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Multiple levels of crosstalk in hormone networks regulating plant defense

Niels Aerts, Marciel Pereira Mendes and Saskia C. M. Van Wees*

Plant-Microbe Interactions, Department of Biology, Science4Life, Utrecht University, P.O. Box 800.56, Utrecht 3408 TB, The Netherlands

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*For correspondence (e-mail s.vanwees@uu.nl).

SUMMARY

Plant hormones are essential for regulating the interactions between plants and their complex biotic and abiotic environments. Each hormone initiates a specific molecular pathway and these different hormone pathways are integrated in a complex network of synergistic, antagonistic and additive interactions. This inter-pathway communication is called hormone crosstalk. By influencing the immune network topology, hormone crosstalk is essential for tailoring plant responses to diverse microbes and insects in diverse environmental and internal contexts. Crosstalk provides robustness to the immune system but also drives specificity of induced defense responses against the plethora of biotic interactors. Recent advances in dry-lab and wet-lab techniques have greatly enhanced our understanding of the broad-scale effects of hormone crosstalk on immune network functioning and have revealed underlying principles of crosstalk mechanisms. Molecular studies have demonstrated that hormone crosstalk is modulated at multiple levels of regulation, such as by affecting protein stability, gene transcription and hormone homeostasis. These new insights into hormone crosstalk regulation of plant defense are reviewed here, with a focus on crosstalk acting on the jasmonic acid pathway in *Arabidopsis thaliana*, highlighting the transcription factors MYC2 and ORA59 as major targets for modulation by other hormones.

Keywords: hormone crosstalk, defense, network, jasmonic acid, salicylic acid, abscisic acid, ethylene, MYC2, ORA59.

INTRODUCTION

Plants in nature and agriculture are constantly interacting with their biotic and abiotic environment. To ensure their survival in different and often hostile conditions they evolved a sophisticated and flexible environmental signaling network that is steered by plant hormones. This elaborate hormone-controlled network finetunes the plants' responses according to highly dynamic and heterogeneous circumstances. Immune signaling is part of this overarching network and can be activated and tweaked by the intricate molecular communication between the plant and the microbe or insect that it encounters. The intertwinement of the immune network with other stress and internal networks allows for adjustments in plant defense responses according to the abiotic conditions, plant developmental stage and time of day (Atkinson and Urwin, 2012; Lu *et al.*, 2017; Nobori and Tsuda, 2019; Figure 1).

Plant hormones are central regulators of plant immunity. Depending on the type of attacker different hormones accumulate in the plant, whereby each hormone regulates its own core pathway in the immune network (Figure 1). The two most studied defense pathways are those regulated by jasmonic acid (JA) and salicylic acid (SA), which form the backbone of the hormone-regulated part of the immune system (Wasternack and Song, 2017; Zhang and Li, 2019). The JA pathway can be subdivided into two branches (Pieterse *et al.*, 2012). The ERF branch of the JA pathway is co-regulated by ethylene (ET) and is activated by infection with pathogens with a necrotrophic lifestyle. The MYC branch of the JA pathway is co-regulated by abscisic acid (ABA) and generally provides protection against chewing insects. The SA pathway is considered to be mostly directed against pathogens with a biotrophic lifestyle. So, the infection or infestation strategy of the attacker determines which hormones accumulate and

which pathways the plant activates to express the appropriate defense responses to the attacker at hand. Moreover, hormone homeostasis is greatly influenced by the status of the plant, being internal, for example age, or being external, for example experiencing other stresses (Berens *et al.*, 2019; Nobori and Tsuda, 2019). Overall, the final hormone balance and responsiveness is a cumulative result of the activation of plant immunity and the context in the plant (Figure 1).

The plant immune system is built on two layers, and hormone signaling is essential for both layers. In the first layer, plants recognize small conserved microbe- or insect-derived molecules, called microbe/pathogen-associated molecular patterns (M/PAMPs) or herbivore-associated molecular patterns (HAMPs). If there is damage caused by an attacker, plant-derived small molecules called damage-associated molecular patterns (DAMPs) are released and these can also be recognized. P/M/HAMPs and DAMPs trigger immune signaling resulting in pattern-triggered immunity (PTI), which wards off most of the non-adapted microbes and insects (Dangl *et al.*, 2013; Erb and Reymond, 2019). However, successful pathogens and insects, which can be pathogenic, beneficial or neutral to the plant, can secrete variable effectors into the host plant to suppress PTI signaling. This is known as effector-triggered susceptibility (ETS) and commonly is established by repression of effective defense hormone pathways (Han and Kahmann, 2019). Resistant plants recognize these effectors or their action, setting off a second layer of immunity called effector-triggered immunity (ETI). In the case of plant interactions with biotrophic pathogens ETI often results in a hypersensitive response (HR), which arrests the invading pathogen (Cui *et al.*, 2015). During PTI, ETS and ETI, plant hormones trigger extensive transcriptional reprogramming and thereby tightly regulate defense responses (Berens *et al.*, 2017). This ultimately leads to elimination of harmful microbes and insects and accommodation of beneficial microbes and insects, which can occur simultaneously in the plant.

It is important for plant health and long-term survival that defense responses are finetuned to turn on effective defenses but switch off ineffective defenses. Moreover, defense responses need to be balanced with general housekeeping and responses to other stresses (Vos *et al.*, 2013a;

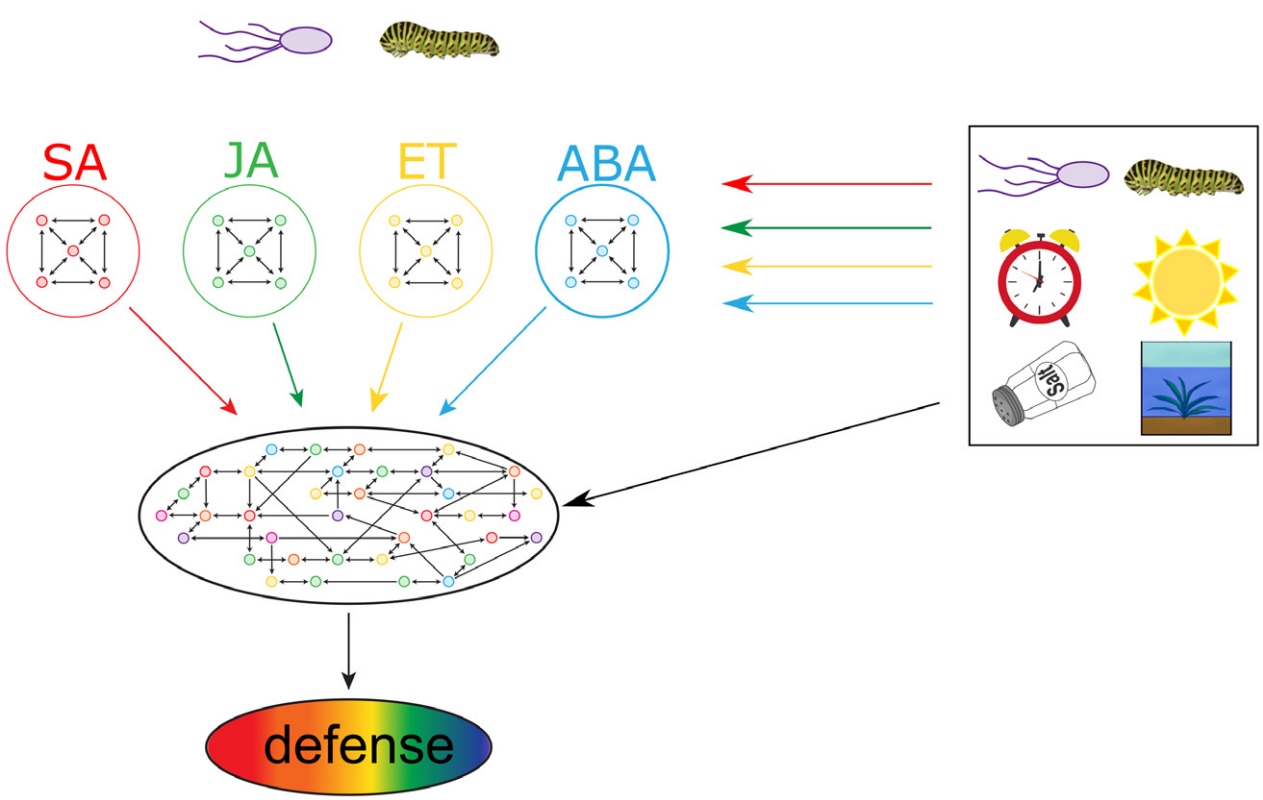

Figure 1. Schematic overview of integration of hormone networks involved in plant defense. Microbes and insects elicit the accumulation of specific blends of hormones. The main hormones involved in the regulation of plant defense responses are salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA). Each hormone regulates its own pathway, but also influences other hormone pathways in a complex mix of synergistic, antagonistic and additional interactions, a phenomenon known as hormone crosstalk. Moreover, accumulation of these hormones and the responsiveness to them can be further modulated by (i) light quality, (ii) time of day, (iii) abiotic stresses such as drought, flooding and salt stress and (iv) prior or simultaneous interactions with other microbes or insects. Integration of the different hormone networks shapes the defense response leading to elimination or accommodation of the microbe or insect in diverse environmental and internal contexts.

Vos *et al.*, 2015; Berens *et al.*, 2017; Van Butselaar and Van den Ackerveken, 2020). To this end, different hormone networks interact in a complex interplay of synergistic, antagonistic and additive interactions, a phenomenon known as hormone crosstalk (Figure 1). Hormone crosstalk is an important component of the architecture of the immune signaling network. Besides finetuning and balancing of responses, antagonistic interactions can also serve to provide robustness to the response. For example, two sectors can positively regulate the same immune response, but negatively regulate each other. That means that if one sector is compromised (for example by manipulation by a pathogen) the other sector is derepressed and can take over the function of the first sector. The classical example of crosstalk in defense regulation is that between the SA and JA pathways. Antagonism between these two pathways is the most studied and prevalent form, although large-scale additive and synergistic interactions have been described as well (Hickman *et al.*, 2019). Additionally, the ERF branch and the MYC branch of the JA pathway have been reported to repress each other (Pieterse *et al.*, 2012; Gimenez-Ibanez and Solano, 2013; Wasternack and Hause, 2013).

A molecular- and systems-level understanding of hormone crosstalk will improve our predictions of effects that disruption or overactivation of parts of the network have on the overall plant response. Implementation of this knowledge can help breeders to engineer crops with a strengthened immune response without undesired traits like enhanced susceptibility to other attackers or decreased plant growth and yield. Here, we review recent advances in hormone crosstalk within the immune network. Different levels of regulation, from network and genome scale to single gene and protein scale, are described. We focus on crosstalk in the JA pathway in *Arabidopsis thaliana* (hereafter Arabidopsis), as a showcase for the multiple regulation levels of pathway interference in hormone defense signaling.

CROSSTALK AT THE NETWORK LEVEL

Studying crosstalk at the network level enables the investigation of crosstalk without defining beforehand all the individual components. This coarse-grain overview can reveal the overall architecture of the hormone-regulated plant immune system. Furthermore, it can provide hypotheses about crosstalk at more fine-grain levels, which can be validated experimentally.

A network approach encompasses gathering information on a genome-wide scale. As RNA sequencing (RNA-seq) is the most widely available genome-wide technique, information on the transcriptome is most commonly used for hormone network-scale analyses until now. However, newly emerging technologies also allow gathering information on other regulation levels such as the proteome and translatome (Lee and Bailey-Serres, 2019; Zander *et al.*, 2020). In addition to these molecular data, relatively simple phenotypical readouts can be used for network analysis. The different types of data are usually gathered from leaves of plants that are given a stimulus such as hormone application or pathogen infection. Comparisons can be made between effects that one stimulus has on various mutants that are impaired in hormone signaling sectors. Alternatively, effects of different hormone treatments on one plant genotype (wild type) can be compared.

Network modeling using hormone mutants

A good example of network-level research is a series of papers that describe how different hormone sectors interact to regulate PTI and ETI (Tsuda *et al.*, 2009; Kim *et al.*, 2014; Hillmer *et al.*, 2017; Mine *et al.*, 2017). The researchers used single and higher-order mutants of key regulators of the SA, JA and ET pathways to understand how each pathway contributes to PTI and ETI. A mutant of PAD4 was added because although PAD4 expression is known to be induced by SA and SA response-eliciting pathogens, PAD4 itself can regulate both SA-dependent and -independent responses (Jirage *et al.*, 1999; Glazebrook *et al.*, 2003). Using bacterial growth as a readout, they found that each of the four sectors alone positively contributes to both PTI and ETI (see also Figure 1). However, interactions between the sectors differ between the PTI and ETI responses (Tsuda *et al.*, 2009). The PTI response involves both synergistic and antagonistic interactions (Tsuda *et al.*, 2009; Kim *et al.*, 2014) and gene expression in this response is almost always influenced by one or multiple interactions between sectors (Hillmer *et al.*, 2017). Robustness during PTI is mostly provided by the JA and ET sectors. This was demonstrated by the finding that in a mutated JA or ET background knocking out another sector had much more impact on MAMP-induced immunity levels against two *Pseudomonas syringae* strains than in the wild-type background (Kim *et al.*, 2014). In contrast, during ETI all of the sectors act antagonistically and can (partially) take over the response if one of the sectors is inactive. This crosstalk mechanism ensures that the ETI response is robust against manipulation or dysfunction of one of the involved network sectors caused by an attacker or another stimulus (Tsuda *et al.*, 2009).

An example of how such network robustness can be achieved was elegantly demonstrated in a follow-up paper by Mine *et al.* (2017). They provided evidence for a robust regulation of SA biosynthesis by interactions between transcriptional regulators of the JA, SA and PAD4 sectors. These regulators form a so-called incoherent type 4 feed-forward loop, in which two components positively regulate one target, but one of these two components also negatively regulates the other component (Mangan and Alon, 2003). In this case, both PAD4 and the JA master regulator MYC2 (Kazan and Manners, 2013) positively regulate *EDS5* (Mine *et al.*, 2017), a gene essential for SA biosynthesis (Rekhter *et al.*, 2019), but MYC2 represses *PAD4* (Mine

et al., 2017). These interactions provide robustness to the system, which was demonstrated by the following. In wild-type plants PAD4 positively contributed to basal and flg22-induced *EDS5* expression, whereas the JA sector had no effect on *EDS5* expression and in fact contributed negatively to SA accumulation (Mine *et al.*, 2017). However, when PAD4 was compromised, which in nature could result from activity of a pathogen effector or high temperature, MYC2 was able to activate *EDS5* and hence, the JA pathway could positively influence SA biosynthesis. This was demonstrated using the *dde2 pad4* mutant (impaired in JA signaling and the PAD4 sector), which, compared to the *pad4* single mutant, exhibited lower levels of flg22-induced *EDS5* expression and free SA (Mine *et al.*, 2017). So, in the case of SA biosynthesis the JA sector can functionally replace the PAD4 sector when the latter is compromised. Moreover, a direct interaction between the MYC2 and PAD4 proteins has been shown to affect free SA accumulation (Cui *et al.*, 2018), as described in the section 'Crosstalk at the hormone homeostasis level'. *Vice versa*, the SA sector stimulates JA biosynthesis during ETI (Liu *et al.*, 2016a), which is described in the section 'Crosstalk by modulation of protein stability'.

Network modeling using time series of hormone treatments of wild-type plants

A complementary approach to the above-described systems biology network studies using mutants as conducted by the Tsuda and Katagiri groups is using hormone applications to wild-type plants. A high-throughput time series set-up can provide extra power to the analysis, as this unveils regulatory connections between different components that shape the dynamic architecture of the network. Such an approach facilitates our understanding of the temporal information flow through the different sectors of the individual hormone regulatory networks, including the interactions with sectors of other hormone networks. This approach was taken for JA (Hickman *et al.*, 2017; Zander *et al.*, 2020), the JA mimic phytotoxin (of *P. syringae*) coronatine (Attaran *et al.*, 2014), SA (Hickman *et al.*, 2019), ET (Chang *et al.*, 2013) and ABA (Song *et al.*, 2016), which followed up on seminal time series papers studying responses to pathogen infection (Windram *et al.*, 2012; Lewis *et al.*, 2015).

Hickman *et al.* (2017) built a gene regulatory network model of the JA response based on a transcriptome study of a time series of 14 time points within 16 after a one-time treatment of mature Arabidopsis with aqueous methyl jasmonate (MeJA), which is converted to JA in the plant. The majority of the differentially expressed genes were detected as such within 2 h after treatment. Correlation analysis of expression at different time points showed that there were six distinct phases of upregulation and four distinct phases of downregulation. Each phase was enriched for different processes and contained specific transcription factors (TFs) that were predicted to regulate genes in subsequent phases, based on enrichment of TF binding motifs in genes differentially expressed during these phases. Intersections of the JA network with other hormone networks were also observed. For example, genes related to the SA pathway were downregulated in early phases (1–2 h) and genes related to the growth hormone auxin were downregulated in later phases (3–4 h).

Zander *et al.* (2020) integrated more data types to elucidate the JA response in etiolated seedlings that had received continuous treatment with gaseous MeJA for up to 24 h. They focused on the role of MYC2 (Boter *et al.*, 2004; Lorenzo *et al.*, 2004; Dombrecht *et al.*, 2007) and MYC3 (Fernández-Calvo *et al.*, 2011) as master regulators of the JA response by conducting chromatin immunoprecipitation–sequencing (ChIP-seq) analysis of these TFs, and ChIP-seq or DNA affinity purification–sequencing (DAP-seq) (basically *in vitro* ChIP-seq) of five of their targets. In addition, six other known JA-related TFs were included in their ChIP-seq/DAP-seq experiments. They also generated proteome and phosphoproteome data, and integrated these with the other data types to infer a regulatory network. This network contained known and new components of the JA regulatory network and pointed to known and novel nodes of crosstalk of the JA pathway with other hormone pathways. An important role for MYC2 and MYC3 in modulation of other hormone pathways was demonstrated by the finding that 37–59% of genes that are annotated as being part of other hormone signaling pathways were bound by MYC2 and MYC3 and that their transcription responded to the MeJA treatment. Furthermore, the JA-induced transcriptional repressor STZ was predicted to suppress genes from several other hormone pathways, including the SA, gibberellin (GA) and brassinosteroid (BR) pathways (Hickman *et al.*, 2019; Zander *et al.*, 2020).

In a follow-up paper of the Hickman *et al.* (2017) paper on individual MeJA treatment, another part of the same experiment was reported, for which plants received a single SA treatment or a combination treatment of MeJA with SA (Hickman *et al.*, 2019). The single SA treatment had a greater impact on the transcriptome than the single MeJA treatment, affecting the expression of more genes and having more prolonged effects. Validation of the built SA gene regulatory network model confirmed that specific TFs regulate specific paths in the network that are biologically relevant for defense against biotrophic pathogens. Comparison of the individual SA and MeJA treatments showed that there is a high level of interplay between the SA and JA networks (see also Figure 1). Of the MeJA-responsive genes 69% was also modulated by the individual SA treatment, and of the SA-responsive genes (which was a greater set) 26% was modulated by MeJA. Contrary to the paradigm of SA/JA antagonism, only half of the overlapping genes were regulated in an opposite manner (upregulated by SA and

downregulated by MeJA or *vice versa*), while the other half of the genes were regulated in a similar direction by the two hormones. Noteworthily, hormone biosynthesis and pathways regulators like *LOX2*, *MYC2*, *EDS1* and *PAD4* were generally upregulated by the respective hormone treatment but downregulated by the other hormone treatment (Figure 2a, see also the section 'Crosstalk at the hormone homeostasis level'). Moreover, many of the SA- and MeJA-co-upregulated genes were canonical JA and SA pathway genes, like *GRX480, ANAC019*, *ANAC055*, some *JAZs* and *RAP2.6*. Many of these genes are also associated with ET and ABA signaling, hinting to their responsiveness to a broad range of hormone-inducing environmental stimuli. The combined SA and MeJA treatment showed that 68% of the MeJA-responsive genes changed its expression when SA was added to the treatment, while this was the case for only 12% of SA-responsive genes. While antagonistic and synergistic effects of the dual treatment were observed, the vast majority of the effects were just additive. Short-term effects by MeJA were overridden by SA effects over time, resulting in a dominance of the SA profile over the MeJA profile (Hickman *et al.*, 2019).

Chang *et al.* (2013) combined ChIP-seq of the ET master regulator TF EIN3 with RNA-seq at five time points within 24 h following continuous ET treatment. ET was found to influence many other hormone pathways besides the JA pathway (Lorenzo *et al.*, 2003; Anderson *et al.*, 2004), as the GA, auxin, BR, ABA, SA and cytokinin pathways were also affected by ET treatment. A study by Song *et al.* (2016) investigated the ABA network based on RNA-seq time series and ChIP-seq experiments with 21 ABA-responsive TFs in the presence of ABA. A complex network of regulation by multiple master regulators, including extensive feedback regulation, was revealed. A thousand genes involved in other hormone pathways were bound by at least one of the investigated ABA-responsive TFs. However, the genes that were bound by a large number of TFs and/or by TFs that showed increased binding after ABA treatment usually belonged to the ABA pathway itself.

CROSSTALK AT THE PROTEIN LEVEL

Proteins can modulate the functioning of other proteins in their own pathway or in other hormone pathways through various mechanisms, such as co-activation, repression, competitive binding to multiple targets and chemical modification (e.g., phosphorylation, ubiquitination, sumoylation, nitrosylation or sulfonylation). Several molecular players in crosstalk have been demonstrated to modulate proteins that act in other hormone pathways. These include hormone receptors and their interactors. In addition, TFs have been implicated as crosstalk mediators at the protein level, with leading roles for the bHLH TF MYC2 and the ERF TF ORA59 as important targets for crosstalk in the JA pathway (Figure 2).

Crosstalk by protein–protein interactions

Protein–protein interactions are of major importance for hormone pathway crosstalk. Recently, an extensive network of protein–protein interactions between members of all hormone pathways in Arabidopsis was revealed using yeast two-hybrid with 1226 genes with probable or genetically demonstrated functions in plant hormone signaling (Altmann *et al.*, 2020). Not only was there high connectivity within each single hormone pathway, but also many inter-pathway contact points were uncovered. Validation of a subset of these inter-pathway contact points suggested that many of these interactions indeed likely represented crosstalk mechanisms. This was demonstrated by the finding that a mutant of one interaction partner influenced the plant phenotype that correlated with the hormone-associated function of the other interaction partner. It should be noted that such validation does not explicitly show that the convergence of two pathways depends on the detected protein interaction. Alternatively, it could be regulated by another factor that acts downstream in the pathway of the mutated gene.

Hormone receptors were especially often found to interact with proteins that were not involved in the canonical hormone pathway of the receptor (Altmann *et al.*, 2020). This suggests that signaling by hormone receptors through non-canonical pathways has a more prominent role in integrated hormone signaling than previously anticipated. One such example was previously shown for the ABA receptor PYL6, which interacts with the JA master regulator MYC2 (Aleman *et al.*, 2016; Figure 2a). In the presence of ABA, the binding of PYL6 to MYC2 is enhanced, which alters the transcriptional specificity of MYC2 from promoting *JAZ6* expression to *JAZ8* expression. The genome-wide implications of this mechanism have yet to be determined. Another example of an interaction between hormone receptors and key components of non-canonical hormone pathways is that of the SA receptors NPR3 and NPR4 with JAZ repressors. This is discussed in the section 'Crosstalk by modulation of protein stability'.

MYC branch/ERF branch antagonistic crosstalk in the JA defense pathway is likely also (partly) regulated by protein–protein interactions. MYC2 can suppress the ERF branch by directly binding to the TF EIN3, which causes reduced binding of EIN3 to the promoter of a target gene (Song *et al.*, 2014; Zhang *et al.*, 2014; Figure 2). This effect was only shown for the promoter of *HLS1*, a gene involved in the formation of the apical hook in etiolated seedlings (Song *et al.*, 2014; Zhang *et al.*, 2014). It is thus unclear if this mechanism also underlies MYC2-mediated antagonism on JA-responsive defense genes in the ERF branch. *Vice versa*, binding of EIN3 and EIL1 to MYC2 represses the transcriptional activity of *MYC2* (Figure 2). This could

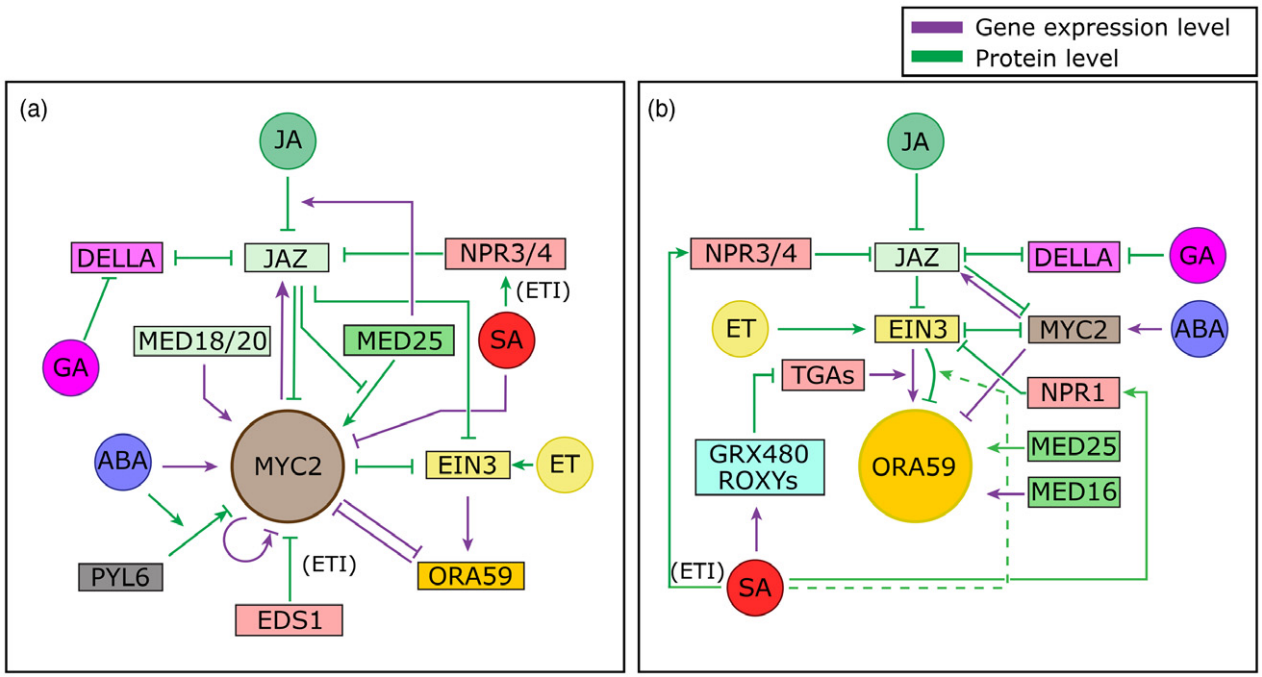

Figure 2. Schematic overview of hormone crosstalk acting on two key transcription factors, MYC2 and ORA59, of the two branches of the jasmonic acid (JA) pathway.

(a) Crosstalk acting on the MYC branch master regulator MYC2. In the context of defense, MYC2 mostly regulates anti-insect responses. MYC2 is repressed by interacting JAZ repressors, and MYC2 itself can induce transcription of these JAZs. JA induces the breakdown of JAZs, thus leading to activation of MYC2. MED25 promotes MYC2 transcriptional activity, but JAZs prevent binding of MED25 to MYC2. MED25 also promotes JAZ breakdown by recruiting COI1, and alters JAZ splicing and thereby JAZ sensitivity to JA. MED18 and MED20 promote transcription of *MYC2*. EIN3 is activated by JA-mediated breakdown of JAZ proteins and ethylene (ET)-mediated stabilization. It binds to and represses MYC2 and *vice versa*. EIN3 also transcriptionally activates *ORA59* and ORA59 can repress *MYC2* transcription and *vice versa* (either directly or indirectly, see also panel b). MYC2 can enhance its own transcription in the short term but represses it in the long term. During AvrRps4-induced ETI, EDS1 can repress MYC2. Furthermore, SA can promote degradation of JAZs via NPR3 and NPR4 during ETI. Generally, SA is an inhibitor of *MYC2* transcription. Abscisic acid (ABA) directly activates transcription of *MYC2* and enhances binding of the ABA receptor PYL6 to MYC2, modulating transcriptional activity of MYC2, which differentially acts on the *JAZ6* and *JAZ8* promoters (leading to repression versus activation). DELLA proteins bind JAZs and thereby JAZs and DELLAs prevent each other from binding to their respective target transcription factors (TFs). Gibberellin (GA) induces breakdown of DELLAs and thus indirectly represses MYC2. (b) Crosstalk acting on the ERF branch master regulator ORA59. ORA59 mostly regulates defense against necrotrophic pathogens. ORA59 is indirectly regulated by both JA and ET through their action on EIN3: JA releases EIN3 from its repression by JAZ and ET stabilizes EIN3. When released from repression and degradation, EIN3 activates transcription of *ORA59*. TGA TFs are also needed for this activation. GRX480 and other ROXYs are induced by SA and repress the transcriptional activity of these TGAs, leading to reduced transcription of *ORA59*. EIN3 also mediates degradation of ORA59. Because this only leads to reduced ORA59 functioning under high SA levels and not under high ET levels we propose that SA specifically modulates EIN3 activity such that it leads to ORA59 degradation (dotted line). SA activates NPR1's activity as a co-transcriptional regulator. NPR1 can interact with EIN3, leading to repression of EIN3 transcriptional activity. We propose that it is unlikely that NPR1 modulates EIN3-mediated *ORA59* activation during SA/JA crosstalk (see the section 'Crosstalk by modulation of protein stability'). EIN3 is further repressed by MYC2 through direct binding and this also occurs the other way around. MYC2 is repressed by interaction of JAZ repressors and itself activates transcription of these JAZs. It is also transcriptionally activated by ABA. *ORA59* expression is inhibited by MYC2, both directly and possibly indirectly via inhibition of EIN3 by MYC2. DELLAs bind to JAZs and thereby they inhibit each other from binding to target TFs in their respective pathways. GA leads to breakdown of DELLAs, thus indirectly repressing ORA59. MED25 interacts with ORA59 and promotes its transcriptional activity, and MED16 promotes *ORA59* transcription. Modulation of JAZ breakdown and JAZ RNA splicing by MED25 is not shown here (see panel a). During ETI SA can promote degradation of JAZs via NPR3 and NPR4. Note that in most cases where EIN3 is mentioned, EIL1 likely has the same function. However, in most research only EIN3 is extensively characterized.

Mechanisms acting on the gene expression level are colored purple and mechanisms acting on the protein level are colored green. Arrows and bar-headed lines indicate positive and negative effects, respectively. Mechanisms acting downstream of MYC2 and ORA59 or at the hormone homeostasis level are not shown.

likely explain the enhanced level of MYC2-regulated *VSP2* expression in the *ein3 eil1* mutant and the reduced growth of a caterpillar that feeds on this mutant (Song *et al.*, 2014). The reciprocal inhibition between MYC2 and ERF branch signaling components is not only regulated at the level of protein–protein interactions but also at the level of transcriptional regulation. This was demonstrated by the direct positive effect of MYC2 on expression of the F-box protein-coding gene *EBF1*, whose protein product targets EIN3 for degradation (Zhang *et al.*, 2014), thus further suppressing ET signaling through a combined effect of MYC2 on ET signaling via transcription, protein stability and protein–protein interaction.

Crosstalk by modulation of protein stability

Modulation of stability of activators and especially repressors is an important regulatory mechanism in many different pathways. The most famous example from hormone

defense signaling is that of JAZ proteins, which form a co-receptor complex with the E3 ubiquitin ligase F-box protein COI1 for JA-Ile, the active form of JA (Fonseca *et al.*, 2009; Sheard *et al.*, 2010). JAZ proteins inhibit transcription within the MYC and ERF branches through direct binding to key TFs, recruitment of co-repressors and inhibition of the interaction of the Mediator subunit MED25 with MYCs (see the section 'Crosstalk by the Mediator complex'). Upon perception of JA-Ile, JAZs are degraded by the 26S proteasome, which releases the previously bound TFs and initiates the JA response (Chini *et al.*, 2007; Thines *et al.*, 2007; Figure 2). The key function of JAZ proteins is substantiated by experiments that identified JAZs as targets for interference of immune signaling by pathogen and insect effectors. For example, HARP1, an effector of the chewing cotton bollworm (*Helicoverpa armigera*) was recently shown to bind to multiple JAZs in Arabidopsis, cotton (*Gossypium hirsutum*) (its preferred host) and tobacco (*Nicotiana benthamiana*) (Chen *et al.*, 2019). This led to an increase in stability of JAZs, likely because it prevented JA-Ile-induced binding of JAZs with COI1. Via this mechanism, HARP1 reduced wound-induced defense signaling and increased plant susceptibility to the insect (Chen *et al.*, 2019). HopX1, an effector from the hemi-biotrophic bacterial pathogen *P. syringae* pv. *tabaci* 11528, was also found to interact with JAZs but in contrast this led to a decrease (rather than an increase) in their stability, in a COI1-independent manner. The resulting activation of the JA pathway in turn led to repression of the SA pathway and thus increased susceptibility to this pathogen (Gimenez-Ibanez *et al.*, 2014). Similarly, the effector HopZ1a from *P. syringae* pv. *syringae* A2 causes degradation of JAZs and consequent activation of the JA pathway and repression of SA signaling. However, in contrast to the effect of HopX1, JAZ degradation by HopZ1a depends on COI1 and likely involves acetylation of JAZs (Jiang *et al.*, 2013). The most studied example of an effector that causes degradation of JAZs, the JA-Ile mimic coronatine, is discussed in the section 'Crosstalk at the hormone homeostasis level'.

Degradation of JAZ proteins is essential for synergistic effects between the JA pathway and the ET, ABA, SA and BR pathways, as we will outline in this paragraph. Synergism between the JA and ET pathways drives activation of the ERF branch of defense. JAZ proteins can physically interact with and repress the ET response TFs EIN3 and EIL1 by recruitment of the chromatin modifier HDA6 as a co-repressor (Zhu *et al.*, 2011). In the presence of JA, the JAZs are degraded and thereby the interaction between HDA6 and EIN3/EIL1 is reduced and thus EIN3/EIL1 transcriptional activity is enhanced. In combination with ET's activity to stimulate EIN3/EIL1 protein accumulation, the de-repression of EIN3/EIL1 by JA enhances transcription of *ERF1* and *ORA59* (Zhu *et al.*, 2011; Figure 2), which are key TFs in the ERF branch of the JA pathway (Pré *et al.*, 2008; Pieterse *et al.*, 2012). ABA/JA synergistic crosstalk is also partly regulated through stability of a JAZ protein. This is mediated by the RING E3 ligase KEG, which is promoted for self-ubiquitination and subsequent degradation by ABA. KEG normally decreases the COI1-mediated degradation of JAZ12, but this is prevented under high ABA conditions due to degradation of KEG, leading to reduced JAZ12 levels (Pauwels *et al.*, 2015). This was associated with an enhanced expression level of the MYC branch marker gene *VSP2* under basal conditions in a *keg* knockdown line (Pauwels *et al.*, 2015). No evidence was found for a role of JAZ (de)stabilization in antagonistic SA/JA crosstalk on JA-responsive gene expression when plants were exogenously treated with SA and/or MeJA (Van der Does *et al.*, 2013; Liu *et al.*, 2016a). However, a role for SA-mediated JAZ degradation was reported in synergistic SA/JA crosstalk occurring during ETI triggered by *P. syringae* pv. *maculicola* (*Psm*) ES4326 carrying the effector AvrRpt2 (Liu *et al.*, 2016a). JAZ proteins were shown to bind to the SA response regulators NPR3 and NPR4, and this binding was enhanced by SA. Being substrate adaptors for Cullin 3 (Cul3) ubiquitin E3 ligases, NPR3 and NPR4 target the JAZs for degradation (Liu *et al.*, 2016a; Figure 2). This results in increased JA signaling, which is necessary for a full HR response. It is unclear why SA-mediated breakdown of JAZ proteins would occur only during ETI triggered by *Psm* ES4326 AvrRpt2 but not after exogenous SA application. In rice (*Oryza sativa*), BR/JA crosstalk is regulated through modulation of OsJAZ4 stability (He *et al.*, 2020). This is mediated by OsGSK2, a kinase that itself is negatively regulated by BR in Arabidopsis through dephosphorylation and degradation (Peng *et al.*, 2008; Kim *et al.*, 2009; He *et al.*, 2020). OsGSK2 was shown to interact with and phosphorylate OsJAZ4, which subsequently leads to disruption of the OsJAZ4–OsNINJA and OsJAZ4–OsJAZ11 interactions and to degradation of OsJAZ4 in an OsCOI1-dependent manner (He *et al.*, 2020). In accordance, high BR levels lead to reduced OsGSK2 levels and activity, which in turn leads to higher OsJAZ4 levels and thus decreased JA signaling, resulting in enhanced susceptibility to the rice black-streaked dwarf virus (He *et al.*, 2017).

SA/JA antagonistic crosstalk is partly regulated through modulation of the stability of the TF ORA59, a master regulator in the ERF branch of the JA pathway (Pré *et al.*, 2008). SA treatment leads to breakdown of ORA59 (Van der Does *et al.*, 2013; He *et al.*, 2017) but not in an *ein3 eil1* mutant (He *et al.*, 2017). Furthermore, SA increases EIN3 protein abundance and co-transfection of *EIN3* and *ORA59* in *N. benthamiana* leads to degradation of ORA59 unless a proteasome inhibitor is added (He *et al.*, 2017). Also, EIN3 interacts with ORA59 (He *et al.*, 2017). Hence, the SA-mediated degradation of ORA59 depends on the interaction of ORA59 with EIN3 and likely also its homolog EIL1 (Figure 2b). However, the underlying molecular mechanism by

which SA can induce EIN3-mediated breakdown of ORA59 is not completely clear. It is unlikely that the SA-increased protein abundance of EIN3 can explain SA-mediated ORA59 degradation, since ET also increases EIN3 protein abundance (Dolgikh *et al.*, 2019) but has a positive effect on ORA59 functioning in the ERF branch of defense (Pré *et al.*, 2008). We propose that SA modulates the activity of EIN3 such that it specifically causes degradation of ORA59 (Figure 2b, dotted line). Recently, it was found that the SA master transcriptional regulator NPR1 can bind to EIN3 and repress its transcriptional activity in the regulation of apical hook formation (Huang *et al.*, 2020; Figure 2b). Hypothetically, an NPR1–EIN3 interaction may also be required for EIN3-mediated breakdown of ORA59 during SA/JA crosstalk. In contrast, under high ET conditions, which would increase EIN3 levels, the SA/JA crosstalk was shown to be independent of NPR1 (Leon-Reyes *et al.*, 2009), making this hypothesis very unlikely. This suggests that another protein that functions in the SA pathway must be the missing link for SA/JA crosstalk via EIN3-mediated ORA59 degradation.

Crosstalk by competitive binding of proteins to multiple other proteins

A regulatory protein can be held inactive if binding to its downstream target protein is prevented due to its bound status to another protein. An example of such a crosstalk mechanism that is based on competitive binding to multiple proteins is provided by the interaction between JAZs and DELLAs, which are repressors of the JA response and the GA response, respectively (Hou *et al.*, 2010). When they are bound to each other, JAZs compete for binding of DELLAs to growth-promoting PIF TFs, and DELLAs compete for binding of JAZs to the JA master regulator MYC2 (Hou *et al.*, 2010; Hou *et al.*, 2013; Figure 2). When plants are attacked by an insect the JA levels rise, causing degradation of JAZs and thus release of MYC2, so that JA-responsive transcription is initiated. At the same time more DELLAs can bind to PIFs and elongation growth is inhibited. Other JA pathway-regulating TFs that physically associate with JAZs, such as EIN3 and EIL1, are likely also indirectly affected by DELLAs, which may impact transcription of *ORA59* (Hou *et al.*, 2013; Figure 2). In contrast, if GA levels are high, such as under far-red light conditions (Franklin, 2008), DELLAs are degraded, thereby releasing PIFs and thus leading to elongation growth, while the JAZs can now bind more MYC2, leading to repression of JA-mediated defense responses (Hou *et al.*, 2013; Figure 2). This crosstalk mechanism between JA and GA signaling is traditionally viewed as important for regulation of the defense–growth trade-off (Hou *et al.*, 2013). However, recently, conflicting results on the role of the JAZ–DELLA interaction in the JA-mediated growth effects were reported and other interaction points of the JA pathway with growth signaling have been pinpointed (Chakraborty *et al.*, 2019; Liu *et al.*, 2019; Major *et al.*, 2020; Ortigosa *et al.*, 2020).

Crosstalk by redox regulation and ROXY glutaredoxins

Redox status is an important determinant of protein functioning and as such plays a role in plant defense hormone signaling, among which hormone crosstalk. The defense hormone SA itself induces cycles of oxidation and reduction in the cell, leading to an increase in the amount of the antioxidant glutathione and changing the ratio between the oxidized and reduced states of glutathione (Spoel and Loake, 2011). The changes in redox potential and the change in glutathione state as a result of SA elevation has consequences for oxidation or reduction of cellular proteins and thereby modulates their function. The increase in glutathione levels was found to coincide with the time frame in which SA could suppress JA signaling. That is, SA treatment prior to MeJA treatment led to a reduction of MeJA-induced *PDF1.2* expression only if MeJA was applied in the time frame when glutathione levels were increased by SA. Additionally, chemical inhibition of glutathione biosynthesis severely diminished the ability of SA to suppress MeJA-induced *PDF1.2* expression (Koornneef *et al.*, 2008). This suggests that the SA-induced shift in redox potential is involved in SA/JA antagonistic crosstalk.

Glutaredoxins are small oxidoreductases that are involved in reduction of oxidative modifications using glutathione (Ströher and Millar, 2012). Four of the CC-type glutaredoxins, which are also known as ROXYs and include GRX480, are induced by SA and can suppress induction of *ORA59* by EIN3, making them potential candidates for SA-mediated crosstalk on the ERF branch (Zander *et al.*, 2012; Figure 2b). Several lines of evidence indicate that under high ET levels group II TGA TFs regulate *ORA59* induction, but that the TGAs recruit ROXYs under high SA levels (Ndamukong *et al.*, 2007; Zander *et al.*, 2010; Zander *et al.*, 2012; Zander *et al.*, 2014). It was believed that this would lead to redox modification of the TGAs, which would cause a decrease of their transcriptional activity. However, the ROXYs were recently shown to recruit the co-repressor TPL through the same motif that was shown to be essential for repression of *ORA59* transcription (Uhrig *et al.*, 2017). This suggests that the effect of GRX480 and other ROXYs on SA/ERF branch crosstalk via suppression of TGA-mediated transcription of *ORA59* is caused by recruitment of a transcriptional co-repressor rather than by redox modification.

CROSSTALK AT THE GENE EXPRESSION LEVEL

Regulation of gene transcription is a major component of hormone crosstalk. In fact, most of the regulation at the protein level that is discussed above eventually leads to altered transcription of downstream target genes. In this

section we discuss that crosstalk can act across multiple regulatory scales of gene expression, from binding of a TF to the promoter of a gene to translation of mRNA to protein.

Crosstalk by binding of TFs to promoters

Most TFs have affinity for binding to a specific DNA motif (Franco-Zorrilla *et al.*, 2014; Weirauch *et al.*, 2014; O'Malley *et al.*, 2016). TF–DNA binding in the promoter region of a gene largely determines activation or repression of transcription. A gene may be subject to crosstalk if, for example, TFs from different hormone pathways compete for binding to the same DNA motif in the promoter of a gene. Also binding of different TFs to different DNA motifs or cooperative binding of different TFs to the promoter of a gene may modulate the expression of that gene by multiple hormones. The increasing amount of available ChIP-seq and DAP-seq data (of currently >500 Arabidopsis TFs) greatly facilitates the identification of DNA motifs and their *trans*-acting TFs (O'Malley *et al.*, 2016; Zander *et al.*, 2020). Besides DNA sequence, many other factors determine where a TF binds to DNA, as demonstrated by the fact that TFs from the same family often bind to very similar motifs, but regulate divergent genes (O'Malley *et al.*, 2016). This can for example be determined by the chromatin structure, which can be more compact or relaxed, depending on, for example, acetylation/methylation/ubiquitination of the histones. This local chromatin status of genomic DNA influences the exposure of *cis*-regulatory DNA elements for proteins and consequently transcription. Additional important factors that determine transcriptional activity are the 3D structure of the DNA (Muiño *et al.*, 2014; Mathelier *et al.*, 2016), methylation of the DNA (O'Malley *et al.*, 2016) and spacing between adjacent DNA motifs (Krawczyk *et al.*, 2002; O'Malley *et al.*, 2016). Although to our knowledge these types of information have not been implicated in hormone crosstalk research yet, they provide an enormous potential to uncover cross-regulatory mechanisms based on differential TF–DNA binding.

Investigations with overexpression and/or knockout lines implied a role for several SA-activated WRKY TFs in SA/JA crosstalk (reviewed by Caarls *et al.* (2015)). The role of the WRKY-bound W-box motif in SA/JA crosstalk was investigated using RNA-seq data derived from time course experiments with MeJA, SA and MeJA + SA combination treatments (see also the section 'Network modeling using time series of hormone treatments of wild-type plants'). The W-box was, as expected, significantly enriched in SA-upregulated genes as well as in MeJA-downregulated genes (Hickman *et al.*, 2017; Hickman *et al.*, 2019). However, there was no enrichment of the W-box in MeJA-upregulated genes that were antagonized by SA in the combination treatment (Hickman *et al.*, 2019). This is in contrast to a previously reported microarray-based study of a single, relatively late time point (28 h), in which the W-box was enriched in a small number of ERF branch response genes that were upregulated by MeJA, but antagonized by the combination with SA (Van der Does *et al.*, 2013). This difference may be explained by the fact that compared to the above-mentioned RNA-seq study the ERF branch was activated to a higher extent by the MeJA treatment in the latter experiment. Thus, WRKY TFs may regulate SA/JA crosstalk through binding to a subset of promoters of MeJA-inducible genes in the ERF branch to repress their expression under certain conditions, but likely do not play such a role in the entire JA pathway, which is in line with the finding that in contrast to the ERF branch response genes, the MYC branch response genes, which are antagonized by SA, were not enriched in the W-box (Van der Does *et al.*, 2013).

Two other motifs, the GCC-box and the G-box, which are mostly known for their role in JA and ET signaling, may also be important in SA-mediated crosstalk. These motifs are bound by ERF TFs, among which ORA59, and bHLH TFs, among which MYC2, respectively. Both motifs are enriched in MeJA-induced genes that are suppressed by SA (Van der Does *et al.*, 2013; Hickman *et al.*, 2019). Moreover, a synthetic promoter only containing four repeats of the GCC-box was demonstrated to be inducible by a single MeJA treatment but repressed by a combination treatment of MeJA and SA (Van der Does *et al.*, 2013). A study by Caarls *et al.* (2017) investigated if this was caused by repressive SA-induced ERFs or EAR domain-containing ERFs that may compete with JA-induced ERF activators for binding to target genes in the ERF branch. Gene expression analyses of 16 *erf* mutants showed that the tested ERFs are not required for SA/JA crosstalk. To the best of our knowledge there are also no known potential JA pathway-repressing bHLH TFs from the SA pathway. It is worth noting that there is a clade of bHLH TFs that repress the JA pathway, consisting of JAM1, JAM2 and JAM3, but they are described as JA-responsive rather than SA-responsive (Sasaki-Sekimoto *et al.*, 2013). Together, these results suggest that it is unlikely that the JA pathway is antagonized through large-scale binding of SA-responsive TFs directly to the promoters of JA-activated genes. Instead, SA may antagonize the JA pathway by inhibiting the transcriptional activity of certain key activator TFs of the JA pathway such as ORA59 and MYC2. In agreement with this hypothesis, SA treatment is known to cause reduced transcription of the *ORA59* gene and degradation of the ORA59 protein (see the sections 'Crosstalk by modulation of protein stability' and 'Crosstalk by redox regulation and ROXY glutaredoxins' and Figure 2b).

There is ample evidence for extensive regulation of ORA59 and MYC2 by several proteins that are involved in hormone crosstalk (see previous and subsequent sections, Figure 2) but the exact underlying mechanisms have not

always been elucidated. For example, Verhage *et al.* (2011) showed that basal and caterpillar-induced *ORA59* mRNA levels were higher in a *myc2* mutant and caterpillar-induced *MYC2* levels were higher in an *ORA59* RNAi line. This suggests that MYC2 and ORA59 directly or indirectly repress each other's transcription (Figure 2), in addition to the reciprocal inhibitory effects of binding between MYC2 and EIN3, the upstream regulator of ORA59 (Figure 2 and the section 'Crosstalk by protein–protein interactions'). Later, it was shown that repression of *ORA59* by MYC2 was mediated by direct binding of MYC2 to a G-box in the *ORA59* promoter (Zhai *et al.*, 2013), but to the best of our knowledge the mechanism underlying the repressive effect of ORA59 on the transcription of *MYC2* has not been elucidated yet. Another example is the finding that *MYC2* is transcriptionally upregulated by both JA and ABA and can itself regulate genes in both JA and ABA signaling (Abe *et al.*, 1997; Abe *et al.*, 2003; Hickman *et al.*, 2017; Figure 2). As such, MYC2 is both an integrator and a regulator of JA and ABA signaling. Possibly, the enhanced *MYC2* expression by ABA treatment is related to ABA-enhanced JA biosynthesis (see the section 'Crosstalk at the hormone homeostasis level'), which not only activates MYC2 transcriptional activity but also enhances transcription of *MYC2* through autoregulation (Wang *et al.*, 2019; Figure 2).

Crosstalk by the Mediator complex

The multiprotein Mediator complex forms the molecular bridge that relays signals from DNA-binding TFs to the transcription machinery (Zhai and Li, 2019). It plays an important role in hormone signaling pathways, including hormone crosstalk. MED25 is the most studied Mediator subunit in defense hormone signaling. It interacts with the TFs MYC2, MYC3, MYC4, ORA59 and ERF1 as well as with the JA receptor component COI1. In doing so MED25 is involved in the transcriptional activity of these TFs in both the MYC and the ERF branch (Çevik *et al.*, 2012; Chen *et al.*, 2012; An *et al.*, 2017; Figure 2). Additionally, MED25 promotes JAZ breakdown by recruiting COI1 to target promoters (An *et al.*, 2017). *Vice versa*, JAZ proteins inhibit the MED25–MYC interaction (Zhang *et al.*, 2015; Figure 2a).

MED25 promotes transcription in the JA pathway via various mechanisms such as recruitment of RNA polymerase II, histone acyltransferase HAC1 and JA-related enhancers to promoters that are targeted by for example MYC2 and ORA59 (Çevik *et al.*, 2012; Chen *et al.*, 2012; An *et al.*, 2017; Wang *et al.*, 2019; You *et al.*, 2019; Figure 2). Moreover, MED25 has a role in alternative splicing of JAZ proteins, which determines their sensitivity to JA (Wu *et al.*, 2020; Figure 2a). This multifaceted role makes MED25 an obvious candidate player in crosstalk regulation. Indeed, *med25* mutant studies and interaction studies of MED25 with MYC2 and ABI5 (key regulators of JA and ABA signaling, respectively) suggest that it plays a positive role in JA signaling but has a negative role in ABA signaling (Chen *et al.*, 2012).

Other Mediator subunits have also been implicated in defense hormone crosstalk. This was mostly based on mutant studies. MED14, MED15 and MED16 were found to be involved in suppression of MYC branch marker genes by SA and ET (Wang *et al.*, 2015; Wang *et al.*, 2016). This suggests that these three Mediator subunits are required for SA/JA and ERF branch/MYC branch crosstalk. In another study, MED18 and MED20 were found to be involved in the activation of transcription of *MYC2* and the MYC branch marker gene *VSP1* by *Fusarium oxysporum* and in the repression of SA pathway marker genes (Fallath *et al.*, 2017; Figure 2a). It is important to note that in all experiments the Mediator subunits were not only found to suppress a certain pathway, but also to activate another pathway that is known to antagonize the suppressed pathway. Therefore, it is possible that the repression by Mediator subunits is not a direct effect on that pathway but rather an indirect effect resulting from activation of a repressor derived from the antagonizing pathway. For example, MED16 was not only found to suppress MYC branch marker gene expression, but also to activate *ORA59* expression (Wang *et al.*, 2015; Figure 2b). ORA59 represses *MYC2* expression (see the section 'Crosstalk by binding of TFs to promoters' and Figure 2a), which may explain how MED16 causes suppression of MYC branch response genes.

Crosstalk affecting mRNA maturation and translation to protein

Above we described the different steps in initiation of transcription. The subsequent steps in gene expression are also potential points of crosstalk. For example, crosstalk may act through modulation of alternative splicing, stability of mRNA, retention of mRNA in the nucleus or translation efficiency of mRNA into protein in the cytosol. In ET and JA signaling extensive regulation of these regulatory steps is suggested by the findings that only a subset of genes that are bound by key TFs show alterations in mRNA levels (Chang *et al.*, 2013; Zander *et al.*, 2020) and that transcript and protein abundance do not match up after MeJA treatment (Zander *et al.*, 2020).

Some JAZ proteins undergo alternative splicing, potentially making them insensitive to JA-Ile-induced breakdown mediated by COI1 (Chung *et al.*, 2010). So far, no evidence for selective favoring of undegradable JAZ isoforms during SA/JA crosstalk has been found (Van der Does *et al.*, 2013). The *myc2* mutant was shown to affect phosphorylation of proteins that act in the spliceosome (Zander *et al.*, 2020). In agreement with this, the isoforms of 151 transcripts were switched after MeJA treatment (Zander *et al.*, 2020). Only two of these genes were related to JA, while the rest was

related to other processes, including ABA signaling. This suggests that MYC2 can influence other signaling pathways by modulation of transcript splicing. However, the importance of this observed JA-induced alternative splicing and the role of MYC2 in this mechanism need further investigation.

Besides the stability of proteins, the stability of mRNA molecules may also serve as a way for hormones to influence each other's pathway activities. A role for RNA-binding proteins and small RNAs (Narsai *et al.*, 2007) in determining mRNA stability during plant immune responses and root nodule symbiosis has been indicated (Staiger *et al.*, 2013; Zanetti *et al.*, 2020). For proper responsiveness to the hormone ET, the mRNA of the F-box protein-coding gene *EBF2* is targeted to decay in P-bodies (Merchante *et al.*, 2015). However, to the best of our knowledge the role of mRNA stability in hormone crosstalk has not been explored yet.

Mature mRNAs can be temporarily disengaged from the translation process by retaining them in the nucleus. There is recent evidence for a role of selective nuclear retention of mRNAs in the control of gene expression activity during adaptation to hypoxia, an abiotic stress. After reaeration the mRNAs are quickly released to the cytosol to be translated into protein (Lee and Bailey-Serres, 2019). However, there is no evidence yet that regulation of plant immunity or hormone crosstalk acts on temporary retention of mRNAs in the nucleus in order to divert all the plant's molecular attention to the most critical response.

The final step in gene expression is that of translation from mRNA to protein. Translation efficiency is influenced by different features of the mRNA (Merchante *et al.*, 2017). The literature on translational regulation of plant immunity, although scarce, points to translational control of specific mRNAs via upstream, short open reading frames (uORFs) during defense activation by the pathogen elicitors AvrRpm1 and elf18 in Arabidopsis (Pajerowska-Mukhtar *et al.*, 2012; Meteignier *et al.*, 2017; Xu *et al.*, 2017). The elicitor treatments transiently alleviate the repressive effect of the uORFs on expression of the main ORF, the heat shock factor gene *TBF1*, so that ribosomes can engage in its translation, leading to activation of the immune system. Furthermore, it has been shown that in the establishment of root nodule symbiosis, small RNAs are crucial by determining stability and translatability of mRNAs (Zanetti *et al.*, 2020). If and how control at the translation level can affect crosstalk between different hormone pathways in defense is not known yet.

CROSSTALK AT THE HORMONE HOMEOSTASIS LEVEL

The previous sections focused on crosstalk by hormones via their interference with responsiveness to other hormones, namely downstream of these other hormones. Here, we describe effects that hormones have on the levels of other hormones. For example, ABA is known to enhance the biosynthesis of JA (Adie *et al.*, 2007; Fan *et al.*, 2009; Wang *et al.*, 2018). This is correlated with the ABA-induced expression of *PLIP2* and *PLIP3*, which encode lipases that catalyze the release of polyunsaturated fatty acids (PUFAs) (Wang *et al.*, 2018), which can be further metabolized to form JA (Wasternack and Hause, 2013). In accordance, overexpression lines of *PLIP2* and *PLIP3* show enhanced JA signaling (Wang *et al.*, 2018). The ERF TF gene *ORA47* is upregulated via MYC2 by JA treatment (Zander *et al.*, 2020) and directly targets promoters of JA and ABA biosynthesis genes, which leads to enhanced JA levels, and upon wounding also to enhanced ABA levels (Pauwels *et al.*, 2008; Chen *et al.*, 2016; Hickman *et al.*, 2017; Zander *et al.*, 2020). Hence, the canonical JA pathway regulator MYC2 acts at multiple levels as an integrator of JA and ABA signaling: MYC2 is itself positively regulated by JA and ABA at the protein and gene expression level (see the sections 'Crosstalk by protein–protein interactions' and 'Crosstalk by binding of TFs to promoters') and subsequently, MYC2 regulates JA and ABA levels. Apart from activating JA biosynthesis genes, MYC2 also activates transcription of JAZ repressors, whose protein products in the long term attenuate the JA response via repression of MYC2 and other JA master regulators (Chini *et al.*, 2007). This form of short-term self-activation and long-term self-inhibition of MYC2 is reinforced by MED25. This Mediator subunit promotes looping of a MYC2 enhancer, which is also bound by MYC2 itself, to the *MYC2* promoter. For unknown reasons this leads to self-activation of the *MYC2* promoter during short-term JA responses but inhibition of the *MYC2* promoter during long-term JA responses (Wang *et al.*, 2019). Besides JA, other hormones also activate or repress transcription of different JAZ genes, which potentially modulates JA responsiveness, resulting in synergism, antagonism or reestablishment of the basal situation when both pathways are elicited in the same cell (Hickman *et al.*, 2019).

SA and JA can also influence each other's levels. RNA-seq analyses after MeJA treatment pointed to repression of JA and SA biosynthesis genes by the respective reciprocal treatments with SA and MeJA (Hickman *et al.*, 2017; Hickman *et al.*, 2019). Several underlying mechanisms for this antagonism in transcriptional activity and the resultant decrease in hormone levels have been elucidated. For example, SA inhibits activity of the catalase CAT2, which leads to reduced activity of the acyl-CoA oxidases ACX2 and ACX3, which are enzymes involved in JA biosynthesis. This effect of SA leads to lower JA levels and reduced defense against *Botrytis cinerea* (Yuan *et al.*, 2017). Additionally, WRKY51, which is transcriptionally activated by SA, inhibits JA biosynthesis by repressing transcription of the JA biosynthesis gene *AOS* (Yan *et al.*, 2018). This repression is mediated by a complex containing WRKY51,

JAV1 and JAZ8. However, during wounding JAV1 is degraded, leading to de-repression of *AOS* and increased JA biosynthesis (Yan *et al.*, 2018). *Vice versa*, JA also has the potential to reduce free SA levels. This is exploited by biotrophic pathogens to reduce effective plant defense responses. For example, *P. syringae* pv *tomato* (*Pst*) DC3000 produces the JA-Ile mimic coronatine (COR), which, via MYC2, MYC3 and MYC4, activates transcription of three NAC TFs, *ANAC019*, *ANAC055* and *ANAC072* (Zheng *et al.*, 2012; Gimenez-Ibanez *et al.*, 2017). These NAC TFs repress expression of the SA biosynthesis gene *ICS1* and activate expression of the SA methyltransferase gene *BSMT1* (Zheng *et al.*, 2012). This leads to lower levels of free SA and reduced defense against *Pst* DC3000. The bacterial effectors HopX1 and HopZ1a (see the section 'Crosstalk by modulation of protein stability') are likely to have the same effect on free SA levels as COR has. This was investigated for HopZ1a, which reduces the transcription level of *ICS1* (Jiang *et al.*, 2013). Another study found that the negative effect of MYC2 on SA accumulation is antagonized by EDS1 during ETI that is triggered by the pathogen effector AvrRps4 (Cui *et al.*, 2018; Figure 2a). This antagonism by EDS1 involves the competitive binding of EDS1 to PAD4, which otherwise binds to MYC2. This results in reduced binding of MYC2 to the *ANAC019* promoter and less *BSMT1* expression, and thus enhanced SA levels (Cui *et al.*, 2018; Bhandari *et al.*, 2019).

Research on the effect of crosstalk on hormone levels has been restricted mostly to measurements of (proven) active compounds, like JA or JA-Ile. However, the concentrations of hormone derivates, resulting from effects on biosynthesis or catabolism, will also change and these may also modulate plant responses. For example, a mutation in the JA biosynthesis gene *OPR3* that completely blocked the canonical JA biosynthesis pathway and led to accumulation of the JA precursors OPDA and dn-ODPDA enabled research on the role of these compounds in the absence of JA (Chini *et al.*, 2018). It was demonstrated that these JA precursors promote thermotolerance through a mechanism independent of COI1 in Arabidopsis as well as in a bryophyte and a charophyte alga (Monte *et al.*, 2020). Whether hormone derivates are targeted by other hormones and whether the derivates themselves could affect other hormone pathways remains to be investigated.

PERSPECTIVES

This review reports on several molecular components in hormone crosstalk that regulate plant defense responses. In most cases their regulation at the transcriptional or protein level has been demonstrated, but the exact mechanisms underlying their role in hormone crosstalk have often not been fully elucidated yet. The integration of data derived from different technologies aiming to address different regulation levels has the potential to unveil these crosstalk mechanisms in different internal and external contexts of the plant.

The network-level understanding of defense hormone crosstalk as a whole is still rudimentary. Until now, most research has been restricted to the use of hormone mutants, single hormone applications, or TF–DNA binding studies under control conditions. These systems approaches gave us a little glimpse of regulatory nodes in hormone signaling networks and their possible role in hormone crosstalk. However, addition of multiple hormones and the integration of multiple data types regarding different levels of regulation are crucial to unveil new crosstalk mechanisms. Such multiomics research is now possible thanks to modern wet-lab technologies as well as advanced data analysis and modeling tools (Zander *et al.*, 2020). A network-level understanding of crosstalk is also necessary to ultimately grasp the impact of hormone signal integration under different conditions for the plant. Therefore, we need to learn not only the 'how', but also the 'when' and 'where' of hormone crosstalk. In addition to interactions with microbes and insects, other environmental conditions of the plant and the internal context in the plant determine its hormone balance and hormone responsiveness (Figure 1). Indeed, the extent of crosstalk between hormone pathways during immune responses has been shown to be influenced by additional stresses, location of the stimulus, and plant age. Likely, different crosstalk mechanisms, such as described above and below in this review, are engaged in different situations and are regulated in a spatiotemporal manner.

The order in which sequential stresses occur and the nature of the stresses determine whether hormone crosstalk is effective. For example, for primed expression of JA-mediated defenses in systemic tissue that expresses *MYC2* after local herbivory by caterpillars of *Pieris rapae*, the ABA pathway needs to be activated by a secondary infestation (Vos *et al.*, 2013b). In contrast, ABA can inhibit the JA pathway in tissue that is primed for dehydration stress: A first experience of dehydration stress leads to induction of the JA pathway, but this does not occur during a second dehydration stress (Ding *et al.*, 2013), which is likely due to a lack of *MYC2* activation during the second stress if ABA levels had already increased previously (Liu *et al.*, 2016b; Avramova, 2019). An RNA-seq experiment studying responsiveness to the sequential stresses drought, herbivory and infection showed that the transcriptome profiles during the sequential stresses rapidly change to largely resemble those of the last stress (Coolen *et al.*, 2016). This shows that many crosstalk mechanisms can be overridden by a second, dominating stress. Nevertheless, it was also found that the first stress leaves a relatively small expression signature, which is enriched for hormone signaling genes, suggesting a lasting effect of induced hormone signaling by the first stress.

A spatial separation between SA and JA signaling has been demonstrated when a local hypersensitive response (HR) is activated during ETI as a result of infection with the avirulent pathogen *Pst* DC3000 carrying AvrRpt2. The zone around the HR-induced cell death is surrounded by a small layer where the SA marker gene *PR1* is highly expressed, followed by a region where the JA marker gene *VSP1* is highly expressed. This demands differential prioritization of SA versus JA antagonism mechanisms. In the SA zone SA-mediated defenses against *Pst* DC3000 AvrRpt2 can be activated, while in the JA zone runaway cell death and secondary infections by necrotrophic pathogens are limited, which could otherwise take advantage of the dead tissue generated by the HR response (Betsuyaku *et al.*, 2018).

Leaf age is another factor that can influence hormone crosstalk. Biotic and abiotic stress responses are differently balanced in older leaves compared to younger leaves (Berens *et al.*, 2019). Abiotic stress suppresses immune responses in older leaves through ABA. This antagonistic effect on immunity is blocked in young leaves, which is dependent on NPR1 as well as on the SA biosynthesis component PBS3, but independent of ICS1. This suggests an SA-independent function of NPR1 and PBS3 in regulating leaf age-dependent crosstalk (Berens *et al.*, 2019).

The above examples illustrate that knowing the 'when' and 'where' of hormone pathway integration in immune networks is important to predict the outcome of immune signaling. To advance our knowledge, future research should focus on the different levels of hormone network regulation under different internal and external conditions. For such biological experimental systems, it would be even more meaningful to use single-cell methods instead of bulk analyses. This will provide a better spatiotemporal resolution, which is particularly powerful when studying plant–microbe interactions, where relevant molecular events are often restricted to localized cell populations (of specific cell types), ranging from being infected themselves, to residing in the same leaf or in distant tissue and not (yet) being infected. Moreover, information on the single-cell level will increase the accuracy of network predictions, as the measured responses are derived from one cell and not diluted by bulk analyses of multiple cells. This will further increase our knowledge of the 'how' of hormone pathway integration. Together, this molecular- and systems-level knowledge is crucial to design crops with a strengthened immune response without undesired side effects like enhanced sensitivity to other stresses or decreased plant growth and yield.

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AUTHOR CONTRIBUTIONS

NA and SCMW wrote the manuscript and designed the figures. MPM helped design the figures and substantially contributed to conception of the paper.

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