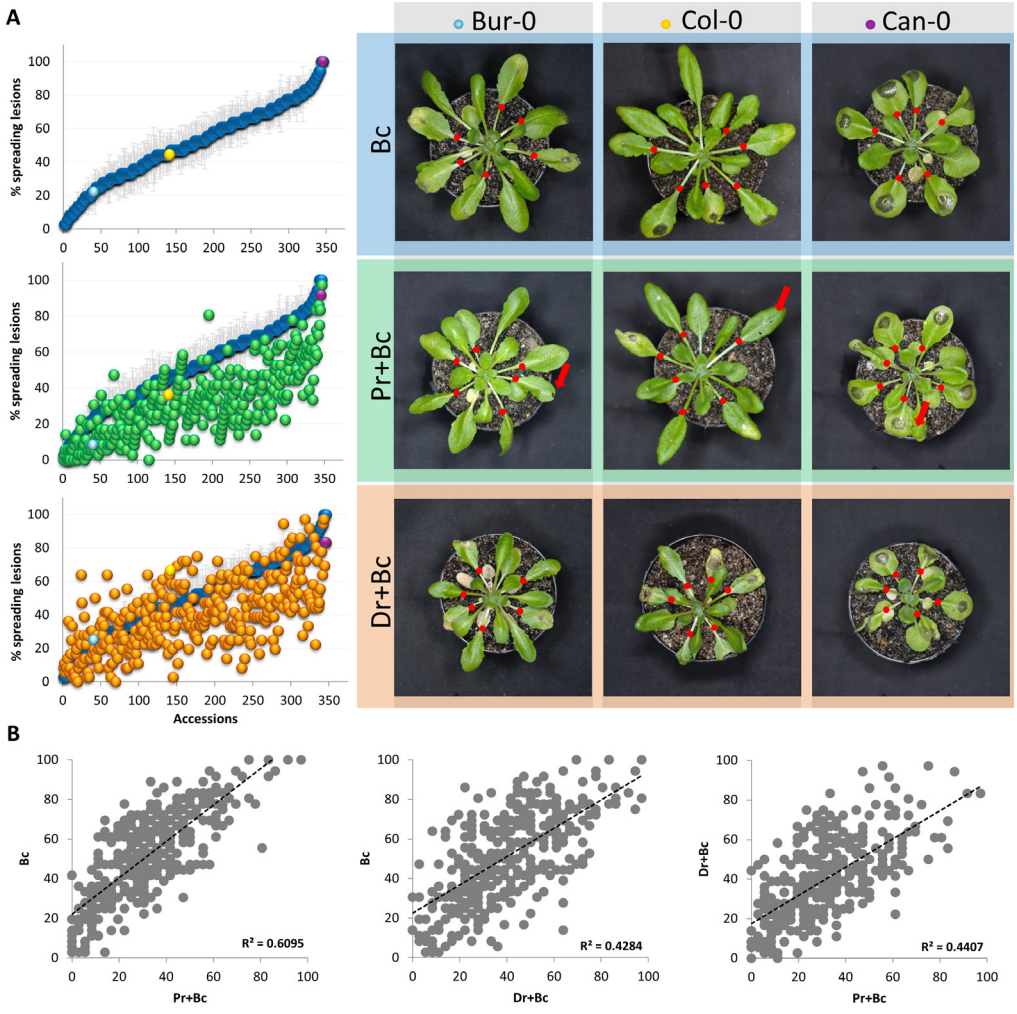


Figure 2. Phenotypic variation in *B. cinerea* resistance and effect of prior herbivory or drought stress in 346 *A. thaliana* accessions. A) *B. cinerea* disease severity in average percentage of spreading lesions per accession (n = 6) at 3 d after pathogen inoculation. The accessions are ordered on the x-axis according to their level of disease severity under the single stress condition (Bc; in blue). The *B. cinerea* disease severity values in the sequential double stress treatments with *P. rapae* infestation (Pr+Bc) or drought (Dr+Bc) followed by *B. cinerea* inoculation are displayed with green and orange dots, respectively. In the panel on the right, photographs of representative plants of 3 contrasting accessions are depicted. The presented accessions are color-coded with light blue, yellow and purple for Bur-0, Col-0 and Can-0, respectively, referring to their position in the graphs on the left. The 6 *B. cinerea*-inoculated leaves per plant are indicated with red dots. Leaves with damage caused by 24 h of *P. rapae* feeding are indicated with red arrows. B) Relationship between the level of *B. cinerea* resistance in the 346 *A. thaliana* accessions in the single versus the sequential double stress conditions and between both sequential stresses. In the plots a linear regression line was fitted with corresponding Pearson correlation (R^2) values.

To identify candidate genes with putative roles in *B. cinerea* resistance and in the effect of herbivory or drought stress on the level of *B. cinerea* resistance, we performed GWA mapping using the ~214 K SNP set that is commonly used for GWA studies in *A. thaliana* (Kim *et al.,* 2007; Atwell *et al.,* 2010; Li *et al.,* 2010a; Horton *et al.,* 2012; Bac-Molenaar *et al.,* 2015b; Kloth *et al.,* 2016; Davila Olivas *et al.,* 2017b; Thoen *et al.,* 2017). To this end, we used the *B. cinerea* disease severity scores and the residual data (Supplementary Table S3) to perform a GWA analysis using the factored spectrally transformed linear mixed models (FaST-LMM) algorithm (Lippert *et al.,* 2011). SNP-trait associations of interest were selected by setting an arbitrary threshold with a logarithm (base 10) of the odds (LOD, $-\log_{10}(p)$) score of 4.0 (Figure 3). Manhattan plots from the GWA analysis show several peaks with single (singleton) or multiple (string) SNP-trait associations (Figure 3 and Table 2). In total 117 SNP-trait associations were identified in 94 loci (14 strings and 80 singletons). Nine loci were shared between two traits, yielding a total of 85 unique loci with SNP-trait associations for *B. cinerea* resistance during single and sequential double stress conditions (Table 2 and Supplementary Table S4). Because the linkage disequilibrium (LD) of the natural population of *A. thaliana* accessions used for GWA mapping is estimated to be 10-50 kb (Nordborg *et al.,* 2005; Kim *et al.,* 2007), we considered all genes within 25 kb up- and down-stream of each identified SNP to be candidates for the observed SNP-trait associations. When taking along a 50-kb window surrounding the 117 significant SNPs in the 85 loci, a total number of 233 candidate genes were identified. Of these 233 genes, 189 were found for only one trait, 43 genes were found for two traits, and 1 gene was found for three traits (Figure 4 and Supplementary Table S5). This latter gene, *AT1G20960* encoding an ATP-dependent RNA helicase, is shared between Pr+Bc sequential double stress, Pr+Bc sequential double stress residuals and Dr+Bc sequential double stress.

Table 1. GWA input data summary. Heritability characteristics of the datasets used for the single and sequential double stress GWA analyses. Bc, single stress *B. cinerea* treatment; Pr+Bc, sequential double stress with *P. rapae* infestation prior to *B. cinerea* inoculation; Dr+Bc, drought stress prior to *B. cinerea* inoculation; Pr+Bc residuals, residual dataset of Pr+Bc; and Dr+Bc, residual dataset of Dr+Bc.

Trait	h^2	CI	va	ve
Bc	0.40	0.13 - 0.74	0.02	0.03
Pr+Bc	0.17	0.03 - 0.56	0.01	0.03
Pr+Bc residuals	0.05	0.00 - 0.91	0.00	0.02
Dr+Bc	0.31	0.08 - 0.68	0.01	0.03
Dr+Bc residuals	0.02	0.00 - 1.00	0.00	0.02

h^2, narrow sense heritability h^2=va/(va+ve); CI, 95% h^2 confidential interval; va, additive genetic variance; ve, residual or enviromental and non-additive variance (Kruijer *et al.,* 2015; Thoen *et al.,* 2017).

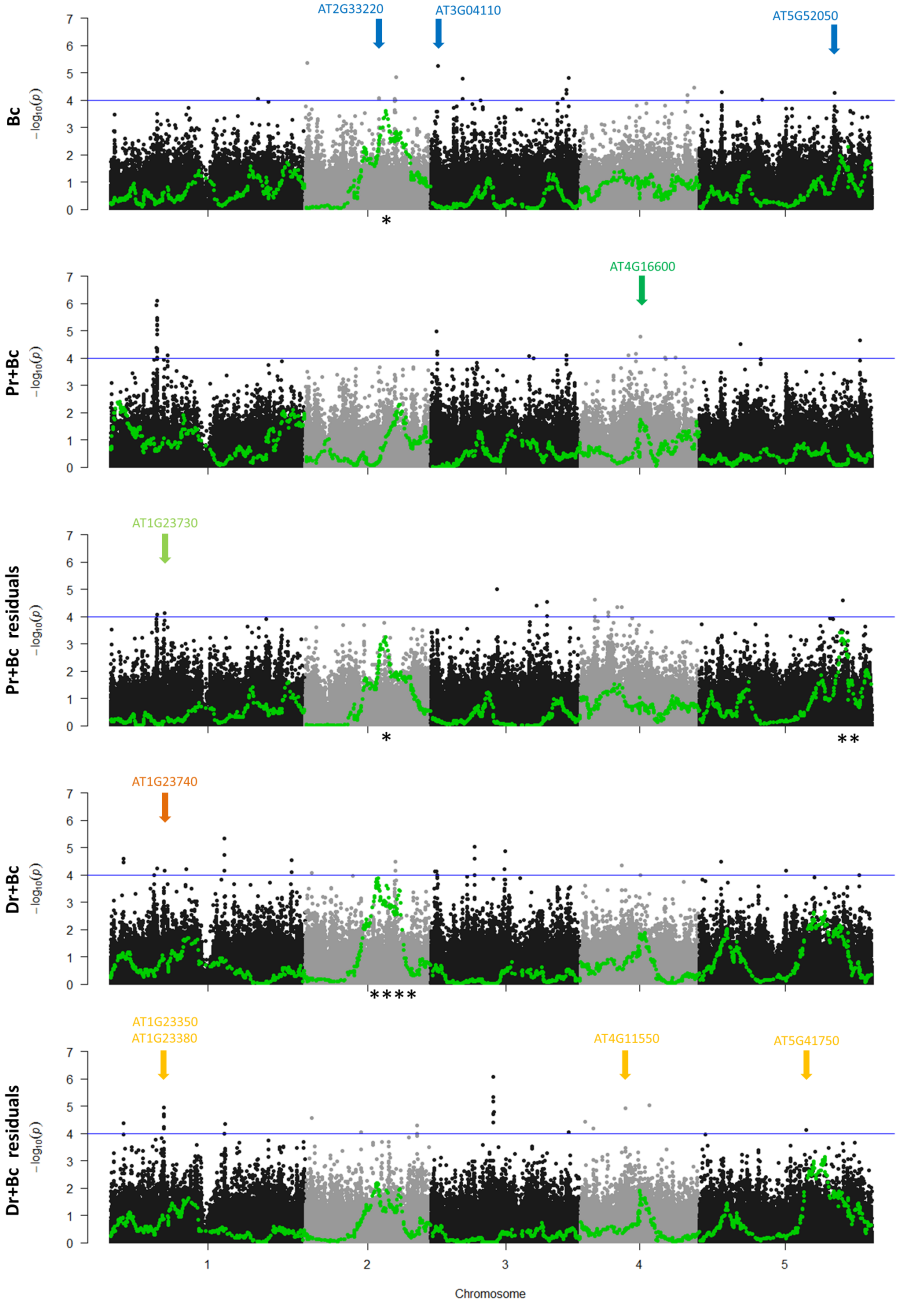


Figure 3. GWA, QTL and fine mapping results of the effect of prior herbivory or drought stress on the level of *B. cinerea* resistance in 346 *A. thaliana* accessions. Manhattan plots (grey and dark grey) showing the $-\log_{10}(p)$ values of the SNP marker-trait associations from the GWA mapping on *A. thaliana* chromosomes 1-5 (x-axis). Manhattan plots are given of the GWA analyses of *B. cinerea* resistance scores in 346 *A. thaliana* accessions exposed to *B. cinerea* alone (Bc), to herbivory by *P. rapae* followed by *B. cinerea* (Pr+Bc), and to drought stress followed by *B. cinerea* (Dr+Bc). The GWA output with the residual data (i.e., the relative effect of the prior stresses herbivory or drought on *B. cinerea* resistance in each accession) are given in Manhattan plots "Pr+Bc residuals" and "Dr+Bc residuals". The dark blue line indicates the arbitrary LOD threshold of 4.0 ($-\log_{10}(p)$=4.0). QTL mapping results with the *A. thaliana* MAGIC lines are indicated in green. Asterisks on the x-axis indicate significant QTLs (Kover *et al.*, 2009; Kover and Mott 2012). Colored arrows indicate the nine GWA loci for which SNP-trait associations (LOD ≥ 4.0) were confirmed via fine mapping using the 50-kb window genome sequences of 164 of the 346 tested *A. thaliana* accessions.

Amongst the candidate genes are several genes previously associated with the adaptive response of *A. thaliana* to either *B. cinerea* infection, herbivory by *P. rapae,* or drought stress. For instance, amongst the candidate genes associated with the *B. cinerea* single stress response is *LysM RLK1-INTERACTING KINASE 1* (*LIK1*), which encodes a LRR-RLK protein that is phosphorylated by the kinase CERK1 in response to chitin perception (Miya *et al.,* 2007; Le *et al.,* 2014). Amongst the candidate genes associated with the residuals of the *P. rapae*/*B. cinerea* sequential double stress response is *LOX2,* which encodes one of the first enzymes of the JA biosynthesis pathway (Wasternack 2015) and is responsive to both herbivory by *P. rapae* (Bell *et al.,* 1995; Reymond *et al.,* 2000; De Vos *et al.,* 2005; Coolen *et al.,* 2016) and infection by *B. cinerea* (AbuQamar *et al.,* 2006; Birkenbihl *et al.,* 2012; Windram *et al.,* 2012; Coolen *et al.,* 2016). Amongst the candidate genes associated with the response to the drought/*B. cinerea* double stress treatment are *YUCCA 7* (*YUC7*) and *HISTIDINE KINASE 5* (*HK5*). YUC7 is implicated in drought tolerance and ABA- and ET-mediated root growth inhibition, while HK5 is involved in stomatal closure and plant resistance against *B. cinerea* (Iwama *et al.,* 2006; Desikan *et al.,* 2008; Lee *et al.,* 2012; Pham *et al.,* 2012). These findings suggest that the followed GWA approach has the potential of identifying novel players in the plant's adaptive response to the single and double stresses applied in this study.

Table 2. Number of SNP-trait associations, loci and candidate genes within a 50-kb window of the SNPs identified by GWA mapping. Summary for the GWA results of the traits *B. cinerea* resistance during single stress (Bc), during *P. rapae*/*B. cinerea* sequential double stress (Pr+Bc), and during drought/*B. cinerea* sequential double stress (Dr+Bc), and of the corresponding residual data (Pr+Bc residuals and Dr+Bc residuals).

SNP-trait associations (LOD ≥4.0)						
Trait	**SNPs**	**Strings**	**Singletons**	**Unique genes**	**Additional genes in 50-kb window**	**Total number of candidate genes**
Bc	14	1	12	13	43	56
Pr+Bc	33	6	17	23	29	52
Pr+Bc residuals	13	1	11	12	12	24
Dr+Bc	30	4	22	26	43	69
Dr+Bc residuals	27	2	18	20	12	32
Total	117	14	80	94	139	233

Support of GWA-identified loci by QTL mapping

To reduce the number of false positives and false negatives that are induced by the correction for population structure that is implemented within our GWA analysis, additional evidence is required to independently support the identified SNP-trait associations in the GWA mapping analyses (Nordborg and Weigel 2008; Bergelson and Roux 2010). Therefore, we performed quantitative trait loci (QTL) mapping using a set of multi-parent advanced generation inter-cross (MAGIC) lines comprised of recombinant inbred lines (RILs) descended from 19 heterogeneously intercrossed parental *A. thaliana* accessions (Kover *et al.,* 2009; Kover and Mott 2012). Of these parental accessions, 12 were also present in the HapMap collection used in this study, representing accessions with levels of *B.*

cinerea resistance that ranged from highly resistant to highly susceptible (Figure S2 and Supplemental Tables S6). In total 431 RILs of the MAGIC population were tested for the level of resistance against *B. cinerea* under conditions of single or sequential double stress. The frequency distribution of the disease severity scores of the MAGIC lines under the different single and sequential double stress conditions (Supplemental Figure S3) shows that the MAGIC lines displayed a large variation in susceptibility towards *B. cinerea.* From the frequency distribution of *B. cinerea* disease severity it is clear that herbivory prior to *B. cinerea* inoculation significantly shifted the disease severity frequency distribution towards the lower disease severity classes (Chi-square, X^2, p=2.23^{-07}), a phenomenon that was also observed in the 346 *A. thaliana* accessions of the HapMap collection (Figure 2). Also the drought pre-treatment resulted in a shift in the *B. cinerea* disease severity frequency distribution, with significantly fewer MAGIC RILs in the lowest disease severity classes (X^2, p=0.05) and significantly more MAGIC RILs in the median disease severity class (X^2, p=0.05). QTL mapping of the obtained disease severity scores using available scripts in R (Kover *et al.,* 2009; Kover and Mott 2012), revealed several significant QTLs that roughly map to positions on the *A. thaliana* genome as do some of the GWA-identified loci (Figure 3, green line and Supplemental Table S8). No overlap was found with the high LOD score-associations found for the GWA mapping results on chromosome 1 (Figure 3). The latter can be explained by the limited genetic variation in the MAGIC lines in comparison to the much larger genetic variation of the HapMap population. What is clear from the QTL mapping results is that there are two genomic regions, one on chromosome 2 and one on chromosome 5, that emerge after GWA mapping of the HapMap data sets and after QTL mapping of the MAGIC RILs dataset and contain several significant marker-trait associations.

Furthermore, two of the significant QTL markers for the residuals of the *P. rapae*/*B. cinerea* sequential double stress dataset are closely located to our GWA mapping results on chromosome 5 (Figure 3), pointing to the locus *AT5G55250* (Supplemental Figure S4) containing the *IAA CARBOXYLMETHYLTRANSFERASE 1* (*IAMT1*) gene, which is known to convert auxin indole-3-acetic acid to its methyl ester form MeIAA.

Fine mapping of GWA SNP-trait associations using genome sequences of 164 accessions

To further support the identified SNP-trait associations in the GWA mapping analyses, we made use of the genetic variation present in the available full genome sequences of the accessions used in this study (total 164 full genomes available) to fine map the SNP-trait associations within the originally identified 85 unique loci of the GWA analysis (Figure 3, Supplemental Table S4). For fine mapping we used a Kruskal-Wallis test for significant SNP-trait associations with a minor allele frequency larger than 5% (MAF > 0.05), where significant associations exceed the false discovery rate (FDR) corrected threshold for significance, and the 164 genomic sequences of each of the 85 GWA loci (spanning the 50-bp windows around the 117 SNPs with LOD ≥ 4.0). While the original GWA mapping procedure makes use of a selection of SNPs within the accessions relative to the reference genome of accession Col-0, the fine mapping approach makes use of all genetic variance within the used genomic regions of the 164 full genome sequences. In total nine loci could be confirmed

with significant SNP-trait associations, three for the *B. cinerea* single stress data set, two for the *P. rapae*/*B. cinerea* sequential double stress data set, and four for the drought/*B. cinerea* sequential double stress data set (Figure 5; Supplemental Figure S5, and Supplemental Table S9).

For the *B. cinerea* single stress data set, three significant loci were identified with fine mapping. The first significant association was found at the interval of AT2G33210 and AT2G33220 (Figure 5), which contains two genes predicted to be transcribed in opposite directions from the point of the fine mapping association. The first gene is *HEAT SHOCK PROTEIN 60-2* (*HSP60-2;* AT2G33210; Table 3), which has been described as being responsive to *B. cinerea* infection (Windram *et al.,* 2012; Coolen *et al.,* 2016). The second gene is *AT2G33220,* which codes for an oxidation-reduction process-related protein with homology to the mammalian cell death-related protein GRIM-19 (Huang *et al.,* 2004). The second significant association was found in the genomic region of AT3G04130, AT3G04140 and AT3G04150, close to the GWA SNP-trait association located in AT3G04110. The first gene (AT3G04130) codes for a tetratricopeptide repeat (TPR)-like superfamily protein, the second gene (AT3G04140) for an ankyrin-repeat family protein, and the third gene (AT3G04150) for a RmlC-like cupins superfamily protein, all with unknown functions. The third significant association was found at position AT5G52040, containing a gene encoding ARGININE/SERINE-RICH SPLICING FACTOR 41 (RS41). RS41 is known to bind primary microRNA (pri-miRNA) *in vivo* and is involved in miRNA biogenesis (Chen *et al.,* 2015). An *A. thaliana* double mutant *rs40,rs41* containing mutations in *RS41* and its closely related homologue *RS40*, was shown to have reduced *DICER LIKE-1* (*DCL1*) expression. Mutation of *DCL1* in the miRNA biogenesis mutant *dcl1-7* was shown to result in enhanced susceptibility to *B. cinerea* (Weiberg *et al.,* 2013).

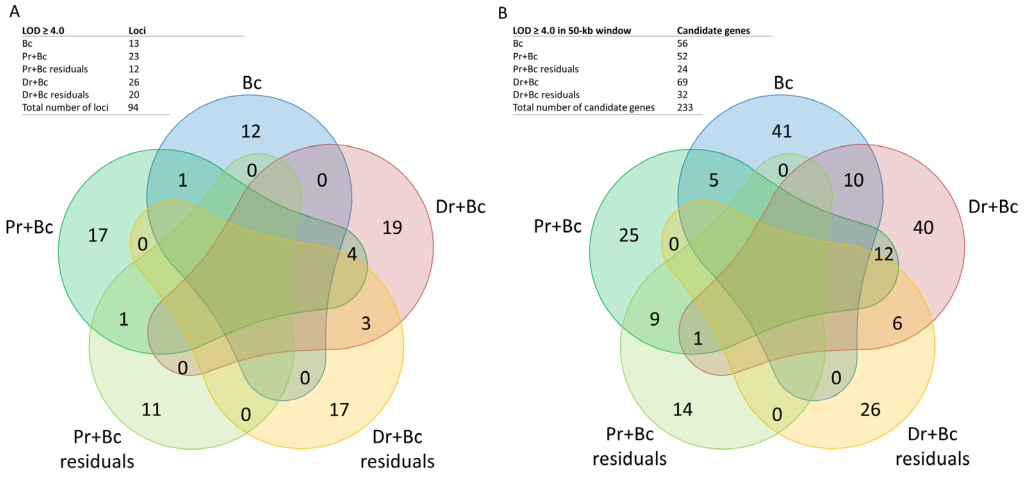


Figure 4. Number of shared loci and candidate genes within a 50-kb window related to the SNP-trait associations obtained with the GWA analyses of *B. cinerea* resistance in single and sequential double stress conditions. Venn diagrams showing the number of (A) shared loci with SNP-trait associations (LOD ≥4.0) and (B) shared candidate genes within a 50-kb window of the SNP-trait associations (LOD ≥ 4.0) identified in the GWA analyses of *B. cinerea* resistance in 346 *A. thaliana* accessions exposed to *B. cinerea* alone (Bc; blue), to herbivory by *P. rapae* followed by *B. cinerea* (Pr+Bc; green), and to drought stress followed by *B. cinerea* (Dr+Bc; orange). The GWA output with the residual data (i.e., the relative effect of the prior stresses herbivory or drought on *B. cinerea* resistance in each accession) are given in "Pr+Bc residuals" (light green) and "Dr+Bc residuals" (yellow).

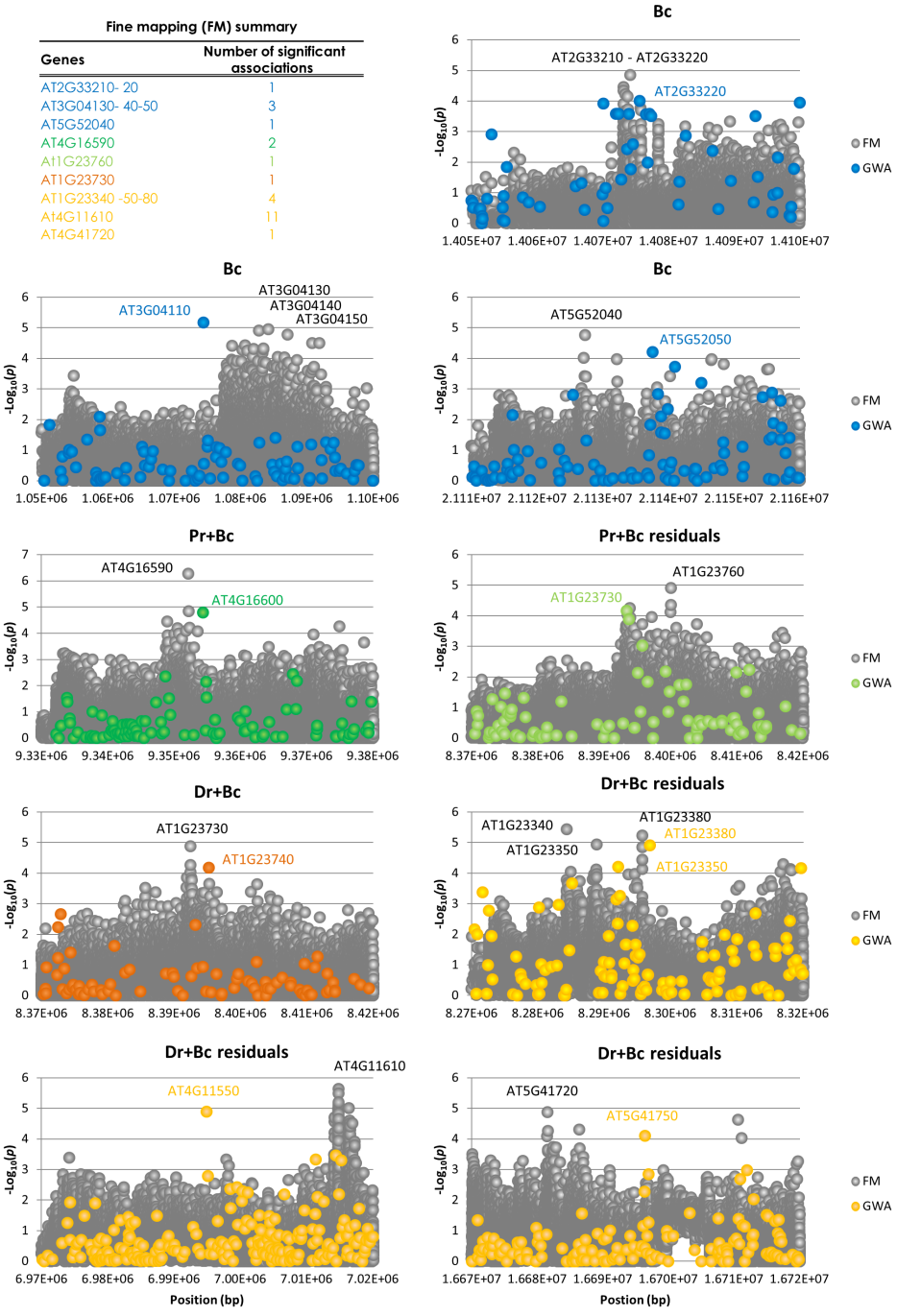


Figure 5. Fine mapping results of confirmed GWA SNP-trait associations. Manhattan plots for the fine mapping results (grey dots) showing the $-\log_{10}(p)$ values of the SNP marker-trait associations of the nine GWA loci for the Bc single stress (blue dots), Pr+Bc sequential double stress (green dots), Pr+Bc residual (light green dots), Dr+Bc sequential double stress (orange dots) and Dr+Bc residuals (yellow dots). On the x-axis the position in base pairs (bp) is shown. Genes indicated in blue, green, light green, orange and yellow show SNP-trait associations obtained with GWA mapping and the gene indicated in black corresponds to the significant (FDR-corrected) fine mapping associations.

For *P. rapae*/*B. cinerea* sequential double stress data a significant association was found with position AT4G16590, representing *CELLULOSE SYNTHASE-LIKE A1* (*CSLA01;* Table 3). Impairment of cellulose synthases was previously shown to enhance disease resistance against necrotrophic fungi such as *Plectosphaerella cucumerina*, confirming its negative effect on necrotrophic fungi (Hernández-Blanco *et al.,* 2007). Furthermore, a significant association was found for the *P. rapae*/*B. cinerea* sequential double stress residual data with locus AT1G23760 containing a gene coding for POLYGALACTURONASE 3 (PG3). PG3 is a pectin-degrading enzyme that is involved in many stages of plant development (Cao 2012). However, pectin degradation is also part of the infection strategy used by *B. cinerea* and deletion of the *B. cinerea PG2* gene was shown to result in delayed primary lesion formation and a strong reduction of virulence on tomato and broad bean (Kars *et al.,* 2005). Furthermore, inhibition of polygalacturonase proteins in *A. thaliana* was shown to increases resistance to the flower fungal pathogen *Fusarium graminearum* (Ferrari *et al.,* 2012).

The drought/*B. cinerea* sequential double stress data yielded a significant association in our fine mapping results with position AT1G23730 representing *BETA CARBONIC ANHYDRASE 3* (*BCA3*; Table 3). BCA3 is implicated in photosynthetic carbon fixation although it is not only localized in the cytosol of foliar tissue, but also in root tissue (Fabre *et al.,* 2007). Furthermore, the expression of *BCA3* was found upregulated 24 h after inoculation in the drought/*B. cinerea* sequential double stress combination (Coolen *et al.,* 2016). For the drought/*B. cinerea* sequential double stress residual data, 3 significant associations were identified at the first locus, corresponding to a gene with unknown function (*AT1G23340*), a gene encoding a plant invertase/pectin methylesterase inhibitor (*AT1G23350*) and *KNOTTED 1-LIKE HOMEOBOX GENE 6* (*KNAT6*, AT1G23380). The plant invertase/pectin methylesterase inhibitor was previously described as an important player in defense against *B. cinerea* infection, as overexpression was shown to restrict infection by *B. cinerea* (Lionetti *et al.,* 2007). Homeodomain transcription factor *KNAT6* was previously shown to be involved in plant flowering and activation of stress pathways that promote JA biosynthesis (Khan *et al.,* 2015). Another strong and significant association for the drought/*B. cinerea* sequential double stress residual data was confirmed at *AT5G11610* on chromosome 5, representing a gene coding for an exostosin family protein with unknown function (Busse-Wicher *et al.,* 2014). Finally, a significant association was found at an F-box associated ubiquitination effector family gene (*AT5G41720*) with unknown function (Lechner *et al.,* 2006).

Expression patterns of the fine mapping-confirmed candidate genes

By looking at the expression patterns of *A. thaliana* (Col-0) in the first 24 h after *B. cinerea* inoculation in the single and sequential double stress treatments (Coolen *et al.,* 2016), the fine mapping-confirmed genes are shown to be responsive to the applied stresses (Figure S6, (Coolen *et al.,* 2016). This may further indicate a role of these genes in the stress phenotypes that we observed. For instance, *HSP60-2* is induced at 12 h after *B. cinerea* inoculation and continues to increase in expression until the last measured time point, indicating its responsiveness to *B. cinerea*. Furthermore, both *P. rapae* and drought stress pretreatments repress the induction of *HSP60-2* until 18 h after *B. cinerea* inoculation,

indicating that both pretreatments change the single stress *B. cinerea* expression pattern, making this an interesting candidate gene to study the possible consequence of the repression of *HSP60-2* during early *B. cinerea* infection when preceded by another stress. Another example is the ankyrin-repeat family protein gene *AT3G04140* that is repressed during the first 24 h of the *B. cinerea infection* and is induced until 12 h after inoculation in the drought/*B. cinerea* sequential double stress combination, pointing to a possible involvement of this gene in the drought/*B. cinerea* sequential double stress. Also *BCA3,* from the drought/*B. cinerea* sequential double stress dataset, shows to be differentially expressed compared to the single stress *B. cinerea* treatment. *BCA3* was shown to be repressed during the first 18 h after *B. cinerea* inoculation in the drought/*B. cinerea* sequential double stress treatment, whereas this is not the case for single stress *B. cinerea*. This is also the case for the exostosin family protein with unknown function, *AT5G11610*, that is induced in the *B. cinerea* single stress treatment from 18 h onwards and is repressed in the drought/*B. cinerea* sequential double stress data for the whole first 24 h after *B. cinerea* inoculation. And lastly, *PG3* that is known to be involved in pectin degradation shows to be strongly repressed in the *P. rapae*/*B. cinerea* sequential double stress combination at 24 h compared to the repression seen in the *B. cinerea* single stress. Since the inhibition of pectin degradation in *A. thaliana* was shown to affect the fungal pathogen *F. graminearum* (Ferrari *et al.,* 2012), it would be interesting to study if the strong repression as observed in the *P. rapae*/*B. cinerea* sequential double stress data also affects *B. cinerea* infection, leading to more resistant plants.

Table 3. Fine mapping-confirmed candidate genes associated with *B. cinerea* resistance during single and sequential double stress.

Trait	Gene	TAIR gene description	Associated before with Necrotroph resistance?	Reference
Bc	AT2G33210	HEAT SHOCK PROTEIN 60-2 (HSP60-2)	Possibly	Windram *et al.*, 2012
	AT2G33220	GRIM-19	No	
	AT3G04130	Tetratricopeptide repeat-like superfamily protein	No	
	AT3G04140	Ankyrin-repeat family protein	No	
	AT3G04150	RmlC-like cupins superfamily protein	No	
	AT5G52040	ARG/SER-RICH SPLICING FACTOR 41 (RS41)	Yes	Weiberg *et al.*, 2013
Pr+Bc	AT4G16590	CELLULOSE SYNTHASE-LIKE A1 (CSLA01)	Possibly	Hernández-Blanco *et al.*, 2007
	AT1G23760	POLYGALACTURONASE 3 (PG3)	Possibly[1]	Kars *et al.*, 2005[1]
Dr+Bc	AT1G23730	BETA CARBONIC ANHYDRASE 3 (BCA3)	Possibly	Coolen *et al.*, 2016
	AT1G23340	Unknown	No	
	AT1G23350	Invertase/pectin methylesterase inhibitor protein	Yes	Lionetti *et al.*, 2007
	AT1G23380	KNOTTED 1-LIKE HOMEOBOX GENE 6 (KNAT6)	Possibly[2]	Khan *et al.*, 2015[2]
	AT5G11610	Exostosin family protein with unknown function	No	
	AT5G41720	F-box associated ubiquitination effector	No	

[1] Polygalacturonases excreted by *B. cinerea* increase pathogen virulence (Kars et al., 2005).
[2] Indicated gene is involved in the regulation of JA biosynthesis (Khan et al., 2015) and may therefore contribute to necrotroph resistance.

All together the combination of GWA mapping, QTL mapping and usage of fine mapping with full genome sequences confirmed nine loci with potential roles in the plant adaptive response to infection by the necrotrophic fungus *B. cinerea* during sequential biotic and abiotic stresses. Several of these genes are already known to be involved in single stress plant responses (Table 3), confirming that the followed approach has the potential of identifying novel players in the plant's adaptive response to the single and double stresses. Further research on the candidate genes will be required to reveal their role in plant stress signaling and adaptation during *B. cinerea* infection under sequential biotic and abiotic stress.

Discussion and conclusion

Plant responses to sequential double (a)biotic stresses

To identify genes that play a crucial role in the plant's adaptive response during sequential double stresses, we performed *B. cinerea* disease resistance bioassays with 346 *A. thaliana* accessions under single stress and sequential double stress conditions. Our screening readout for spreading *B. cinerea* lesions was obtained 3 d after pathogen inoculation, a so called 'endpoint' measurement. Using this approach, one may expect to find genes that influence the outcome of the defense response in early and late stages of the infection process. By inoculating the leaves with *B. cinerea* immediately after another stress (herbivory or drought stress), signals from the previous stresses are still present and can thus have impact on the response of the secondary stress (Atkinson *et al.,* 2013; Coolen *et al.,* 2016; Davila Olivas *et al.,* 2016). In a previous study focused on the transcriptional dynamics of *B. cinerea*-inoculated *A. thaliana* leaves during multistress conditions, we showed that both herbivory and drought stress had an impact on *B. cinerea*-induced transcriptional reprogramming, especially within the first 12 h after *B. cinerea* inoculation (Coolen *et al.,* 2016). Phenotypic analysis of the impact of prior herbivory or drought stress on the level of *B. cinerea* resistance in 346 *A. thaliana* accessions showed that a prior stress indeed significantly changed the level of *B. cinerea* resistance in many of the tested *A. thaliana* accessions (Figure 2 and Supplementary Tabel S2). These findings suggest that there is natural variation that can potentially be mined for yet unknown key players in the plants ability to adapt to changing environmental conditions, such as tested here with sequential double (a)biotic stresses in combination with *B. cinerea*.

Although timing and severity of stresses are expected to greatly influence the outcome of the plants adaptive response, a first stress encounter can leave a detectable and permanent imprint, e.g. at the level of gene transcription (Coolen *et al.,* 2016), or at the level of second stress resistance. Prior herbivory, which is often only a bite of less than a millimeter in size in our setup, facilitates major changes in the whole-plant transcriptional profile (Coolen *et al.,* 2016), in the majority of the accessions resulting in a reduced susceptibility to *B. cinerea* (Figure 2). This phenomenon was previously described to be the result of wound-induced resistance, and could partially be explained by increased and more rapid accumulation of camalexin (Chassot *et al.,* 2008). These findings, together with the strong correlation that we observed between the level of resistance in the 346 *A.*

thaliana accessions under conditions of single stress (Bc) and sequential double stress (Pr+Bc; Figure 2B), suggest that the plant's response towards *P. rapae* and *B. cinerea* make use of similar signaling components that act synergistically in mounting defense against *B. cinerea*. Both *P. rapae* and *B. cinerea* abundantly trigger JA-dependent defenses (Reymond *et al.,* 2000; Verhage *et al.,* 2011; Windram *et al.,* 2012; Vos *et al.,* 2013b; Coolen *et al.,* 2016), making it likely that components of the JA response contribute to the synergistic effect of prior herbivory on the level of *B. cinerea* resistance in the majority of the *A. thaliana* accession.

For the drought sequential double stress, a moderate correlation between the level of *B. cinerea* resistance under single and double stress conditions was observed in the 346 *A. thaliana* accessions (Figure 2B). This points to the contribution of accession-specific components on the effect of prior drought stress on the level of *B. cinerea* resistance. These accession-specific components may be related to water usage and drought stress tolerance amongst the accessions (Supplemental Figure S1). Drought is one of the most frequently experienced abiotic environmental stresses by plants. They are well able to sense a reduction in the availability of water in their rhizosphere and in response transmit a chemical signal to the shoots to reduce stomatal conduction and leaf growth (Schachtman and Goodger 2008). The plant hormone ABA is currently the best candidate for such chemical signal (Leckie *et al.,* 1998). ABA not only regulates the adaptive plant response to drought, it also modulates the relative strength of the MYC-branch of the JA response (predominantly effective against insect herbivory) and the ERF-branch of the JA response (predominantly effective against necrotrophic pathogens) (Verhage *et al.,* 2011; Pieterse *et al.,* 2012). Hence, differences in drought resistance amongst *A. thaliana* accessions may impact the level of *B. cinerea* resistance via components of the ABA signaling pathway.

Candidate genes for plant resistance to *B. cinerea* during sequential (a)biotic stresses

By using the phenotypic variation in plant resistance to *B. cinerea* during sequential double (a) biotic stresses, GWA mapping pinpointed genomic regions that are potentially responsible for the observed phenotypic variance amongst the accessions (Figure 2). Using a combination of mapping approaches rises the probability that a real contributing factor can be identified, especially when traits are genetically complex (Nordborg and Weigel 2008). Therefore, to pinpoint truly interesting SNP-trait associations, we used a combined approach of GWA mapping, QTL mapping and fine mapping using full genome sequences. QTL mapping identified a number of map positions that were linked to the GWA mapping-identified mapping positions (Figure 3). However, several GWA-mapping positions, including ones associated with candidate genes previously implicated in *B. cinerea* resistance (e.g. *LIK1 and LOX2*), were not identified by QTL mapping. This can be explained by the fact that there is limited natural variation within these genes or within the natural genetic variation of the MAGIC lines (RILs of 19 *A. thaliana* accessions) used for QTL mapping, compared to the 346 *A. thaliana* accessions used for GWA mapping. Nevertheless, QTL mapping did result in several significant loci of which one QTL island for the *P. rapae*/*B. cinerea* sequential double stress residual data mapped close to GWA locus pointing to the primary auxin indole-3-acetic acid (IAA)

methyltransferase gene *IAMT1* (Figure 3; Supplemental Figure S10). IAMT is known to be involved in the regulation of plant development and auxin homeostasis through IAA methylation (Spiess *et al.,* 2014). Although this locus could not be confirmed with fine mapping, both GWA mapping and QTL mapping supported involvement of this locus in the effect of *P. rapae* herbivory on *B. cinerea* resistance. Interestingly, IAMT1 was shown to be induced by *P. rapae* feeding, indicating its involvement in plant responses to herbivory (Coolen *et al.,* 2016). Furthermore, IAA was previously implicated in the defense response of *Nicotiana attenuate* to *Manduca sexta* herbivory, where herbivory-induced IAA was shown to regulate a subset of systemic JA-dependent secondary metabolites involved in defense against herbivores (Machado *et al.,* 2013; Machado *et al.,* 2016).

Fine mapping of the 85 GWA-mapped loci resulted in the confirmation of 9 loci with significantly SNP-trait associations. For *B. cinerea* as a single stress, three loci were identified pointing to candidate genes *HSP60-2* or *GRIM-16,* a tetratricopeptide repeat (TPR)-like superfamily gene, an ankyrin repeat family gene, an RmlC-like cupins superfamily gene, and *RS41* (Table 3). Of these genes, *HSP60-2* has been shown to be stress-inducible and is induced 24 h after *B. cinerea* inoculation (Windram *et al.,* 2012; Coolen *et al.,* 2016). Moreover, the miRNA biogenesis gene *RS41* has previously been implicated in *B. cinerea* resistance as the double mutant *rs40,rs41* displays reduced *DCL-1* expression and the *A. thaliana dcl1-7* mutant shows enhanced susceptibility to *B. cinerea* (Weiberg *et al.,* 2013; Chen *et al.,* 2015). *A. thaliana* DCL-1 processes hairpin mRNA into miRNA that target specific mRNA transcripts for destruction. Since miRNAs were shown to be important for plant defense against *B. cinerea*, impairment of miRNA production is likely to results in enhanced disease susceptibility to *B. cinerea* (Jin and Wu 2015).

For the *P. rapae/B. cinerea* sequential double stress condition, the cell wall biosynthesis genes *CSLA01* emerged as a candidate affecting the level of *B. cinerea* resistance after prior exposure to herbivory (Table 3). This gene was previously found to be up-regulated in response to *P. rapae* feeding (Coolen *et al.,* 2016). Furthermore, mutation of cellulose synthase genes *cesa4*, *cesa7* and *cesa8* was shown to enhance disease resistance against necrotrophic fungi such as *Plectosphaerella cucumerina* (Hernández-Blanco *et al.,* 2007; Coolen *et al.,* 2016; Davila Olivas *et al.,* 2016). Conversely, *B. cinerea* has been shown to downregulate the activity of plant cellulose synthases to increase virulence, highlighting the role of cellulose biosynthesis and breakdown in the interaction between *A. thaliana* and necrotrophs (Ramírez *et al.,* 2014). Additionally, the polygalacturonase gene *PG3* was found to be associated with a GWA- and fine mapping-verified locus linked to herbivory-mediated changes in the level of *B. cinerea* resistance. As a pectin-degrading enzyme PG3 is involved in many stages of plant development (Cao 2012) and since pectin degradation is also part of the infection strategy of necrotrophs, *B. cinerea* might make use of the plant's PGs for plant invasion. Indeed, deletion of the *B. cinerea PG2* gene was shown to result in delayed primary lesion formation and a strong reduction of virulence on tomato and broad bean (Kars *et al.,* 2005), and inhibition of PG proteins in *A. thaliana* was shown to increase resistance to *Fusarium graminearum* (Ferrari *et al.,* 2012).

For the drought/*B. cinerea* sequential double stress combination, four loci were confirmed with fine mapping pointing to the candidate genes *BCA3*, a plant invertase/pectin methylesterase inhibitor

gene, *KNAT6*, an exostosin gene, and an F-box associated ubiquitination effector family gene (Table 3). BCA3 was found to be induced at 24 h after inoculation in the drought/*B. cinerea* sequential double stress combination, suggesting a possible involvement in drought mediated plant responses to *B. cinerea* (Coolen *et al.,* 2016). *KNAT6* is known to be involved in plant flowering activated stress pathways that promote JA biosynthesis (Khan *et al.,* 2015), potentially influencing JA-induced defenses against *B. cinerea*. Furthermore, the plant invertase/pectin methylesterase inhibitor was previously described in having an important role in *B. cinerea* resistance, as overexpression was shown to restrict infection by *B. cinerea* (Lionetti *et al.,* 2007). Moderate drought stress, as we applied in this study, was previously shown to induce expression of plant invertase/pectin methylesterase inhibitors, potentially affecting *B. cinerea* in our setup (Clauw *et al.,* 2015).

Besides the 9 GWA loci that were confirmed by fine mapping, many of the other GWA loci showed a similar pattern of SNP-trait associations in the GWA and fine mapping approach, but because of the lack of power did not exceed the FDR-corrected threshold for significance (Figure S5). Below, we will name a few that are worthwhile mentioning. For instance, *RESISTANCE TO PSEUDOMONAS SYRINGAE PV MACULICOLA 1* (*RPM1*) *INTERACTING PROTEIN 13* (*RIN13,* AT2G20310), which emerged from the drought/*B. cinerea* sequential double stress data. RIN13 has been described to positively enhance RPM1 (NBS-LRR resistance protein) mediated resistance to *P. syringae* while restricting the RPM1-induced hypersensitive response (HR) (Boyes *et al.,* 1998; Al-Daoude *et al.,* 2005). This could benefit *B. cinerea* as the hypersensitive response induced by *P. syringae* pv. *tomato* DC3000 (*avrRMP1*) was shown to promote *B. cinerea* disease development in *A. thaliana* (Govrin and Levine 2000; Schouten *et al.,* 2007; Rossi *et al.,* 2011), suggesting that *RIN13* could be involved in HR-mediated disease development triggered by *B. cinerea*. Another candidate gene that emerged from the drought/*B. cinerea* sequential double stress data is the putative TIR-NBS-LRR class disease resistance gene *AT5G41750*. Also for the other stress combinations, this locus displays several SNP-trait associations, albeit non-significant (Figure S7). Furthermore, *AT5G41750* was found to be induced by *B. cinerea* infection and shows similar expression patterns when preceded by drought or *P. rapae* feeding, suggesting that this gene is responsive to *B. cinerea* independent of the prior stresses (Coolen *et al.,* 2016).

Concluding remarks

The aim of this study was to reveal gene candidates that are involved in the plants adaptive response during sequential stresses with *B. cinerea*. Our GWA mapping approach yielded several candidate genes, some of which with known or expected effects on the level of *B. cinerea* resistance. Future research will be directed towards elucidating the roles of these candidate genes in the modulation of the defense response to *B. cinerea*. Ideally, we will identify functions that enhance the plant's capacity to cope with multiple stresses at the same time or in sequence. To this end, we aim to test T-DNA knockout and knockdown mutants of the genes listed in Table 3 for their level of *B. cinerea* resistance under single and sequential double stress. The disadvantage of this strategy is that most T-DNA knockout mutants are in the Col-0 background, which due to its intermediate position in the

spectrum of phenotypes is not an ideal background for the anticipated experiments. Therefore, we also aim to test overexpressors of the genes listed in Table 3 in *B. cinerea* resistance assays.

Acknowledgements

We are thankful to Willem Kruijer for his help with GWA mapping and data handling, Dmitry Lapin for helping with fine mapping and Anja van Dijken, Christos Zamioudis and Colette Broekgaarden for their support and advice. This work was supported by the Netherlands Organization for Scientific Research (NWO) through the Dutch Technology Foundation (STW) STW Perspective Program 'Learning from Nature' [STW10988]. This study was part of a meta-analysis study that aimed to identify genes underlying resistance to multiple biotic and abiotic stresses (Thoen *et al.,* 2017). Furthermore, the stress combinations tested in this study were also tested in different sequence by Davila Olivas *et al.* (Davila Olivas *et al.,* 2017b).

Experimental procedures

Plant material

In this study 346 *A. thaliana* (L.) Heynh. accessions (Table S2) of the HapMap population were included, which are genotyped for 250K bi-allelic SNPs (Baxter *et al.,* 2010; Platt *et al.,* 2010; Chao *et al.,* 2012). After quality control and imputation this SNP-set was reduced to a set of 214.051 SNPs (Thoen *et al.,* 2017). Furthermore, a set of 431 MAGIC line RILs (Table S6) was included that are genotyped for 1,260 SNP markers (Kover *et al.,* 2009; Kover and Mott 2012).

Plant growing conditions

Seeds of *A. thaliana* were sown in cultivation containers filled with autoclaved river sand. Sand was supplied with half-strength Hoagland solution containing sequestrene as described (Wees *et al.*, 2013). In order to attain a high relative humidity (RH) for germination, cultivation containers were enclosed in a tray with water and covered with a transparent lid. Seeds were stratified for 2 days at 4 °C in the dark to ensure a homogeneous germination after which the tray was moved to a growth chamber with an 8-h day/16-h night rhythm, a temperature of 21°C, and a light intensity of 100 µmol m^{-2} sec^{-1}. After 8 days, the lids of the trays were slightly opened and gradually removed over a 2-day period to adjust to the 70% RH present in the growth chamber. Ten-day-old seedlings were transplanted to individual pots containing an autoclaved mixture of river sand and potting soil (1:1 (v:v)). Pots were supplied with water from the bottom up three times per week. At an age of 3 weeks the plants were supplied once with half-strength Hoagland solution.

Rearing of *P. rapae* caterpillars

P. rapae caterpillars were reared on cabbage plants (*Brassica oleracea* convar. capitata var. alba) under greenhouse conditions (24°C, with natural daylight). Butterflies were supplied with flowering plants such as *Lantana camara* for their (nectar) food. When flowers were scarce, additional food (solution of 20% honey and 10% sucrose) was offered to the butterflies. Inbreeding of the population was minimalized by adding wild butterflies and caterpillars from the Dutch Flevopolder to the existing population. First-instar (L1) larvae were placed on Arabidopsis leaves using a fine paint brush as described by Van Wees *et al.* (2013).

Cultivation of *B. cinerea*

B. cinerea strain B05.10 (Staats and Van Kan 2012) was grown on half-strength Potato Dextrose Agar (PDA; Difco BD Diagnostics, Franklin Lakes, N.J., U.S.A.) plates containing penicillin (100 µg ml^{-1}) and streptomycin (200 µg ml^{-1}) for 2 weeks at room temperature. Spores were collected, filtered through glass wool, and re-suspended in half-strength Potato Dextrose Broth (PDB; Difco BD Diagnostics) to a final density of 1 x 10^5 spores ml^{-1}. After a 3-h incubation period, the spores were used for inoculation by applying 5-µl droplets on *A. thaliana* leaves as described (Van Wees *et al.,* 2013).

Stress treatments and experimental design

Single and sequential double stress treatments were applied according to the schedule shown in Figure 1. Pre-treatment with drought was started when plants were 4 weeks old by withholding water for 7 days, after which plants were re-watered and allowed to recover for 1 day before plants were inoculated with *B. cinerea*. *P. rapae* pretreatment was started 1 day prior to *B. cinerea* inoculation by placing a single *P. rapae* L1 caterpillar on each plant and allowing it to feed for 1 day after which it was removed again. All plants were simultaneously inoculated with *B. cinerea* and were kept under 100% RH for the remaining time period until disease development was registered after 3 days of *B. cinerea* infection. As disease measurement, the occurrence of spreading lesions was recorded. Lesions that did not exceed the size of the inoculation droplet of 5-µl were scored as non-spreading. In total 6 leaves per plant were inoculated and the 3 treatments were screened simultaneously. For our GWA experiments, each treatment was performed on six plants (biological replicates) of each accession. Furthermore, plants were screened in 11 experiments of approximately 35 randomly chosen accessions and Col-0 was present in all experiments as a reference. For the QTL analysis experiments, 341 RILs were screened individually in two experiments.

GWA mapping

GWA mapping was performed on genotypic means that were calculated from the raw disease severity data (Supplementary Table S3). The original disease severity scores per plant of each of the 346 accessions (n = 6 plants per accession) were corrected for the variation within the 11

experiments (with ~35 randomly selected accessions each). This was done by subtracting the mean of each experiment from the scored values in that experiment. Subsequently, the corrected data were arcsine transformed to a normal distribution, as was described for this dataset in an meta-analysis by Thoen *et al.* (2017). Residuals were calculated by regressing the disease severity scores of the prior stress/*B. cinerea* treatments on the predicted disease severity scores of the single stress (*B. cinerea*), that were obtained from a linear regression (Table S3). The observed prior stress/*B. cinerea* scores minus the predicted observations from the *B. cinerea* single stress data represent the residual data. GWAS was performed by employing FaST-LMM software in R (Lippert *et al.,* 2011; Thoen *et al.,* 2017). A minor allele frequency (MAF) of > 0.05 was used together with an arbitrary threshold with a LOD ($-\text{Log}_{10}(p)$) score of 4.0 to determine SNP-trait associations of interest. Linkage disequilibrium (LD) was taken into account by including all SNPs within a 50-kb window surrounding the SNP of interest. Narrow sense heritability (h^2) was estimated using the 'heritability' R package (Kruijer *et al.,* 2015).

QTL mapping

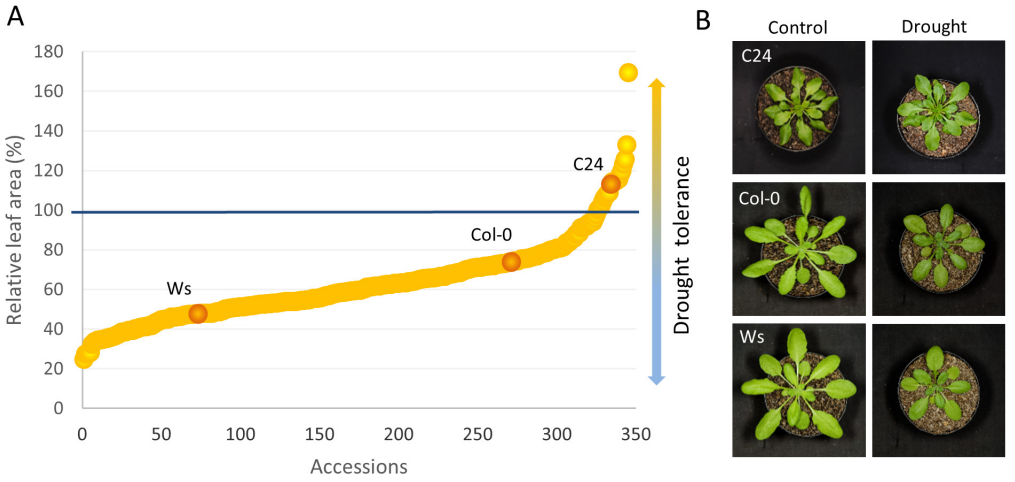
QTL mapping was performed on transformed raw disease severity data of 431 tested MAGIC line RILs. The original mean disease severity scores per plant were divided by 100 and transformed using an arcsine-square root transformation where 0.00 was replaced by 0.04 (Table S7). Residuals of the sequential double stress data regressed on the *B. cinerea* single stress were calculated for obtaining the relative effect of the sequential double stress on the single stress. QTL mapping was performed using publically available R-scripts for performing QTL mapping with the MAGIC line RILs (http://mtweb.cs.ucl.ac.uk/mus/www/magic/). Significant QTLs exceed the genome-wide permutation threshold with *p*-value < 0.1 (Kover *et al.,* 2009; Kover and Mott 2012).

Fine mapping

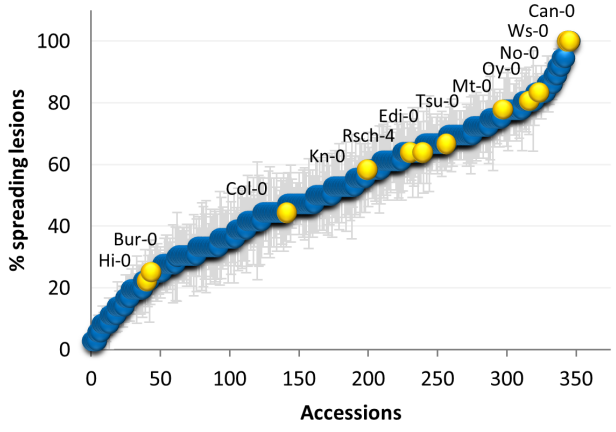
Fine mapping was performed using full genome sequences of 164 available accessions from the 1001 genomes project (http://signal.salk.edu/atg1001/3.0/gebrowser.php, Table S9). Genome sequences surrounding the SNP of interest with a 50-kb window were downloaded and aligned using Jalview (http://www.jalview.org/; Waterhouse *et al.,* 2009). Fine mapping was performed using GWA mapping phenotypic input data (Table S3). Furthermore, a MAF of > 0.05 and a Kruskal–Wallis test was used for obtaining significant, FDR-corrected, SNP-trait associations using R and the 'p. adjust' function with the Benjamini and Hochberg (BH) method (Benjamini and Hochberg 1995).

Supporting information

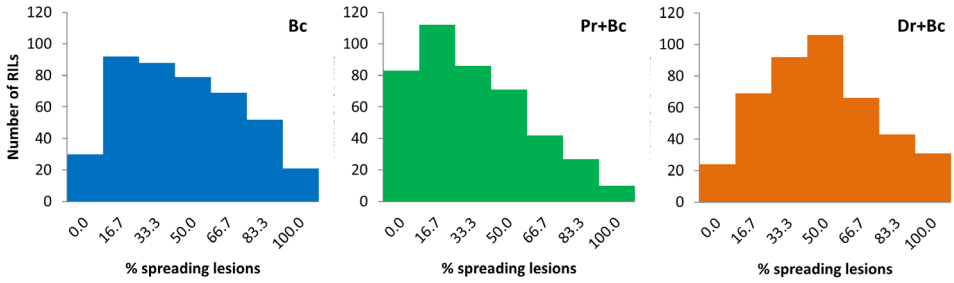
Additional Supporting information on this chapter.



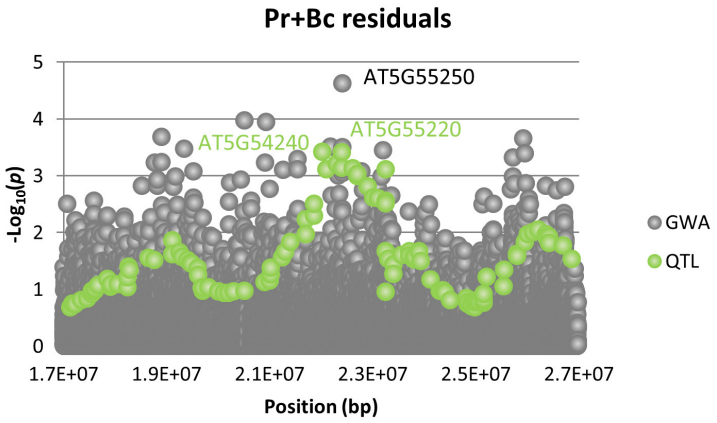
Supplemental Figure S1. Natural variation of drought stress tolerance amongst 346 *A. thaliana* accessions. A) Level of drought tolerance of all 346 tested *A. thaliana* HapMap accessions. Of each accession, photographs were taken from representative 4-week-old plants that were normally-watered or withheld from water for 7 days. Subsequently, total leaf area per plant was measured using the Adobe Design and Web Premium (CS6) Analysis tool. The leaf area of normally-watered plants is set at 100% for each accession (blue horizontal line), the leaf area of drought-stressed plants of each accession are plotted relative to that. B) Visual effects of drought stress on the drought-tolerant accession C24 (CS76106), and the drought-sensitive accessions Col-0 (CS76113) and Ws (CS28823), 7 d after water withheld.



Supplemental Figure S2. *B. cinerea* disease severity in 12 MACIG parental accessions within the population of 346 *A. thaliana* accessions of the HapMap collection. Dotplot showing the level of *B. cinerea* disease severity in average percentage of spreading lesions per accession (n = 6) at 3 d after pathogen inoculation. The 346 accessions of the *A. thaliana* HapMap collection are ordered on the x-axis according to their level of *B. cinerea* disease severity in the single stress condition. The parental accessions of the MAGIC RILs are indicated in orange. Error bars represent the Standard Error (SE).

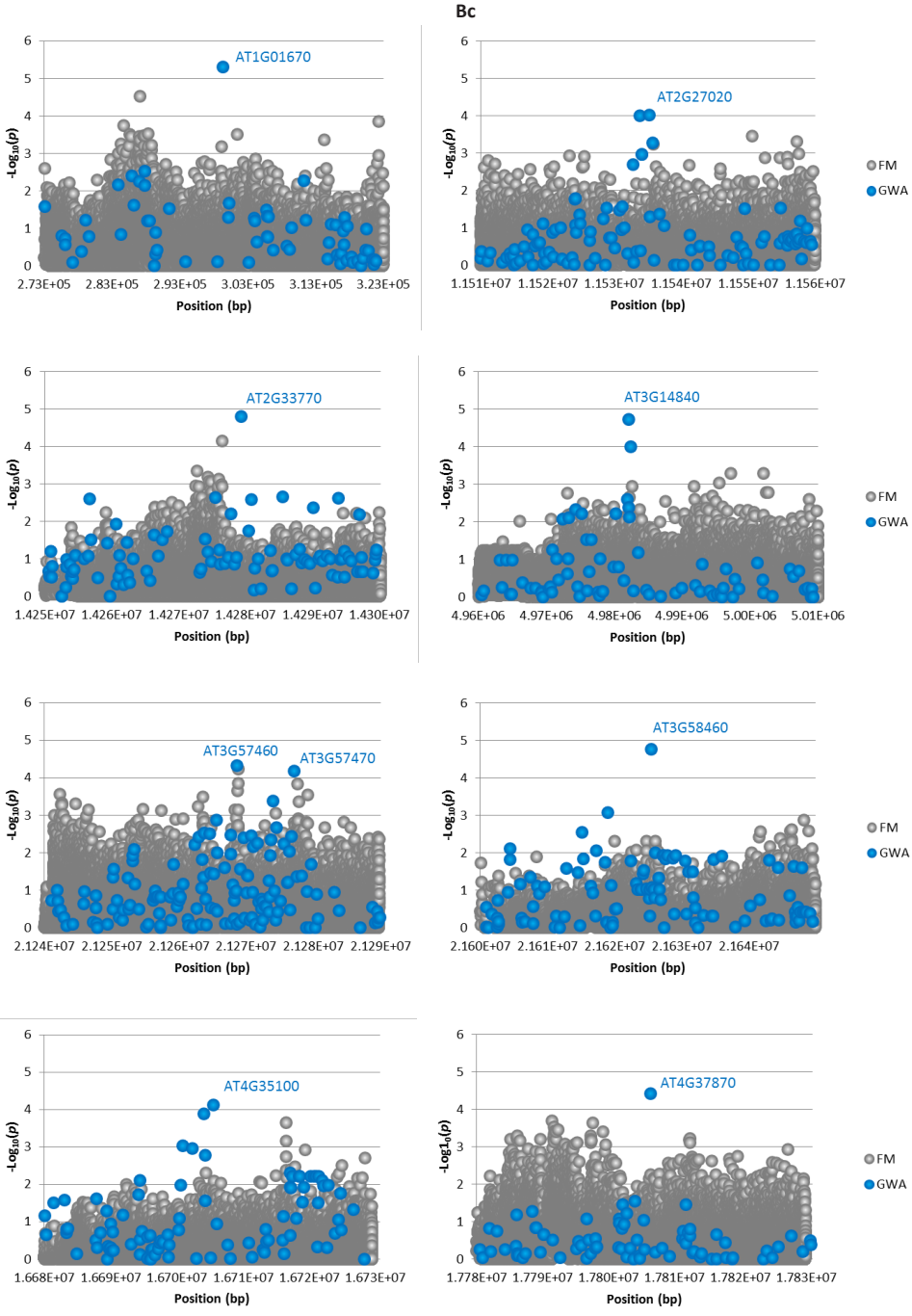


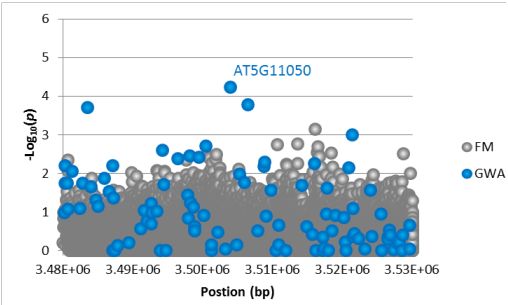
Supplemental Figure S3. Phenotypic variation in the level of *B. cinerea* resistance in the 431 RILs of the MAGIC population under conditions of single and sequential stress. The graphs show the frequency distribution of the disease severity scores of the 431 RILs (*n*=1) over the indicated disease severity classes. Disease severity classes reflect the average percentage of spreading lesions scored on 6 leaves per plant at 3 d after inoculation with *B. cinerea*. Bc, *B. cinerea* single stress (blue); Pr+Bc, prior herbivory by *P. rapae* followed by *B. cinerea* inoculation (green); and Dr+Bc, prior drought stress followed by *B. cinerea* inoculation (orange).



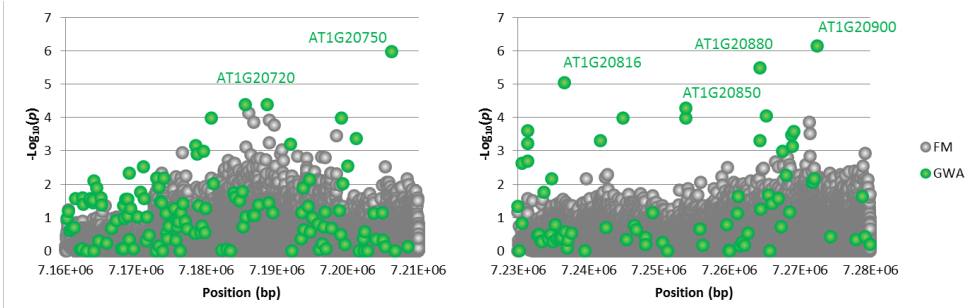
Supplemental Figure S4. GWA and QTL mapping results of the Pr+Bc residuals dataset around locus *AT5G55250*. Manhattan plots (grey dots) showing the $-\log_{10}(p)$ values of the SNP marker-trait associations from the GWA mapping of the Pr+Bc residual data around the GWA LOD ≥ 4.0 SNP-trait association at position AT5G55250 on *A. thaliana* chromosome 5 (x-axis; position in base pairs (bp)). QTL mapping results with the *A. thaliana* MAGIC lines are indicated with green dots. Genes indicated in green are significant SNP-trait associations obtained with QTL mapping.

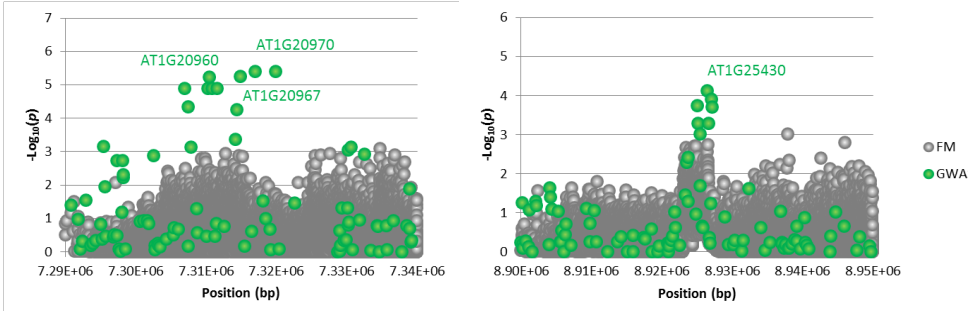
Figure S5. Non-significant fine mapping results of GWA SNP-trait associations. Manhattan plots for the fine mapping results (grey dots) showing the $-\log_{10}(p)$ values of the SNP marker-trait associations of the GWA loci for the Bc single stress (blue dots), Pr+Bc sequential double stress (green dots), Pr+Bc residual (light green dots), Dr+Bc sequential double stress (orange dots) and Dr+Bc residuals (yellow dots). On the x-axis the position in base pairs (bp) is shown. Genes indicated in blue, green, light green, orange and yellow show genes obtained from the GWA mapping SNP trait associations.

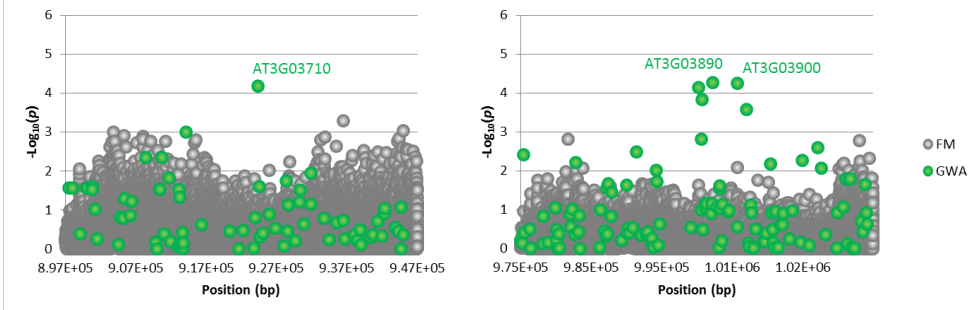


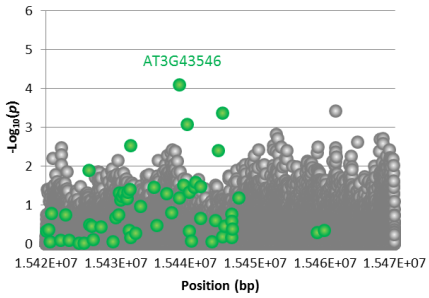

AT5G11050
-Log10(p)
Postion (bp)
3.48E+06
3.49E+06
3.50E+06
3.51E+06
3.52E+06
3.53E+06
FM
GWA

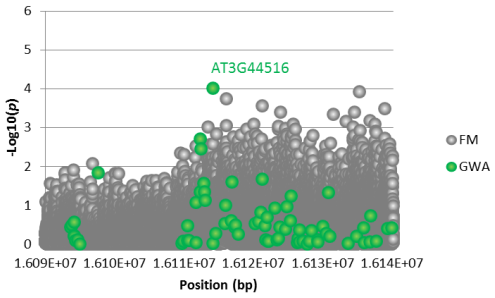
Pr+Bc

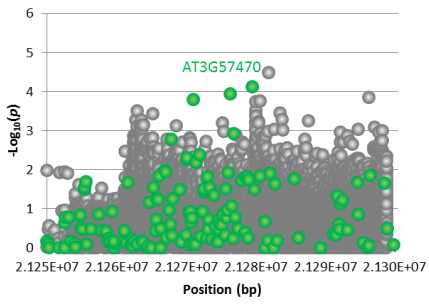

AT1G20750
AT1G20720
AT1G20900
AT1G20880
AT1G20816
AT1G20850
-Log10(p)
Position (bp)
7.16E+06
7.17E+06
7.18E+06
7.19E+06
7.20E+06
7.21E+06
7.23E+06
7.24E+06
7.25E+06
7.26E+06
7.27E+06
7.28E+06
FM
GWA

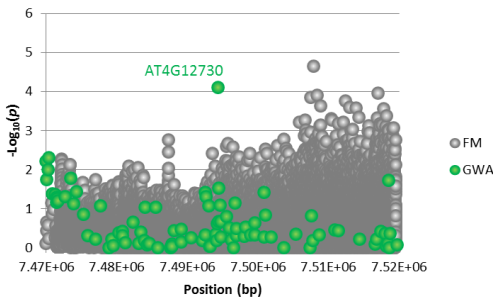

AT1G20970
AT1G20960
AT1G20967
AT1G25430
-Log10(p)
Position (bp)
7.29E+06
7.30E+06
7.31E+06
7.32E+06
7.33E+06
7.34E+06
8.90E+06
8.91E+06
8.92E+06
8.93E+06
8.94E+06
8.95E+06
FM
GWA

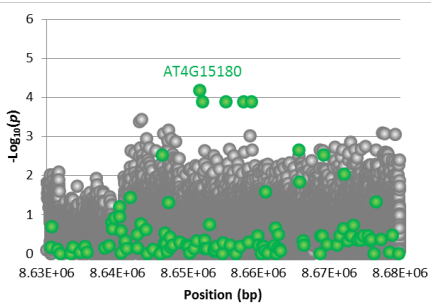

AT3G03710
AT3G03890
AT3G03900
-Log10(p)
Position (bp)
8.97E+05
9.07E+05
9.17E+05
9.27E+05
9.37E+05
9.47E+05
9.75E+05
9.85E+05
9.95E+05
1.01E+06
1.02E+06
FM
GWA

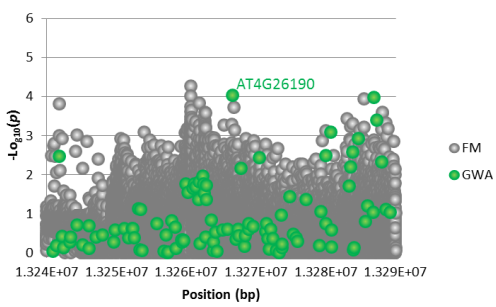

AT3G43546
-Log10(p)
Position (bp)

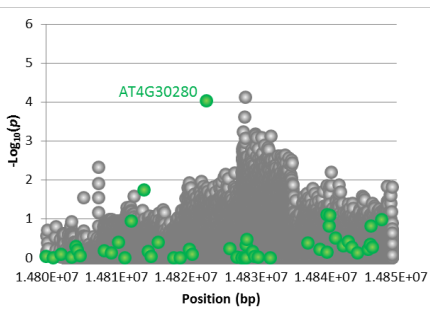

AT3G44516
-Log10(p)
Position (bp)
FM
GWA

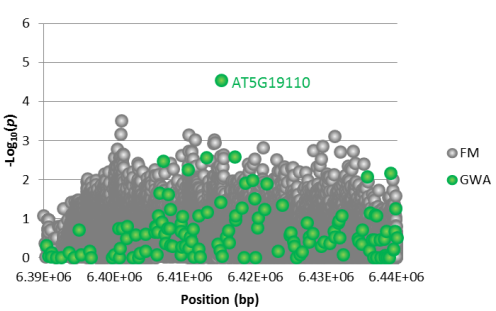

AT3G57470
-Log10(p)
Position (bp)

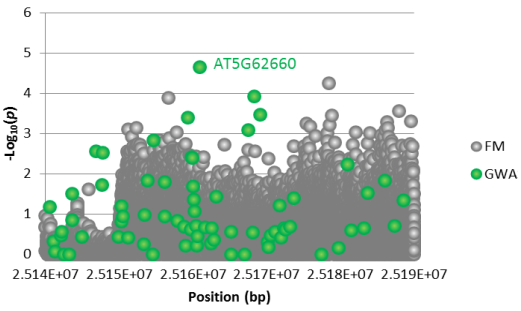

AT4G12730
-Log10(p)
Position (bp)
FM
GWA


AT4G15180
-Log10(p)
Position (bp)

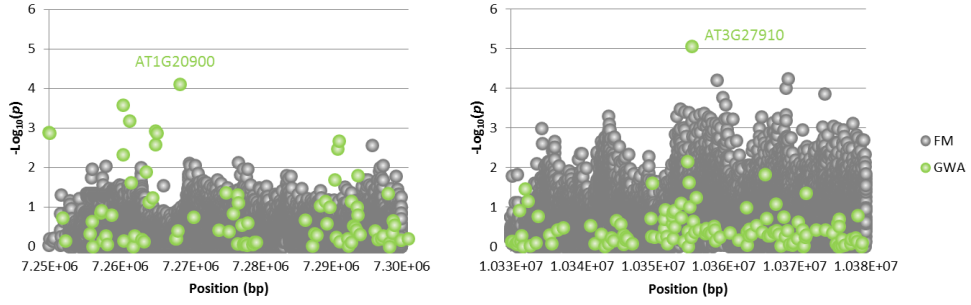

AT4G26190
-Log10(p)
Position (bp)
FM
GWA

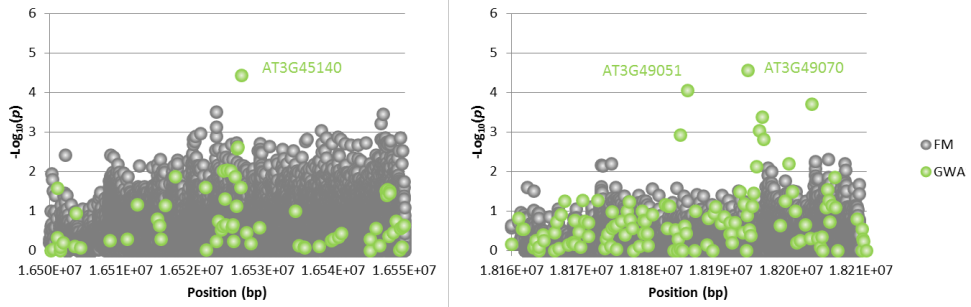

AT4G30280
-Log10(p)
Position (bp)

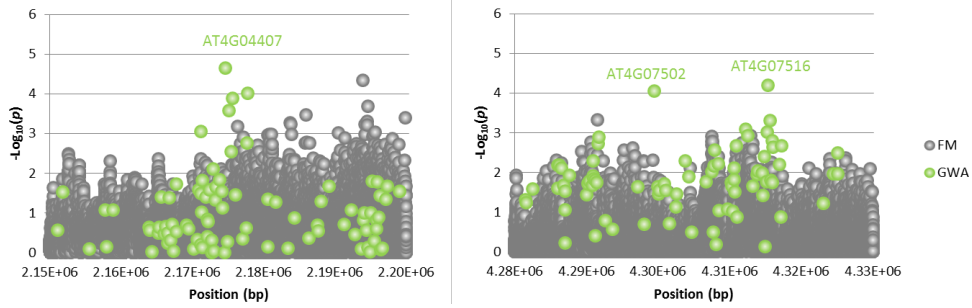

AT5G19110
-Log10(p)
Position (bp)
FM
GWA

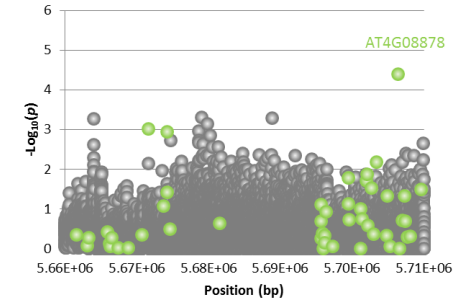


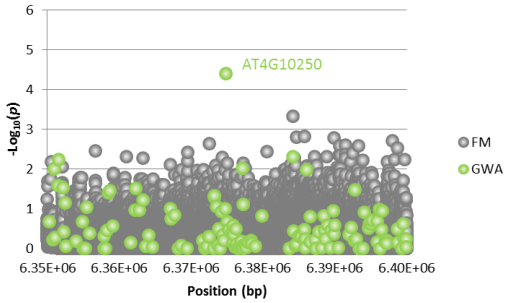
Pr+Bc residuals

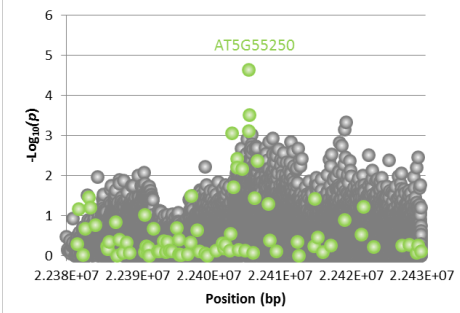




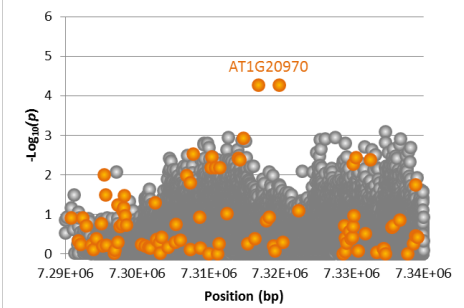


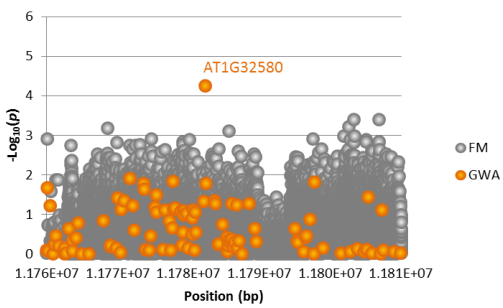

AT4G08878
-Log10(p)
Position (bp)
5.66E+06
5.67E+06
5.68E+06
5.69E+06
5.70E+06
5.71E+06

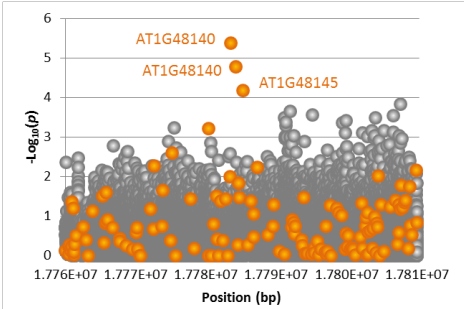

AT4G10250
-Log10(p)
Position (bp)
6.35E+06
6.36E+06
6.37E+06
6.38E+06
6.39E+06
6.40E+06
FM
GWA

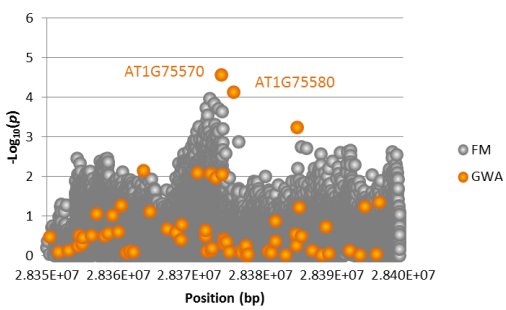

AT5G55250
-Log10(p)
Position (bp)
2.238E+07
2.239E+07
2.240E+07
2.241E+07
2.242E+07
2.243E+07
FM
GWA

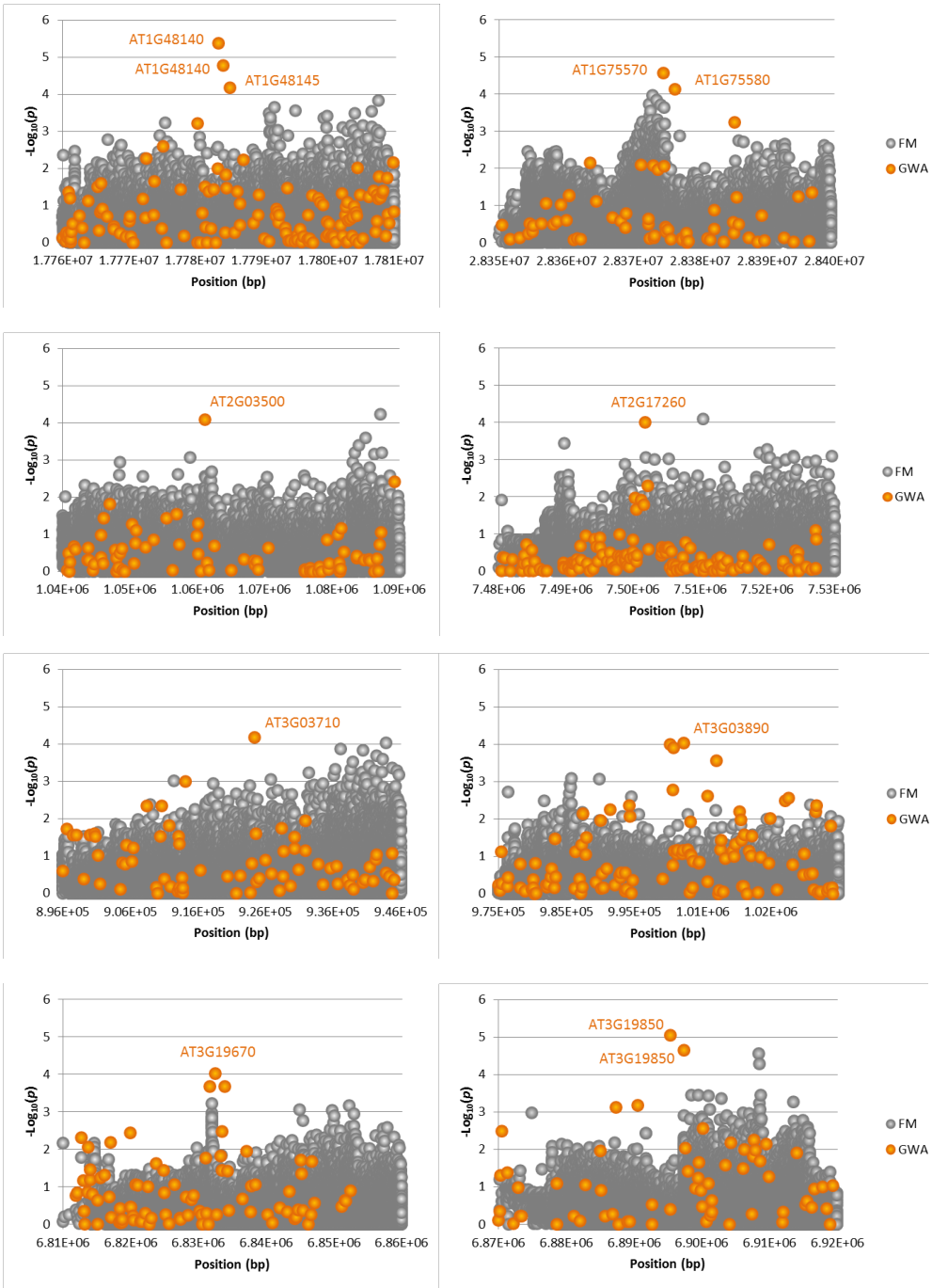
Dr+Bc

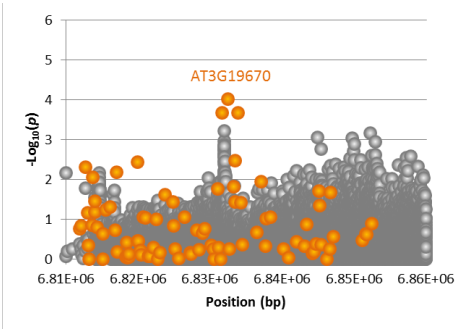

AT1G20970
-Log10(p)
Position (bp)
7.29E+06
7.30E+06
7.31E+06
7.32E+06
7.33E+06
7.34E+06

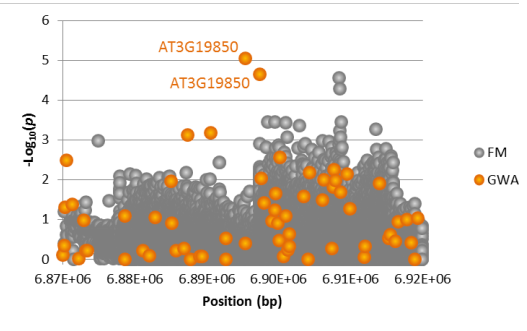

AT1G32580
-Log10(p)
Position (bp)
1.176E+07
1.177E+07
1.178E+07
1.179E+07
1.180E+07
1.181E+07
FM
GWA

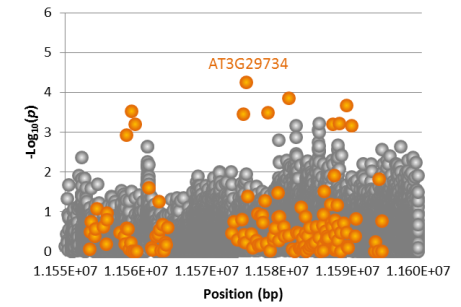

AT1G48140
AT1G48140
AT1G48145
-Log10(p)
Position (bp)
1.776E+07
1.777E+07
1.778E+07
1.779E+07
1.780E+07
1.781E+07

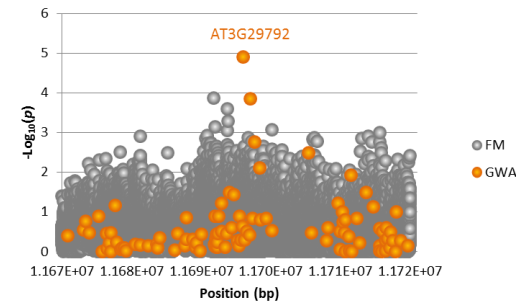

AT1G75570
AT1G75580
-Log10(p)
Position (bp)
2.835E+07
2.836E+07
2.837E+07
2.838E+07
2.839E+07
2.840E+07
FM
GWA

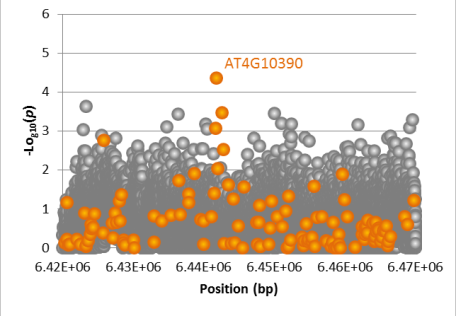

AT1G48140
AT1G48140
AT1G48145
AT1G75570
AT1G75580
AT2G03500
AT2G17260
AT3G03710
AT3G03890
AT3G19670
AT3G19850
AT3G19850
-Log10(p)
Position (bp)
FM
GWA

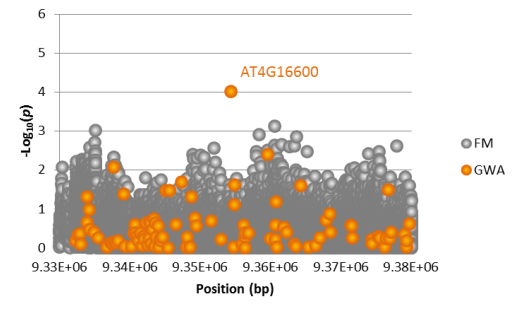

AT3G19670
-Log10(p)
Position (bp)
6.81E+06 6.82E+06 6.83E+06 6.84E+06 6.85E+06 6.86E+06

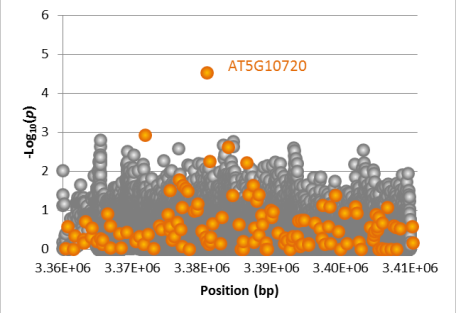

AT3G19850
AT3G19850
-Log10(p)
Position (bp)
6.87E+06 6.88E+06 6.89E+06 6.90E+06 6.91E+06 6.92E+06
FM
GWA

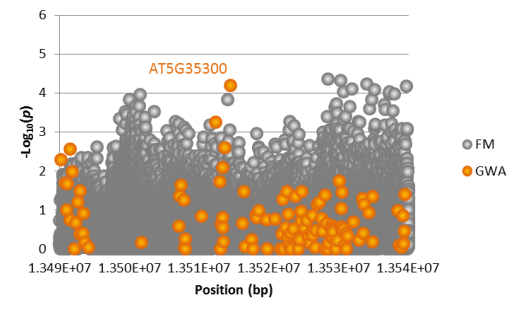

AT3G29734
-Log10(p)
Position (bp)
1.155E+07 1.156E+07 1.157E+07 1.158E+07 1.159E+07 1.160E+07

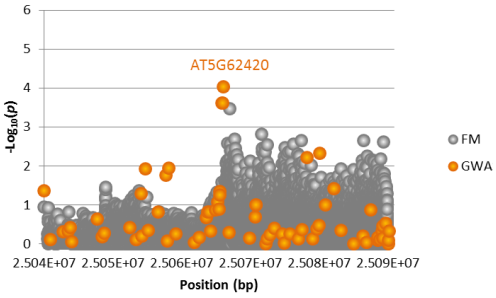

AT3G29792
-Log10(p)
Position (bp)
1.167E+07 1.168E+07 1.169E+07 1.170E+07 1.171E+07 1.172E+07
FM
GWA


AT4G10390
-Log10(p)
Position (bp)
6.42E+06 6.43E+06 6.44E+06 6.45E+06 6.46E+06 6.47E+06

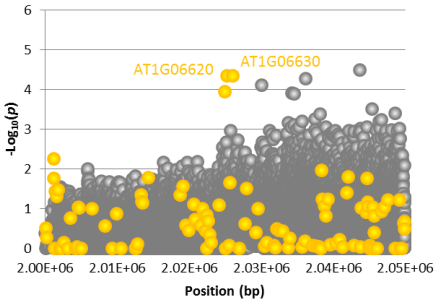

AT4G16600
-Log10(p)
Position (bp)
9.33E+06 9.34E+06 9.35E+06 9.36E+06 9.37E+06 9.38E+06
FM
GWA

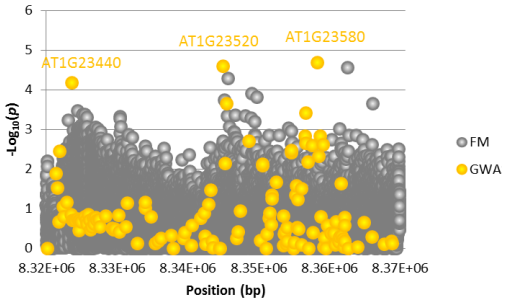

AT5G10720
-Log10(p)
Position (bp)
3.36E+06 3.37E+06 3.38E+06 3.39E+06 3.40E+06 3.41E+06

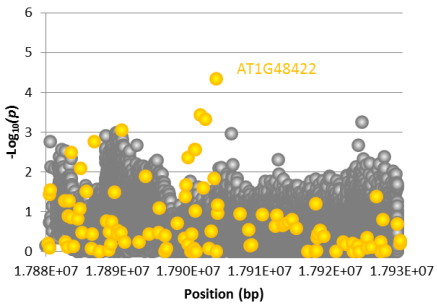

AT5G35300
-Log10(p)
Position (bp)
1.349E+07 1.350E+07 1.351E+07 1.352E+07 1.353E+07 1.354E+07
FM
GWA

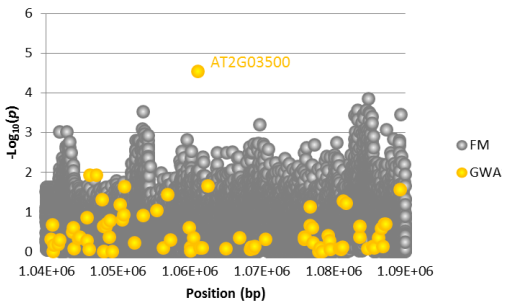


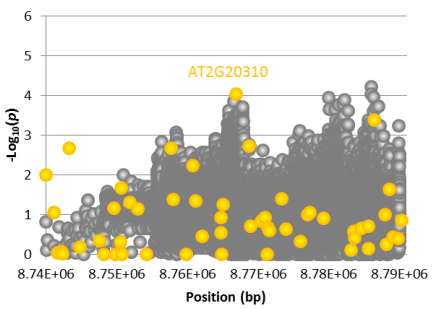
Dr+Bc residuals

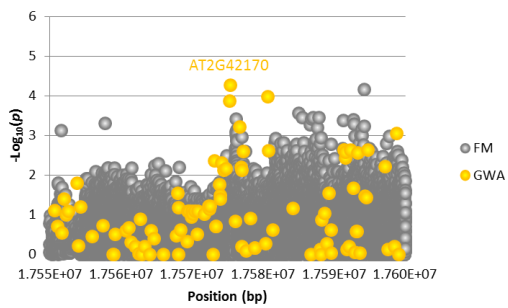


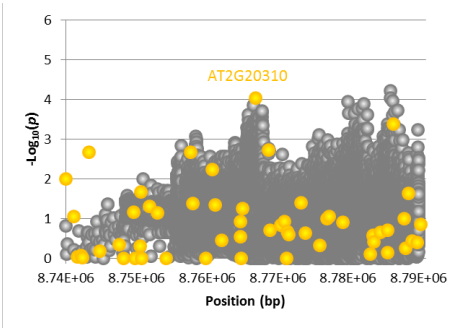


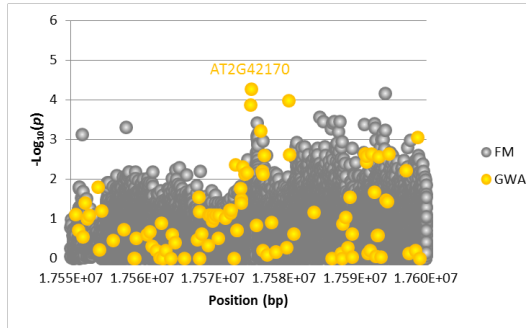


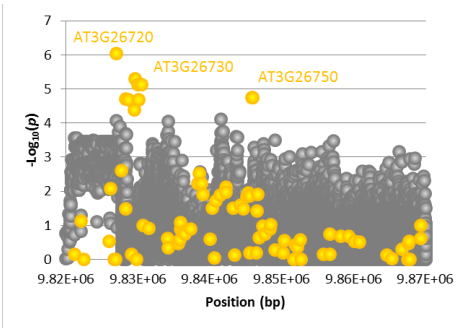


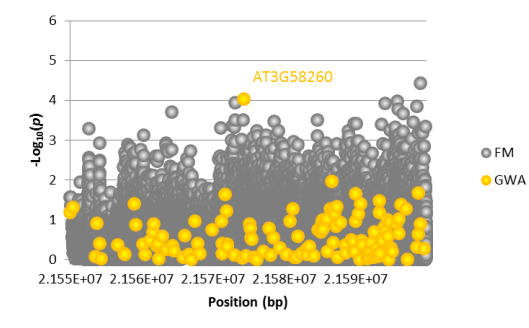


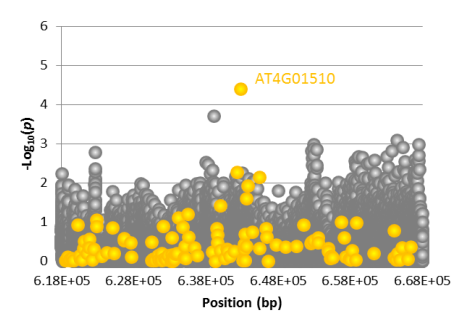


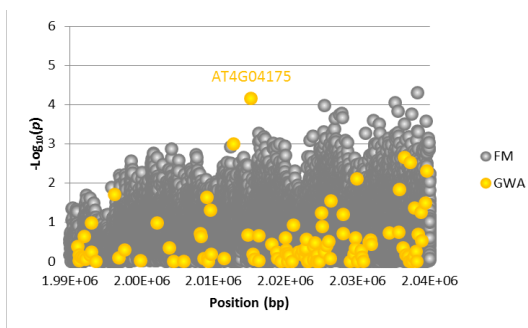

AT2G20310
-Log10(p)
Position (bp)
8.74E+06 8.75E+06 8.76E+06 8.77E+06 8.78E+06 8.79E+06

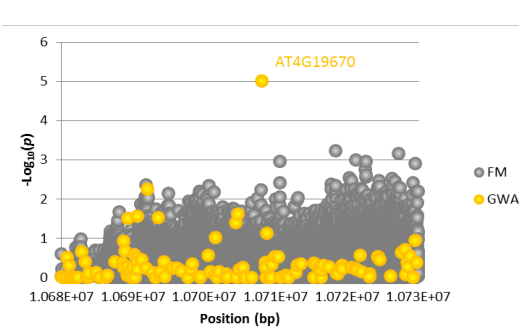

AT2G42170
-Log10(p)
Position (bp)
1.755E+07 1.756E+07 1.757E+07 1.758E+07 1.759E+07 1.760E+07
FM
GWA


AT3G26720
AT3G26730
AT3G26750
-Log10(p)
Position (bp)
9.82E+06 9.83E+06 9.84E+06 9.85E+06 9.86E+06 9.87E+06


AT3G58260
-Log10(p)
Position (bp)
2.155E+07 2.156E+07 2.157E+07 2.158E+07 2.159E+07
FM
GWA


AT4G01510
-Log10(p)
Position (bp)
6.18E+05 6.28E+05 6.38E+05 6.48E+05 6.58E+05 6.68E+05


AT4G04175
-Log10(p)
Position (bp)
1.99E+06 2.00E+06 2.01E+06 2.02E+06 2.03E+06 2.04E+06
FM
GWA


AT4G19670
-Log10(p)
Position (bp)
1.068E+07 1.069E+07 1.070E+07 1.071E+07 1.072E+07 1.073E+07
FM
GWA

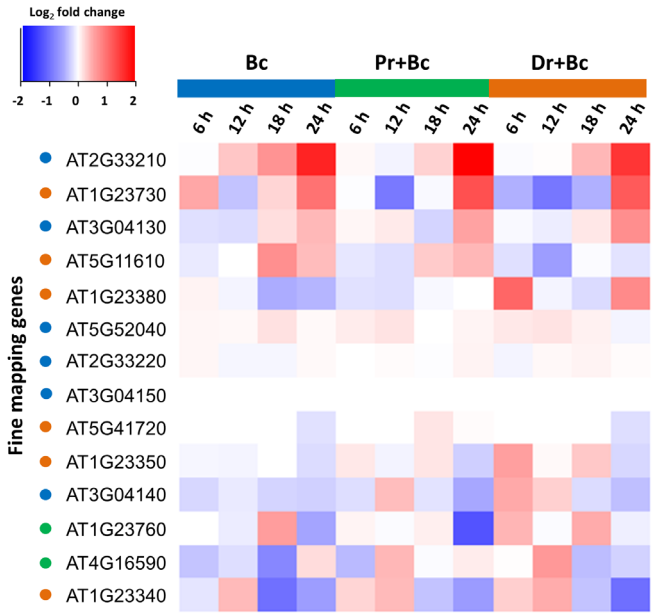


Figure S6. Expression patterns of fine mapping-confirmed candidate genes. Heatmap showing gene expression patterns of the 13 fine mapping-confirmed candidate genes (Table 3) with putative roles in the plant response to *B. cinerea* infection during single or sequential double stress conditions. Gene expression profiles are based on RNA-seq data obtained from Coolen *et al.* (2016) and represent the $\log_2$ fold-change in gene expression, with addition of a pseudocount of 0.0001, during the first 24 h after inoculation with *B. cinerea* in the single and sequential double stress treatments. Colored dots indicate association of gene with single or sequential double stress treatment. Bc (blue dot), *B. cinerea* single stress; Pr+Bc (green dot), *P. rapae*/*B. cinerea* sequential double stress; Dr+Bc (orange dot), drought/*B. cinerea* sequential double stress. The candidate genes were clustered using hclust. Blue–red color key show the change in gene expression level: -2.0 > $\log_2$ fold change >2.0.

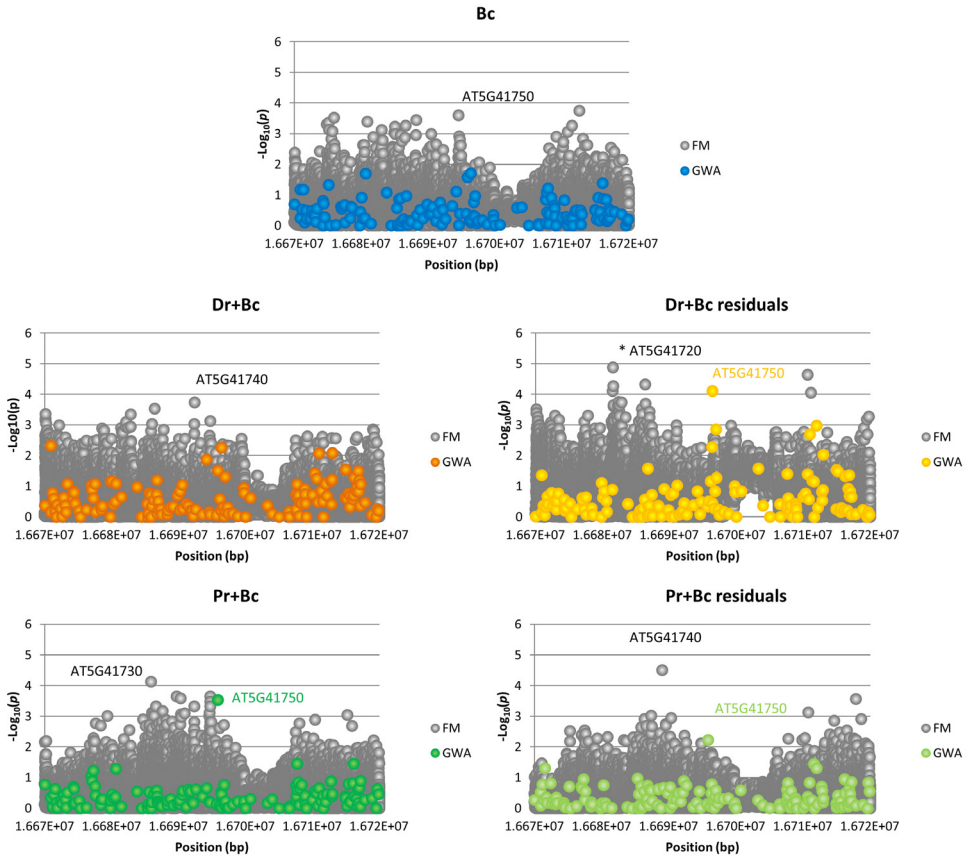


Figure S7. Fine mapping results of non-confirmed GWA SNP-trait associations at the TIR-NBS-LRR *R*-gene locus AT5G41750. Manhattan plots for the fine mapping results (grey dots) showing the $-\log_{10}(p)$ values of the SNP marker-trait associations of the GWA R-gene *AT5G41750* locus for the Bc single stress (blue dots), Pr+Bc sequential double stress (green dots), Pr+Bc residual (light green dots), Dr+Bc sequential double stress (orange dots) and Dr+Bc residuals (yellow dots). On the x-axis the position in base pairs (bp) is shown. Genes indicated in blue, green, light green, orange and yellow show SNP-trait associations obtained with GWA mapping and the genes indicated in black corresponds to the non-significant fine mapping associations. The significant (FDR-corrected) fine mapping SNP-trait association for the Dr+Bc residual data (Figure 4) is marked with an asterisk.

Supporting tables. https://www.dropbox.com/preview/Proefschrift%20Silvia/Proefschrift%20Silvia%20Coolen%20Supplemental%20Tables%20Chapter%203.xlsx?role=personal

Table S1. Leaf areas of 346 normally-watered control plants and drought-stressed plants that were witheld from watering for 7 days

Table S2. *B. cinerea* disease severity in 346 *A. thaliana* accessions of the HapMap collection under conditions of single and sequential stress

Table S3. Phenotypic input data for GWA mapping

Table S4. *A. thaliana* loci of of SNP-trait associations and underlying candidate genes within 50-kb window of each SNP

Table S5. Venn diagram data of shared genes between datasets presented in Figure 4

Table S6. *B. cinerea* disease severity in 431 *A. thaliana* RILs of the MAGIC lines under conditions of single and sequential stress

Table S7. QTL mapping phenotypic input data

Table S8. Significant QTL mapping SNP-trait associations

Table S9. Accessions used for fine mapping

Chapter 4

Genetic architecture of plant stress resistance: multi-trait genome-wide association mapping

Manus P. M. Thoen[1,2,3*], Nelson H. Davila Olivas[1*], Karen J. Kloth[1,2,3*], Silvia Coolen[4*], Ping-Ping Huang[5*], Mark G. M. Aarts[5], Johanna A. Bac-Molenaar[2,5], Jaap Bakker[6], Harro J. Bouwmeester[2], Colette Broekgaarden[4,7], Johan Bucher[7], Jacqueline Busscher-Lange[1,2,3], Xi Cheng[2], Emilie F. Fradin[2], Maarten A. Jongsma[3], Magdalena M. Julkowska[8,9], Joost J. B. Keurentjes[5], Wilco Ligterink[2], Corné M. J. Pieterse[4], Carolien Ruyter-Spira[2], Geert Smant[6], Christa Testerink[8,9], Björn Usadel[10], Joop J. A. van Loon[1], Johan A. van Pelt[4], Casper C. van Schaik[6], Saskia C. M. van Wees[4], Richard G. F. Visser[7], Roeland Voorrips[7], Ben Vosman[7], Dick Vreugdenhil[2], Sonja Warmerdam[6], Gerrie L. Wiegers[1,3], Joost van Heerwaarden[11], Willem Kruijer[11], Fred A. van Eeuwijk[11] and Marcel Dicke[1]

* These authors contributed equally to this work

New Phytologist 213, 1346-1362 (2017)

1. Laboratory of Entomology, Wageningen University and Research, PO Box 16, 6700 AA Wageningen, the Netherlands

2. Laboratory of Plant Physiology, Wageningen University and Research, PO Box 16, 6700 AA Wageningen, the Netherlands

3. Business Unit Bioscience, Wageningen Plant Research, Wageningen University and Research, PO Box 16, 6700 AA Wageningen, the Netherlands

4. Plant–Microbe Interactions, Department of Biology, Utrecht University, PO Box 800.56, 3508 TB Utrecht, the Netherlands

5. Laboratory of Genetics, Wageningen University and Research, PO Box 16, 6700 AA Wageningen, the Netherlands

6. Laboratory of Nematology, Wageningen University and Research, PO Box 8213, 6700 ES Wageningen, the Netherlands

7. Wageningen University and Research Plant Breeding, Wageningen University and Research, PO Box 386, 6700 AJ Wageningen, the Netherlands;

8. Section of Plant Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, PO Box 94215, 1090 GE Amsterdam, the Netherlands

9. Section of Plant Cell Biology, Swammerdam Institute for Life Sciences, University of Amsterdam, PO Box 94215, 1090 GE Amsterdam, the Netherlands

10. Institute for Biology I, RWTH Aachen University, Worringer Weg 3, 52074 Aachen, Germany

11. Biometris, Wageningen University and Research, PO Box 16, 6700 AA Wageningen, the Netherlands

Summary

Plants are exposed to combinations of various biotic and abiotic stresses, but stress responses are usually investigated for single stresses only. Here, we investigated the genetic architecture underlying plant responses to 11 single stresses and several of their combinations by phenotyping 350 *Arabidopsis thaliana* accessions. A set of 214 000 single nucleotide polymorphisms (SNPs) was screened for marker-trait associations in genome-wide association (GWA) analyses using tailored multi-trait mixed models. Stress responses that share phytohormonal signaling pathways also share genetic architecture underlying these responses. After removing the effects of general robustness, for the 30 most significant SNPs, average quantitative trait locus (QTL) effect sizes were larger for dual stresses than for single stresses. Plants appear to deploy broad-spectrum defensive mechanisms influencing multiple traits in response to combined stresses. Association analyses identified QTLs with contrasting and with similar responses to biotic vs abiotic stresses, and below-ground vs above-ground stresses. Our approach allowed for an unprecedented comprehensive genetic analysis of how plants deal with a wide spectrum of stress conditions.

Introduction

In nature, plants face variable environments that impose a wide range of biotic and abiotic stresses. These include, for example, below-ground and above-ground stresses, stresses imposed by unicellular and multicellular organisms, and short- and long-lasting stresses. Under natural conditions, these stresses do not occur in isolation but are commonly present simultaneously (Rizhsky *et al.,* 2004; Bergelson and Roux 2010; Mittler and Blumwald 2010; Vile *et al.,* 2012; Prasch and Sonnewald 2013; Rasmussen *et al.,* 2013; Kissoudis *et al.,* 2014; Rivero *et al.,* 2014; Sewelam *et al.,* 2014; Suzuki *et al.,* 2014). Thus, plants are under strong selection to adapt to local conditions and have evolved sophisticated mechanisms to withstand multiple adverse environmental conditions(Howe and Jander 2008; Bergelson and Roux 2010; Pieterse *et al.,* 2012; Stam *et al.,* 2014; Brachi *et al.,* 2015; Julkowska and Testerink 2015; Kerwin *et al.,* 2015). Yet, investigating this in a targeted experimental way is a major challenge owing to the complexity of multiple stress exposure. To gain an insight into the adaptation of plants to the wide variety of stress-inducing conditions they face, genetic variation and mechanisms underlying stress resistance should be studied (Alonso-Blanco *et al.,* 2009; Brachi *et al.,* 2015; Kerwin *et al.,* 2015). The responses of plants to stresses have traditionally been investigated for individual stresses (Howe and Jander 2008), but the research focus is currently shifting towards plant responses to combinations of stresses (Holopainen and Gershenzon 2010; Pierik and Testerink 2014; Stam *et al.,* 2014; Suzuki *et al.,* 2014; Kissoudis *et al.,* 2015). The emerging picture is that responses to stress combinations cannot be predicted reliably from the responses to individual stresses (De Vos *et al.,* 2006b; Makumburage *et al.,* 2013). For instance, the majority of transcriptional responses of Arabidopsis to combinations of two abiotic stresses could not be predicted from responses to the individual stresses (Rasmussen *et al.,* 2013). Moreover, phenotype expression in response to two biotic stresses could not be predicted on the basis of existing information regarding interactions between underlying signaling pathways (De Vos *et al.,* 2006b). Phytohormones are major players in

a signaling network, mediating responses to both biotic and abiotic stresses (Pieterse *et al.,* 2009). For instance, chewing insect herbivores particularly elicit the jasmonic acid (JA), abscisic acid (ABA) and ethylene (ET) signaling pathways; phloem-sucking insects and biotrophic microbial pathogens particularly elicit the salicylic acid (SA) pathway; and drought elicits the ABA pathway (Pieterse *et al.,* 2009). The phytohormonal responses exhibit extensive crosstalk, resulting in specific changes in plant phenotype in response to individual stresses(De Vos *et al.,* 2005; Pieterse *et al.,* 2012).

In plant breeding, resistance and tolerance to multiple stresses are a common selection target (Braun *et al.,* 1996). A well-known strategy to achieve resistance and tolerance is by evaluation of candidate varieties in multi-environment trials, that is, field trials at multiple locations during several years (Van Eeuwijk *et al.,* 2010; Malosetti *et al.,* 2013). In such trials, multiple stresses can occur, but their occurrence and the intensity with which they occur are not guaranteed and, therefore, plant breeders developed the concept of managed stress trials in which specific and well-defined stress conditions are imposed for a single stress or a small number of stresses (Cooper and Hammer 1996; Cooper *et al.,* 2014). Recently, the urge to manage environmental factors even more precisely has led to the development of phenotyping platforms, where, again, mainly single stresses are investigated (Fiorani and Schurr 2013; Granier and Vile 2014; Kloth *et al.,* 2015).

Most studies, outside plant breeding, that have examined plant responses to multiple stresses included only one or a few genotypes (Holopainen and Gershenzon 2010; Rasmussen *et al.,* 2013; Pierik and Testerink 2014; Stam *et al.,* 2014; Suzuki *et al.,* 2014; Kissoudis *et al.,* 2015). To obtain a further understanding of the genetic architecture of complex traits such as plant adaptation to a diversity of stresses, extensive study of the natural genetic variation within a species is instrumental. Genome-wide association (GWA) analysis is an important tool for this, requiring a large number of well-genotyped plant accessions. Yet, although the interest in natural variation and GWA mapping is rapidly increasing (Wijnen and Keurentjes 2014; Ogura and Busch 2015), a large-scale evaluation of natural genetic variation in resistance of plants to the full diversity of stresses to which they are exposed, including pathogens, herbivores and abiotic stresses and their interactions, has not been done to date. To elucidate the genetic architecture of plant stress resistance, an integrated approach is needed that models the genetics of responses to a range of single and combined stresses, including the interaction between those responses. Here, we have adopted a comprehensive and integrated approach to investigate the genetics underlying plant responses to 15 carefully standardized single stresses or stress combinations (Table 1), making use of a global population of 350 Arabidopsis accessions that have been genotyped for 214 000 single nucleotide polymorphisms (SNPs) (Baxter *et al.,* 2010; Li *et al.,* 2010a). The standardization of these 15 stress conditions is an important element of the study, because it allows for phenotyping of well-defined stress responses. We developed a tailored multi-trait GWA analysis that allowed the identification of candidate genes associated with plant responses to multiple stresses that were validated by gene expression and mutant analyses.

Materials and Methods

Arabidopsis thaliana population

In this study we included 350 *Arabidopsis thaliana* (L.) Heynh. accessions from the HapMap population (http://bergelson.uchicago.edu/wp-content/uploads/2015/04/Justins-360-lines.xls). The HapMap population has been genotyped for 250 000 bi-allelic SNPs (Baxter *et al.,* 2010; Platt *et al.,* 2010; Chao *et al.,* 2012) and after quality control and imputation this SNP set was reduced to a set of 214 051 SNPs.

Definition of the target traits

For every experiment, the target traits were derived from the individual plant data using the following strategy. First, when residuals deviated from normality, a logarithmic, arcsine or square root transformation was applied to the original observations. Second, genotypic (accession) means for each treatment were calculated using a mixed model to account for design effects. Different mixed models were used in the experiments, reflecting the different designs. In all cases, accession effects were modeled as fixed, and the accession means were the best linear unbiased estimator (BLUE) of these effects. Third, for traits measured in treatment and control conditions, differences or residuals (when regressing treatment on control values) were defined, in order to obtain a measure of stress tolerance that was corrected for the expression of the same trait under control conditions. Finally, within each experiment, the traits were replaced by the first principal component if the latter explained more than half of the variation in all traits in this experiment; in all other cases, the original traits were retained. An overview of final traits and their corresponding sections in the Supporting Information Methods can be found in Table 1. In case of replacement by the first principal component, original traits and the variance explained by the first principal component are listed (Supporting Information Methods Tables M1–M5 in Methods S1–S3, S7 & S9). In total, phenotypic data for 73 individual traits were obtained by 10 different research groups. All calculations were performed in R, unless stated otherwise. Mixed-model analysis was performed with the R package ASREML (Butler *et al.,* 2009). In all equations, the term *E* denotes residual error. All other terms represent fixed effects unless stated otherwise. A colon (:) is used to define interactions between terms.

Statistics

Genetic correlation networks and heritability. Pairwise marker-based genetic correlations between traits, genomic correlations, were estimated using a multi-trait mixed model (MTMM; Korte *et al.,* 2012). Residuals were assumed to be uncorrelated for traits that were measured on different plants. For some pairs of traits, the likelihood was monotone, which can also occur in single-trait mixed models (Kruijer *et al.,* 2015). In this case, the genetic correlation was estimated by the (Pearson) correlation between the univariate G-BLUPs (De Los Campos *et al.,* 2013) estimated for these traits. A network between predefined groups of traits was constructed by connecting groups whose average genetic correlation across pairs of traits was > 0.2.

Narrow sense heritability (Table S1) was estimated using the mixed model $Y_i=\mu+A_i+E_i$ where Y_i represents the phenotypic means of accessions (i=1,..,350), and A_i and E_i are random genetic and residual effects. The vector of additive genetic effects follows a multivariate normal distribution with covariance $\sigma_A^2 K$,K being a marker-based relatedness matrix. The residual errors are independent, with variance σ_E^2. We obtained restricted maximum likelihood (REML) estimates of σ_A^2 and σ_E^2, estimated heritability as $h^2 = \sigma_A^2/(\sigma_A^2 + \sigma_E^2)$. This is an estimate of narrow-sense heritability, as the model for the genetic effects only captures additive effects, and σ_E^2 is the sum of environmental and non-additive genetic effects (see e.g. (Kruijer *et al.,* 2015)).

Multi-trait mixed models. Following Zhou and Stephens (2014), we assume the MTMM, $Y=XB+G+E$, with Y being the genotypes by traits (n x p) matrix of phenotypic observations. The terms XB, G and E stand for, respectively, the fixed effects (including trait-specific intercepts and SNP effects) and the random genetic and environmental effects. G follows a zero mean matrix variate normal distribution with row-covariance (marker-based kinship) matrix K and column (trait) covariance matrix V_g. V_g is a $p\ x\ p$ matrix modeling the genetic correlations between traits. This is equivalent to $g = vec\ (G)$ (the vector containing the columns of G) being multivariate normal with a covariance matrix defined by the Kronecker product $V_g \otimes K$ (Zhou and Stephens 2014). Similarly, $vec(E)$ follows a zero mean normal distribution with covariance $V_e \otimes I_n$, where V_e accounts for the non-genetic correlations between traits.

Factor-analytic models. As V_g and V_e contain a total of $p(p + 1)$ parameters, the MTMM becomes difficult to fit for > 10 traits (Zhou and Stephens 2014). For V_g we therefore assumed a factor analytic model, which is well known in the context of quantitative trait locus (QTL) mapping for experimental populations with limited numbers of markers (Boer *et al.,* 2007), but has not been used in the context of multivariate GWA studies (GWAS). As almost all traits were derived from measurements on different plants, a diagonal model $Ve=diag(\sigma_{e,1}^2,...,\sigma_{e,p}^2)$ was chosen for the environmental covariances. For V_g, a second-order factor analytic structure was chosen $V_g = \sigma_g^2(\lambda\lambda^t+diag(\tau_1^2,...,\tau_p^2))$, where σ_g^2 represents a scale parameter, the magnitude of genetic effects, the $p\ x\ 2$ matrix λ contains the trait-specific scores belonging to the factor analytic part of the model that provides a rank two variance-covariance structure between traits, and $diag(\tau_1^2,...,\tau_p^2)$ provides trait-specific residual genetic variances (Piepho 1997; Meyer 2009). The model was fitted with the R package ASRREML (Butler *et al.,* 2009).

Compressed kinship. Factor analytic models have been successfully applied to experimental populations with a simple genetic relatedness structure (Boer *et al.,* 2007; Alimi *et al.,* 2013; Malosetti *et al.,* 2013), but currently available software could not perform REML estimation for the HapMap population. The kinship matrix was therefore replaced with a compressed kinship matrix (Bradbury *et al.,* 2007; Zhang *et al.,* 2010c), modeling the genetic relatedness between a number of internally homogeneous groups. Assuming there are m such groups, containing $n_1,...,n_m$ accessions each, the original kinship matrix K is replaced by ZK_cZ^t, where K_c is the kinship matrix for the groups, and Z is the $n\ x\ m$ incidence matrix assigning each of the n accessions to one of the m groups. The groups were

created by a procedure that restricted the marker data to be linear combinations of environmental covariates representing the conditions at the place of origin of the accessions, as explained later.

Compressed kinship was calculated as the average kinship within genetic groups. Genotypes were assigned to k genetic groups by performing Ward clustering based on the squared Euclidean distance along the first $k-1$ principal components calculated from a matrix of standardized SNP scores, followed by cutting the resulting dendrogram into k distinct clusters (Van Heerwaardena *et al.,* 2012; Odong *et al.,* 2013; Van Heerwaarden *et al.,* 2013).

The use of a compressed kinship matrix requires a choice of the degree of compression, as determined by the number of genetic groups over which the individual kinship is averaged. This choice needs to balance the gain in computational efficiency with model fit (Zhang *et al.,* 2010c) and the ability of the compressed matrix to capture the correlation between genetic dissimilarity and phenotypic differences, which is ultimately the reason for including a kinship matrix in the association model. There are currently no standard methods to determine the optimum degree of compression, at least not when used in a multi-trait setting. We determined the appropriate degree of compression for each association model based on the model likelihood, convergence and correspondence between kinship and phenotypic and geographical similarity. The latter was quantified as the Frobenius norm of the difference between the complement of the compressed kinship matrix, expanded to a block matrix of full rank, and the Euclidean distance matrix of phenotypic traits or geographic coordinates. We considered a range of four to 100 groups. Correspondence with phenotypic and geographical dissimilarity increased steeply from four to 35 groups, after which correspondence with geographic distance increased more slowly and the correspondence with phenotypic distance showing a local decrease until 58 groups. Model likelihood was relatively stable above four groups, but convergence was erratic depending on the modeled contrasts. For each model the number of groups was therefore chosen to be the minimum number of groups needed to achieve a degree of correspondence approximating that found at 35 groups, under condition of model convergence.

Multi-trait GWAS. Traits (columns of Y) were standardized. Along the genome, MTMMs of the type $Y=XB+G+E$ were fitted with initially for each marker trait-specific QTL effects $\beta_1,\ldots,\beta_p$ (contained in B). To identify general QTLs with trait-specific effects, for individual markers, the null hypothesis $\beta_1=\beta_2=\ldots\beta_p=0$ was tested by a Wald test against the alternative hypothesis that at least one of the trait-specific effects was nonzero (Zhou and Stephens 2014). To identify consistent QTLs, the null hypothesis $\beta_1=\beta_2=\ldots\beta_p=\beta\neq0$ was tested. To identify potentially adaptive QTLs, contrasts defined on the trait-specific QTL effects were tested. For example, suppose the first $p1$ of the full set of p traits represents responses measured under abiotic stresses, while the second traits represent responses under biotic stresses. A contrast can now be defined to test the hypothesis of whether the QTL effect for abiotic stresses differs from that for biotic stresses: $\beta_1=\beta_2=\ldots\beta_{p1}=\alpha_{abiotic}$;$\beta_{p1+1}=\beta_{p1+2}=\ldots\beta_p=\alpha_{biotic}$ and H_0:$\alpha_{abiotic}=\alpha_{abiotic}$ vs H_a:$\alpha_{abiotic}\neq\alpha_{biotic}$. For the Wald test for the hypothesis $\beta_1=\cdots=\beta_p$ we first fit the MTMM $Y=XB+G+E$ with XB only containing trait-specific means $\mu_1,\ldots,\mu_p$, and then we test hypotheses on the marker effects. The contrast is defined

through a partitioning of the traits in two groups (e.g. resistance against biotic or abiotic stress). Using the R package ASREML (Butler *et al.,* 2009) we perform Wald tests for the following hypotheses:

1 H_0:β=0, in the constrained model β_1=...=β_p=β.

2 H_0:α_1=α_2, in the constrained model where α_1 is the effect on all traits in the first group, and α_2 is the effect on traits in the second group.

Simulations to compare power for full MTMM, contrast MTMM and univariate analysis. We further compared the different Wald tests using simulations, described in more detail in Methods S12. Specifically, we compared the performance of the general MTMM (i.e. testing the hypothesis β_1=β_2=... β_p=0) with the MTMM used for the contrasts (i.e. H_0:α_{group1}=α_{group2}, where, within two predefined groups of traits, all SNP effects equal α_{group1} and α_{group2}, respectively). We simulated phenotypic data for given genotypic data, either assuming the SNP effects were positive (but not equal) within one group of traits and negative for the other (scenario A), or choosing the sign of each SNP effect randomly (scenario B). The simulation results as presented in Fig. S11 (see later) clearly indicate that the Wald test for the contrast has superior power under scenario A, while the general MTMM performs best under scenario B. In both cases, univariate analysis of the trait with the highest heritability is outperformed by at least one of the MTMM analyses. As a consequence, univariate GWAS and GWAS with the general and contrast MTMM give different rankings of SNPs.

Selecting candidate genes

A significance threshold of $P < 0.0001$ was chosen after implementation of genomic control (see below in the section 'Correction for genomic inflation'). For MTMM this resulted in 43 SNPs meeting this criterion. Such a threshold of 0.0001 is not uncommon in studies involving single-trait GWAS (e.g. (El-Soda *et al.,* 2015; Van Rooijen *et al.,* 2015; Kooke *et al.,* 2016). Given the total number of SNPs analyzed (i.e. 199 589 SNPs having a minor allele frequency > 0.05) and under the null hypothesis of no QTLs and independence of the markers, we arrive at a naïve estimate for the expected number of false positives of c. 20, which is considerably smaller than the 43 SNPs with $P < 0.0001$ recorded in the full MTMM, suggesting that about half of the significant SNPs must be true positives. Furthermore, following the procedure described by Benjamini and Hochberg (1995), we estimated the false discovery rate to be 0.45, a number very comparable to our naive estimate earlier. SNPs within a 20 kb region were considered to be part of one linkage disequilibrium (LD) block. This resulted in 30 genomic regions. For presentation purposes, each LD block was represented in figures and heat maps by the SNP with the strongest (absolute) effect, on average, across all traits. For the GWA contrast analyses, the same procedure was followed to define LD blocks and representative SNPs.

Correcting for genomic inflation

The Wald test is known to suffer from some inflation (Zhou and Stephens 2014), which we correct for using genomic control (Devlin and Roeder 1999; Devlin *et al.,* 2001), which divides the observed test statistics $T_1,...,T_p$ by the genomic inflation factor. For both the unconstrained MTMM and the MTMM for contrasts described earlier, we observed inflation for small as well as large *P*-values (i.e. also more *P*-values close to 1 than expected). Consequently, the usual genomic control procedures based on the observed vs expected median of test statistics gave overly optimistic inflation factors. We therefore applied an alternative genomic control procedure, in which we regress the observed $-\log_{10}(P)$ values on the expected ones, and correct the observed $-\log_{10}(P)$ values for the slope. The genomic inflation factor was 1.24 for the full MTMM, with similar values for the other MTMM analyses (between 1.07 and 1.38). For the full MTMM without correction for population structure (i.e. taking the kinship to be the identity matrix), the inflation factor was 2.36.

Results

The phenotypic response of a population of 350 Arabidopsis accessions to an extensive set of stress-inducing conditions was quantified relative to the respective control treatments. Correcting for the respective control means that in the residual signal for a trait, effects of earliness, flowering time, general robustness, vigor, and so on, have been removed already. Therefore, the traits as analyzed represent a kind of stress *per se* response from which all kinds of disturbances have already been eliminated. Thirty traits, including, for example, root length, number of damaged leaves or number of pathogen-inflicted spreading lesions (Table 1), were quantified when the plants were exposed to 15 different stresses, that is, four abiotic stresses (drought, salt stress, osmotic stress and heat), seven biotic stresses (parasitic plant, phloem-feeding aphid, phloem-feeding whitefly, cell-content feeding thrips, leaf-chewing caterpillar, root-feeding nematode and necrotrophic fungus) and four stress combinations (fungus and caterpillar, drought and fungus, drought and caterpillar, caterpillar and osmotic stress). For detailed information on the carefully standardized stress treatments, the trait definitions and phenotyping, see Supporting Information Methods S1–S10.

Heritability of responses to biotic and abiotic stresses

The phenotypic analysis resulted in a wide range of marker-based, narrow-sense heritability (Kruijer *et al.,* 2015) estimates with 15 traits of low ($h^2 < 0.2$), 10 of moderate heritability ($0.2 < h^2 < 0.5$) and five of high ($h^2 > 0.5$) heritability (Fig. S1). The number of abiotic stress traits per heritability category was similar, while the number of traits related to biotic and combined stresses decreased with increasing heritability class. The most heritable traits were responses to feeding damage by thrips (Thrips_1; $h^2 = 0.8$), nematodes ($h^2 = 0.7$) and responses to salt (Salt_1 and Salt_3; resp. $h^2 = 0.6$ and $h^2 = 0.7$) and heat (Heat; $h^2 = 0.6$) (Table S1). The traits related to combined stresses have predominantly low heritabilities; however, it should be emphasized that the combined stresses particularly relate to combinations involving fungal and caterpillar stress.

Table 1. Phenotypes assessed

	Stress	Trait name	Section of Supporting Information	Trait phenotype	Treatment
Abiotic stresses	Salt	Salt_1	Methods S1	Main root length, number of lateral roots and straightness	75 mM NaCl
		Salt_2	Methods S1	Main root length	125 mM NaCl
		Salt_3	Methods S1	Number of lateral roots	125 mM NaCl
		Salt_4	Methods S1	Main root angle	125 mM NaCl
		Salt_5	Methods S2	Biomass	25 mM NaCl
	Drought	Drought_1	Methods S2	Biomass	Drought
		Drought_2	Methods S3	Biomass	Drought
	Osmotic	Osmotic	Methods S2	Biomass	PEG8000
	Heat	Heat	Methods S2	Number of siliques	35°C
Biotic stresses	Parasitic plant	Parasitic plant	Methods S4	Attachments	*Phelipanche ramosa*
	Nematode	Nematode	Methods S5	Offspring, egg mass	*Meloidogyne incognita*
	Whitefly	Whitefly_1	Methods S6	Survival, whiteflies	*Aleyrodes proletella*
		Whitefly_2	Methods S6	Reproduction, eggs	*A. proletella*
	Aphid	Aphid_1	Methods S7	Behavior T1, probing	*Myzus persicae*
		Aphid_2	Methods S7	Behavior T2, probing	*M. persicae*
		Aphid_3	Methods S7	Offspring, aphids	*M. persicae*
	Thrips	Thrips_1	Methods S8	Feeding damage	*Frankliniella occidentalis*
		Thrips_2	Methods S8	Behavior T1	*F. occidentalis*
		Thrips_3	Methods S8	Behavior T2	*F. occidentalis*
	Caterpillar	Caterpillar_1	Methods S9	Leaf area consumed	*Pieris rapae*
		Caterpillar_2	Methods S3	Biomass	*P. rapae*
		Caterpillar_3	Methods S3	Number of damaged leaves and feeding sites	*P. rapae*
	Fungus	Fungus	Methods S10	Number of spreading lesions	*Botrytis cinerea*
	Double stress	Fungus and caterpillar_1	Methods S3	Biomass	*B. cinerea* and *P. rapae*
		Fungus and caterpillar_2	Methods S3	Number of damaged leaves and feeding sites	*B. cinerea* and *P. rapae*
		Caterpillar and fungus	Methods S10	Number of spreading lesions	*P. rapae* and *B. cinerea*
Abiotic and biotic stress	Double stress	Drought and fungus	Methods S10	Number of spreading lesions	Drought and *B. cinerea*
		Drought and caterpillar	Methods S3	Number of damaged leaves and feeding sites	Drought and *P. rapae*
		Caterpillar and osmotic_1	Methods S9	Projected leaf area	*P. rapae* and PEG8000
		Caterpillar and osmotic_2	Methods S9	Biomass	*P. rapae* and PEG8000

The dataset contains three plant stress categories applied to *Arabidopsis thaliana*: abiotic stress, biotic stress and combinations of both abiotic and biotic stress. Phenotype assessments that were performed under similar environmental conditions have similar background shading (light and dark gray). 'Phenotype' refers to different phenotypic assessments (in some cases the first principal component of a group of phenotypes). 'Treatment' refers to the sort of stress that was applied. Additional information on traits can be found in Supporting Information Methods S1–S10. Yellow, abiotic stress; green, biotic stress; blue, combinations of biotic and abiotic stress.

Genetic commonality underlying responses to different stresses

To analyze the phenotypic variation between Arabidopsis accessions as a function of molecular marker variation, we used various mixed-model approaches (see the Materials and Methods section). We estimated marker-based genetic correlations, that is, correlations based on the genome-wide commonality of SNP effects underlying pairs of traits (see the Materials and Methods section), to investigate the magnitude of genetic commonality underlying resistance mechanisms in response to a range of biotic and abiotic stresses. For brevity, we will refer to these marker-based genetic correlations as genetic correlations. Such genetic correlations can be interpreted as upper bounds to the joint determination of pairs of traits by genetic factors. Genetic correlation analysis revealed a strong connection between the responses to parasitic plants and to aphids (r= 0.8), which were both negatively associated with other stress responses (Fig. 1). Parasitic plants and aphids have in common that they target phloem and xylem tissue (Tjallingii and Hogen Esch 1993; Dorr and Kollmann 1995), and induce the SA phytohormonal pathway (De Vos *et al.*, 2005; Runyon *et al.*, 2008). By contrast, the biotic stress responses that were negatively associated with the responses to

parasitic plants and aphids, that is, responses to necrotrophic fungi, caterpillars, and thrips, represent JA-inducing stresses (De Vos *et al.,* 2005; Pieterse *et al.,* 2009; Pieterse *et al.,* 2012). Because the SA and JA pathways predominantly interact through negative crosstalk (Pieterse *et al.,* 2009), the two main clusters resulting from the genetic correlation analysis represent different phytohormonal signaling response mechanisms. We also observed a strong genetic correlation between plant responses to osmotic stress and root-feeding nematodes. This supports the notion that root-knot nematodes trigger a differentiation of root cells to multinucleate giant cells with severely altered water potential and osmotic pressure (Baldacci-Cresp *et al.,* 2015). While the correlations between traits at the phenotypic level were generally rather low, the genetic correlation analysis revealed a common genetic basis underlying the responses to sets of single and combined stresses (Fig. S2).

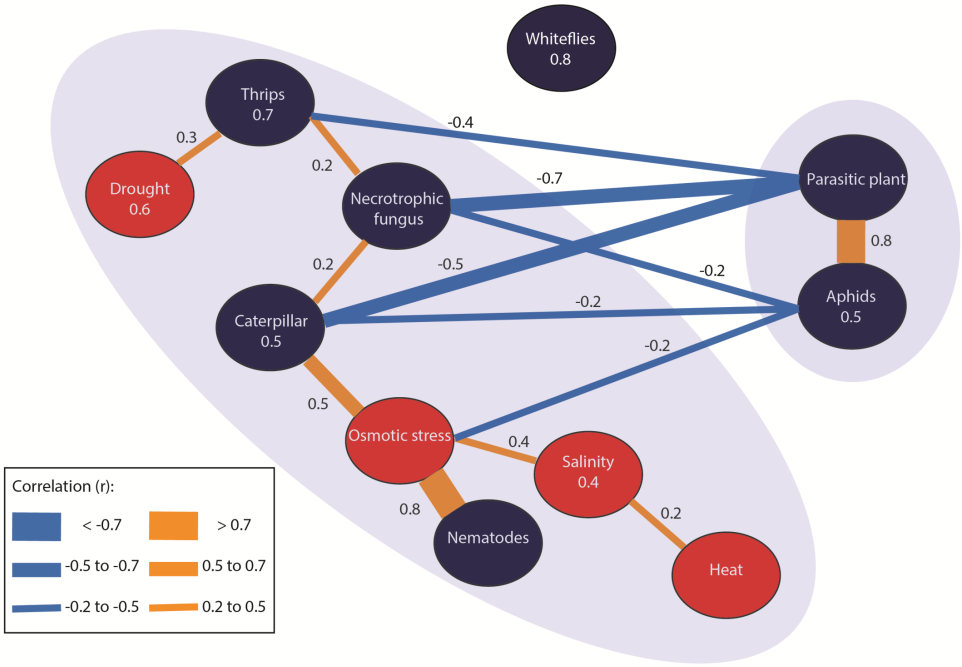


Fig. 1 Mean genetic correlations between responses of *Arabidopsis thaliana* to abiotic (red) and biotic (dark blue) plant stresses. The thickness of lines represents the strength of mean genome-wide correlations, annotated with *r*-values (orange, positive; blue, negative correlation). The more shared genetic associations between stresses, the higher the absolute genetic correlation. Correlations are negative when alleles have opposite effects, that is, resulting in increased resistance to one stress, but decreased resistance to the other stress. Values in balloons represent mean within-group correlation (not shown for groups consisting of a single trait). Mean between-group correlations are not shown if they are below an absolute value of $r = 0.2$. Two clusters can be distinguished: parasitic plants and aphids; and the other stresses, except whiteflies.

Candidate genes underlying responses to stresses

To identify individual candidate genes that contributed most to the pattern of genetic correlations, we fitted multi-trait QTL mixed models (MTMMs) to the total set of 30 traits, using a 214 000 SNP set that is commonly used for GWAS in Arabidopsis (Kim *et al.,* 2007; Atwell *et al.,* 2010; Li *et al.,* 2010a; Horton *et al.,* 2012; Bac-Molenaar *et al.,* 2015a). Our multi-trait GWA approach closely follows the modeling framework developed by Zhou & Stephens (2014) and generalizes the use of MTMMs as described previously (Boer *et al.,* 2007; Malosetti *et al.,* 2008; Alimi *et al.,* 2013) for classical bi-parental offspring populations to association panels. This GWA analysis identified 30 chromosome regions with multiple, significant SNP–trait associations. From each of those regions, the significant SNP with the strongest effect was chosen to represent the locus (Fig. 2; Table S2). Clustering of stresses by estimated SNP-effect profiles (Fig. 2) indicates that multiple SNPs were associated with response to more than one stress. Stress combinations induced large QTL allele substitution effects in the MTMM mapping (Fig. 2; Table S2), indicating that combinations of stresses trigger broad-spectrum defensive mechanisms. A total of 125 genes were in LD with the 30 most significant SNPs from the GWA analysis. Twenty of these genes were stress-related according to gene ontology annotation data (Table S3). Of these 20 genes, six have been functionally characterized by at least one study (Table 2a). For these six genes, we explored expression data to evaluate the biological relevance of these genes in stress-responsive mechanisms of Arabidopsis (Fig. S3). Of special interest were SNPs chr5.7493620, chr5.22041081 and chr4.6805259, which were in LD with *WRKY38* (encoding a WRKY transcription factor involved in SA-dependent disease resistance) (Kim *et al.,* 2008), *AtCNGC4* (involved in pathogen resistance) (Chin *et al.,* 2013) and *RMG1* (coding for disease resistance protein; Yu *et al.,* 2013), respectively.

Phytohormonal signaling underlying contrasts in stress responses

The MTMM framework allowed constraints to be imposed on the values of the estimated QTL effects, thereby providing a powerful testing framework for QTLs that have a common effect for the stresses belonging to one particular group of stresses, as contrasted with the effect for another group of stresses (see the Materials and Methods section 'Multi-trait GWAS'). We investigated whether polymorphisms for genes involved in SA and JA biosynthesis or genes responsive to signals from these pathways were the cause of the negative genetic correlations between the groups of traits sharing one or the other phytohormonal signaling pathway. To this end, we performed multi-trait GWA mapping to test the contrast between parasitic plant and aphid response vs the most negatively correlated traits, that is, fungus, caterpillar, thrips and drought response (Fig. 1). Fifteen SNPs were significantly associated with contrasting effects between the two trait clusters (Fig. S4). Seven of these SNPs were in LD with one or more genes known to be involved in JA-, SA- or resistance-related signal transduction (Table S4). Among these genes are *LOX5*, whose product is involved in facilitating aphid feeding (Nalam *et al.,* 2012; Nalam *et al.,* 2013), *MYB107* encoding a transcription factor responsive to SA (Stracke *et al.,* 2001; Yanhui *et al.,* 2006), the JA inducible genes *TPS02* and *TPS03* encoding terpene synthases (Huang *et al.,* 2010), and *MES16*, encoding a methyl jasmonate esterase (Christ *et al.,* 2012). Using TAIR10 annotations, we found that in total there are 371 genes

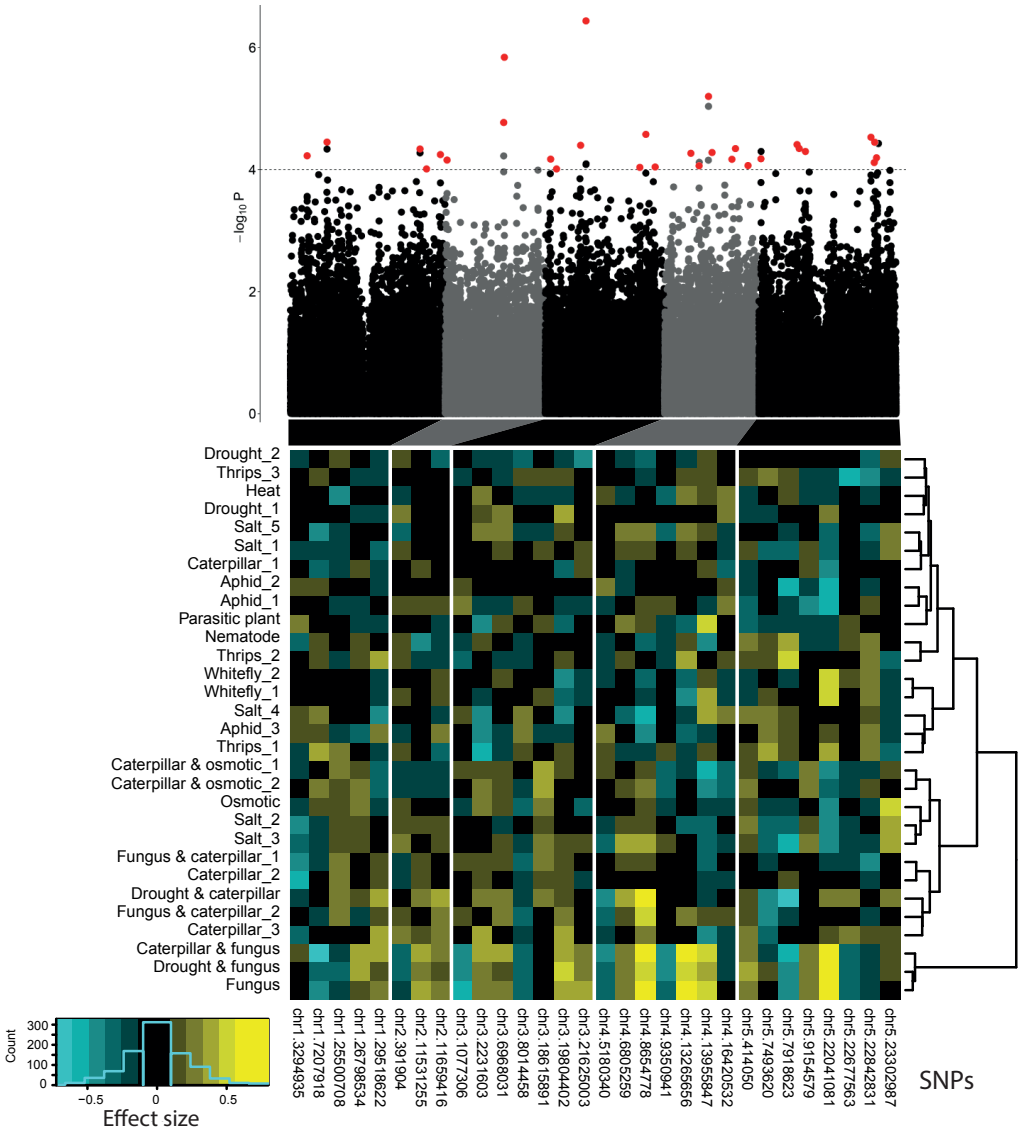


Fig. 2 Multi-trait mixed-model (MTMM) genome-wide association (GWA) mapping with 30 different stress responses of *Arabidopsis thaliana*. The top panel shows the 214 000 single nucleotide polymorphisms (SNPs) with their corresponding -$\log_{10}(P)$ values for the five chromosomes. The lower panel depicts the trait-specific effect sizes of the rare alleles for significant SNPs ($P < 0.0001$) as estimated by the full MTMM. When several SNPs were located within the 20 kb linkage disequilibrium half-windows around the most significant SNP in a region, the effects for the SNP with the strongest absolute average effects are shown (red-flagged in the Manhattan plot). SNPs are named by chromosome number and position on the chromosome. Negative effect sizes (blue) correspond to reduced plant resistance as a result of the rare allele, and positive effect sizes (yellow) correspond to increased resistance as a result of the rare allele. Stress responses were clustered hierarchically according to their effect, using Ward's minimum variance method. The key shows the frequency distribution for the effect sizes of the SNPs.

Table 2. Candidate *Arabidopsis thaliana* genes resulting from (a) multi-trait mixed-model (MTMM) analysis of all 30 stress responses as presented in Fig. 2; and (b) contrast-specific analysis with MTMM for contrasting effects of biotic and abiotic stresses as presented in Fig. 3.

Marker*	Gene in LD	Gene name	Gene description†	Responsiveness	References
(a)					
chr2.11659416	*AT2G27250*	*CLV3*	One of the three *CLAVATA* genes controlling the size of the shoot apical meristem (SAM) in *Arabidopsis*	Unknown	Clark *et al.* (1996); Fletcher *et al.* (1999); Shinohara & Matsubayashi (2010)
chr3.19804402	*AT3G53420*	*PIP2*	A member of the plasma membrane intrinsic protein subfamily PIP2	Heat, salt and heat, heat and silwet	Martiniere *et al.* (2012); Peret *et al.* (2012); Rasmussen *et al.* (2013); Sanchez-Romera *et al.* (2014)
chr4.6805259	*AT4G11170*	*RMG1*	Encodes RMG1 (Resistance Methylated Gene 1), an NB-LRR disease resistance protein with a Toll/interleukin-1 receptor (TIR) domain at its N terminus	Flagellin	Yu *et al.* (2013)
chr5.7493620	*AT5G22570*	*WRKY38*	Member of WRKY Transcription Factor; Group III	SA, *Pseudomonas*	Mare *et al.* (2004); Kim *et al.* (2008)
chr5.22041081	*AT5G54250*	*CNGC4*	Member of cyclic nucleotide gated channel family, a downstream component of the signaling pathways leading to hypersensitive response (HR) resistance. Mutant plants exhibit gene-for-gene disease resistance against avirulent *Pseudomonas syringae* despite the near-complete absence of the HR. Salicylic acid accumulation in *dnd2* mutants is completely *PAD4*-independent	Cold, flagellin	Jurkowski *et al.* (2004); Keisa *et al.* (2011); Chin *et al.* (2013); Rasmussen *et al.* (2013)
chr5.23302987	*AT5G57560*	*TCH4*	Encodes a cell wall modifying enzyme, rapidly up-regulated in response to environmental stimuli	Heat, heat and silwet, heat and salt, heat and high light, high light, high light and cold, high light and salt	Braam & Davis (1990); Xu *et al.* (1996); Purugganan *et al.* (1997); Iliev *et al.* (2002); Rasmussen *et al.* (2013)
(b)					
chr1.30381439	*AT1G80820*	*CCR2*	*CINNAMOYL COA REDUCTASE*. Encodes a cinnamoyl CoA reductase isoform. Involved in lignin biosynthesis	Cold and flagellin and silwet	Luderitz & Grisebach (1981); Lauvergeat *et al.* (2001); Zhou *et al.* (2010); Rasmussen *et al.* (2013)
chr1.30381439	*AT1G80840*	*WRKY40*	Pathogen-induced transcription factor. Binds W-box sequences *in vitro*. Forms protein complexes with itself and with WRKY60. Coexpression with *WRKY18* or *WRKY60* made plants more susceptible to both *P. syringae* and *Botrytis*	Cold and flagellin and silwet	Chen *et al.* (2010a); Pandey *et al.* (2010); Liu *et al.* (2012); Rasmussen *et al.* (2013)
chr1.6038270	*AT1G17610*	*CHS1*	*CHILLING SENSITIVE 1*, mutant accumulates steryl-esters at low temperature	Cold and high light	Rasmussen *et al.* (2013); Wang *et al.* (2013); Zbierzak *et al.* (2013)
chr5.171177	*AT5G17640*	*ASG1*	*ABIOTIC STRESS GENE 1*; expression of this gene is induced by ABA and salt stress	ABA, salt	Coste *et al.* (2008); Batelli *et al.* (2012)
chr5.23247572	*AT5G57380*	*VIN3*	Encodes a plant homeodomain protein *VERNALIZATION INSENSITIVE 3* (*VIN3*). *In planta* VIN3 and VRN2, VERNALIZATION 2, are part of a large protein complex that can include the polycomb group (PcG) proteins *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*), *CURLY LEAF* (*CLF*), and *SWINGER* (*SWN* or *EZA1*). The complex has a role in establishing *FLC* (*FLOWERING LOCUS C*) repression during vernalization	Cold	Sung *et al.* (2007); Bond *et al.* (2009); Finnegan *et al.* (2011)
chr5.23293119	*AT5G57560*	*TCH 4*	Encodes a cell wall-modifying enzyme	Heat, heat and silwet, heat and salt, heat and high light, high light, high light and cold, high light and salt	Braam & Davis (1990); Xu *et al.* (1996); Purugganan *et al.* (1997); Iliev *et al.* (2002); Rasmussen *et al.* (2013)
chr5.23293870	*AT5G57490*	*VDAC4*	Encodes a voltage-dependent anion channel (VDAC: AT3G01280/VDAC1)	*Pseudomonas*	Lee *et al.* (2009); Tateda *et al.* (2011)
chr5.23366252	*AT5G57685*	*GDU3*	Encodes a member of the GDU (glutamine dumper) family proteins involved in amino acid export: At4g31730 (GDU1)	Unknown	Chen *et al.* (2010b)

NB-LRR, nucleotide binding site–leucine-rich repeat.
*Markers derived from MTMM analysis (see Fig. 2).
†Based on information on http://www.arabidopsis.org/tools/bulk/go/index.jsp.

JA and SA signaling (JA-SA genes). Our GWA analysis identified significant SNPs inside or in a 20 kb neighborhood of five of those. In the remainder of the genome (i.e. non JA-SA), we identified 162 that have an annotation related to genes close to or with significant SNPs. So, in candidate regions for JA-SA, we had a ratio of 5/371 = 1.35% significant genes, while in non-candidate regions, we found 162/ 27863 = 0.58%. This is an enrichment of 2.33 times, significant at a = 0.05 (Fisher's exact probability test, mid-*P* value < 0.046; Rivals *et al.,* 2007). Following Atwell *et al.* (2010), an upper bound for the false discovery rate is then 1/2.33 = 0.43. In addition to screening for SNPs with contrasting effects, we screened for SNPs with a similar effect across the earlier mentioned trait clusters (Fig. S5) and found candidate genes involved in oxidative stress and plant responses to salinity and pathogens (Table S5).

QTLs underlying contrasts in responses to biotic and abiotic stresses

We expected a negative correlation between the responses to abiotic and biotic stresses as a result of antagonistic interactions between ABA and the SA and JA-ET pathways (Anderson *et al.,* 2004; Fujita *et al.,* 2006; De Torres-Zabala *et al.,* 2009; Kissoudis *et al.,* 2015). Testing for this contrast within the GWA analysis using our MTMM approach significantly identified 43 SNPs with a QTL effect that changed sign between biotic and abiotic conditions. For presentation purposes, traits were grouped by a cluster analysis across SNPs, while SNPs were grouped by clustering across traits. Fig. 3 shows the SNPs with the strongest overall effects, identified in 18 LD intervals. The minor alleles of nine of these SNPs displayed a positive effect on biotic stress response traits and a negative effect on abiotic response traits. The remaining nine SNPs displayed the opposite effect (Fig. 3). Several candidate genes were identified in LD with the SNPs that are specific for plant responses to either abiotic or biotic stresses (Table 2b), such as *TCH4* (encoding a cell wall-modifying enzyme), *AtCCR2* (involvement in lignin biosynthesis) and *ASG1* (a gene induced by ABA and salt stress). Transcription data (Fig. S6) support the notion that these genes play a contrasting role in responses to abiotic and biotic stresses and reveal an antagonistic responsiveness between ABA and JA treatment (*TCH4*) or a specific responsiveness to either ABA (*AtCCR2*, *ASG1*, *ATVDAC4*) or JA (*ATWRKY40*). This is in line with the hypothesis that there are antagonistic effects between abiotic stress responses, predominantly involving the ABA pathway, and wound and biotic stress responses involving the JA-ET or SA pathways (Kissoudis *et al.,* 2015). Previous studies have, however, also revealed an overlap in abiotic and biotic plant responses, such as similar transcriptomic perturbations after salinity and pathogen stress (Ma *et al.,* 2006). A screen for QTLs with similar effects on resistance to biotic and abiotic stress (Fig. S7) identified three genes annotated to be responsive to stress stimuli (Table S6). Transcriptional data show that these genes respond differentially to different (a)biotic stresses and phytohormones (Fig. S8). *ARGAH2*, encoding an arginase enzyme with a role in the metabolism of polyamines and nitric oxide, is involved in both SA- and JA-mediated resistance to both biotrophic and necrotrophic pathogens, and is also responsive to abiotic stimuli such as temperature, salt and light intensity (Fig. S8) (Jubault *et al.,* 2008; Gravot *et al.,* 2012; Rasmussen *et al.,* 2013). *PKS1* is known to be involved in adaptation in plant growth in response to light (Fankhauser *et al.,* 1999; Molas and Kiss 2008), but also seems to be responsive to Botrytis (Fig. S8). These genes are promising candidates for consistent effects across biotic and abiotic stresses.

QTLs underlying contrasts in responses to below- and above-ground stresses

We expected a negative correlation between responses to below- and above-ground stresses. A strong QTL signal was found on chromosome 1 for this contrasting response (Fig. S9). The associated marker (chr1.13729757) had 12 genes in LD with it, of which 11 are annotated as pseudogenes. Transcriptional data on abiotic stresses for the only protein coding gene (*AT1G36510*) show an up-regulation in above-ground tissues, yet a down-regulation in the root tissues (Winter *et al.,* 2007). Marker chr5.16012837 showed the strongest signal for similar effects on responses to below- and above-ground stresses (Fig. S10) for which the pathogenesis related thaumatin superfamily protein (*AT5G40020*) is the most promising candidate gene.

Validation of identified QTLs

To obtain experimental support for the most interesting QTLs resulting from the MTMM, we tested homozygous T-DNA insertion lines for candidate genes *RMG1* and *WRKY38* (both resulting from the MTMM analysis), and *TCH4* (from MTMM analysis on biotic vs abiotic contrast) for several of the stresses addressed in this study. Two independent *rmg1* T-DNA insertion lines showed a phenotype that was different from the wildtype (Col-0) for some of the stress conditions (Fig. 4; Methods S11), being more resistant to caterpillar feeding and osmotic stress (Fig. 4). *RMG1* (AT4G11170) encodes a nucleotide binding site–leucine-rich repeat disease resistance protein, which acts as a pattern-recognition receptor that recognizes evolutionarily conserved pathogen-derived signatures, and transcription is induced by the bacterial peptide flg22 (Yu *et al.,* 2013). The rare allele of the corresponding marker chr4.6805259 is associated with enhanced resistance to salt stress and the combined stresses 'caterpillar and drought' and 'caterpillar and fungus' and with enhanced susceptibility to drought stress. Gene expression data show that *RMG1* is up-regulated by several abiotic and biotic stresses (Fig. 4). In addition, gene ontology enrichment analysis of the co-expression network of *RMG1* shows an overrepresentation of genes involved in immune responses and maintenance of ion homeostasis. The latter is based upon co-expression with five genes encoding glutamate receptors (*GLR1.2, GLR1.3, GLR2.5, GLR2.8, and GLR2.9*), putatively involved in ion-influx-mediated long-distance signaling of wound, pathogen and salt stress (Ma *et al.,* 2006; Mousavi *et al.,* 2013; Choi *et al.,* 2014; Kissoudis *et al.,* 2015). T-DNA insertion lines for *TCH4* and *WRKY38* did not show a phenotype different from the wild-type (Col-0) for any of the tested stress conditions. Whether this is dependent on the genetic background used remains to be investigated.

Summarizing, our multi-trait GWA methodology facilitated a detailed analysis of the genetic architecture of resistance in Arabidopsis to a wide diversity of biotic and abiotic stresses. Application of this methodology revealed novel candidate genes associated with multiple stress responses, where specific contrasts were identified with some genes positively associated with the resistance to one set of stresses while being negatively associated with another set of stresses. In plant breeding (Brady et al., 2005; Ballesteros et al., 2015), such genes are classified as adaptive. Alternatively, other genes were identified with consistent effects across a wide spectrum of stress conditions. Such

genes are labeled as constitutive in the plant breeding literature (Brady et al., 2005; Ballesteros et al., 2015). Both adaptive and constitutive QTLs are important factors to contribute to improved stress resistance and tolerance in commercial crop species (Brady et al., 2005; Ballesteros et al., 2015).

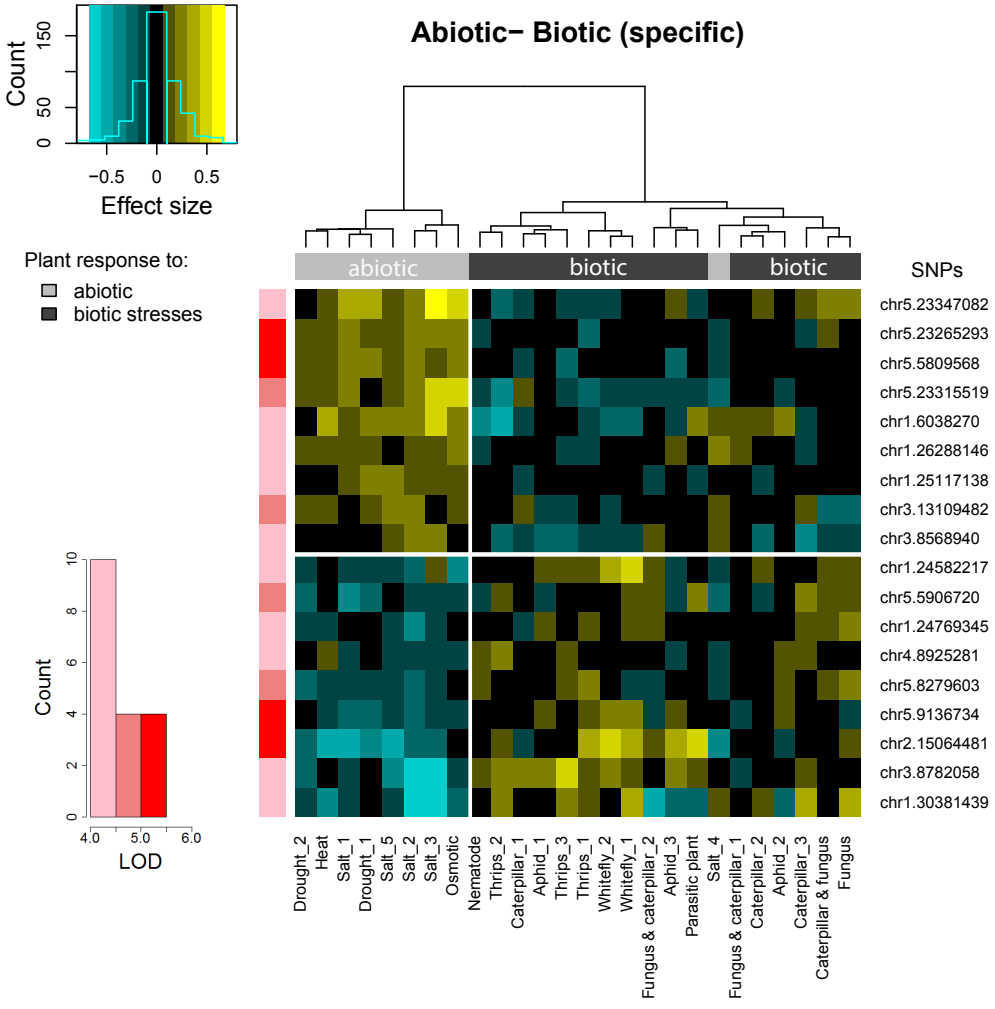


Fig. 3 Genetic associations specific for contrasting responses of *Arabidopsis thaliana* to abiotic and biotic stresses. Genetic associations (in red) were estimated with a contrast-specific genome-wide association analysis using a multi-trait mixed model (MTMM). For exploratory purposes, significant single nucleotide polymorphisms (SNPs) ($P \leq 10^{-4}$) for the biotic–abiotic contrast were clustered on their trait-specific effect sizes as estimated in the full MTMM, that is, without imposing a contrast restriction on the SNP effects. If there was another SNP in LD that had a higher effect size, this SNP was used as a representative of the LD block. Negative effects (blue) were cases where the rare allele was associated with a detrimental effect on the plants, while positive effects (yellow) were cases where the rare allele was associated with increased resistance to the stress. The rare alleles of the top nine SNPs are associated with enhanced resistance to abiotic stresses and reduced resistance to biotic stresses; the bottom nine SNPs show the inverse. Stresses were clustered on the basis of SNP effects using Ward's minimum variance method. The key shows the frequency distribution of SNPs across effect sizes.

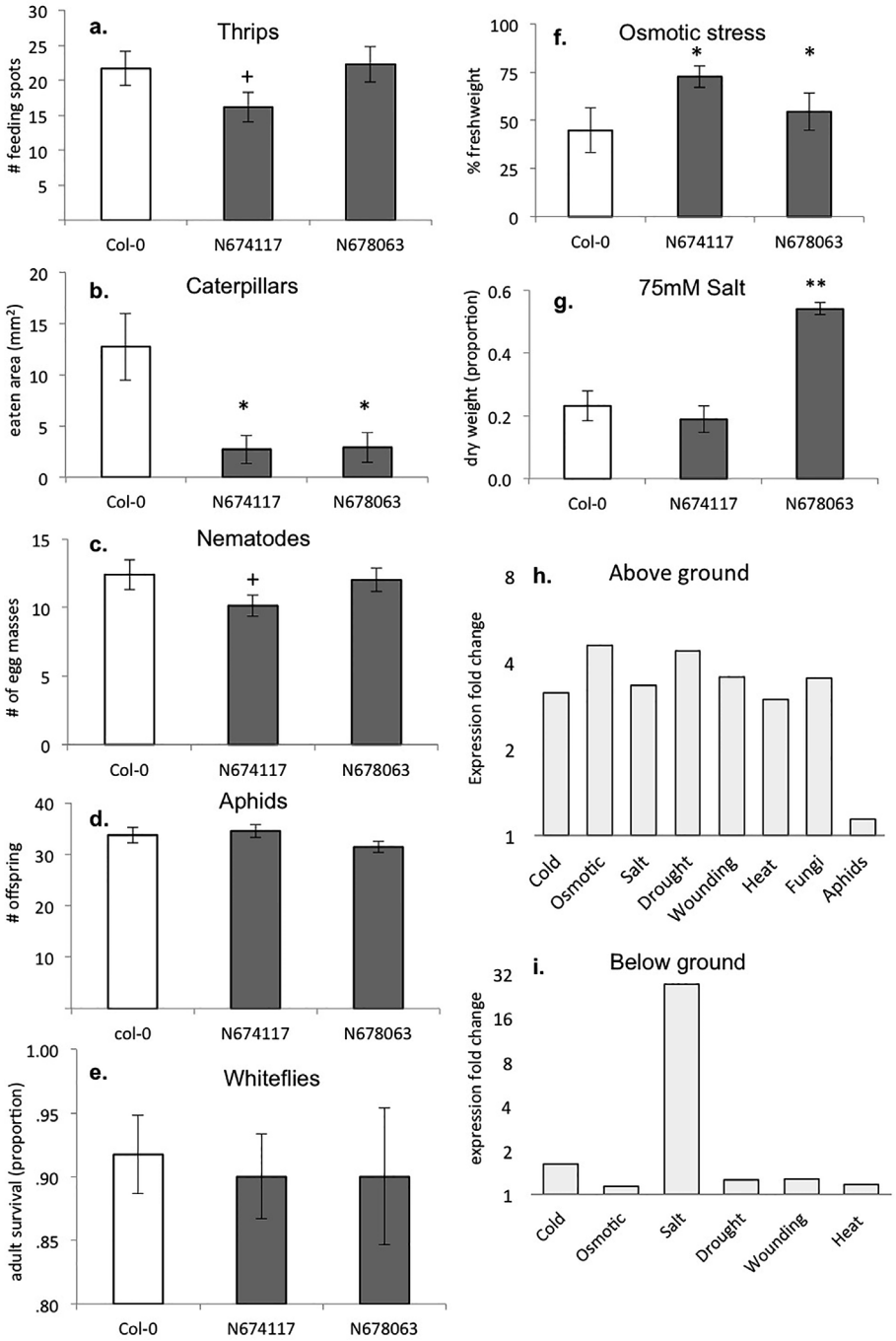


Fig. 4 Phenotypes of *RMG1* T-DNA mutant screenings for *Arabidopsis thaliana*. Phenotypes are given for two T-DNA lines in the *RMG1* gene and for Col-0 as control. (a) Number of thrips feeding spots on a detached leaf at 6 d post-infestation (n = 24). (b) Leaf area consumed by *Pieris rapae* caterpillars (n = 6). (c) Number of nematode egg masses (n = 23). (d) Number of *Myzus persicae* aphid offspring (n = 10 -17). (e) Percentage survival of adult whiteflies (*Aleyrodes proletella*) (n = 10). (f) Plant FW after osmotic treatment in comparison with control (% relative to control) (n = 4). (g) Plant DW after 75mM salt treatment in comparison with control (ratio) (n = 7–10); mean ± SE; *, $P < 0.05$; **, $P < 0.01$ (difference in comparison with Col-0). (h, i) Relative expression fold-change for *RMG1* compared with untreated control plants in above-ground (h) and below-ground (i) tissue. Expression data from Arabidopsis eFP browser (http://bbc.botany.utoronto.ca).

Discussion

We developed a novel mixed-model approach to multi-trait GWA mapping with a special feature for testing contrasts between groups of stresses to identify the genetic architecture underlying a total of 30 stress response traits in Arabidopsis. The strength of our statistical approach was that our multi-trait mixed model accounted simultaneously for dependencies between genotypes and between traits, providing a natural and appropriate correction for multiple testing, while maximizing power for the detection of QTLs for the stress contrast under study. As we addressed a large number of stresses, our phenotyping experiments were distributed across a series of laboratories and were not performed simultaneously. To mitigate as much as possible the occurrence of QTLs induced purely by experiment-specific differences in plant management and environmental control, our phenotypic responses were defined in terms of control-corrected responses. This type of correction will emphasize QTLs for resistance and tolerance *per se* and will decrease detection power for QTLs related to development and viability. The extensive phenotyping executed in this study was done under carefully controlled conditions in climate chambers. Ideally, phenotyping should be done in nature because that is where genetic variation is exposed to natural selection (Bergelson and Roux 2010; Brachi *et al.,* 2010; Brachi *et al.,* 2013). Here, we have phenotyped the plant population to 15 different stresses under laboratory conditions and our data show an interesting pattern based on genetic correlations that matches with phytohormonal signaling underlying stress responses (Fig. 1). This indicates that the genetic architecture recorded here is biologically relevant. Drought and salt stress responses share signal transduction mechanisms (Zhu 2002) which are represented by the genetic correlations recorded (Fig. 1). Insect damage is commonly associated with drought or osmotic stress and this is also clear from overlap in underlying phytohormonal signaling (Pieterse *et al.,* 2012). Fig. 1 shows that drought stress and osmotic stress correlate with insect stresses. Extending studies of genetic variation and the genetic architecture underlying responses to multiple stresses to natural conditions will be an important next step (Bergelson and Roux 2010).

Through the approach developed here, candidate genes for stress responses were identified that are involved in contrasting responses when comparing biotic and abiotic stresses, above- and below-ground stresses, and attack by phloem feeders vs other biotic stresses. Among these genes many are involved in phytohormone-mediated processes, supporting the notion that the phytohormonal regulatory network plays an important role in plant stress responses (Pieterse *et al.,* 2012). The MTMM approach further showed that certain SNPs were associated with multiple stress responses and that transcriptional patterns of genes to which the SNPs were linked, as well as the phenotype expressed upon knocking out one of these genes, matched the observed stress responses of the plants. The *RMG1* gene that was identified through this procedure has relevant effects on plant phenotype in the context of responses to individual stresses. *RMG1* is a bacterium-inducible resistance gene whose activity is modulated by the plant through RNA-directed DNA methylation (RdDM) (Yu *et al.,* 2013). *RMG1* expression activates the SA pathway (Yu *et al.,* 2013). Thus, the increased resistance against caterpillars in rmg1 mutants may be the result of elimination of SA-mediated interference with JA-induced resistance to caterpillars (Pieterse *et al.,* 2012). *RMG1* appears to be inducible by

several stresses and deserves further in-depth analysis for its role in plant response to multiple stresses. Our data show that for the 30 most significant SNPs resulting from the MTMM analysis, the average absolute effect size for double stresses is higher, on average, than that for single stresses ($P < 0.007$, Table S2). This suggests that resistance mechanisms involved in countering dual stresses are of a more general nature, in contrast to the rather specific resistance mechanisms involved in single stress responses. However, the combined stresses included in this study particularly involve fungal and caterpillar stresses. Future studies including other combined stresses are needed to further investigate the suggested pattern.

The MTMM framework that we used for GWA mapping provides unbiased estimates for QTL allele substitution effects together with correct standard errors for these effects. Within the same framework we developed unique facilities to test hypotheses on QTL 9 stress interactions in multi-trait models, which are not available in competing meta-analysis approaches (Zhu *et al.,* 2015). The variance-covariance structure that we used for the polygenic term protects against inflated type I error, that is, too many false-positive SNP–trait associations, as a consequence of population structure and kinship on the genotypic side and genetic correlations between traits on the trait side. The inclusion of trait correlations will, for most QTLs, improve the power of detection in comparison to single-trait GWA mapping (Korte *et al.,* 2012; Zhou and Stephens 2014), 2014; see 'Multi-trait GWAS' in the Materials and Methods section). For a comparison of the MTMM analysis with single-trait analyses, see 'Simulations to compare power for full MTMM, contrast MTMM and univariate analysis' in the Materials and Methods section, Methods S12 and Figs S11 and S12. Our choice for the variance-covariance structure of the polygenic term as a Kronecker product of a compressed kinship on the genotypes with an approximated unstructured variance-covariance model on the environments is sometimes used in plant breeding for genomic prediction models (Burgueño *et al.,* 2012). However, implementation of such models in GWA mapping and especially on the scale that we present here, with 30 traits, is unprecedented and is practically far from straightforward. It required substantial work on preparatory phenotypic analyses as well as fine-tuning of the genotypic and trait variance-covariance structures to achieve convergence of the mixed models.

The MTMM analyses identified candidate genes associated with contrasting responses to biotic and abiotic stresses. Stress combinations appeared to have a strong influence on the MTMM outcome, indicative for significant interactions between different stresses when occurring simultaneously, and underlining the importance of studying the resistance of plants to combinations of stress. Transcriptional data and phenotyping of mutants provide initial support for the role of several of the candidate genes identified. Studies of plant responses to a diverse set of biotic stresses show that the transcriptional pattern is stress-specific and that phytohormonal signaling pathways can explain up to 70% of the induced gene regulation (De Vos *et al.,* 2005). Taking the outcome of the MTMM analyses to investigate the involvement of identified candidate genes in the resistance of plants to several stresses, not only in Arabidopsis but also in related crop species, such as, for example, Brassica species, will be valuable in the breeding by design of future crops to protect them against combinations of stresses, including biotic and abiotic stresses. This will be of great value for next-generation crops.

Acknowledgements

This research is supported by the Perspective Program 'Learning from Nature' of the Dutch Technology Foundation STW, which is part of the Netherlands Organisation for Scientific Research (NWO) and is partly funded by the Ministry of Economic Affairs.

Author contributions

M.P.M.T., N.H.D.O., K.J.K., S.C., P-P.H., J.A.B-M., C.B., J. Bucher, J.B-L., X.C., E.F.F., M.M.J., W.L., J.A.v.P., S.W. and G.L.W. phenotyped the plants; M.P.M.T., N.H.D.O., K.J.K., S.C., P-P.H., M.G.M.A., J.A.B-M., J. Bakker, H.J.B., J. Bucher, C.B., X.C., E.F.F., M.A.J., M.M.J., J.J.B.K., W.L., C.M.J.P., C.R-S., G.S., C.T., J.J.A.v.L., J.A.v.P., C.C.v.S., S.C.M.v.W., R.G.F.V., R.V., B.V., D.V., S.W., G.L.W. and M.D. designed the phenotyping experiments and made initial analyses; W.K., J.v.H. and F.A.v.E. developed the multi-trait mixed model; W.K., J.v.H., M.P.M.T., N.H.D.O., K.J.K., S.C., P-P.H., B.U., F.A.v.E. and M.D. analyzed the total dataset. M.D. and F.A.v.E. coordinated the study as a whole. M.P.M.T., N.H.D.O., K.J.K., S.C., P-P.H., W.K., J.v.H., F.A.v.E. and M.D. wrote the manuscript with input from J.J.B.K., C.J.M.P. and M.G.M.A. and all authors proofread the final version of the manuscript.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article http://onlinelibrary.wiley.com/doi/10.1111/nph.14220/abstract.

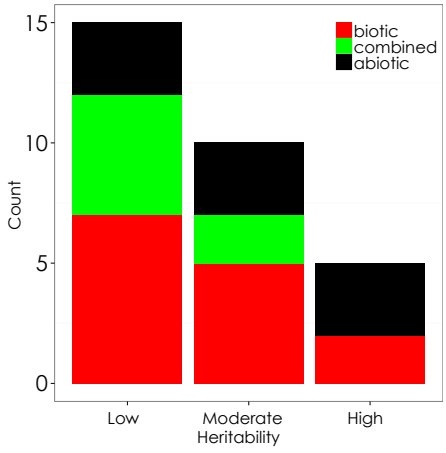


Fig. S1 Narrow sense heritability for *Arabidopsis thaliana* resistance to abiotic and biotic stresses. Narrow sense heritability values were estimated using the 'heritability' R package. Traits were classified in three biological categories: resistance to abiotic, biotic and double stresses. These biological categories were grouped based on their heritability in low (h^2<0.2), moderate (0.2<h^2<0.5) and high (h^2>0.5) heritability classes.

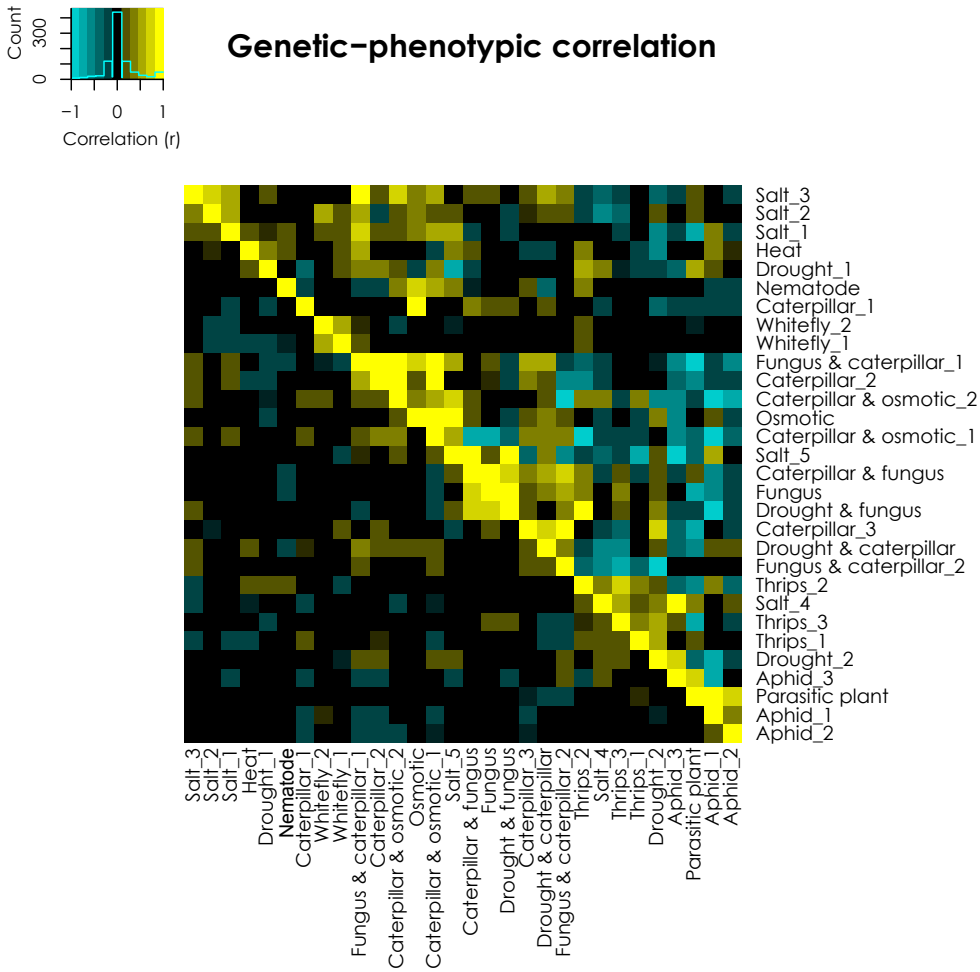


Fig. S2 Genetic and phenotypic correlation matrix. Heatmap displaying phenotypic correlations below the diagonal and genetic correlations above the diagonal. The genetic correlations shown above the diagonal are the same ones as those used for the construction of Fig. 1 in the main text. Phenotypic correlations were calculated using Spearman's correlation coefficient *rho*, whereas the genome-wide genetic correlations were estimated bivariately and with correction for population structure (on full kinship matrix). For Whitefly_1 and Whitefly_2 the maximum likelihood estimates were not available so genetic correlations were estimated using G-BLUP. Traits were clustered according to Ward's minimum variance method for the genetic correlation coefficient values.

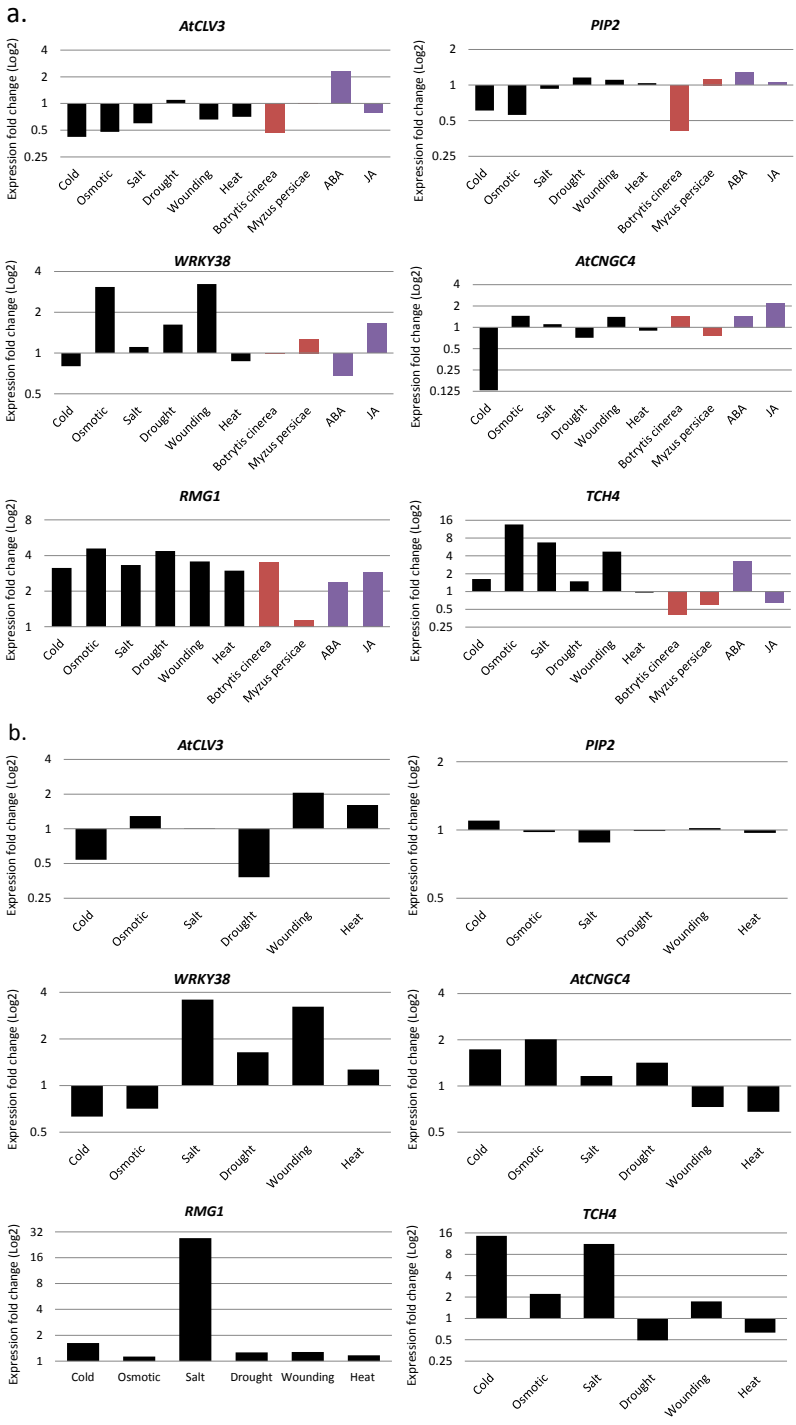


Fig. S3 Expression data of six candidate genes (resulting from MTMM, see Table 2a) in plants exposed to biotic or abiotic stress factors, relative to control conditions. (a) Shoot tissues and (b) root tissues. Black bars represent abiotic stresses, red bars represent biotic stresses and purple bars represent phytohormonal treatments. Expression data from Arabidopsis eFP browser (http://bbc.botany.utoronto.ca).

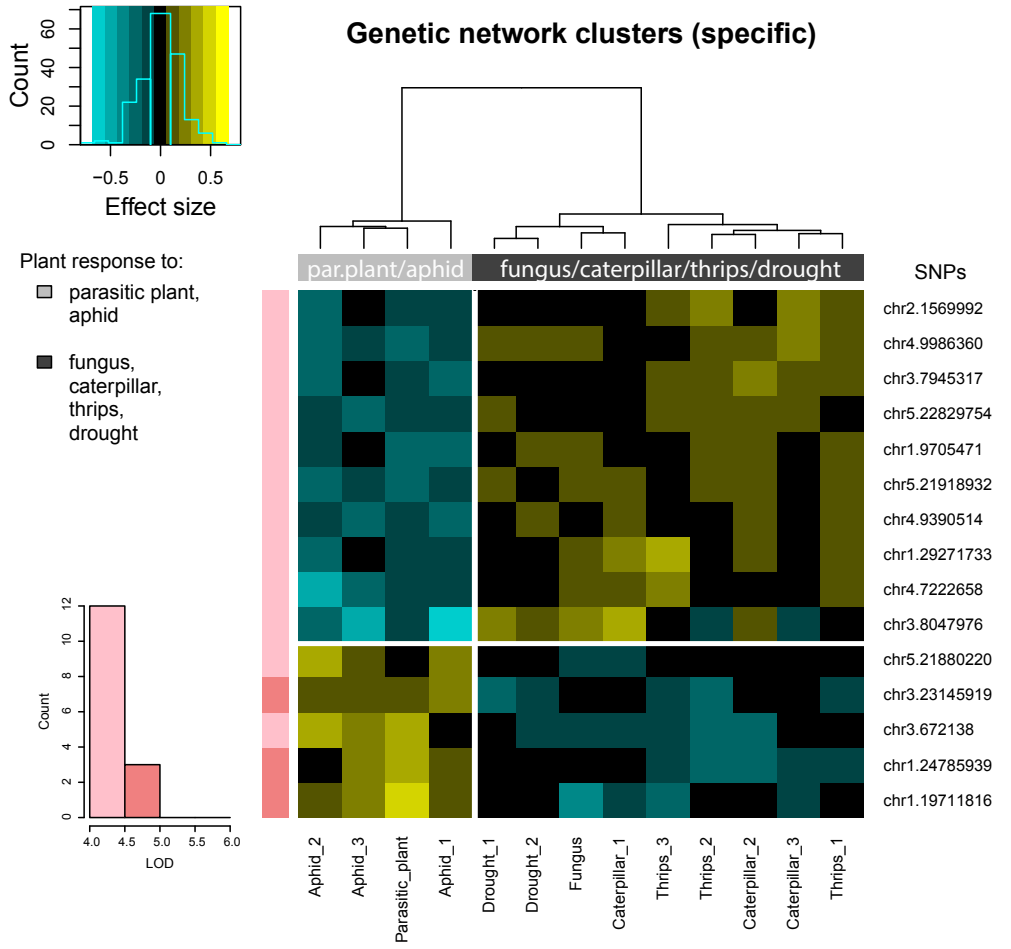


Fig. S4 Genetic associations specific for plant responses to the main clusters of the genetic correlation network (see Fig. 1): parasitic plant and aphid vs fungus, caterpillar, thrips and drought. Genetic associations were estimated with a contrast-specific analysis using MTMM. Significant SNPs ($P \leq 10^{-4}$) for the contrast are clustered according to trait-specific effects estimated from the full MTMM. If there was another SNP in LD that had a higher effect size, this SNP was used as representative for the LD block. Negative effect sizes (blue) were cases where the rare allele was associated with a detrimental effect on the plants, positive effect sizes (yellow) were cases where the rare allele was associated with increased resistance to the stress. The rare alleles of the top 10 SNPs are associated with enhanced resistance to fungus, caterpillar, thrips and drought stresses and reduced resistance to stresses inflicted by parasitic plants and aphids; the bottom 5 SNPs show the inverse. Stresses are clustered according to effect size, using Ward's minimum variance method. If SNPs were located within a 20 kb half-window of each other, only the SNP with the highest absolute cumulative effect size was included. The key shows the frequency distribution of SNPs across effect sizes.

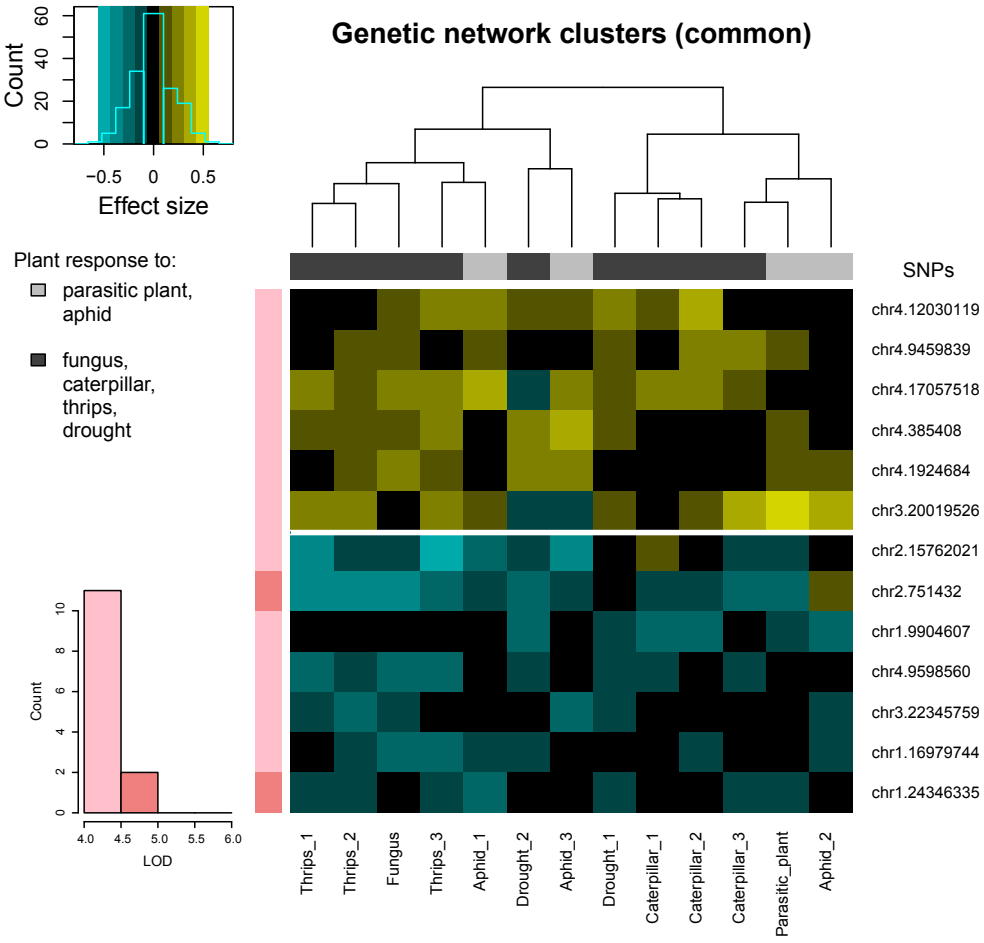


Fig. S5 Genetic associations common for plant response to the main clusters of the genetic correlation network: parasitic plant and aphid on the one hand vs fungus, caterpillar, thrips and drought on the other hand. Genetic associations were estimated with a contrast analysis using MTMM. Significant SNPs ($P \leq 10^{-4}$) for the common response are clustered according to trait-specific effects estimated from the full MTMM. If there was another SNP in LD that had a higher effect size, this SNP was used as representative for the LD block. Negative effect sizes (blue) were cases where the rare allele was associated with a detrimental effect on the plants, positive effect sizes (yellow) were cases where the rare allele was associated with increased resistance to the stress. The rare alleles of the top 6 SNPs are associated with enhanced resistance to abiotic stresses and reduced resistance to biotic stresses; the bottom 7 SNPs show the inverse. Stresses are clustered according to SNP effect size, using Ward's minimum variance method. If SNPs were located within a 20 kb half-window of each other, only the SNP with the highest absolute cumulative effect size was included. The key shows the frequency distribution of SNPs across effect sizes.

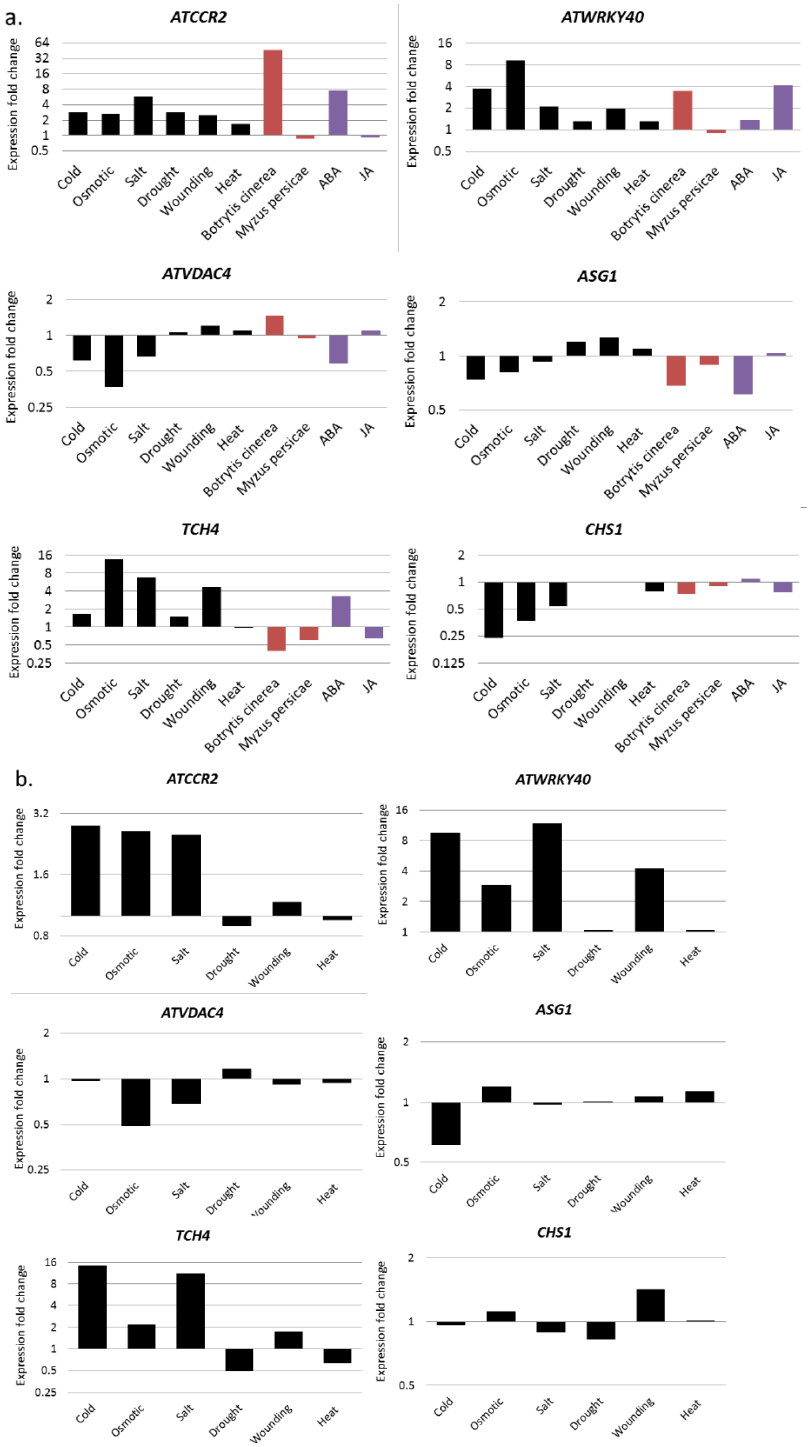


Fig. S6 Expression data of six candidate genes (resulting from MTMM analysis, see Table 2b) in plants exposed to biotic or abiotic stress factors, relative to control conditions. (a) Shoot tissues and (b) root tissues. Black bars represent abiotic stresses, red bars represent biotic stresses and purple bars represent phytohormonal treatments. Expression data from Arabidopsis eFP browser (http://bbc.botany.utoronto.ca).

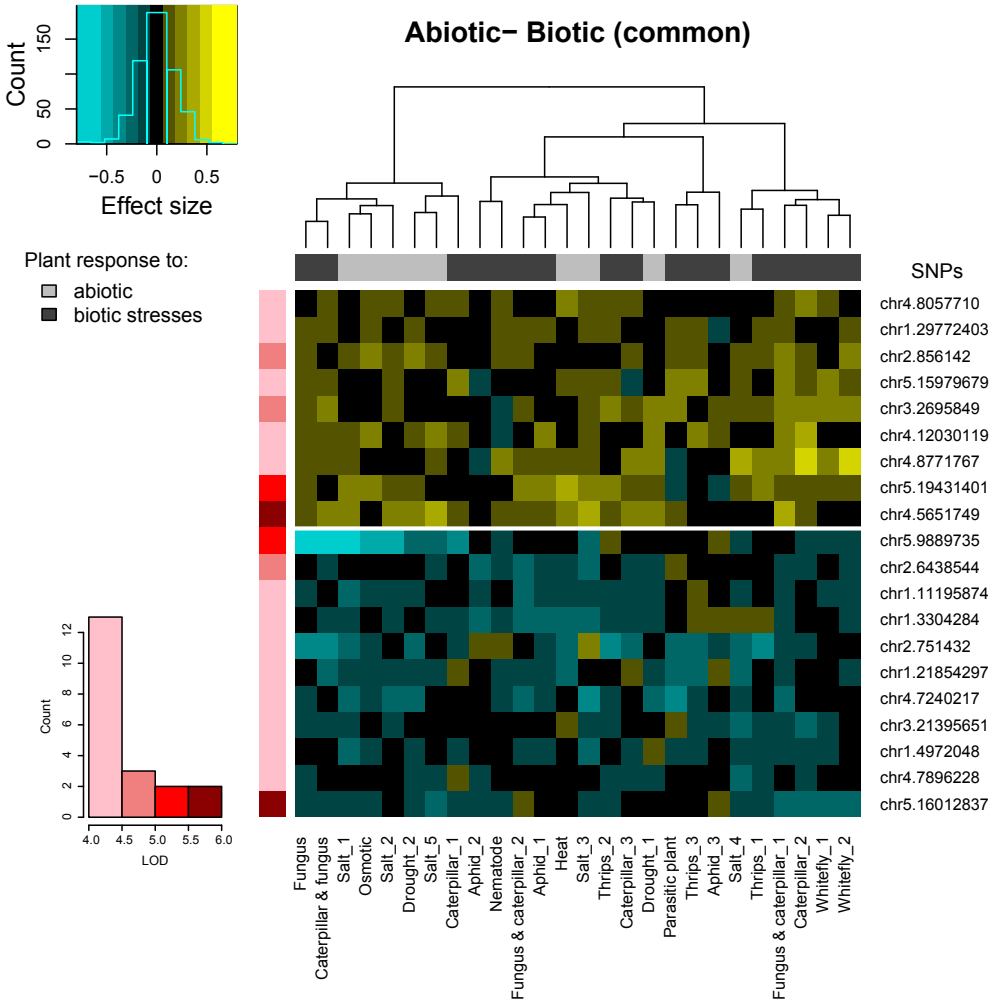


Fig. S7 Genetic associations common for plant responses to abiotic and biotic stresses. Genetic associations were estimated with a contrast analysis using MTMM. Significant SNPs ($P \leq 10^{-4}$) for the common response are clustered according to trait-specific effects estimated from the full MTMM. If there was another SNP in LD that had a higher effect size, this SNP was used as representative for the LD block. Negative effect sizes (blue) were cases where the rare allele was associated with a detrimental effect on the plants, positive effect sizes (yellow) were cases where the rare allele was associated with increased resistance to the stress. The rare alleles of the top 9 SNPs are associated with enhanced resistance to abiotic and biotic stresses; the bottom 11 SNPs are associated with reduced resistance to abiotic and biotic stresses. Stresses are clustered according to SNP effect size, using Ward's minimum variance method. If SNPs were located within a 20 kb half-window of each other, only the SNP with the highest absolute cumulative effect size was included. The key shows the frequency distribution of SNPs across effect sizes.

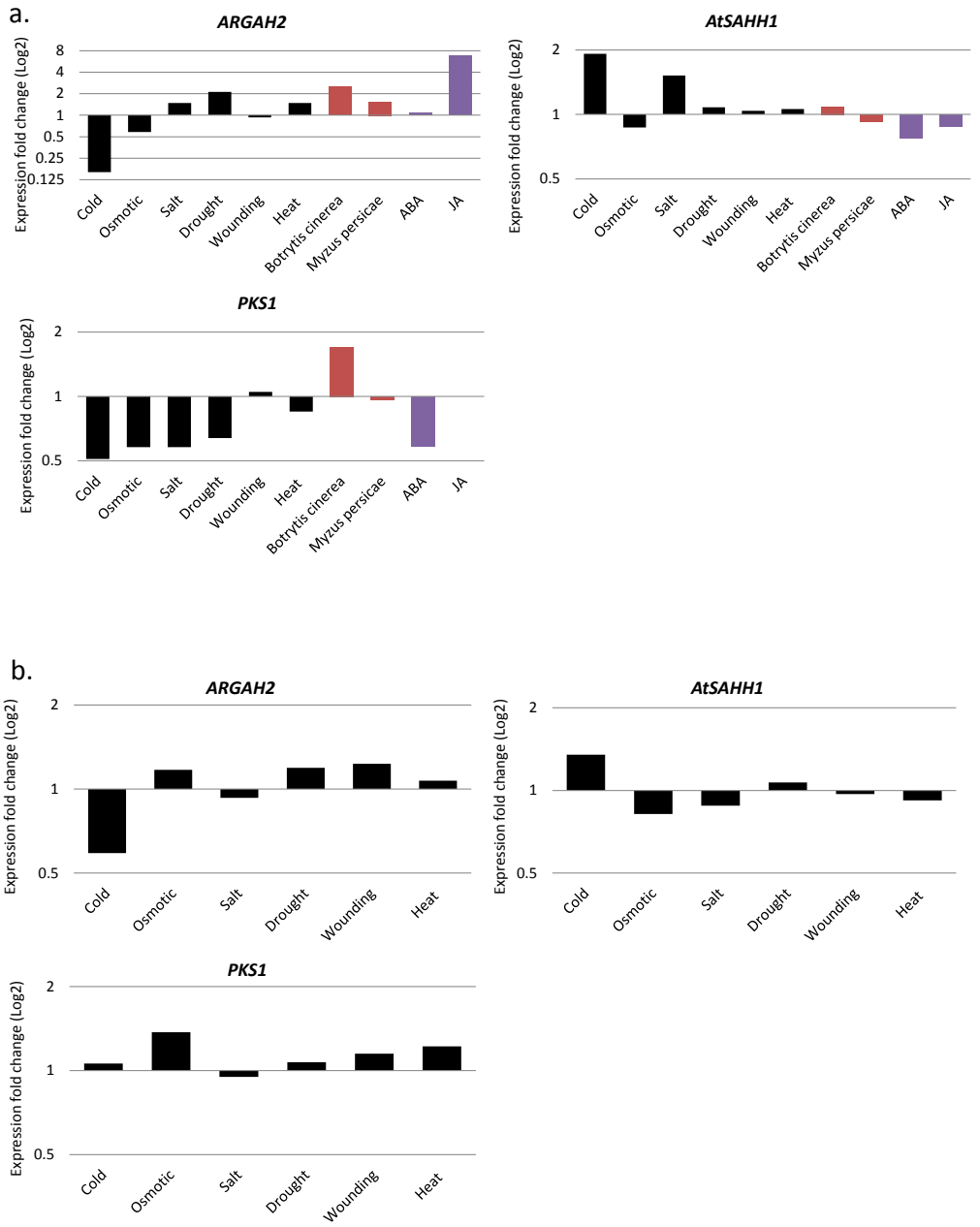


Fig. S8 Expression data of three candidate genes (resulting from MTMM, see Table S6) in plants exposed to biotic or abiotic stress factors, relative to control conditions. (a) Shoot tissues and (b) root tissues. Black bars represent abiotic stresses, red bars represent biotic stresses and purple bars represent phytohormonal treatments. Expression data from Arabidopsis eFP browser (http://bbc.botany.utoronto.ca).

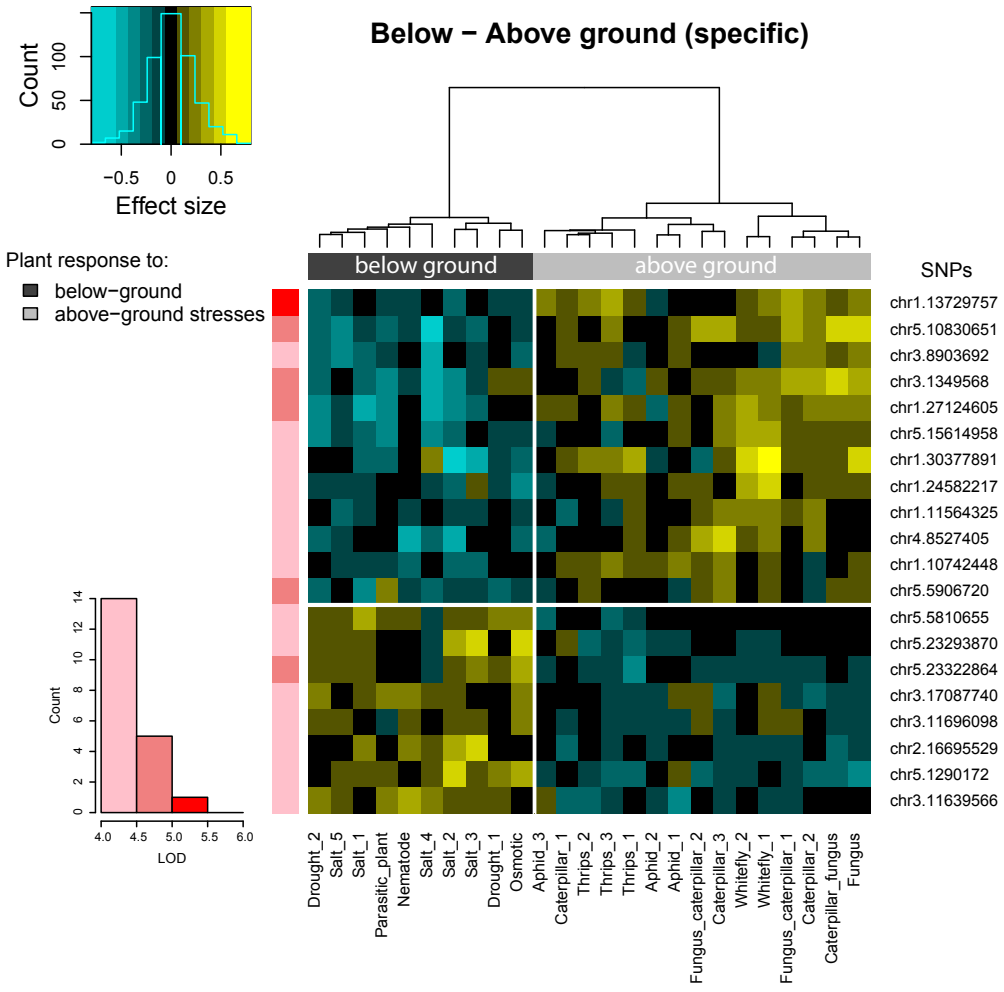


Fig. S9 Genetic associations specific for plant responses to either below- or aboveground stress. Genetic associations were estimated with a contrast analysis using MTMM. Significant SNPs ($P \leq 10^{-4}$) for the belowground-aboveground contrast are clustered according to trait-specific effects estimated from the full MTMM. If there was another SNP in LD that had a higher effect size, this SNP was used as representative for the LD block. Negative effect sizes (blue) were cases where the rare allele was associated with a detrimental effect on the plants, positive effect sizes (yellow) were cases where the rare allele was associated with increased resistance to the stress. The rare alleles of the top 12 SNPs are associated with enhanced resistance to aboveground stresses and reduced resistance to belowground stresses; the bottom 8 SNPs show the inverse. Stresses are clustered according to SNP effect size, using Ward's minimum variance method. If SNPs were located within a 20 kb half-window of each other, only the SNP with the highest absolute cumulative effect size was included. The key shows the frequency distribution of SNPs across effect sizes.

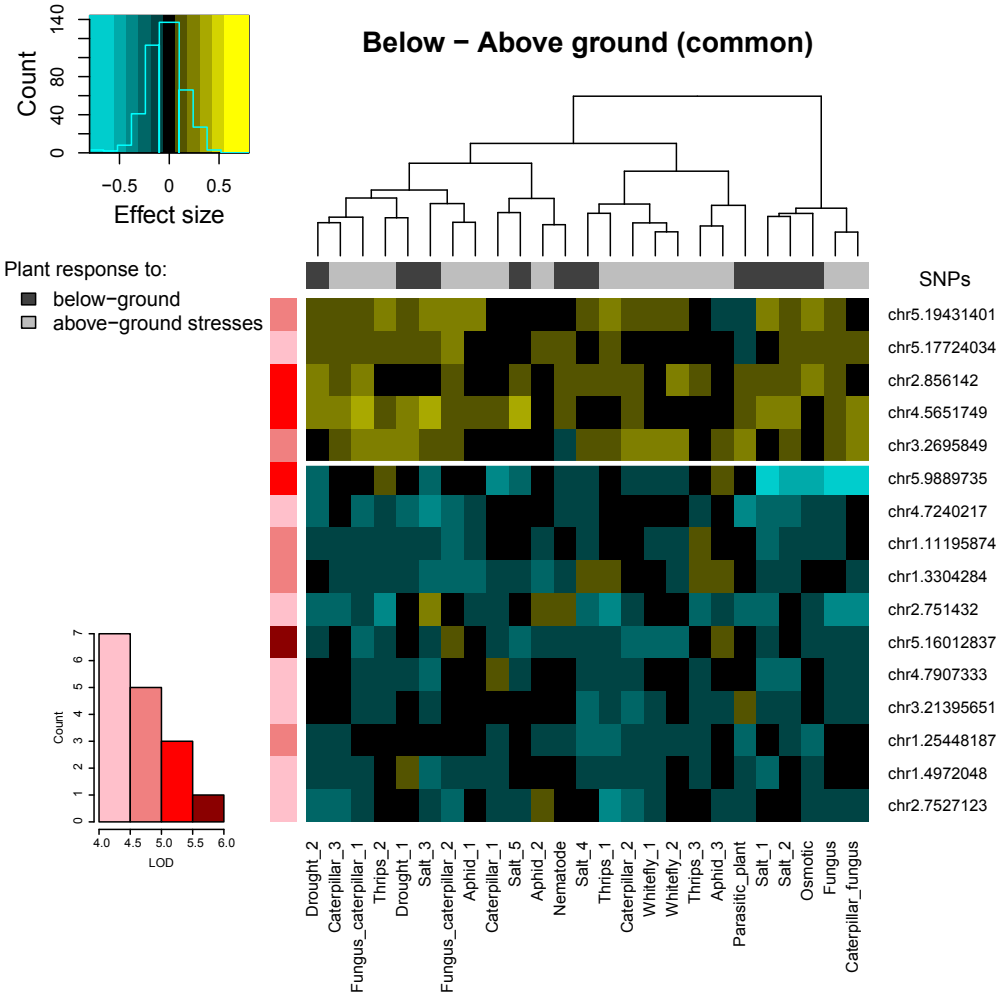


Fig. S10 Genetic associations common for plant responses to below- and aboveground stresses. Genetic associations were estimated with a contrast analysis using MTMM. Significant SNPs ($P \leq 10^{-4}$) for the common response are clustered according to trait-specific effects estimated from the full MTMM. If there was another SNP in LD that had a higher effect size, this SNP was used as representative for the LD block. Negative effect sizes (blue) were cases where the rare allele was associated with a detrimental effect on the plants, positive effect sizes (yellow) were cases where the rare allele was associated with increased resistance to the stress. The rare alleles of the top 5 SNPs are associated with enhanced resistance to above- and belowground stresses; the bottom 11 SNPs are associated with reduced resistance to above- and belowground stresses. Stresses are clustered according to SNP effect size, using Ward's minimum variance method. If SNPs were located within a 20 kb half-window of each other, only the SNP with the highest absolute cumulative effect size was included. The key shows the frequency distribution of SNPs across effect sizes.

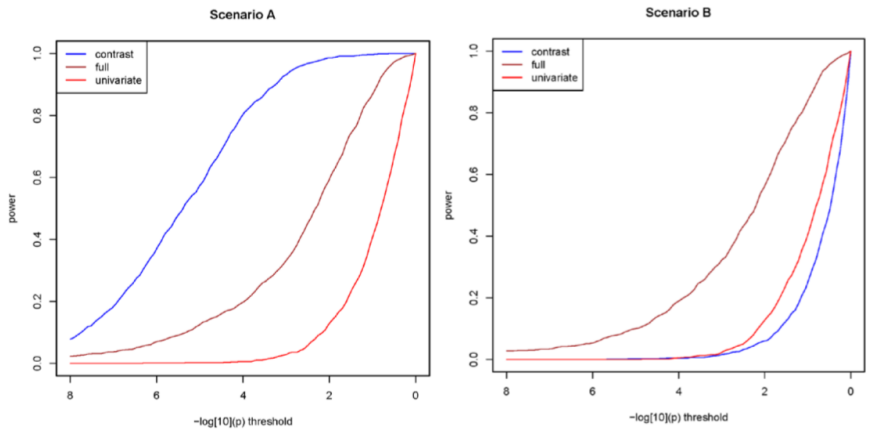


Fig. S11 Power of MTMM in simulations. Power of the full MTMM (brown), contrast MTMM (blue) and univariate analysis (red) as a function of *P*-value thresholds, in case of contrasting SNP-effects (Scenario A) and SNP-effects with random sign (Scenario B). Power was estimated based on 1000 simulations, which were performed as described in Methods S12.

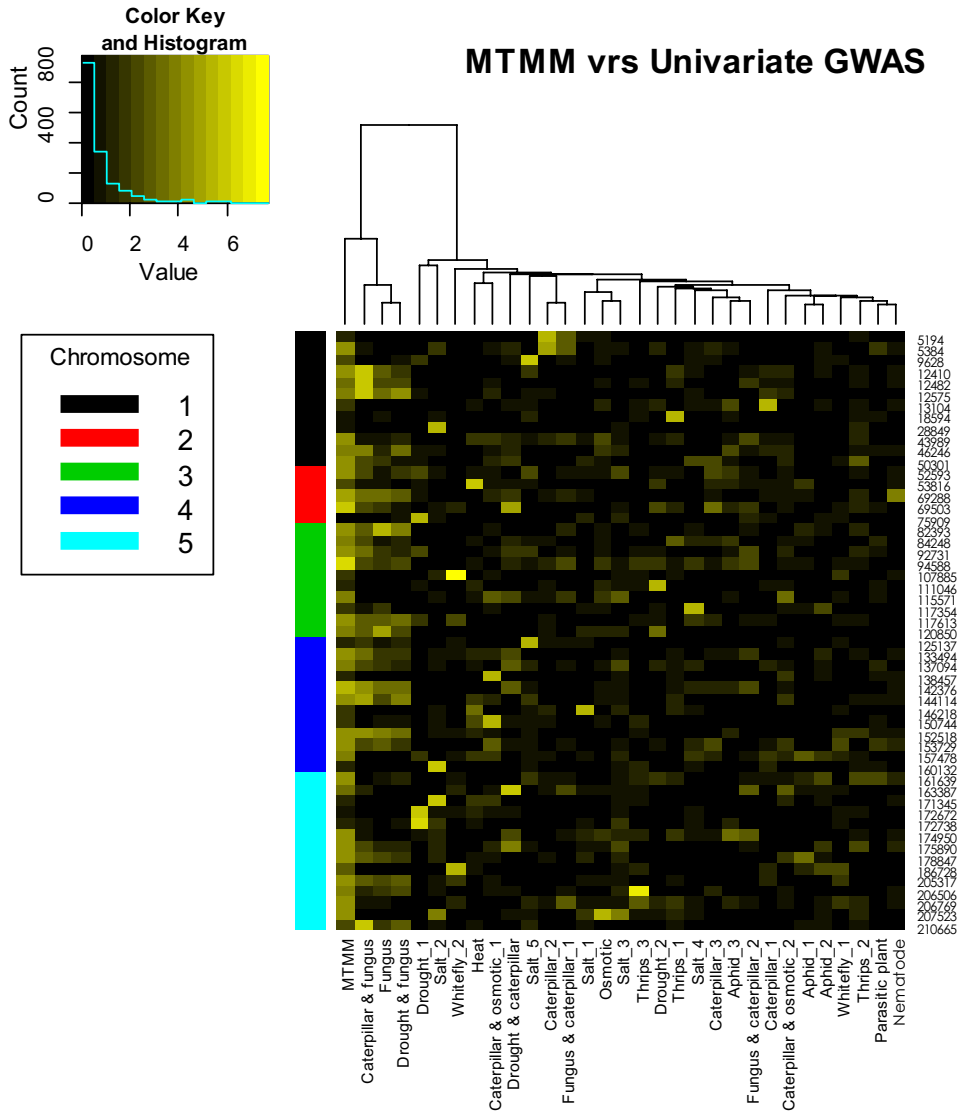


Fig. S12 Comparison of SNPs identified by MTMM and Univariate GWAS. Heatmap displaying *P*-values at -$\log_{10}(P)$ scale of SNPs that were among the most significant in either MTMM (first column) or at least one of the univariate GWAS (subsequent columns). For MTMM a significance threshold $P \leq 0.0001$ was used. For univariate GWAS a significance threshold of $P \leq 4 \times 10^{-6}$ was used, following a multiple testing Bonferroni correction. Chromosomes are displayed with different colours in the left panel of the figure. Traits were clustered using hierarchical clustering (Ward). Per LD block only the marker with the highest effect size is shown. Markers within 20 kb half window size of each other were considered to be in LD.

Table S1 Data overview on phenotyping the 350 *Arabidopsis thaliana* accessions of the HapMap collection

Table S2 Summed effect sizes of 30 most significant SNPs in MTMM per trait

Table S3 125 candidate genes derived from the Multitrait Mixed Model analysis

Table S4 Genes in linkage with SNPs with $-\log_{10}(P)$ score > 4 (20 kb half-window size) in the contrast-specific GWA mapping of parasitic plants and aphids, on the one hand, vs fungus, caterpillar, thrips and drought on the other

Table S5 Candidate genes in linkage with SNPs with $-\log_{10}(P)$ score > 4 (20 kb half-window size) that have common effects on plant response to parasitic plants and aphids, on the one hand, vs fungus, caterpillar, thrips and drought on the other

Table S6 Candidate genes in linkage with SNPs with $-\log_{10}(P)$ score > 4 (20 kb half-window size) that have common effects on biotic and abiotic stress responses

Methods S1 Salt.

Methods S2 Abiotic.

Methods S3 Caterpillar – combinatory stress.

Methods S4 Parasitic plants.

Methods S5 Nematodes.

Methods S6 Whiteflies.

Methods S7 Aphids.

Methods S8 Thrips.

Methods S9 Drought – combinatory stress.

Methods S10 Fungus – combinatory stress.

Methods S11 Screening of T-DNA lines.

Methods S12 Simulations to compare power for full MTMM, contrast MTMM and univariate analysis.

Chapter 5

Natural genetic variation in *Arabidopsis thaliana* for oviposition preference by the small cabbage white butterfly *Pieris rapae*

Silvia Coolen, Johan A. Van Pelt, Saskia C.M. Van Wees and Corné M.J. Pieterse

Plant-Microbe Interactions, Department of Biology, Utrecht University, P.O. Box 800.56, 3508 TB, Utrecht, the Netherlands

Abstract

Insect herbivores are amongst the most destructive plant pests, damaging both naturally occurring and domesticated plants. As sessile organisms, plants make use of structural and chemical barriers to counteract herbivores. However, herbivores that are well adapted to their host's defenses are generally difficult to refrain. By actively antagonizing the number of insect eggs deposited on plants, future damage by the herbivore's offspring can be limited. Therefore, it is important to understand which plant traits influence attractiveness for oviposition, especially for insects that are well adapted to their host plants. In this study, we investigated the oviposition preference of *Pieris rapae* butterflies by offering them the choice between 350 different naturally-occurring *Arabidopsis thaliana* accessions for laying their eggs. Using genome-wide association mapping of the oviposition data and subsequent fine mapping with full genome sequences of 164 accessions, we identified loci in the *A. thaliana* genome with candidate genes associated with plant growth, regulation of respiration, plant defense, stress signaling, and jasmonic acid biosynthesis. Our findings suggest that *P. rapae* butterflies are capable of selecting specific plant genotypes for their offspring and that plant genotype can influence oviposition.

Introduction

Insect herbivores consume considerable amounts of plant biomass, causing major crop losses world-wide. Plants evolved different strategies to counteract insect herbivores, such as the formation of structural and chemical barriers, and the emission of volatile organic compounds (VOCs) that attract parasitoid enemies of the attacking herbivores (Pieterse and Dicke 2007). A first barrier that can ward off insect herbivores consists of trichomes. Trichomes contain defensive secondary metabolites, such as toxic glucosides, which are released upon damage to hinder the attacking insect (Kliebenstein *et al.,* 2001). Trichomes of *Arabidopsis thaliana* contain anti-microbial and anti-herbivore glucosinolate compounds (Reymond *et al.,* 2004; Frerigmann *et al.,* 2012). When the constitutive and structural barriers are ineffective, a second line of defense can be activated. In this induced defense response, recognition of the insect herbivore that feeds on the plant triggers the production of one or more of the phytohormones jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA), which subsequently leads to the induction of defenses that ward off the invading insect (De Vos *et al.,* 2006b; Howe and Jander 2008; Verhage *et al.,* 2011; Vos *et al.,* 2015). Besides the aforementioned glucosinolates, these induced defenses can comprise the production of other anti-insecticidal toxins, feeding deterrents, and proteinase inhibitors that impair the activity of digestive proteases in the insect gut (Howe and Jander 2008). Insect-induced defense signaling extends systemically to undamaged plant parts, therewith protecting the plant against future herbivore damage (De Vos *et al.,* 2006b; Bodenhausen and Reymond 2007; Howe and Jander 2008; Soler *et al.,* 2013; Vos *et al.,* 2013b).

Amongst the most destructive pests on cruciferous plants is the small cabbage white butterfly *P. rapae*, a herbivore belonging to the order of the Lepidoptera. *P. rapae* is well adapted to its host's defenses, either by inactivating or evading them (Hopkins *et al.,* 2009). Plant responses to *P. rapae*

feeding lead to the production of lipoxygenases that are involved in the biosynthesis of JA and other oxylipins, which activate downstream defenses but can also be directly toxic to herbivores (Kessler *et al.,* 2004; Howe and Jander 2008; Dąbrowska *et al.,* 2009; Shabab *et al.,* 2014). To counteract these plant defenses, *P. rapae* larvae have elicitors in their oral secretions that can modulate the plant's hormone-regulated defense response for its own advantage (Verhage *et al.,* 2011; Broekgaarden *et al.,* 2015). Aside from phytohormones, secondary metabolites, such as glucosinolates, play an important role in the interaction between *P. rapae* and Brassicaceae. Glucosinolates are potent toxins that inhibit growth of insect larvae that feed on the plant. However, *P. rapae* is adapted to these host defenses with a gut enzyme that degrades indole-glucosinolates, resulting in the formation of less toxic nitriles. *P. rapae* uses these secondary metabolites as feeding and oviposition stimulants because non-adapted insect herbivores do not favor to feed or oviposite on glucosinolate-producing plants (De Vos *et al.,* 2008; Mumm *et al.,* 2008; Hopkins *et al.,* 2009; Ali and Agrawal 2012). Before being in direct contact with a host plant, *P. rapae* butterflies use visual and volatile cues to detect a potential host plant during flight (Hern *et al.,* 1996; Smallegange *et al.,* 2006; Zheng *et al.,* 2010). It has been suggested that *P. rapae* relies on plant color to induce landings, as plant color may be connected to the plant's nutritional status, an important prerequisite for oviposition (Myers 1985; Renwick and Radke 1988; Hwang *et al.,* 2008). Furthermore, *P. rapae* is able to learn which optical traits correspond to suitable host plants by a contact-chemosensory reward with plant oviposition stimulants such as glucosinolates (Traynier and Truscott 1991; Bukovinszky *et al.,* 2005).

After egg deposition by *P. rapae* butterflies, *A. thaliana* plants were shown to recognize egg-derived elicitors, resulting in the subsequent induction local SA-dependent defenses (Little *et al.,* 2007; Bruessow *et al.,* 2010; Verhage *et al.,* 2010; Fatouros *et al.,* 2012). SA antagonizes JA-dependent defenses, hence this egg-mediated induction of SA-JA crosstalk gives the newly born caterpillars a head start by suppressing the JA-dependent defenses that are activated when the larvae start to feed (Bruessow *et al.,* 2010). In black mustard (*Brassica nigra*), insect eggs were shown to activate a rapid local cell death underneath the deposited eggs, resulting in effective removal of the insect eggs (Shapiro and De Vay 1987; Fatouros *et al.,* 2016). Hence, both *P. rapae* and its host plants have adapted several mechanisms to counteract each other.

With the ultimate aim to discover novel plant traits that make a plant (un)favorable for host selection by specialist herbivores such as *P. rapae*, we mined the natural genetic variation amongst 350 naturally occurring *A. thaliana* accessions for genomic regions that are related to oviposition discrimination by *P. rapae*. To this end, we offered *P. rapae* butterflies the choice of 350 randomly placed *A. thaliana* accessions and scored the number of eggs laid per accession. The obtained data were subsequently used in a genome-wide association study (GWAS). GWA mapping has been used in many studies to gain insight into naturally evolved plant adaptive responses, revealing genes with important functions in diverse processes of plant growth and survival (Atwell *et al.,* 2010; Baxter *et al.,* 2010; Kloth *et al.,* 2016; Davila Olivas *et al.,* 2017b; Thoen *et al.,* 2017). Together with supportive fine mapping, using full genome sequences, we revealed several genomic regions with plant genes potentially involved in host selection by *P. rapae* butterflies.

Results

Oviposition preference of *P. rapae* butterflies on 350 *A. thaliana* accessions

To study the genetic basis of host selection for oviposition by *P. rapae* butterflies, we investigated their oviposition preference on 350 naturally occurring accessions of the *A. thaliana* haplotype map (HapMap) collection (Baxter *et al.,* 2010; Platt *et al.,* 2010). Butterflies were allowed to oviposit on the total collection of randomly distributed, homogeneously spaced *A. thaliana* accessions for 2-3 days in a cage setup, resulting in a total number of 622-1879 eggs deposited per experiment (Figure 1A and Supplemental Table S1 and S2). In total 7 independent randomized experiments were conducted. *P. rapae* is known to oviposit predominantly on sunny and warm days during the morning and early afternoon (Root and Kareiva 1984). Because the experimental cage was positioned in a greenhouse with natural daylight, we anticipated that the position of the plants within the cage would influence the choice of host plant of the butterflies. Figure 1B shows that in our cage setup most eggs were deposited on the East side of the cage, i.e. the side where the sunlight was coming from in the morning. Oviposition preference was also influenced by the corners and the edges of the cage setup, as the majority of the eggs were deposited in these zones of the cage (Figure 1B). Natural flight and search behavior of *P. rapae* butterflies was previously described to cause 'edge effects' under field conditions (Root 1973; Jones 1977; Muriel and Grez 2002), which could explain the observed skewed egg distribution over the cage. To correct for these cage position effects, egg counts were normalized with the average distribution effects per experiment, resulting in a normalized egg distribution that ranges from 320-351 per experiment. Because the total number of eggs deposited in the cage differed over the 7 experiments, we also normalized the egg counts per accession to equal total egg counts per experiment.

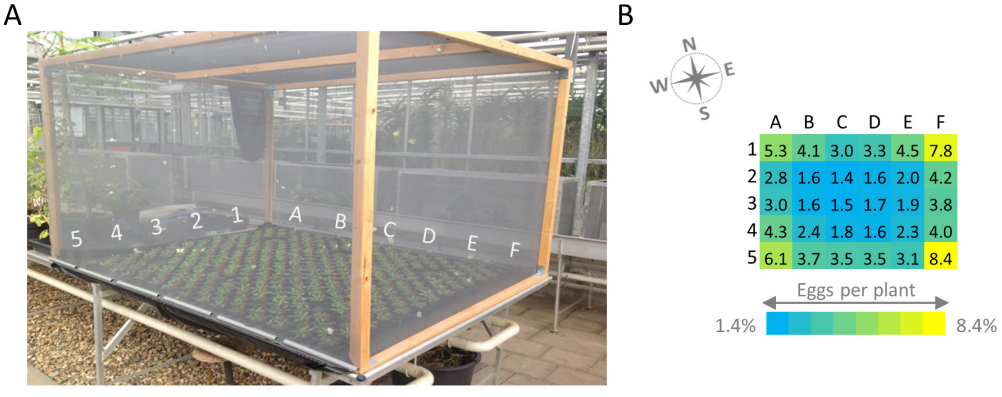


Figure 1. Experimental setup and oviposition distribution within the cage setup. (A) All 350 *A. thaliana* accessions were randomly assigned a position in one of the 30 plots in the cage, after which a mixed group of 20-30 female and male butterflies were placed in the cage. Accessions were evenly spaced throughout the cage and randomized within 7 replicated experiments to correct for cage position effects. Butterflies were allowed to feed on feeding solutions supplied within the cage. The position of the cage is indicated by the compass. Female butterflies were allowed to oviposit freely for 2-3 days. (B) Oviposition distribution in the cage over 7 experiments. Heatmap indicates the average number of eggs deposited per plant in the respective plots.

Differences in oviposition preference by *P. rapae* for 350 *A. thaliana* accessions

To analyze the effect of plant genotype on the oviposition preference of *P. rapae* butterflies, we first normalized the egg counts per accession for the average cage-position effects in each experiment and subsequently normalized that data for the total egg counts per experiment (Supplemental Table S2). The resulting normalized average egg counts per accession are depicted in Figure 2. Amongst the 350 accessions that were used we observed two glabrous, trichome-less, accessions, Est-0 and Br-0 (Hauser *et al.,* 2001; Barth *et al.,* 2002; Bloomer *et al.,* 2012). Previously, Reymond *et al.* (2004) demonstrated that *P. rapae* caterpillars performed better on glabrous plants, hence we hypothesized that butterflies may anticipate on this and prefer to oviposit on plants without trichomes. With a normalized egg score of 1.05 and 1.21 respectively, Br-0 and Est-0 indeed belong to the accessions that received above median numbers of egg per plant of tested *A. thaliana* accessions. However, additional experiments with trichome-containing Col-0 and glabrous Col-5 plants showed that *P. rapae* butterflies do not prefer glabrous plants over plants with trichomes (Supplemental Figure S1), although caterpillar performance (weight) was shown to be higher on glabrous Col-5 (Verhage *et al.,* 2011).

Amongst the 350 accessions tested, 14 accessions developed spontaneous chlorosis and necrosis, which was described by Todesco *et al.* (2010) as being late onset necrosis. Of these accessions none with moderate or severe late onset necrosis were found amongst the top 25% of most-preferred accessions for oviposition, whereas 6 of them were amongst the top 25% of least-preferred accessions (Supplemental Table S2). This suggests that spontaneous necrosis of the plant is an unfavorable trait for host selection by *P. rapae* butterflies.

Plant size also differed amongst the 350 accessions. To test if this influenced oviposition preference, we categorized the accessions of 4 experiments into 3 size classes that correspond to plant rosette diameter respective to the pot size: small (rosette fully within the pot boundary), medium (max. 4 rosette leaves exceeding the pot boundary), and large (more than 4 rosette leaves exceeding the pot boundary). The average plant size within each category positively correlated with the normalized average number of eggs per corresponding classes (Pearson correlation; R = 0.90), with medium and large plants clearly receiving more eggs than small plants (Figure 3).

Previously, Kliebenstein *et al.* (2001) measured aliphatic- and indole-glucosinolate levels in a range of *A. thaliana* accessions, 15 of which were also present amongst the 350 accessions tested in this study (Supplemental Table S3). Comparing the normalized average number of eggs deposited on these accessions with the aliphatic- and the indole-glucosinolate levels reported by Kliebenstein *et al.* (2001), revealed a weak positive correlation (R = 0.36) between egg number and indole-glucosinolate levels, confirming previous findings showing that indole-glucosinolates such as indole-3-carbinol are attractive for oviposition by *P. rapae* (Huang and Renwick 1994; De Vos *et al.,* 2008; Müller *et al.,* 2010). Conversely, we found a moderate negative correlation (R = -0.51) between of egg number and aliphatic-glucosinolate levels, which suggests that this class of glucosinolates deters rather than attracts *P. rapae* butterflies for oviposition.

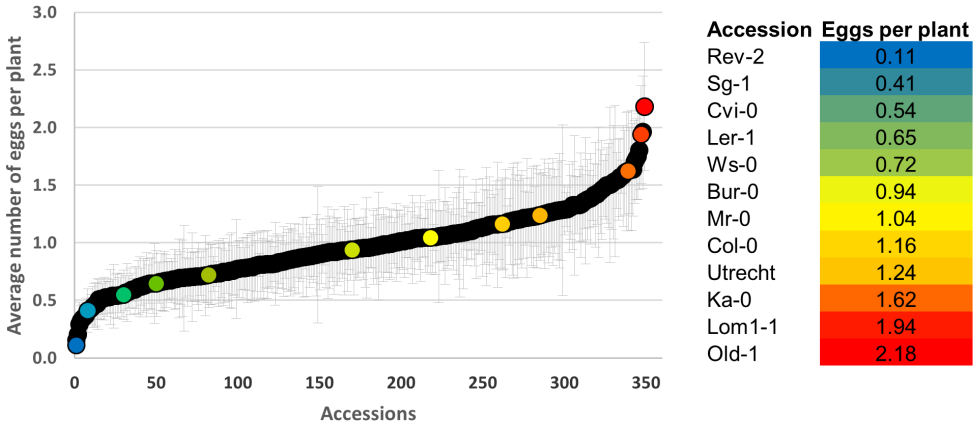


Figure 2. Natural variation in oviposition preference of *P. rapae* butterflies on *A. thaliana* accession. The graph shows the normalized average number of eggs deposited by *P. rapae* butterflies on 350 different *A. thaliana* accessions. Data are the average of 7 independent experiments on the total set of 350 accessions, each experiment containing one randomly positioned plant per accession. In each experiment, 10-15 female *P. rapae* butterflies were allowed to freely oviposit for 2-3 days on the offered population of 350 plants. Error bars show standard errors (SE). In the color gradient on the right, specific accessions with distinct normalized average eggs counts are highlighted.

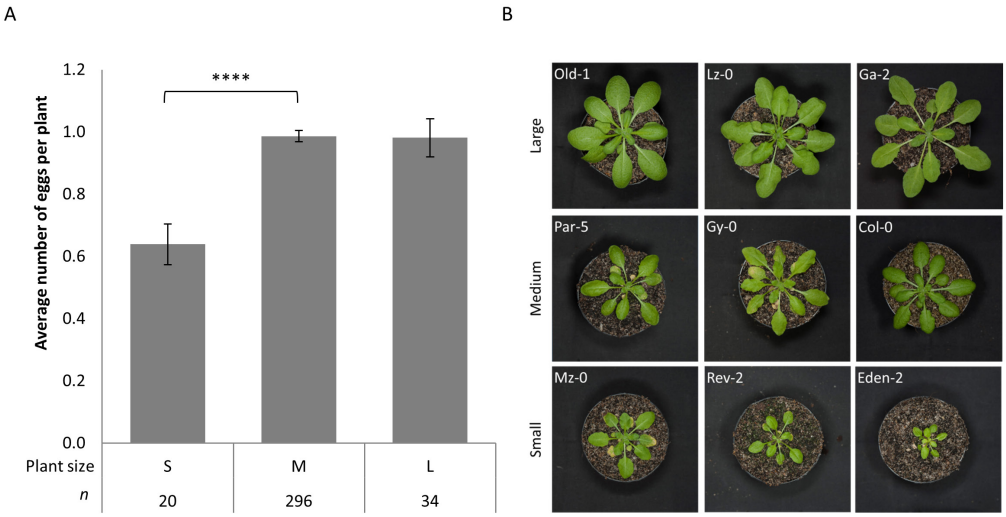


Figure 3. Relationship between plant size and normalized number of eggs deposited per plant. A) The graph shows the normalized average number of eggs deposited per plant (n= 4) on small (S), medium (M), and large (L) plants. In 4 experiments all 350 4-week-old plants were assigned to the rosette diameter category small (rosette fully within the pot boundary), medium (max 4 rosette leaves exceeding the pot boundary), and large (more than 4 rosette leaves exceeding the pot boundary). Significance was calculated with a Student's t-test ($p < 0.0001$). B) Examples of 4-week-old *A. thaliana* accessions in the plant size categories.

Genome-wide association mapping of loci associated with *P. rapae* oviposition preference

Next, we mined the natural genetic variation in egg deposition among 346 of the tested *A. thaliana* accessions (excluding accessions L*er*-1, Cnt-1, Ct-1 and Belmonte-4-94) for genetic components that contribute to the observed oviposition differences. To this end, we performed a GWAS on the setup-corrected and transformed data of 7 experiments (Supplemental Table S2 and S4). Narrow sense heritability was estimated to be very low (h^2 = 0.03 with a 95% confidence interval of 0.0-1.0, (Kruijer *et al.,* 2015)), suggesting that there is little additive genetic variance causing the choice for host plants. This low narrow sense heritability can be caused by the use of average values of 7 independent experiments, which results in large h^2 standard errors (Kruijer *et al.,* 2015). Another explanation is the presence of associations with plant life history traits that are essential for fitness and survival (Mousseau and Roff 1987). Because of the lack of genetic variation due to extensive selection during evolution, these plant traits hardly change over time. Although not particularly interesting for breeding purposes due to their limited heritable variance, these traits can provide insight into essential plant survival or fitness mechanisms that contribute to host selection by *P. rapae* butterflies.

To identify candidate genes with putative roles in *P. rapae* oviposition preference, we performed GWA mapping using the factored spectrally transformed linear mixed models (FaST-LMM) algorithm (Lippert *et al.,* 2011) and a set of ~214 K SNPs (Baxter *et al.,* 2010; Platt *et al.,* 2010; Chao *et al.,* 2012; Thoen *et al.,* 2017). SNP-trait associations of interest were selected by setting an arbitrary threshold with a logarithm (base 10) of the odds (LOD, $-\log_{10}(p)$) score of 4.0. GWA mapping results revealed 14 SNP-trait associations for a total of 12 unique genes (Figure 3A and Table 1) and 50 SNPs in linkage disequilibrium (LD; estimated to be 10-50 kb (Nordborg *et al.,* 2005; Kim *et al.,* 2007)) accounting for an additional number of 23 genes (Supplemental Table S5). The genes within the loci that exceeded the threshold for gene selection have not previously been directly connected with plant traits affecting herbivory, host selection by *P. rapae* butterflies, nor with stress signaling or general defensive mechanisms. We found a RING/U-box gene with unknown function, several copia-like retrotransposons, genes with unknown functions, an RNA-directed DNA polymerase, *ACTIN-DEPOLYMERIZING FACTOR 1* (*ADF1*), a Sec23/Sec24 protein transport gene, *AUXIN RESPONSE FACTOR 16* (*ARF16*) and *TATA-BINDING PROTEIN (TBP)-ASSOCIATED FACTOR 4* (*TAF4*). ADFs were previously shown to have different roles in plant defense. For instance, ADF3 was shown to be required in controlling infestation by phloem feeding *Myzus persicae* and *Triticum aestivum*, while ADF4 has been implicated in resistance against stripe rust (*Puccinia striiformis* f. sp. *Tritici;* Zhang *et al.,* 2017; Mondal *et al.,* 2018)). ADF1 was shown to be phosphorylated by CALCIUM-DEPENDENT PROTEIN KINASE (CDPK6, also known as CPK3), which is involved in defense signaling induced by generalist herbivore *Spodoptera littoralis* (Nagamangala Kanchiswamy *et al.,* 2010; Boudsocq and Sheen 2013; Dong and Hong 2013). Hence, activation of ADF1 can be a result of herbivory-induced signaling and may be involved in defense against herbivores. The Sec23/Sec24 protein transport gene was found to be ET-regulated (De Paepe *et al.,* 2004). ET is known to be induced in plants by oral secretions upon herbivory by *P. rapae* and is involved in rewiring the plants defense

response to a more favorable JA- and ET-mediated response (Koornneef *et al.,* 2008). This suggests that the Sec23/Sec24 protein transport gene might act downstream of herbivore-induced ET production. ARFs were recently shown to be responsive to herbivory in chickpea (*Cicer arietinum* L.) after induction with oral secretions of gram pod borer (*Helicoverpa armigera*) and simultaneous wounding (Pandey *et al.,* 2017). This suggests a possible role for ARF16 in herbivore-induced plant signaling in *A. thaliana*. TAF4 was shown to interact with TAF15b of which a T-DNA insertion line was shown to partially compensate the constitutive SA accumulation found in the *suppressor of npr1-1, constitutive 1* (*snc1*) mutant. Since SA was found locally induced by egg-derived elicitors (Little et al., 2007; Bruessow et al., 2010; Verhage et al., 2010; Fatouros et al., 2012), TAF4 might play a role in the plant's response to *P. rapae* eggs (Dong *et al.,* 2016).

Table 1. GWAS candidate genes associated with *P. rapae* oviposition preference.

Gene	LOD score	TAIR gene description	SNPs
AT1G62370	4.36-5.09	RING/U-box superfamily protein	2
AT2G34100	4.12	Unknown protein	1
AT3G20990	4.16	Copia-like retrotransposon family	1
AT3G25725	4.02-4.42	Copia-like retrotransposon family	2
AT3G25727	4.08	RNA-directed DNA polymerase (reverse transcriptase)	1
AT3G43460	4.20	Unknown protein	1
AT3G46010	4.05	Actin-depolymerizing factor 1 (ADF1)	1
AT4G04426	4.07	Copia-like retrotransposon family	1
AT4G14160	4.16	Sec23/Sec24 protein transport family protein	1
AT4G29310	4.01	Protein of unknown function (DUF1005)	1
AT4G30080	4.31	Auxin response factor 16 (ARF16)	1
AT5G43130	5.05	TBP-associated factor 4 (TAF4)	1

Fine mapping of GWA SNP-trait associations using genome sequences of 164 accessions

To further investigate and strengthen the GWA mapping results, fine mapping of the identified loci was performed using full genome sequences of 164 accessions (Supplemental Table S6) available via the 1001 genomes project (Weigel and Mott 2009). The fine mapping procedure makes use of all genetic variance within the used genomic regions of the 164 full genome sequences compared to a selection of SNPs within the accessions relative to the reference genome of accession Col-0 in case of GWA mapping. Because LD in *A. thaliana* is estimated to be 10-50 kb (Nordborg *et al.,* 2005; Kim *et al.,* 2007), a 50-kb window surrounding the SNPs of interest was included for finding genes of interest. Fine mapping was based on a Kruskal-Wallis test for trait associations with a minor allele frequency larger than 5% (MAF > 0.05).

Fine mapping results show that there are several significant false discovery rate (FDR)-corrected associations that correspond to loci identified with GWA mapping (Figure 3B and Supplemental Figure S2). On the locus surrounding transposable element gene *AT3G25725,* associations were found in an upstream cluster containing 4 genes involved in ET signaling and JA biosynthesis

(Figure 3A). Just upstream of *ETHYLENE RESPONSE DNA BINDING FACTOR 3* (*EDF3*, *AT3G25730*) and downstream of JA biosynthesis gene *ALLENE OXIDE CYCLASE 2* (*AOC2, AT3G25770*) *and AOC3* (*AT3G25780*), two significant SNP peaks were observed with fine mapping (Figure 3B), pointing to *METHIONINE AMINOPEPTIDASE 1B* (*MAP1B, AT3G25740*) and *AOC1* (*AT3G25760*). Of these, *AOC1* encodes an enzyme essential for JA biosynthesis and was shown to be rapidly upregulated within 3 h after *P. rapae* feeding on *A. thaliana* plants (Coolen *et al.,* 2016). JA biosynthesis and signaling significantly impacts the performance of insect herbivores, including *P. rapae*, on *A. thaliana* (De Vos *et al.,* 2006a; Little *et al.,* 2007; Verhage *et al.,* 2011; Vos *et al.,* 2013b). Interestingly, Bruinsma *et al.* (2007) showed that *P. rapae* butterflies lay more eggs on control plants over JA treated plants, suggesting that JA levels influence oviposition preference of *P. rapae*. The developmental time from larval hatching until pupation was shown to be delayed on JA-treated plants (De Vos et al., 2006), which may be an incentive for *P. rapae* butterflies to avoid oviposition on plants with high JA levels. Downstream of *AOC1*, a significant association was found with *MAP1B*. *MAP1B* was shown to be a potential targeted of miRn5998, a microRNA responsive to JA treatment (Zhang *et al.,* 2012), again suggesting involvement of JA in host choice for oviposition by *P. rapae*. On chromosome 4 a significant association was observed at the interval of transcription factor *WRKY42* (AT4G04450) and *PUTATIVE ASPARTIC PROTEINASE A3* (*PASPA3*, AT4G04460). *WRKY42* was found to be repressed 3 h after *P. rapae* caterpillar introduction onto *A. thaliana* plants and was shown to be involved in plant phosphate (Pi) homeostasis (Su *et al.,* 2015). Plant nutrient status was previously shown to influence caterpillar performance and plants that experience Pi deficiency where shown to induce the JA pathway and enhance their defense against insect herbivory (Hwang *et al.,* 2008; Khan *et al.,* 2016). *PASPA3* was shown to be upregulated 6 h after the introduction of a *P. rapae* caterpillar onto an *A. thaliana* plant. Furthermore, PASPA3 was shown to be a potential target of VASCULAR-RELATED NAC-DOMAIN 7 (VND7), which was reported to be involved in plant drought tolerance and found upregulated 12 h after introducing *P. rapae* caterpillars onto *A. thaliana* plants (Yamaguchi *et al.,* 2011; Reusche *et al.,* 2012; Coolen *et al.,* 2016).

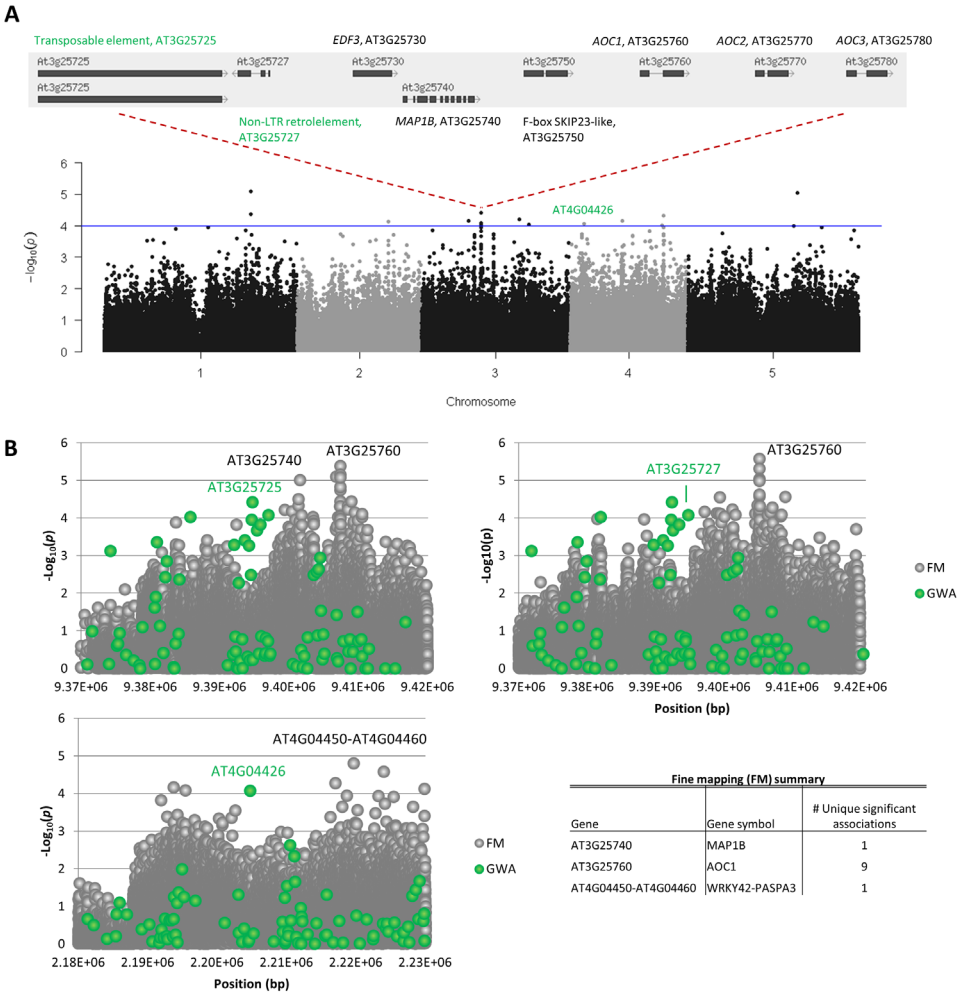


Figure 3. GWA mapping and fine mapping results for oviposition preference of *P. rapae* on 346 *A. thaliana* accessions. A) Manhattan plot (grey and dark grey) showing the $-\log_{10}(p)$ values of the SNP-trait associations from the GWA mapping results on *A. thaliana* chromosomes 1-5 (x-axis). In green three GWA loci are indicated for which SNP-trait associations (LOD ≥ 4.0) were confirmed via fine mapping. The blue line indicates the arbitrary LOD threshold of 4.0 ($-\log_{10}(p)$=4.0) for selection of SNPs. Above the Manhattan plot a gene cluster is depicted that was found upstream of the transposable element gene *AT3G25725* in which SNPs above the threshold were found by GWA mapping. B) Fine mapping (FM; grey dots) of three SNP-trait associations that were identified by GWA mapping (green dots), using the 50-kb window around the GWA SNPs from the genome sequences of 164 of the tested *A. thaliana* accessions. The graphs show the $-\log_{10}(p)$ values of the SNP-trait associations on the y-axis and the chromosome position of the SNPs in base pairs (bp) on the x-axis. Significant (FDR-corrected) FM associations are shown in black ATG-numbers along with the number of significant associations in the FM summary.

Discussion and conclusion

To study the genetic contribution of plant genes to oviposition preferences by *P. rapae* butterflies we studied the natural genetic variation in the model plant *A. thaliana*. Previously, it was questioned whether there has been an evolutionary arms race between *A. thaliana* and *P. rapae* because of their separation in seasonal occurrence (Harvey *et al.,* 2007). However, especially summer annuals within the collection might have experienced selective pressure by herbivores such as *P. rapae* (Pigliucci 1998; Johanson *et al.,* 2000; Koornneef *et al.,* 2004; Edger *et al.,* 2015; Davila Olivas *et al.,* 2017a). Notwithstanding the fact that in nature *A. thaliana* and *P. rapae* hardly interact, both species clearly possess general traits that determine the outcome of such plant-insect interactions and which can be studied by mining the natural genetic variation for such traits in the *A. thaliana* HapMap collection. Host selection is one of the crucial steps in which plant traits can affect both plant and insect herbivore survival. For insect herbivores such as *P. rapae*, both visual and non-visual plant traits can affect host selection as was shown by many studies (Traynier and Truscott 1991; Hern *et al.,* 1996; Bukovinszky *et al.,* 2005; Smallegange *et al.,* 2006; Zheng *et al.,* 2010). In a search for plant traits that determine plant attractiveness for oviposition by *P. rapae* butterflies, we conducted 7 independent experiments in which butterflies were allowed to oviposit on 350 different *A. thaliana* accessions. Depending on the weather conditions, being warm and sunny preferably, butterflies were allowed to oviposit on the plants for 2-3 days. Because of this relatively long host selection time, learning behavior and triggered plant defenses can play a role in the host selection procedure beside visual and non-visual cues that can be obtained fairly quickly. Results from the host selection experiments show that butterflies responded strongly to edges and especially corners in our cage setup. These effects were shown to be even stronger on the East side of the setup where natural daylight entered during the mornings when *P. rapae* is most active with oviposition (Root and Kareiva 1984). Furthermore, the overall number of eggs deposited on the 350 plants differed per experiment, ranging between 622 and 1879 eggs per experiment. These results show how important it is to carefully monitor experimental setups and insect behavior before interpreting the data and using it for further study. To limit the plant-genotype unrelated effects, we randomized the 350 accessions over all 7 experiments and normalized the egg counts per plant for the overall cage position effects and the total number of eggs deposited. After normalization, we still observed differences in the number of eggs that were deposited on the accessions, suggesting that the genetic background of the accessions influenced *P. rapae* oviposition preference.

Based on a number of general observations in the collection of 350 *A. thaliana* accessions, we asked the data set a number of specific questions related to the effect of plant size, spontaneous lesion development, and the role of trichomes on the oviposition preference of *P. rapae*. Firstly, we tested the effect of trichomes on oviposition preference. For the specialist herbivore *Plutella xylostella* a negative relationship was found between trichome density and egg number on *A. thaliana* plants (Handley *et al.,* 2005). In our study, glabrous accessions Est-0 and Br-0 indeed belonged to the accessions that received above median numbers of egg per plant. However, we found no difference in oviposition preference between trichomed Col-0 and glabrous Col-5, suggesting that

trichomes are not an important host selection cue for *P. rapae* oviposition in our experimental setup. This might be due to the fact that *P. rapae* butterflies deposit their eggs predominantly at the abaxial side of the leaf where no trichomes are present.

Secondly, we found that small plants received significantly fewer eggs than medium and large plants. This can be explained by the fact that larger plants have simply more leaf surface so by chance would have a higher probability of being chosen by the butterflies. However, larger plants may also intentionally be more attractive, because they would provide the offspring with more food.

Thirdly, we found that plants with spontaneous chlorotic or necrotic lesions were less attractive for *P. rapae* oviposition. The reason for this is unclear. Lesion-forming plants may be visually unattractive or exhibit unfavorable defenses that can be sensed by the butterflies. In black mustard (*Brassica nigra* L.), egg-induced necrosis can cause detachment from plant leaves, preventing herbivory after hatching (Shapiro and De Vay 1987). Although we did not observe egg-induced necrosis in the 350 *A. thaliana* accessions, *P. rapae* butterflies apparently seem not to prefer to deposit eggs on plants displaying visual chlorotic or necrotic spots.

Fourthly, we found a weak positive correlation between egg counts and indole-glucosinolate levels as determined by Kliebenstein *et al.* (2001), confirming previous findings that *P. rapae* is attracted by glucosinolates for host selection and feeding (Müller *et al.,* 2010). It also validates our experimental setup as being capable of assessing oviposition preference of *P. rapae*. While the correlation between egg counts and indole-glucosinolate levels were positive, the correlation with aliphatic-glucosinolate levels was moderate negative. A possible explanation is that plants that predominantly have indole-glucosinolates are attractive for oviposition by *P. rapae*, resulting in less oviposition on plants that predominantly have aliphatic-glucosinolates (Huang and Renwick 1994; Kliebenstein *et al.,* 2001; De Vos *et al.,* 2008; Müller *et al.,* 2010).

To find plant genes that contribute to host selection preferences for oviposition by *P. rapae* butterflies, GWA mapping was conducted on data obtained from 7 independent experiments in which *P. rapae* was allowed to oviposit on 350 *A. thaliana* accessions at once. The obtained associations revealed 12 candidate genes have not previously been connected with plant traits affecting herbivory, host selection by *P. rapae* butterflies, nor with stress signaling or general defensive mechanisms. To further strengthen our GWAS findings, fine mapping was performed providing additional evidence for the observed associations. These include genes known to be involved in or associated with defense signaling mediated by JA and plant defenses against insect herbivores. In particular the association found with *AOC1* is very interesting. *AOC1* encodes an allene oxide cyclase that is essential for the biosynthesis of JA and its oxylipin derivatives (Wasternack and Hause 2013). JA biosynthesis is known to be an essential step in defense against insect herbivores and our data support the possibility that *P. rapae* butterflies are able to detect differences caused by allelic variations between the accessions in this essential JA biosynthesis gene. Perhaps the gene variants of *AOC1* within the HapMap collection correspond to differences in basal levels of JA and its derivatives that can be detected by butterflies and may affect their choice for a host plant. Indirectly, butterflies may

also detect differences in glucosinolates as a result of altered JA biosynthesis. For instance, it was shown that impairment of JA responses in the *coronatine insensitive 1* (*coi1*) mutant had much lower constitutive levels of aliphatic glucosinolates than wildtype (Mewis *et al.,* 2006).

Future research will be focused on obtaining definite evidence for the role of *AOC1* and the other identified candidate genes in host selection of *P. rapae* for oviposition. Knowledge on the genetic basis of *P. rapae* oviposition preference may be used in breeding strategies that are aimed at reducing the attractiveness of crop plants for insect herbivores.

Acknowledgements

We are thankful to Rutger Baar, Mirjam de Vries, Sjon Hartman, Roderick Bouwman, Jason Banda and Tom Broere for their help with the experiments, Willem Kruijer for his help with GWA mapping and Dmitry Lapin for his help with fine mapping. This work was supported by the Netherlands Organization for Scientific Research (NWO) through the Dutch Technology Foundation (STW) STW Perspective Program 'Learning from Nature' [STW10988].

Materials and methods

Plant material

In this study 350 *A. thaliana* (L.) Heynh. accessions of the HapMap population (http://bergelson.uchicago.edu/wp-content/uploads/2015/04/Justins-360-lines.xls) were used. The Hapmap population has been genotyped for 250K bi-allelic SNPs (Baxter *et al.,* 2010; Platt *et al.,* 2010; Chao *et al.,* 2012) and after quality control and imputation this SNP-set was reduced to a set of 214.051 SNPs (Thoen *et al.,* 2017).

Plant growth conditions

A. thaliana seeds were sown in cultivation containers filled with autoclaved river sand supplied with half-strength Hoagland solution containing sequestrene as described (Wees *et al.*, 2013). Cultivation containers were enclosed in a tray with water and covered with a transparent lid to attain a high relative humidity (RH) for germination. Seed stratification was performed in the dark for 2 days at 4°C to ensure a homogeneous germination. After stratification, the containers were moved to a growth chamber with an 8-h day/16-h night rhythm, a temperature of 21°C, and a light intensity of 100 µmol m^{-2} sec^{-1}. Container lids were slightly opened after 8 days and gradually removed over a 2-day period to adjust to the 70% RH present in the growth chamber. Seedlings were transplanted to individual pots containing an autoclaved mixture of river sand and potting soil (1:1 (v:v)). Plants were supplied with water from the bottom up three times per week and at an age of 3 weeks the plants were supplied once with half-strength Hoagland solution.

Rearing of *P. rapae*

P. rapae was reared on cabbage plants (*Brassica oleracea* convar. capitata var. alba) under greenhouse conditions (24°C, with natural daylight). Butterflies were supplied with flowering plants such as *Lantana camara* for their (nectar) food and additionally with a solution of 20% honey and 10% sucrose. Inbreeding of the population was minimalized by regularly adding wild butterflies and caterpillars from the Dutch Flevopolder to the existing population.

Experimental conditions and experimental design

Oviposition by *P. rapae* was performed in 7 independent experiments, each with a single 4-week-old plant of all 350 *A. thaliana* accessions. The 350 plants were randomized and assigned to 1 of the 30 square 27.5x27.5cm plots (A1-F5) in the 2.0x1.6m (1.2m high) insect cage in the greenhouse (Figure 1). Accession were evenly spaced in the cage. A mix of approximately 20-30 male and female butterflies were released into the cage and females were allowed to oviposit freely on the 350 accessions for 2-3 days, depending on the weather conditions. Butterfly feeding sites consisted of a solution containing 20% (v/v) honey and 10% (w/v) sucrose, which were positioned in the middle of cage locations B2, E2, B4 and E4. After 2-3 days the butterflies were removed from the cage after which the number of eggs was recorded by counting all the eggs on both the plant and the corresponding pot.

Plant size categories

Plant sizes were evaluated for 4 out of the in total 7 experiments. Plants were categorized in 3 size classes: small (rosette fully within the pot boundary), medium (max 4 rosette leaves exceeding the pot boundary) or large (more than 4 rosette leaves exceeding the pot boundary). To average the plant size parameters over the 4 experiments, the categories small, medium and large were assigned the numbers 1, 2, and 3, respectively, after which the average plant size per accession was calculated. For the correlation analysis with egg counts, accessions with an average plant size ≤1.5 were placed in bin "small", accessions with average plant size >1.5 and ≤2.5 were placed in bin "medium", and accessions with average plant size >2.5 were placed in bin "large".

Oviposition preference test on *A. thaliana* genotypes with or without trichomes

For the experiment in which the preference of *P. rapae* butterflies was tested on *A. thaliana* genotypes with or without trichomes, five small 30x30 cm (54 cm high) insect cages were used, each containing two Col-0 plants (with trichomes) and two glabrous Col-5 plants. The plants were placed crosswise in a square position in the middle of the cage with feeding solution in the middle. After three days the number of eggs on each plant and pot was recorded.

GWAS

GWAS was performed using data of 346 accessions on the average number of deposited eggs per plant that was normalized for the cage position effects and the total number of eggs deposited per experiment, and subsequently transformed to a normal distribution, using an arcsine transformation (Supplemental Table S4). The transformed phenotype was defined as arcsin(square root(average normalized number of eggs per plant/6)). GWAS was employed using Fast-LMM software (Lippert *et al.,* 2011) with a minor allele frequency (MAF) of > 0.05 together with an arbitrary threshold with a LOD ($-\log_{10}(p)$) score of 4 to determine SNP associations of interest. Linkage disequilibrium was taken into account by including all SNPs within 25 kb up and downstream of the SNP of interest. Narrow sense heritability was estimated using the 'heritability' R package (Kruijer *et al.,* 2015).

Fine mapping

Fine mapping was performed using full genome sequences available from the 1001 genomes project ((Weigel and Mott 2009; http://signal.salk.edu/atg1001/3.0/gebrowser.php). Genome sequences were converted to .bcl formats using Jalview (http://www.jalview.org/; Waterhouse *et al.,* 2009) after which the files were used in .csv format for fine mapping. Locus specific mapping was performed using a MAF of > 0.05. A Kruskal–Wallis test was used for obtaining significant, false discovery rate (FDR)-corrected, SNP-trait associations using R and the 'p. adjust' function with the Benjamini and Hochberg (BH) method (Benjamini and Hochberg 1995).

Supporting information

Additional Supporting Information for this Chapter.

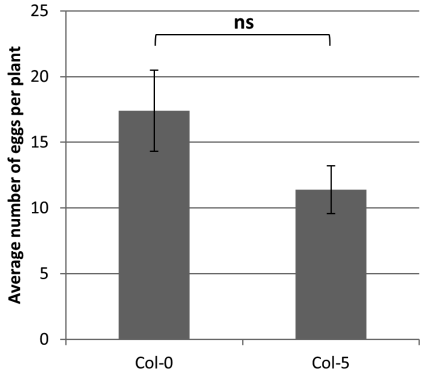
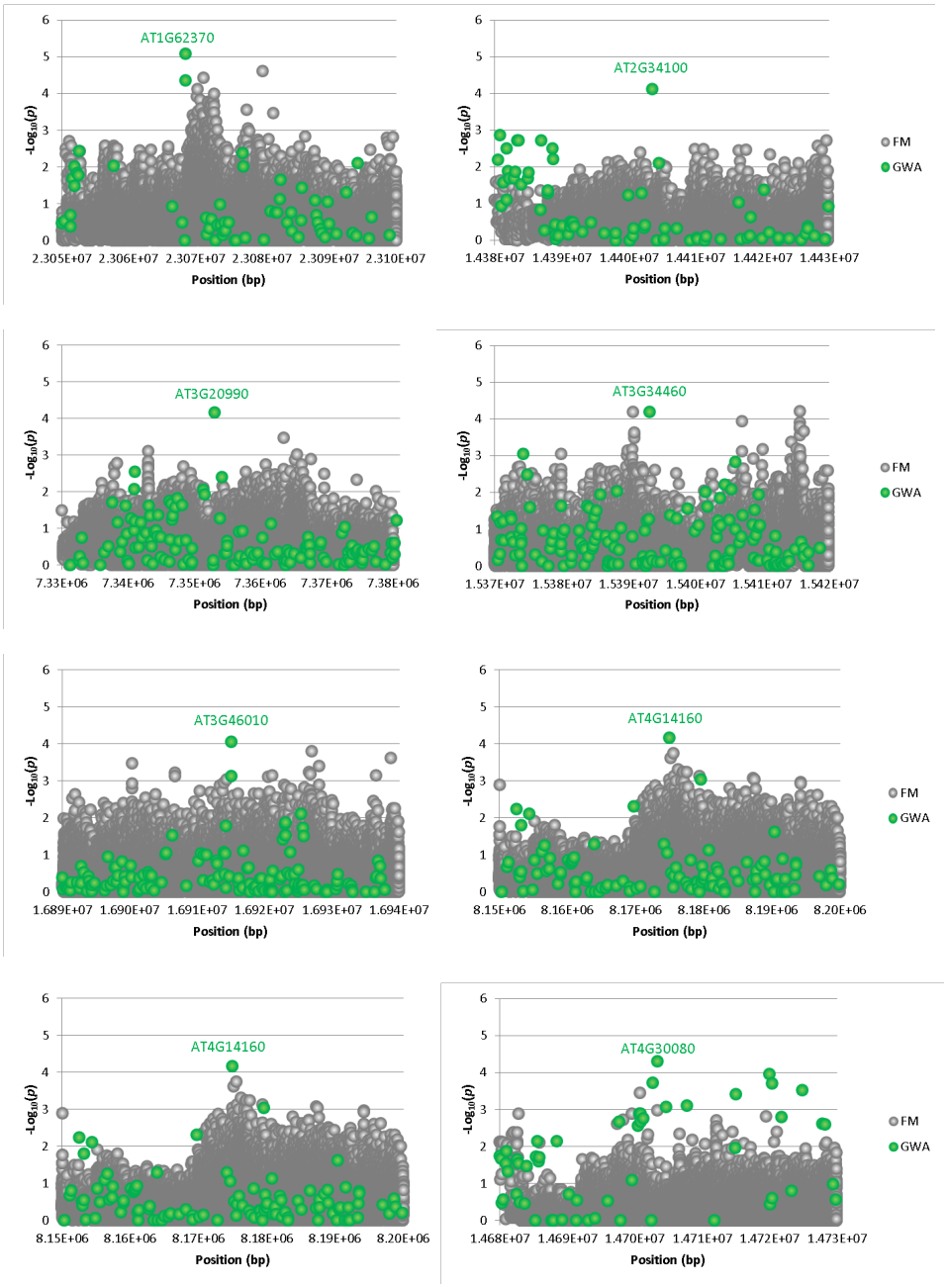
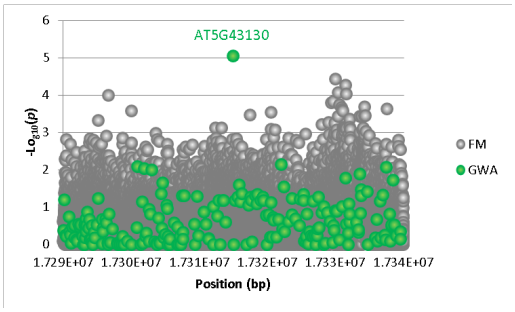


Figure S1. Oviposition preference of *P. rapae* butterflies on trichomed Col-0 versus glabrous Col-5 *A. thaliana* plants. In a setup with 5 cages, each containing 2 trichome-containing Col-0 and 2 glabrous Col-5 plants, 2 female butterflies were allowed to oviposit for 3 days. Bars represent the average number of eggs deposited per plant (n=10). Error bars represent the standard error. Significance was calculated with a Student's *t*-test ($p > 0.05$ = non-significant, ns).

Figure S2. Fine mapping results of GWA SNP-trait associations. Manhattan plots showing the $-\log_{10}(p)$ values of the SNP-trait associations for the fine mapping results (grey dots) of 9 of the 12 loci that with GWA mapping (green dots) were associated with the oviposition preference of *P. rapae* (normalized average number of egg depositions per plant. On the x-axis the position in base pairs (bp) is shown. Genes indicated in green are those obtained from the GWA mapping SNP-trait associations.





Supporting Tables. https://www.dropbox.com/preview/Proefschrift%20Silvia/Proefschrift%20Silvia%20Coolen%20Supplemental%20Tables%20Chapter%205.xlsx?role=personal

Table S1. Average number of eggs depositied per plant on each position within the experimental setup (Figure 1)

Table S2. Avarage number of eggs deposited per plant for 350 *A. thaliana* accessions of the HapMap collection

Table S3. Correlation between oviposition by *P. rapae* butterflies and aliphatic- and indole-glucosinolate levels in 15 *A. thaliana* accessions.

Table S4. Input data for GWA mapping

Table S5. *A. thaliana* loci of SNP-trait associations and underlying candidate genes within 50-kb window of each SNP

Table S6. Accessions used for fine mapping

Chapter 6

Summarizing discussion

In nature plants are often challenged by exposure to a multitude of abiotic and biotic stresses that occur sequentially or simultaneously. Because plants have evolved in diverse environmental settings, they provide a wealth of genetic variation that can be used for studying naturally existing plant adaptive responses. Plant responses to diverse (a)biotic stresses are known to involve extensive signaling that is predominantly mediated by the phytohormones ABA, SA, JA and ET (Pieterse *et al.,* 2012). These phytohormonal signaling pathways are known to interact, allowing the plant to carefully balance its stress response (Audenaert *et al.,* 2002; Anderson *et al.,* 2004; Laurie-Berry *et al.,* 2006; Mur *et al.,* 2006; Mao *et al.,* 2007; Mohr and Cahill 2007; De Vleesschauwer *et al.,* 2010; Leon-Reyes *et al.,* 2010; Gao *et al.,* 2011; Birkenbihl *et al.,* 2012; Pieterse *et al.,* 2012; Caarls *et al.,* 2015; Kloth *et al.,* 2016; Yuan *et al.,* 2017). However, a growing body of evidence has shown that plant responses to combinations of different stresses are distinct from the sum of the individual stress responses (Atkinson and Urwin 2012; Rasmussen *et al.,* 2013; Santino *et al.,* 2013; Kissoudis *et al.,* 2014; Coolen *et al.,* 2016; Davila Olivas *et al.,* 2016; Davila Olivas *et al.,* 2017b), suggesting a complexity of antagonistic and synergistic interactions between stress responsive pathways. However, still little is known about plant responses during simultaneous or sequential stress encounters and with our rapidly changing climate, that facilitates the co-occurrence of unique combinations of diverse abiotic and biotic stresses, serious problems for our food security are predicted (Mittler 2006; Grieve *et al.,* 2010; Mittler and Blumwald 2010; Chakraborty and Newton 2011; Newton *et al.,* 2011; Nicol *et al.,* 2011; Atkinson and Urwin 2012; Bebber *et al.,* 2013; Garrett *et al.,* 2013; Teixeira *et al.,* 2013; Syvertsen and Garcia-Sanchez 2014). To tackle these problems, knowledge on the fundaments of plant adaptations to multi-stress environments is essential and will provide tools that can be used by plant breeders for ensuring optimal and sustainable food production in the future.

In this thesis we aimed at unraveling plant multi-stress resistance mechanisms by studying plant stress responses and natural genetic diversity of *A. thaliana*. We studied plant responses to sequential combinations of drought stress, herbivory by *P. rapae* caterpillars and infection by the necrotrophic fungal pathogen *B. cinerea*, which all form great threats to plants worldwide (Ciais *et al.,* 2005; Hopkins *et al.,* 2009; Dean *et al.,* 2012). Because it was previously demonstrated that the plant hormones ABA, JA and ET are involved in the plant's adaptive response to these stresses (Thomma *et al.,* 1998; Thomma *et al.,* 1999; Shinozaki *et al.,* 2003; Bodenhausen and Reymond 2007; Howe and Jander 2008; Huang *et al.,* 2008; Vos *et al.,* 2013b), our hypothesis was that a combination of these stresses would require signal interactions that could potentially affect the outcome of the response to the second stress. To this end we firstly investigated the plants transcriptional dynamics during sequential (a)biotic stress, to learn how a first stress encounter influences the plants response to a subsequent stress. Secondly, we studied the natural genetic variation of plant responses amongst 346 *A. thaliana* accessions to sequential (a)biotic stress to mine naturally occurring gene adaptations that are potentially involved in plant multi-stress responses. Thirdly, we performed a meta-analysis on multiple (a)biotic stress phenotypes of 11 (a)biotic stresses and 4 combined sequential stresses of 350 *A. thaliana* accessions to reveal shared components that could potentially contribute to plant multi-stress resistance. And lastly, host selection preference by *P. rapae* butterflies was assessed to reveal plant traits that could prevent egg deposition and subsequent herbivory.

Stress-mediated transcriptional adaptations affect plant responses to sequential stress

To cope with a multitude of different stresses, plants rely on a well-balanced and cross-communicating signaling system that allows them to rewire their response whenever required (Pieterse *et al.,* 2012; Caarls *et al.,* 2015). To investigate how plants cope with multi-stress conditions and integrate signals from different stress-driven phytohormonal pathways, we analyzed the dynamics of whole-transcriptome profiles of *A. thaliana* plants exposed to six sequential double stresses inflicted by combinations of drought, herbivory by *P. rapae* and infection with *B. cinerea* (Chapter 2). Our results show that *A. thaliana* is capable of swiftly adapting its transcriptional response to the second stress irrespective of the first stress. The plant's response to the second stress was shown to contain clear first-stress signatures, although over time the second stress-induced transcriptome is highly similar to that of plants that did not receive a first stress. Still, the eventual outcome of plant sequential stress interaction was shown to be significantly different from that of plants that encountered only one stress.

We showed that *P. rapae* infestation renders Col-0 plants less susceptible to *B. cinerea* infection. This effect is likely to be caused by wound-induced resistance to *B. cinerea* that was previously described as being a result of priming of the phytoalexin camalexin (Ferrari *et al.,* 2003; Chassot *et al.,* 2008). Since herbivory by *P. rapae* is known to induce expression of the cytochrome P450 gene *CYP79B2*, involved in both glucosinolate and phytoalexin biosynthesis (Reymond *et al.,* 2004), it is tempting to speculate that the wound-induced resistance mechanism as describe by Chassot *et al.* (2008) could potentially cause resistance to *B. cinerea* in our setup. However, no evidence for the involvement of camalexin was found, as camalexin biosynthetic genes *CYP79B2*, *CYP71A13*, *ANTHRANILATE SYNTHASE 1*(*ASA1*), *PHYTOALEXIN DIFFICIENT* 2 (*PAD2*) and *PAD3* gene expression was found to be unaffected by prior *P. rapae* exposure. Still, it is possible that non-transcriptionally regulated priming of camalexin biosynthesis plays a role in the observed induced resistance to *B. cinerea* as a result of prior herbivory. Therefore, it would be informative to measure camalexin accumulation during *B. cinerea* infection with prior *P. rapae* herbivory in future experiments.

Transcriptional differences that could explain the *P. rapae*-induced plant resistance to *B. cinerea* infection in our setup are likely to be found in the first 6-12 h after applying *B. cinerea,* since in time the sequential stress response gradually becomes highly similar to the plants response to single stress *B. cinerea*. This suggests that transcriptional rewiring during the first 12 h after *B. cinerea* inoculation is responsible for the phenotypic difference in plant resistance to *B. cinerea* observed 3 days after inoculation. This demonstrates the impact a previous stress encounter can have on a subsequent plant-stress interaction, even though the transcriptional effect is only of limited duration. Indeed, the plant's response to ET was shown to be significantly delayed in the combination with prior *P. rapae* infestation followed by *B. cinerea* infection, suggesting that the plant's responsiveness to ET may play an important role in the plant's susceptibility to *B. cinerea*. Indeed, ET was previously shown to play an important role in plant resistance to *B. cinerea.* Application of exogenous ET was shown to increase plant resistance to *B. cinerea* in tomato and inhibition of ET perception was shown to result in increased susceptibility (Diaz *et al.,* 2002). Moreover, the *A. thaliana ein2* mutant was shown to be

more susceptible to *B. cinerea* than wild type plants, again indicating the importance of ET signaling in plant resistance to *B. cinerea*. Thus, also here it would be informative to have more information on ET accumulation in multi-stress combinations to see whether ET accumulation is also altered by prior herbivory.

In contrast to prior herbivory, drought prior to *B. cinerea* infection leads to repression of several plant defense pathways at 6 h after inoculation, including plant responses to ET. ET biosynthetic processes on the other hand are induced earlier in the drought stress and *B. cinerea* sequential stress combination, indicating that even though no significant differences are observed in terms of the number of spreading *B. cinerea* lesions, drought stress clearly affects the plants response to *B. cinerea* infection. Since we observed that *B. cinerea* lesion size seem to be increased compared to the single stress *B. cinerea* treatment, it will be interesting to see whether prior drought stress affects lesion spreading, which could be explained by the difference in the plant's transcriptional response. Moreover, previous studies have shown that ABA, which is produced under drought stress (Huang *et al.,* 2008), was shown to negatively impact plant resistance to *B. cinerea* in tomato (Audenaert *et al.,* 2002), suggesting that drought stress could have a visible negative effect on plant resistance to *B. cinerea*.

Another interesting observation is that although the plant's transcriptional response to multi-dimensional cell growth is repressed in both drought stress and *B. cinerea* infection, the combination of drought stress followed by *B. cinerea* yields induction of this response in the first 18 h after inoculation. An explanation for this observation might be that drought stressed plants compensate for the drought induced growth retardation in their recovery period and during subsequent *B. cinerea* infection. However, this seems conflicting since simultaneous investment in growth and defense is likely to come with a price (e.g. increased susceptibility to *B. cinerea;* Vos *et al.,* 2013a)). On the other hand, *P. rapae* is shown to induce transcription related to multi-dimensional cell growth, possibly benefitting caterpillars by providing them with more plant material and thus food resources (Machado *et al.,* 2013; Davila Olivas *et al.,* 2016; Machado *et al.,* 2016). However, *P. rapae* infestation was shown to result in plant resistance to *B. cinerea*, suggesting that in this case the investment in growth does not affect plant defenses and that there is no tradeoff of the simultaneous investment. Future research on the role of ET in *P. rapae* induced resistance to *B. cinerea*, the possible role for ET in drought-induced increased *B. cinerea* lesion spreading and the role of *P. rapae* induced plant growth-related gene expression is needed to further investigated their contribution in plant multi-stress resistance.

GWAS reveals genes potentially involved in plant adaptive responses during sequential stress

Natural genetic variation in plants provides a wealth of information on plant adaptive responses that can be mined for knowledge on naturally occurring plant adaptations and resistance breeding (Atwell *et al.,* 2010; Baxter *et al.,* 2010; Rasmussen *et al.,* 2013; Kloth *et al.,* 2016; Davila Olivas *et al.,* 2017b; Thoen *et al.,* 2017). Within the HapMap collection of naturally occurring *A. thaliana* accessions natural adaptive responses were previously described for the Portuguese *A. thaliana*

accession C24 that was shown to be tolerant to drought, submergence and *Pseudomonas syringae* pv. tomato DC3000 infection (Ton *et al.,* 1999; Bechtold *et al.,* 2010; Vashisht *et al.,* 2011), suggesting that additional adaptive responses to multiple stresses are likely to be found in *A. thaliana* accessions. In Chapter 3 we mined the natural existing genetic variation of 346 *A. thaliana* accessions of the HapMap collection (Baxter *et al.,* 2010; Platt *et al.,* 2010) for naturally occurring adaptations that allow plants to cope with sequential (a)biotic stress combinations, drought or herbivory followed by *B. cinerea* infection. Like with the Col-0 accession used for our transcriptome study in Chapter 2, *P. rapae* infestation was shown to render plants more resistant to *B. cinerea* infection in a large group of accessions within the HapMap collection. Also drought prior to *B. cinerea* infection resulted in enhanced resistance in many of the accessions. The overall phenotypic variation of plants subjected to the sequential stresses was shown to partly depend on the level of *B. cinerea* resistance observed in the single stress condition, indicating that both the prior and secondly applied stress play an important role in the observed sequential stress plant phenotypes. This was also found for our transcriptome analysis on Col-0 as shown in Chapter 2. Although the transcriptional response was shown to be highly similar to single stress *B. cinerea*, this first stress signature significantly affected the plant's resistance to *B. cinerea* as was shown by prior *P. rapae* infestation. We used the obtained phenotypic data to mine the genetic basis of phenotypic differences in *B. cinerea* disease severity with a univariate GWAS analysis and found *LIK1, LOX2* and *YUC7 and HK5* that are known to be involved in plant responses to *B. cinerea*, *P. rapae* and drought stress respectively (Bell *et al.,* 1995; Reymond *et al.,* 2000; De Vos *et al.,* 2005; AbuQamar *et al.,* 2006; Iwama *et al.,* 2006; Miya *et al.,* 2007; Desikan *et al.,* 2008; Birkenbihl *et al.,* 2012; Lee *et al.,* 2012; Pham *et al.,* 2012; Windram *et al.,* 2012; Le *et al.,* 2014; Wasternack 2015; Coolen *et al.,* 2016). These findings support that our experimental setup was capable of identifying existing and potential novel players in the plant's adaptive response to the single and double sequential stresses that were applied.

To further support our GWAS findings, additional QTL mapping was performed on MAGIC RILs (Nordborg and Weigel 2008; Kover *et al.,* 2009; Bergelson and Roux 2010; Kover and Mott 2012). Although these RILs were expected to cover only a minor part of the genetic variation found in the HapMap collection it was very reassuring to see that the QTL mapping results roughly followed our GWAS results. Although the SNPs used in our GWAS analysis are markers that describe differences between accessions, they are highly unlikely to cause phenotypic differences in disease severity. However, these SNPs represent a locus with SNPs that are potentially in LD up to 10-50 kb (Nordborg *et al.,* 2005; Kim *et al.,* 2007), leaving this area to be investigated for causative SNPs. Therefore, we performed fine mapping using full genome sequences of 164 available accessions surrounding the SNP of interest with a 50-kb window to cover the potential LD area. Although the 164 accessions have limited genetic variation in comparison to the accessions of the HapMap collection, we were able to find significant (FDR-corrected) fine mapping associations that pointed to genes in close proximity to our GWAS SNPs. These genes were found in 9 loci containing 25 significant associations that code for 14 unique genes and will require further validation for their role in plant resistance to *B. cinerea* during sequential (a)biotic stress. Their transcriptional response to the applied sequential (a)biotic stresses, performed in Chapter 2, was shown to further strengthen their role in plant responses

to *B. cinerea* under sequential stress. Furthermore, when additional full genome sequences will become available of the accessions within the HapMap collection, additional candidate genes could potentially be identified with fine mapping. Loci that showed non-significant fine mapping associations, such as found at two *R*-gene loci, could potentially be linked to plant responses to *B. cinerea* in single or sequential (a)biotic stress, opening a new door for *R*-gene mediated signaling as response to necrotrophic pathogens such as *B. cinerea*.

Genetic commonalities underlying plant responses to different (a)biotic stresses

The ultimate breeding strategy for food producing crops will be finding and implementing plant multi-stress resistance mechanisms that equip plants with tools that allow them to withstand diverse (a)biotic stresses and still have a highly efficient food production. However, investment in constitutive defenses often leaves plants with a dwarfed phenotype (Bowling *et al.,* 1994; Clarke *et al.,* 1998; Jirage *et al.,* 2001) that is unfavorable for efficient food production (Vos *et al.,* 2013a). Moreover, since plant defense pathways can negatively influence one another (Pieterse *et al.,* 2012; Caarls *et al.,* 2015), constitutive activation of a specific signaling pathway may leave plants highly susceptible to stresses that require other pathways. Therefore, a search for favorable plant adaptive responses to diverse (a) biotic stresses was started in Chapter 4. We studied the genetic commonalities of 30 plant stress traits that cover 11 (a)biotic stresses and four sequential stresses to reveal potential multi-stress tolerance mechanisms. To find common genetic components that are required for plant multi-stress tolerance several approaches were taken to analyze the information obtained by the diverse phenotypic screens. In contrast to Chapter 3, the plant stress phenotypes were studied using a multivariate GWAS approach instead of an univariate analyses. In our study we showed that the multivariate approach has more power of identifying SNPs associated with all tested traits than a univariate GWAS. However, it was also shown that the power for detection can be influenced by both very strong associations found for specific stresses and weak associations found for multiple stresses, making the multivariate approach a mix of stress specific and shared associations.

Phenotypic correlations between traits were found to be low, presumably caused by differences in experimental setups and environmental factors. Still, quite high genetic correlations between the tested traits were observed, revealing that there are genetic commonalities. The genetic correlations between different stresses were shown to correspond to plant phytohormonal regulation, explaining opposing interactions between stresses that are known to induce plant stress responsive pathways that are known to negatively regulate one another (Laurie-Berry *et al.,* 2006; Mur *et al.,* 2006; Pieterse *et al.,* 2012). As expected from the plants SA-JA crosstalk antagonism, we found that SA-inducers (aphid *Myzus persicae* and parasitic plant *Phelipanche ramose*) are negatively correlated with JA-inducers (herbivory by *P. rapae* caterpillars, infection with necrotrophic fungus *B. cinerea* and infestation with western flower thrips *Frankliniella occidentalis*). Genes involved in plant multi-stress resistance in these contrasting pathways are therefore unlikely to be identified. Still, a gene that was found with our GWAS analysis, *RMG1*, showed to be involve in multiple stress responses: osmotic stress, salt stress tolerance and resistance to *P. rapae* feeding, indicating that with our approach

it is possible to find genes involved in plant tolerance to diverse (a)biotic stresses. In addition, our GWAS analyses showed that traits with contrasting origins, such as abiotic and biotic stresses, can yield SNPs associated with stress specific and shared effects on plant tolerance, suggesting that it is possible to find plant multi-stress tolerance mechanisms within the tested traits. Our results show that with the help of a multivariate GWAS analysis it is possible to find genes involved in plant tolerance to diverse stresses. The next challenge will be to use this approach to identify naturally adaptive responses of plants that lead to plant multi-stress tolerance and plants that can swiftly adapt to simultaneous or sequential (a)biotic stresses.

Traits involved in plant attractiveness for oviposition by *Pieris rapae*

Insect herbivores form an enormous threat to plants as they consume large amounts of tissue, sometimes consuming the whole plant. In this case preventing infestation by insect herbivores has priority since these plant remaining's are unlikely to encounter a subsequent stress. By studying what makes plants unattractive for insect herbivores such as *P. rapae*, future crop plants might be equipped with traits to prevent crop damage or complete loss of plants. To investigate if we could identify genes involved in host selection for oviposition by *P. rapae*, 350 *A. thaliana* accessions were screened in an experimental setup in which *P. rapae* butterflies were allowed to choose between all 350 accession of the HapMap collection for oviposition (Chapter 5). Because *P. rapae* is a specialist on Brassicacea species, such as *A. thaliana*, and is thought to be relatively insensitive to their defense systems, finding plant traits that could affect host selection by *P. rapae* butterflies was expected to be challenging. From previous studies it is known that *P. rapae* caterpillars degrade toxic plant indole-glucosinolates, resulting in the formation of less toxic nitriles. These secondary metabolites also act as feeding and oviposition stimulants because non-adapted insect herbivores do not favor to feed or oviposite on glucosinolate-producing plants (De Vos *et al.,* 2008; Mumm *et al.,* 2008; Hopkins *et al.,* 2009; Ali and Agrawal 2012). We observed that the oviposition behavior of *P. rapae* was influenced by daylight and oviposition was preferred on plants at the edges and corners of our experimental cage setup. This effect could be the result of the natural flight and search behavior of *P. rapae* butterflies that was previously described to be predominantly cause 'edge effects' under field conditions (Root 1973; Jones 1977; Muriel and Grez 2002). Furthermore, *P. rapae* was shown previously to be most active with oviposition during the morning, explaining the skewed egg distribution on the East side of the cage (Root and Kareiva 1984). In addition, more eggs depositions were found on medium and large plants compared to small plants and spontaneous plant chlorosis and necrosis was found to be an unfavorable trait for host selection by *P. rapae* butterflies, presumably influencing caterpillar growth and ensuring safe egg attachment respectively. Aliphatic-glucosinolates, as described for 15 of our tested accessions by Kliebenstein *et al.* (2001), showed a moderate negative correlation with the number of eggs deposited on plants together with a weak positive correlation with indole-glucosinolates. The latter were previously described as oviposition and feeding stimulants (De Vos *et al.,* 2008; Mumm *et al.,* 2008; Hopkins *et al.,* 2009; Ali and Agrawal 2012).

In order to find plant genes involved in host selection by *P. rapae*, an univariate GWAS analysis was performed on data obtained from 7 independent experiments in which female butterflies were allowed to freely oviposit on all accessions of the HapMap collection at once. The obtained data yielded 12 candidate genes with hardly any connection to plant defenses or related to herbivory. However, subsequent fine mapping results showed significant associations with a JA biosynthesis gene, *AOC1*. Since JA is known to be an important plant defense hormone involved in resistance to *P. rapae* (De Vos *et al.,* 2006a; Little *et al.,* 2007; Verhage *et al.,* 2011; Vos *et al.,* 2013b), our result is well supported by previous studies. However, how specific alterations in *AOC1* and the other identified candidates influence the oviposition preference by *P. rapae* butterflies remains to be functionally validated.

Future prospects

In the work presented in this thesis we explored adaptive plant responses to biotic and abiotic stress combinations in order to find plant multi-stress resistance mechanisms that can be used for plant breeding in the future. We found that plants are very well capable of swiftly adapting their transcriptional response to the last encountered stress, suggesting that they are able to cope with multi-stress environments. Still, a first stress encounter leaves a clear transcriptional signature that can have great impact on plant stress resistance. We showed that there is great potential in mining natural adaptive responses of plants by studying naturally occurring variation in plant genotypes. By studying how natural genetic variation influences the plant's response to (sequential) (a)biotic stresses, several genes were identified that could potentially play a role in plant adaptive responses during multi-stress situations and attractiveness for oviposition by *P. rapae* butterflies. This brings us one step closer to validating and translating these findings into crop plants to ensure a sustainable food production in the future.

With our rapidly increasing human population and changing environment that supports a dramatic increase of complex multi-stress conditions, serious problems for our food security are predicted (Grieve *et al.,* 2010; Mittler and Blumwald 2010; Chakraborty and Newton 2011; Newton *et al.,* 2011; Nicol *et al.,* 2011; Atkinson and Urwin 2012; Bebber *et al.,* 2013; Garrett *et al.,* 2013; Teixeira *et al.,* 2013; Syvertsen and Garcia-Sanchez 2014), A dramatic increase in efficient food production will be required to meet with future food demands (UN 2011; FAO 2012). To reach these demands, sustainable pest management and usage and improvement of natural adaptive mechanisms of plants through resistance breeding will become even more important in the future (Gilden *et al.,* 2010; Meissle *et al.,* 2010; Gressel 2011). Innovative approaches that enhance plant tolerance for multiple (a)biotic stresses without adversely affecting our ecological footprint are required for a sustainable increase in crop production. Furthermore, the conservation of wild plants that harbor naturally adaptive responses will not only be crucial for maintaining natural diversity, but will also be essential for mining naturally existing traits that can be used for crop improvement. Extending our knowledge on naturally occurring plant adaptations that allow for plant multi-stress resistance will be an important milestone in a sustainable future.

References

Abe, H., Tomitaka, Y., Shimoda, T., Seo, S., Sakurai, T., Kugimiya, S., Tsuda, S. and Kobayashi, M. (2012). Antagonistic plant defense system regulated by phytohormones assists interactions among vector insect, thrips and a tospovirus. **Plant Cell Physiol. 53,** 204-212.

Abro, M., Lecompte, F., Bardin, M. and Nicot, P. (2014). Nitrogen fertilization impacts biocontrol of tomato gray mold. **Agron. Sustain. Dev. 34,** 261-267.

AbuQamar, S., Chen, X., Dhawan, R., Bluhm, B., Salmeron, J., Lam, S., Dietrich, R. and Mengiste, T. (2006). Expression profiling and mutant analysis reveals complex regulatory networks involved in Arabidopsis response to Botrytis infection. **Plant J. 48,** 28-44.

Agrawal, A. A. (2011). Current trends in the evolutionary ecology of plant defence. **Funct. Ecol. 25,** 420-432.

Al-Babili, S. and Bouwmeester, H. J. (2015). Strigolactones, a novel carotenoid-derived plant hormone. **Annu. Rev. Plant. Biol. 66,** 161-186.

Al-Daoude, A., De Torres Zabala, M., Ko, J.-H. and Grant, M. (2005). RIN13 is a positive regulator of the plant disease resistance protein RPM1. **Plant Cell 17,** 1016-1028.

Ali, J. G. and Agrawal, A. A. (2012). Specialist versus generalist insect herbivores and plant defense. **Trend. Plant Sci. 17,** 293-302.

Alimi, N. A., Bink, M. C., Dieleman, J. A., Magan, J. J., Wubs, A. M., Palloix, A. and van Eeuwijk, F. A. (2013). Multi-trait and multi-environment QTL analyses of yield and a set of physiological traits in pepper. **Theor. Appl. Genet. 126,** 2597-2625.

Alonso-Blanco, C., Aarts, M. G. M., Bentsink, L., Keurentjes, J. J. B., Reymond, M., Vreugdenhil, D. and Koornneef, M. (2009). What has natural variation taught us about plant development, physiology, and adaptation? **Plant Cell 21,** 1877-1896.

Alonso, J., Stepanova, A., Solano, R., Wisman, E., Ferrari, S., Ausubel, F. and Ecker, J. (2003). Five components of the ethylene-response pathway identified in a screen for weak ethylene-insensitive mutants in Arabidopsis. **Proc. Natl. Acad. Sci. U.S.A. 100,** 2992-2997.

Anders, S., Pyl, P. and Huber, W. (2014). HTSeq-a Python framework to work with high-throughput sequencing data. **Bioinformatics 31,** 166-169.

Anderson, J. P., Badruzsaufari, E., Schenk, P. M., Manners, J. M., Desmond, O. J., Ehlert, C., Maclean, D. J., Ebert, P. R. and Kazan, K. (2004). Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in Arabidopsis. **Plant Cell 16,** 3460-3479.

Anuradha, C., Gaur, P., Pande, S., Gali, K., Ganesh, M., Kumar, J. and Varshney, R. (2011). Mapping QTL for resistance to botrytis grey mould in chickpea. **Euphytica 182,** 1–9.

Appel, H. M., Fescemyer, H., Ehlting, J., Weston, D., Rehrig, E., Joshi, T., Xu, D., Bohlmann, J. and Schultz, J. (2014). Transcriptional responses of *Arabidopsis thaliana* to chewing and sucking insect herbivores. **Front. Plant Sci. 5**.

Asai, T., Tena, G., Plotnikova, J., Willmann, M. R., Chiu, W. L., Gómez-Gómez, L., Boller, T., Ausubel, F. M. and Sheen, J. (2002). MAP kinase signalling cascade in Arabidopsis innate immunity. **Nature 415,** 977-983.

Atkinson, N. J., Lilley, C. J. and Urwin, P. E. (2013). Identification of genes involved in the response of Arabidopsis to simultaneous biotic and abiotic stresses. **Plant Physiol. 162,** 2028-2041.

Atkinson, N. J. and Urwin, P. E. (2012). The interaction of plant biotic and abiotic stresses: from genes to the field. **J. Exp. Bot. 63,** 3523-3543.

Atwell, S., Huang, Y. S., Vilhjálmsson, B. J., Willems, G., Horton, M., Li, Y., Meng, D., Platt, A., Tarone, A. M., Hu, T. T., Jiang, R., Muliyati, W. N., Zhang, X., Amer, M., Baxter, I., Brachi, B., Chory, J., Dean, C., Debieu, M., De Meaux, J., Ecker, J. R., Faure, N., Kniskern, J. M., Jones, J. D. G., Michael, T., Nemri, A., Roux, F., Salt, D. E., Tang, C., Todesco, M., Traw, B. M., Weigel, D., Marjoram, P., Borevitz, J. O., Bergelson, J. and Nordborg, M. (2010). Genome-wide association study of 107 phenotypes in *Arabidopsis thaliana* inbred lines. **Nature 465,** 627-631.

Audenaert, K., De Meyer, G. B. and Höfte, M. M. (2002). Abscisic acid determines basal susceptibility of tomato to *Botrytis cinerea* and suppresses salicylic acid-dependent signaling mechanisms. **Plant Physiol. 128,** 491-501.

Bac-Molenaar, J. A., Fradin, E. F., Becker, F. F. M., Rienstra, J. A., Van der Schoot, J., Vreugdenhil, D. and Keurentjes, J. J. B. (2015a). Genome-wide association mapping of fertility reduction upon heat stress reveals developmental stage-specific QTLs in *Arabidopsis thaliana*. **Plant Cell 27,** 1857-1874.

Bac-Molenaar, J. A., Fradin, E. F., Rienstra, J. A., Vreugdenhil, D. and Keurentjes, J. J. B. (2015b). GWA mapping of anthocyanin accumulation reveals balancing selection of *MYB90* in *Arabidopsis thaliana*. **PLoS ONE 10,** e0143212.

Baldacci-Cresp, F., Maucourt, M., Deborde, C., Pierre, O., Moing, A., Brouquisse, R., Favery, B. and Frendo, P. (2015). Maturation of nematode-induced galls in *Medicago truncatula* is related to water status and primary metabolism modifications. **Plant Sci. 232,** 77-85.

Ballesteros, D. C., Mason, R. E., Addison, C. K., Andrea Acuña, M., Nelly Arguello, M., Subramanian, N., Miller, R. G., Sater, H., Gbur, E. E., Miller, D., Griffey, C. A., Barnett, M. D. and Tucker, D. (2015). Tolerance of wheat to vegetative stage soil waterlogging is conditioned by both constitutive and adaptive QTL. **Euphytica 201,** 329-343.

Balmer, O., Géneau, C. E., Belz, E., Weishaupt, B., Förderer, G., Moos, S., Ditner, N., Juric, I. and Luka, H. (2014). Wildflower companion plants increase pest parasitation and yield in cabbage fields: Experimental demonstration and call for caution. **BioControl 76,** 19-27.

Barth, S., Melchinger, A. E. and Lübberstedt, T. (2002). Genetic diversity in *Arabidopsis thaliana* L. Heynh. investigated by cleaved amplified polymorphic sequence (CAPS) and inter-simple sequence repeat (ISSR) markers. **Mol. Ecol. 11**, 495–505.

Baxter, I., Brazelton, J. N., Yu, D., Huang, Y. S., Lahner, B., Yakubova, E., Li, Y., Bergelson, J., Borevitz, J. O., Nordborg, M., Vitek, O. and Salt, D. E. (2010). A coastal cline in sodium accumulation in *Arabidopsis thaliana* is driven by natural variation of the sodium transporter AtHKT1;1. **PLoS Genet. 6,** e1001193.

Bebber, D. P., Ramotowski, M. A. T. and Gurr, S. J. (2013). Crop pests and pathogens move polewards in a warming world. **Nat. Clim. Change 3,** 985-988.

Bechtold, U., Lawson, T., Mejia-Carranza, J., Meyer, R. C., Brown, I. R., Altmann, T., Ton, J. and Mullineaux, P. M. (2010). Constitutive salicylic acid defences do not compromise seed yield, drought tolerance and water productivity in the Arabidopsis accession C24. **Plant Cell Environ. 33,** 1959-1973.

Bell, E., Creelman, R. A. and Mullet, J. E. (1995). A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in Arabidopsis. **Proc. Natl. Acad. Sci. 92,** 8675-8679.

Benikhlef, L., L'Haridon, F., Abou-Mansour, E., Serrano, M., Binda, M., Costa, A., Lehmann, S. and Métraux, J.-P. (2013). Perception of soft mechanical stress in Arabidopsis leaves activates disease resistance. **BMC Plant Biol. 13,** 113.

Benjamini, Y. and Hochberg, Y. (1995). Controlling the false discovery rate - a practical and powerful approach to multiple testing. **J. Royal Statistical Soc. Series B 57,** 289-300.

Bergelson, J. and Roux, F. (2010). Towards identifying genes underlying ecologically relevant traits in *Arabidopsis thaliana*. **Nat. Rev. Genet. 11,** 867-879.

Berrocal-Lobo, M., Molina, A. and Solano, R. (2002). Constitutive expression of *ETHYLENE-RESPONSE-FACTOR1* in Arabidopsis confers resistance to several necrotrophic fungi. **Plant J. 29,** 23-32.

Bessire, M., Chassot, C., Jacquat, A.-C., Humphry, M., Borel, S., Petétot, J., Métraux, J.-P. and Nawrath, C. (2007). A permeable cuticle in Arabidopsis leads to a strong resistance to *Botrytis cinerea*. **EMBO J. 26,** 2158–2168.

Bhargava, A., Ahad, A., Wang, S., Mansfield, S. D., Haughn, G. W., Douglas, C. J. and Ellis, B. E. (2013). The interacting MYB75 and KNAT7 transcription factors modulate secondary cell wall deposition both in stems and seed coat in Arabidopsis. **Planta 237,** 1199–1211.

Bi, D., Cheng, Y. T., Li, X. and Zhang, Y. (2010). Activation of plant immune responses by a gain-of-function mutation in an atypical receptor-like kinase. **Plant Physiol. 153,** 1771-1779.

Birkenbihl, R. P., Diezel, C. and Somssich, I. E. (2012). Arabidopsis WRKY33 is a key transcriptional regulator of hormonal and metabolic responses toward *Botrytis cinerea* infection. **Plant Physiol. 159,** 266-285.

Blanco-Ulate, B., Morales-Cruz, A., Amrine, K. C. H., Labavitch, J. M., Powell, A. L. T. and Cantu, D. (2014). Genome-wide transcriptional profiling of *Botrytis cinerea* genes targeting plant cell walls during infections of different hosts. **Front. Plant Sci. 5,** 435.

Bloomer, R. H., Juenger, T. E. and Symonds, V. V. (2012). Natural variation in GL1 and its effects on trichome density in *Arabidopsis thaliana*. **Mol. Ecol. 21,** 3501-3515.

Bodenhausen, N. and Reymond, P. (2007). Signaling pathways controlling induced resistance to insect herbivores in Arabidopsis. **Mol. Plant Microbe Interact. 20,** 1406-1420.

Boer, M. P., Wright, D., Feng, L., Podlich, D. W., Luo, L., Cooper, M. and Van Eeuwijk, F. A. (2007). A mixed-model quantitative trait loci (QTL) analysis for multiple-environment trial data using environmental covariables for QTL-by-environment interactions, with an example in maize. **Genetics 177,** 1801-1813.

Boter, M., Ruiz-Rivero, O., Abdeen, A. and Pratt, S. (2004). Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and Arabidopsis. **Genes Dev. 18,** 1577-1591.

Boudsocq, M. and Sheen, J. (2013). CDPKs in immune and stress signaling. **Trends Plant Sci. 18,** 30-40.

Boutrot, F., Segonzac, C., Chang, K. N., Qiao, H., Ecker, J. R., Zipfel, C. and Rathjen, J. P. (2010). Direct transcriptional control of the *Arabidopsis* immune receptor FLS2 by the ethylene-dependent transcription factors EIN3 and EIL1. **Proc. Natl. Acad. Sci. U.S.A. 107,** 14502-14507.

Bowling, S. A., Guo, A., Cao, H., Gordon, A. S., Klessig, D. F. and Dong, X. (1994). A mutation in Arabidopsis that leads to constitutive expression of systemic acquired resistance. **Plant Cell 6,** 1845-1857.

Boyes, D. C., Nam, J. and Dangl, J. L. (1998). The *Arabidopsis thaliana RPM1* disease resistance gene product is a peripheral plasma membrane protein that is degraded coincident with the hypersensitive response. **Proc. Natl. Acad. Sci. U.S.A. 95,** 15849-15854.

Boyle, E. I., Weng, S. A., Gollub, J., Jin, H., Botstein, D., Cherry, J. M. and Sherlock, G. (2004). GO::TermFinder - open source software

for accessing gene ontology information and finding significantly enriched gene ontology terms associated with a list of genes. **Bioinformatics 20,** 3710-3715.

Brachi, B., Faure, N., Bergelson, J., Cuguen, J. and Roux, F. (2013). Genome-wide association mapping of flowering time in *Arabidopsis thaliana* in nature: genetics for underlying components and reaction norms across two successive years. **Acta Bot. Gallica 160,** 205-219.

Brachi, B., Faure, N., Horton, M., Flahauw, E., Vazquez, A., Nordborg, M., Bergelson, J., Cuguen, J. and Roux, F. (2010). Linkage and association mapping of *Arabidopsis thaliana* flowering time in nature. **PLoS Genet. 6,** e1000940.

Brachi, B., Meyer, C. G., Villoutreix, R., Platt, A., Morton, T. C., Roux, F. and Bergelson, J. (2015). Coselected genes determine adaptive variation in herbivore resistance throughout the native range of *Arabidopsis thaliana*. **Proc. Natl. Acad. Sci. 112,** 4032-4037.

Bradbury, P. J., Zhang, Z., Kroon, D. E., Casstevens, T. M., Ramdoss, Y. and Buckler, E. S. (2007). TASSEL: software for association mapping of complex traits in diverse samples. **Bioinformatics 23,** 2633-2635.

Brady, K. U., Kruckeberg, A. R. and Bradshaw Jr, H. D. (2005). Evolutionary ecology of plant adaptation to serpentine soils. **Annu. Rev. Ecol. Evol. Syst. 36,** 243-266.

Brauc, S., Vooght, E., Claeys, M., Geuns, J., Höfte, M. and Angenon, G. (2012). Overexpression of arginase in *Arabidopsis thaliana* influences defence responses against *Botrytis cinerea*. **Plant Biol. 14,** 39-45.

Braun, H.-J., Rajaram, S. and Van Ginkel, M. (1996). CIMMYT's approach to breeding for wide adaptation. **Euphytica 92,** 175-183.

Bray, E. A. (1997). Plant responses to water deficit. **Trends Plant Sci. 2,** 48-54.

Broekgaarden, C., Caarls, L., Vos, I. A., Pieterse, C. M. J. and Van Wees, S. C. M. (2015). Ethylene: traffic controller on hormonal crossroads to defense. **Plant Physiol. 169,** 2371-2379.

Bruessow, F., Gouhier-Darimont, C., Buchala, A., Metraux, J.-P. and Reymond, P. (2010). Insect eggs suppress plant defence against chewing herbivores. **Plant J. 62,** 876-885.

Bruinsma, M., Dam, N. M., Loon, J. J. A. and Dicke, M. (2007). Jasmonic acid-induced changes in *Brassica oleracea* affect oviposition preference of two specialist herbivores. **J. Chem. Ecol. 33,** 655-668.

Bukovinszky, T., Potting, R. P. J., Clough, Y., Lenteren, J. C. and Vet, L. E. M. (2005). The role of pre- and post- alighting detection mechanisms in the responses to patch size by specialist herbivores. **Oikos 109,** 435-446.

Burgueño, J., de los Campos, G., Weigel, K. and Crossa, J. (2012). Genomic prediction of breeding values when modeling genotype × environment interaction using pedigree and dense molecular markers. **Crop Sci. 52,** 707-719.

Burow, M., Atwell, S., Francisco, M., Kerwin, R. E., Halkier, B. A. and Kliebenstein, D. J. (2015). The glucosinolate biosynthetic gene *AOP2* mediates feed-back regulation of jasmonic acid signaling in Arabidopsis. **Mol. Plant. 8,** 1201-1212.

Busse-Wicher, M., Wicher, K. B. and Kusche-Gullberg, M. (2014). The extostosin family: proteins with many functions. **Matrix Biol. 35,** 25-33.

Caarls, L., Pieterse, C. M. J. and Van Wees, S. C. M. (2015). How salicylic acid takes transcriptional control over jasmonic acid signaling. **Front. Plant Sci. 6,** 170.

Cabanne, C. and Donèche, B. (2002). Purification and characterization of two isozymes of polygalacturonase from *Botrytis cinerea*. Effect of calcium ions on polygalacturonase activity. **Microbiol. Res. 157,** 183-189.

Cao, J. (2012). The pectin lyases in *Arabidopsis thaliana*: evolution, selection and expression profiles. **PLoS ONE 7,** e46944.

Cao, Y., Liang, Y., Tanaka, K., Nguyen, C. T., Jedrzejczak, R. P., Joachimiak, A. and Stacey, G. (2014). The kinase LYK5 is a major chitin receptor in Arabidopsis and forms a chitin-induced complex with related kinase CERK1. **eLife 3,** e03766.

Chakraborty, S. and Newton, A. C. (2011). Climate change, plant diseases and food security: an overview. **Plant Pathol. 60,** 2-14.

Chao, D.-Y., Silva, A., Baxter, I., Huang, Y., Nordborg, M., Danku, J., Lahner, B., Yakubova, E. and Salt, D. (2012). Genome-wide association studies identify heavy metal ATPase3 as the primary determinant of natural variation in leaf cadmium in *Arabidopsis thaliana*. **PLoS Genet. 8,** e1002923.

Chassot, C., Buchala, A., Schoonbeek, H. j., Métraux, J. P. and Lamotte, O. (2008). Wounding of Arabidopsis leaves causes a powerful but transient protection against Botrytis infection. **Plant J. 55,** 555-567.

Chen, T., Cui, P. and Xiong, L. (2015). The RNA-binding protein HOS5 and serine/arginine-rich proteins RS40 and RS41 participate in miRNA biogenesis in Arabidopsis. **Nucleic Acids Res. 43,** 8283–8298.

Chen, X., Wang, Y., Lv, B., Li, J., Luo, L., Lu, S., Zhang, X., Ma, H. and Ming, F. (2014). The NAC family transcription factor OsNAP confers abiotic stress response through the ABA pathway. **Plant Cell Physiol. 55,** 604-619.

Cheng, Y. T., Li, Y., Huang, S., Huang, Y., Dong, X., Zhang, Y. and Li, X. (2011). Stability of plant immune-receptor resistance proteins is

controlled by SKP1-CULLIN1-F-box (SCF)-mediated protein degradation. **Proc. Natl. Acad. Sci. 108,** 14694-14699.

Chin, K., DeFalco, T. A., Moeder, W. and Yoshioka, K. (2013). The Arabidopsis cyclic nucleotide-gated ion channels AtCNGC2 and AtCNGC4 work in the same signaling pathway to regulate pathogen defense and floral transition. **Plant Physiol. 163,** 611-624.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nurnberger, T., Jones, J. D., Felix, G. and Boller, T. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. **Nature 448,** 497-500.

Choi, J., Huh, S. U., Kojima, M., Sakakibara, H., Paek, K. H. and Hwang, I. (2010). The cytokinin-activated transcription factor ARR2 promotes plant immunity via TGA3/NPR1-dependent salicylic acid signaling in Arabidopsis. **Dev. Cell 19,** 284-295.

Choi, W. G., Toyota, M., Kim, S. H., Hilleary, R. and Gilroy, S. (2014). Salt stress-induced Ca^{2+} waves are associated with rapid, long-distance root-to-shoot signaling in plants. **Proc. Natl. Acad. Sci. U.S.A. 111,** 6497-6502.

Christ, B., Schelbert, S., Aubry, S., Sussenbacher, I., Muller, T., Krautler, B. and Hortensteiner, S. (2012). MES16, a member of the methylesterase protein family, specifically demethylates fluorescent chlorophyll catabolites during chlorophyll breakdown in Arabidopsis. **Plant Physiol. 158,** 628-641.

Ciais, P., Reichstein, M., Viovy, N., Granier, A., Ogee, J., Allard, V., Aubinet, M., Buchmann, N., Bernhofer, C., Carrara, A., Chevallier, F., De Noblet, N., Friend, A. D., Friedlingstein, P., Grunwald, T., Heinesch, B., Keronen, P., Knohl, A., Krinner, G., Loustau, D., Manca, G., Matteucci, G., Miglietta, F., Ourcival, J. M., Papale, D., Pilegaard, K., Rambal, S., Seufert, G., Soussana, J. F., Sanz, M. J., Schulze, E. D., Vesala, T. and Valentini, R. (2005). Europe-wide reduction in primary productivity caused by the heat and drought in 2003. **Nature 437,** 529-533.

Cipollini, D., Enright, S., Traw, M. B. and Bergelson, J. (2004). Salicylic acid inhibits jasmonic acid-induced resistance of *Arabidopsis thaliana* to *Spodoptera exigua*. **Mol. Ecol. 13,** 1643-1653.

Clarke, J. D., Liu, Y., Klessig, D. F. and Dong, X. (1998). Uncoupling *PR* gene expression from NPR1 and bacterial resistance: characterization of the dominant Arabidopsis *cpr6-1* mutant. **Plant Cell 10,** 557-569.

Clauw, P., Coppens, F., Beuf, K., Dhondt, S., Daele, T., Maleux, K., Storme, V., Clement, L., Gonzalez, N. and Inzé, D. (2015). Leaf responses to mild drought stress in natural variants of Arabidopsis. **Plant Physiol. 167,** 800-816.

Coll, N. S., Epple, P. and Dangl, J. L. (2011). Programmed cell death in the plant immune system. **Cell Death Differ. 18,** 1247-1256.

Coolen, S., Proietti, S., Hickman, R., Davila Olivas, N. H., Huang, P. P., Van Verk, M. C., Van Pelt, J. A., Wittenberg, A. H. J., De Vos, M., Prins, M., Loon, J. J. A., Aarts, M., Dicke, M., Pieterse, C. M. J. and Wees, S. C. M. (2016). Transcriptome dynamics of Arabidopsis during sequential biotic and abiotic stresses. **Plant J. 86,** 249-267.

Cooper, M., Messina, C. D., Podlich, D., Totir, L. R., Baumgarten, A., Hausmann, N. J., Wright, D. and Graham, G. (2014). Predicting the future of plant breeding: complementing empirical evaluation with genetic prediction. **Crop Pasture Sci. 65,** 311-336.

Couto, D. and Zipfel, C. (2016). Regulation of pattern recognition receptor signalling in plants. **Nat. Rev. Immunol. 16,** 537-552.

Cui, F., Brosché, M., Sipari, N., Tang, S. and Overmyer, K. (2013). Regulation of ABA dependent wound induced spreading cell death by MYB108. **New Phytol. 200,** 634-640.

Curvers, K., Seifi, H., Mouille, G., de Rycke, R., Asselbergh, B., Van Hecke, A., Vanderschaeghe, D., Höfte, H., Callewaert, N., Van Breusegem, F. and Höfte, M. (2010). Abscisic acid deficiency causes changes in cuticle permeability and pectin composition that influence tomato resistance to *Botrytis cinerea*. **Plant Physiol. 154,** 847-860.

Cyr, H. and Pace, M. L. (1993). Magnitude and patterns of herbivory in aquatic and terrestrial ecosystems. **Nature 361,** 148-150.

Dąbrowska, P., Freitak, D., Vogel, H., Heckel, D. G. and Boland, W. (2009). The phytohormone precursor OPDA is isomerized in the insect gut by a single, specific glutathione transferase. **Proc. Natl. Acad. Sci. 106,** 16304–16309.

Dat, J. F., Lopez, D., Foyer, C. H. and Scott, I. M. (1998). Parallel changes in H_2O_2 and catalase during thermotolerance induced by salicylic acid or heat acclimation in mustard seedlings. **Plant Physiol. 116,** 1351-1357.

Davila Olivas, N. H., Coolen, S., Huang, P., Severing, E., Van Verk, M. C., Hickman, R., Wittenberg, A. H. J., de Vos, M., Prins, M., Loon, J. J. A., Aarts, M. G. M., Van Wees, S. C. M., Pieterse, C. M. J. and Dicke, M. (2016). Effect of prior drought and pathogen stress on Arabidopsis transcriptome changes to caterpillar herbivory. **New Phytol. 210,** 1344–1356.

Davila Olivas, N. H., Frago, E., Thoen, M. P. M., Kloth, K. J., Becker, F. F. M., Van Loon, J. J. A., Gort, G., Keurentjes, J. J. B., Van Heerwaarden, J. and Dicke, M. (2017a). Natural variation in life history strategy of *Arabidopsis thaliana* determines stress responses to drought and insects of different feeding guilds. **Mol. Ecol. 26,** 2959-2977.

Davila Olivas, N. H., Kruijer, W., Gort, G., Wijnen, C. L., Van Loon, J. J. A. and Dicke, M. (2017b). Genome-wide association analysis reveals distinct genetic architectures for single and combined stress responses in *Arabidopsis thaliana*. **New Phytol. 213,** 838-851.

Davis, J., Yu, D., Evans, W., Gokirmak, T., Chetelat, R. and Stotz, H. (2009). Mapping loci from *Solanum lycopersicoides* conferring resistance or susceptibility to *Botrytis cinerea* in tomato. **Theor. Appl. Genet. 119,** 305-314.

Davis, K. R., Darvill, A. G., Albersheim, P. and Dell, A. (1986). Host-pathogen interactions XXIX. Oligogalacturonides released from sodium polypectate by endopolygalacturonic acid lyase are elicitors of phytoalexins in soybean. **Plant Physiol. 80,** 568-577.

Davis, K. R. and Hahlbrock, K. (1987). Induction of defense responses in cultured parsley cells by plant cell wall fragments. **Plant Physiol. 84,** 1286-1290.

De Los Campos, G., Hickey, J. M., Pong-Wong, R., Daetwyler, H. D. and Calus, M. P. (2013). Whole-genome regression and prediction methods applied to plant and animal breeding. **Genetics 193,** 327-345.

De Ollas, C., Hernando, B., Arbona, V. and Gómez-Cadenas, A. (2013). Jasmonic acid transient accumulation is needed for abscisic acid increase in citrus roots under drought stress conditions. **Physiol. Plant. 147,** 296-306.

De Paepe, A., Vuylsteke, M., Van Hummelen, P., Zabeau, M. and Van Der Straeten, D. (2004). Transcriptional profiling by cDNA-AFLP and microarray analysis reveals novel insights into the early response to ethylene in Arabidopsis. **Plant J. 39,** 537-559.

De Torres-Zabala, M., Bennett, M. H., Truman, W. H. and Grant, M. R. (2009). Antagonism between salicylic and abscisic acid reflects early host-pathogen conflict and moulds plant defence responses. **Plant J. 59,** 375-386.

De Vleesschauwer, D., Yang, Y., Cruz, C. V. and Hofte, M. (2010). Abscisic acid-induced resistance against the brown spot pathogen *Cochliobolus miyabeanus* in rice involves MAP kinase-mediated repression of ethylene signaling. **Plant Physiol. 152,** 2036-2052.

De Vos, M., Denekamp, M., Dicke, M., Vuylsteke, M., Loon, V. L. C., Smeekens, S. C. M. and Pieterse, C. (2006a). The *Arabidopsis thaliana* transcription factor AtMYB102 functions in defense against the insect herbivore *Pieris rapae*. **Plant Signal. Behav. 1,** 305-311.

De Vos, M., Kriksunov, K. L. and Jander, G. (2008). Indole-3-acetonitrile production from indole glucosinolates deters oviposition by *Pieris rapae*. **Plant Physiol. 146,** 916-926.

De Vos, M., Van Oosten, V. R., Van Poecke, R. M., Van Pelt, J. A., Pozo, M. J., Mueller, M. J., Buchala, A. J., Métraux, J.-P. P., Van Loon, L. C., Dicke, M. and Pieterse, C. M. M. (2005). Signal signature and transcriptome changes of Arabidopsis during pathogen and insect attack. **Mol. Plant Microbe Interact. 18,** 923-937.

De Vos, M., Van Zaanen, W., Koornneef, A., Korzelius, J. P., Dicke, M., Van Loon, L. C. and Pieterse, C. M. J. (2006b). Herbivore-induced resistance against microbial pathogens in Arabidopsis. **Plant Physiol. 142,** 352-363.

Dean, R., Kan, J. A. L., Pretorius, Z. A., Hammond-Kosack, K. E., Pietro, A. D. I., Spanu, P. D., Rudd, J. J., Dickman, M., Kahmann, G., Ellis, J. and Foster, G. D. (2012). The Top 10 fungal pathogens in molecular plant pathology. **Mol. Plant Pathol. 13,** 414–430.

Delessert, C., Kazan, K., Wilson, I. W., Van Der Straeten, D., Manners, J., Dennis, E. S. and Dolferus, R. (2005). The transcription factor ATAF2 represses the expression of pathogenesis-related genes in Arabidopsis. **Plant J. 43,** 745-757.

Denby, K. J., Kumar, P. and Kliebenstein, D. J. (2004). Identification of *Botrytis cinerea* susceptibility loci in *Arabidopsis thaliana*. **Plant J. 38,** 473-486.

Desikan, R., Horák, J., Chaban, C., Mira-Rodado, V., Witthöft, J., Elgass, K., Grefen, C., Cheung, M.-K., Meixner, A. J., Hooley, R., Neill, S., Hancock, J. and Harter, K. (2008). The histidine kinase AHK5 integrates endogenous and environmental signals in Arabidopsis guard cells. **PLoS ONE 3,** e2491.

Devlin, B. and Roeder, K. (1999). Genomic control for association studies. **Biometrics 55,** 997-1004.

Devlin, B., Roeder, K. and Wasserman, L. (2001). Genomic control, a new approach to genetic-based association studies. **Theor. Popul. Biol. 60,** 155-166.

Diaz, J., Ten Have, A. and Van Kan, J. A. L. (2002). The role of ethylene and wound signaling in resistance of tomato to *Botrytis cinerea*. **Plant Physiol. 129,** 1341-1351.

Dicke, M. (2016). Plant phenotypic plasticity in the phytobiome: a volatile issue. **Curr. Opin. Plant Biol. 32,** 17-23.

Ding, Y., Tao, Y. and Zhu, C. (2013). Emerging roles of microRNAs in the mediation of drought stress response in plants. **J. Exp. Bot. 64,** 3077-3086.

Dobra, J., Motyka, V., Dobrev, P., Malbeck, J., Prasil, I. T., Haisel, D., Gaudinova, A., Havlova, M., Gubis, J. and Vankova, R. (2010). Comparison of hormonal responses to heat, drought and combined stress in tobacco plants with elevated proline content. **J. Plant Physiol. 167,** 1360-1370.

Dong, C. H. and Hong, Y. (2013). Arabidopsis CDPK6 phosphorylates ADF1 at N-terminal serine 6 predominantly. **Plant Cell Rep. 32,** 1715-1728.

Dong, J. X., Chen, C. H. and Chen, Z. X. (2003). Expression profiles of the Arabidopsis WRKY gene superfamily during plant defense response. **Plant Mol. Biol. 51,** 21-37.

Dong, O. X., Meteignier, L. V., Plourde, M. B., Ahmed, B., Wang, M., Jensen, C., Jin, H., Moffett, P., Li, X. and Germain, H. (2016). Arabidopsis TAF15b localizes to RNA processing bodies and contributes to snc1-mediated autoimmunity. **Mol. Plant Microbe Interact. 29,** 247-257.

Dorr, I. and Kollmann, R. (1995). Symplasmic sieve element continuity between Orobanche and its host. **Bot. Acta 108,** 47–55.

Du, H., Liu, H. and Xiong, L. (2013). Endogenous auxin and jasmonic acid levels are differentially modulated by abiotic stresses in rice. **Front. Plant Sci. 4,** 397.

Du, L., Ali, G. S., Simons, K. A., Hou, J., Yang, T., Reddy, A. S. N. and Poovaiah, B. W. (2009). Ca^{2+}/calmodulin regulates salicylic-acid-mediated plant immunity. **Nature 457,** 1154-1158.

Dubiella, U., Seybold, H., Durian, G., Komander, E., Lassig, R., Witte, C.-P., Schulze, W. X. and Romeis, T. (2013). Calcium-dependent protein kinase/NADPH oxidase activation circuit is required for rapid defense signal propagation. **Proc. Natl. Acad. Sci. 110,** 8744–8749.

Duran-Flores, D. and Heil, M. (2016). Sources of specificity in plant damaged-self recognition. **Curr. Opin. Plant Biol. 32,** 77-87.

Edger, P. P., Heidel-Fischer, H. M., Bekaert, M., Rota, J., Glöckner, G., Platts, A. E., Heckel, D. G., Der, J. P., Wafula, E. K., Tang, M., Hofberger, J. A., Smithson, A., Hall, J. C., Blanchette, M., Bureau, T. E., Wright, S. I., de Pamphilis, C. W., Schranz, E. M., Barker, M. S., Conant, G. C., Wahlberg, N., Vogel, H., Pires, C. J. and Wheat, C. W. (2015). The butterfly plant arms-race escalated by gene and genome duplications. **Proc. Natl. Acad. Sci. 112,** 8362-8366.

El-Soda, M., Kruijer, W., Malosetti, M., Koornneef, M. and Aarts, M. G. (2015). Quantitative trait loci and candidate genes underlying genotype by environment interaction in the response of *Arabidopsis thaliana* to drought. **Plant Cell Environ. 38,** 585-599.

El Oirdi, M., Abd El Rahman, T., Rigano, L., El Hadrami, A., Rodriguez, M. C., Daayf, F., Vojnov, A. and Bouarab, K. (2011). *Botrytis cinerea* manipulates the antagonistic effects between immune pathways to promote disease development in tomato. **Plant Cell 23,** 2405-2421.

Fabre, N., Reiter, I. M., Becuwe-Linka, N., Genty, B. and Rumeau, D. (2007). Characterization and expression analysis of genes encoding alpha and beta carbonic anhydrases in Arabidopsis. **Plant, Cell Environm. 30,** 617–629.

Fankhauser, C., Yeh, K.-C., Lagarias, J. C., Zhang, H., Elich, T. D. and Chory, J. (1999). PKS1, a substrate phosphorylated by phytochrome that modulates light signaling in Arabidopsis. **Science 284,** 1539-1541.

Fatouros, N. E., Cusumano, A., Danchin, E. and Colazza, S. (2016). Prospects of herbivore egg-killing plant defenses for sustainable crop protection. **Ecol. Evol. 6,** 6906-6918.

Fatouros, N. E., Lucas-Barbosa, D., Weldegergis, B. T., Pashalidou, F. G., Van Loon, J. J. A., Dicke, M., Harvey, J. A., Gols, R. and Huigens, M. E. (2012). Plant volatiles induced by herbivore egg deposition affect insects of different trophic levels. **PLoS ONE 7,** e43607.

Felton, A., Nilsson, U., Sonesson, J., Felton, A. M., Roberge, J. M., Ranius, T., Ahlstrom, M., Bergh, J., Bjorkman, C., Boberg, J., Drossler, L., Fahlvik, N., Gong, P., Holmstrom, E., Keskitalo, E. C., Klapwijk, M. J., Laudon, H., Lundmark, T., Niklasson, M., Nordin, A., Pettersson, M., Stenlid, J., Stens, A. and Wallertz, K. (2016). Replacing monocultures with mixed-species stands: Ecosystem service implications of two production forest alternatives in Sweden. **Ambio 45 Suppl 2,** 124-139.

Ferrari, S., Galletti, R., Denoux, C., De Lorenzo, G., Ausubel, F. M. and Dewdney, J. (2007). Resistance to *Botrytis cinerea* induced in Arabidopsis by elicitors is independent of salicylic acid, ethylene, or jasmonate signaling but requires *PHYTOALEXIN DEFICIENT3*. **Plant Physiol. 144,** 367-379.

Ferrari, S., Plotnikova, J. M., De Lorenzo, G. and Ausubel, F. M. (2003). Arabidopsis local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires *EDS4* and *PAD2*, but not *SID2*, *EDS5* or *PAD4*. **Plant J. 35,** 193-205.

Ferrari, S., Savatin, D., Sicilia, F., Gramegna, G., Cervone, F. and Lorenzo, G. (2013). Oligogalacturonides: plant damage-associated molecular patterns and regulators of growth and development. **Front. Plant Sci. 4,** 49.

Ferrari, S., Sella, L., Janni, M., Lorenzo, D. G., Favaron, F. and D'Ovidio, R. (2012). Transgenic expression of polygalacturonase-inhibiting proteins in Arabidopsis and wheat increases resistance to the flower pathogen *Fusarium graminearum*. **Plant Biol. 14,** 31-38.

Fiorani, F. and Schurr, U. (2013). Future scenarios for plant phenotyping. **Annu. Rev. Plant Biol. 64,** 267-291.

Frerigmann, H., Böttcher, C., Baatout, D. and Gigolashvili, T. (2012). Glucosinolates are produced in trichomes of *Arabidopsis thaliana*. **Front. Plant Sci. 3,** 242.

Frerigmann, H., Pislewska-Bednarek, M., Sanchez-Vallet, A., Molina, A., Glawischnig, E., Gigolashvili, T. and Bednarek, P. (2016). Regulation of pathogen-triggered tryptophan metabolism in *Arabidopsis thaliana* by MYB transcription factors and indole glucosinolate conversion products. **Mol. Plant 9,** 682-695.

Fujita, M., Fujita, Y., Maruyama, K., Seki, M., Hiratsu, K., Ohme-Takagi, M., Phan Tran, L. S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2004). A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. **Plant J. 39,** 863-876.

Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006). Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. **Curr. Opin. Plant Biol. 9,** 436-442.

Galletti, R., Denoux, C., Gambetta, S., Dewdney, J., Ausubel, F. M., De Lorenzo, G. and Ferrari, S. (2008). The AtrbohD-mediated oxidative burst elicited by oligogalacturonides in Arabidopsis is dispensable for the activation of defense responses effective against

Botrytis cinerea. **Plant Physiol. 148,** 1695-1706.

Gao, Q.-M., Venugopal, S., Navarre, D. and Kachroo, A. (2011). Low oleic acid-derived repression of jasmonic acid-inducible defense responses requires the WRKY50 and WRKY51 proteins. **Plant Physiol. 155,** 464-476.

Gao, X., Chen, X., Lin, W., Chen, S., Lu, D., Niu, Y., Li, L., Cheng, C., McCormack, M., Sheen, J., Shan, L. and He, P. (2013). Bifurcation of Arabidopsis NLR immune signaling via Ca^{2+}-dependent protein kinases. **PLoS Pathog. 9,** e1003127.

Garrett, K. A., Dobson, A. D. M., Kroschel, J., Natarajan, B., Orlandini, S., Tonnang, H. E. Z. and Valdivia, C. (2013). The effects of climate variability and the color of weather time series on agricultural diseases and pests, and on decisions for their management. **Agr. For. Meteorol. 170,** 216-227.

Geraats, B. P. J., Bakker, P. A. H. M. and Van Loon, L. C. (2002). Ethylene insensitivity impairs resistance to soilborne pathogens in tobacco and *Arabidopsis thaliana*. **Mol. Plant Microbe Interact. 15,** 1078-1085.

Gilden, R. C., Huffling, K. and Sattler, B. (2010). Pesticides and health risks. **J. Obstet. Gynecol. Neonatal. Nurs. 39,** 103–110.

Giron, D., Frago, E., Glevarec, G., Pieterse, C. M. J. and Dicke, M. (2013). Cytokinins as key regulators in plant–microbe–insect interactions: connecting plant growth and defence. **Funct. Ecol. 27,** 599-609.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. **Annu. Rev. Phytopathol. 43,** 205-227.

Gols, R. and Harvey, J. A. (2009). Plant-mediated effects in the Brassicaceae on the performance and behaviour of parasitoids. **Phytochem. Rev. 8,** 187-206.

Govrin, E. M. and Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. **Curr. Biol. 10,** 751-757.

Granier, C. and Vile, D. (2014). Phenotyping and beyond: modelling the relationships between traits. **Curr. Opin. Plant Biol. 18,** 96-102.

Gravino, M., Locci, F., Tundo, S., Cervone, F., Savatin, D. V. and De Lorenzo, G. (2017). Immune responses induced by oligogalacturonides are differentially affected by AvrPto and loss of BAK1/BKK1 and PEPR1/PEPR2. **Mol. Plant Pathol. 18,** 582-595.

Gravot, A., Deleu, C., Wagner, G., Lariagon, C., Lugan, R., Todd, C., Wendehenne, D., Delourme, R., Bouchereau, A. and Manzanares-Dauleux, M. J. (2012). Arginase induction represses gall development during clubroot infection in Arabidopsis. **Plant Cell Physiol. 53,** 901-911.

Gressel, J. (2011). Low pesticide rates may hasten the evolution of resistance by increasing mutation frequencies. **Pest Manag. Sci. 67,** 253-257.

Grieve, C. M., Poss, J. A., Grattan, S. R., Suarez, D. L. and Smith, T. E. (2010). The combined effects of salinity and excess boron on mineral ion relations in broccoli. **Sci. Hortic. 125,** 179-187.

Grinnan, R., Carter Jr, T. E. and Johnson, M. T. J. (2013). The effects of drought and herbivory on plant–herbivore interactions across 16 soybean genotypes in a field experiment. **Ecol. Entomol. 38,** 290–302.

Groen, S. C., Whiteman, N. K., Bahrami, A. K., Wilczek, A. M., Cui, J., Russell, J. A., Cibrian-Jaramillo, A., Butler, I. A., Rana, J. D., Huang, G.-H., Bush, J., Ausubel, F. M. and Pierce, N. E. (2013). Pathogen-triggered ethylene signaling mediates systemic-induced susceptibility to herbivory in Arabidopsis. **Plant Cell 25,** 4755-4766.

Gutbrodt, B., Mody, K. and Dorn, S. (2011). Drought changes plant chemistry and causes contrasting responses in lepidopteran herbivores. **Oikos 120,** 1732-1740.

Handley, R., Ekbom, B. and Ågren, J. (2005). Variation in trichome density and resistance against a specialist insect herbivore in natural populations of *Arabidopsis thaliana*. **Ecol. Entomol. 30,** 284–292.

Harvey, J. A., Witjes, L. M. A., Benkirane, M., Duyts, H. and Wagenaar, R. (2007). Nutritional suitability and ecological relevance of *Arabidopsis thaliana* and *Brassica oleracea* as foodplants for the cabbage butterfly, *Pieris rapae*. **Plant Ecol. 189,** 117-126.

Hasanuzzaman, M., Nahar, K., Alam, M. M., Roychowdhury, R. and Fujita, M. (2013). Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. **Int. J. Mol. Sci. 14,** 9643-9684.

Hauser, M.-T., Harr, B. and Schlötterer, C. (2001). Trichome distribution in *Arabidopsis thaliana* and its close relative *Arabidopsis lyrata*: molecular analysis of the candidate gene *GLABROUS1*. **Mol. Biol. Evol. 18,** 1754–1763

Heil, M. and Karban, R. (2010). Explaining evolution of plant communication by airborne signals. **Trends Ecol. Evol. 25,** 137-144.

Hentrich, M., Bottcher, C., Duchting, P., Cheng, Y., Zhao, Y., Berkowitz, O., Masle, J., Medina, J. and Pollmann, S. (2013). The jasmonic acid signaling pathway is linked to auxin homeostasis through the modulation of *YUCCA8* and *YUCCA9* gene expression. **Plant J. 74,** 626-637.

Herbers, K., Takahata, Y., Melzer, M., Mock, H. P., Hajirezaei, M. and Sonnewald, U. (2000). Regulation of carbohydrate partitioning during the interaction of potato virus Y with tobacco. **Mol. Plant Pathol. 1,** 51-59.

Hern, A., Edwards-Jones, G. and McKinlay, R. G. (1996). A review of the cabbage white pre-oviposition behaviour of the small butterfly, *Pieris rapae* (Lepidoptera: Pieridae). **Ann. appl. Biol. 128,** 349-371.

Hernández-Blanco, C., Feng, D., Hu, J., Sánchez-Vallet, A., Deslandes, L., Llorente, F., Berrocal-Lobo, M., Keller, H., Barlet, X., Sánchez-Rodríguez, C., Anderson, L., Somerville, S., Marco, Y. and Molina, A. (2007). Impairment of cellulose synthases required for Arabidopsis secondary cell wall formation enhances disease resistance. **Plant Cell 19,** 890-903.

Hevia, M., Canessa, P., Müller-Esparza, H. and Larrondo, L. (2015). A circadian oscillator in the fungus *Botrytis cinerea* regulates virulence when infecting *Arabidopsis thaliana*. **Proc. Natl. Acad. Sci. U.S.A. 112,** 8744-8749.

Hickman, R., Van Verk, M. C., Van Dijken, A. J. H., Mendes, M. P., Vroegop-Vos, I. A., Caarls, L., Steenbergen, M., Van der Nagel, I., Wesselink, G. J., Jironkin, A., Talbot, A., Rhodes, J., De Vries, M., Schuurink, R. C., Denby, K., Pieterse, C. M. J. and Van Wees, S. C. M. (2017). Architecture and dynamics of the jasmonic acid gene regulatory network. **Plant Cell 29,** 2086-2105.

Holopainen, J. K. and Gershenzon, J. (2010). Multiple stress factors and the emission of plant VOCs. **Trends Plant Sci. 15,** 176-184.

Hong, G. J., Xue, X. Y., Mao, Y. B., Wang, L. J. and Chen, X. Y. (2012). Arabidopsis MYC2 interacts with DELLA proteins in regulating sesquiterpene synthase gene expression. **Plant Cell 24,** 2635-2648.

Hopkins, R. J., Van Dam, N. M. and Van Loon, J. J. A. (2009). Role of glucosinolates in insect-plant relationships and multitrophic interactions. **Ann. Rev. Entomol. 54,** 57-83.

Horton, M. W., Hancock, A. M., Huang, Y. S., Toomajian, C., Atwell, S., Auton, A., Muliyati, W. N., Platt, A., Sperone, G. F., Vilhjálmsson, B. J., Nordborg, M., Borevitz, J. O. and Bergelson, J. (2012). Genome-wide patterns of genetic variation in worldwide *Arabidopsis thaliana* accessions from the RegMap panel. **Nat. Genet. 44,** 212-216.

Howe, G. A. and Jander, G. (2008). Plant immunity to insect herbivores. **Annu. Rev. Plant Biol. 59,** 41-66.

Huang, D. Q., Wu, W. R., Abrams, S. R. and Cutler, A. J. (2008). The relationship of drought-related gene expression in *Arabidopsis thaliana* to hormonal and environmental factors. **J. Exp. Bot. 59,** 2991-3007.

Huang, G., Lu, H., Hao, A., Ng, D. C., Ponniah, S., Guo, K., Lufei, C., Zeng, Q. and Cao, X. (2004). GRIM-19, a cell death regulatory protein, is essential for assembly and function of mitochondrial complex I. **Mol. Cell. Biol. 24,** 8447-8456.

Huang, M., Abel, C., Sohrabi, R., Petri, J., Haupt, I., Cosimano, J., Gershenzon, J. and Tholl, D. (2010). Variation of herbivore-induced volatile terpenes among Arabidopsis ecotypes depends on allelic differences and subcellular targeting of two terpene synthases, TPS02 and TPS03. **Plant Physiol. 153,** 1293-1310.

Huang, X. and Renwick, J. A. A. (1994). Relative activities of glucosinolates as oviposition stimulants for *Pieris rapae* and *P. napi oleracea*. **J. Chem. Ecol. 20,** 1025-1037.

Huberty, A. F. and Denno, R. F. (2004). Plant water stress and its consequences for herbivorous insects: a new synthesis. **Ecology 85,** 1383-1398.

Hwang, S.-Y., Liu, C.-H. and Shen, T.-C. (2008). Effects of plant nutrient availability and host plant species on the performance of two Pieris butterflies (Lepidoptera: Pieridae). **Biochem. Syst. Ecol. 36,** 505-513.

Ingle, R., Stoker, C., Stone, W., Adams, N., Smith, R., Grant, M., Carré, I., Roden, L. and Denby, K. (2015). Jasmonate signalling drives time-of-day differences in susceptibility of Arabidopsis to the fungal pathogen *Botrytis cinerea*. **Plant J. 84,** 937–948.

Ingram, J. and Bartels, D. (1996). The molecular basis of dehydration tolerance in plants. **Annu. Rev. Plant Physiol. Plant Mol. Biol. 47,** 377-403.

Iwama, A., Yamashino, T., Tanaka, Y., Sakakibara, H., Kakimoto, T., Sato, S., Kato, T., Tabata, S., Nagatani, A. and Mizuno, T. (2006). AHK5 histidine kinase regulates root elongation through an ETR1-dependent abscisic acid and ethylene signaling pathway in *Arabidopsis thaliana*. **Plant Cell Physiol. 48,** 375-380.

Janda, T., Szalai, G., Tari, I. and Páldi, E. (1999). Hydroponic treatment with salicylic acid decreases the effects of chilling injury in maize (*Zea mays* L.) plants. **Planta 208**.

Jensen, M. K., Lindemose, S., de Masi, F., Reimer, J. J., Nielsen, M., Perera, V., Workman, C. T., Turck, F., Grant, M. R., Mundy, J., Petersen, M. and Skriver, K. (2013). ATAF1 transcription factor directly regulates abscisic acid biosynthetic gene *NCED3* in *Arabidopsis thaliana*. **FEBS Open Bio 3,** 321-327.

Jeworutzki, E., Roelfsema, M. R., Anschutz, U., Krol, E., Elzenga, J. T., Felix, G., Boller, T., Hedrich, R. and Becker, D. (2010). Early signaling through the Arabidopsis pattern recognition receptors FLS2 and EFR involves Ca^{2+}-associated opening of plasma membrane anion channels. **Plant J. 62,** 367-378.

Jin, W. and Wu, F. (2015). Characterization of miRNAs associated with *Botrytis cinerea* infection of tomato leaves. **BMC Plant Biol. 15,** 1.

Jirage, D., Zhou, N., Cooper, B., Clarke, J. D., Dong, X. and Glazebrook, J. (2001). Constitutive salicylic acid-dependent signaling in *cpr1* and *cpr6* mutants requires PAD4. **Plant J. 26,** 395-407.

Johanson, U., West, J., Lister, C., Michaels, S., Amasino, R. and Dean, C. (2000). Molecular analysis of *FRIGIDA*, a major determinant of natural variation in Arabidopsis flowering time. **Science 290,** 344-347.

Johnson, M. T. J. (2011). Evolutionary ecology of plant defences against herbivores. **Funct. Ecol. 25,** 305-311.

Johnson, S. M., Lim, F.-L., Finkler, A., Fromm, H., Slabas, A. R. and Knight, M. R. (2014). Transcriptomic analysis of *Sorghum bicolor* responding to combined heat and drought stress. **BMC Genomics 15,** 1-19.

Jones, J. D. G. and Dangl, J. L. (2006). The plant immune system. **Nature 444,** 323-329.

Jones, R. E. (1977). Movement patterns and egg distribution in cabbage butterflies. **J. Animal Ecol. 46,** 95-212.

Jubault, M., Hamon, C., Gravot, A., Lariagon, C., Delourme, R., Bouchereau, A. and Manzanares-Dauleux, M. J. (2008). Differential regulation of root arginine catabolism and polyamine metabolism in clubroot-susceptible and partially resistant Arabidopsis genotypes. **Plant Physiol. 146,** 2008-2019.

Julkowska, M. M. and Testerink, C. (2015). Tuning plant signaling and growth to survive salt. **Trends Plant Sci. 20,** 586-594.

Kachroo, P., Shanklin, J., Shah, J., Whittle, E. J. and Klessig, D. F. (2001). A fatty acid desaturase modulates the activation of defense signaling pathways in plants. **Proc. Natl. Acad. Sci. 98,** 9448-9453.

Kadota, Y., Sklenar, J., Derbyshire, P., Stransfeld, L., Asai, S., Ntoukakis, V., Jones, J. D., Shirasu, K., Menke, F., Jones, A. and Zipfel, C. (2014). Direct regulation of the NADPH oxidase RBOHD by the PRR-associated kinase BIK1 during plant immunity. **Mol. Cell 54,** 43-55.

Kanani, H., Dutta, B. and Klapa, M. (2010). Individual vs. combinatorial effect of elevated CO_2 conditions and salinity stress on *Arabidopsis thaliana* liquid cultures: comparing the early molecular response using time-series transcriptomic and metabolomic analyses. **BMC Syst. Biol. 4,** 177.

Kars, I., Krooshof, G. H., Wagemakers, L., Joosten, R., Benen, J. and Kan, J. (2005). Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. **Plant J. 43,** 213-225.

Kathiria, P., Sidler, C., Golubov, A., Kalischuk, M., Kawchuk, L. M. and Kovalchuk, I. (2010). Tobacco mosaic virus infection results in an increase in recombination frequency and resistance to viral, bacterial, and fungal pathogens in the progeny of infected tobacco plants. **Plant Physiol. 153,** 1859-1870.

Kazan, K. and Lyons, R. (2014). Intervention of phytohormone pathways by pathogen effectors. **Plant Cell 26,** 2285-2309.

Kazan, K. and Manners, J. M. (2009). Linking development to defense: auxin in plant-pathogen interactions. **Trends Plant Sci. 14,** 373-382.

Kerwin, R., Feusier, J., Corwin, J., Rubin, M., Lin, C., Muok, A., Larson, B., Li, B., Joseph, B., Francisco, M., Copeland, D., Weinig, C. and Kliebenstein, D. J. (2015). Natural genetic variation in *Arabidopsis thaliana* defense metabolism genes modulates field fitness. **elife 4,** e05604.

Kessler, A., Halitschke, R. and Baldwin, I. T. (2004). Silencing the jasmonate cascade: induced plant defenses and insect populations. **Science 305,** 665-668.

Kettles, G. J., Drurey, C., Schoonbeek, H. J., Maule, A. J. and Hogenhout, S. A. (2013). Resistance of *Arabidopsis thaliana* to the green peach aphid, *Myzus persicae*, involves camalexin and is regulated by microRNAs. **New Phytol. 198,** 1178-1190.

Khan, G. A., Vogiatzaki, E., Glauser, G. and Poirier, Y. (2016). Phosphate deficiency induces the jasmonate pathway and enhances resistance to insect herbivory. **Plant Physiol. 171,** 632-644.

Khan, M., Ragni, L., Tabb, P., Salasini, B. C., Chatfield, S., Datla, R., Lock, J., Kuai, X., Després, C., Proveniers, M., Yongguo, C., Xiang, D., Morin, H., Rullière, J.-P. P., Citerne, S., Hepworth, S. R. and Pautot, V. (2015). Repression of lateral organ boundary genes by PENNYWISE and POUND-FOOLISH is essential for meristem maintenance and flowering in Arabidopsis. **Plant Physiol. 169,** 2166–2186.

Khan, S. and Stone, J. M. (2007). *Arabidopsis thaliana* GH3.9 influences primary root growth. **Planta 226,** 21-34.

Kidokoro, S., Watanabe, K., Ohori, T., Moriwaki, T., Maruyama, K., Mizoi, J., Myint Phyu Sin Htwe, N., Fujita, Y., Sekita, S., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2015). Soybean DREB1/CBF-type transcription factors function in heat and drought as well as cold stress-responsive gene expression. **Plant J. 81,** 505-518.

Kim, K. C., Lai, Z., Fan, B. and Chen, Z. (2008). Arabidopsis WRKY38 and WRKY62 transcription factors interact with histone deacetylase 19 in basal defense. **Plant Cell 20,** 2357-2371.

Kim, S., Plagnol, V., Hu, T. T., Toomajian, C., Clark, R. M., Ossowski, S., Ecker, J. R., Weigel, D. and Nordborg, M. (2007). Recombination and linkage disequilibrium in *Arabidopsis thaliana*. **Nature Genet. 39,** 1151-1155.

Kissoudis, C., Chowdhury, R., Van Heusden, S., Van de Wiel, C., Finkers, R., Visser, R. G. F., Bai, Y. and Van der Linden, G. (2015). Combined biotic and abiotic stress resistance in tomato. **Euphytica 202,** 317-332.

Kissoudis, C., Van de Wiel, C., Visser, R. G. F. and Van der Linden, G. (2014). Enhancing crop resilience to combined abiotic and biotic stress through the dissection of physiological and molecular crosstalk. **Front. Plant Sci. 5,** 207.

Kliebenstein, D. J., Kroymann, J., Brown, P., Figuth, A., Pedersen, D., Gershenzon, J. and Mitchell-Olds, T. (2001). Genetic control of natural variation in Arabidopsis glucosinolate accumulation. **Plant Physiol. 126,** 811-825.

Kliebenstein, D. J., Rowe, H. C. and Denby, K. J. (2005). Secondary metabolites influence Arabidopsis/*Botrytis* interactions: variation in host production and pathogen sensitivity. **Plant J. 44,** 25-36.

Kloth, K. J., Ten Broeke, C. J., Thoen, M. P., Hanhart-Van den Brink, M., Wiegers, G. L., Krips, O. E., Noldus, L. P., Dicke, M. and Jongsma, M. A. (2015). High-throughput phenotyping of plant resistance to aphids by automated video tracking. **Plant Methods 11,** 4.

Kloth, K. J., Wiegers, G. L., Busscher-Lange, J., Van Haarst, J. C., Kruijer, W., Bouwmeester, H. J., Dicke, M. and Jongsma, M. A. (2016). AtWRKY22 promotes susceptibility to aphids and modulates salicylic acid and jasmonic acid signalling. **J. Exp. Bot. 67,** 3383-3396.

Kocal, N., Sonnewald, U. and Sonnewald, S. (2008). Cell wall-bound invertase limits sucrose export and is involved in symptom development and inhibition of photosynthesis during compatible interaction between tomato and *Xanthomonas campestris* pv *vesicatoria*. **Plant Physiol. 148,** 1523-1536.

Kooke, R., Kruijer, W., Bours, R., Becker, F., Kuhn, A., van de Geest, H., Buntjer, J., Doeswijk, T., Guerra, J., Bouwmeester, H., Vreugdenhil, D. and Keurentjes, J. J. (2016). Genome-wide association mapping and genomic prediction elucidate the genetic architecture of morphological traits in Arabidopsis. **Plant Physiol. 170,** 2187-2203.

Koornneef, A., Leon-Reyes, A., Ritsema, T., Verhage, A., Den Otter, F. C., Van Loon, L. C. and Pieterse, C. M. J. (2008). Kinetics of salicylate-mediated suppression of jasmonate signaling reveal a role for redox modulation. **Plant Physiol. 147,** 1358-1368.

Koornneef, M., Alonso-Blanco, C. and Vreugdenhil, D. (2004). Naturally occurring genetic variation in *Arabidopsis thaliana*. **Ann. Rev. Plant Biol. 55,** 141-172.

Korte, A., Vilhjalmsson, B. J., Segura, V., Platt, A., Long, Q. and Nordborg, M. (2012). A mixed-model approach for genome-wide association studies of correlated traits in structured populations. **Nat. Genet. 44,** 1066-1071.

Koussevitzky, S., Suzuki, N., Huntington, S., Armijo, L., Sha, W., Cortes, D., Shulaev, V. and Mittler, R. (2008). Ascorbate peroxidase 1 plays a key role in the response of *Arabidopsis thaliana* to stress combination. **J. Biol. Chem. 283,** 34197-34203.

Kover, P. and Mott, R. (2012). Mapping the genetic basis of ecologically and evolutionarily relevant traits in *Arabidopsis thaliana*. **Curr. Opin. Plant Biol. 15,** 212-217.

Kover, P., Valdar, W., Trakalo, J., Scarcelli, N., Ehrenreich, I., Purugganan, M., Durrant, C. and Mott, R. (2009). A multiparent advanced generation inter-cross to fine-map quantitative traits in *Arabidopsis thaliana*. **PLoS Genet. 5,** e1000551.

Kruijer, W., Boer, M. P., Malosetti, M., Flood, P. J. J., Engel, B., Kooke, R., Keurentjes, J. J. and Van Eeuwijk, F. A. (2015). Marker-based estimation of heritability in immortal populations. **Genetics 199,** 379-398.

Laluk, K. and Mengiste, T. (2010). Necrotroph attacks on plants: wanton destruction or covert extortion? **Arabidopsis Book 8,** e0136.

Larkindale, J., Hall, J. D., Knight, M. R. and Vierling, E. (2005). Heat stress phenotypes of Arabidopsis mutants implicate multiple signaling pathways in the acquisition of thermotolerance. **Plant Physiol. 138,** 882-897.

Larkindale, J. and Knight, M. R. (2002). Protection against heat stress-induced oxidative damage in Arabidopsis involves calcium, abscisic acid, ethylene, and salicylic acid. **Plant Physiol. 128,** 682-695.

Laurie-Berry, N., Joardar, V., Street, I. H. and Kunkel, B. N. (2006). The Arabidopsis thaliana *JASMONATE INSENSITIVE 1* gene is required for suppression of salicylic acid-dependent defenses during infection by *Pseudomonas syringae*. **Mol. Plant Microbe Interact. 19,** 789-800.

Le, M., Cao, Y., Zhang, X.-C. and Stacey, G. (2014). LIK1, a CERK1-interacting kinase, regulates plant immune responses in Arabidopsis. **PLoS ONE 9,** e102245.

Lechner, E., Achard, P., Vansiri, A., Potuschak, T. and Genschik, P. (2006). F-box proteins everywhere. **Curr. Opinion Plant Biol. 9,** 631-638.

Leckie, C. P., McAinsh, M. R., Allen, G. J., Sanders, D. and Hetherington, A. M. (1998). Abscisic acid-induced stomatal closure mediated by cyclic ADP-ribose. **Proc. Natl. Acad. Sci. U.S.A. 95,** 15837-15842.

Lee, M., Jung, J.-H., Han, D.-Y., Seo, P., Park, W. and Park, C.-M. (2012). Activation of a flavin monooxygenase gene *YUCCA7* enhances drought resistance in Arabidopsis. **Planta 235,** 923-938.

Leon-Reyes, A., Du, Y., Koornneef, A., Proietti, S., Körbes, A. P., Memelink, J., Pieterse, C. M. J. and Ritsema, T. (2010). Ethylene signaling renders the jasmonate response of Arabidopsis insensitive to future suppression by salicylic acid. **Mol. Plant Microbe Interact. 23,** 187-197.

Li, W., Zhang, C., Lu, Q., Wen, X. and Lu, C. (2011). The combined effect of salt stress and heat shock on proteome profiling in *Suaeda salsa*. **J. Plant Physiol. 168,** 1743-1752.

Li, Y., Huang, Y., Bergelson, J., Nordborg, M. and Borevitz, J. O. (2010a). Association mapping of local climate-sensitive quantitative

trait loci in *Arabidopsis thaliana*. **Proc. Natl. Acad. Sci. 107,** 21199-21204.

Li, Y., Li, S., Bi, D., Cheng, Y., Li, X. and Zhang, Y. (2010b). SRFR1 negatively regulates plant NB-LRR resistance protein accumulation to prevent autoimmunity. **PLoS Pathog. 6,** e1001111.

Li, Y., Tessaro, M. J., Li, X. and Zhang, Y. (2010c). Regulation of the expression of plant resistance gene *SNC1* by a protein with a conserved BAT2 domain. **Plant Physiol. 153,** 1425-1434.

Lin, R.-C., Park, H.-J. and Wang, H.-Y. (2008). Role of Arabidopsis RAP2.4 in regulating light- and ethylene-mediated developmental processes and drought stress tolerance. **Mol. Plant 1,** 42-57.

Lionetti, V., Raiola, A., Camardella, L., Giovane, A., Obel, N., Pauly, M., Favaron, F., Cervone, F. and Bellincampi, D. (2007). Overexpression of pectin methylesterase inhibitors in Arabidopsis restricts fungal infection by *Botrytis cinerea*. **Plant Physiol. 143,** 1871–1880.

Lippert, C., Listgarten, J., Liu, Y., Kadie, C. M., Davidson, R. I. and Heckerman, D. (2011). FaST linear mixed models for genome-wide association studies. **Nat. Methods 8,** 833-835.

Little, D., Gouhier-Darimont, C., Bruessow, F. and Reymond, P. (2007). Oviposition by pierid butterflies triggers defense responses in Arabidopsis. **Plant Physiol. 143,** 784-800.

Liu, J. and Wang, X.-J. (2006). An integrative analysis of the effects of auxin on jasmonic acid biosynthesis in *Arabidopsis thaliana*. **J. Integr. Plant Biol. 48,** 99-103.

Liu, S., Kracher, B., Ziegler, J., Birkenbihl, R. P. and Somssich, I. E. (2015). Negative regulation of ABA signaling by WRKY33 is critical for Arabidopsis immunity towards *Botrytis cinerea* 2100. **eLife 4,** e07295.

Liu, T., Liu, Z., Song, C., Hu, Y., Han, Z., She, J., Fan, F., Wang, J., Jin, C., Chang, J., Zhou, J. M. and Chai, J. (2012). Chitin-induced dimerization activates a plant immune receptor. **Science 336,** 1160-1164.

Liu, Y. and Zhang, S. (2004). Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by MPK6, a stress-responsive mitogen-activated protein kinase, induces ethylene biosynthesis in Arabidopsis. **Plant Cell 16,** 3386-3399.

Liu, Z., Wu, Y., Yang, F., Zhang, Y., Chen, S., Xie, Q., Tian, X. and Zhou, J. M. (2013). BIK1 interacts with PEPRs to mediate ethylene-induced immunity. **Proc. Natl. Acad. Sci. U.S.A. 110,** 6205-6210.

Lorenzo, O., Chico, J. M., Sanchez-Serrano, J. J. and Solano, R. (2004). *JASMONATE-INSENSITIVE1* encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. **Plant Cell 16,** 1938-1950.

Love, M. I., Huber, W. and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. **Genome Biol. 15,** 550.

Lozano-Durán, R., Bourdais, G., He, S. Y. and Robatzek, S. (2014). The bacterial effector HopM1 suppresses PAMP-triggered oxidative burst and stomatal immunity. **New Phytol. 202,** 259-269.

Lu, H. and Higgins, V. J. (1999). The effect of hydrogen peroxide on the viability of tomato cells and of the fungal pathogen *Cladosporium fulvum*. **Physiol. Mol. Plant Pathol. 54,** 131–143.

Lu, K., Liang, S., Wu, Z., Bi, C., Yu, Y.-T., Wang, X.-F. and Zhang, D.-P. (2016). Overexpression of an Arabidopsis cysteine-rich receptor-like protein kinase, CRK5, enhances abscisic acid sensitivity and confers drought tolerance. **J. Exp. Bot. 67,** 5009-5027.

Ma, S., Gong, Q. and Bohnert, H. J. (2006). Dissecting salt stress pathways. **J. Exp. Bot. 57,** 1097-1107.

Machado, R. A. R., Ferrieri, A. P., Robert, C. A. M., Glauser, G., Kallenbach, M., Baldwin, I. T. and Erb, M. (2013). Leaf-herbivore attack reduces carbon reserves and regrowth from the roots via jasmonate and auxin signaling. **New Phytol. 200,** 1234-1246.

Machado, R. A. R., Robert, C. A. M., Arce, C. C. M., Ferrieri, A. P., Xu, S., Jimenez-Aleman, G. H., Baldwin, I. T. and Erb, M. (2016). Auxin is rapidly induced by herbivore attack and regulates a subset of systemic, jasmonate-dependent defenses. **Plant Physiol. 172,** 521-532.

Makumburage, G. B., Richbourg, H. L., LaTorre, K. D., Capps, A., Chen, C. and Stapleton, A. E. (2013). Genotype to phenotype maps: multiple input abiotic signals combine to produce growth effects via attenuating signaling interactions in maize. **G3 (Bethesda) 3,** 2195-2204.

Malosetti, M., Ribaut, J. M. and van Eeuwijk, F. A. (2013). The statistical analysis of multi-environment data: modeling genotype-by-environment interaction and its genetic basis. **Front Physiol. 4,** 44.

Malosetti, M., Ribaut, J. M., Vargas, M., Crossa, J. and Van Eeuwijk, F. A. (2008). A multi-trait multi-environment QTL mixed model with an application to drought and nitrogen stress trials in maize (*Zea mays* L.). **Euphytica 161,** 241-257.

Manabe, Y., Nafisi, M., Verhertbruggen, Y., Orfila, C., Gille, S., Rautengarten, C., Cherk, C., Marcus, S. E., Somerville, S., Pauly, M., Knox, P. J., Sakuragi, Y. and Scheller, H. (2011). Loss-of-function mutation of *REDUCED WALL ACETYLATION2* in Arabidopsis leads to reduced cell wall acetylation and increased resistance to *Botrytis cinerea*. **Plant Physiol. 155,** 1068-1078.

Mandal, S., Mallick, N. and Mitra, A. (2009). Salicylic acid-induced resistance to *Fusarium oxysporum* f. sp. *lycopersici* in tomato. **Plant**

Physiol. Biochem. 47, 642-649.

Mann, R. S., Ali, J. G., Hermann, S. L., Tiwari, S., Pelz-Stelinski, K. S., Alborn, H. T. and Stelinski, L. L. (2012). Induced release of a plant-defense volatile 'deceptively' attracts insect vectors to plants infected with a bacterial pathogen. **PLoS Pathog. 8,** e1002610.

Mäntylä, E., Lang, V. and Palva, T. E. (1995). Role of abscisic acid in drought-induced freezing tolerance, cold acclimation, and accumulation of LT178 and RAB18 proteins in *Arabidopsis thaliana*. **Plant Physiol. 107,** 141-148.

Mao, G., Meng, X., Liu, Y., Zheng, Z., Chen, Z. and Zhang, S. (2011). Phosphorylation of a WRKY transcription factor by two pathogen-responsive MAPKs drives phytoalexin biosynthesis in Arabidopsis. **Plant Cell 23,** 1639-1653.

Mao, P., Duan, M., Wei, C. and Li, Y. (2007). WRKY62 transcription factor acts downstream of cytosolic NPR1 and negatively regulates jasmonate-responsive gene expression. **Plant Cell Physiol. 48,** 833-842.

Meissle, M., Mouron, P., Musa, T., Bigler, F., Pons, X., Vasileiadis, V. P., Otto, S., Antichi, D., Kiss, J., Pálinkás, Z., Dorner, Z., Weide, V. R., Groten, J., Czembor, E., Adamczyk, J., Thibord, J. B., Melander, B., Nielsen, C. G., Poulsen, R. T., Zimmermann, O., Verschwele, A. and Oldenburg, E. (2010). Pests, pesticide use and alternative options in European maize production: current status and future prospects. **J. Appl. Entomol. 134,** 357–375.

Mengiste, T., Chen, X., Salmeron, J. and Dietrich, R. (2003). The *BOTRYTIS SUSCEPTIBLE1* gene encodes an R2R3MYB transcription factor protein that is required for biotic and abiotic stress responses in Arabidopsis. **Plant Cell 15,** 2551-2565.

Mersmann, S., Bourdais, G., Rietz, S. and Robatzek, S. (2010). Ethylene signaling regulates accumulation of the FLS2 receptor and is required for the oxidative burst contributing to plant immunity. **Plant Physiol. 154,** 391-400.

Mewis, I., Tokuhisa, J. G., Schultz, J. C., Appel, H. M., Ulrichs, C. and Gershenzon, J. (2006). Gene expression and glucosinolate accumulation in *Arabidopsis thaliana* in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways. **Phytochemistry 67,** 2450-2462.

Meyer, K. (2009). Factor-analytic models for genotype x environment type problems and structured covariance matrices. **Genet. Sel. Evol. 41,** 21.

Miller, G., Suzuki, N., Rizhsky, L., Hegie, A., Koussevitzky, S. and Mittler, R. (2007). Double mutants deficient in cytosolic and thylakoid ascorbate peroxidase reveal a complex mode of interaction between reactive oxygen species, plant development, and response to abiotic stresses. **Plant Physiol. 144,** 1777-1785.

Mittler, R. (2006). Abiotic stress, the field environment and stress combination. **Trend. Plant Sci. 11,** 15-19.

Mittler, R. and Blumwald, E. (2010). Genetic engineering for modern agriculture: challenges and perspectives. **Annu. Rev. Plant Biol. 61,** 443-462.

Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V. B., Vandepoele, K., Gollery, M., Shulaev, V. and Van Breusegem, F. (2011). ROS signaling: the new wave? **Trends Plant Sci. 16,** 300-309.

Miura, K., Okamoto, H., Okuma, E., Shiba, H., Kamada, H., Hasegawa, P. M. and Murata, Y. (2013). SIZ1 deficiency causes reduced stomatal aperture and enhanced drought tolerance via controlling salicylic acid-induced accumulation of reactive oxygen species in Arabidopsis. **Plant J. 73,** 91-104.

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H. and Shibuya, N. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. **Proc. Natl. Acad. Sci. U.S.A. 104,** 19613-19618.

Mizoi, J., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2012). AP2/ERF family transcription factors in plant abiotic stress responses. **Biochim. Biophys. Acta 1819,** 86-96.

Mohr, P. G. and Cahill, D. M. (2003). Abscisic acid influences the susceptibility of *Arabidopsis thaliana* to *Pseudomonas syringae* pv. *tomato* and *Peronospora parasitica*. **Funct. Plant Biol. 30,** 461-469.

Mohr, P. G. and Cahill, D. M. (2007). Suppression by ABA of salicylic acid and lignin accumulation and the expression of multiple genes, in Arabidopsis infected with *Pseudomonas syringae* pv. *tomato*. **Funct. Integr. Genomics 7,** 181-191.

Molas, M. L. and Kiss, J. Z. (2008). PKS1 plays a role in red-light-based positive phototropism in roots. **Plant Cell Environ. 31,** 842-849.

Mondal, H. A., Louis, J., Archer, L., Patel, M., Nalam, V. J., Sarowar, S., Sivapalan, V., Root, D. D. and Shah, J. (2018). Arabidopsis *ACTIN-DEPOLYMERIZING FACTOR3* is required for controlling aphid feeding from the phloem. **Plant Physiol. 176,** 879-890.

Mousavi, S. A. R., Chauvin, A., Pascaud, F., Kellenberger, S. and Farmer, E. E. (2013). *GLUTAMATE RECEPTOR-LIKE* genes mediate leaf-to-leaf wound signalling. **Nature 500,** 422-426.

Mousseau, T. A. and Roff, D. A. (1987). Natural selection and the heritability of fitness components. **Heredity 59,** 181-197.

Müller, R., de Vos, M., Sun, J. Y., Sønderby, I. E., Halkier, B. A., Wittstock, U. and Jander, G. (2010). Differential effects of indole and aliphatic glucosinolates on Lepidopteran herbivores. **J. Chem. Ecol. 36,** 905-913.

Mumm, R., Burow, M., Bukovinszkine'Kiss, G., Kazantzidou, E., Wittstock, U., Dicke, M. and Gershenzon, J. (2008). Formation of simple

nitriles upon glucosinolate hydrolysis affects direct and indirect defense against the specialist herbivore, *Pieris rapae*. **J. Chem. Ecol. 34,** 1311-1321.

Mur, L. A. J., Kenton, P., Atzorn, R., Miersch, O. and Wasternack, C. (2006). The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. **Plant Physiol. 140,** 249-262.

Muriel, S. B. and Grez, A. A. (2002). Effect of plant patch shape on the distribution and abundance of three lepidopteran species associated with *Brassica oleracea*. **Agr. For. Entomol. 4,** 179-185.

Myers, J. H. (1985). Effect of physiological condition of the host plant on the ovipositional choice of the cabbage white butterfly, *Pieris rapae*. **J. Animal Ecol. 54,** 193-204.

Nagamangala Kanchiswamy, C., Takahashi, H., Quadro, S., Maffei, M. E., Bossi, S., Bertea, C., Atsbaha Zebelo, S., Muroi, A., Ishihama, N., Yoshioka, H., Boland, W., Takabayashi, J., Endo, Y., Sawasaki, T. and Arimura, G.-i. (2010). Regulation of Arabidopsis defense responses against *Spodoptera littoralis* by CPK-mediated calcium signaling. **BMC Plant Biology 10,** 97.

Nagpal, P., Ellis, C. M., Weber, H., Ploense, S. E., Barkawi, L. S., Guilfoyle, T. J., Hagen, G., Alonso, J. M., Cohen, J. D., Farmer, E. E., Ecker, J. R. and Reed, J. W. (2005). Auxin response factors ARF6 and ARF8 promote jasmonic acid production and flower maturation. **Development 132,** 4107-4118.

Nakata, M., Mitsuda, N., Herde, M., Koo, A., Moreno, J. E., Suzuki, K., Howe, G. A. and Ohme-Takagi, M. (2013). A bHLH-type transcription factor, ABA-INDUCIBLE BHLH-TYPE TRANSCRIPTION FACTOR/JA-ASSOCIATED MYC2-LIKE1, acts as a repressor to negatively regulate jasmonate signaling in Arabidopsis. **Plant Cell 25,** 1641-1656.

Nalam, V. J., Keereetaweep, J. and Shah, J. (2013). The green peach aphid, *Myzus persicae*, acquires a LIPOXYGENASE5-derived oxylipin from *Arabidopsis thaliana*, which promotes colonization of the host plant. **Plant Signal. Behav. 8,** e22735.

Nalam, V. J., Keeretaweep, J., Sarowar, S. and Shah, J. (2012). Root-derived oxylipins promote green peach aphid performance on Arabidopsis foliage. **Plant Cell 24,** 1643-1653.

Newton, A. C., Johnson, S. N. and Gregory, P. J. (2011). Implications of climate change for diseases, crop yields and food security. **Euphytica 179,** 3-18.

Nordborg, M., Hu, T. T., Ishino, Y., Jhaveri, J., Toomajian, C., Zheng, H., Bakker, E., Calabrese, P., Gladstone, J., Goyal, R., Jakobsson, M., Kim, S., Morozov, Y., Padhukasahasram, B., Plagnol, V., Rosenberg, N. A., Shah, C., Wall, J. D., Wang, J., Zhao, K., Kalbfleisch, T., Schulz, V., Kreitman, M. and Bergelson, J. (2005). The pattern of polymorphism in *Arabidopsis thaliana*. **PLoS Biol. 3,** e196.

Nordborg, M. and Weigel, D. (2008). Next-generation genetics in plants. **Nature 456,** 720-723.

O'Brien, J. A. and Benková, E. (2013). Cytokinin cross-talking during biotic and abiotic stress responses. **Front Plant Sci. 4,** 451.

Odong, T. L., Van Heerwaarden, J., Van Hintum, T. J. L., Van Eeuwijk, F. A. and Jansen, J. (2013). Improving hierarchical clustering of genotypic data via principal component analysis. **Crop Sci. 53,** 1546–1554.

Ogura, T. and Busch, W. (2015). From phenotypes to causal sequences: using genome wide association studies to dissect the sequence basis for variation of plant development. **Curr. Opin. Plant Biol. 23,** 98-108.

Osakabe, Y., Mizuno, S., Tanaka, H., Maruyama, K., Osakabe, K., Todaka, D., Fujita, Y., Kobayashi, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2010). Overproduction of the membrane-bound receptor-like protein kinase 1, RPK1, enhances abiotic stress tolerance in Arabidopsis. **J. Biol. Chem. 285,** 9190-9201.

Osbourn, A. (1996). Saponins and plant defence - a soap story. **Trends Plant Sci. 1,** 4-9.

Pandey, S. P., Srivastava, S., Goel, R., Lakhwani, D., Singh, P., Asif, M. H. and Sane, A. P. (2017). Simulated herbivory in chickpea causes rapid changes in defense pathways and hormonal transcription networks of JA/ethylene/GA/auxin within minutes of wounding. **Sci. Rep. 7,** 44729.

Pel, M. J. C. and Pieterse, C. M. J. (2013). Microbial recognition and evasion of host immunity. **J. Exp. Bot. 64,** 1237-1248.

Peleg, Z. and Blumwald, E. (2011). Hormone balance and abiotic stress tolerance in crop plants. **Curr. Opinion Plant Biol. 14,** 290-295.

Petek, M., Rotter, A., Kogovsek, P., Baebler, S., Mithofer, A. and Gruden, K. (2014). Potato virus Y infection hinders potato defence response and renders plants more vulnerable to Colorado potato beetle attack. **Mol. Ecol. 23,** 5378-5391.

Pham, J., Liu, J., Bennett, M. H., Mansfield, J. W. and Desikan, R. (2012). Arabidopsis histidine kinase 5 regulates salt sensitivity and resistance against bacterial and fungal infection. **New Phytol. 194,** 168-180.

Phan Tran, L.-S., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2010). Role of cytokinin responsive two-component system in ABA and osmotic stress signalings. **Plant Signal. Behav. 5,** 148-150.

Piepho, H.-P. (1997). Analyzing genotype-environment data by mixed models with multiplicative terms. **Biometrics 53,** 761-766.

Pierik, R. and Testerink, C. (2014). The art of being flexible: how to escape from shade, salt, and drought. **Plant Physiol. 166,** 5-22.

Pieterse, C. M. J. and Dicke, M. (2007). Plant interactions with microbes and insects: from molecular mechanisms to ecology. **Trends Plant Sci. 12,** 564-569.

Pieterse, C. M. J., Leon-Reyes, A., Van der Ent, S. and Van Wees, S. C. M. (2009). Networking by small-molecule hormones in plant immunity. **Nature Chem. Biol. 5,** 308-316.

Pieterse, C. M. J., Van der Does, D., Zamioudis, C., Leon-Reyes, A. and Wees, S. C. M. (2012). Hormonal modulation of plant immunity. **Annu. Rev. Cell Dev. Biol. 28,** 489-521.

Pieterse, C. M. J., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C. M. and Bakker, P. A. H. M. (2014). Induced systemic resistance by beneficial microbes. **Annu. Rev. Phytopathol. 52,** 347–375.

Pigliucci, M. (1998). Ecological and evolutionary genetics of Arabidopsis. **Trend. Plant Sci. 3,** 485-489.

Platt, A., Horton, M., Huang, Y. S., Li, Y., Anastasio, A. E., Mulyati, N., Agren, J., Bossdorf, O., Byers, D., Donohue, K., Dunning, M., Holub, E. B., Hudson, A., Corre, V., Loudet, O., Roux, F., Warthmann, N., Weigel, D., Rivero, L., Scholl, R., Nordborg, M., Bergelson, J. and Borevitz, J. O. (2010). The scale of population structure in *Arabidopsis thaliana*. **PLoS Genet. 6,** e1000843.

Poelman, E. H., Broekgaarden, C., Van Loon, J. J. A. and Dicke, M. (2008). Early season herbivore differentially affects plant defence responses to subsequently colonizing herbivores and their abundance in the field. **Mol. Ecol. 17,** 3352-3365.

Polanski, K., Rhodes, J., Hill, C., Zhang, P. J., Jenkins, D. J., Kiddle, S. J., Jironkin, A., Beynon, J., Buchanan-Wollaston, V., Ott, S. and Denby, K. J. (2014). Wigwams: identifying gene modules co-regulated across multiple biological conditions. **Bioinformatics 30,** 962-970.

Pombo, M., Rosli, H., Martínez, G. and Civello, P. (2011). UV-C treatment affects the expression and activity of defense genes in strawberry fruit (*Fragaria×ananassa*, Duch.). **Postharvest Biol. Technol. 59,** 94-102.

Prasch, C. M. and Sonnewald, U. (2013). Simultaneous application of heat, drought, and virus to Arabidopsis plants reveals significant shifts in signaling networks. **Plant Physiol. 162,** 1849-1866.

Qin, F., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2011). Achievements and challenges in understanding plant abiotic stress responses and tolerance. **Plant Cell Physiol. 52,** 1569-1582.

Qiu, Z., Guo, J., Zhu, A., Zhang, L. and Zhang, M. (2014). Exogenous jasmonic acid can enhance tolerance of wheat seedlings to salt stress. **Ecotoxicol. Environ. Saf. 104,** 202-208.

Ramegowda, V. and Senthil-Kumar, M. (2015). The interactive effects of simultaneous biotic and abiotic stresses on plants: mechanistic understanding from drought and pathogen combination. **J. Plant Physiol. 176,** 47-54.

Ramel, F., Sulmon, C., Bogard, M., Couee, I. and Gouesbet, G. (2009). Differential patterns of reactive oxygen species and antioxidative mechanisms during atrazine injury and sucrose-induced tolerance in *Arabidopsis thaliana* plantlets. **BMC Plant Biol. 9,** 28.

Ramírez, V., García-Andrade, J. and Vera, P. (2014). Enhanced disease resistance to *Botrytis cinerea* in *myb46* Arabidopsis plants is associated to an early down-regulation of *CesA* genes. **Plant Signal. Behav. 6,** 911-913.

Rampino, P., Mita, G., Fasano, P., Borrelli, G. M., Aprile, A., Dalessandro, G., De Bellis, L. and Perrotta, C. (2012). Novel durum wheat genes up-regulated in response to a combination of heat and drought stress. **Plant Physiol. Biochem. 56,** 72-78.

Rao, M. V., Lee, H.-I., Creelman, R. A., Mullet, J. E. and Davis, K. R. (2000). Jasmonic acid signaling modulates ozone-induced hypersensitive cell death. **Plant Cell 12,** 1633-1646.

Rasmann, S., Vos, M., Casteel, C. L., Tian, D., Halitschke, R., Sun, J. Y., Agrawal, A. A., Felton, G. W. and Jander, G. (2012). Herbivory in the previous generation primes plants for enhanced insect resistance. **Plant Physiol. 158,** 854-863.

Rasmussen, S., Barah, P., Suarez-Rodriguez, M. C., Bressendorff, S., Friis, P., Costantino, P., Bones, A. M., Nielsen, H. B. and Mundy, J. (2013). Transcriptome responses to combinations of stresses in Arabidopsis. **Plant Physiol. 161,** 1783-1794.

Rehrig, E. M., Appel, H. M., Jones, A. D. and Schultz, J. C. (2014). Roles for jasmonate- and ethylene-induced transcription factors in the ability of Arabidopsis to respond differentially to damage caused by two insect herbivores. **Front. Plant Sci. 5,** 407.

Rejeb, I. B., Pastor, V. and Mauch-Mani, B. (2014). Plant responses to simultaneous biotic and abiotic stress: molecular mechanisms. **Plants 3,** 458-475.

Ren, D., Liu, Y., Yang, K.-Y., Han, L., Mao, G., Glazebrook, J. and Zhang, S. (2008). A fungal-responsive MAPK cascade regulates phytoalexin biosynthesis in Arabidopsis. **Proc. Natl. Acad. Sci. 105,** 5638–5643.

Renwick, J. A. A. and Radke, C. D. (1988). Sensory cues in host selection for oviposition by the cabbage butterfly, *Pieris rapae*. **J. Insect Physiol. 34,** 251-257.

Reusche, M., Thole, K., Janz, D., Truskina, J., Rindfleisch, S., Drubert, C., Polle, A., Lipka, V. and Teichmann, T. (2012). Verticillium infection triggers VASCULAR-RELATED NAC DOMAIN7-dependent de novo xylem formation and enhances drought tolerance in Arabidopsis. **Plant Cell 24,** 3823-3837.

Reymond, P., Bodenhausen, N., Dicke, M. and Farmer, E. E. (2004). A conserved transcript pattern in response to a specialist and a

generalist herbivore. **Plant Cell 16,** 3132–3147.

Reymond, P. and Farmer, E. E. (1998). Jasmonate and salicylate as global signals for defense gene expression. **Curr. Opin. Plant Biol. 1,** 404-411.

Reymond, P., Weber, H., Damond, M. and Farmer, E. E. (2000). Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis. **Plant Cell 12,** 707–720.

Riera, M., Valon, C., Fenzi, F., Giraudat, J. and Leung, J. (2005). The genetics of adaptive responses to drought stress: abscisic acid-dependent and abscisic acid-independent signalling components. **Physiol. Plant. 123,** 111-119.

Rivals, I., Personnaz, L., Taing, L. and Potier, M. C. (2007). Enrichment or depletion of a GO category within a class of genes: which test? **Bioinformatics 23,** 401-407.

Rivero, R. M., Mestre, T. C., Mittler, R., Rubio, F., Garcia-Sanchez, F. and Martinez, V. (2014). The combined effect of salinity and heat reveals a specific physiological, biochemical and molecular response in tomato plants. **Plant Cell Environ. 37,** 1059-1073.

Rizhsky, L., Liang, H., Shuman, J., Shulaev, V., Davletova, S. and Mittler, R. (2004). When defense pathways collide. The response of Arabidopsis to a combination of drought and heat stress. **Plant Physiol. 134,** 1683-1696.

Robert-Seilaniantz, A., Grant, M. and Jones, J. D. G. (2011). Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. **Annu. Rev. Phytopathol. 49,** 317-343.

Rodriguez-Saona, C. R., Musser, R. O., Vogel, H., Hum-Musser, S. M. and Thaler, J. S. (2010). Molecular, biochemical, and organismal analyses of tomato plants simultaneously attacked by herbivores from two feeding guilds. **J. Chem. Ecol. 36,** 1043–1057.

Root, R. B. (1973). Organization of a plant-arthropod association in simple and diverse habitats: the faunaof collards (*Brassica oleracea*). **Ecological Monographs 43,** 95-124.

Root, R. B. and Kareiva, P. M. (1984). The search for resources by cabbage butterflies (*Pieris rapae*): ecological consequences and adaptive significance of markovian movements in a patchy environment. **Ecology 65,** 147-165.

Rossi, F. R., Gárriz, A., Marina, M., Romero, F. M., Gonzalez, M. E. E., Collado, I. G. and Pieckenstain, F. L. (2011). The sesquiterpene botrydial produced by *Botrytis cinerea* induces the hypersensitive response on plant tissues and its action is modulated by salicylic acid and jasmonic acid signaling. **Mol. Plant Microbe Interact. 24,** 888-896.

Rowe, H. and Kliebenstein, D. (2008). Complex genetics control natural variation in *Arabidopsis thaliana* resistance to *Botrytis cinerea*. **Genetics 180,** 2237-2250.

Rowe, H. C., Walley, J. W., Corwin, J., Chan, E. K. F., Dehesh, K. and Kliebenstein, D. J. (2010). Deficiencies in jasmonate-mediated plant defense reveal quantitative variation in *Botrytis cinerea* pathogenesis. **PLoS Pathogens 6,** e1000861.

Runyon, J. B., Mescher, M. C. and De Moraes, C. M. (2008). Parasitism by *Cuscuta pentagona* attenuates host plant defenses against insect herbivores. **Plant Physiol. 146,** 987-995.

Sailaja, B., Voleti, S., Subrahmanyam, D., Sarla, N., Prasanth, V., Bhadana, V. and Mangrauthia, S. (2014). Prediction and expression analysis of miRNAs associated with heat stress in *Oryza sativa*. **Rice Sci. 21,** 3-12.

Santino, A., Taurino, M., De Domenico, S., Bonsegna, S., Poltronieri, P., Pastor, V. and Flors, V. (2013). Jasmonate signaling in plant development and defense response to multiple (a)biotic stresses. **Plant Cell Rep. 32,** 1085-1098.

Saruhan, N., Saglam, A. and Kadioglu, A. (2012). Salicylic acid pretreatment induces drought tolerance and delays leaf rolling by inducing antioxidant systems in maize genotypes. **Acta Physiol. Plant. 34,** 97-106.

Scascighini, N., Mattiacci, L., D'Alessandro, M., Hern, A., Rott, A. S. and Dorn, S. (2005). New insights in analysing parasitoid attracting synomones: early volatile emission and use of stir bar sorptive extraction. **Chemoecology 15,** 97-104.

Schachtman, D. P. and Goodger, J. Q. D. (2008). Chemical root to shoot signaling under drought. **Trends Plant Sci. 13,** 281-287.

Schenke, D., Böttcher, C. and Scheel, D. (2011). Crosstalk between abiotic ultraviolet-B stress and biotic (flg22) stress signalling in Arabidopsis prevents flavonol accumulation in favor of pathogen defence compound production. **Plant, Cell Environm. 34,** 1849-1864.

Schenke, D., Cai, D. and Scheel, D. (2014). Suppression of UV-B stress responses by flg22 is regulated at the chromatin level via histone modification. **Plant, Cell Environm. 37,** 1716-1721.

Schlaeppi, K., Bodenhausen, N., Buchala, A., Mauch, F. and Reymond, P. (2008). The glutathione-deficient mutant *pad2-1* accumulates lower amounts of glucosinolates and is more susceptible to the insect herbivore *Spodoptera littoralis*. **Plant J. 55,** 774-786.

Schouten, A., Van Baarlen, P. and Van Kan, J. A. L. (2007). Phytotoxic Nep1-like proteins from the necrotrophic fungus *Botrytis cinerea* associate with membranes and the nucleus of plant cells. **New Phytol. 177,** 493–505.

Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., Masakazu, S., Akiyama, K., Taji, T., Yamaguchi-Shinozaki, K., Carninci, P., Kawai, J., Hayashizaki, Y. and Shinozaki, K. (2002). Monitoring the expression profiles

of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. **Plant J. 31,** 279-292.

Sekmen, A. H., Ozgur, R., Uzilday, B. and Turkan, I. (2014). Reactive oxygen species scavenging capacities of cotton (*Gossypium hirsutum*) cultivars under combined drought and heat induced oxidative stress. **Environ. Exp. Bot. 99,** 141-149.

Seo, P. and Park, C. M. (2010). MYB96-mediated abscisic acid signals induce pathogen resistance response by promoting salicylic acid biosynthesis in Arabidopsis. **New Phytol. 186,** 471-483.

Sewelam, N., Oshima, Y., Mitsuda, N. and Ohme-Takagi, M. (2014). A step towards understanding plant responses to multiple environmental stresses: a genome-wide study. **Plant Cell Environ. 37,** 2024-2035.

Shabab, M., Khan, S. A., Vogel, H., Heckel, D. G. and Boland, W. (2014). OPDA isomerase GST16 is involved in phytohormone detoxification and insect development. **FEBS J. 281,** 2769-2783.

Shaik, R. and Ramakrishna, W. (2013). Genes and co-expression modules common to drought and bacterial stress responses in Arabidopsis and rice. **PLoS ONE 8,** e77261.

Sham, A., Moustafa, K., Al-Ameri, S., Al-Azzawi, A., Iratni, R. and AbuQamar, S. (2015). Identification of Arabidopsis candidate genes in response to biotic and abiotic stresses using comparative microarrays. **PLoS ONE 10,** e0125666.

Shapiro, A. M. and De Vay, J. E. (1987). Hypersensitivity reaction of *Brassica nigra* L. (Cruciferae) kills eggs of Pieris butterflies (Lepidoptera: Pieridae). **Oecologia 71,** 631-632.

Shi, H., Wang, X., Ye, T., Cheng, F., Deng, J., Yang, P., Zhang, Y. and Chan, Z. (2014). The Cys2/His2-type zinc finger transcription factor *ZAT6* modulates biotic and abiotic stress responses by activating salicylic acid-related genes and *CBFs* in Arabidopsis. **Plant Physiol. 165,** 1367–1379.

Shin, D. H., Choi, M. G., Lee, H. K., Cho, M., Choi, S. B., Choi, G. and Park, Y. I. (2013). Calcium dependent sucrose uptake links sugar signaling to anthocyanin biosynthesis in Arabidopsis. **Biochem. Biophys. Res. Commun. 430,** 634-639.

Shinozaki, K., Yamaguchi-Shinozaki, K. and Seki, M. (2003). Regulatory network of gene expression in the drought and cold stress responses. **Curr. Opin. Plant Biol. 6,** 410-417.

Shinya, T., Yamaguchi, K., Desaki, Y., Yamada, K., Narisawa, T., Kobayashi, Y., Maeda, K., Suzuki, M., Tanimoto, T., Takeda, J., Nakashima, M., Funama, R., Narusaka, M., Narusaka, Y., Kaku, H., Kawasaki, T. and Shibuya, N. (2014). Selective regulation of the chitin-induced defense response by the Arabidopsis receptor-like cytoplasmic kinase PBL27. **Plant J. 79,** 56-66.

Sicher, R. C. and Barnaby, J. Y. (2012). Impact of carbon dioxide enrichment on the responses of maize leaf transcripts and metabolites to water stress. **Physiol. Plant. 144,** 238-253.

Smallegange, R. C., Everaarts, T. C. and Loon, J. A. (2006). Associative learning of visual and gustatory cues in the large cabbage white butterfly, *Pieris brassicae*. **Animal Biol. 56,** 157-172.

Soler, R., Erb, M. and Kaplan, I. (2013). Long distance root-shoot signalling in plant-insect community interactions. **Trends Plant Sci. 18,** 149-156.

Spiess, G. M., Hausman, A., Yu, P., Cohen, J. D., Rampey, R. A. and Zolman, B. K. (2014). Auxin input pathway disruptions are mitigated by changes in auxin biosynthetic gene expression in Arabidopsis. **Plant Physiol. 165,** 1092-1104.

Spoel, S. H., Johnson, J. S. and Dong, X. (2007). Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. **Proc. Natl. Acad. Sci. U.S.A. 104,** 18842-18847.

Staats, M. and Van Kan, J. A. L. (2012). Genome update of *Botrytis cinerea* strains B05.10 and T4. **Eukar. Cell 11,** 1413-1414.

Stam, J. M., Kroes, A., Li, Y., Gols, R., van Loon, J., Poelman, E. H. and Dicke, M. (2014). Plant interactions with multiple insect herbivores: from community to genes. **Annu. Rev. Plant Biol. 65,** 689-713.

Stefanato, F. L., Abou-Mansour, E., Buchala, A., Kretschmer, M., Mosbach, A., Hahn, M., Bochet, C. G., Métraux, J. P. and Schoonbeek, H. j. (2009). The ABC transporter BcatrB from *Botrytis cinerea* exports camalexin and is a virulence factor on *Arabidopsis thaliana*. **Plant J. 58,** 499-510.

Stracke, R., Werber, M. and Weisshaar, B. (2001). The *R2R3-MYB* gene family in *Arabidopsis thaliana*. **Curr. Opinion Plant Biol. 4,** 447-456.

Su, L. T., Li, J. W., Liu, D. Q., Zhai, Y., Zhang, H. J., Li, X. W., Zhang, Q. L., Wang, Y. and Wang, Q. Y. (2014). A novel MYB transcription factor, GmMYBJ1, from soybean confers drought and cold tolerance in *Arabidopsis thaliana*. **Gene 538,** 46-55.

Su, T., Xu, Q., Zhang, F. C., Chen, Y., Li, L. Q., Wu, W. H. and Chen, Y. F. (2015). WRKY42 modulates phosphate homeostasis through regulating phosphate translocation and acquisition in Arabidopsis. **Plant Physiol. 167,** 1579-1591.

Sun, C., Gao, X., Fu, J., Zhou, J. and Wu, X. (2015). Metabolic response of maize (*Zea mays* L.) plants to combined drought and salt stress. **Plant Soil 388,** 99–117.

Suzuki, N., Bassil, E., Hamilton, J. S., Inupakutika, M. A., Zandalinas, S. I., Tripathy, D., Luo, Y., Dion, E., Fukui, G., Kumazaki, A., Nakano, R., Rivero, R. M., Verbeck, G. F., Azad, R. K., Blumwald, E. and Mittler, R. (2016). ABA is required for plant acclimation to a combination of

salt and heat stress. **PLoS ONE 11,** e0147625.

Suzuki, N., Miller, G., Salazar, C., Mondal, H. A., Shulaev, E., Cortes, D. F., Shuman, J. L., Luo, X., Shah, J., Schlauch, K., Shulaev, V. and Mittler, R. (2013). Temporal-spatial interaction between reactive oxygen species and abscisic acid regulates rapid systemic acclimation in plants. **Plant Cell 25,** 3553-3569.

Suzuki, N., Rivero, R., Shulaev, V., Blumwald, E. and Mittler, R. (2014). Abiotic and biotic stress combinations. **New Phytol. 203,** 32-43.

Syvertsen, J. P. and Garcia-Sanchez, F. (2014). Multiple abiotic stresses occurring with salinity stress in citrus. **Environ. Exp. Bot. 103,** 128-137.

Teixeira, E. I., Fischer, G., Van Velthuizen, H., Walter, C. and Ewert, F. (2013). Global hot-spots of heat stress on agricultural crops due to climate change. **Agr. For. Meteorol. 170,** 206-215.

Thoen, M. P. M., Davila Olivas, N. H., Kloth, K. J., Coolen, S., Huang, P. P., Aarts, M. G. M., Bac-Molenaar, J. A., Bakker, J., Bouwmeester, H. J., Broekgaarden, C., Bucher, J., Busscher-Lange, J., Cheng, X., Fradin, E. F., Jongsma, M. A., Julkowska, M. M., Keurentjes, J. J. B., Ligterink, W., Pieterse, C. M. J., Ruyter-Spira, C., Smant, G., Testerink, C., Usadel, B., Loon, J. J. A., Van Pelt, J. A., Schaik, C. C., Wees, S. C. M., Visser, R. G. F., Voorrips, R., Vosman, B., Vreugdenhil, D., Warmerdam, S., Wiegers, G. L., Van Heerwaarden, J., Kruijer, W., Van Eeuwijk, F. A. and Dicke, M. (2017). Genetic architecture of plant stress resistance: multi-trait genome-wide association mapping. **New Phytol. 213,** 1346-1362.

Thomma, B. P., Eggermont, K., Penninckx, I. A., Mauch-Mani, B., Vogelsang, R., Cammue, B. P. and Broekaert, W. F. (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. **Proc. Natl. Acad. Sci. U.S.A. 95,** 15107-15111.

Thomma, B. P. H. J., Eggermont, K., Tierens, K. F. M. and Broekaert, W. F. (1999). Requirement of functional *ethylene-insensitive 2* gene for efficient resistance of Arabidopsis to infection by *Botrytis cinerea*. **Plant Physiol. 121,** 1093-1102.

Tiryaki, I. and Staswick, P. E. (2002). An Arabidopsis mutant defective in jasmonate response is allelic to the auxin-signaling mutant *axr1*. **Plant Physiol. 130,** 887-894.

Tjallingii, W. F. and Hogen Esch, T. (1993). Fine structure of aphid stylet routes in plant tissues in correlation with EPG-signals. **Physiol. Entomol. 18,** 317-328.

Todesco, M., Balasubramanian, S., Hu, T. T., Traw, B. M., Horton, M., Epple, P., Kuhns, C., Sureshkumar, S., Schwartz, C., Lanz, C., Laitinen, R. A. E., Huang, Y., Chory, J., Lipka, V., Borevitz, J. O., Dangl, J. L., Bergelson, J., Nordborg, M. and Weigel, D. (2010). Natural allelic variation underlying a major fitness trade-off in *Arabidopsis thaliana*. **Nature 465,** 632-636.

Ton, J., Pieterse, C. M. J. and Van Loon, L. C. (1999). Identification of a locus in Arabidopsis controlling both the expression of rhizobacteria-mediated induced systemic resistance (ISR) and basal resistance against *Pseudomonas syringae* pv. tomato. **Mol. Plant Microbe Interact. 12,** 911–918.

Torres-Vera, R., Garcia, J. M., Pozo, M. J. and Lopez-Raez, J. A. (2014). Do strigolactones contribute to plant defence? **Mol. Plant Pathol. 15,** 211-216.

Trapnell, C., Pachter, L. and Salzberg, S. L. (2009). TopHat: discovering splice junctions with RNA-Seq. **Bioinformatics 25,** 1105-1111.

Traynier, R. M. M. and Truscott, R. J. W. (1991). Potent natural egg-laying stimulant for cabbage butterfly *Pieris rapae*. **J. Chem. Ecol. 17,** 1371-1380.

Van Eeuwijk, F. A., Bink, M. C., Chenu, K. and Chapman, S. C. (2010). Detection and use of QTL for complex traits in multiple environments. **Curr. Opin. Plant Biol. 13,** 193-205.

Van Heerwaarden, J., Odong, T. L. and Van Eeuwijk, F. A. (2013). Maximizing genetic differentiation in core collections by PCA-based clustering of molecular marker data. **Theor. Appl. Genet. 126,** 763–772.

Van Heerwaardena, J., Hufforda, M. B. and Ross-Ibarra, J. (2012). Historical genomics of North American maize. **Proc. Natl. Acad. Sci. 109,** 12420–12425.

Van Kan, J. A. L. (2006). Licensed to kill: the lifestyle of a necrotrophic plant pathogen. **Trends Plant Sci. 11,** 247-253.

Van Oosten, V., Bodenhausen, N., Reymond, P., Pelt, J. A., Van Loon, L. C., Dicke, M. and Pieterse, C. M. J. (2008). Differential effectiveness of microbially induced resistance against herbivorous insects in Arabidopsis. **Mol. Plant Microbe Interact. 21,** 919-930.

Van Rooijen, R., Aarts, M. G. and Harbinson, J. (2015). Natural genetic variation for acclimation of photosynthetic light use efficiency to growth irradiance in Arabidopsis. **Plant Physiol. 167,** 1412-1429.

Van Verk, M. C., Bol, J. F. and Linthorst, H. J. M. (2011). WRKY transcription factors involved in activation of SA biosynthesis genes. **BMC Plant Biol. 11,** 89.

Van Verk, M. C., Hickman, R., Pieterse, C. M. J. and Van Wees, S. C. M. (2013). RNA-Seq: revelation of the messengers. **Trends Plant Sci. 18,** 175-179.

Van Wees, S. C. M., Van Pelt, J. A., Bakker, P. A. H. M. and Pieterse, C. M. J. (2013). Bioassays for assessing jasmonate-dependent defenses triggered by pathogens, herbivorous insects, or beneficial rhizobacteria. **Meth. Mol. Biol. 1011,** 35-49.

Vanderauwera, S., Zimmermann, P., Rombauts, S., Vandenabeele, S., Langebartels, C., Gruissem, W., Inze, D. and Van Breusegem, F. (2005). Genome-wide analysis of hydrogen peroxide-regulated gene expression in Arabidopsis reveals a high light-induced transcriptional cluster involved in anthocyanin biosynthesis. **Plant Physiol. 139,** 806-821.

Vashisht, D., Hesselink, A., Pierik, R., Ammerlaan, J. M., Bailey-Serres, J., Visser, E. J., Pedersen, O., van Zanten, M., Vreugdenhil, D., Jamar, D. C., Voesenek, L. A. and Sasidharan, R. (2011). Natural variation of submergence tolerance among *Arabidopsis thaliana* accessions. **New Phytol. 190,** 299-310.

Verhage, A., Van Wees, S. C. M. and Pieterse, C. M. J. (2010). Plant immunity: It's the hormones talking, but what do they say? **Plant Physiol. 154,** 536-540.

Verhage, A., Vlaardingerbroek, I., Raaymakers, C., Van Dam, N., Dicke, M., Van Wees, S. C. M. and Pieterse, C. M. J. (2011). Rewiring of the jasmonate signaling pathway in Arabidopsis during insect herbivory. **Front. Plant Sci. 2,** 47.

Verhagen, B., Trotel-Aziz, P., Couderchet, M., Höfte, M. and Aziz, A. (2010). *Pseudomonas* spp.-induced systemic resistance to *Botrytis cinerea* is associated with induction and priming of defence responses in grapevine. **J. Exp. Bot. 61,** 249-260.

Veronese, P., Nakagami, H., Bluhm, B., AbuQamar, S., Chen, X., Salmeron, J., Dietrich, R., Hirt, H. and Mengiste, T. (2006). The membrane-anchored BOTRYTIS-INDUCED KINASE 1 plays distinct roles in Arabidopsis resistance to necrotrophic and biotrophic pathogens. **Plant Cell 18,** 257-273.

Vile, D., Pervent, M., Belluau, M., Vasseur, F., Bresson, J., Muller, B., Granier, C. and Simonneau, T. (2012). Arabidopsis growth under prolonged high temperature and water deficit: independent or interactive effects? **Plant Cell Environ. 35,** 702-718.

Vos, I. A., Moritz, L., Pieterse, C. M. J. and Wees, S. C. M. (2015). Impact of hormonal crosstalk on plant resistance and fitness under multi-attacker conditions. **Front. Plant Sci. 6**.

Vos, I. A., Pieterse, C. M. J. and Wees, S. C. M. (2013a). Costs and benefits of hormone-regulated plant defences. **Plant Pathol. 62,** 43-55.

Vos, I. A., Verhage, A., Schuurink, R. C., Watt, L. G., Pieterse, C. M. J. and Wees, S. C. M. (2013b). Onset of herbivore-induced resistance in systemic tissue primed for jasmonate-dependent defenses is activated by abscisic acid. **Front. Plant Sci. 4,** 539.

Wang, C., Guo, L., Li, Y. and Wang, Z. (2012). Systematic comparison of C3 and C4 plants based on metabolic network analysis. **BMC Syst. Biol. 6(Suppl 2),** S9.

Wang, D., Pajerowska-Mukhtar, K., Culler, A. H. and Dong, X. (2007). Salicylic acid Inhibits pathogen growth in plants through repression of the auxin signaling pathway. **Curr. Biol. 17,** 1784-1790.

Wang, W. and Wang, Z.-Y. (2014). At the intersection of plant growth and immunity. **Cell Host Microbe 15,** 400-402.

Ward, E. W. B., Cahill, D. M. and Bhattacharyya, M. K. (1989). Influence of abscisic acid and the abscisic acid biosynthesis inhibitor, norflurazon, on interactions between *Phytophthora sojae* and soybean (*Glycine max*). **Plant Physiol. 91,** 23-27.

Wasternack, C. (2015). How jasmonates earned their laurels: past and present. **J. Plant Growth Regul. 34,** 761-794.

Wasternack, C. and Hause, B. (2013). Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. **Ann. Bot. 111,** 1021-1058.

Waterhouse, A. M., Procter, J. B., Martin, D. M. A., Clamp, M. and Barton, G. J. (2009). Jalview version 2-a multiple sequence alignment editor and analysis workbench. **Bioinformatics 25,** 1189-1191.

Waters, M. T., Gutjahr, C., Bennett, T. and Nelson, D. C. (2017). Strigolactone signaling and evolution. **Annu. Rev. Plant. Biol. 68,** 291-322.

Weiberg, A., Wang, M., Lin, F.-M., Zhao, H., Zhang, Z., Kaloshian, I., Huang, H.-D. and Jin, H. (2013). Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. **Science 342,** 118-123.

Weigel, D. and Mott, R. (2009). The 1001 genomes project for *Arabidopsis thaliana*. **Genome Biol. 10,** 107.

Wijnen, C. L. and Keurentjes, J. J. (2014). Genetic resources for quantitative trait analysis: novelty and efficiency in design from an Arabidopsis perspective. **Curr. Opin. Plant Biol. 18,** 103-109.

Willmann, R., Lajunen, H. M., Erbs, G., Newman, M.-A., Kolb, D., Tsuda, K., Katagiri, F., Fliegmann, J., Bono, J.-J., Cullimore, J. V., Jehle, A. K., Götz, F., Kulik, A., Molinaro, A., Lipka, V., Gust, A. A. and Nürnberger, T. (2011). Arabidopsis lysin-motif proteins LYM1 LYM3 CERK1 mediate bacterial peptidoglycan sensing and immunity to bacterial infection. **Proc. Natl. Acad. Sci. 108,** 19824–19829.

Windram, O., Madhou, P., McHattie, S., Hill, C., Hickman, R., Cooke, E., Jenkins, D. J., Penfold, C. A., Baxter, L., Breeze, E., Kiddle, S. J., Rhodes, J., Atwell, S., Kliebenstein, D. J., Kim, Y. s., Stegle, O., Borgwardt, K., Zhang, C., Tabrett, A., Legaie, R., Moore, J., Finkenstadt, B., Wild, D. L., Mead, A., Rand, D., Beynon, J., Ott, S., Buchanan-Wollaston, V. and Denby, K. J. (2012). Arabidopsis defense against *Botrytis cinerea*: chronology and regulation deciphered by high-resolution temporal transcriptomic analysis. **Plant Cell 24,** 3530-3557.

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G. V. and Provart, N. J. (2007). An "electronic fluorescent pictograph" browser for exploring and analyzing large-scale biological data sets. **PLoS One 2,** e718.

Yamada, K., Yamaguchi, K., Shirakawa, T., Nakagami, H., Mine, A., Ishikawa, K., Fujiwara, M., Narusaka, M., Narusaka, Y., Ichimura, K., Kobayashi, Y., Matsui, H., Nomura, Y., Nomoto, M., Tada, Y., Fukao, Y., Fukamizo, T., Tsuda, K., Shirasu, K., Shibuya, N. and Kawasaki, T. (2016). The Arabidopsis CERK1-associated kinase PBL27 connects chitin perception to MAPK activation. **EMBO J. 35,** 2468-2483.

Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006). Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. **Annu. Rev. Plant Biol. 57,** 781-803.

Yamaguchi, M., Mitsuda, N., Ohtani, M., Ohme-Takagi, M., Kato, K. and Demura, T. (2011). VASCULAR-RELATED NAC-DOMAIN7 directly regulates the expression of a broad range of genes for xylem vessel formation. **Plant J. 66,** 579-590.

Yamaguchi, Y., Huffaker, A., Bryan, A. C., Tax, F. E. and Ryan, C. A. (2010). PEPR2 is a second receptor for the Pep1 and Pep2 peptides and contributes to defense responses in Arabidopsis. **Plant Cell 22,** 508-522.

Yang, D. L., Yao, J., Mei, C. S., Tong, X. H., Zeng, L. J., Li, Q., Xiao, L. T., Sun, T. P., Li, J. G., Deng, X. W., Lee, C. M., Thomashow, M. F., Yang, Y. N., He, Z. H. and He, S. Y. (2012). Plant hormone jasmonate prioritizes defense over growth by interfering with gibberellin signaling cascade. **Proc. Natl. Acad. Sci. U.S.A. 109,** E1192-E1200.

Yang, T., Chaudhuri, S., Yang, L., Du, L. and Poovaiah, B. W. (2010). A calcium/calmodulin-regulated member of the receptor-like kinase family confers cold tolerance in plants. **J. Biol. Chem. 285,** 7119-7126.

Yanhui, C., Xiaoyuan, Y., Kun, H., Meihua, L., Jigang, L., Zhaofeng, G., Zhiqiang, L., Yunfei, Z., Xiaoxiao, W., Xiaoming, Q., Yunping, S., Li, Z., Xiaohui, D., Jingchu, L., Xing-Wang, D., Zhangliang, C., Hongya, G. and Li-Jia, Q. (2006). The MYB transcription factor superfamily of Arabidopsis: expression analysis and phylogenetic comparison with the rice MYB family. **Plant Mol. Biol. 60,** 107-124.

Yasuda, M., Ishikawa, A., Jikumaru, Y., Seki, M., Umezawa, T., Asami, T., Maruyama-Nakashita, A., Kudo, T., Shinozaki, K., Yoshida, S. and Nakashita, H. (2008). Antagonistic interaction between systemic acquired resistance and the abscisic acid-mediated abiotic stress response in Arabidopsis. **Plant Cell 20,** 1678-1692.

Yu, A., Lepere, G., Jay, F., Wang, J., Bapaume, L., Wang, Y., Abraham, A. L., Penterman, J., Fischer, R. L., Voinnet, O. and Navarro, L. (2013). Dynamics and biological relevance of DNA demethylation in Arabidopsis antibacterial defense. **Proc. Natl. Acad. Sci. U.S.A. 110,** 2389-2394.

Yu, H., Chen, X., Hong, Y.-Y., Wang, Y., Xu, P., Ke, S.-D., Liu, H.-Y., Zhu, J.-K., Oliver, D. J. and Xiang, C.-B. (2008). Activated expression of an Arabidopsis HD-START protein confers drought tolerance with improved root system and reduced stomatal density. **Plant Cell 20,** 1134–1151.

Yuan, H. M., Liu, W. C. and Lu, Y. T. (2017). CATALASE2 coordinates SA-mediated repression of both auxin accumulation and JA biosynthesis in plant defenses. **Cell Host Microbe 21,** 143-155.

Zhang, B., Hua, Y., Wang, J., Huo, Y., Shimono, M., Day, B. and Ma, Q. (2017). TaADF4, an actin-depolymerizing factor from wheat, is required for resistance to the stripe rust pathogen *Puccinia striiformis* f. sp. *tritici*. **Plant J. 89,** 1210-1224.

Zhang, B., Xie, D. and Jin, Z. (2012). Global analysis of non-coding small RNAs in Arabidopsis in response to jasmonate treatment by deep sequencing technology. **J. Integr. Plant Biol. 54,** 73-86.

Zhang, P. J., Broekgaarden, C., Zheng, S. J., Snoeren, T. A. L., van Loon, J. J. A., Gols, R. and Dicke, M. (2013). Jasmonate and ethylene signaling mediate whitefly-induced interference with indirect plant defense in *Arabidopsis thaliana*. **New Phytol. 197,** 1291-1299.

Zhang, S., Cai, Z. and Wang, X. (2009). The primary signaling outputs of brassinosteroids are regulated by abscisic acid signaling. **Proc. Natl. Acad. Sci. 106,** 4543–4548.

Zhang, Y., Xu, S., Ding, P., Wang, D., Cheng, Y., He, J., Gao, M., Xu, F., Li, Y., Zhu, Z., Li, X. and Zhang, Y. (2010a). Control of salicylic acid synthesis and systemic acquired resistance by two members of a plant-specific family of transcription factors. **Proc. Natl. Acad. Sci. 107,** 18220-18225.

Zhang, Y., Yang, Y., Fang, B., Gannon, P., Ding, P., Li, X. and Zhang, Y. (2010b). Arabidopsis *snc2-1D* activates receptor-like protein-mediated immunity transduced through WRKY70. **Plant Cell 22,** 3153-3163.

Zhang, Z., Ersoz, E., Lai, C. Q., Todhunter, R. J., Tiwari, H. K., Gore, M. A., Bradbury, P. J., Yu, J., Arnett, D. K., Ordovas, J. M. and Buckler, E. S. (2010c). Mixed linear model approach adapted for genome-wide association studies. **Nat. Genet. 42,** 355-360.

Zheng, S.-J., Snoeren, T. A. L., Hogewoning, S. W., van Loon, J. J. A. and Dicke, M. (2010). Disruption of plant carotenoid biosynthesis through virus-induced gene silencing affects oviposition behaviour of the butterfly *Pieris rapae*. **New Phytol. 186,** 733-745.

Zheng, X.-Y., Spivey, N., Zeng, W., Liu, P.-P., Fu, Z., Klessig, D. F., He, S. and Dong, X. (2012). Coronatine promotes *Pseudomonas syringae* virulence in plants by activating a signaling cascade that inhibits salicylic acid accumulation. **Cell Host Microbe 11,** 587-596.

Zhou, X. and Stephens, M. (2014). Efficient multivariate linear mixed model algorithms for genome-wide association studies. **Nat. Methods 11,** 407-409.

Zhu, J. K. (2002). Salt and drought stress signal transduction in plants. **Annu. Rev. Plant Biol. 53,** 247-273.

Zhu, X., Feng, T., Tayo, B. O., Liang, J., Young, J. H., Franceschini, N., Smith, J. A., Yanek, L. R., Sun, Y. V., Edwards, T. L., Chen, W., Nalls, M., Fox, E., Sale, M., Bottinger, E., Rotimi, C., Consortium, C. B., Liu, Y., McKnight, B., Liu, K., Arnett, D. K., Chakravati, A., Cooper, R. S. and Redline, S. (2015). Meta-analysis of correlated traits via summary statistics from GWASs with an application in hypertension. **Am. J. Hum. Genet. 96,** 21-36.

Zhu, Z., Xu, F., Zhang, Y., Cheng, Y., Wiermer, M., Li, X. and Zhang, Y. (2010). Arabidopsis resistance protein SNC1 activates immune responses through association with a transcriptional corepressor. **Proc. Natl. Acad. Sci. 107,** 13960-13965.

Zinta, G., AbdElgawad, H., Domagalska, M. A., Vergauwen, L., Knapen, D., Nijs, I., Janssens, I. A., Beemster, G. and Asard, H. (2014). Physiological, biochemical, and genome-wide transcriptional analysis reveals that elevated CO_2 mitigates the impact of combined heat wave and drought stress in *Arabidopsis thaliana* at multiple organizational levels. **Glob. Change Biol. 20,** 3670-3685.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J. D. G., Boller, T. and Felix, G. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. **Cell 125,** 749-760.

Summary

In nature plants are often exposed to a multitude of biotic and abiotic stress factors (e.g. drought, herbivory and infections) that occur sequentially or simultaneously. To withstand these stresses plants have evolved defense mechanisms that allow them to cope with diverse stresses. However, still little is known about how these adaptive mechanisms cooperate to maximize plant survival under multi-stress conditions.

Our research reveals that plants are well-capable of adjusting their defense strategy when encountering multiple stresses. To investigate how plants cope with multi-stress conditions, we analyzed the dynamics of whole-transcriptome profiles of *Arabidopsis thaliana* exposed to three individual and six sequential double stresses inflicted by combinations of: (i) infection by the necrotrophic fungus *Botrytis cinerea*, (ii) herbivory by caterpillars of *Pieris rapae*, and (iii) drought stress. Each of these stresses induced specific expression profiles over time, in which one-third of all differentially expressed genes was shared by at least two single stresses. Still, a first stress encounter leaves a transcriptional stress mark that can adversely affect plant defenses to a second stress event, indicating that plant multi-stress interactions are affected by simultaneous or sequential stresses.

Aside from a flexible defense response, we also find natural variation in globally collected *A. thaliana* plants for their resistance to *B. cinerea* as individual stress or when preceded by drought stress or herbivory by *P. rapae*. In search for the genetics underlying the variation in plant susceptibility, we found genes that are potentially involved in plant adaptive responses to the applied sequential stresses. Genes with both known and putative roles in defense against each of the applied stresses were identified. In a subsequent study, we compared the tolerance of plants to diverse (a)biotic stresses and sequential stress combinations in order to find genes involved in plant multi-stress resistance. Plants that are resistant to multiple stresses are well-adapted to grow in diverse environmental settings and can therefore ensure efficient and sustainable food production in the future. With a genetic study we revealed genes involved in one or more (a)biotic stresses. Validation of one of the identified genes revealed its involvement in tolerance to multiple stresses, indicating that with our approach it is possible to find genes involved in plant multi-stress tolerance.

To minimalize crop damage, prevention of stress encounters is another approach of reducing crop losses instead of looking for plant adaptive responses that allow plants to cope with multiple stresses. For instance, by preventing egg-deposition by butterflies, subsequent herbivory by their offspring is avoided. In search of plant traits that are unfavorable for *P. rapae*, we studied plant selection for egg-deposition on naturally occurring *A. thaliana* plants. We observed variation in egg-deposition that was partially influenced by daylight, the experimental cage setup, plant size, spontaneous chlorosis and necrosis and the presence of secondary metabolites. A genetic study revealed genes of which one gene was shown to significantly associate with plant defense against caterpillars, jasmonic acid induced defenses. Butterflies might be capable of detecting differences in jasmonic acid defenses and select host plants that will benefit their offspring the best. Future functional analysis of the JA biosynthesis gene and the other identified candidate genes will be required to reveal their role in host selection for oviposition by *P. rapae*.

In sum, this research has shown that plants have an incredible ability to adapt to and integrate diverse stress signals into an effective defense response. Knowledge on these natural adaptive capabilities and the natural genetic variation hereof forms a valuable basis for the sustainable development of stress resilient future crops.

Samenvatting

In de natuur ervaren planten regelmatig verschillende biotische en abiotische stressen, zoals droogte, vraat en infecties, zowel tegelijkertijd als opeenvolgend. Om deze stressen te kunnen weerstaan hebben planten gedurende de evolutie mechanismen ontwikkeld die hen in staat stellen om met deze verschillende bedreigingen om te gaan. Er is echter nog weinig bekend over het aanpassingsvermogen van planten gedurende combinaties van verschillende stressfactoren en hoe planten deze mechanismen inzetten om de kans op overleving te vergroten.

Uit ons onderzoek komt naar voren dat planten in staat zijn om hun afweermechanismen snel aan te passen wanneer er zich twee opeenvolgende stressfactoren aandienen. Dit hebben we onderzocht door naar de reactie van de zandraket (*Arabidopsis thaliana*) plant te kijken na blootstelling aan drie individuele stressen en zes verschillende opeenvolgende dubbele stress combinaties: (i) infectie met de necrotrofe schimmel *Botrytis cinerea*, (ii) vraat door rupsen van het kleine koolwitje *Pieris rapae*, en (iii) droogte stress. Door in de tijd naar het expressiepatroon van alle Arabidopsis genen te kijken zagen we dat elke afzonderlijke stress een specifiek genexpressiepatroon in de plant in gang zet om zich specifiek te wapenen tegen de desbetreffende stressfactor. Indien een eerste stress werd opgevolgd door een tweede stress, bleek de plant snel over te schakelen naar het genexpressiepatroon van de laatste stressfactor. De signatuur van de eerste stress bleef echter wel zichtbaar wat mogelijk de adaptatie aan de tweede stress kan beïnvloeden.

Naast deze flexibiliteit in stressadaptatie is er natuurlijke genetische variatie in het vermogen van een plant om zich aan te passen aan één of meer stressfactoren. Om inzicht te krijgen in hoe planten zich aan kunnen passen aan opeenvolgende stressen hebben we de natuurlijke genetische variatie die in Arabidopsis aanwezig is onderzocht met een techniek die 'genome-wide association (GWA) mapping' wordt genoemd. Hiertoe hebben we 346 genetisch verschillende ecotypen van Arabidopsis geïnfecteerd met de schimmel *B. cinerea*, al dan niet na een periode van droogtestress of insectenvraat. Vervolgens hebben we in de enkele en dubbele stress combinaties het niveau van resistentie tegen *B. cinerea* bepaald. M.b.v. GWA mapping hebben we vervolgens Arabidopsis genen geïdentificeerd die mogelijk een rol spelen in de afweerreactie van de plant tegen deze ziekteverwekker in combinatie met droogtestress of insectenvraat. In een hierop volgende studie hebben we de gevoeligheid van planten voor diverse biotische en abiotische stressen met elkaar vergeleken met als doel genen te vinden die planten ongevoelig maken voor meerdere stressfactoren. Planten die ongevoelig zijn voor diverse stressfactoren zijn immers breed inzetbaar in diverse omgevingen en kunnen een duurzame en efficiënte voedselproductie in de toekomst waarborgen. Door middel van een genetische analyse vonden we genen die mogelijk betrokken zijn bij plantenafweer tegen diverse stressen. De functionele validatie van één van de geïdentificeerde genen bevestigde inderdaad de betrokkenheid bij planttolerantie voor meerdere stressen.

Om schade aan gewassen te minimaliseren kan er, naast te zoeken naar planten die goed met stress kunnen omgaan, ook gezocht worden naar plant-eigenschappen die schade kunnen voorkomen. Vraatschade door rupsen kan wellicht voorkomen worden door plant-eigenschappen die onaantrekkelijk zijn voor ei-afzetting door vlinders. In onze zoektocht naar deze plant-eigenschappen

hebben we gekeken naar plantselectie voor ei-afzetting door *P. rapae* vlinders op de set van 350 natuurlijk-voorkomende Arabidopsis ecotypen. We zagen variatie in ei-afzetting op de verschillende ecotypen welke onder meer beïnvloed werd door daglicht, de vlinderkooi, plant grootte, spontane vergeling en necrose en de aanwezigheid van secundaire metabolieten. GWA mapping onthulde genen waarvan één gen significant geassocieerd was met jasmonzuur-geïnduceerde afweer tegen rupsen. Mogelijk kunnen vlinders verschillen in jasmonzuur-afweer detecteren en zo planten met de meest gunstige eigenschappen voor hun nakomelingen selecteren om hun eitjes op te leggen. Een functionele analyse van het jasmonzuur gen en de andere potentiële genen zal in de toekomst moeten uitwijzen wat hun rol is in plantselectie door vlinders.

Dit onderzoek heeft laten zien dat planten over een indrukwekkend aanpassingsvermogen beschikken dat hen in staat stelt verschillende stress-signalen te integreren in een effectieve afweerrespons. Kennis over deze natuurlijke eigenschappen en natuurlijke genetische variatie in het adaptatievermogen van planten aan combinaties van stressen vormen een waardevolle basis voor de ontwikkeling van weerbare gewassen en duurzame voedselproductie in de toekomst.

Acknowledgements

This is it, the completion of my thesis….. finally! It has been a beautiful journey in which I learned so much and became an independent and passionate researcher. As in every learning process there are ups and downs and although it was far from a straight forward and easy project, I would do it all over again without any doubt. Of course I would not have been able to do this without the help and support of others that I received along the way.

First of all I would like to thank Corné Pieterse and Saskia van Wees, my promotor and co-promotor, for giving me the opportunity to work and learn under their supervision. Thank you for your patience and for giving me the freedom to find my own path. Corné, I really appreciate the way you taught me how to be independent and critical. You always knew how to guide me in the right direction without giving me too much information about the answers. Furthermore, I really admire the enthusiasm with which you know how to motivate everyone. Saskia, my daily supervisor. We didn't see each other that often, but when we had a meeting I was sure that it would take a couple of hours. I really appreciate the time you took to make sure that we thought of everything and explaining me your hypotheses. Besides being a supervisor to many, you make everyone laugh. I always knew when you were around because we could hear you joking and laughing throughout the whole corridor.

Secondly, I would like to thank the whole Plant-Microbe Interactions group for creating a superb atmosphere where I felt at home. Thank you all for the good times, the nice discussions and for creating an inspiring environment. Although my project was very different from most of the work within the PMI group you all contributed to the completion of my thesis. I would like to thank a few, or actually many, colleagues in special. Christos Zamioudis, thank you for being there when I needed advice. Although you repeatedly joked to me to quit when I still could, I eventually finalized my thesis. Also a special thanks to Dmitry Lapin. I don't know all the details about all the weird things you did during Thursday evening drinks, but the vibrant stories on your 'frikandellen' record lived on for a long time. With your help I was able to perform fine mapping and to get my first GWAS running, that took my computer more than a day to complete. Also the 'labuitje' that we planned together was a blast, going to a farm, making cheese and milking a cow. Also a special thanks goes to Ientse van der Sluis. You were the basis of our Arabidopsis seed collection and a great companion during coffee breaks and lunches at the greenhouse. Your choice of words was often Chinese to my ears, which you really enjoyed of course. I would also like to thank Marcel van Verk and Richard Hickman for their help and support with the RNA-Seq data. Both of you are the experts when it comes to complicated bioinformatics and large datasets. Also Silvia Proietti, thank you for joining me on the combined stress project and supporting me with getting our first work published. I hope you can continue working on your promising combined stress project in Italy that was derived from the work that we started in Utrecht. Of course also a warm thank you to Anja van Dijk, the stable factor when it comes to molecular work. Thank you so much for helping me with finalizing some crucial PCRs. Also a big thank you to all the students that joined me in my work. Thank you Mirjam de Vries, Rutger Baar, Iko Koevoets, Bregje Loverbos, Maartje de Graaf, Sjon Hartman, Roderick Bouwman, Jason Banda and Tom Broere for all your help with the work on combined stresses and the oviposition work. I would also like to thank the people of the Botanical Garden at the Utrecht University campus for

the great times. I specially want to thank Fred Siesling for all his help and support in the greenhouse.

A warm thank you for the lovely years to all the LFN combined stress team members. Thank you Marcel Dicke, Mark Aarts and Joop van Loon for your support and input on the project and critical discussions. Marcel, the driving force behind the LFN project, thank you for all your input and hard work on the LFN paper. Mark, thank you and yes you were right. It is indeed better to test the whole population of accessions at once instead of cutting it up into pieces and trying to correct for in-between experiment effects afterwards. I will never make that mistake again. Joop van Loon, thank you for being such a pleasant contact for our oviposition work, for the communications on *P. rapae* rearing together with Leon Westerd and for the collaboration with Ana Pineda and Nina Fatouros on oviposition research.

I would also like to thank the whole LFN consortium and the people form the Wageningen University , my second home, for all their input, nice discussions and fruitful collaborations. A special thanks goes out to the LFN core team and the supervisors that wrote the paper on our joined meta-analysis that is finally published in The New Phytologist. Thank you Willem Kruijer for taking a big part in the analysis. Although you were sometimes hard to follow for us as biologists, you were the driving force of the analysis together with Joost van Heerwaarden and Fred van Eeuwijk. Of course also a warm thank you for everything to Manus Thoen and Karen Kloth. Both of you are very determined and hardworking researchers and were an essential part of the core team. I would also like to thank the company representatives Marcel Prins (Keygene), Paul Passarinho (Genetwister technologies) and Sjoukje Heimovara (Royal van Zanten) for their input during our meetings and Alexander Wittenberg and Martin De Vos (Keygene) for performing RNA sequencing for us.

And then my companions, Pingping Huang and Nelson Davila Olivas. We had some rough times during our project. We have seen gray hairs growing from frustration and deep dark circles under our eyes. Yet we made it and can all be very proud of what we have accomplished. Both of you even managed to finish your thesis's before I did, well done. We had many meetings together, many discussions and even slept at the greenhouse for our big RNA-Seq experiment. I wish both of you all the best in life and in your careers and hope we will occasionally meet up again.

Last and certainly not least I would like to thank my family and friends for all their support. Hans van Pelt, my partner in crime and my partner. We were already really good friends from the time I was still an undergraduate student. After tough and emotional changes to both our lives we found support in each other and decided to make a rather drastic but beautiful turn. Although not directly accepted by some we eventually made it to our dream house, a small farm in the countryside of 'de Achterhoek'. There we live our lives with our animal family and spend our time working and enjoying our farm life. Also a big thank you to my sister Madalena, you always know how to cheer me up, often resulting in unstoppable laughter's. Also a warm thank you to Ale Ockhuijsen, my stepdad, and my best friend Sabine Kwaks for always being there for me. And then the most important person in my life, my mother, Maria Eduarda da Conçeicao Brites. I dedicated this booklet to you because you are the most kind, loving, caring, motivating and strong person I have ever known. It is because

of you that I have made it this far. It is because of you that I am able to fight my way through hard times and have managed to become the scientist that I always dreamed of. I hope that I can continue to make you proud and hope that my journey may inspire others to fight for their dreams.

- Silvia Coolen –

About the author

Curriculum vitae

Silvia Coolen was born on 8 November 1985 in Deurne, the Netherlands. In 2002 she left the St. Aloysius MAVO in Hilversum with a diploma after which she obtained her HAVO diploma in 2004 at the A. Roland Host college in Hilversum. In 2006 she obtained her propedeuse in biology and medical laboratory research at the Hogeschool Utrecht. During her bachelor study she was a trainee for 6 months at the Sanquin blood bank in Amsterdam in the Immunoglobulins research group of prof. dr. Rob Aalberse. Under supervision of Ninotska Derksen and Theresa Guhr, she studied how antibodies derived from donor blood can be effective in treatment against rheumatic arthritis. Her second internship was a 9 month research project at the Academic Medical Centre (AMC) in Amsterdam in the research group Protein Degradation and Aggregation of dr. Eric Reits. Under supervision of Judith Gillis, she studied how poly-glutamine aggregation, as in Huntington's disorder, disrupts normal cell functioning. After her internship she prolonged her stay at the AMC as research technician until her new challenge started. In 2008 she received her bachelor's diploma in biology and medical laboratory research with a specialization in medical molecular biology. Subsequently she applied for a master study in plant biology at the Utrecht University and was a trainee in the Plant-Microbe Interactions group of prof. dr. ir. Corné Pieterse for 9 months. Under supervision of dr. Sjoerd van der Ent she studied the bacterial enzyme alkaline protease A (AprA) that breaks down bacterial flagellin and thereby prevents plants from recognizing flagellin containing bacteria. During her internship she was a student assistant for the bachelor course 'Microbial interactions' under supervision of dr. Peter Bakker of the Plant-Microbe Interactions group. In 2009 she worked as a trainee in a 6 month project at the University Medical Center (UMC) Utrecht in a collaboration between the Plant-Microbe Interactions group of the Utrecht University and the Immune Evasion group of prof. dr. Jos van Strijp. Here she continued to study the bacterial AprA enzyme and studied how modifications of the bacterial flagellin molecule affect recognition by human cells and plants. She wrote her master's thesis on prevention of host immunity by pathogenic and non-pathogenic pseudomonad bacteria under supervision of prof. dr. ir. Corné Pieterse and received her master's diploma with the adjudication cum laude in 2010. In October of 2010 she started as PhD student at the Plant-Microbe Interactions group under supervision of prof. dr. ir. Corné Pieterse and dr. Saskia van Wees. The results of her PhD research are presented in this dissertation. After her PhD project she worked as a researcher in seed physiology, phytopathology and genetics at Schoneveld Breeding for almost two years. Currently, she has a position in tree and fruit research at Proeftuin Randwijk of Wageningen University & Research.

List of publications

Judith Gillis, Sabine Schipper-Krom, Katrin Juenemann, Anna Gruber, **Silvia Coolen**, Rian Van den Nieuwendijk, Henk Van Veen, Hermen Overkleeft, Joachim Goedhart, Harm H. Kampinga and Eric A. Reits (2013). The DNAJB6 and DNAJB8 protein chaperones prevent intracellular a of polyglutamine peptides. ***The Journal of Biological Chemistry 288***, 17225-17237

Silvia Coolen, Silvia Proietti, Richard Hickman, Nelson H. Davila Olivas, Ping-Ping Huang, Marcel C. Van Verk, Johan A. Van Pelt, Alexander H.J. Wittenberg, Martin De Vos, Marcel Prins, Joop J.A. Van Loon, Mark G.M. Aarts, Marcel Dicke, Corné M.J. Pieterse and Saskia C.M. Van Wees (2016). Transcriptome dynamics of Arabidopsis during sequential biotic and abiotic stresses. ***The Plant Journal 86***, 249–267

Manus P.M. Thoen, Nelson H. Davila Olivas, Karen J. Kloth, **Silvia Coolen**, Ping-Ping Huang, Mark G.M. Aarts, Johanna A. Bac-Molenaar, Jaap Bakker, Harro J. Bouwmeester, Colette Broekgaarden, Johan Bucher, Jacqueline Busscher-Lange, Xi Cheng, Emilie F. Fradin, Maarten A. Jongsma, Magdalena M. Julkowska, Joost J.B. Keurentjes, Wilco Ligterink, Corné M.J. Pieterse, Carolien Ruyter-Spira, Geert Smant, Christa Testerink, Björn Usadel, Joop J.A. Van Loon, Johan A. Van Pelt, Casper C. Schaik, Saskia C.M. Van Wees, Richard G.F. Visser, Roeland Voorrips, Ben Vosman, Dick Vreugdenhil, Sonja Warmerdam, Gerrie L. Wiegers, Joost Van Heerwaarden, Willem Kruijer, Fred A. Van Eeuwijk, Marcel Dicke (2016). Genetic architecture of plant stress resistance: multi-trait genome-wide association mapping. ***New Phytologist 213***, 1346–1362

Nelson H. Davila Olivas, **Silvia Coolen**, Pingping Huang, Edouard Severing, Marcel C. Van Verk, Richard Hickman, Alexander H.J. Wittenberg, Martin De Vos, Marcel Prins, Joop J.A. Van Loon, Mark G.M. Aarts, Saskia C.M. Van Wees, Corné M.J. Pieterse, Marcel Dicke (2016). Effect of prior drought and pathogen stress on Arabidopsis transcriptome changes to caterpillar herbivory. ***New Phytologist 210***, 1344–1356

Silvia Coolen, Johan. A. Van Pelt, Saskia C. M Van Wees and Corné M. J. Pieterse. Mining the natural genetic variation in *Arabidopsis thaliana* for adaptation to sequential abiotic and biotic stresses. ***Submitted to Plant, Cell & Environment***

Silvia Proietti, Lotte Caarls, **Silvia Coolen**, Johan A. Van Pelt, Saskia C.M. Van Wees and Corné M.J. Pieterse. Genome-wide association study reveals novel players in defense hormone crosstalk in Arabidopsis. ***Submitted to Plant, Cell & Environment***

Silvia Coolen, Johan. A. Van Pelt, Saskia C. M Van Wees and Corné M. J. Pieterse. Natural genetic variation in *Arabidopsis thaliana* for oviposition preference by the small cabbage white butterfly *Pieris rapae*. ***In prep.***