Flooding resilience is determined by leaf age in *Arabidopsis thaliana*

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Flooding tolerance is determined by leaf age in Arabidopsis thaliana

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(met een samenvatting in het Nederlands)

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Chapter 1

Introduction

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Flooding poses a threat to crop yields

The occurrence of weather extremes is increasing due to climate change (Stott, 2016). This includes flooding caused by extreme precipitation events, which have increased in frequency in recent years (Coumou and Rahmstorf, 2012; Alfieri et al., 2015). Flooding of agricultural areas is typically caused by local precipitation excesses or by flooding of river basins or coastlines. This is a major cause of crop losses worldwide, and can severely limit the yield of an agricultural system (Bailey-Serres et al., 2012, 2019; Reyes and Elias, 2019; Venkatappa et al., 2021). The term flooding can refer to multiple types of stress (Sasidharan et al., 2017). Flooding of the root system is called waterlogging. Partial or complete submergence refers to situations where the shoot system is also submerged, and this is typically the most severe stress for a plant. The risk of complete submergence is greatest in small seedlings, and in plants with a rosette habit like *Arabidopsis thaliana*, beet, and lettuce. Developing new crop varieties with greater flooding tolerance is essential to reduce flooding-induced yield losses, and promising quantitative trait loci have been identified in multiple species (Mustroph, 2018). Of all major crops rice is the most tolerant to partial submergence, and tolerance of rice to complete submergence has been greatly improved by introgressing the *SUB1* locus from wild rice into commercial cultivars (Bailey-Serres et al., 2010). This locus contains the submergence tolerance gene *SUB1A*. SUB1A is a transcription factor that induces a quiescence strategy involving conservative energy usage and enhanced reactive oxygen species (ROS) amelioration which boosts submergence survival. However there are few examples of similar success stories of improved tolerance to complete submergence in other crop species beyond rice (Mustroph, 2018).

Flooding is a combination of multiple stresses

Flooding is a compound stress, in which several stressors converge on a plant. To aid survival, affected plants must respond to these stresses at the optimal moment and in relevant tissues. The limited gas diffusion under flooded conditions and reduced photosynthesis due to muddy floodwaters reducing light reaching a plant, lead to the onset of hypoxia in flooded tissues. Hypoxic conditions severely limit ATP production via oxidative phosphorylation. Aerobic respiration of glucose normally yields 30-36 moles of ATP. The switch to anaerobic metabolism upon flooding-induced hypoxia limits this to only 2-4 moles of ATP per mole of glucose (Bailey-Serres and Voesenek, 2008). The combination of impaired photosynthesis and a reduced efficiency in using existing carbon reserves due to hypoxia leads to severe energy starvation during flooding. The anaerobic production of ethanol leads to carbon loss but is unlikely to cause injury to plants as ethanol concentrations typically remain low (Jackson et al., 1982). Rather, the energy starvation coupled with cytoplasmic acidosis due to acids leaking from the vacuole seems to play a bigger role in plant mortality upon flooding (Roberts et al., 1984; Loreti et al., 2016). Flooded soils are also rapidly depleted of oxygen, a process escalated by the activity of soil microorganisms (Francioli et al., 2021). The respiration of these microorganisms further decreases the oxygen content of the soil, exacerbating the severity of the stress upon a plant.

Another consequence of limited gas diffusion upon flooding is the rapid accumulation of the volatile hormone ethylene. This accumulation of ethylene is one of the first signals that a flooded plant senses. Ethylene triggers several physiological and morphological adaptations to flooding, like hyponasty in Arabidopsis and *Rumex palustris* (Sasidharan and Voesenek, 2015). In response to ethylene accumulation during flooding, rice induces the formation of aerenchyma tissue in the root and stem cortex, which improves internal aeration (Yamauchi and Nakazono, 2022; Steffens et al., 2011). Several mechanisms exist to overcome the reduction in gas exchange between the flooded plant and its environment. Rice leaves have a hydrophobic wax layer, which upon submergence, causes a retention of surface gas films which enhances underwater gas exchange and photosynthesis (Pedersen et al., 2009; Kurokawa et al., 2018).

Ethylene also induces the expression of the nitric oxide (NO) scavenger *PHYTOGLOBIN1*, which leads to NO depletion. NO depletion stabilizes the group VII ETHYLENE RESPONSE FACTORs (ERFVIIs), which are essential regulators of the low-oxygen response (Hartman et al., 2019; Gibbs et al., 2011; Licausi et al., 2011). ERFVIIs induce the transcription accumulation of enzymes involved in hypoxic metabolism, like *ALCOHOL DEHYDROGENASE1* (*ADH1*), *PYRUVATE DECARBOXYLAXE1* (*PDC1*) and *PDC2* (Paul et al., 2016; Yang et al., 2011a).

The post-submergence phase is a second round of compound stress

The end of submergence is not yet the culmination of a stressful period for a submerged plant. Instead it marks the transition to the post-submergence phase which presents another set of stresses (Yeung et al., 2019). Sudden reillumination and reoxygenation of plants already stressed by submergence will lead to a burst in ROS production. Damaged light harvesting complexes and saturated mitochondrial electron transport chains (mETCs) leak electrons, which leads to ROS formation (Pospíšil, 2016; Chang et al., 2012). Plants can use the alternative mETC, which contains type II NAD(P) dehydrogenases (NDs), to bypass the saturated mETC and prevent excessive ROS production. The expression of *NDs* is induced upon reoxygenation by the transcription factors ANAC016 and ANAC017, which improves hypoxia tolerance (Jethva et al., 2022). Furthermore, ROS production is

increased during anoxia and during the post-submergence phase by the NADPH oxidase RESPIRATORY BURST OXIDASE HOMOLOGUE D (RBOHD) (Yeung et al., 2018; Pucciariello et al., 2012). Interestingly, chemical or genetic inhibition of post-submergence ROS production was demonstrated to improve post-submergence recovery of an Arabidopsis accession that accumulated excessive ROS upon desubmergence but not that of another accession with much lower ROS production (Yeung et al., 2018). This suggests that although excessive ROS production is harmful during the post-submergence phase, some controlled ROS production is essential, possibly via a signaling role. To reduce the damage caused by excess ROS production in the post-submergence phase, the transcription factor MYB30 induces the expression of the antioxidants *VITAMIN C DEFECTIVE1* (*VTC1*) and *GLUTATHIONE SYNTHETASE1* (*GSH1*) (Xie et al., 2022).

The post-submergence phase not only involves potential reillumination stress for the shoot, it also entails a transition from a wet to dry environment. Although the root systems of desubmerged plants still have access to water, low root hydraulic conductivity coupled with the high transpiration in the leaves ultimately leads to rapid dehydration. This reduced water uptake has been linked to a ROS-mediated repression of aquaporins, which occurs in roots of *Brassica napus* during the post-submergence phase (Liu and Zwiazek, 2022). To limit this post-submergence dehydration, a plant must be able to rapidly close its stomata. Although stomatal closure is promoted by ABA, an ABA-regulated mechanism has been demonstrated to do the opposite. Expression of the protein phosphatase 2C *SENESCENCE-ASSOCIATED GENE 113* (*SAG113*) is increased by ABA and leads to stomatal closure in the submergence-sensitive Arabidopsis accession Bay-0 but not in the submergence-tolerant Lp2-6 (Yeung et al., 2018). *SAG113* is a senescence-associated gene that by triggering stomatal opening not only promotes water loss in Bay-0 during the post-submergence phase, but also during natural leaf senescence (Zhang et al., 2012). If senescence has been initiated during submergence, this will often further progress during the post-submergence phase. Reducing senescence during this phase by knocking out the NAC domain transcription factor *ORESARA1* (*ORE1*) has been shown to improve submergence tolerance (Yeung et al., 2018).

The response to flooding depends on the developmental state of a plant and its tissues

Plants are complex multicellular organisms that, unlike mammals, do not have a predetermined body plan. This helps to compensate for their sessile lifestyle since it permits greater plasticity in adapting to environmental changes. This increased plasticity encompasses the ability for differential stress responses within the same plant, where the optimal response is determined by organ or tissue identity, age, and the developmental state of the entire plant. Changes occurring during plant and organ development strongly influence the way a plant interacts with its environment. Age-related resistance (ARR) to pests and pathogens (also called ontogenic resistance) is a well-established phenomenon and generally increases with age, at both the whole-plant and organ level (Hu and Yang, 2019; Develey-Rivière and Galiana, 2007). However, age-related changes in abiotic stress resilience although equally common, are not as well studied. Here the current knowledge on age-dependent flooding stress resilience of leaves is discussed, and how this largely depends on morphological plasticity, the onset of premature leaf senescence, ROS homeostasis, and changes in stomatal conductance.

Morphological plasticity

The capacity for stress-induced plastic morphological responses depends on the leaf developmental age. However, these responses are also strongly related to the age of the whole plant. When deepwater rice is confronted with flooding, it elongates its internodes in an attempt to grow above water, a trait common to many amphibious plant species (van Veen and Sasidharan, 2021). Elongation of the youngest internodes can only be induced when at least six leaves have been formed (Ayano et al., 2014). Similar patterns are observed for the submergence-induced petiole elongation of *Rumex palustris*, where older plants display more vigorous elongation of the youngest leaves (Groeneveld and Voesenek, 2003; Huber et al., 2012). Breaking down older leaves is essential to fuel the high carbon and energy requirement of strong underwater petiole elongation (Groeneveld and Voesenek, 2003). Having convertible biomass available can be advantageous to frequently flooded species, since larger individuals endure flooding stress longer (Nabben et al., 1999).

Flooding-induced elongation can often be linked to age-dependent molecular and hormonal signatures. For example, during salt (El-Iklil et al., 2000) and drought stress (Abou Hadid et al., 1986) in tomato, ethylene-induced epinasty appears stronger in young leaves, while ethylene production is higher in older leaves. Waterlogging stress, unlike salt and drought stress, induces a stronger epinasty of old leaves than young leaves (Geldhof et al., 2022). During flooding, petiole elongation occurs via the ethylene-induced shade avoidance machinery (van Veen et al., 2013). Although ethylene entrapment and accumulation occurs in all submerged tissues, underwater elongation occurs primarily in younger tissues (Jackson, 2008; Alpuerto et al., 2022). Both examples show a discrepancy between the plastic morphological changes during stress and actual ethylene levels or sensitivity, pinpointing toward an age-specific regulation of petiole epinasty and elongation.

Aging-related quantitative differences can be caused by resource availability. For example, young leaves of both young and old plants display flood-induced petiole elongation, the magnitude of which depends on resource availability (Groeneveld and Voesenek, 2003). Ethylene is also key to rice internode elongation. In young plants, a developmental brake blocks elongation, independent from resource availability or ethylene levels. Underwater internode elongation of older plants phenotypically resembles the microRNA172 and GA-regulated flowering-induced internode elongation (Patil et al., 2019). Internode elongation in deepwater rice is regulated by the GA-responsive gene *ACCELERATOR OF INTERNODE ELONGATION 1* (*ACE1*) (Nagai et al., 2020). Paddy rice, which lacks a functional *ACE1* allele, never elongates during submergence, whereas deepwater rice is able to elongate after it has reached the four-leaf stage. The mechanisms underlying *ACE1*-mediated internode elongation have not yet been identified, but its function is conserved among monocots.

Stress-induced senescence

Abiotic stresses typically accelerate leaf senescence, with older leaves being the first to show visible signs of chlorosis. Several factors are implicated in accelerating senescence in older leaves. ROS levels typically increase upon exposure to abiotic stress, causing double-stranded breaks (DSBs) in DNA (Hu et al., 2016). Aging leaves lose their ROS scavenging and DSB repair capacities, resulting in accumulation of DSBs in older leaves during stress, which leads to senescence. Furthermore, ROS/DSB-induced senescence is prevented in young leaves by ATAXIA TELANGIECTASIA MUTATED (ATM), which serves as a transducer between sensors of DSBs and chromatin remodelers that repress the induction of senescence-associated transcription factors (Li et al., 2020a). Expression of ATM gradually decreases as leaves age. This, in combination with a reduced DSB repair capacity, might make older leaves more sensitive to stress-induced senescence.

Senescence induced by abiotic stress typically involves increased expression of NAC domain transcription factors (Kim et al., 2016). For example, darkness-induced senescence is regulated by stay-green-related NAC ANAC032 and ANAC046, whereas ANAC083/VNI2 and ANAC092/ORE1 are involved in salt-induced senescence (Mahmood et al., 2016; Oda-Yamamizo et al., 2016; Yang et al., 2011b; Balazadeh et al., 2010). However, a subset of NACs can also function as senescence brakes by inhibiting the expression of other NACs during leaf development (Kim et al., 2018). The mechanisms controlling age-related expression remain unidentified for most NACs. An exception is the mechanism of ORE1, where high miR164 levels degrade *ORE1* mRNA in young leaves, linking susceptibility to stress-induced senescence to the genetic regulation of leaf development (Kim et al., 2009).

One of the major hormones regulating the onset of senescence is ethylene. Whether or not exposure to ethylene induces senescence in a leaf largely depends on the age of a leaf (dela Fuente and Leopold, 1968; Jing et al., 2005). As a leaf ages, transcript levels of the ethylene signaling integrator *ETHYLENE INSENSITIVE3* (*EIN3*) gradually goes up (Li et al., 2013). This increases the sensitivity of older leaves to ethylene exposure, which triggers a stronger induction of senescence-associated genes in old leaves (Li et al., 2013; Qiu et al., 2015). This, together with the lifting of the repression of *ORE1* by miR164 with leaf age, contributes to the age-dependent sensitivity of leaves to ethylene.

The accumulation of ethylene in flooded plant tissues not only activates responses that help keep tissues alive, it can also induce leaf senescence. In rice, treating seedlings with the ethylene perception inhibitor 1-methylcyclopropene (1-MCP) reduces the expression of chlorophyll-degrading enzymes during submergence (Ella et al., 2003). In tomato, partial submergence leads to a reduction in chlorophyll content and photosynthetic activity, this reduction is smaller in ethylene-insensitive plants (De Pedro et al., 2020). There are examples in several plant species where reduced leaf senescence during flooding stress and the post-submergence phase correlates with an increased flooding tolerance (Alpuerto et al., 2016; Krishnan et al., 1999; Yeung et al., 2018; Campbell et al., 2015; Buraschi et al., 2020). In Arabidopsis, *ORE1* expression remains high during the post-submergence phase in the sensitive accession Bay-0 whereas its expression is low in the flooding-tolerant accession Lp2-6. The high expression of *ORE1* is controlled by ethylene signaling, as application of the ethylene perception inhibitor 1-MCP reduces the expression of *ORE1* in Bay-0 and improves its flooding tolerance.

Senescence has the potential to recycle valuable nutrients and, therefore, stress-induced senescence of older leaves could provide for younger leaves and the meristem during stress. However, this has not yet been unequivocally established. Arabidopsis expressing the ABA receptor *PYL9* under the stress-responsive *RD29A* promoter showed increased expression of senescence-associated genes and sugar transporters in older leaves. This coincided with elevated solute concentrations in younger leaves, suggesting that carbon resources were being remobilized from senescing older leaves toward younger leaves to enhance resilience (Zhao et al., 2016). By contrast, several studies showed that knocking out senescence-activating NACs or overexpressing senescence-repressing NACs limited senescence yet improved stress tolerance (Yeung et al., 2018; Yang et al., 2011b; Sakuraba et al., 2014; Lee et al., 2012; Wu et al., 2012). This variation in the effect of delayed senescence (stay-green phenotypes) on stress tolerance is not limited to Arabidopsis, but is

also observed in (monocot) crops. There are numerous examples where delaying senescence improves stress tolerance, but there are also cases where this reduces stress tolerance (Gregersen et al., 2013). These studies suggest that maintaining older leaves to act as source tissue upon stress recovery is more valuable than sacrificing leaves to mobilize nutrients toward sink tissue during stress. Ultimately, stress severity, and the age at which the point of no return toward senescence occurs, could determine the success of either strategy.

ROS detoxification

ROS management strategies vary with leaf and plant age. During bolting, and especially during senescence, leaves partially lose their scavenging potential against ROS, resulting in their accumulation (Zimmermann et al., 2006). However, this process is carefully regulated in time and is related to the degeneration of the photosynthetic machinery and its protection against photo-oxidative damage (Juvany et al., 2013). An important regulator of the H_2O_2 pool is glutathione reductase, encoded by two genes (*GR1-2*) in Arabidopsis. *GR2* expression increases in mature leaves (Ding et al., 2016b), which protects photosystem II (PSII) from H_2O_2 damage caused by excess light (Ding et al., 2016a). By contrast, newly emerging leaves are more susceptible to photoinhibition because their PSII is still being assembled (Juvany et al., 2013; Jiang et al., 2005). To cope with the excess of energy, the alternative xanthophyll cycle-mediated non-photochemical quenching (NPQ) is still highly active in young soybean (*Glycine max*) leaves, protecting them from photodamage caused by ROS (Jiang et al., 2005). In addition, multiple antioxidant enzymes, such as superoxide dismutase, peroxidase, ascorbate peroxidase, and glutathione reductase, appear to be relatively more active during early stages of leaf development (Jiang et al., 2005). However, this age-related protection is highly species-specific and other factors might come into play during different adverse conditions. In the case of cadmium toxicity, young soybean leaves can no longer protect their photosynthetic apparatus from ROS damage (Xue et al., 2014). In Arabidopsis, drought appeared to protect older leaves more from ROS damage by means of higher NPQ, upregulation of antioxidant enzymes, and production of antioxidants (Jung, 2004). By contrast, young leaves maintained their Fv/Fm and prevented photo-oxidative stress via alternative strategies, such as light shielding by anthocyanins (Jung, 2004). By comparison, oxidative damage associated with chilling stress was more severe in young leaves of cucumber (*Cucumis sativus*) (Zhang et al., 2014) and tea plants (*Camellia sinensis*) (Li et al., 2018a). These examples demonstrate that redox homeostasis undergoes an age-specific regulation and influences the fate and survival of leaves under certain abiotic stress conditions. Through the integration of these ROS signals with hormonal pathways, plants can alter their growth and adjust certain morphological features to facilitate abiotic stress resilience (Potters et al., 2009). The ability to handle the ROS burst induced by reoxygenation depends on the antioxidant status of the leaf before and during submergence. There is age-dependent variation in the ROS detoxification capacity of a leaf, and although little is known on the degree to which this plays a role during submergence stress, it is likely to be involved here as well given the importance of ROS management during the post-submergence phase (Yeung et al., 2018).

Changes in stomatal conductance

As a leaf expands, stomatal density decreases and substomatal cavities still continue to expand during later stages. The resulting microstructure determines leaf gas exchange with the environment (Baillie and Fleming, 2020; Lundgren et al., 2019). Leaf transpiration also depends on the leaf cuticle, which acts as a boundary layer and thickens as leaves age (Pantin et al., 2012). The biosynthesis and composition of this protective layer vary with leaf age and are regulated by factors such as abscisic acid (ABA) (Martin et al., 2017). Water loss of a leaf in a fluctuating environment is largely controlled by the regulation of stomatal aperture. The transcription factor AtNAP can induce expression of *SAG113*, which negatively regulates ABA-mediated stomatal closure in older leaves, thus leading to higher water loss and accelerated senescence (Zhang et al., 2012; Zhang and Gan, 2012). In addition, a higher production of ethylene during senescence antagonizes ABA-mediated stomatal closure (Watkins et al., 2014), potentially explaining the reduced sensitivity to ABA-mediated stomatal closure observed in older wheat (*Triticum aestivum*) leaves (Chen et al., 2013). These two age-related mechanisms might partly explain the discrepancy between increasing ABA levels (Breeze et al., 2011) and reduced stomatal closure in older leaves. During the post-submergence phase, plants often experience dehydration of their leaves (Yeung et al., 2019). This dehydration is controlled by ABA signaling, as ABA-mediated stomatal closure is essential in reducing water loss from the leaves after desubmergence (Yeung et al., 2018). Although little is known on how this varies with leaf age, given the strong age-dependency of ABA responses it is likely that the age of a leaf and its developmental state play a role here.

Outline of this thesis

Stress resilience varies with plant age, tissue identity, and between leaves of a different age on the same plant. This is also the case for flooding stress. As flooding is a compound stress, age-dependent responses to this stress are likely the result of a combination of age-dependent responses to component stressors.

Well-characterized responses to flooding and the post-flooding phase, like petiole elongation, aerenchyma formation, leaf senescence, and stomatal movement are all subject to tissue- and age-dependent regulation. However, while the regulation of these traits during flooding stress is well-studied, less is known on how these traits are differentially regulated throughout the plant.

The overarching goal of this thesis was to investigate the mechanisms mediating differential flooding resilience of leaves of different ages. The model plant species *Arabidopsis thaliana* was used as it has a short generation time, an extensively characterized genome, and a large collection of available mutant lines. Furthermore, previous research using natural accessions of Arabidopsis which vary in their submergence tolerance has already yielded valuable insight in how flooding stress responses are regulated in this species (Vashisht et al., 2011; Yeung et al., 2018). When submerged, Arabidopsis plants exhibit sequential leaf death throughout their rosette: the old leaves are the first to die and the young leaves and the shoot apical meristem are the last. This characteristic phenotype provided an ideal experimental system to probe age-dependent submergence resilience mechanisms.

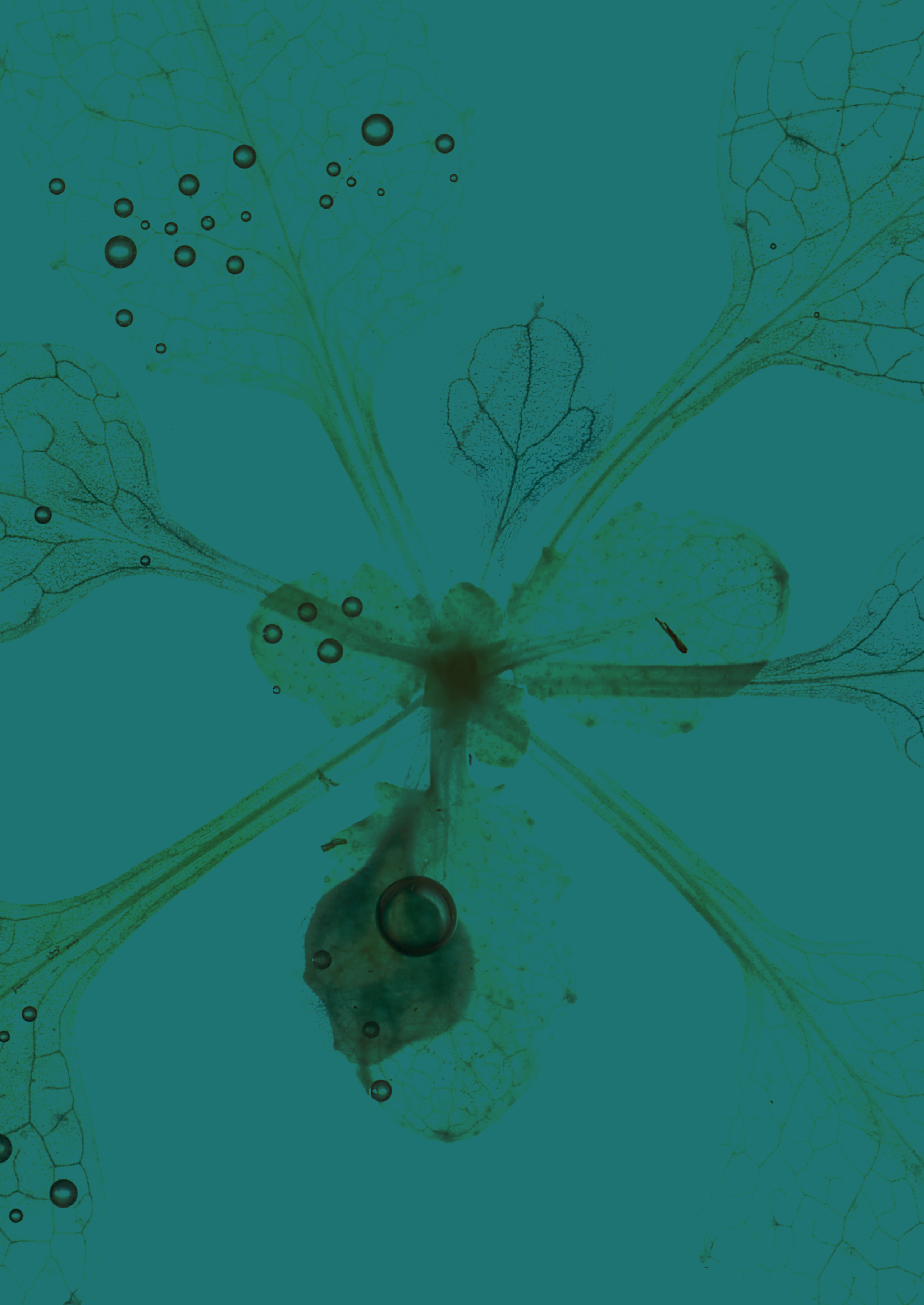
In **Chapter 2**, mRNAseq was used to characterize and compare the transcriptomes of old and young Arabidopsis leaves before, during, and after submergence. This unbiased approach identified the age-dependent transcriptional regulation of many genes involved in processes that have previously been associated with flooding and the post-submergence phase, like carbon starvation, ethylene signaling, and ABA signaling, as well as newly identified processes like endoplasmic reticulum stress.

One of the processes identified in Chapter 2 and governed by leaf age, was the stronger induction in old leaves of ABA signaling during the post-submergence phase, which is explored further in **Chapter 3**. Older leaves experienced more dehydration following desubmergence, which was in part due to a reduced sensitivity of their stomata to ABA. Although transcriptome data from whole leaves suggested that old leaves responded more strongly to ABA during the post-submergence phase, their stomatal physiology did not reflect this.

As an established principle regulator of submergence responses, the role of ethylene in age-dependent resilience was also probed. Whereas wild-type Arabidopsis plants showed a clear gradient in age-dependent leaf senescence during flooding stress, this was lost in ethylene-insensitive mutants. The consequences of this perturbed gradient in leaf senescence are explored in **Chapter 4**. Ethylene induced senescence via the NAC domain transcription factor ORE1, which accumulated independently of leaf age during submergence. Knocking out *ORE1* reduced senescence of old leaves and improved plant performance under flooding stress conditions.

Although ethylene accumulation during submergence induced a systemic induction of ORE1, ORE1-mediated senescence was always initiated in the old leaves. The mechanism controlling the age-dependent activity of ORE1 was further investigated and is described in **Chapter 5**. Knocking out *ORE1* had a stronger effect on the transcription of its target genes in old leaves than in young leaves. This age-dependent ORE1 activity was controlled by its age-dependent phosphorylation during submergence. Ethylene induced age-dependent phosphorylation of ORE1 and thereby limited its activity to old leaves, which contributed to a gradient in senescence with leaf age.

The results of this thesis help in understanding the variation in flooding tolerance observed with leaf age in Arabidopsis. **Chapter 6** summarizes these findings, discusses their potential applications in developing flooding-tolerant crop varieties, and highlights some promising hypotheses for future research.



Chapter 2

Transcriptomic characterization of leaf age-dependent responses to submergence and recovery

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Abstract

The responses and resilience to abiotic stress is highly dependent on leaf age. This is evident in submerged Arabidopsis rosettes where older leaves are the most stress sensitive. Here, mRNA sequencing was used to characterize the underlying leaf age-dependent transcriptomic responses to submergence and recovery and characterize potential determinants of age-dependent tolerance. The third and seventh leaf of a 10-leaf Arabidopsis plant were used as a proxy for old and young leaves. Leaf blades were harvested at several submergence and recovery time points. This permitted assessment of the effects of submergence and recovery separately, together with the effect of the sequential exposure to these phases. Most age-dependent differences in gene expression were in the magnitude or speed of changes in transcript abundance, rather than the direction in which the abundance of transcripts was induced. During the submergence phase, senescence-related processes were induced faster in old leaves, and eventually also in young leaves after prolonged submergence. Processes related to growth and photosynthesis were systemically repressed in the submergence phase but were resumed faster in young leaves during recovery. Age-dependent changes in the expression of transcription factors did not always reflect changes of their target genes, suggesting extensive age-dependent regulation of transcription factors beyond their mRNA levels. This dataset provides a framework to dissect the molecular mechanisms of how the response to flooding stress is regulated in a leaf age-dependent manner.

Introduction

Complete submergence exposes the entire plant to the stress (Sasidharan et al., 2017). The aerial parts of a plant consist of a heterogeneous collection of different organs, each composed of various cell types. The distinct identity of plant tissues can cause substantial differences in stress sensing and response. This variation can be a consequence of differences in physical properties, but also due to differences in the molecular regulation of stress responses and can ultimately lead differential stress resilience (Rankenberg et al., 2021). Previous research has demonstrated both shared and unique responses to hypoxia and submergence stress between different cell types. For example, profiling of 21 different cell types of Arabidopsis seedlings exposed to hypoxia has shown that production of mRNAs encoding heat shock proteins is specifically induced in phloem companion cells (Mustroph et al., 2009). The development of aerenchyma in maize roots during waterlogging is limited to cortical cells. Waterlogging induces the expression of cell wall modifying enzymes, but only in the root cortex and not in the stele (Rajhi et al., 2011). Submergence can also elicit organ-specific responses. Roots and shoots of different Arabidopsis accessions (van Veen et al., 2016) displayed both unique and shared responses to complete submergence. Organ-specific responses included changes in the expression of development-associated transcripts in shoots, and surprisingly the transcription of photosynthesis-associated genes in roots (van Veen et al., 2016). These studies compared cell types or organs that fulfill very different roles within a plant. Root cortex cells, for example, provide structural support and store starch, whereas the stele is involved in transport of water and nutrients.

However variation in submergence responses can also be determined by plant age. Submergence tolerance was found to decline as an Arabidopsis plant aged from two-week old seedlings to five-week adult plants. This decrease in tolerance stemmed from a reduction in the ability of ERFVIIs and the NAC transcription factor ANAC017 to activate their targets (Giuntoli et al., 2017; Bui et al., 2020). Another study on Arabidopsis seedlings found that the hypoxia tolerance of root tips decreased with age from four days old to seven days old, via an unknown mechanism (Liu, 2019). During submergence, the activity of the ERFVII RAP2.12 is limited by the trihelix transcription factor HYPOXIA RESPONSE ATTENUATOR1 (HRA1) (Giuntoli et al., 2014). HRA1 binds to RAP2.12 and reduces its activity as a transcriptional activator, which attenuates the induction of anaerobic metabolism by RAP2.12. The repression of RAP2.12 activity by HRA1 is strongest in young leaves and meristems, where it likely serves to limit the utilization of carbon resources during hypoxia. Limiting carbon consumption during hypoxia is not only beneficial to preserve energy for post-stress recovery but also prevents premature activation of metabolic adjustments to hypoxia, since meristems are already hypoxic under non-flooded conditions (Weits et al., 2019).

These studies have thus clearly demonstrated how differential regulation of principle hypoxia response regulators such as ERFVIIs, HRA1 and ANAC017 lead to age-associated changes in submergence tolerance. However, since flooding is a compound stress during which multiple signals converge on a plant (Sasidharan et al., 2018), it could be that transcription factors associated with age-dependent tolerance to other stresses also play a role in age-dependent flooding tolerance. The decrease in flooding tolerance with age is observed for both whole plants and individual leaves. Changes in stress tolerance of tissues with age can simply be a consequence of changes in physiology but can also be an actively regulated process. Given that several specific transcription factors have recently been linked to changes in age-dependent stress tolerance (D'Alessandro et al., 2018; Zhao et al.,

2022) suggests that age-dependent stress responses are often actively regulated at a molecular level.

A commonly found pattern in age-dependent stress responses is a decrease in stress tolerance with leaf age (Rankenberg et al., 2021). This can be caused by the inability of old leaves to properly respond to a stress (D'Alessandro et al., 2018), but it can also be caused by the faster induction of stress-induced senescence in old leaves (Schippers, 2015). Actively allowing an old leaf to die by speeding up the onset of senescence while a young leaf remains alive can be beneficial to a stressed plant, as nutrients can be transported from dying old tissues to young tissues and meristems to fuel their survival (Yu et al., 2015). Prioritizing the survival of more expendable tissues to keep meristems alive could permit plant reproductive success during stressful conditions. The priorities set by a plant might, however, not be the ones that are most desirable from a human perspective. Whether or not exposure to environmental stress leads to leaf senescence depends on the age of the leaf. As a leaf ages it acquires the competence to senesce, although few mechanisms controlling this competence have been identified (Schippers, 2015). Understanding how plants respond to stress in an age-dependent manner is essential for developing crops that minimize yield losses under stressful conditions.

Despite the observed age dependence of stress tolerance in many species, the underlying molecular mechanisms have been underexplored. Most studies tend to neglect the age dimension focusing instead on bulk tissues where such differences might be lost. Here we capitalized on the very clear age directed senescence gradient induced in Arabidopsis leaves upon complete submergence.

To identify the processes and regulators that play a role in this age-dependent response to submergence stress, we used mRNAseq to characterize and compare the transcriptomes of an old and a young leaf of Arabidopsis to both complete submergence and the post-submergence phase. Since old leaves show more severe visual signs of stress upon submergence stress, we expected a stronger increase in stress-associated genes in old leaves. In young leaves on the other hand, we hypothesized a stronger activation of stress protective mechanisms.

Our results provide an overview of the age-dependent and -independent transcriptional responses of Arabidopsis leaves to submergence and desubmergence. Shared transcriptional responses to submergence between old and young leaves included the general repression of translation and growth. Enriched in old leaves was the increased expression of genes associated with leaf senescence, during both the submergence and post-submergence phase. However, prolonged submergence treatment eventually also induced the expression of senescence associated genes in young leaves. Young leaf- specific responses included a stronger induction of some immunity-related processes during submergence than old leaves. Some responses were specific to the post-submergence phase. This included a strong desiccation transcriptome signature in old leaves and a stronger induction of endoplasmic reticulum (ER) stress genes in young leaves.

Results

Submerged Arabidopsis rosettes show a pattern of age-dependent sequential leaf death

As the duration of a submergence treatment increased, leaves went into senescence. This was observed in rosettes of Arabidopsis Col-0 plants submerged in darkness for varying durations (Figure 1A). Visible senescence and leaf death was initiated in the oldest leaves and progressed towards younger leaves. Plants recovering from sub-lethal submergence durations showed further deterioration following desubmergence. Senescence and leaf dehydration were accelerated in the post-submergence phase, being more severe in old and intermediate leaves than in young leaves (Figure 1B). The age-dependent effects of the submergence and post-submergence phases led to age-dependent leaf death: the old leaves died faster than young leaves (Figure 1C). To characterize the molecular responses and potential regulatory genes and processes underlying this differential age effect, an mRNAseq approach was used. Blades of leaf three and seven of a 10-leaf Arabidopsis plant were sampled at eight timepoints (Figure 1D). Leaf three was chosen as a representative 'old' leaf. It looks like most other rosette leaves unlike leaves one and two, which have a rounder shape and die faster than the rest (Figure 1C). Leaf number seven was the representative 'young' leaf. It is the youngest leaf at this growth stage that has an easily distinguishable petiole. Despite their physiological similarities, these two leaves died at different rates following submergence (Figure 1C). Sampling for mRNAseq occurred before submergence (t0_00), after two (t2_00), four (t4_00), and six (t6_00) days of submergence in complete darkness. Leaf samples were also harvested after four days of submergence at one (t4_01), three (t4_03), six (t4_06), and twenty-four (t4_24) hours in the light after desubmergence (Figure 1D). Only the young leaf was sampled at the six days submergence timepoint, as the old leaf was often dead at this time. Gene expression was calculated at: each submergence timepoint compared to the non-submerged plants (the submergence effect); each post-submergence phase time point relative to four days submerged plants (the

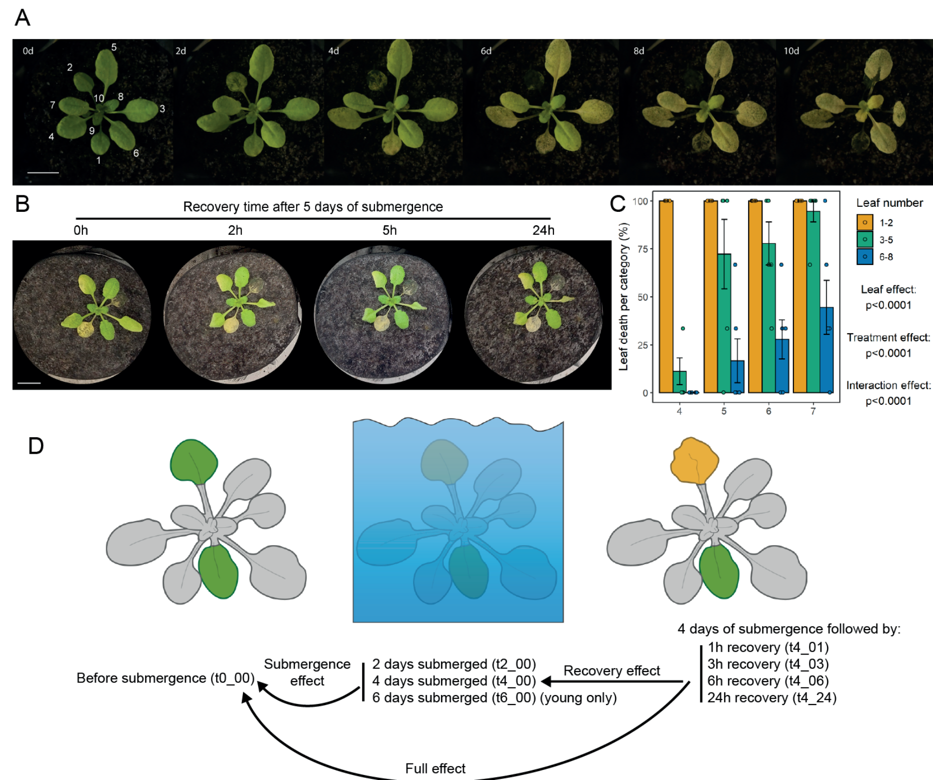


Figure 1 | Rosettes of submerged Arabidopsis plants show an age-dependent sequential leaf death pattern

A) **Arabidopsis wild type (accession Col-0) plants submerged in darkness and recovering in light show age-dependent leaf death.** Images are of a representative plant submerged for the duration indicated in the top-left of each image. Numbers in the first image indicate leaf numbers. Scale bar indicates 1cm.

B) **A representative Arabidopsis plant recovering from five days of dark submergence in the light showing age-dependent leaf death.** Scale bar indicates 1cm.

C) **Submergence causes age-dependent leaf death.** Leaf death per age category was scored three days after desubmergence after the indicated submergence durations. Significance was determined by two-way ANOVA (leaf age*time). Leaf numbers are as indicated in 1A.

D) **mRNAseq harvesting schematic.** Laminas of old (leaf three) and young (leaf seven) leaves of 10-leaf-stage Arabidopsis plants were harvested before submergence and after the indicated treatments. Gene expression was calculated for each submergence timepoint relative to non-submerged conditions (submergence effect), for the post-submergence timepoints relative to the last submerged timepoint before desubmergence (recovery effect), and for the post-submergence timepoints relative to the non-submerged conditions (full effect).

recovery effect); each post-submergence timepoint compared to the non-submerged plants (the full effect). The first two approaches allowed us to investigate the specific response to the submergence and post-submergence phases without including the effect of the sequential exposure to these phases.

The submergence and post-submergence phases together have a stronger effect on the transcriptome of old leaves than of young leaves

Illumina sequencing of cDNA libraries from mRNA isolated from harvested samples resulted in between 30 million and 47 million high-quality paired end reads per library (Figure S1A-B). These reads were then trimmed of adapter sequences and aligned to the Araport11 transcriptome yielding high mapping percentages (Figure S1C). The mapping percentage was notably lower for young leaves harvested after six days of submergence. A brief analysis of the origin of reads that did not map to Arabidopsis using BLAST revealed that they originated from multiple oomycetes, some viruses, as well as some unidentified sources. This suggests that the harvested Arabidopsis plants were not infected by one specific virus, as is sometimes observed in RNAseq datasets (Verhoeven et al., 2023).

Multidimensional scaling of the 2000 most variable genes showed that transcriptomes of old and young leaves were relatively similar before submergence (t0_00, Figure 2A). However, the submergence timepoints (t2_00, t4_00, and t6_00) all clustered far away from the pre-submergence timepoints, suggesting massive transcriptional reprogramming during submergence in both old (circles) and young leaves (triangles). The post-submergence timepoints (t4_01, t4_03, t4_06, and t4_24) of old leaves revealed a second phase of transcriptional reprogramming, where the transcriptomes 24h after desubmergence were still far removed from the t0_00 transcriptomes. This was in contrast with the pattern of the post-submergence timepoints of the young leaf, where the last post-submergence timepoint clustered very close to the t0_00 timepoint. We initially calculated differential gene expression for old and young leaves separately, comparing each sampled timepoint to non-submerged old or young leaves (the full effect, see Figure 1D). The number of differentially expressed genes (DEGs) detected for old and young leaves at the different sampling points was consistent with the results of the multidimensional scaling (MDS) plot. DEG numbers were similar at submergence time points (t2_00 and t4_00) but diverged during the post-submergence phase (t4_01, t4_03, t4_06, t4_24) with lower numbers in young than in old leaves, genes were generally repressed and induced at a similar rate (Figure 2B). These patterns were consistent with observed leaf phenotypes after the post-submergence phase: the old leaf was

generally barely alive and severely damaged, whereas the young leaf fully recovered and continued to grow.

We also calculated differential gene expression between old and young leaves based on changes in the difference between old and young leaves over time: leaf age-dependent changes in gene expression. At each of the six sampling timepoints during the submergence and post-submergence phases at which old and young leaves were harvested, more than 5000 genes showed a leaf age-dependent response to the treatment (Figure 2C). This number was highest at the three-hour post-submergence timepoint, where 8710 genes showed an age-dependent response. Although many genes responded in an age-dependent manner to submergence, the correlation in gene expression between old and young leaves was relatively high (Figure 2D). This correlation was largely lost during the post-submergence phase, indicating an age-dependent divergence of the transcriptomes during this phase.

Old and young leaves exhibit shared responses to the submergence and post-submergence phases

To explore the response to submergence that is age-independent, we first focused on the common stress responses between old and young leaves. We did this by selecting all genes whose expression was significantly different from t0_00 at two or more timepoints of the full time course (submergence and post-submergence), and selecting the genes in which the difference in response between old and young leaves was never significant (an FDR-adjusted p-value > 0.05 and/or an absolute $\log_2$ fold change < 1). This approach led to a selection of genes that change in expression during the submergence and post-submergence treatments, but in an age-independent way. The resulting list of 1629 genes was then divided over six clusters by fuzzy k-means clustering, which allowed us to identify common patterns in gene expression. Genes with a maximum cluster membership lower than 0.50 were then omitted to reduce the noise in the dataset (Figure 3A). The remaining 1482 genes, whose response to the treatments was independent from leaf age, were then further analyzed by testing which gene ontology (GO) terms are enriched per cluster (Figure 3B).

Several of the GO terms significantly enriched in cluster K1 were related to immune responses, like GO:0010200 “response to chitin” and GO:0002679 “respiratory burst involved in defense response”. Immune responses are known to be induced by submergence treatment (Hsu et al., 2013). This was consistent with the expression pattern of genes in cluster K1, which were upregulated gradually during submergence and slightly further upregulated during the post-submergence

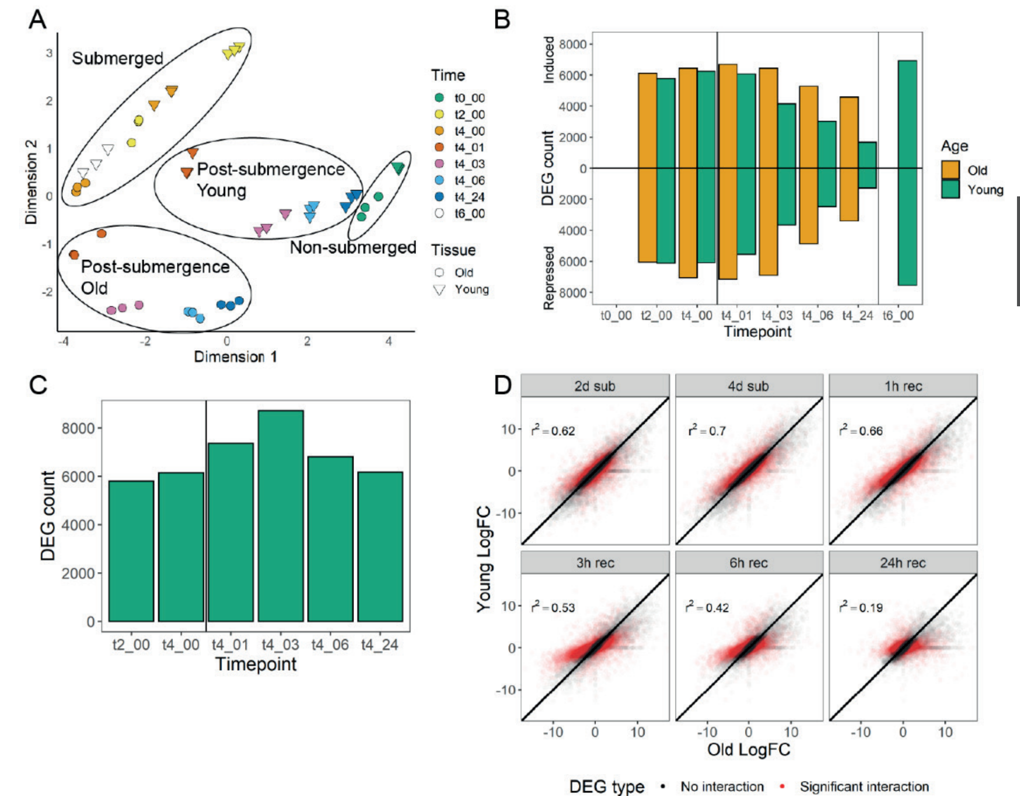


Figure 2 | The transcriptomic response to the submergence and post-submergence phases is age-dependent

A) MDS plot of transcriptomes of old and young leaves before, during, and after submergence. Each shape represents one biological replicate, the 2000 genes that were most responsive to the treatment were used to calculate distances between samples.

B) DEG count for genes that responded significantly at the submergence or post-submergence timepoints compared to their non-submerged expression for old and young leaves separately. The first vertical line separates the submergence phase from the post-submergence phase. The second vertical line separates t6_00 from the samples recovering from four days of submergence (see Figure 1D).

C) DEG count for genes of which the difference in expression between old and young leaves changes significantly at each timepoint compared to non-submerged conditions. The vertical line indicates the separation between the submergence and post-submergence phases.

D) The correlation between the response of old and young leaves is stronger during the submergence phase than during the post-submergence phase (Pearson’s r^2). Red dots represent genes with a significant interaction between the effects of the indicated timepoint compared to expression before submergence and leaf age (the DEGs reported in 2C).

Differences in gene expression were deemed as significant when the FDR-adjusted p-values was below 0.05 and the absolute $\log_2$ fold change was greater than 1.

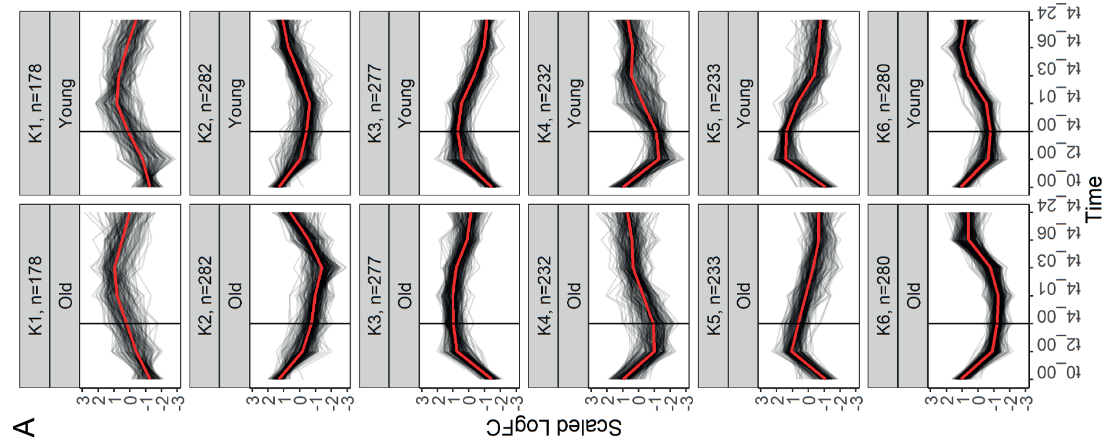


Figure 3 | The age-independent response to the submergence and post-submergence phase
A) Fuzzy clustering of genes whose expression was different from non-submerged conditions in at least two samples, that never show an age-dependent response. 1629 genes were divided over six clusters, yielding 1482 genes with a minimum cluster membership of 50%. Each black line represents a single gene, each red line represents the median expression in each cluster. The vertical black line indicates the separation between the submergence and the post-submergence phase.
B) Gene ontology enrichment of biological process terms in each of the six clusters shown in Figure 3A. All shown terms are significantly overrepresented in their respective cluster with an FDR-corrected p-value below 0.05.

phase. Cluster K1 was also enriched for genes associated with endoplasmic reticulum stress, like those in categories GO:0034976 "response to endoplasmic reticulum stress" and GO:0006457 "protein folding". Lastly, cluster K1 was enriched for genes associated with ethylene-related GO terms, like GO:0009723 "Response to ethylene". The age-independent response to ethylene was likely a result of systemic accumulation of ethylene in submerged plants (Sasidharan & Voesenek, 2013).

Many of the GO terms enriched in cluster K2 were related to growth and photosynthesis, processes that are paused during submergence and reactivated during the post-submergence phase. This pattern of downregulation during submergence and reactivation during the post-submergence phase was also found in cluster K6, although the downregulation was much steeper here. Cluster K6 was enriched for GO terms involved in translation, consistent with previous reports that have found a rapid general shutdown of this process during low-oxygen stress although translation of specific transcripts is increased (Sachs et al., 1980; Juntawong et al., 2014; Cho et al., 2022). Clusters K2 and K6 were the two gene clusters with the most members (282 and 280 members, respectively), which highlights how common the shutdown of processes associated with growth and development was during submergence stress.

Surprisingly, we did not observe the enrichment of GO terms associated with hypoxia stress, like GO:0001666 "Response to hypoxia". The expression of multiple core hypoxia genes varied significantly between old and young leaves, although there was no trend towards a stronger response in either leaf during submergence (Figure S2). During the post-submergence phase, however, expression of core hypoxia genes was reduced faster in young leaves than in old leaves (Figure S2).

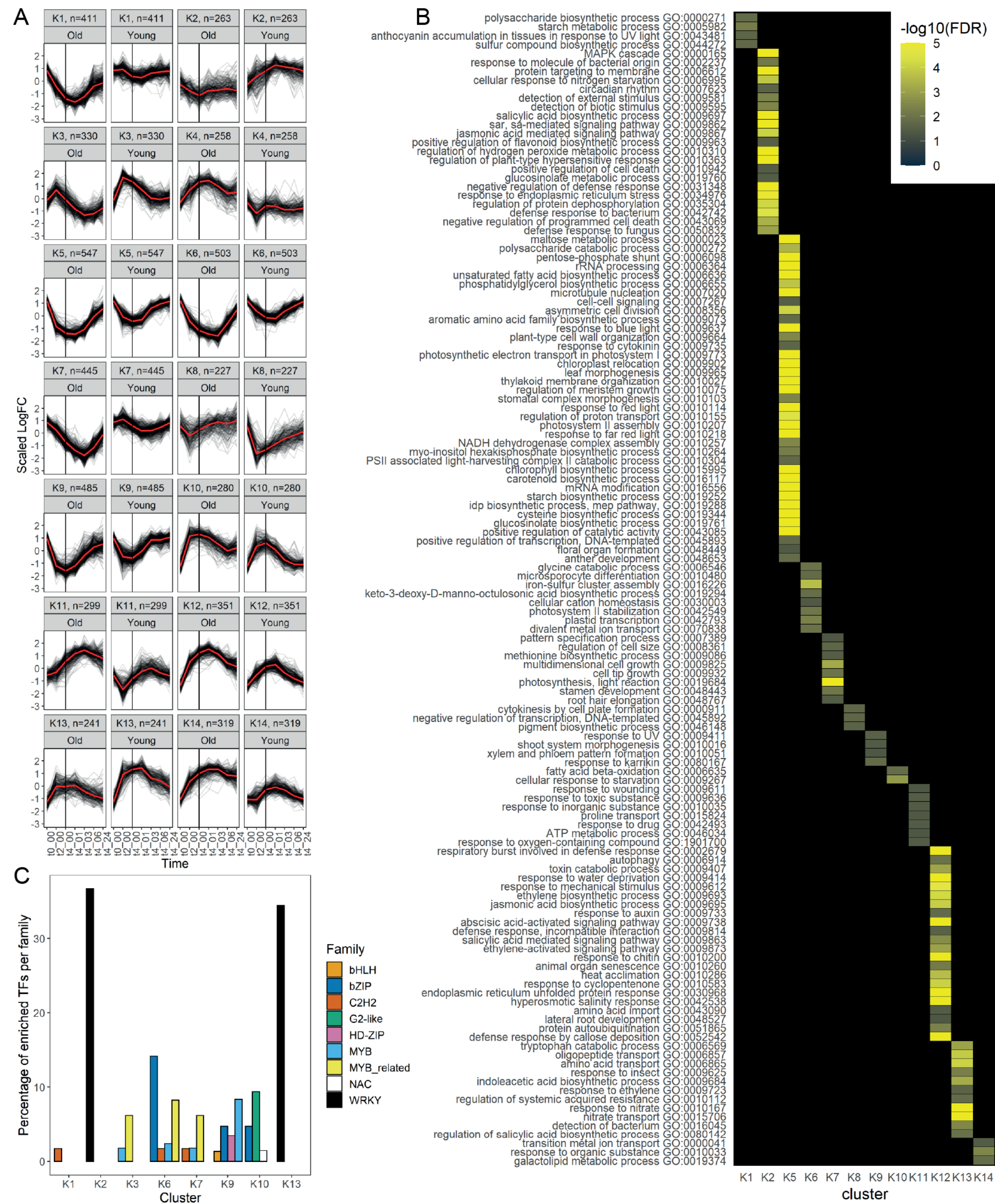


Figure 4 | The age-dependent response to the submergence and post-submergence phase

A) Clustering of genes whose expression showed an age-dependent response at a minimum of four timepoints. 4977 genes were divided over 14 clusters, yielding 4959 genes with a minimum highest cluster membership of 20%. Each black line represents a single gene, each red line represents the median expression in each cluster. The vertical black line indicates the separation between the submergence and post-submergence phase.

B) Gene ontology enrichment of the biological process terms in each of the 14 clusters shown in Figure 4A. All shown terms were significantly overrepresented in their respective cluster with an FDR-corrected p-value below 0.05.

C) Enrichment of targets of transcription factors in each cluster, grouped by transcription factor family. Enrichment was calculated by hypergeometric tests, corrected for multiple testing. Families with fewer than 2 transcription factors with overrepresented targets were omitted.

Old and young leaves show age-dependent responses to the submergence and post-submergence phases

The results discussed above describe the responses that are shared between leaves of different ages during submergence and the post-submergence phase. Transcriptomic data suggested that downregulation of growth and translation occurred in both young and old leaves, and that responses to ethylene were induced in both old and young leaves.

Under control conditions, there were already 5345 genes differentially expressed between old and young leaves. Of these 5345 DEGs, 2494 were expressed higher in old leaves and 2851 were expressed higher in young leaves. The genes with higher expression in old leaves were primarily enriched for GO categories associated with immunity (e.g. GO:0050842 "Defense to fungus") and transport (e.g. GO:0015706 "Nitrate transport") (Figure S3). Genes with higher expression in young leaves were enriched for processes related to cell division and development like GO:0009965 "Leaf morphogenesis" and GO:0006275 "Regulation of DNA replication". These baseline differences reflect differences between them in developmental status, and the enrichment of GO terms related to immunity could be an example of the development of immunity with leaf age (Hu and Yang, 2019).

To identify the age-dependent responses with a potential role in stress tolerance, genes that showed an age-dependent response to the submergence or post-submergence phase at a minimum of four out of the six timepoints at which both old and young leaves were sampled were selected. This number was used as the threshold to include genes that are different between old and young leaves

during the four post-submergence phase timepoints but not the two submergence timepoints. Significance was defined as an FDR-corrected p-value lower than 0.05 and an absolute log_2 fold change value greater than 1, yielding a total of 4799 genes. These were visualized by dividing them into 14 clusters by k-means clustering, omitting the ones with a maximum cluster membership lower than 20% to reduce noise (Figure 4A).

GO analysis of these clusters showed that the defense response was more strongly activated in young leaves than in old leaves (Figure 4B, clusters K2 & K13), the induction of a defense response during submergence is consistent with previous reports (Hsu et al., 2013). The enrichment of genes associated with GO terms connected to defense and ethylene responses was also seen in the analysis of responses shared between old and young leaves (Figure 3). Overlapping the shared GO terms between age-independent (Figure 3, cluster K1) and age-dependent (Figure 4, clusters K2 & K13) revealed that broader GO terms associated with defense responses, like GO:0009697 “Salicylic acid biosynthetic process” and GO:0010363 “Regulation of plant-type hypersensitive response” were enriched in both sets of genes. Among the GO terms specific to age-independent or -dependent responses (labeled as “Only common” or “Only different” in Table S1) were several GO terms associated with more specific aspects of defense responses. This shows that some aspects of the connection between submergence stress and immune responses vary with leaf age, whereas others are independent of leaf age.

Although clustering of the age-independent response showed that old and young leaves downregulate the expression of genes associated with photosynthesis and growth (Figure 3A), clustering the age-dependent response showed that the halting of these processes was faster and the restarting during the post-submergence phase was slower in old leaves than young leaves (Figure 4A, clusters K5-7). Lastly, the induction of genes associated with senescence-related GO categories was stronger in old leaves than in young leaves (clusters K10 & K12).

Changes in gene expression are often the result of regulation by transcription factors. To see which transcription factors are likely to play a central role in the responses to the submergence and post-submergence phases, we measured the enrichment of targets of transcription factors in each cluster. This revealed that the clusters enriched for defense-related genes (clusters K2 & K13) were strongly enriched for targets of WRKY transcription factors (Figure 4C). This is consistent with the role of WRKY transcription factors in the defense response (Birkenbihl et al., 2017). Targets of NAC transcription factors, which are often associated with leaf senescence (Kim et al., 2016), were enriched only in cluster K10. Cluster K6 was enriched for targets of bZIP transcription factors. GO term analysis revealed that this cluster was enriched for genes involved in chloroplast development, a process that is partially controlled by bZIP transcription factors (Cackett et al., 2022).

Of the transcription factors that themselves were differentially expressed between young and old leaves at all timepoints beyond t0_00, the majority responded stronger in old leaves than in young leaves (Figure S4). This reflects the pattern seen in Figure 4A, where most clusters contain genes that responded stronger in old leaves than in young leaves. Of the transcription factors with an age-dependent response, a set of NAC domain transcription factors that have been connected to leaf senescence (*ANAC010*, *ANAC075*, and *ANAC100* (Kim et al., 2016)) showed a stronger increase in old leaves than young leaves during submergence. Two clusters that were strongly enriched for the targets of WRKY transcription factors showed a stronger increase in gene expression in young leaves in the submergence phase (Figure 4, clusters K2 & K13). In contrast with this, there were no WRKY transcription factors showing a stronger induction in young leaves than in old leaves (Figure S4). This could point towards an important role for the age-dependent regulation of WRKY transcription factors beyond their transcript abundance. Although many transcription factors showed an age-dependent response, only few of them showed opposite responses in old and young leaves. One of the few transcription factors that does show a contrasting pattern between these leaves is *MYB102* (Figure S4). *MYB102* has been shown to stimulate ethylene production (Zhu et al., 2018), and the increased expression of *MYB102* in old leaves during the post-submergence phase might contribute to their increased senescence in this phase. Another transcription factor with an opposite transcriptional response between old and young leaves is *ASYMMETRIC LEAVES2-LIKE 9* (*ASL9*), which was induced in old leaves specifically during the post-submergence phase but was repressed in young leaves. ASL9 is a cytokinin-responsive transcription factor of the LBD family that induces secondary root growth (Naito et al., 2007; Ye et al., 2021). Although increasing cytokinin production in senescing leaves has been associated with improved flooding tolerance in Arabidopsis (Zhang et al.), it is currently unclear if this is connected to the expression pattern of *ASL9* observed here.

In addition to the transcription factors described above, several other genes that have previously been associated with flooding tolerance also show age-dependent expression patterns (Figure S5). Expression of *ORE1* was highest during submergence in old and young leaves, this expression remained high during the post-submergence phase in old leaves. *ORE1* is a positive regulator of senescence that contributes to leaf

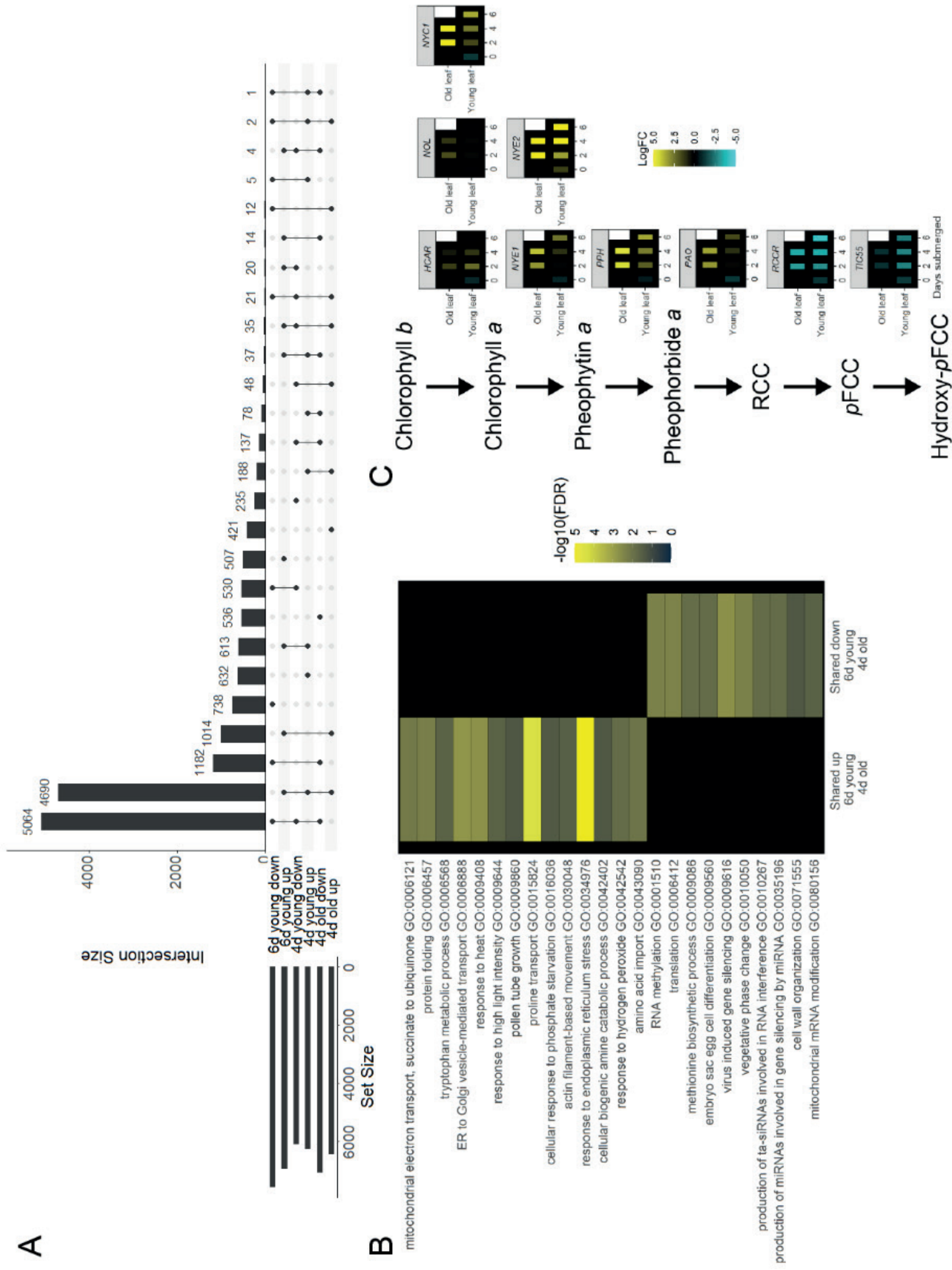


Figure 5 | Prolonged submergence makes the transcriptome of a young leaf similar to that of an old leaf
A) UpSet plot showing the intersection between genes induced or repressed after four or six days of submergence in young leaves and genes induced or repressed after four days of submergence in old leaves. Dots show which categories are intersected, numbers above each vertical bar indicate the size of each intersect. Horizontal bars indicate the size of each individual category.
B) Significantly enriched biological process GO categories among genes that were up- or downregulated in old leaves after four days of submergence and young leaves after six days of submergence. These were the last harvested submergence timepoints for old and young leaves. All shown terms were significantly overrepresented in their respective overlapping dataset with an FDR-corrected p-value below 0.05.
C) Expression of enzymes involved in chlorophyll catabolism in chloroplasts via the PAO/phyllobilin pathway during submergence in old and young leaves (Kuai et al., 2018). RCC: Red Chlorophyll Catabolite; pFCC: primary Fluorescent Chlorophyll Catabolite

senescence during the post-submergence phase (Kim et al., 2009; Yeung et al., 2018). Expression of *RBOHD*, involved in ROS production upon reoxygenation, is highest one hour after desubmergence in both old and young leaves, although its expression was always higher in old leaves than in young leaves (Yeung et al., 2018). Lastly, expression of *SAG113* increased gradually during submergence in both old and young leaves, and increased further during the first hour of the post-submergence phase. At later post-submergence timepoints expression of *SAG113* remained high in old leaves, whereas it went down in young leaves. *SAG113* is involved in the desiccation of senescing leaves, and its age-dependent expression pattern could contribute to age-dependent water loss during the post-submergence phase (Zhang et al., 2012; Yeung et al., 2018).

Prolonged submergence makes the transcriptome of a young leaf similar to that of an old leaf

For all sampling timepoints (Figure 1C) both an old leaf and a young leaf were harvested. The exception was the six days submergence timepoint, where only the young leaf was sampled since the old leaf was generally dead after six days of submergence. It was of interest to establish whether the transcriptome of a ‘near-death’ young leaf after six days of submergence was like that of a ‘near-death’ old leaf after four days of submergence. For this the young leaf submergence effect DEGs after four days (6246 upregulated and 6094 downregulated genes) and six days (6934 upregulated and 7555 downregulated) of submergence were overlapped with the old leaf submergence effect DEGs after four days of submergence (6431 upregulated and 7053 downregulated). The majority of DEGs analyzed were significantly down- or upregulated in all three of these sets of genes (Figure 5A). Of the genes that were not shared between all three sets (3652 for upregulated

DEGs, 3635 for downregulated DEGs), most overlap was between the transcriptomes of old leaves of plants submerged for four days and young leaves of plants submerged for six days, with 1182 shared downregulated genes and 1014 shared upregulated genes, respectively. This was also consistent with the MDS plot in Figure 2A, in which the transcriptomes of old leaves submerged for four days and young leaves submerged for six days were located close to each other. Gene ontology analysis of these two sets of genes showed that processes commonly downregulated were often involved in fundamental cellular processes like transcription and translation (Figure 5B). Shared upregulated genes between old and young leaves at their last submergence timepoint were often involved in transport-related process, suggesting export of the last nutrients out of the dying leaf to use them in other parts of the plant. The high degree of similarity between the "near-death" transcriptomes of old and young leaves showed that a prolonged submergence treatment eventually had the same transcriptomic result in these leaves. The difference between the two leaves was in the time it took for them to reach this point.

Stress-induced aging of a leaf is often accompanied by a reduction in chlorophyll content. This was also reflected in the phenotype of plants submerged for prolonged period: old leaves turned yellow faster than young leaves (Figure 1A). Consistent with this, several genes in the chlorophyll catabolic pathway (Kuai et al., 2018) were induced faster in old leaves than in young leaves (Figure 5C). The activity of Peophorbide a Oxidase (PAO) has been shown to be oxygen-dependent (Hörtensteiner et al., 1998). Interestingly, the expression of enzymes downstream of *PAO* (*RCCR* and *TIC55*) was repressed rather than induced during submergence (Figure 5C). However, the downregulation of *RCCR* and *TIC55* was consistent with previous reports on the regulation of chlorophyll catabolism during senescence when oxygen is available (Aubry et al., 2020). Furthermore, it has been shown that both *RCCR* and *TIC55* are not essential for the accumulation of non-fluorescent chlorophyll catabolites (NCCs), which are formed further downstream in the pathway (Pružinská et al., 2007; Hauenstein et al., 2016). After chlorophyll is broken down into hydroxy-pFCC, this is transported to the vacuole and degraded into non-fluorescent chlorophyll catabolites (NCCs) (Kuai et al., 2018). The further degradation of NCCs in the vacuole is highly similar to that of toxic compounds (Hinder et al., 1996; Frelet-Barrand et al., 2008). This would explain the enrichment of GO terms associated with exposure to toxic compounds like GO:0009636 "Response to toxic substance" and GO:0042493 "Response to drug" in cluster K11, which consisted of genes that only went up in old leaves during submergence (Figure 4A-B).

The transcriptomic response specific to the post-submergence phase

Our initial approach, outlined above, to identify the age-independent and age-dependent responses to the submergence and post-submergence phases was to calculate differential gene expression at each timepoint compared to that of non-submerged plants. An inevitable consequence of this approach is that it can dilute the individual effects of the submergence and post-submergence phases. To address this limitation, gene expression was calculated in old and young leaves relative to the last timepoint before desubmergence (t4_00) to determine the post-submergence response (The recovery effect, Figure 1D). This approach allowed us to isolate the transcriptional response to the post-submergence phase, without taking the response to the preceding submergence phase into account. The age-dependent response to the post-submergence phase reached a plateau of roughly 5000 age-dependent DEGs three hours after desubmergence (Figure 6A). While the total amount of DEGs with an age-dependent response to the post-submergence phase did not change much, the strength of the response of these DEGs increased further at later post-submergence timepoints (Figure 6B). The gradual increase in the strength of the response to desubmergence was accompanied by an increased correlation between the response of old and young leaves. This was different from the analysis of the combined effect of the submergence and post-submergence phases, where this correlation increased during the submergence phase but decreased during the post-submergence phase (Figure 2D). These results highlight how the response to individual phases of a stress period can be similar in different tissues, but exposure to multiple sequential stress phases can lead to a divergence in the response.

Age-dependent regulation of transcriptional responses specific to the post-submergence phase

To identify age dependent processes specific to the post-submergence phase, DEGs with a significant interaction effect for Time*Age at a minimum of three out of the four post-submergence timepoints were selected. These 1848 genes were then divided over 14 clusters by fuzzy k-means clustering, and genes with a maximum cluster membership below 20% were again removed. Consistent with the results in Figure 4, young leaves induced the transcription of genes related to photosynthesis and growth faster during the post-submergence phase than old leaves (Figure 7B, clusters K1, K4, & K14). Also consistent with Figure 4, cluster K5 showed that senescence-associated processes were shut down faster in young leaves than old leaves during the post-submergence phase.

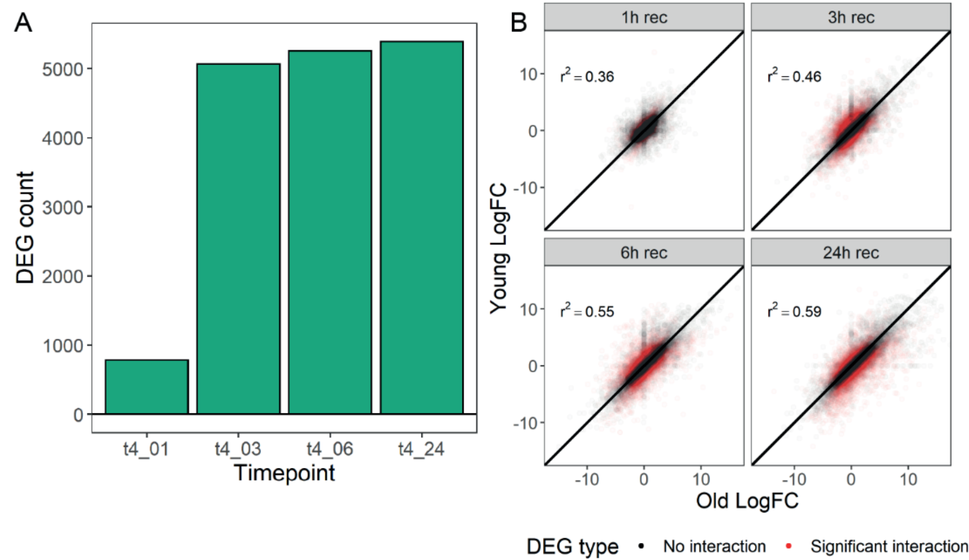
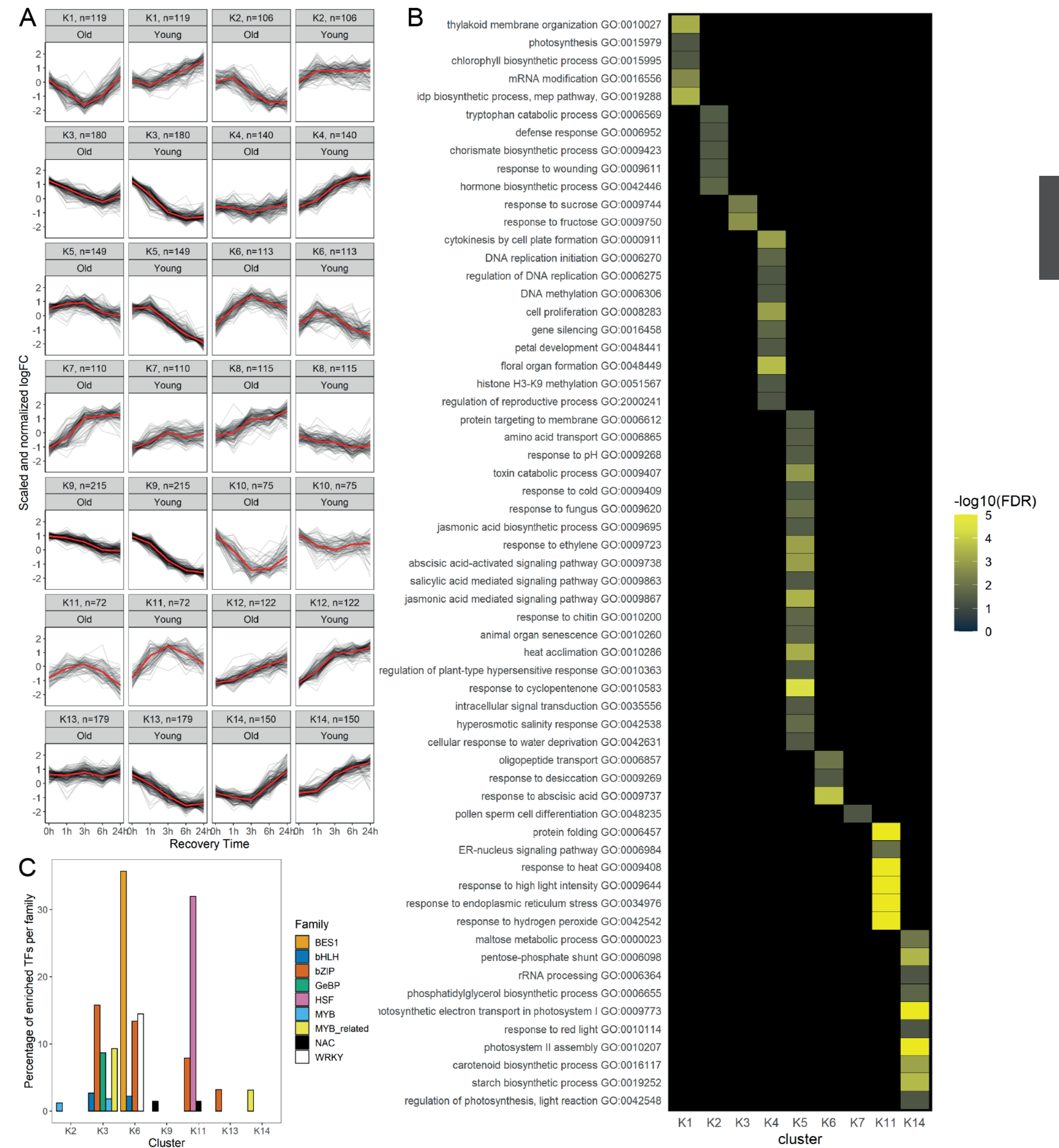


Figure 6 | The transcriptomic response to the post-submergence phase
A) DEG count for genes of which the difference between expression in old leaves and young leaves changes significantly between the last submergence timepoint (t4_00) and the indicated post-submergence timepoints.
B) Correlation between gene expression in old and young leaves during the post-submergence phase. Although the number of genes showing an age-dependent response to desubmergence (6A) did not change much beyond the first three hours of the post-submergence phase, the strength of the response of these genes continues to increase during twenty-four hours of this phase. The correlation between gene expression in old and young leaves also increased with time (as determined by Pearson's r^2). Red dots represent genes with a significant interaction between the effects of the indicated timepoint compared to expression before submergence and leaf age.
Differences in gene expression were deemed as significant when the FDR-adjusted p-values was below 0.05 and the absolute $\log_2$ fold change was greater than 1.

Figure 7 (right page) | Age-dependent regulation of transcriptional responses specific to the post-submergence phase
A) Clustering of genes whose expression showed an age-dependent response at a minimum of three post-submergence timepoints, compared to their expression just before desubmergence. 1848 genes were divided over 14 clusters, yielding 1845 genes with a minimum cluster membership of 20%. Each black line represents a single gene, each red line represents the median expression in each cluster.
B) Gene ontology enrichment of biological process terms in each of the 14 clusters shown in Figure 7A. All shown terms are significantly overrepresented in their respective cluster with an FDR-corrected p-value below 0.05.
C) Enrichment of targets of transcription factors in each cluster, grouped by transcription factor family.



Clustering of the age-dependent responses to the post-submergence phase revealed a stronger and more sustained induction of genes associated with the GO categories "Response to desiccation" and "response to abscisic acid" in old leaves (Figure 7B, cluster K6). This was consistent with the visual phenotype of plants recovering from submergence, where there was more desiccation in old than in young leaves (Figure 1B). ABA-mediated responses to dehydration are controlled by ABA-RESPONSIVE ELEMENT BINDING FACTOR (ABF) transcription factors of the bZIP family (Uno et al., 2000). The targets of several ABFs, as well as those of other bZIP transcription factors, were overrepresented among the genes in cluster K6 (Figure 7C). Cluster K5 was enriched for genes associated with GO categories associated with ethylene signaling and senescence, like GO:0009723 "Response to ethylene" and GO:0010260 "Animal organ senescence" (Figure 7B). Expression of these genes was rapidly reduced in young leaves during the post-submergence phase but remained high in old leaves (Figure 7A). Although leaf senescence can in some instances be reversible (Thomas et al., 2003), multiple genetic feed-forward loops have been identified that ensure that senescence continues once it has started (Kim et al., 2016). The continuously high expression of genes related to senescence during the post-submergence phase in old leaves suggests they had passed a "point of no return", whereas young leaves were still able to undo the transcriptional onset of senescence.

Cluster K11 showed a strong enrichment for multiple GO categories associated with protein misfolding and high light stress, two processes that are often connected (Nawkar et al., 2017). Genes in this cluster went up further in young leaves than in old leaves during the post-submergence phase. Similar GO categories were also enriched in cluster K2 of the full time course, which consisted of genes that show an overall stronger response in young leaves than old leaves (Figure 4A). Targets of HEAT SHOCK FACTOR (HSF) and bZIP transcription factors were overrepresented in cluster K11 of genes responding to the post-submergence phase, which was consistent with the involvement of transcription factors of these families in these processes (Figure 7C, (Ko and Brandizzi, 2022; Jung et al., 2013)). Of the HSF transcription factors whose targets are overrepresented in cluster K11, some had clear age-dependent patterns (Figure S6). The expression of both *HSFA6A* and *HSFA6B* was higher in young leaves than in old leaves during the post-submergence phase. These two closely related transcription factors play a role in the interaction between ABA signaling and ROS signaling (Wenjing et al., 2020). Their higher expression in young leaves could facilitate their faster response to desubmergence, as ABA signaling and ROS signaling are both important during the post-submergence phase (Yeung et al., 2019). In maize, the expression of a homolog of Arabidopsis *HSFA6B*, *HSFTF13*, is induced by bZIP60 during heat stress (Li et al., 2020b). Under heat stress, HSFTF13 induces the expression of HEAT SHOCK PROTEINS (HSPs), which function as chaperones in cleaning up accumulated misfolded proteins under stress conditions. This is also consistent with the overrepresentation of genes associated with ER stress and protein folding in cluster K11 (Figure 7B).

Discussion

In this study we mapped the leaf-age-dependent transcriptomic responses of *Arabidopsis thaliana* to the submergence and post-submergence phases. Previous studies have explored the specific responses of plant organs or cell types to submergence and hypoxia, but none have addressed the effect of leaf age (van Veen et al., 2016; Mustroph et al., 2009). Unsurprisingly, our results show that while old and young leaves shared common responses to the submergence and post-submergence phases, there were significant age-dependent responses as well. Some of the latter could potentially explain the faster onset of submergence-induced senescence and post-submergence desiccation in old leaves of submerged plants that leads to their eventual death.

The age-independent response to the submergence and post-submergence phases included an increase in expression of genes associated with several stress-associated hormones, like ethylene, salicylic acid, jasmonic acid, and ABA (Figure 3). The general signature of stress hormones, and several other stress-related GO terms, showed that old and young leaves both experience stress during the submergence and post-submergence phases. Transcripts associated with growth and photosynthesis, on the other hand, were repressed in both old and young leaves (Figure 3). Since Arabidopsis does not store substantial carbon reserves, it is unable to sustain its growth during dark submergence when photosynthesis is limited (Gibon et al., 2004; Sand-Jensen, 1989).

Senescence was triggered faster in old leaves during early submergence, but was also induced in young leaves during prolonged submergence

The similarity between the transcriptomes of a 4d-submerged old leaf and a 6d-submerged young leaf suggested that the difference between old and young leaves in their response to submergence was in part determined by the speed of their response (Figure 5). Submergence treatment induced "transcriptomic aging" of a leaf, and old leaves were more sensitive to this induction than young leaves. This was also reflected in the position of transcriptomes of old and young leaves

during submergence in the MDS plot (Figure 2A). All 15 of these transcriptomes fell on the same diagonal line, but those of old leaves were further along it than young leaves that were harvested at the same timepoint. At the latest submerged timepoint (four days for old leaves, six days for young leaves), leaves induced the transcription of genes associated with amino acid transport and transfer of electrons from succinate to ubiquinone in the mitochondrial electron transport chain. This pattern was consistent with previous studies on the transcriptomes of leaves experiencing developmental leaf senescence (Chrobok et al., 2016). Genes involved in chlorophyll catabolism, a process typically associated with leaf senescence, were induced earlier in old leaves, although their expression was also increased after six days of submergence in young leaves (Figure 5B). An important regulator of chlorophyll catabolism in Arabidopsis is the transcription factor ORE1 (Qiu et al., 2015). Although the expression of *ORE1* was strongly induced in both old and young leaves during submergence, its expression went down again during the post-submergence phase in young leaves but not in old leaves (Figure S5).

Processes related to senescence were induced faster in old leaves than in young leaves, but they were not enriched among genes with higher expression in old leaves under control conditions (Figure S3). This suggests that the earlier onset of submergence-induced senescence in old leaves was not merely due to baseline differences in development but caused by differences in the speed at which senescence was activated. Age-related differences in the onset of senescence are associated with age-related changes (ARCs) that control when a leaf is sensitive to senescence-inducing signals (Jibran et al., 2013; Kanojia et al., 2020; Schippers, 2015). The gradual derepression of *ORE1* by miR164 with leaf age is a prime example of these ARCs (Kim et al., 2009), but few other mechanisms have been identified.

The comparison between leaves of different developmental stages during flooding stress has recently been made in rice plants (Alpuerto et al., 2022). Whereas in Arabidopsis young leaves were more tolerant to submergence, in rice plants the young leaves are the least tolerant. This is due to excessive elongation of the youngest leaves during submergence, which can be repressed by introducing the *SUB1A* tolerance gene (Alpuerto et al., 2022). *SUB1A* can serve as a brake on submergence-induced elongation and carbon consumption of young rice leaves and thereby improve their survival. The stronger induction of genes associated with carbon starvation in old leaves in our dataset (Cluster K10, Figure 4A-B) suggested that a similar mechanism might be beneficial to the survival of old leaves of Arabidopsis. Reducing the carbon consumption in these leaves might delay their starvation and eventually prolong their survival under flooding stress. Whether or not a leaf goes into senescence is in part controlled by its energy status (Wingler et al., 2006). Since old leaves showed a stronger starvation response than young leaves during submergence, it could be that this contributed to the age-dependent gradient in senescence during flooding stress. Another flooding-associated signal that plays a role in leaf senescence is ethylene, which accumulates in flooded plants (Grbić and Bleecker, 1995; Koyama, 2014; Voesenek and Sasidharan, 2013). Chapters 4 and 5 explore how systemic ethylene accumulation leads to age-dependent leaf senescence via ORE1.

During the post-submergence phase, old leaves showed a stronger induction of dehydration-related genes, young leaves showed a stronger induction of ER stress-related genes

During the post-submergence phase, the transcriptomes of young leaves were largely reset to their original state whereas the transcriptomes of old leaves remained more different from their non-submerged state (Figure 2B). We questioned whether this return of the transcriptome to its original state was the cause or the effect of the increased submergence tolerance of young leaves compared to old leaves. Plants recovering from submergence stress typically experience physiological drought, as their damaged root systems are unable to compensate for water lost via the leaves (Yeung et al., 2019; Liu and Zwiazek, 2022). Our results suggest that plants experienced more severe drought stress in older leaves during the post-submergence phase, as drought-related genes were induced stronger in these leaves. Furthermore, gene clusters enriched for drought-related genes was also enriched for the targets of bZIP-type transcription factors (Cluster K6, Figure 7C). bZIPs are known to play a role in drought responses. This highlights how the physiologically similar processes of drought stress and the post-submergence phase are both regulated by similar molecular mechanisms. In Arabidopsis, the Protein Phosphatase 2C *SAG113* inhibits stomatal closure in plants recovering from submergence or plants experiencing drought stress (Zhang et al., 2012; Yeung et al., 2018). The role of *SAG113* in the post-submergence phase has been identified by comparing the accessions Bay-0 and Lp2-6, which also vary in their drought tolerance (Morales et al., 2022; Yeung et al., 2018). The higher expression of *SAG113* in old leaves during the post-submergence phase (Figure S5) suggested that it could play a role in the age-dependent drought response during this phase by keeping the stomata of old leaves open and thereby facilitating desiccation of old leaves. Chapter 3 further explores the connection between the stronger transcriptional response of desiccation-related genes in old leaves and their dehydration phenotype during the post-submergence phase.

Our analysis of the response specific to the post-submergence phase confirmed the induction of several pathways that have previously been shown to be involved during this recovery period. Post-submergence dehydration and ROS accumulation contribute to the difference in submergence tolerance of the Arabidopsis accessions Bay-0 and Lp2-6 (Yeung et al., 2018). The response to these stimuli was induced more strongly in young leaves than old leaves (Figure 7). In addition to this, young leaves showed a stronger induction of genes associated with ER stress and the unfolded protein response (UPR) (Figure 7). ER stress occurs when misfolded proteins accumulate in the ER lumen, which can be the result of environmental stress (Howell, 2013). Upon ER stress, the UPR is activated to clean up the accumulated misfolded proteins. The induction of transcripts related to ER stress is consistent with previous observations that ER stress-associated transcripts are induced upon hypoxia, and that Arabidopsis mutants that lack parts of the ER stress response machinery have reduced flooding tolerance (Zhou et al., 2021). It will be interesting to see whether the stronger signature of ER stress-related genes in young leaves reflects more severe ER stress, or if it reflects a greater capacity to respond to ER stress. Transcripts associated with translation were enriched in a cluster of genes that was repressed during submergence and induced during the post-submergence phase in both old and young leaves (Figure 3). However, this cluster of genes was most strongly induced six hours after desubmergence, which is later than the peak of expression of genes associated with ER stress, three hours after desubmergence (Figure 7). This would suggest that the accumulation of misfolded proteins in the ER might already start during the submergence phase. Although some elements of the ER stress response pathway were induced in an age-dependent manner during the post-submergence phase, the enrichment of ER stress genes seen in cluster K1 of Figure 3 shows that there are also age-independent aspects of the ER stress response. A more in-depth analysis is required to disentangle the importance of ER stress signaling during the submergence and post-submergence phases, and to which degree this is regulated in an age-dependent manner.

mRNAseq of leaves of different ages during the submergence and post-submergence phases revealed responses that were shared and unique between leaves

The results described here show that although some responses to the submergence and post-submergence phases were independent of leaf age, many responses were controlled in an age-dependent manner. Whereas old and young leaves both repressed processes associated with growth and photosynthesis during submergence, young leaves were able to restart these processes faster upon desubmergence. Age-dependent responses included the stronger induction of senescence- and desiccation-related genes in old leaves, and a stronger response to ER stress and a faster induction of growth-related transcripts in young leaves. The transcriptional responses of many transcription factors were regulated in an age-dependent manner, although there were surprisingly few transcription factors that exhibited opposite expression patterns between old and young leaves. This also applied to non-transcription factor genes: differences between old and young leaves were mostly in the strength of the response rather than the direction of the response. Further identification of the causes and effects of age-dependent expression of specific genes will help in explaining why young leaves of Arabidopsis show a greater resilience to the submergence and post-submergence phases than old leaves.

Materials and methods

Plant growth and treatments

Seeds were sowed on Primasta soil mix and stratified in the dark for three to four days before being transferred to a short-day condition climate chamber (20°C, 9h light 15h dark, 70% relative humidity, ~140-180 µmol/m²/s photosynthetically active radiation of either LED or fluorescent light). After germinating for nine days, seedlings were transplanted to individual (5.5cm diameter, 5cm height) pots consisting of 2:1 perlite:soil mix, which were covered with a black mesh to prevent soil from floating out of the pot during submergence. One liter of 0.5x Hoagland medium was added to each tray of 42 pots. When plants reached the 10-leaf stage they were submerged in complete darkness at 20°C for the indicated amount of time, and were left to recover for the indicated amount of time in the original climate chamber. Leaf survival was scored three days after desubmergence, leaves were scored as dead when approximately more than half of the lamina area had desiccated.

mRNAseq

For mRNAseq between 8 and 16 leaf laminas were pooled per replicate and snap-frozen in liquid nitrogen at the indicated timepoints. Biological replicates were harvested independently. RNA was isolated using the RNeasy Plant Mini Kit (Qiagen), residual genomic DNA was digested using on-column DNase-I digestion (Ambion). RNA was quantified using a Nanodrop spectrophotometer, quality was analyzed by measuring A260/A280 and A230/A280 values on a Nanodrop and by running 1µl of RNA on an agarose gel. Library preparation was performed commercially by Macrogen Korea using the TruSeq Stranded mRNA LT Sample Prep Kit. The cDNA libraries were sequenced by paired-end Illumina sequencing on an Illumina Novaseq6000 platform, yielding approximately 30 million paired-end 150bp reads

per library. Reads have been deposited at the European Nucleotide Archive under accession number PRJEB57289.

Read alignment and DEG calculation

FASTQ files were cleaned up using cutadapt (Martin, 2011) to remove some residual sequencing primers, and the quality was assessed with FastQC (Babraham Bioinformatics). Cleaned up FASTQ files were aligned to the Arabidopsis Araport11 transcriptome using Kallisto (Bray et al., 2016) on the Utrecht Bioinformatics Cluster. Differential gene expression was calculated in R using Bioconductor packages edgeR and limma (Robinson et al., 2010; Ritchie et al., 2015). Only transcripts that were present at a minimum of 20 reads in three or more samples were included and different transcript isoforms encoded from the same gene were merged, yielding a total of 20588 genes. Genes were determined to be differentially expressed when they had an absolute $\log_2$ fold change above 1 and an FDR-corrected p-value below 0.05. Lists of differentially expressed genes can be found at https://www.biorxiv.org/content/10.1101/2022.11.23.517613v1.

Gene ontology and transcription factor enrichment

Gene Ontology enrichment was calculated using the R package GOseq (Young et al., 2010). Enrichment of transcription factor targets was done using the PlantRegMap database (Tian et al., 2020). Enrichment of the targets of each transcription factor per cluster was tested by hypergeometric test, adjusted for multiple testing. For visualization, transcription factors were grouped together based on their family and the percentage of overrepresented transcription factors per family was shown to account for differences in transcription factor family sizes.

Supplemental figures

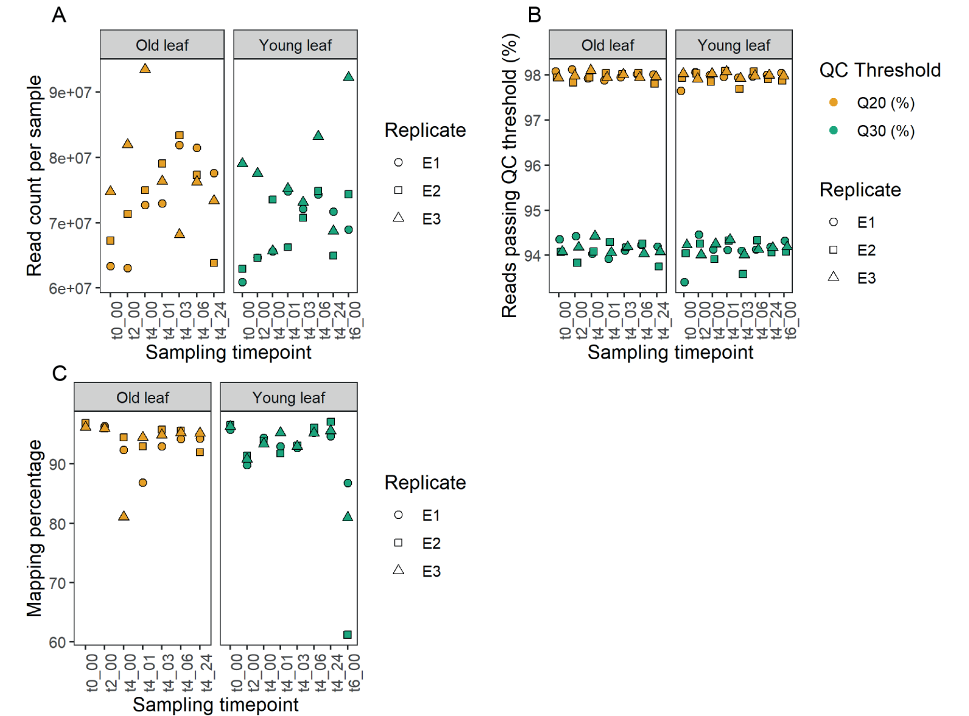


Figure S1 | Quality control of mRNAseq

A) Number of sequenced reads per library of old and young leaves, shapes indicate different biological replicates.

B) Percentage of reads of each library passing the Q20 (99% accuracy) and Q30 (99.9% accuracy) Phred quality thresholds.

C) Percentage of reads per library mapped to the Araport11 transcriptome.

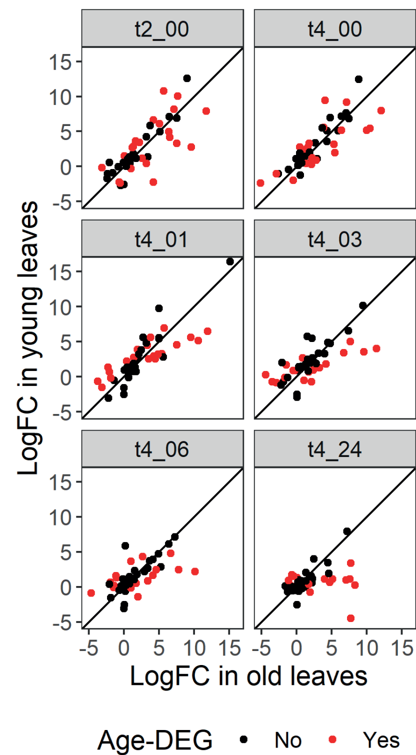
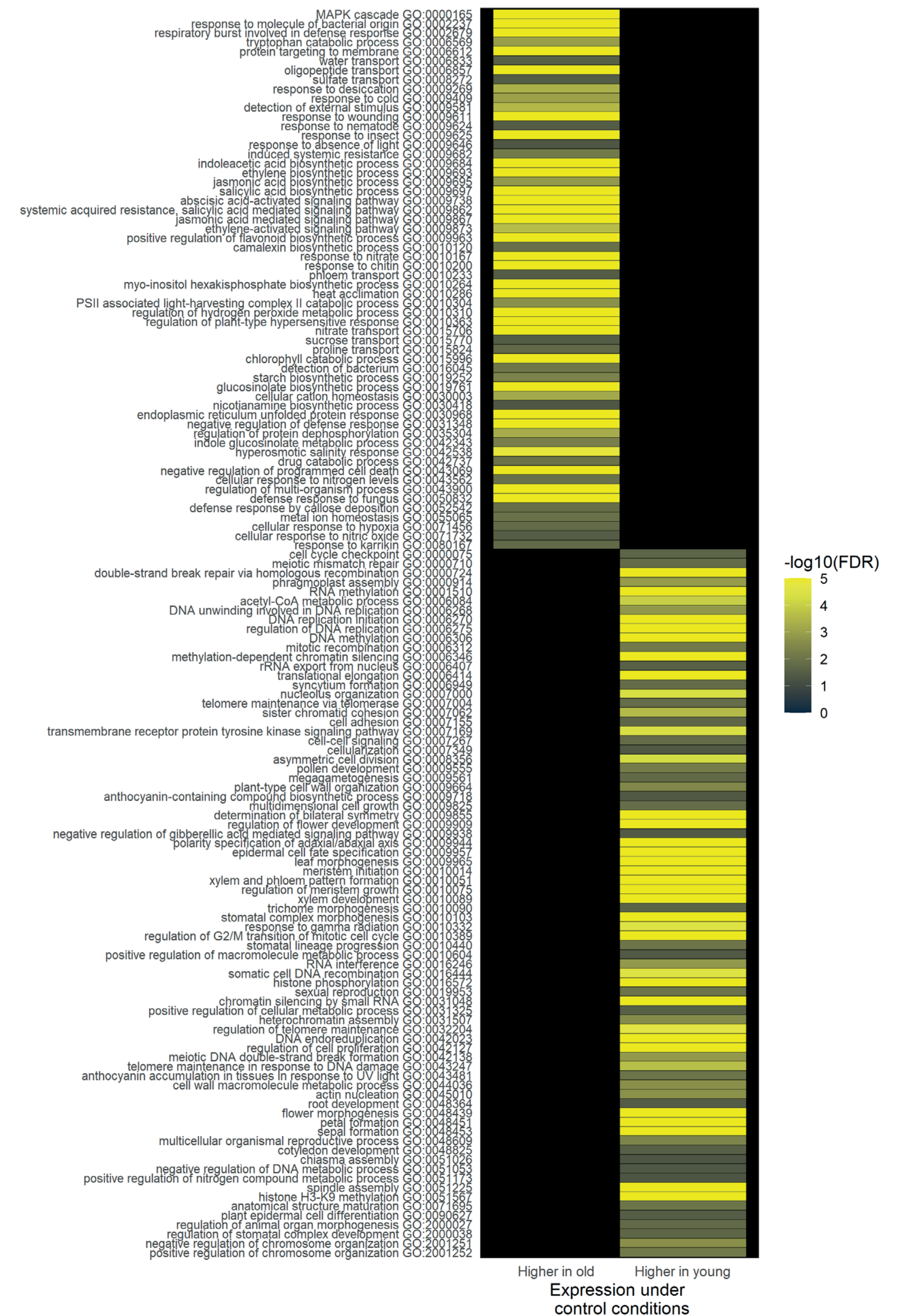


Figure S2 | Core hypoxia genes in mRNAseq data

The response of core hypoxia genes (Mustroph et al., 2009) to the indicated treatments. Genes with a significantly age-dependent response to the submergence or post-submergence treatments are indicated in red. The diagonal line indicates where the response in old leaves (X-axis) is the same as that in young leaves (Y-axis).

Figure S3 (Right page) | Differences between old and young leaves under control conditions

GO enrichment of the genes that were significantly differentially expressed between old and young leaves under control conditions, separated into those that were higher expressed in old leaves (n=2494) and those higher expressed in young leaves (n=2851). Differences in gene expression were deemed as significant when the FDR-adjusted p-values was below 0.05 and the absolute $\log_2$ fold change was greater than 1. All shown GO terms are significantly overrepresented in their respective set of genes, with an FDR-corrected p-value below 0.05.



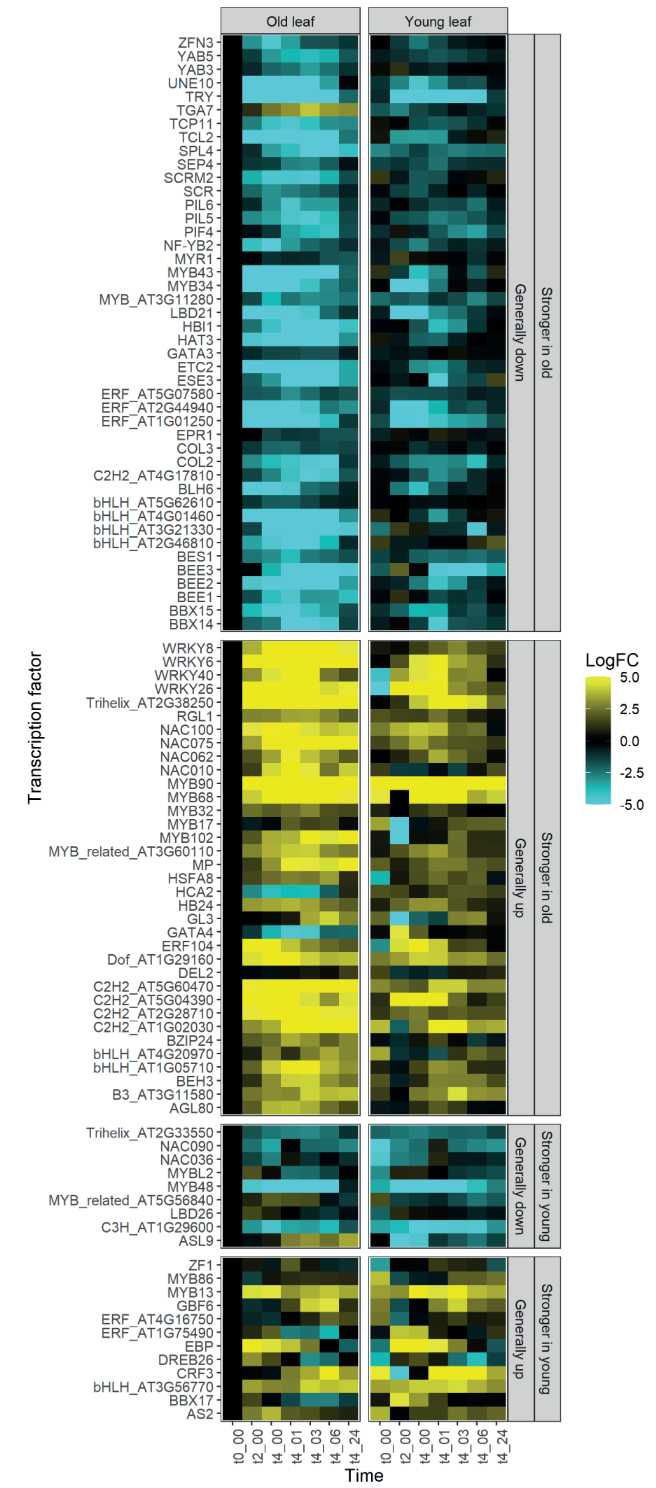


Figure S4 | Transcription factors showing age-dependent responses
All Arabidopsis transcription factors were selected that were differentially expressed between old and young leaves at all six timepoints after t0_00. These were then divided into those that responded stronger in old or young leaves and those that generally showed an increase or decrease of transcript levels. Genes were classified as stronger in old when the absolute mean $\log_2$ fold change across all old samples was higher than the absolute mean $\log_2$ fold change across all young samples. Genes were classified as generally up when the median $\log_2$ fold change across all samples was above zero, others were classified as generally down.

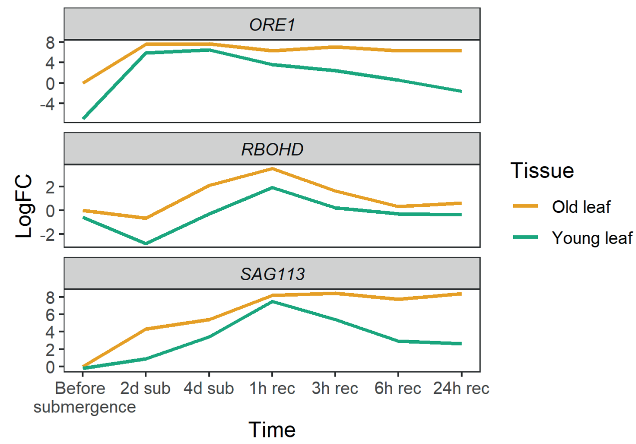


Figure S5 | Expression of genes identified in Yeung et al., 2018
Expression of *ORE1*, *RBOHD*, and *SAG113* before submergence, during submergence, and during the post-submergence phase. Expression was calculated relative to that in old leaves before submergence.

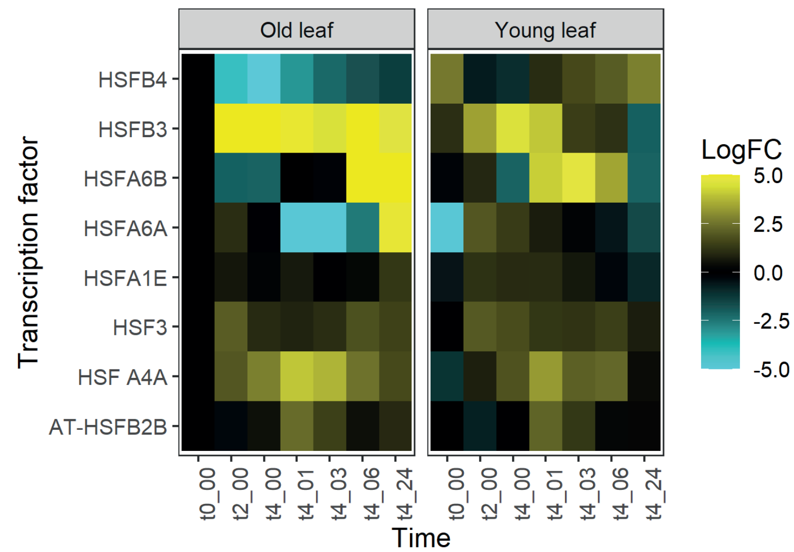
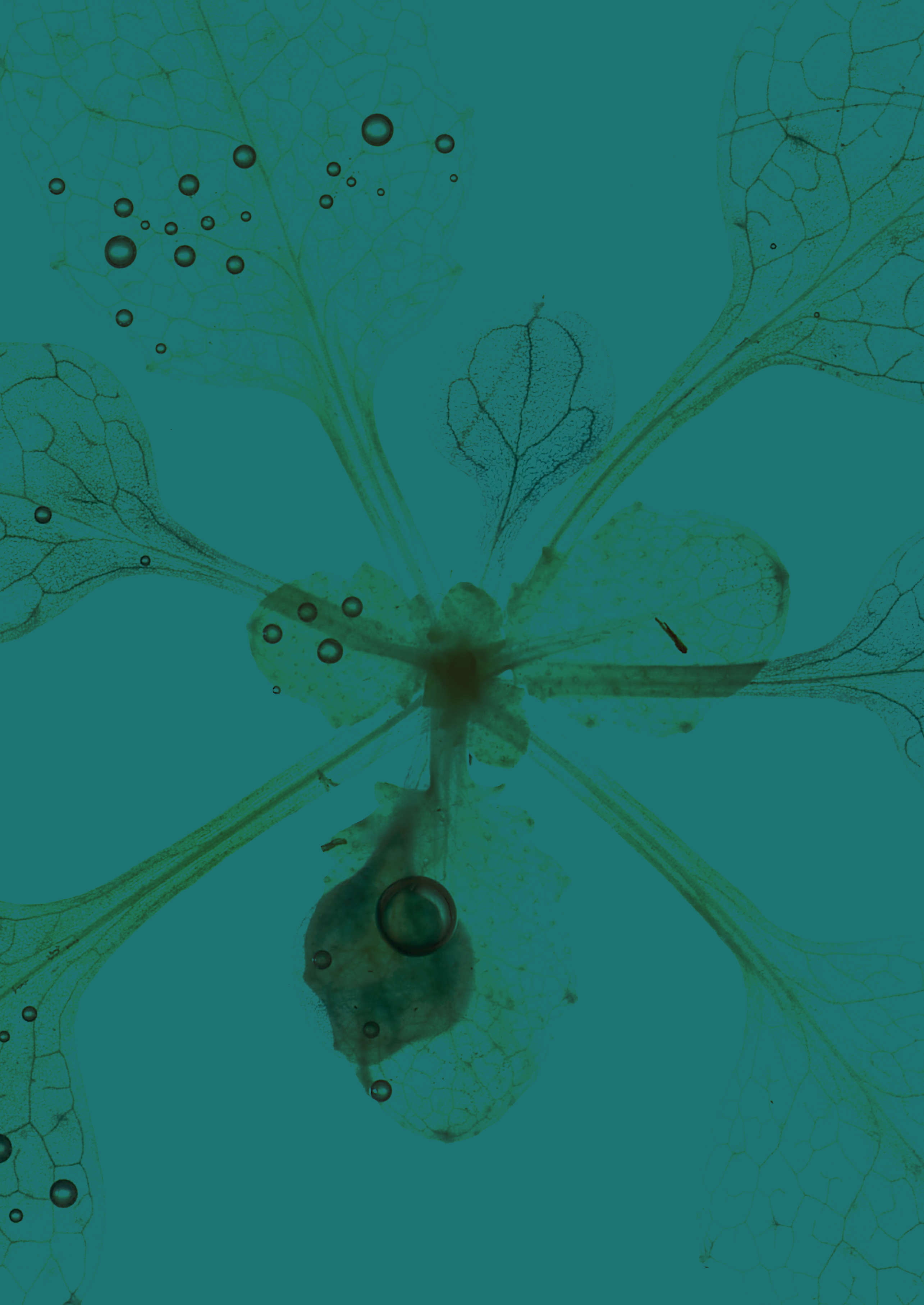


Figure S6 | Expression of HSF transcription factors
Expression of the eight transcription factors of the HSF family whose targets are enriched in cluster K11 in Figure 7. Expression was calculated relative to that of non-submerged old leaves.



Chapter 3

Submergence reduces the ABA sensitivity of old Arabidopsis leaves, leading to age-dependent dehydration during recovery

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Abstract

Submergence imposes limitations on plant metabolism and photosynthesis, leading to an energy crisis. The post-submergence phase poses a second round of stress, where plants are exposed to sudden reoxygenation and reillumination. A visible effect of this phase is the rapid dehydration of old leaves. A transcriptome survey of leaf-age specific submergence responses suggested a role for ABA in this dehydration response. Here we investigated potential mechanisms, including the role of ABA in mediating the observed leaf-age dependent water loss. Age-dependent differences in post-submergence water retention capacity could not be explained by variation in stomatal aperture or in cuticular permeability. Rather, the sensitivity of stomatal closure to exogenous ABA application was reduced in old leaves but not in young leaves during the post-submergence phase. This suggests that although old leaves were still sensitive to ABA at the transcriptional level after a submergence treatment and had a smaller stomatal aperture relative to non-submerged conditions, their stomata were unable to sufficiently reduce their aperture to prevent water loss. The mechanism controlling age-dependent water loss was not established, but potential regulators are discussed.

Introduction

Even after the water levels decline after a flooding event, the soil remains wet for an extended period. Despite this, plants recovering from flooding display drought-like symptoms such as leaf wilting. This “physiological drought” is attributed to flood damaged root systems that are unable to compensate for the water lost through the leaves via evapotranspiration. In soybean (*Glycine max*), seven days of waterlogging lead to an average reduction in root dry weight of 26.1% and a reduction in root surface area of 57.5% (Sakazono et al., 2014). In a flooding-intolerant rice accession, seven days of complete submergence lead to a reduction in root length by 68% (Ismail et al., 2009). The inability of the smaller root systems to take up water also leads to symptoms associated with drought stress in Arabidopsis plants recovering from submergence (Yeung et al., 2018). In a study comparing two Arabidopsis accessions, shoot dehydration following desubmergence was more severe in the sensitive accession Bay-0 relative to the more tolerant Lp2-6. Grafting experiments showed that this variation stemmed from differences between the shoots of these accessions rather than their root systems, indicating an important role for leaf responses in limiting post-submergence water loss (Yeung et al., 2018). Higher water loss in sensitive Bay-0 was associated with a re-opening of stomata upon desubmergence. This correlated with increased expression of the phosphatase *SAG113* which inhibits ABA-mediated stomatal closure (Yeung et al., 2018).

In the rice cultivar IR42, water loss during post-submergence recovery was caused by a loss of hydraulic conductivity of leaf cells (Setter et al., 2010). The major regulator of rice submergence responses SUB1A also contributes to post-submergence recovery by mitigating this water loss (Fukao et al., 2011). During submergence SUB1A limits carbohydrate consumption and thereby induces the low-oxygen quiescence strategy (Perata and Voesenek, 2007). During post-submergence recovery SUB1A increases the sensitivity of leaves to ABA, which improves their water retention capacity via an unidentified mechanism (Fukao et al., 2011). Increased sensitivity to ABA by overexpression of ABA receptors has been shown to improve drought tolerance in Arabidopsis, poplar, soybean, rice, and wheat (Kim et al., 2014; Verma et al., 2019; Zhao et al., 2016; Yu et al., 2017; Cao et al., 2017; Mao et al., 2022).

This effect of ABA in mitigating water loss is likely to involve stomatal regulation. Gas exchange (including CO_2 uptake and water loss), between a leaf and its environment largely occurs via the stomata. The mechanisms controlling stomatal development are highly conserved across plant species (Peterson et al., 2010). By rapidly changing the turgor pressure of stomatal guard cells, leaves can change the size of their stomatal pores. Activation of ion channels on the plasma membrane of guard cells induces a rapid efflux of anions out of the cell, leading to stomatal closure (Vahisalu et al., 2008). Plants regulate stomatal movement and thus gas exchange in response to various environmental stimuli. For example, pathogen exposure triggers stomatal closure and restricts their entry into the plant (Zeng et al., 2010). Leaves can also close their stomata to prevent water loss under drought conditions (Murata et al., 2015). Drought-induced stomatal closure is largely controlled by ABA. When roots sense water deficit, ABA is produced and is transported via the vasculature to the leaves (Kuromori et al., 2018). Besides transport of ABA itself, roots can also induce the transport of small signaling peptides, which induce the local biosynthesis of ABA in leaves (Takahashi et al., 2018). In addition to these mobile signals, guard cells themselves also produce ABA, which was demonstrated to facilitate quick local responses to changes in humidity (Bauer et al., 2013). Although stomatal closure can be beneficial for a plant to reduce water loss, it also limits the CO_2 uptake required for carbon fixation.

Stomatal signaling and drought responses are both affected by leaf age (Pantin et al., 2012; Rankenberg et al., 2021). In *Arbutus pineda*, drought treatment lead to a stronger reduction in the photosynthesis rate and the stomatal conductance of

young leaves than old leaves (Nadal et al., 2020). In Arabidopsis, however, drought treatment elicited a greater decrease in photosynthetic efficiency of old leaves than of young leaves (Jung, 2004). To further complicate the relationship between leaf age and drought tolerance, young leaves of *Artemisia ordosica* were more tolerant to mild drought than old leaves but were less tolerant to severe drought (Wang et al., 2021). Although drought often has an age-dependent effect on photosynthetic performance of a leaf and changes in stomatal aperture (Pantin et al., 2012), the exact mechanisms mediating this are poorly defined.

Characterisation of the transcriptome responses of old and young leaves to the post-submergence phase revealed a stronger induction of genes associated with ABA signaling in old leaves. This correlated with the faster dehydration of old leaves following desubmergence. In this chapter we further investigated the mechanism regulating this. We investigated whether the increased water loss in old leaves was related to stomatal traits. The role of ABA signaling in regulating post-submergence water loss was probed using mutants. While we could confirm the role of ABA in minimizing water loss, we found that old leaves closed their stomata further than young leaves. However, ABA sensitivity of old leaf guard cells was reduced in the post-submergence phase and could explain the greater water loss. The mechanism controlling the age-dependent reduction in the sensitivity of guard cells to ABA is unknown, but potential regulatory components are discussed.

Results

Old leaves exhibit greater dehydration than young leaves during the post-submergence phase

In the post-submergence phase, older leaves visibly dehydrated faster than young leaves (Chapter 2, Figure 1B). This trend was also confirmed from fresh weight and water content measurements of old and young leaves recovering from different submergence durations (Figure 1A-B). Leaves were labelled as dehydrated when their water content (defined as (fresh weight – dry weight) / fresh weight * 100%) was lower than 90% of the mean water content of control leaves. A reduction of relative water content of 10% or more is typically associated with a stressful water deficit (Zhang and Bartels, 2018). Another indication of the higher water loss of old leaves was their greater reduction in leaf fresh weight (Figure 1B). Here, leaves with a fresh weight below 40% of the mean of non-submerged leaves were designated as stressed, as this was the lower boundary of the variation in fresh weight of untreated plants. These two parameters of leaf dehydration, thus demonstrated more frequent dehydration in old leaves than in young leaves ($p<0.05$, Fisher's exact test). There was a positive correlation between dehydration and submergence duration. This was likely due to the inability of leaves that were already severely damaged by the submergence phase to respond properly to the post-submergence phase.

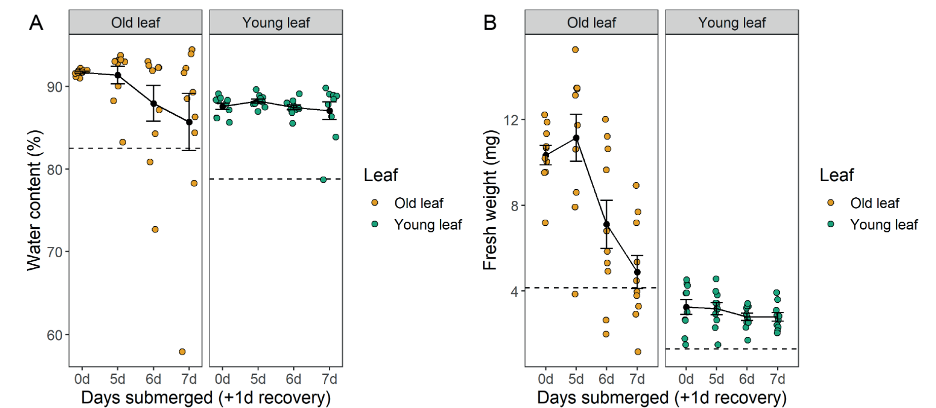

Figure 1 | Age-dependent leaf dehydration in the post-submergence phase
A) Water content (the percentage of water per leaf weight) of old (yellow circles) and young leaves (green circles) one day after the indicated submergence durations. The dashed line indicates 90% of the mean water content of leaves of non-submerged plants, leaves with a lower water content were designated as dehydrated. This happened more often in old leaves than in young leaves ($p<0.05$, Fisher's exact test), n = 10 leaves per timepoint.
B) Fresh weight of old and young leaves one day after the indicated submergence durations. The dashed line indicates 40% of the fresh weight of leaves under control conditions, leaves with a lower fresh weight were designated as dehydrated. This happened more often in old leaves than in young leaves ($p<0.05$, Fisher's exact test), n=10 leaves per timepoint.

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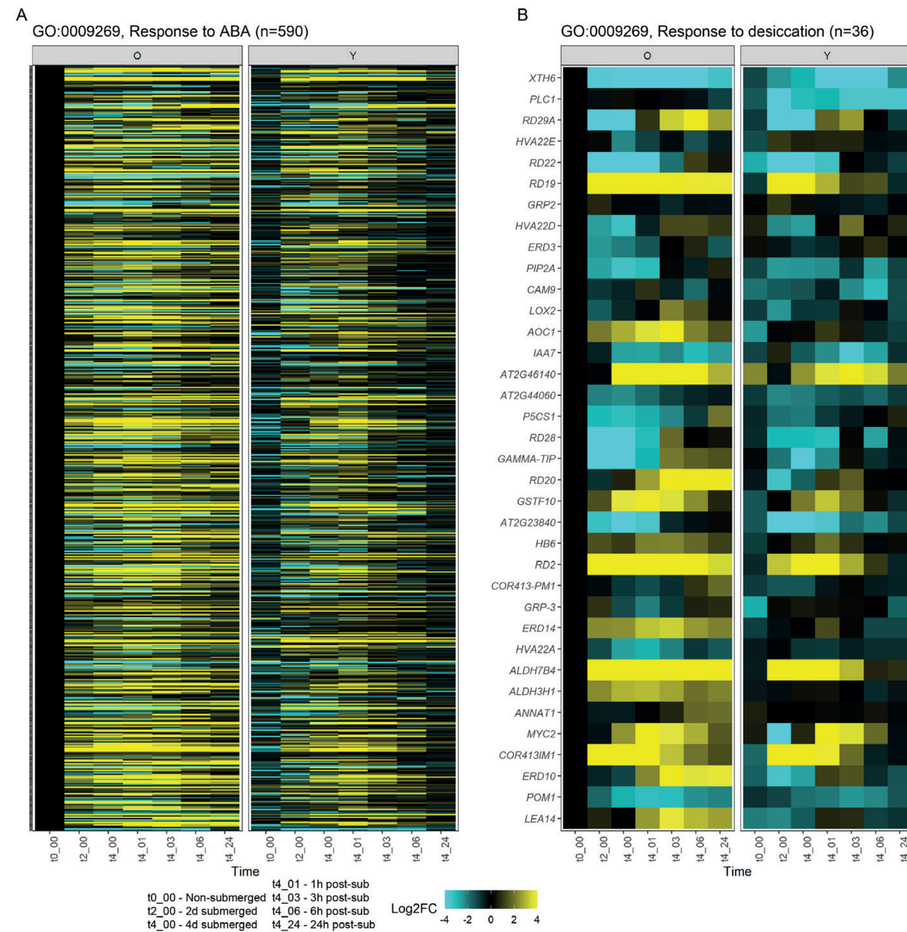


Figure 2 | A stronger transcriptomic signature of ABA and desiccation-related genes is observed in old leaves
Heat maps showing the expression of all genes in the "Response to ABA" (A) and "Response to desiccation" (B) GO categories, which were overrepresented in a cluster of leaf-age specific DEGs with higher upregulation in old leaves than young leaves during the post-submergence phase (Chapter 2, Figure 7, Cluster K6). Expression was calculated relative to that of non-submerged old leaves.

As expected based on the greater dehydration of old leaves, mRNAseq analysis of age-dependent responses to the submergence and post-submergence phases revealed a stronger induction of transcripts associated with ABA signaling and desiccation (the final stage of dehydration, as defined in (Zhang and Bartels, 2018)) relative to young leaves (Chapter 2, Figure 7, cluster K6). This cluster of 113 genes showing a stronger induction in old leaves than in young leaves during the post-submergence phase was enriched for the GO terms "Response to ABA" (GO:0009737) and "Response to desiccation" (GO:0009269). Plotting the individual genes associated with these GO terms in these clusters revealed that while genes associated with ABA were generally induced in both old and young leaves during the submergence phase, they were further induced during the post-submergence phase only in old leaves (Figure 2A). Genes associated with the response to desiccation showed a less homogenous expression pattern, some were downregulated whereas others were induced during the submergence and post-submergence phases (Figure 2B). Among the genes showing a stronger induction in old leaves than in young leaves were some well-characterized markers for drought stress, like *RESPONSE TO DESICCATION* (*RD*) *20* and *RD29A*, and *EARLY RESPONSE TO DESICCATION* (*ERD*) *10* and *ERD14* (Kiyosue et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1993; Takahashi et al., 2000). This stronger dehydration signature in old leaves was consistent with the dehydration phenotype of plants recovering from submergence stress, and indicated a role for ABA in mediating post-submergence leaf water loss (Figure 1). We thus investigated its role further.

ABA signaling is active in both old and young leaves during recovery

ABA binding to the PYR/PYL/RCAR receptors inhibits dephosphorylation of SnRK2 by PP2C phosphatases. Phosphorylation of SnRK2 activates its ability to phosphorylate ABF transcription factors, which bind to genes with an *ABRE* (*ABA-RESPONSIVE ELEMENT*) in their promoter and induce their transcription (Nakashima and Yamaguchi-Shinozaki, 2013) (Figure 3A). Of the 113 genes in cluster K6 (Chapter 2, Figure 7A) 36 contained a motif similar to the *ABRE* binding motif of ABF1/2/3/4, in their promoters. This was a significant overrepresentation ($p=8.0*10^{-21}$) (Figure 3B). Among the genes with an *ABRE* element in their promoter were multiple that have been previously implicated in drought and ABA responses, like *NINE-CIS EPOXYCAROTENOID DIOGYNASE* (*NCED3*), *ABI FIVE-BINDING PROTEIN1* (*AFP1*), and *ERD10*.

To further investigate the induction of ABA signaling in response to desubmergence, we used the *ABRE:GUS* reporter line, which expresses a GUS enzyme driven by six copies of the *ABRE* from *RD29A* (Wu et al., 2018). In non-submerged plants faint GUS staining was visible in the middle of leaf laminas (Figure 3C). In plants that were submerged for five days GUS staining was only observed in the outer edges of laminas of young leaves and the shoot apex. However, desubmergence had the strongest effect. When plants were left to recover in light for three hours, GUS staining spread throughout the entire rosette. These results were consistent with the mRNAseq data showing the induction of ABA signaling in plants recovering from submergence, but deviated from the age-dependent pattern revealed by the mRNAseq data. This could be a consequence of the difficulty in determining quantitative and temporal differences in gene expression via GUS staining, as constitutively low promoter

activity and a brief burst of high promoter activity can both yield the same amount of accumulated GUS enzyme (Kim et al., 2006).

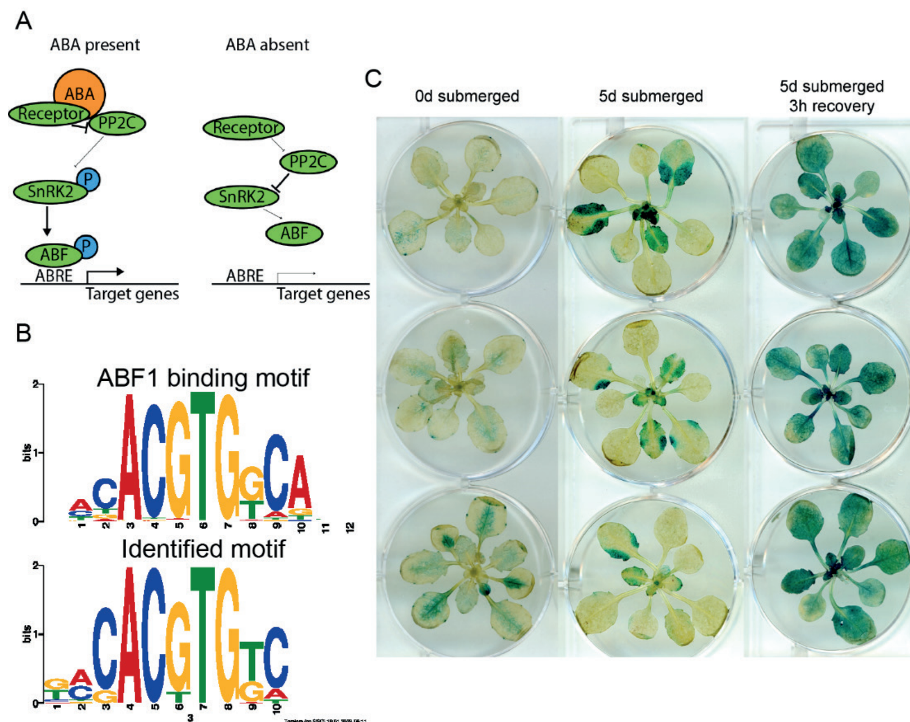


Figure 3 | ABA signaling is strongly activated in the post-submergence phase in both old and young leaves

A) Schematic of ABA-mediated signaling. When ABA binds to its receptors, this sequesters the PP2C phosphatases that normally dephosphorylate SnRK2. SnRK2 can then autophosphorylate and phosphorylate the ABF transcription factors. Phosphorylated ABFs bind the ABRE motif in the promoters of their target genes and induce their transcription. The relative thickness of a line indicates whether an interaction occurs in the presence or absence of ABA.

B) The motif overrepresented ($p=8.0*10^{-21}$) in the promoters of genes in cluster K6 (Chapter 2, Figure 7A), as identified using MEME (Bailey and Elkan, 1994). This motif was compared to known Arabidopsis transcription factor binding motifs using TOMTOM (Gupta et al., 2007; Castro-Mondragon et al., 2022), p-values for similarity of this motif to the binding sites of ABF1/2/3/4 ranged between $1.44*10^{-4}$ (ABF4) and $8.48*10^{-8}$ (ABF1). The similarity between the binding motif of ABF1 and the identified motif is shown as an example.

C) Representative images of ABRE:GUS plants before submergence, after five days of submergence in darkness, and after three hours of recovery in light. Per time point three rosettes were stained and all showed a similar trend across two experimental replicates.

ABA signaling contributes to limiting post-submergence water loss

Following the establishment of ABA signaling activation during the post-submergence phase we next explored its role in limiting water loss. For this, we assessed the speed of water loss of rosettes lacking ABA signaling components and compared it to Col-0 rosettes. Under control conditions, *abaQ* (lacking ABA receptors *PYR1/PYL1/PYL2/PYL4*), *arebQ* (lacking *ABF1/2/3/4*), and *snrk2.2snrk2.3* all dehydrated faster than Col-0 (Figure 4A). The difference was biggest for the *abaQ* mutants, which was expected as these lack signaling components that are the highest up in the cascade. In plants that were submerged for four days, water loss was faster in all genotypes, although the *abaQ* and *snrk2.2snrk2.3* plants still performed significantly worse than Col-0 at most timepoints. The increased water loss of all genotypes recovering from submergence compared to those under control conditions suggested that the water retention capacity of plants was reduced when they had previously been stressed by a submergence treatment. Although these experiments were performed using excised entire rosettes rather than individual leaves still attached to the plant, it did reflect the same pattern as that observed in Figure 1, where longer submergence treatments led to greater dehydration in the post-submergence phase.

ABA is an important regulator of stomatal movement during the post-submergence phase, but ABA also plays a role in several other processes related to flooding tolerance (Yeung et al., 2018; Zhao et al., 2021; González-Guzmán et al., 2021). The inhibition of growth and the induction of senescence, for example, are both regulated by ABA (Chen et al., 2020). To disentangle the stomatal and non-stomatal effects of ABA we compared the dehydration phenotypes of the ABA biosynthesis mutant *aba3* with that of *MYB60:ABA3 aba3*, in which ABA biosynthesis has been restored in the stomata. Under control conditions and after five days of submergence, water loss was increased in the *aba3* mutant compared to Col-0 as expected (Figure 3B). This confirmed our previous results (Figure 3A), that ABA is important in limiting water loss under control conditions and in the post-submergence phase. The *MYB60:ABA3 aba3* plants, in which ABA biosynthesis was restored specifically in the guard cells, showed an intermediate phenotype between Col-0 and *aba3* under control conditions (Figure 3B). Although the *MYB60:ABA3 aba3* lines lost more water than Col-0 plants, they did not dehydrate as quickly as *aba3* mutants did. In plants that were submerged for five days the speed of water loss was again increased in all genotypes but the difference between Col-0 and *MYB60:ABA3 aba3* was lost. However, both *MYB60:ABA3 aba3* and Col-0 still dehydrated slower than *aba3*. These results suggested that stomatal ABA is important for reducing water loss in plants recovering from submergence.

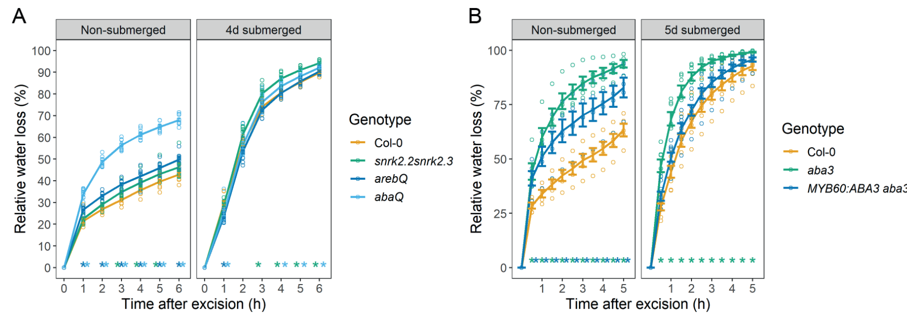


Figure 4 | ABA signaling and biosynthesis contribute to limiting post-submergence dehydration

A) Relative water loss of excised rosettes of ABA signaling mutants snrk2.2snrk2.3, arebQ, and abaQ is increased compared to Col-0. Plants were submerged for four days and then rosettes were excised immediately following desubmergence. Rosettes were then weighed every hour, n=10 plants per genotype.

B) Relative water loss of the ABA biosynthesis mutant aba3, and MYB60:ABA3 aba3 plants in which ABA biosynthesis is restored in the guard cells. Rosettes were weighed every 30 minutes, n=5-8 plants per genotype.

Relative water loss was calculated using the formula $1-((FW_{tX}-DW)/(FW_{t0}-DW))*100\%$, where FW_{tX} is the fresh weight at a given timepoint, FW_{t0} is the fresh weight immediately after excision of the rosette, and DW is the dry weight.

Asterisks indicate significant differences from Col-0 at the indicated timepoint (One-way ANOVA with Dunnett's post-hoc test).

Old leaves have a smaller stomatal aperture and density than young leaves

Despite the importance of stomatal ABA signaling in limiting dehydration (Figure 4) and the increased transcriptional signature of ABA responses in old leaves (Figure 2 - 3), water loss was greater in old leaves than in young leaves in the post-submergence phase (Figure 1).

Differences in water loss can either be caused by variation in stomatal aperture or density, or variation in cuticular water loss. We hypothesized that age-dependent differences in stomatal aperture in the post-submergence phase could be responsible for the greater post-submergence water loss of old leaves, as this trait is controlled by ABA and can change the fastest in response to environmental changes.

We found that the stomatal aperture of old leaves was significantly smaller than that of young leaves under control conditions (Figure 5A). In plants recovering from four days of submergence, the stomatal aperture of both old and young leaves was reduced, although the stomata of young leaves remained further open compared to old leaves. The relative change in the stomatal aperture was significantly greater in young leaves ($p<0.05$, two-way ANOVA), but the difference here was small: the average stomatal aperture of old leaves after a submergence treatment was 70% of that of untreated old leaves, whereas it was 65% for young leaves. The density of mature stomata in old leaves was also significantly lower than that of young leaves (Figure 5B). The greater variation in stomatal density of young leaves can be attributed to the greater variation in leaf expansion of young leaves, whereas old leaves are typically fully expanded.

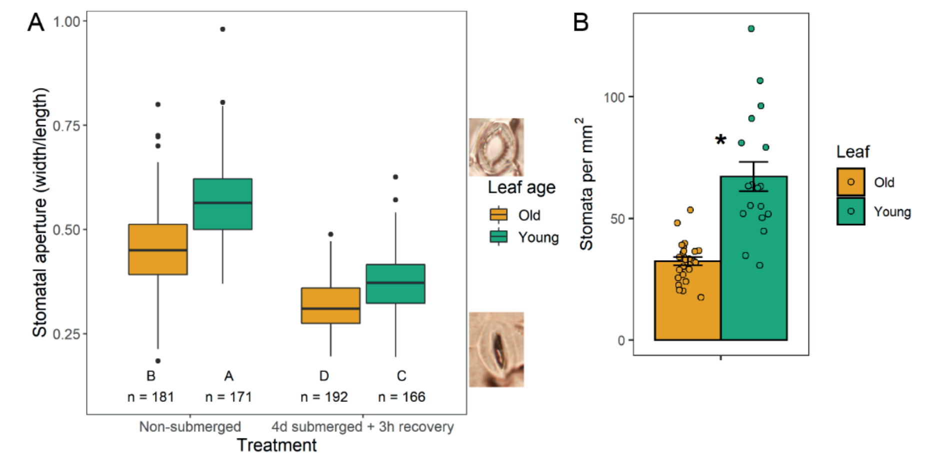


Figure 5 | Old leaves have a smaller stomatal aperture and density than young leaves

A) Stomatal aperture of old and young leaves before and after four days of submergence followed by three hours of recovery. The images on the right of the plot are representative stomatal pores with apertures of 0.6 (top image) and 0.2 (bottom image). Sample sizes are indicated underneath each boxplot, different letters indicate significant differences as indicated by a two-way ANOVA followed by Tukey's post-hoc test.

B) Stomatal density on the adaxial side of old and young leaves. Asterisk indicates significant differences as determined by Welch's test, n=18-25 images per leaf type.

Submergence does not induce age-dependent changes in cuticular permeability

Although the majority of water lost through a leaf is via the stomata, non-stomatal water loss also occurs. Plants typically limit non-stomatal water loss by developing a cuticle, a waxy layer on the epidermis that prevents water from diffusing across cell membranes (Domínguez et al., 2017). The cuticle plays a role in preventing post-submergence dehydration, as mutants lacking a cuticle lose more water during post-

submergence recovery (Xie et al., 2020). Under control conditions, the conductance of the cuticle only makes up a small proportion of the total conductance of a leaf (Duursma et al., 2019). Submergence treatments have been reported to lead to a thinner leaf cuticle to increase the gas exchange capacity of a leaf (Mommer et al., 2007). We hypothesized that this might happen in an age-dependent manner in Arabidopsis, which could increase the dehydration of old leaves during the post-submergence phase. Cuticle permeability was measured by determining toluidine blue uptake of leaves, and quantifying this via spectrophotometry (Tanaka et al., 2004; Zhang et al., 2022). This was done after various submergence durations to determine if this treatment increased the cuticular permeability in an age-dependent manner. We found that although there was an increase in toluidine blue uptake in submerged plants, indicating a thinner cuticle, this effect was not greater in old leaves than in young leaves (Figure 6). Submergence treatments of intermediate lengths increased the toluidine blue uptake of leaves, consistent with other studies (Xie et al., 2020). Notably, a reduced toluidine blue uptake was observed plants submerged for six or seven days. A potential explanation for this is that once cells are severely damaged by extended submergence durations, they will dissolve into the water in which they are submerged and can no longer absorb toluidine blue.

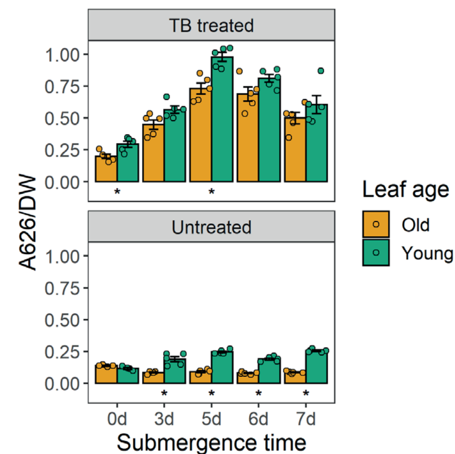


Figure 6 | Age-dependent cuticular permeability
Cuticular permeability as measured by toluidine blue (TB) uptake of old and young leaves. Absorbance was measured at 626nm, values were normalized by leaf dry weight. Leaves not treated with TB were used to control for baseline differences in absorbance at 626nm. n=4-5 per group, each consisting of two old or young leaves from different plants pooled together. Asterisks indicate significant differences between old and young leaves (paired t-test).

Stomatal sensitivity to ABA is reduced in old leaves after submergence but not in young leaves

Although old leaves had a smaller stomatal aperture and lower stomatal density than young leaves (Figure 5), they lost more water during post-submergence recovery (Figure 1). A possible explanation for this is that due to the thinner lamina of old leaves they are more susceptible to water loss than young leaves. It would be beneficial for old leaves to have stomata that are more sensitive to ABA to compensate for this. To determine stomatal sensitivity to ABA of old and young leaves control plants and plants recovering from four days of submergence were sprayed with 0µM, 50µM, or 100µM of ABA and the stomatal aperture measured. Old leaves showed a clear pattern of reduced stomatal aperture with increasing ABA concentrations, but this sensitivity was reduced in plants recovering from submergence (Figure 7A). Young leaves, on the other hand, showed the same pattern of decreasing stomatal aperture in response to increasing ABA concentrations, in both control plants and plants recovering from submergence. This suggests that stomatal sensitivity to ABA was reduced in an age-dependent manner after a submergence treatment.

Although the stomatal sensitivity of old leaves to ABA was reduced in the post-submergence phase, old leaves still showed a stronger ABA transcriptome response signature during this phase than young leaves (Figure 2A). It could be that this stronger transcriptional response was the consequence of the overrepresentation of non-guard cell tissue in the whole leaves harvested for mRNAseq. Since the majority of cells in a leaf are mesophyll cells and epidermal cells, the majority of the RNA used in bulk mRNAseq will come from these tissues as well. Guard cells, on the other hand, only make up less than 3% of the total amount of cells in an Arabidopsis leaf (Xia et al., 2022). To determine the guard cell-specific transcriptional response to submergence recovery in old and young leaves we used a previously identified set of ABA-responsive genes that are expressed specifically in guard cells (Bauer et al., 2013). These 29 genes were divided into two groups, based on their repression (n=20) or induction (n=9) in response to ABA treatment (Bauer et al., 2013). Genes repressed in response to ABA treatment were already repressed in response to submergence in old leaves and their expression remained low during the recovery phase (Figure 7B). In young leaves these genes did not exhibit a strong response to submergence or recovery although their expression was generally higher than in old leaves. Genes that were induced in response to ABA showed a repression in response to submergence in both old and young leaves, and a further induction during the recovery phase. Several of these genes have been connected to ABA responses, like *SUCROSE SYNTHASE3* (*SUS3*) and *LATE EMBRYOGENESIS ABUNDANT 4-5* (*LEA4-5*) (Daloso et al., 2016; Olvera-Carrillo et al., 2010), and were induced faster and

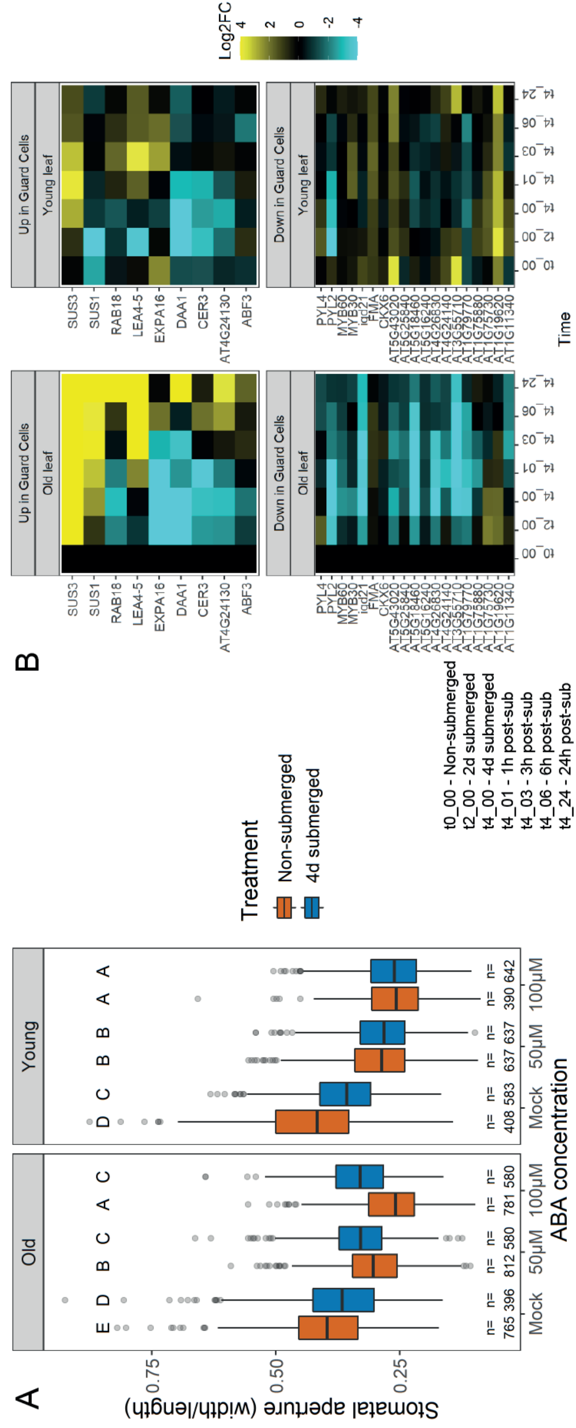


Figure 7 | Stomatal sensitivity to ABA is reduced in old leaves after submergence but not in young leaves
A) Stomatal aperture of old and young leaves before and after four days of submergence after treatment with 0µM (Mock), 50µm, or 100µM ABA. The indicated number of stomata (below the boxplots) was measured from three to five leaves per group. Different letters (above the boxplots) indicate significant differences, as determined by a two-way ANOVA followed by Tukey's post-hoc test.
B) Expression of genes that respond to 25µM ABA treatment specifically in guard cells (Bauer et al., 2013) in mRNAseq data. Genes were divided into those that are repressed (n=20) or induced (n=9) in guard cells in response to ABA treatment (Bauer et al., 2013).

for longer in old leaves compared to young leaves during the post-submergence phase. These results show that the stronger induction of ABA-responsive genes in old leaves during the post-submergence phase likely also happened in guard cells.

Discussion

Dehydration tolerance in the post-submergence phase varies with leaf age

Our results show that old leaves exhibited a stronger induction of ABA-related transcripts but also lost more water during the post-submergence phase than young leaves. We expected that this was either due to a greater stomatal aperture or a greater cuticular conductivity of old leaves. Surprisingly, there were no differences in the permeability of the cuticle, and young leaves had a greater stomatal aperture than old leaves. In contrast with this, the stomatal movement of old leaves was less sensitive to exogenously applied ABA during post-submergence recovery than that of young leaves. Although old leaves closed their stomata further than young leaves, it was not enough to prevent water loss.

In *Xanthium strumarium*, drought treatment induces stress symptoms in the old leaves first and in the youngest leaves last (Cornish and Zeevaart, 1984). Although young leaves are more drought-tolerant than old leaves, their water potential during drought is higher and they accumulate less ABA than old leaves. *Nicotiana tabacum* also shows a greater drought tolerance of young leaves, although these leaves also accumulate more ABA than in old leaves under drought stress (Havlová et al., 2008). Thus there is no clear correlation between ABA levels and drought tolerance. Transcriptome analysis of the responses of leaf primordia, expanding leaves, and mature leaves of Arabidopsis revealed that there are large developmental differences between these tissues in the response to mannitol treatments (Skirycz et al., 2010). A mannitol treatment effectively imposes a water deficit in plants, as the mannitol in the medium reduces its water potential. Mature leaves showed a strong response of genes typically associated with dehydration stress, expanding leaves and primordia showed this to a lesser extent (Skirycz et al., 2010). Interestingly, younger leaves showed a stronger response of ethylene-associated genes than older leaves and ethylene-insensitive *ein2-5* plants exhibited reduced mannitol tolerance compared to Col-0 plants, suggesting a role for ethylene signaling in developmental stage-dependent responses to mannitol (Skirycz et al., 2010).

The effect of leaf anatomy on water loss

Our experimental approach to investigate the mechanisms underlying age-dependent water loss during post-submergence recovery focused on age-dependent transcriptional regulation. A potential cause for age-dependent water loss independent of transcriptional differences is the difference in leaf anatomy between old and young leaves. Old leaves in our system were typically close to or at full expansion, whereas young leaves were not. As an Arabidopsis leaf expands, its specific leaf area (SLA, area per dry mass) and the surface-to-weight ratio increases (Lawrence et al., 2021). This increased surface area of older leaves means that there is more area via which water can be lost to the environment. In groundnut (*Arachis hypogaea*), there is an inverse correlation between SLA and drought tolerance (Nautiyal et al., 2002). In natural populations of *Arabidopsis lyrata*, a close relative of *Arabidopsis thaliana*, accessions with a lower SLA also have greater drought tolerance (Sletvold and Ågren, 2012). The effect of differences in SLA on age-dependent water loss will be hard to overcome, as they are a direct result of the changes in the physical form of a developing leaf rather than a result of genetically encoded responses to the environment. It is theoretically possible to develop plants that are more drought- and post-submergence dehydration tolerant due to an increased leaf thickness, but this will always have secondary effects as SLA is highly connected to other physiological traits (Poorter et al., 2009).

Enrichment of cis-regulatory elements helps in identifying transcriptional regulators

Transcription factors are often controlled post-translationally, which can make interpretation of their importance based on their mRNA abundance alone difficult (Mazzucotelli et al., 2008). A better tool to assess the importance of transcription factors is via the abundance of their direct targets. There is a plethora of datasets on the binding motifs and target genes of transcription factors, especially in Arabidopsis but also in other species (Tian et al., 2020). Scanning the promoters of genes which showed a stronger increase in expression during recovery in old leaves than young leaves revealed the enrichment of a motif that significantly resembles the binding site of multiple ABA-RESPONSIVE ELEMENT BINDING FACTOR (ABF) transcription factors (Figure 3). ABFs are phosphorylated by the kinase SnRK2 upon ABA exposure (Furihata et al., 2006). Although the binding motifs of ABF1-4 were enriched in the promoters of a cluster of genes showing age-dependent gene expression, the expression of the *ABFs* themselves did not show a strong age-dependent pattern (Figure S1). Here, assaying the enrichment of transcription factor binding sites in the promoters of genes with shared expression patterns has helped in identifying signaling pathways induced by submergence recovery.

Stomatal sensitivity to ABA is dependent on age and ethylene

The ABA sensitivity of guard cells in young, newly emerging Arabidopsis leaves is typically low. As leaves develop, they gradually acquire an increased sensitivity to ABA (Pantin et al., 2013; Hõrak et al., 2021) and this is lost towards the end of the lifecycle of a leaf (Willmer et al., 1988; Zhang et al., 2012). This loss of sensitivity is controlled by the protein phosphatase 2C SAG113 and is proposed to accelerate senescence and death of old, expendable leaves (Zhang and Gan, 2012; Zhang et al., 2012). The increased expression of *SAG113* with leaf age renders stomata of old leaves insensitive to ABA, and facilitates leaf desiccation during both leaf senescence and the post-submergence phase (Yeung et al., 2018).

The strong transcriptional signature of ABA-related genes during the post-submergence phase in old leaves could be indicative of high ABA concentrations in these leaves. The Arabidopsis accession Bay-0 has been shown to keep its stomata open during the post-submergence phase, leading to excessive water loss (Yeung et al., 2018). ABA levels were high in leaves of Bay-0, but the induction of *SAG113* by these high ABA levels led to an inhibition of stomatal closure. Measuring leaf-specific ABA levels in the post-submergence phase would show whether the increased transcriptional response to ABA of old leaves could be a consequence of their high endogenous ABA levels.

Interestingly, the expression of *SAG113* is induced upon ABA exposure (Zhang et al., 2012). Whereas ABA exposure normally leads to stomatal closure, an ABA-mediated induction of *SAG113* can prevent stomatal closure. During the post-submergence phase, inhibiting ABA signaling via ABA ANTAGONIST1 (AA1) led to a decrease in expression of *SAG113* and a reduction in stomatal aperture (Yeung et al., 2018). *SAG113* has also been identified as a target of the ethylene signaling regulator EIN3 (Chang et al., 2013), and applying the ethylene perception inhibitor 1-MCP to Arabidopsis in the post-submergence phase reduced the expression of *SAG113* as well as the stomatal aperture of leaves (Yeung et al., 2018).

These results are consistent with previous reports that treatments of either ABA or ethylene induce stomatal closure, but that the combined application of ABA and ethylene can inhibit stomatal closure (Tanaka et al., 2005; Desikan et al., 2006; Chang et al., 2013; Beguerisse-Dıaz et al., 2012). Although the exact mechanism behind this antagonistic interaction between ABA and ethylene has not been identified, it could be that *SAG113* plays a role here as it inhibits stomatal closure and is induced by both ABA and ethylene.

The expression of ABA-responsive and ethylene-responsive genes, including *SAG113*, is induced during submergence in both old and young leaves, but remains high during recovery only in old leaves (Figure 2; Chapter 2, Figure 7, cluster K5; Chapter 2 Figure S5). As old leaves experience more leaf senescence than young leaves during submergence and post-submergence recovery (See Chapters 5 & 6), it could be that this state of increased senescence and ethylene signaling reduced the effect of ABA signaling on stomatal closure in an age-dependent manner. Given the age-dependent roles of ethylene and ABA, it is likely that the interaction between these two hormones in regulating stomatal aperture also depends on leaf age.

Sugar as a regulator of stomatal closure

One of the guard cell-specific transcripts exhibiting a stronger induction in old leaves during the post-submergence phase was *SUCROSE SYNTHASE 3* (*SUS3*) (Figure 7B). *SUS3* encodes one of the six sucrose synthase enzymes in Arabidopsis catalysing the cleavage of sucrose into UDP-glucose and fructose (Baud et al., 2004). *SUS1* and *SUS4* are both hypoxia-inducible genes, and plants lacking these two isoforms have a lower waterlogging tolerance than wild-type plants (Santaniello et al., 2014). Guard cell-specific overexpression of *SUS3* in tobacco has been shown to increase the aperture of dark-treated stomata upon reillumination and reduced the sucrose content of epidermal cells (Daloso et al., 2016). Consistent with this, reducing the expression of *SUS3* using antisense RNAs led to a decreased stomatal conductance and an increased sucrose content in potato plants (Antunes et al., 2012). Exogenous application of sucrose, glucose, and fructose all lead to the closure of stomata in Arabidopsis, via an unknown mechanism (Kelly et al., 2013; Li et al., 2018b).

Although old leaves showed a stronger carbon starvation response than young leaves during the submergence and post-submergence phases (Chapter 2, Figure 4, cluster K10; Figure S2), they also showed a more sustained response to sucrose and fructose during the post-submergence phase (Chapter 2, Figure 7, cluster K3). The response to sucrose was also enriched in a cluster of genes that was repressed in both old and young leaves during the submergence phase (Chapter 2, Figure 3, cluster K4). Clusters K3 and K4 contained genes with different expression patterns and were both enriched for the same GO term, but there was no overlap between these clusters in the genes associated with this GO term (Figure S3). Among age-independently downregulated genes in cluster K4 associated with the response to sucrose were several that encode enzymes that use UDP-glucose as a substrate, like *UDP-GLUCOSYL TRANSFERASE 78D2* (*UGT78D2*), *UGT84A1*, and *UDP-GLUCOSE DEHYDROGENASE4* (*UDG4*). Of the genes with a high expression in old and young leaves during submergence but sustained induction only in old leaves during the post-submergence phase were several associated with senescence and amino acid metabolism, like *SENESCENCE-ASSOCIATED GENE1* (*SEN1*) and *BRANCHED CHAIN AMINO ACID TRANSFERASE 2* (*BCAT2*). These results suggest that both old and young leaves reduced the expression of genes involved in sugar-based metabolism during the submergence phase, but that old leaves retain high expression of enzymes associated with the metabolism of amino acids during the post-submergence phase. Directly measuring the sugar content of old and young leaves will be essential to determine whether age-dependent changes in carbon metabolism could play a role in regulating differences in stomatal aperture with leaf age. In addition to this, mutants impaired in specific metabolic pathways could be used to assay the role of carbon metabolism in controlling stomatal dynamics. The induction of genes involved in amino acid metabolism could be controlled by the increased stomatal ABA signaling of old leaves, as ABA has been shown to induce the expression of *BCAT2*, *ASN1*, and other enzymes related to this process (Hartmann et al., 2015; Urano et al., 2009).

Stomatal movement in response to ABA is important to limit leaf water loss in the post-submergence phase. Although old leaves showed a strong transcriptional response to ABA-related genes after a submergence treatment, stomatal movement was less sensitive to ABA. This reduction in sensitivity to ABA of old leaves likely contributed to the gradient with age in post-submergence water loss. Potential causes of this variation between leaves of different ages are differences in their carbon metabolism, and differences in the expression of the protein phosphatase 2C *SAG113*.

Materials and methods

Plant growth conditions

See chapter 2

Stomatal aperture

Adaxial sides of leaves were pressed into President dental paste at the indicated timepoints. Imprints were made of the adaxial side, as the abaxial side of the leaf was close to the wet soil and variation in humidity across the leaf microclimate can affect stomatal behaviour (Pantin et al., 2013). After the imprints had hardened, leaves were removed and clear nail polish was applied to the imprints. Dried nail polish was mounted on microscope slides and was imaged on a Zeiss Axioskop II macroscope with a 100X magnification setting. Stomatal aperture was determined

based on these pictures by measuring the ratio between the length and width of the stomatal pore using ImageJ (NIH).

Water loss

Rosettes were detached from the root system either before submergence or after the indicated submergence duration, rosettes cut off after submergence were gently blotted dry with a paper towel. Rosettes were kept on petri dishes at a relative humidity of approximately 55% and a temperature of 21°C and were weighed on a Sartorius MX-5 scale at the indicated timepoints. After the last timepoint the plants were left at 80°C for two days to dry out. Water loss at each timepoint was calculated as $1-((FW_{tX}-DW)/(FW_{t0}-DW))*100\%$.

GUS staining

Plants expressing the beta-glucuronidase enzyme under the ABA responsive promoter element from the *RD29B* gene (Wu et al., 2018) were grown to the 10-leaf stage and submerged for the indicated amount of time. Whole rosettes were detached from the root system and fixed in 90% acetone for 20 minutes at -20°C. Plants were washed twice with GUS washing solution (0.1M pH=7 phosphate buffer, 10mM EDTA, 2mM $K_3Fe(CN)_6$) under vacuum for 10 minutes and then stained with GUS staining solution (0.1M pH=7 phosphate buffer, 10mM EDTA, 1mM $K_3Fe(CN)_6$, 1mM $K_4Fe(CN)_6$ and 1mg/ml X-Gluc) for 10m under vacuum at room temperature and then overnight at 37°C. Staining was stopped by incubating the plants in 3:1 acetic acid:ethanol for 1h and then cleared by washing several times in 70% ethanol.

Chemical treatments

ABA was applied to plants by dissolving ABA in ethanol and dissolving that in distilled water to a final concentration of 50µM or 100µM ABA, 0.1% Tween-20 was added to facilitate uptake of ABA. The mock solution contained 0.1% ethanol and 0.1% Tween-20 in distilled water. Plants recovering in the light from submergence in darkness were sprayed (200µl per spray) with either the ABA solutions or the mock twice directly after desubmergence, once 30 minutes after desubmergence, and once 1.5h after desubmergence. Stomatal aperture was determined using stomatal imprints three hours after desubmergence.

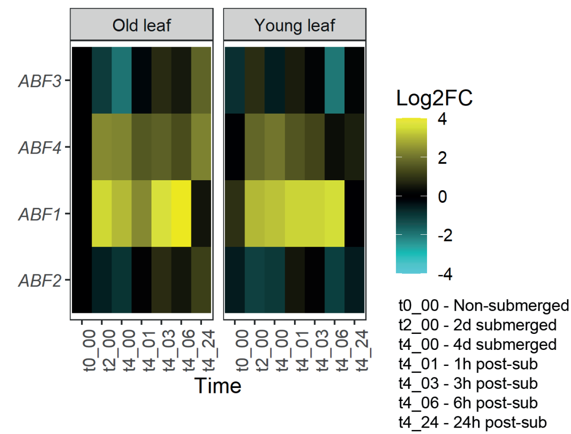
Toluidine blue staining

Old and young leaves were cut off at the indicated timepoints, and two pooled leaves of each were submerged in either 0.05% (w/v) Toluidine Blue solution or water for two minutes. Plants were washed three times in tap water to rinse off excess staining solution and were incubated for four hours in 80% ethanol at room temperature in the dark to let all absorbed toluidine blue leach out of the leaf. 200µl of each ethanol solution was then measured for its absorbance at 626nm in a Biotek HT plate reader. Leaves were dried for 48 hours at 80°C and were weighed to normalize absorbance at 626nm by dry weight.

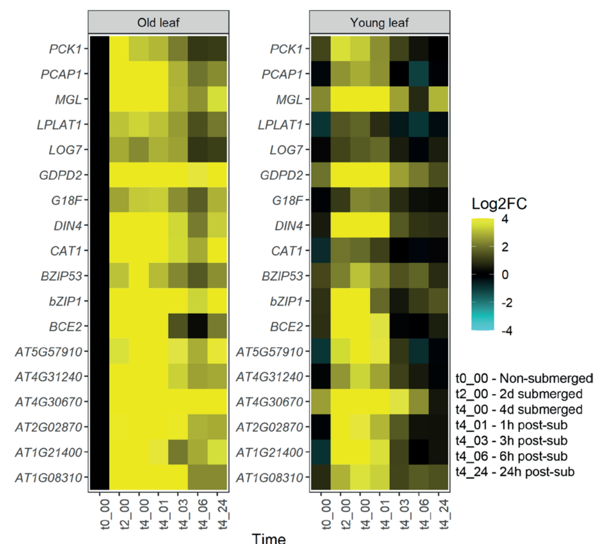
Promoter analysis

Promoters were analysed by extracting 500bp upstream and 100bp downstream of the transcriptional start site of genes of interest using the R package GenomicRanges and the TAIR9 genomic sequence (Lawrence et al., 2013). These DNA sequences were analysed using MEME, with a maximum motif width of 12 nucleotides using the "zero or one occurrences per sequence" setting (Bailey and Elkan, 1994). Selected resulting motifs were compared to the JASPAR 2022 non-redundant database of plant transcription factor binding profiles (Castro-Mondragon et al., 2022) using TOMTOM with the default settings (Gupta et al., 2007).

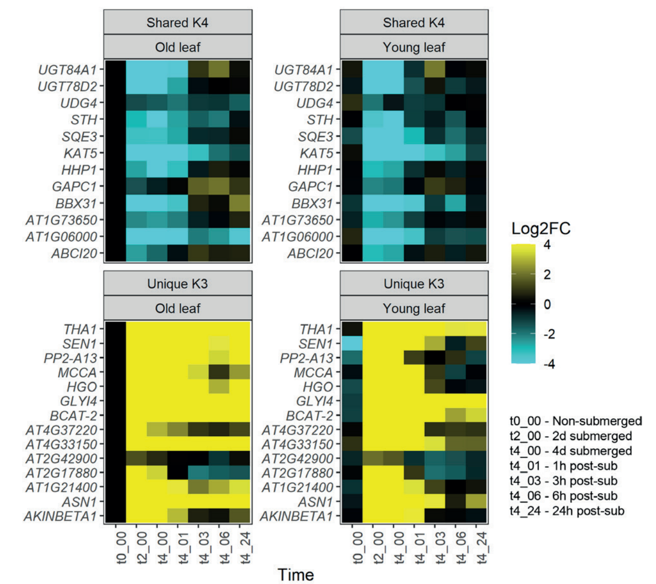
Supplemental figures



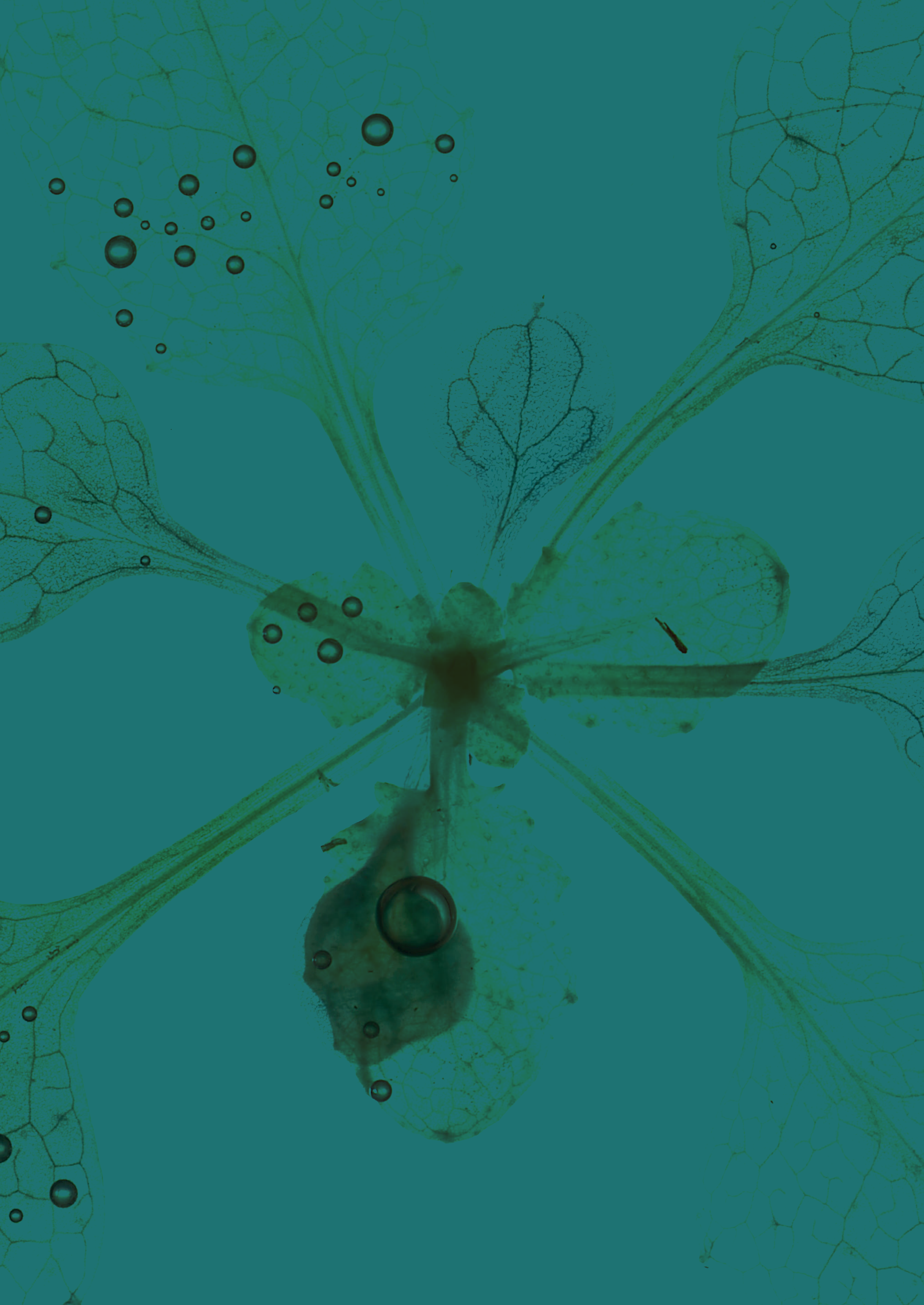
Supplemental Figure 1 | Expression of *ABF1-4*
Expression of ABA-RESPONSIVE ELEMENT BINDING FACTOR transcription factors does not vary strongly between old and young leaves, despite the enrichment of their target genes in a cluster of genes with an age-dependent expression pattern (Figure 3B). Expression was calculated relative to that of non-submerged old leaves.



Supplemental Figure 2 | Age-dependent expression of starvation-related genes
Expression of genes associated with the GO term "Cellular response to starvation" (GO:0009267) in cluster K10 (Chapter 2, Figure 4A). Expression was calculated relative to that of non-submerged old leaves



Supplemental Figure 3 | Sucrose-responsive genes
Expression of genes associated with the GO category GO:0009744, "response to sucrose" in clusters K4 (Chapter 2, Figure 3, genes showing an age-independent response to the submergence and post-submergence phases) and cluster K3 (Chapter 2, Figure 7, genes showing an age-dependent response to the post-submergence phase). Both of these clusters were enriched for genes associated with this GO term, although they consisted of genes with different expression patterns. Expression was calculated relative to that of non-submerged old leaves.



Chapter 4

Leaf age-dependent senescence during submergence involves ethylene-mediated ORESARA1 accumulation

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Abstract

When completely submerged, limitations in gas diffusion leads to ethylene entrapment throughout a plant. Ethylene is an important driver of leaf senescence. However, submerged plants still show a typical sequential leaf death pattern starting with the oldest leaves and progressing gradually towards younger leaves. Here we investigated how indiscriminate senescence of leaves is prevented in submerged shoots despite systemic ethylene accumulation. In flooded ethylene-insensitive mutants the sequential leaf death pattern was lost suggesting a role for ethylene signaling. Notably, we could rule out the involvement of the N-degron oxygen sensing pathways, as related mutants still exhibited a gradient in leaf senescence. Ethylene signaling led to senescence via the induction of the master regulator of senescence, the NAC domain transcription factor *ORESARA1* (*ORE1*). Loss of *ORE1* led to reduced senescence of old leaves and improved plant performance during and after flooding stress. Premature accumulation of ORE1 protein in young leaves is typically prevented by high levels of microRNA164, which induces the cleavage of *ORE1* mRNA. However, we found that this mechanism was not sufficient in flooded plants, as *ORE1* mRNA and protein accumulated in both old and young leaves during submergence. As ORE1-mediated senescence was more severe in old leaves, a potential explanation is that the activity of ORE1 is regulated post translationally.

Introduction

Ethylene is a gaseous plant hormone that controls many aspects of plant development and the interactions between plants and their environment (Dubois et al., 2018; Sasidharan and Voesenek, 2015). When ethylene is present, the transcription factor EIN3 is stabilized, which induces the expression of genes involved in the response to ethylene (Chang et al., 2013; Binder, 2020).

The ethylene-induced stabilization of EIN3 is used by plants as an early flooding signal. The severely limited gas diffusion under submerged conditions ensures that ethylene accumulates rapidly in plant tissues (Xie et al., 2015; Hartman et al., 2019; Voesenek and Sasidharan, 2013). This passive ethylene accumulation influences plant performance during flooding in a variety of ways. In Arabidopsis, ethylene signaling aids hypoxic transcriptional responses, inhibits growth, and modulates damage caused by ROS to enhance hypoxia survival (Peng et al., 2001; Liu et al., 2022; Hartman et al., 2019; Tsai et al., 2014). Post-submergence ethylene causes early senescence and dehydration of Arabidopsis leaves, negatively affecting plant performance (Yeung et al. 2018). In deepwater rice, ethylene signaling induces stem elongation during submergence by inducing gibberellin biosynthesis and signaling (Métraux and Kende, 1983; Kuroha et al., 2018; Hattori et al., 2009). In lowland rice on the other hand, ethylene signaling represses shoot elongation and carbohydrate consumption via SUB1A (Xu et al., 2006; Fukao et al., 2006; Fukao and Bailey-Serres, 2008).

The characterization of the functions of ethylene in plant flooding responses has focused on responses that aid survival of plants or their tissues. However, ethylene is also a positive regulator of senescence and ethylene accumulation could accelerate leaf senescence during submergence. Leaf senescence is often considered a marker for flooding sensitivity, as flooding-intolerant accessions of rice, Arabidopsis, maize, and *Lotus japonicus* display more severe leaf senescence during flooding and post-flooding recovery compared to tolerant accessions (Alpuerto et al., 2016; Krishnan et al., 1999; Yeung et al., 2018; Campbell et al., 2015; Buraschi et al., 2020). Furthermore, Arabidopsis mutants with reduced senescence exhibit improved performance after submergence compared to wild-type plants (Zhang et al.; Yeung et al., 2018).

Given the relevance of senescence for plant performance both during and after flooding, a thorough understanding of the regulatory mechanisms underlying senescence is of prime interest. The response of a plant tissue to ethylene strongly depends on the age of the tissue (Doubt, 1917; Chen et al., 2013; Ceusters and Van de Poel, 2018). Ethylene treatment induces leaf senescence much faster in older leaves than in younger leaves (dela Fuente and Leopold, 1968; Jing et al., 2005). Some mechanisms have been identified that contribute to the age-dependent response to ethylene. As a leaf ages, *EIN3* transcription gradually increases, which increases the strength of the response to endogenous ethylene (Li et al., 2013). EIN3 induces the transcription of the master regulator of senescence *ORE1* a NAC transcription factor. Premature senescence in young leaves is prevented due to the degradation of *ORE1* mRNA by the microRNA *miR164* (Kim et al., 2009). As a leaf ages, the abundance of *miR164* decreases, which leads to a gradual derepression of *ORE1*. This gradient in *miR164* works as a buffer that prevents premature senescence in young leaves during ethylene signaling.

Here we investigated the role of ethylene in the age-dependent leaf senescence observed in submerged Arabidopsis plants (Chapter 2). We expected that despite systemic ethylene accumulation and upregulation of *ORE1* during submergence, young leaves avoid premature senescence via post-transcriptional regulation of *ORE1* due to higher levels of *miR164*. Surprisingly, we found this is not true. Instead,

our results demonstrate that leaf age-independent EIN3 accumulation led to age-independent ORE1 accumulation, bypassing the established *miR164*-dependent mechanism. Although ORE1 protein accumulated to similar levels in old and young leaves during submergence, disrupting *ORE1* had a much stronger effect on the senescence phenotype of old leaves and improved post-submergence performance. Our results thus point towards other regulatory mechanisms controlling ORE1 activity and enforcing the observed sequential leaf-age dependent senescence during complete submergence.

Results

Ethylene signaling during submergence is systemic but mediates age dependent leaf death

The analysis of the leaf age-dependent transcriptome response to submergence and post-submergence recovery revealed that ethylene can be involved in both systemic and age-dependent responses (Chapter 2, Figures 3 & 4). The induction of senescence and cellular catabolism, which is partly controlled by ethylene signaling, was stronger in old leaves than in young leaves during submergence (Chapter 2, Figure 4). To determine the role of ethylene in age-dependent submergence responses, we compared the leaf death pattern of wild type Col-0 with that of ethylene-insensitive *ein3eil1* and *ein2-5* plants. We found that in both mutants the typical age dependent sequential leaf death observed in wild type plants was lost (Figure 1A-B).

It has previously been established that ethylene accumulates quickly in flooded tissues (Sasidharan and Voesenek, 2015) resulting in rapid stabilization of EIN3 (Xie et al., 2015; Hartman et al., 2019). EIN3 is a transcription factor that is a key regulator of downstream transcriptional responses to ethylene (Chao et al., 1997; Chang et al., 2013). However, considering that the ethylene-dependent leaf death was not uniformly triggered across the submerged Arabidopsis rosettes, we wanted to establish whether ethylene signaling was indeed systemic. For this the levels of EIN3 protein were monitored in representative old (leaf 3) and young (leaf 7) leaves. Submergence enhanced EIN3 levels in both old and young leaves already within a few hours consistent with the expected rapid accumulation of ethylene (Figure 1C). Interestingly, while EIN3 was stabilized rapidly following submergence, levels decreased thereafter during the first 24 hours of submergence. This was in agreement with previous observations of EIN3 as a hit-and-run transcription factor, binding briefly to its downstream targets, after which their transcription is maintained by other regulators (Alvarez et al., 2021; Chang et al., 2013).

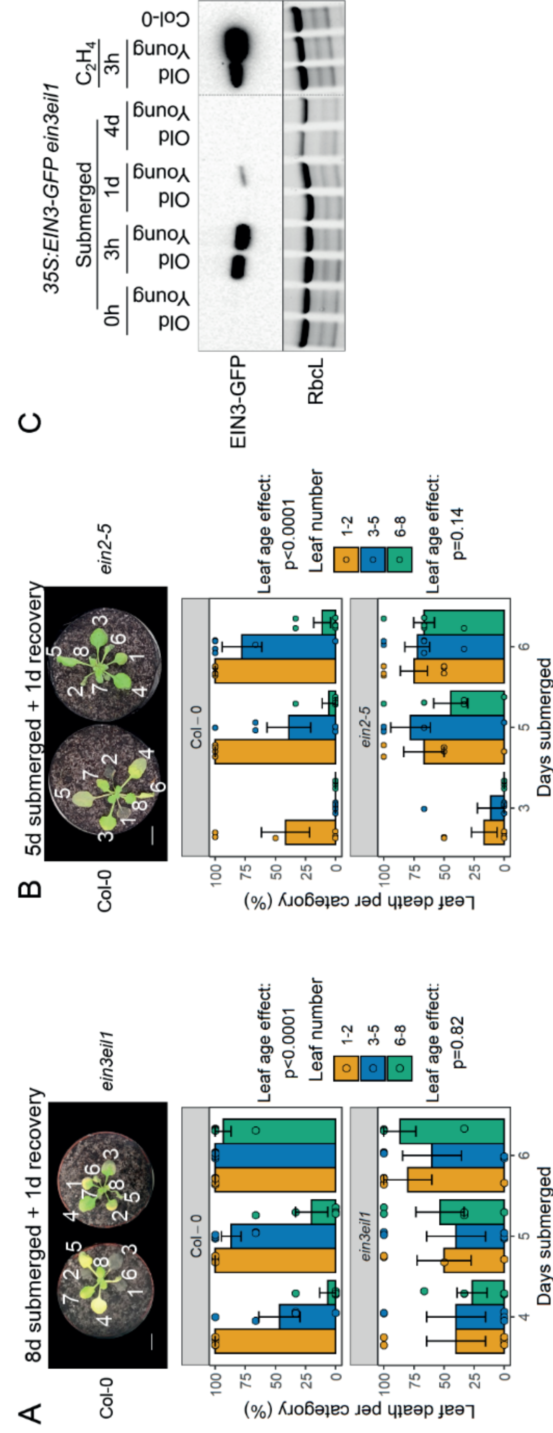


Figure 1 | Ethylene is required for age-dependent leaf death in submerged plants
A) Age-dependent leaf death after submergence followed by recovery is lost in ethylene-insensitive ein3eil1 plants. Above: Representative images of wild-type Col-0 and ein3eil1 plants after eight days of submergence followed by one day of recovery, numbers indicate leaf number. Below: quantification of age-dependent leaf death in Col-0 and ein3eil1 plants. P-values indicate the effect of leaf age on the proportion of dead leaves per genotype, determined by a two-way ANOVA (leaf age * submergence duration). n=5 plants per genotype per timepoint.
B) Age-dependent leaf death after submergence followed by recovery is lost in ethylene-insensitive ein2-5 plants. Above: Representative images of wild-type Col-0 and ein2-5 plants after five days of submergence followed by one day of recovery, numbers indicate leaf number. Below: quantification of age-dependent leaf death in Col-0 and ein2-5 plants. P-values indicate the effect of leaf age on the proportion of dead leaves per genotype, determined by a two-way ANOVA (leaf age * submergence duration). n=6 plants per genotype per timepoint.
C) Immunoblot analyses showing that EIN3-GFP accumulates within three hours of submergence or ethylene treatment in both old (leaf three) and young (leaf seven) leaves of transgenic 35S:EIN3-GFP ein3eil1 plants. Samples were run on the same gel; vertical line indicates where samples were cropped out. Stain-free imaging of the large subunit of Rubisco (RbcL) served as a loading control.
Scale bars indicate 1cm

In addition to ethylene, submergence especially during a light limited flooding event also causes a significant decline in tissue oxygen levels (hypoxia). To probe the role of hypoxia signaling in regulating the observed pattern of leaf death during submergence we tested whether sequential leaf death requires hypoxia sensing. For this we used the Arabidopsis mutants *erfvii* and *pco124*, which lack important components of the plant oxygen sensing machinery (Abbas et al., 2015; Masson et al., 2019). *erfvii* mutants lack the five group VII ERF transcription factors (*RAP2.2*, *RAP2.3*, *RAP2.12*, *HRE1*, & *HRE2*) which induce the transcription of anaerobic genes under hypoxia, *pco124* mutants lack three out of the five *PLANT CYSTEINE OXIDASES* (*PCO1*, *PCO2*, & *PCO4*) that induce the N-terminal cysteine oxidation and subsequent proteasomal degradation of ERFVIIs under normoxia. In both these mutants, submergence still triggered the sequential leaf death pattern as observed in the wild type, suggesting that this response does not require the N-degron pathway of oxygen sensing (Figure 2A-B).

To further investigate the contributions of ethylene and hypoxia in age-dependent leaf senescence during flooding, plants were exposed to combinations of ethylene and hypoxia in darkness. The effect of four days of these treatments on leaf senescence was quantified by measuring the median hue of old and young leaves. The hue (colour) was determined by converting images from an RGB (red, green, blue) image format to an HSV (hue, saturation, value) format. The combination of ethylene and darkness induced age-dependent leaf yellowing, and hypoxia slowed down the yellowing (Figure 2C). However, hypoxia or darkness alone failed to trigger sequential leaf yellowing.

Based on these results we conclude that despite its systemic activation, ethylene signaling induced a pattern of age-dependent leaf death during flooding stress. The mechanisms underlying this observation were of interest to probe further.

Submergence-induced senescence is primarily controlled by the ethylene responsive NAC-domain transcription factor ORE1

The regulatory networks underpinning ethylene-mediated chlorophyll degradation leading to leaf senescence and death are well established (Woo et al., 2019). Of relevance for submergence-induced senescence is the activation by ethylene of the NAC domain transcription factor ORE1 (Qiu et al., 2015; Yeung et al., 2018). ORE1 is a positive regulator of leaf senescence. Since the functioning of the EIN3-ORE1 regulon during leaf senescence is well-established, we used this as a system to investigate how ethylene mediated leaf senescence is coordinated in an age-dependent manner during submergence (Kim et al., 2009; Li et al., 2013; Qiu et al., 2015).

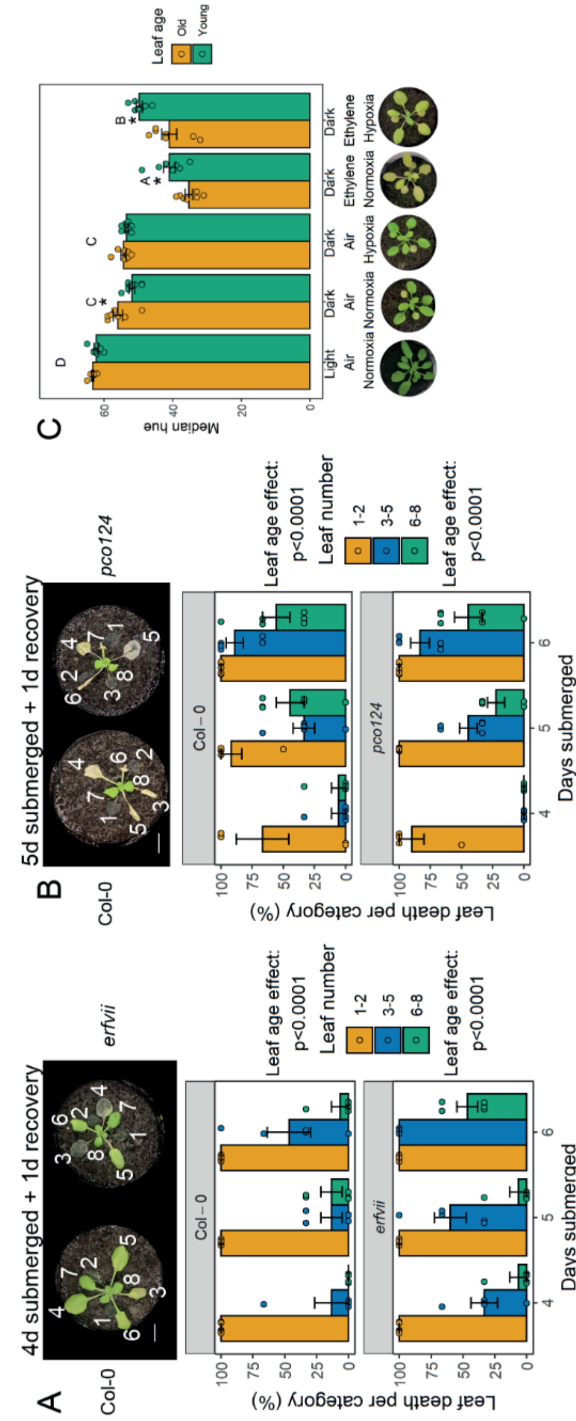


Figure 2 | Age-dependent leaf death during submergence does not require N-degron-mediated oxygen sensing
A) Age-dependent leaf death after submergence followed by recovery is not lost in erfvii plants. Above: Representative images of wild-type Col-0 and erfvii plants after four days of submergence followed by one day of recovery, numbers indicate leaf number. Below: quantification of age-dependent leaf death in Col-0 and erfvii plants. P-values indicate the effect of leaf age on the proportion of dead leaves per genotype, determined by a two-way ANOVA (leaf age * submergence duration). n=6 plants per genotype per timepoint.
B) Age-dependent leaf death after submergence followed by recovery is not lost in ethylene-insensitive pco124 plants. Above: Representative images of wild-type Col-0 and pco124 plants after five days of submergence followed by one day of recovery, numbers indicate leaf number. Below: quantification of age-dependent leaf death in Col-0 and pco124 plants. P-values indicate the effect of leaf age on the proportion of dead leaves per genotype, determined by a two-way ANOVA (leaf age * submergence duration). n=6 plants per genotype per timepoint.
C) The effect of four days of treatment with different flooding cues on leaf yellowing in wild-type Arabidopsis plants. Yellowing is indicated by the median hue of old (leaf three) and young (leaf seven) leaves. Ethylene induced age-dependent leaf yellowing and this process was slowed down by hypoxia. Images below each bar show representative plants from each treatment. Asterisks indicate differences between old and young leaves (paired t-test), different letters indicate significant differences between treatments (two-way ANOVA + Tukey's post-hoc test). n=7 plants per treatment.
Scale bars indicate 1cm

Consistent with previous reports, ethylene-mediated senescence was reduced in *ore1-1* mutants (Figure 3A-B; (Li et al., 2013; Qiu et al., 2015)) and ethylene exposure in darkness triggered a strong increase in *ORE1* transcripts (Figure 3C). Interestingly, this increase was observed in both old and young leaves.

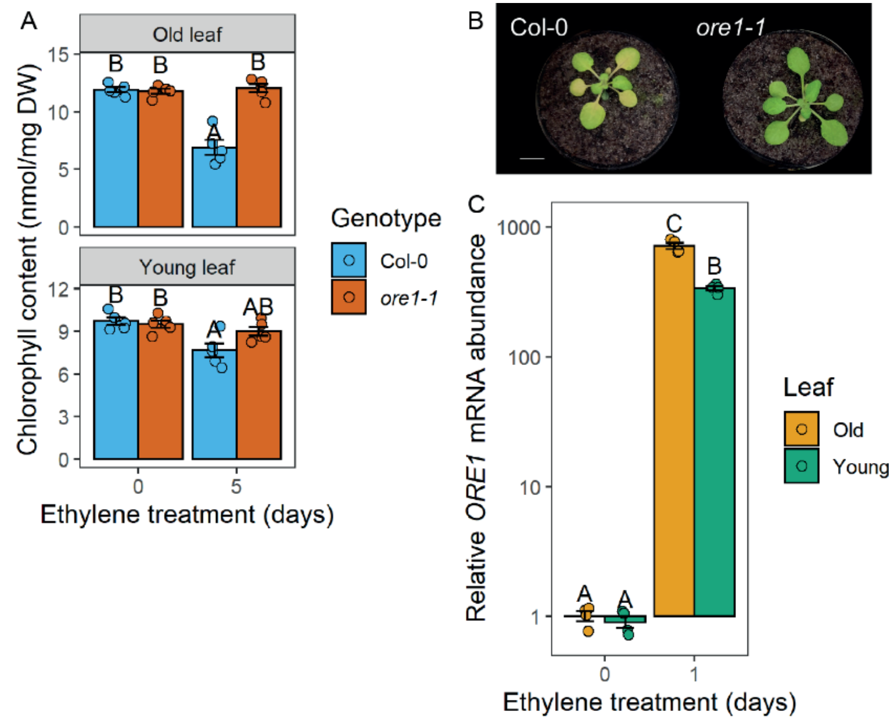


Figure 3 | ORE1 mediates ethylene-induced senescence
A) Chlorophyll content of old and young leaves of Col-0 and ore1-1 plants before and after five days of ethylene treatment. n=5 leaves per sample.
B) Representative images of Col-0 and ore1-1 plants after five days of ethylene treatment in darkness. Scale bar indicates 1cm
C) ORE1 mRNA abundance increased in both old and young leaves after one day of ethylene treatment. n=4 leaves per sample, each consisting of two old or young leaves from different plants pooled together. Expression levels were normalized to those in old leaves of non-submerged plants.
Different letters indicate significant differences between groups (two-way ANOVA + Tukey's post-hoc test).

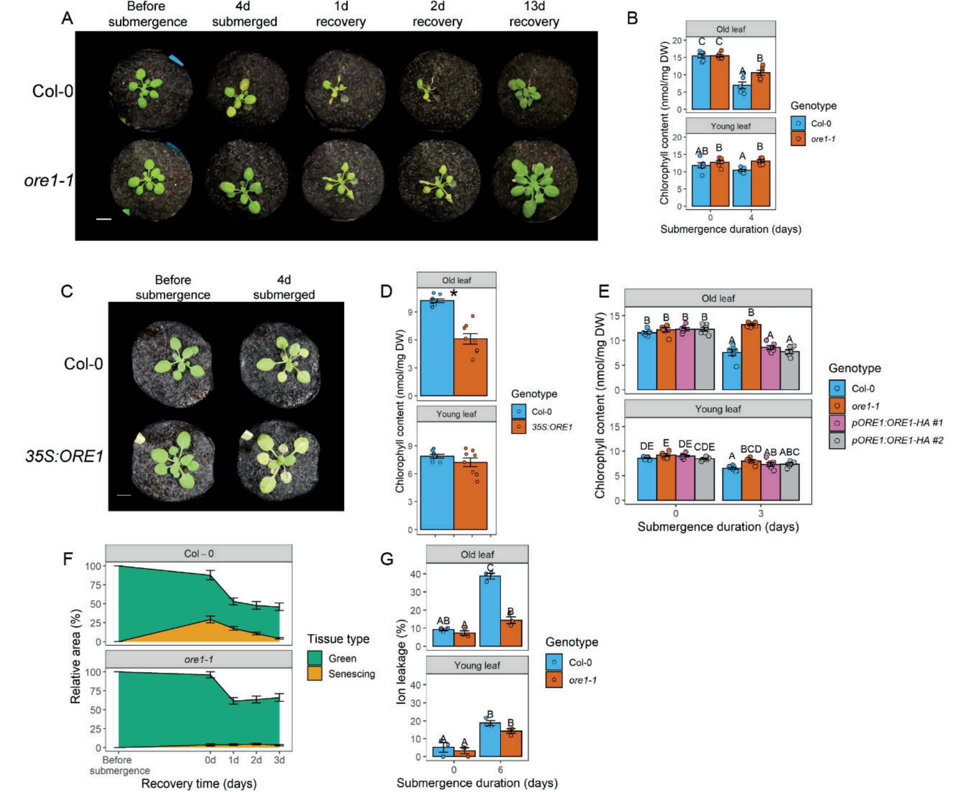


Figure 4 | ORE1 mediates submergence-induced senescence
A) *ore1-1* mutants showed reduced yellowing of old leaves after four days of submergence. Representative images of Col-0 and *ore1-1* plants before and after submergence, and after the indicated recovery periods.
B) Chlorophyll content of old and young leaves of Col-0 and *ore1-1* before and after four days of submergence. n=6 leaves per sample.
C) *35S:ORE1* plants showed increased leaf senescence after four days of submergence. Representative images of Col-0 and *35S:ORE1* plants before and after four days of submergence.
D) Chlorophyll content of old and young leaves of Col-0 and *35S:ORE1* plants after four days of submergence. Asterisks indicate significant differences (t-test), n=8 leaves per sample.
E) Chlorophyll content of Col-0, *ore1-1*, and two independent *pORE1:ORE1-HA ore1-1* lines before and after three days of submergence. n=6 leaves per sample.
F) Relative green and senescing areas of Col-0 and *ore1-1* before and after four days of submergence, and at the indicated recovery timepoints, as quantified by PlantCV. n=17 (Col-0), n=20 (*ore1-1*).
G) Ion leakage of Col-0 and *ore1-1* before and after six days of submergence. n=3 pools of five old or young leaves from different plants per sample.
Different letters indicate significant differences between groups (two-way ANOVA with Tukey's *post-hoc* test). Scale bars indicate 1cm.

Next, we set out to establish that ORE1 is indeed a principal regulator of submergence-induced senescence. In accordance with its role as a positive regulator of senescence, submergence-induced

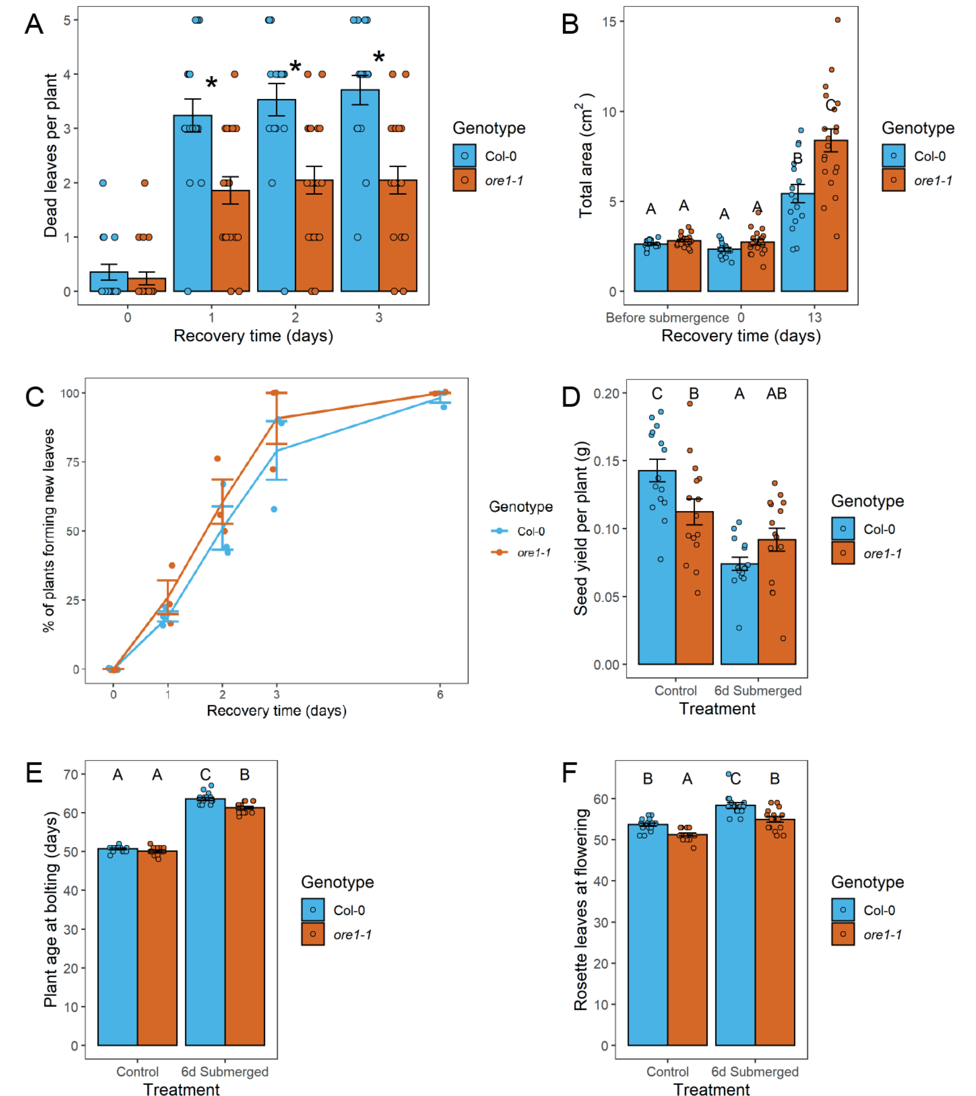
senescence was significantly reduced and enhanced in *ore1* mutants and overexpressors, respectively (Figure 4A-D, Figure S1A). Moreover, the higher chlorophyll retention phenotype of *ore1-1* mutants during submergence could be reverted to wild-type by complementation with *ORE1* (ORE1 fused to an HA tag driven by its own 1.6kb promoter) (Figure 4E). The higher chlorophyll content of *ore1-1* mutants was reflected in the greater proportion of their rosettes that was green upon desubmergence (Figure 4F). Electrolyte leakage of old leaves was reduced in *ore1-1* mutants compared to Col-0 plants, after six days of submergence (Figure 4G).

ORE1 plays a role in dark-induced senescence of detached leaves (Kim et al., 2018). We did not detect visual signs of senescence in whole plants treated with darkness (Figure S2A) and the effect of darkness on rosette area was not different between Col-0 and *ore1-1* (Figure S2B). This showed that the dark submergence phenotype of *ore1-1* mutants was not merely caused by darkness.

ore1 mutants have improved post-submergence recovery

Reduced leaf senescence in *ore1* mutants led to a smaller number of leaves dying during the recovery phase (Figure 5A, S1B). The greater leaf area retention of *ore1-1* mutants correlated with their post-submergence growth, which was higher than

Figure 5 (Right page)| Loss-of-function *ore1* mutants have improved post-submergence recovery
A) Number of dead leaves in Col-0 and *ore1-1* plants during recovery from four days of submergence. Leaves were scored as dead or alive at each of the indicated timepoints, n=17-21 plants per genotype.
B) Total living rosette area of Col-0 and *ore1-1* before and after four days of submergence, and after 13 days of recovery. Images of plants were categorised into dead, senescing, and healthy pixels using PlantCV. Senescing and healthy pixels were combined for each plant and converted to an area in cm^2. n=16-20 plants per sample.
C) The rate of new leaf formation was not affected in *ore1-1* plants after five days of submergence (t-test). Dots represent individual experiments, representing 8-21 plants each.
D) Seed yield of Col-0 and *ore1-1* plants under control conditions and of plants that were submerged for six days. n=15 per group.
E) Flowering time, as measured in days to bolting, in Col-0 and *ore1-1*, under control conditions and after six days of submergence. n=15 per group.
F) Flowering time, as measured in main rosette leaves at bolting, in Col-0 and *ore1-1***,** under control conditions and after six days of submergence. n=15 per group.
Different letters indicate significant differences between groups (two-way ANOVA + Tukey's post-hoc test). Asterisks indicate significant differences between Col-0 and *ore1-1* per timepoint (t-test).



that of Col-0 plants (Figure 5B). The greater increase in rosette area of *ore1-1* mutants was not due to differences in meristem activity directly after desubmergence, as this was not different between *ore1-1* mutants and wild-type plants (Figure 5C). Flooding treatment reduced the seed yield of Col-0 plants, but not of *ore1-1* mutants (Figure 5D). Under control conditions, *ore1-1* mutants did have a significant reduction in seed yield compared to wild-type plants. This can be attributed to delayed leaf senescence in *ore1-1* mutants. Leaf senescence plays a vital role in remobilizing nutrients from dying leaves for seed production at the end of a plant's lifecycle

(Havé et al., 2017). Flowering time of *ore1-1* mutants was typically shorter than that of Col-0 under submerged conditions (Figure 5E-F). This could be explained by the need for Col-0 plants to compensate for the higher number of leaves lost during the submergence treatment.

Notably, we found that the phenotype of *ore1* mutants was age-dependent: the reduction in leaf senescence during flooding was most visible in old leaves (Figure 4A, 4C, S2A). Consistent with this visual observation, the decrease in chlorophyll content and cell membrane integrity was the greatest in old leaves of Col-0 plants (Figure 4B, 4E, 4G). We thus set out to investigate the regulation of ORE1 and determine how it is activated in an age-dependent manner.

Leaf-age dependent regulation of ORE1

Submergence strongly enhanced *ORE1* transcript levels in whole rosettes and this effect was maintained during recovery. While darkness also triggered upregulation of *ORE1,* transcript levels quickly dropped as plants were placed back in the light (Figure 6A).

Surprisingly, submergence led to increased transcript levels of *ORE1* in both old and young leaves, in an *ein3eil1*-dependent manner (Figure 6B). This was further confirmed using a transgenic line in which the 1.6kb promoter of *ORE1* was fused to a GUS enzyme. GUS staining patterns in both young and old leaves confirmed age-independent *ORE1* promoter activity during flooding (Figure 6C). Next, we examined whether age-dependent differences in ORE1 occur at the protein level. To do so, we used the *ore1-1* mutant line complemented by an HA-tagged version of ORE1, driven by its native 1.6kb promoter. In the *pORE1:ORE1-HA ore1-1* lines, there was an accumulation of ORE1 protein in both old and young leaves during submergence both at one and three days of submergence (Figure 6D).

Ethylene is known to enhance *ORE1* mRNA abundance via two routes – a direct transcriptional induction and via inhibition of its post-transcriptional repressor *miR164* (Kim et al., 2009). The latter mode is associated with age-dependent ethylene-induced senescence. Young leaves typically have high levels of *miR164* which decline with age. This ensures that *ORE1* mRNA is degraded when its transcription is induced by EIN3 and protects young leaves from premature senescence (Kim et al., 2009; Li et al., 2013). We did not observe significantly higher expression of *miR164b* in young leaves compared to old leaves (Figure S3), and the similar accumulation of ORE1 protein in both old and young leaves (Figure 6D) suggests that the degradation of *ORE1* mRNA by *miR164* is not sufficient to prevent premature accumulation of ORE1 protein during submergence. This indicated other mechanisms mediating the tissue-specific activation of ORE1 and subsequent senescence upon submergence.

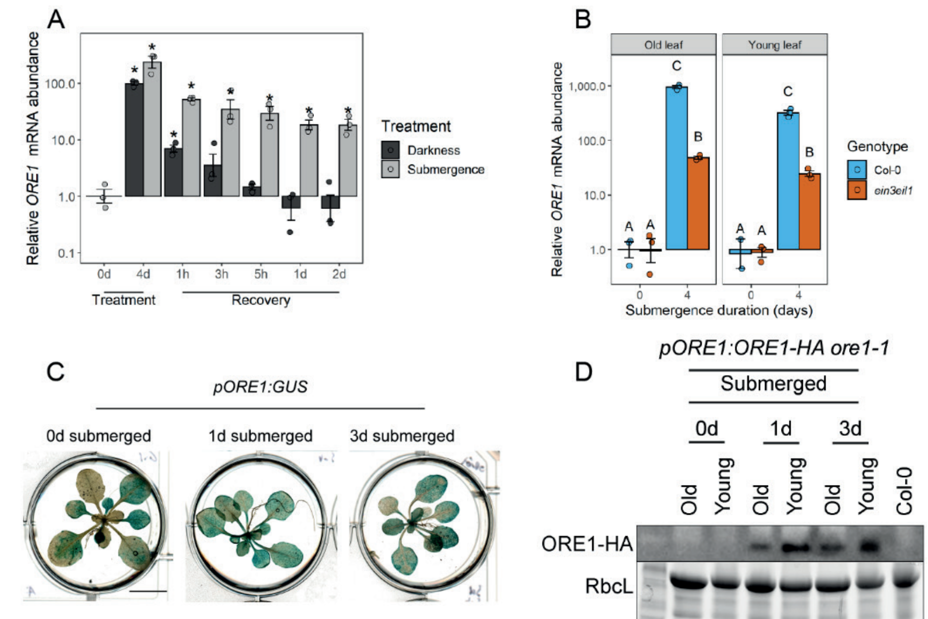


Figure 6 | ORE1 accumulation during submergence is age-independent

A) *ORE1* mRNA abundance in whole rosettes before and after darkness or dark submergence. Asterisks indicate significant differences compared to untreated plants (One-way ANOVA + Dunnett's post-hoc test). Expression was normalized to that of untreated plants. n=3, each sample consists of one rosette.

B) *ORE1* mRNA abundance in old and young leaves before and after four days of submergence, in Col-0 and *ein3eil1*. Different letters indicate significant differences between groups (two-way ANOVA + Tukey's post-hoc test). Expression was normalized to that of non-submerged old leaves of Col-0. n=2 for young, non-submerged Col-0 leaves, n=3 for the rest; each sample consisted of two leaves pooled together from different plants.

C) GUS staining of *pORE1:GUS* shows that *ORE1* promoter activity was limited to old leaves and cotyledons in non-submerged plants but was induced in all leaves upon submergence. Scale bar indicates 1cm.

D) Immunoblots showing ORE1-HA protein abundance in old and young leaves before and after one and three days of submergence, using an antibody against HA. Each *pORE1:ORE1-HA ore1-1* sample consisted of five old or young leaves pooled together from different plants. Proteins of the Col-0 sample are extracted from one whole rosette. Stain-free imaging of the large subunit of Rubisco (RbcL) served as a loading control.

Discussion

The severely reduced diffusion of gases in water means that ethylene will accumulate rapidly in any plant tissue that is completely submerged (Figure 1E). This property of ethylene makes it an ideal flood warning cue mediating many flood-adaptive traits (Sasidharan and Voesenek, 2015). However, exposure to high concentrations of ethylene commonly induces senescence of leaves. Therefore, a mechanism that prevents the simultaneous indiscriminate breakdown of all leaf tissue in such a situation is essential to prolong leaf survival. During natural plant ageing, the genetically coordinated process of chlorophyll breakdown during senescence serves to remobilize nutrients towards seed and tuber filling (Yu et al., 2015). The ability to retain chlorophyll has been found to correlate with higher tolerance to submergence and improved post-submergence photosynthesis (Alpuerto et al., 2016; Yeung et al., 2018). For example, the submergence tolerance gene *SUB1A* delays leaf senescence. Similar to *ORE1*, the expression of *SUB1A* is regulated by ethylene (Fukao et al., 2006). Whereas ORE1 induces chlorophyll degradation, *SUB1A* inhibits it during both submergence and darkness, and thereby contributes to a quiescence strategy during flooding (Xu et al., 2006; Fukao et al., 2006, 2012). However, during prolonged submergence as energy reserves become increasingly limited, senescence would be a beneficial mechanism for survival of the whole plant. In such a situation, a sequential dismantling of older leaves would make energy and nutrient reserves available that can be redirected to sustain younger leaves and the meristem. This sacrificial use of older leaves would serve to enhance growth and photosynthesis recovery when floodwaters subside.

Leaf senescence has an important function in the lifecycle of both entire organisms and their individual organs (Woo et al., 2019). The importance of senescence for seed production was highlighted by the lower seed yield of *ore1-1* mutants under control conditions (Figure 5D). Although *ore1-1* plants had a lower seed yield than Col-0 plants under control conditions, this difference was lost in plants that were submerged for six days. This was consistent with the observation that the relationship between the timing of senescence and the yield of a crop is highly variable and depends on environmental conditions (Gregersen et al., 2013). Understanding how plants coordinate which tissues are broken down under stressful conditions could help in developing more stress-tolerant crop varieties, as the role of NAC domain transcription factors, like ORE1, in senescence is conserved across many plant species (Podzimska-Sroka et al., 2015).

Since ethylene-insensitive mutants do not induce ORE1-mediated senescence of old leaves during flooding, their old leaves die slower than those of wild-type plants. The young leaves of ethylene-insensitive mutants, on the other hand, die faster than those of wild-type plants. This could be an effect of the impaired ability of ethylene mutants in responding to reactive oxygen species that accumulate during submergence recovery or of other unidentified roles of ethylene in submergence survival (Tsai et al., 2014; Liu et al., 2022). This highlights how ethylene signaling can lead to either the death or survival of a leaf during flooding stress, depending on the age of the leaf. The gradient of age-dependent leaf death during flooding stress was independent of oxygen sensing via the N-degron pathway, as mutants which lacked components of this pathway still showed age-dependent leaf death during flooding (Figure 2).

Ethylene can freely diffuse across cell membranes and does not require specific transporters to move between cells. The lack of control of ethylene movement requires a plant to have a highly tissue-specific response system to ethylene. This has been described for different cell types (Vaseva et al., 2018; Polko et al., 2011; Cao et al., 1999; Rajhi et al., 2011), but also for similar tissues in different developmental stages (Jing et al., 2005; dela Fuente and Leopold, 1968). Tissue-specific regulation of ethylene responsiveness occurs on many different levels of the ethylene signaling cascade (Stepanova and Alonso, 2009). Ethylene-mediated leaf senescence is controlled at a tissue-specific levels via an age-dependent gradient in *miR164* (Kim et al., 2009). In young leaves, expression levels of *miR164* are high, these levels decrease as a leaf ages. *miR164* can bind to mRNA of *ORE1* and induce its cleavage, which prevents the induction of ORE1-mediated senescence in young leaves upon ethylene exposure. In our system however, we observed an accumulation of *ORE1* mRNA and protein in both old and young leaves (Figures 6B, 6D). This suggests that the *miR164*-mediated inhibition of ORE1 is not sufficient to prevent the premature accumulation of ORE1 in young leaves. Since the effects of disrupting *ORE1* on the phenotype of submerged plants are strongly age-dependent (Figure 4), we hypothesize that there are other unidentified mechanisms that control ORE1 in an age-dependent manner.

Materials and methods

Plant material

ore1-1 (SALK_090154): described in Yeung et al., 2018
ore1-2 (SAIL_694_C04): described in Kim et al., 2020. Ordered from NASC
35S:ORE1: described in Matallana-Ramirez et al., 2013. Gift from Salma Balazadeh
ein2-5: described in Alonso et al., 1999. Ordered from NASC
ein3eil1: described in Alonso et al., 2003. Ordered from NASC
pco124: described in Masson et al., 2019. Gift from Daan Weits
erfvii: described in Abbas et al., 2015. Gift from Daan Weits
35S:EIN3-GFP ein3eil1: described in Xie et al., 2015. Gift from Shi Xiao
pORE1:ORE1-HA ore1-1: this study
pORE1:GUS: this study

Generating transgenic lines

Genomic DNA from a Col-0 leaf was extracted using phenol:chloroform:isoamyl alcohol. The *ORE1* genomic region, including introns, 5'UTR, and a 1624bp promoter, was amplified from this DNA using primers 5383 & 5384 (Supplemental table 1) and inserted into the pJET1.2 vector (Thermo Fisher, K1231). For the *pORE1:ORE1-HA* line the entire fragment without the stop codon was amplified from this vector using primers 5383 & 5510, and the HA tag was added using primers 5383 & 5783. For the *pORE1:GUS* line the *ORE1* promoter was amplified using the primers 5383 & 5712. Adapters for LIC vector pPLV01 and pPLV13 were added to the *pORE1:ORE1-HA* and *pORE1* fragments using primers 5804 & 5761 and 5739 & 5740, respectively (De Rybel et al., 2011). The fragments were inserted into their respective vectors as described in (De Rybel et al., 2011). These vectors were inserted into *Agrobacterium tumefaciens* strain AGL-1 via electroporation, and *ore1-1* and Col-0 Arabidopsis plants were transformed using the floral dip method (Logemann et al., 2006). Independent T_1 transformants were selected on plates containing 50µM Basta/PPT, homozygous T_3 or T_4 lines were used in all experiments.

Plant growth and treatments

Plants were grown as described in chapter 2. Ethylene treatments were done in 22.5l desiccators as described in (Hartman et al., 2019). Hypoxia treatments were done by mixing N_2 and air to a concentration of 5% O_2, which was flushed through a desiccator for one hour. Desiccator valves were then closed and 5-10ppm ethylene was injected with a syringe.

Green and senescing leaf area quantification

Pictures of Col-0 and *ore1-1* plants were taken with a Nokia 8 phone camera. Individual pixels in each picture were classified into either "green", "senescing", "dead", or "background" using the "Naïve Bayes Multiclass" module within PlantCV (Fahlgren et al., 2015). ImageJ was used to count the number of pixels in the green and senescing categories, and this was plotted relative to the amount of green pixels before the start of treatment for Figure 4F. For Figure 5B the amount of green and senescing pixels of the same plants were added together and converted into an area in cm^2.

Seed yield

For seed yield measurements, plants were either kept in short-day control conditions or submerged for six days in darkness and then returned to control conditions. Watering was stopped once the first siliques started to dry out and plants were left to dry out until all siliques had ripened.

Chlorophyll quantification

For chlorophyll measurements, individual old or young leaf blades of the indicated genotypes were cut off and put into 1.5ml Eppendorf tubes containing 1ml DMSO at the indicated timepoints. Tubes were incubated in a shaking water bath at 60°C for 30 minutes in darkness, and were then left to cool down to room temperature for another 30 minutes in darkness. 200µl of each DMSO solution was pipetted into a 96-well plate and absorbance was measured at 647nm, 664nm, and 750nm using a spectrophotometer plate reader (Synergy HT Multi-Detection Microplate Reader; BioTek Instruments). Chlorophyll A was calculated as 13.71*(664nm-750nm)-2.858*(647nm-750nm), chlorophyll B was calculated as 22.39*(647nm-750nm)-5.42*(664nm-750nm). Leaves were dried at 80°C for 48h before measuring their dry weight on a Mettler-Toledo MX5 microbalance. Total chlorophyll was calculated by adding chlorophyll A and B together and dividing them by the measured dry weight.

Ion leakage

The indicated tissues were placed in a 15ml tube containing 3ml distilled water, and were gently shaken for three hours. The concentration of ions in the solution was measured using a Horiba EC-33 conductivity meter. Plant tissue was then boiled for 20 minutes to destroy all membranes, and ion leakage was measured again to determine the total ion content. Relative ion leakage was calculated as the proportion of the conductivity before boiling to the conductivity after boiling.

Gene expression

RNA was extracted from the indicated tissues using the Qiagen plant RNeasy mini kit, including an on-column DNAse treatment, according to the manufacturer's instructions. qPCR data shown in Figures 6A and S3 was done using the Spectrum RNA extraction kit (Sigma-Aldrich), followed by DNase treatment using AMPD1 DNase I (Sigma-Aldrich) to ensure that *miR164b* would not be excluded by the size exclusion limit of the Qiagen kit.

Extracted RNA was converted into cDNA using RevertAid H Minus Reverse Transcriptase (Thermo Scientific). For qPCR 20ng of cDNA was used per 5µl reaction, using SYBR Green master mix (BioRad) and the primers indicated in Supplemental Table 1.

GUS staining

Whole rosettes of 10-leaf *pORE1:GUS* plants were cut off at the indicated timepoints and were fixated in 90% acetone for 20 minutes at -20°C. Plants were then washed twice for 10 minutes in GUS washing buffer (0.1M phosphate buffer pH=7, 10mM EDTA, 2mM $K_3Fe(CN)_6$) under vacuum and then stained with GUS washing solution (0.1M phosphate buffer pH=7, 10mM EDTA, 1mM $K_3Fe(CN)_6$, 1mM $K_4Fe(CN)_6$*3H2O, 0.5mg/ml X-Gluc) for 10 minutes under vacuum, followed by 20 hours at 37°C. Staining was stopped by incubating the plants with 3:1 acetic acid:ethanol for one hour, and were cleared by washing with 70% ethanol. Plants were scanned using an Epson V800 scanner.

Western blotting

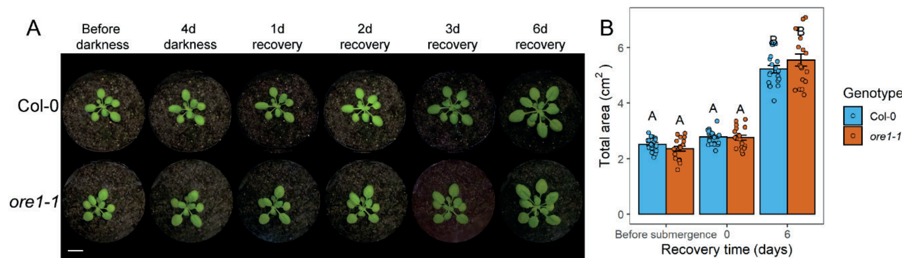
Five leaves of the indicated age were pooled together after the indicated treatment and frozen in liquid nitrogen. Protein was extracted using radioimmunoprecipitation assay (RIPA) buffer (Hartman et al., 2019) and quantified using a Pierce BCA kit. 20-50µg of protein was loaded onto a stain-free 4-15% gel (BioRad), the Rubisco large subunit was visualized using stain-free gel imaging. Proteins were transferred from the gel to a 0.2µm PVDF membrane using a Bio-Rad trans-blot system for 7 minutes, efficient transfer was verified by imaging the stain-free blot afterwards. The blot was blocked overnight at 4°C in TBS-T + 5% milk. Primary antibody (1:1000, anti-GFP (Roche, #11814460001) or anti-HA-HRP (Thermo Fisher, 26183-HRP)) was incubated for one hour at room temperature. The blot was washed four times 10 minutes with TBS-T. In the case of anti-GFP blots, the membrane was incubated with a secondary antibody (1:2500 rabbit anti-mouse, Cell Signaling #7076) for one hour at room temperature and the membrane was washed 3x with TBS-T and 2x with TBS, five minutes each. The membrane was incubated with Femto (Thermo Fisher) and imaged under a ChemiDoc imaging system (BioRad) to visualize HRP activity.

Statistical analysis

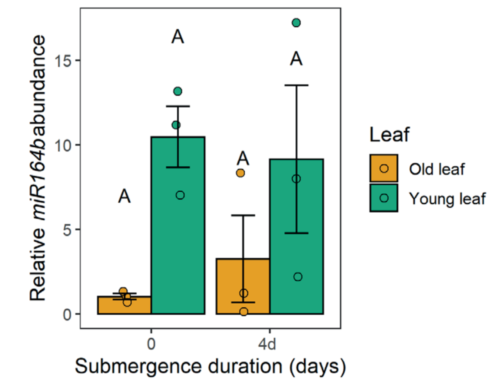
All statistical tests were done in R version 3.6.1 using the indicated statistical tests, differences were deemed significant at $p<0.05$.

Supplemental figures

Supplemental Figure 1 | *ore1-2* mutants showed delayed submergence-induced senescence
A) *ore1-2* mutants showed reduced yellowing of old leaves after recovery from four days of submergence. Representative images of plants (Col-0 and *ore1-2*) that were submerged in darkness for four days followed by recovery under normal growth conditions in the light.
B) Dead leaves of Col-0 and *ore1-2* during recovery from five days of submergence. Asterisks indicate significant differences (t-test), n=8-10 plants per genotype.



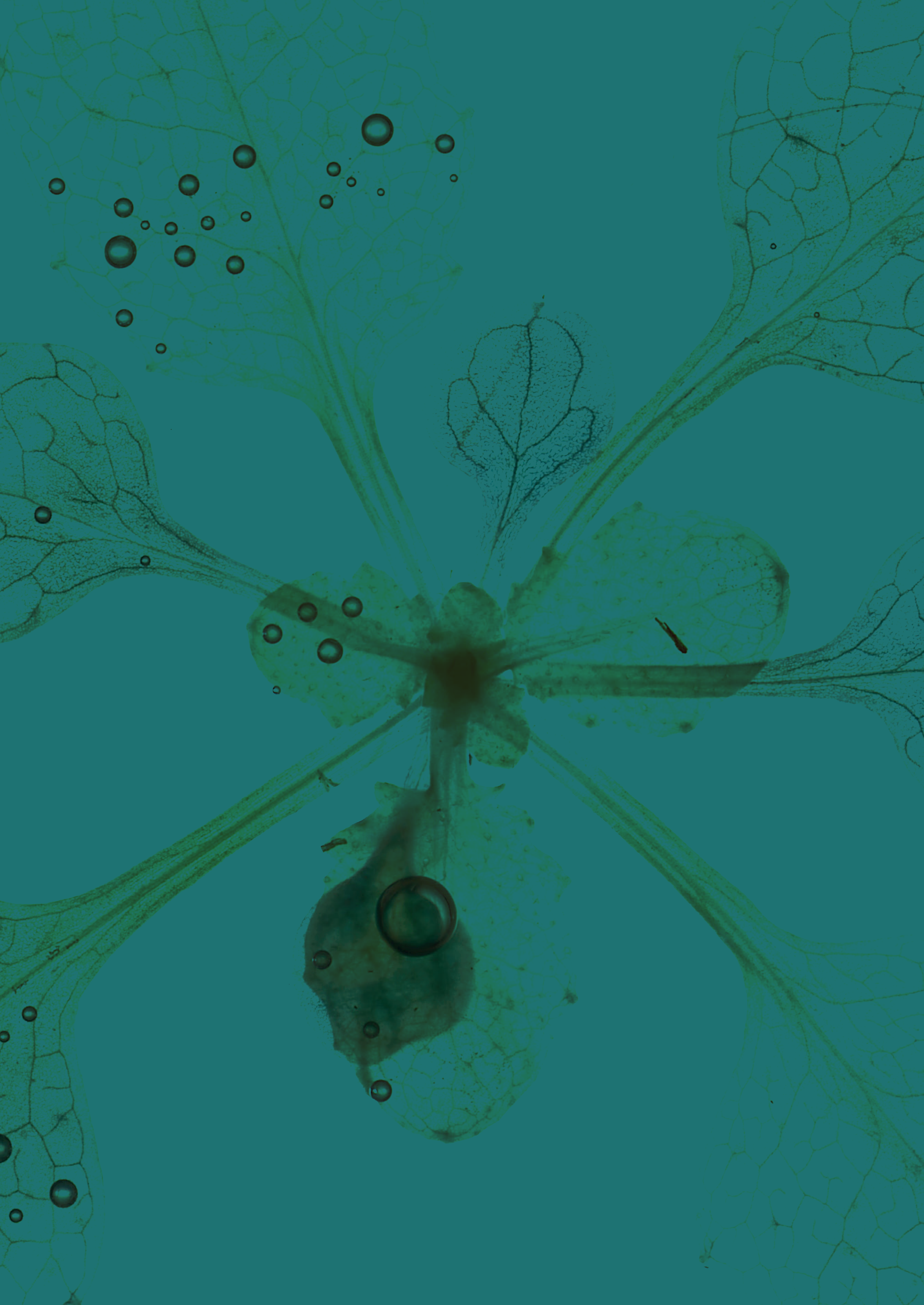
Supplemental Figure 2 | Four days of darkness did not induce leaf senescence
A) Four days of darkness did not induce visual signs of leaf senescence in Col-0 or *ore1-1*. Representative images of plants (Col-0 and *ore1-1*) that were placed in darkness for four days followed by recovery under normal growth conditions in the light.
B) The effect of four days of darkness on rosette area was not different between Col-0 and *ore1-1*. Different letters indicate significant differences between groups (two-way ANOVA + Tukey's post-hoc test), n = 16-20 plants per genotype.



Supplemental Figure 3 | Age-dependent expression of *miR164b*
Abundance of the pri-miRNA *miR164b* was not significantly different between old and young leaves, before or after four days of dark submergence. Different letters indicate significant differences (two-way ANOVA with Tukey's post-hoc test). n=3, each consisting of two old or young leaves from different plants pooled together.

Supplemental Table 1 | Primers used in this chapter

Primer name	Sequence (5' to 3')	Purpose	AGI
ACT F	TTCGTGGTGGTGAGTTTGTT	qPCR	AT3G18780
ACT R	GCATCATCACAAGCATCCTAA	qPCR	AT3G18780
ORE1 F	TCTGCTACTGCCATTGGTGAAGT	qPCR	AT5G39610
ORE1 R	TCGGGTATTTCCGGTCTCTCAC	qPCR	AT5G39610
miR164b F	ATGAGTTAGTTCTTCATGTGCCCATC	qPCR	AT5G01747
MIR164B R	ATATTCCCACGTGCGTAACACTTG	qPCR	AT5G01747
5383	CGACGACCCGAGTAATTGAT	Cloning	
5384	TCAGAAATTCCAAACGCAATC	Cloning	
5510	GAAATTCCAAACGCAATCCA	Cloning	
5738	TTACGCATAGTCAGGAACATCGTATGGGTAGAAATTCCAAACGCAATCCA	Cloning	
5712	GACGAAGAGGGAAGGGTTTT	Cloning	
5804	TTATGGAGTTGGGTTCTTACGCATAGTCAGGAACATCGTATGG	Cloning	
5761	TAGTTGGAATGGGTTCCGACGACCCGAGTAATTGATAAAAATTTAACC	Cloning	
5739	TAGTTGGAATGGGTTCCGACGACCCGAGTAATTGATAAAAATTTAACC	Cloning	
5740	TTATGGAGTTGGGTTGTTCTTTAGAACAATTATTTGTATTGAAATTCAAGAGTTTTACCTAC	Cloning	



Chapter 5

Ethylene accumulation during submergence induces leaf age-dependent ORE1 phosphorylation

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5

Abstract

Rapid ethylene accumulation in submerged tissues is a signal for a flooded plant to activate a wide array of adaptive responses, but it also accelerates senescence. This process is activated in a clear leaf-age dependent manner, starting with the oldest leaves and is mediated by the ethylene-inducible transcription factor ORESARA (ORE1). Premature ORE1 activation and subsequent senescence in young leaves is typically prevented by post transcriptional repression of *ORE1* via high levels of microRNA164. However, in submerged plants ORE1 protein accumulated to similar levels in both old and young leaves indicating a bypass of this mechanism. Here we investigated how systemic ORE1 accumulation upon submergence led to the age-dependent induction of leaf senescence. We hypothesized that the activity of ORE1 was controlled posttranslationally. mRNAseq was used to identify which genes were induced by ORE1 during submergence in old leaves. Among these were several previously established target genes involved in leaf senescence and this set was expanded to four others by verifying that ORE1 could bind to their promoters, both *in vitro* and in *vivo*. We found that ORE1 activation of these target genes was limited to older leaves and required ORE1 phosphorylation, which occurred earlier in old leaves than young leaves. In *ORE1* overexpressors lacking multiple previously identified phosphorylation sites transcription of target genes was absent. These plants also did not show the early-senescence phenotype of plants overexpressing a native form of ORE1. The phosphorylation-mediated activation of ORE1 has been shown to be controlled by CALCIUM DEPENDENT PROTEIN KINASE1 (CPK1) but *cpk1* mutants lacked a delayed senescence phenotype, suggesting that one or more other unidentified kinases were involved in the posttranslational regulation of ORE1 during flooding stress.

Introduction

Spatiotemporal regulation of gene expression in response to environmental stresses involves multiple layers of regulation in signaling cascades, at both mRNA and protein levels (Alvarez et al., 2021). Chapter 4 describes how the transcription factor ORE1 mediates age-dependent leaf senescence, despite systemic accumulation during flooding stress. This suggests that ORE1 activity is potentially post-translationally regulated.

Posttranslational modification of transcription factors is a central element in plant responses to hypoxia, which often occurs upon flooding (Gibbs et al., 2011; Licausi et al., 2011; Bailey-Serres et al., 2012). Oxygen sensing in plants is mediated by the constitutively produced ERFVIIs. Under normoxic conditions ERFVIIs are continually degraded in the 26S proteasome after ubiquitination by the E3-ligase PRT6. This process is oxygen-dependent, so under oxygen-limited conditions the ERFVIIs are stabilized and can induce the transcription of their targets. The expression of the Arabidopsis ERFVII *RAP2.2* is induced by the transcription factor WRKY33. WRKY33 is also regulated at the posttranslational level: phosphorylation of WRKY33 by MPK3/6 increases its ability to transactivate *RAP2.2* (Liu et al., 2021a; Mao et al., 2011). Although phosphorylated WRKY33 has increased transactivation activity compared to non-phosphorylated WRKY33, it is also less stable as phosphorylation induces its degradation via the E3 ligase SR1. SR1 protein accumulates upon reoxygenation, which allows the plant to rapidly shut off the WRKY33-mediated response to hypoxia when desubmerged (Liu et al., 2021a).

ORE1 is a part of the family of NAC (NO APICAL MERISTEM, ARABIDOPSIS THALIANA ACTIVATION FACTOR1, CUP-SHAPED COTYLEDON2) domain transcription factors, which is one of the largest in Arabidopsis, consisting of over 100 members (Ooka et al., 2003). Many genes are targeted by NAC transcription factors, and some major themes can be identified in the processes regulated by NACs. One of these is xylem development. Several NAC domain transcription factors of the VND-type (VASCULAR-RELATED NAC DOMAIN) have been shown to induce xylem vessel formation, whereas this process is repressed by other NACs (Yamaguchi et al., 2010, 2008; Kubo et al., 2005). Although the moss *Physcomitrella patens* lacks xylem tissue, it uses NAC domain transcription factors to control the development of water-conducting cells, indicating a strong evolutionary conservation of the function of NAC transcription factors (Xu et al., 2014). Differentiated xylem tissue typically consists of dead cells (Van Hautegem et al., 2015). To induce the death of developing xylem cells, NAC domain transcription factors induce the expression of a wide set of enzymes involved in catabolism of cellular macromolecules (Ohashi-Ito et al., 2010). NAC transcription factors not only induce cell death during xylem development but also during leaf senescence. Senescence is another process in which NAC transcription factors play a major role, as more than 30 NACs have been implied in its regulation (Kim et al., 2016). Leaf senescence can either be triggered developmentally at the end of a leaf's lifecycle, or by exogenous stress signals. These two signals also interact with each other, as older leaves are more sensitive to senescence-inducing signals than young leaves.

The role of NAC transcription factors in leaf senescence, like their role in the development of water-conducting tissue, is strongly conserved. There are examples

in many different crop species of NAC transcription factors that control the onset of senescence, either in response to developmental or exogenous signals (Podzimska-Sroka et al., 2015). *ORE1* has been described as the most important regulator of senescence in Arabidopsis (Liebsch and Keech, 2016). The closest relative of *ORE1* in rice, *OsNAC2*, is similar to *ORE1* in multiple ways: it is a target of miR164-mediated degradation (Jiang et al., 2018; Fang et al., 2014; Kim et al., 2009) and it is involved in ABA-induced leaf senescence (Mao et al., 2017; Garapati et al., 2015).

Structurally NAC transcription factors can be divided into two groups: membrane-bound and non-membrane bound transcription factors (Kim et al., 2007). The membrane-bound NACs are tethered to the ER via a transmembrane domain in their C-terminus. This is cleaved off in response to environmental changes after which the cleaved protein moves to the nucleus. Multiple membrane-bound NACs are implicated in the fast response to changes in the cellular redox status likely involving rapid changes in subcellular localization of NACs (Ng et al., 2013; De Clercq et al., 2013). Non-membrane-bound NACs, like ORE1, often have a disordered region in their C-terminus, which is involved in protein-protein interactions (O'Shea et al., 2015; Jensen et al., 2010). Although non-membrane-bound NACs are generally not regulated via their subcellular localization, there are multiple other examples of posttranslational regulation. ORE1 is phosphorylated by the kinase CALCIUM-DEPENDENT PROTEIN KINASE1 (CPK1) (Durian et al., 2020). Phosphorylation of ORE1 is not required for its DNA binding activity, which is controlled by its N-terminal region, but it does affect its transactivation activity (Durian et al., 2020). This has also been demonstrated for NAC domain transcription factors ZmNAC84 in maize and NST1 in Arabidopsis where phosphorylation was important for transactivation of downstream targets (Zhu et al., 2016; Liu et al., 2021b). Phosphorylation-mediated activation during stress is not limited to NAC domain transcription factors. During osmotic stress for example, the kinase SnRK2 can phosphorylate the bZIP-class ABF transcription factors, which allows them to induce the transcription of their target genes (Chapter 3, Figure 3A, (Fujita et al., 2013)).

Results in Chapter 4 demonstrated that age-dependent leaf senescence during flooding stress is controlled by ethylene signaling. Ethylene, via EIN3, induces *ORE1* transcription. We found that loss of *ORE1* strongly reduced submergence-induced senescence and improved post-submergence plant performance. Surprisingly, although *ORE1*-mediated senescence was activated sooner in old rather than young leaves, *ORE1* mRNA and protein accumulation was age-independent. Furthermore, our results suggested that the previously described age-dependent control over *ORE1* via *miR164* was bypassed (Kim et al., 2009). In this chapter we investigated how age-independent ORE1 accumulation leads to a leaf age-dependent phenotype (senescence). Since the loss of *ORE1* had age-dependent effects, despite the systemic accumulation of ORE1 protein, we hypothesized that the activity of ORE1 was controlled post-translationally. We found that ethylene not only controls ORE1 protein accumulation, but also its phosphorylation state. ORE1 phosphorylation specifically in old leaves lead to its activation in these tissues and thus leaf age-dependent senescence. This age-dependent activation of ORE1 did not require the previously identified kinase CPK1.

Results

Loss of *ORE1* has an age-dependent effect on the transcriptome of submerged plants

Although ORE1 protein accumulated in both old and young leaves during submergence, ORE1 induced senescence predominantly in old leaves (Chapter 4). To get a global and unbiased overview of whether this was the result of age-dependent activation of ORE1 targets, an mRNAseq experiment was performed. Old and young leaves of Col-0 and *ore1-1* were harvested before submergence, after four days of submergence, and after six hours of recovery (Figure 1A). This experimental design permitted three comparisons: 1) the submergence response obtained by comparing the 4d submergence to the 0h time point 2) the recovery response obtained by comparing 6h recovery to the 4d submergence 3) the combined response encompassing the response to the combination of submergence and recovery, obtained by comparing 6h recovery to the 0h time point. 877 differentially expressed genes (DEGs) were detected between old leaves of Col-0 and *ore1-1* for the submergence response (FDR-corrected p-value < 0.05; absolute $\log_2$ fold change in expression > 1), compared to only 87 DEGs between the young leaves of these genotypes (Figure 1B). Interestingly, no genotype-specific combined response DEGs were detected. Of the 720 genotype-specific recovery DEGs, 428 were already differentially expressed after 4 days of submergence. This suggests that the loss of *ORE1* predominantly affected the transcriptome of old leaves during submergence rather than during recovery.

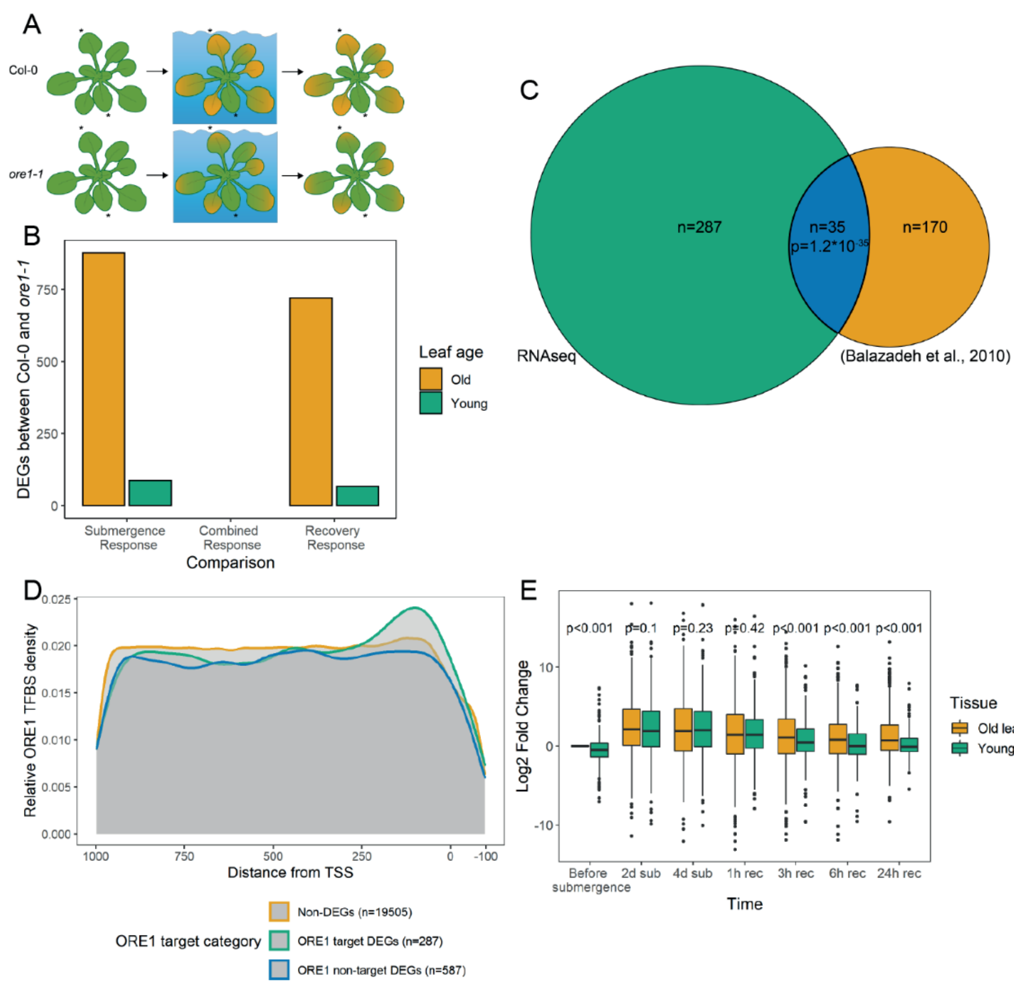


Figure 1 | Loss of *ORE1* has a leaf age-dependent effect on the transcriptome of submerged plants
A) Schematic depicting the mRNAseq experimental design. Old and young leaf blade samples (indicated by asterisks, the top asterisk on each rosette marks the old leaf, the bottom marks the young leaf) of Col-0 and *ore1-1* were harvested before submergence, after four days of submergence, and after six hours of recovery.
B) The number of differentially expressed genes (DEGs) (up and downregulated) that show a genotype-dependent response to four days of submergence was greater in old leaves than in young leaves. No DEGs showed a genotype-dependent effect in their response to submergence followed by recovery (the combined response). Most (428/720) DEGs showing a genotype-specific response to post-submergence recovery showed the opposite pattern during the submergence phase.
C) Overlap between genes showing an *ORE1*-dependent induction during submergence and genes induced by estradiol-mediated induction of *ORE1* (Balazadeh et al., 2010). Significance was determined by hypergeometric test.
D) Density of ORE1 binding sites (TDRCGTRHD/VMGTRN$_{5\text{-}6}$YACR, Olsen et al., 2005; Matallana-Ramirez et al., 2013) in the promoters of Non-DEGs (orange line), DEGs with lower expression in *ore1-1* than Col-0 in old leaves (ORE1 target DEGs) (green line), and all other DEGs between Col-0 and *ore1-1* in old leaves (ORE1 non-target DEGs; blue line).
E) Expression of EIN3 target genes (n=375) (Chang et al., 2013) was similar in old and young leaves during submergence but was higher in old leaves before and after submergence (Wilcoxon Rank Sum test).

Of the genotype-specific submergence DEGs in the old leaves, the subset that showed a significantly smaller increase in expression during submergence in *ore1-1* than in Col-0 (labeled “ORE1 target DEGs”) contained several previously identified targets of ORE1 including *BIFUNCTIONAL NUCLEASE1* (*BFN1*) and *NON-YELLOWING1* (*NYE1*). This set of potential ORE1 target DEGs also showed significant overlap with a set of genes previously shown to be upregulated after estradiol-mediated induction of *ORE1* (Balazadeh et al., 2010) (Figure 1C). The promoters of these putative ORE1 target DEGs were enriched for binding sites of ORE1 (VMGTRN$_{5\text{-}6}$YACR or TDRCGTRHD, (Matallana-Ramirez et al., 2013; Olsen et al., 2005)), which was not the case for genes that were not differentially expressed between the two genotypes or differentially expressed genes that were not classified as ORE1 target DEGs (Figure 1D).

Consistent with our observation that EIN3 accumulation during submergence was age-independent (Chapter 4), the induction of EIN3 target genes (Chang et al., 2013) during submergence was similar in old and young leaves (Figure 1E). For this analysis we used all timepoints at which old and young leaves of Col-0 plants were sampled for mRNAseq (see Chapter 2). The *ORE1*-dependent response during submergence did not seem to involve hypoxia signaling, as none of the 47 out of the 51 core hypoxia genes (HRGs) (Mustroph et al., 2009) detected in our dataset were different between Col-0 and *ore1-1* in either old or young leaves (Figure S1).

ORE1 induces the transcription of genes involved in senescence-associated processes during submergence

The mRNAseq results supported the hypothesis that ORE1 induces the expression of its target genes in an age-dependent manner. The connection between ORE1 and several of its downstream targets has been verified for several targets (Rauf et al., 2013; Matallana-Ramirez et al., 2013; Qiu et al., 2015; Zhang et al., 2021). We expanded this set of genes by confirming that ORE1 can bind the promoters of the protease *METACASPASE9* (*MC9*), the NAC domain transcription factor *ANAC010*, the nuclease *DEFECTIVE IN POLLEN ORGANELLE DNA DEGRADATION 1* (*DPD1*), and the chloroplast-degrading protein *CHLOROPLAST VESICULATION* (*CV*) *in vitro* via electrophoretic mobility shift assay (EMSA) (Figure 2A). These novel target genes were selected because of their role in processes typically associated with leaf senescence (Lim et al., 2007). Binding of ORE1 to these promoters could be reduced by adding a competitor oligo. *In vivo* binding of ORE1 to these promoters was confirmed via ChIP-qPCR using *pORE1:ORE1-HA* plants that were submerged for one day in darkness (Figure 2B). The binding of ORE1 to all newly identified putative targets was significantly enriched, when compared to the binding of ORE1 to the promoter of the negative control (AT4G22180) (Figure 2B).

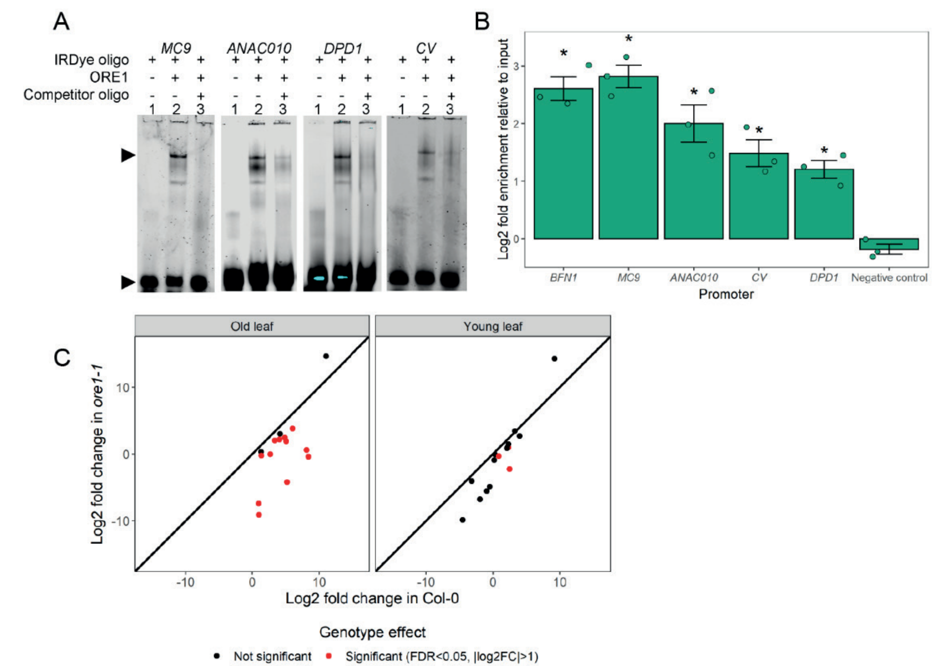


Figure 2 | ORE1 induces the transcription of genes involved in senescence-associated processes during submergence

A) Electrophoretic Mobility Shift Assay (EMSA) showing *in vitro* binding of recombinant ORE1-GST to the promoters of *MC9*, *ANAC010*, *DPD1*, and *CV*. From left to right in each image: lane 1, labelled probe (5’-DY862-labeled double-stranded oligonucleotides); lane 2, labelled probe plus ORE1-GST protein; lane 3, labelled probe, ORE1-GST protein, and competitor (unlabeled oligonucleotide containing an ORE1 binding site; 200x molar access). The top arrowhead indicates protein-DNA complex, the bottom arrowhead indicates free DNA probes.

B) ChIP-qPCR showing *in vivo* binding of ORE1 to the promoters of *MC9*, *ANAC010*, *DPD1*, and *CV*, asterisks indicate significant enrichment relative to the negative control (AT2G22180) (one-way ANOVA + Dunnett’s post-hoc test). Chromatin was extracted from immunoprecipitated samples from whole *pORE1:ORE1-HA ore1-1* rosettes submerged for one day, n=3.

C) Changes in expression after four days of submergence of *in vivo* confirmed ORE1 target genes (n=15 Supplemental Table 1) differed more between Col-0 and *ore1-1* in old leaves than young leaves. Red dots correspond to genes which are regulated during submergence in an *ORE1*-dependent manner in either old or young leaves, black dots correspond to those that are not affected by the loss of *ORE1*.

Out of a set of fifteen in vivo-verified ORE1 targets, from this study and others (Supplemental Table 1), an ORE1-dependent induction was observed in old leaves for twelve (Figure 2C). In young leaves, only three of the fifteen verified targets showed an ORE1-dependent induction during submergence. Taken together, these results demonstrate age-dependent target activation by ORE1 during submergence.

In public transcriptome datasets of hypoxia-treated plants, the expression of *ORE1* and these fifteen targets was generally repressed, rather than induced (Figure S1B) (Branco-Price et al., 2005; Licausi et al., 2011; Chang et al., 2012; Lee and Bailey-Serres, 2019; Liu et al., 2022). This confirmed that *ORE1* expression and downstream target activation was not induced by hypoxia.

ORE1 phosphorylation is age-dependent and controls senescence of old leaves

Following the verification of age-dependent ORE1 activation and the non-involvement of the miRNA-mediated control of *ORE1*, we probed alternative mechanisms that control the activity of ORE1. A recent study has shown that the transactivation activity of ORE1 is increased upon its phosphorylation by the kinase CALCIUM-DEPENDENT PROTEIN KINASE1 (CPK1) (Durian et al., 2020). The ORE1 protein is 285 amino acids long and contains a NAC domain in its N-terminal region involved in DNA binding (Figure S2A). The C-terminal half of ORE1 is largely disordered, and contains a stretch of seventeen amino acids which can be phosphorylated up to sixfold ((Jensen et al., 2010; Durian et al., 2020), Figure S2A-B). Phosphorylation of ORE1 does not affect its DNA binding activity, but is necessary for the capacity of ORE1 to induce transcription of its downstream targets (Durian et al., 2020).

We decided to explore this post-translational modification as a potential mechanism mediating differential ORE1 activation in submerged plants. Protein extracts from leaves of submerged *pORE1:ORE1-HA* plants run on an SDS-PAGE gel containing 50µM PhosTag revealed a slower migration of ORE1-HA from old leaves. This suggested the presence of phosphorylated ORE1-HA in old, submerged leaves supporting our hypothesis of age-dependent ORE1 activation via phosphorylation during submergence (Figure 3A). To further validate this, we used transgenic plants overexpressing a modified ORE1 missing the peptide containing potential phosphorylation sites (*35S:ORE1Δ17*). These *35S:ORE1Δ17* plants showed a dominant-negative phenotype under submergence stress (Figure 3B-C).

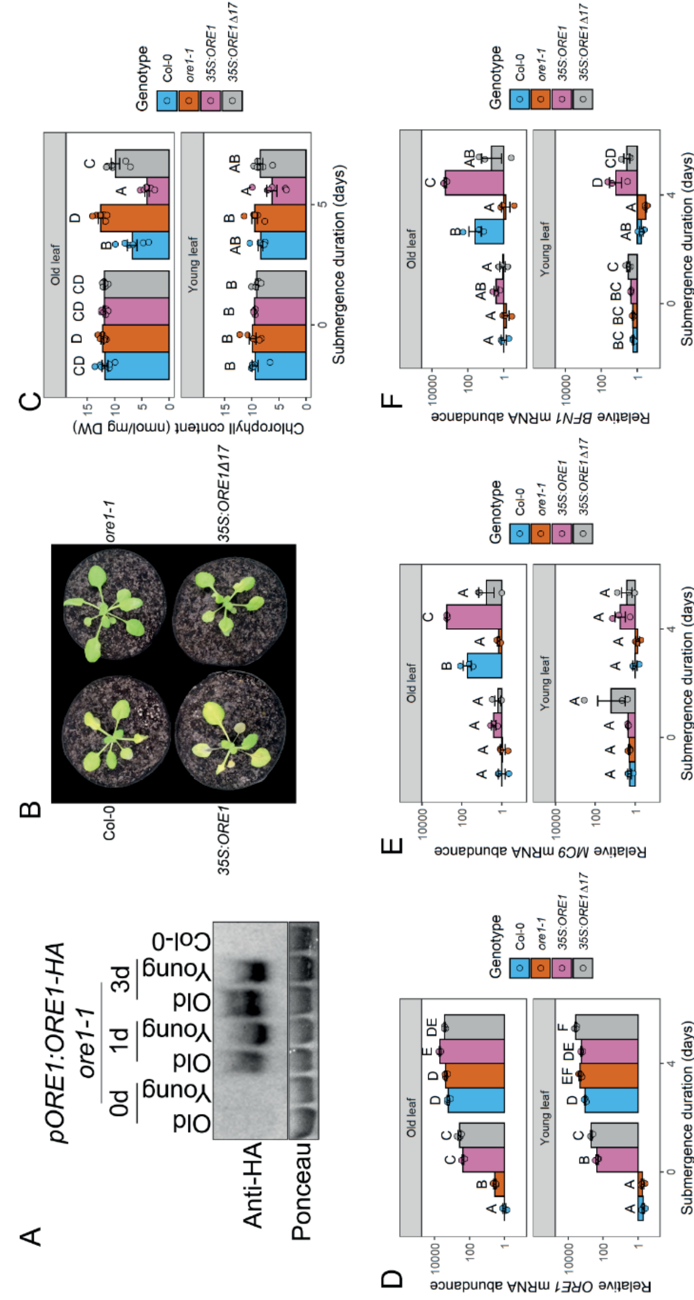


Figure 3 | ORE1 phosphorylation is age-dependent and controls senescence of old leaves
A) *pORE1:ORE1-HA ore1-1* protein samples from submerged old leaves move slower through a PhosTag gel than samples from young leaves, indicating age-specific phosphorylation of ORE1. Five old or young leaves were pooled together from different plants per *pORE1:ORE1-HA ore1-1* sample, the Col-0 sample was from one whole rosette.
B) Representative images of Col-0, *ore1-1*, *35S:ORE1*, and *35S:ORE1Δ17* plants after five days of submergence followed by one day of recovery. Scale bar indicates 1cm.
C) Chlorophyll content of Col-0, *ore1-1*, *35S:ORE1*, and *35S:ORE1Δ17* plants before and immediately after five days of submergence.
D-F) Expression of *ORE1*, *MC9*, and *BFN1* in Col-0, *ore1-1*, *35S:ORE1*, and *35S:ORE1Δ17* before and after four days of submergence. Expression was normalized to that of non-submerged old leaves of Col-0. Two old or young leaves from different plants were pooled together per sample. Different letters indicate significant differences at p<0.05, ANOVA+Tukey's post-hoc test

As expected, the expression of *ORE1* was already high before submergence in *35S:ORE1* and *35S:ORE1Δ17* plants and was induced in both old and young leaves of Col-0 and *ore1-1* plants during submergence (Figure 3D). The *ore1-1* mutant is a true null mutant containing a T-DNA insertion in the last exon. The primer pair used here spans the first intron, explaining the increase in *ORE1* transcript levels in *ore1-1* (Durian et al., 2020; Balazadeh et al., 2010). Although expression of *ORE1* was high during submergence in both old and young leaves of all four genotypes used here, the downstream target genes *MC9* and *BFN1* were only induced in the old leaves of Col-0 and *35S:ORE1* (Figure 3E-F). Taken together, these results showed that age-dependent phosphorylation of ORE1 is required for the activation of its downstream target genes.

Ethylene controls age-dependent ORE1 phosphorylation

Plants with impaired ethylene signaling did not show age-dependent leaf death during submergence and treating plants with ethylene in darkness induced age-dependent leaf death (Chapter 4, Figures 1-2). This could not be explained by *ORE1* transcript levels since ethylene treatment and submergence both caused leaf-age independent *ORE1* induction (Chapter 4, Figures 3 & 6). This held true also for ORE1 protein levels (Chapter 4, Figure 5; This chapter, Figure 4A). While the combination of ethylene and darkness was both essential and sufficient for induction of ORE1 protein to similar levels as during submergence, this occurred in both old and young leaves (Figure 4A). However, considering that during submergence ORE1 phosphorylation and activation occurred in old leaves, we hypothesized that ethylene might be the underlying submergence signal (Figure 3B). In accordance with this, ethylene in darkness was already sufficient to induce age-dependent ORE1 phosphorylation in one day (Figure 4B). Ethylene signaling in darkness thus induced both the accumulation of ORE1 protein and its activation by phosphorylation. This is consistent with the previous observation that ethylene treatment, rather than hypoxia, is sufficient to induce age-dependent leaf yellowing (Chapter 4). Furthermore, treating plants with ethylene in darkness had a similar effect on the senescence phenotype of Col-0 plants as submerging them in darkness (Figure 4C-D). This induction of senescence during dark submergence was lost in ethylene-insensitive *ein3eil1* plants (Figure 4C-D).

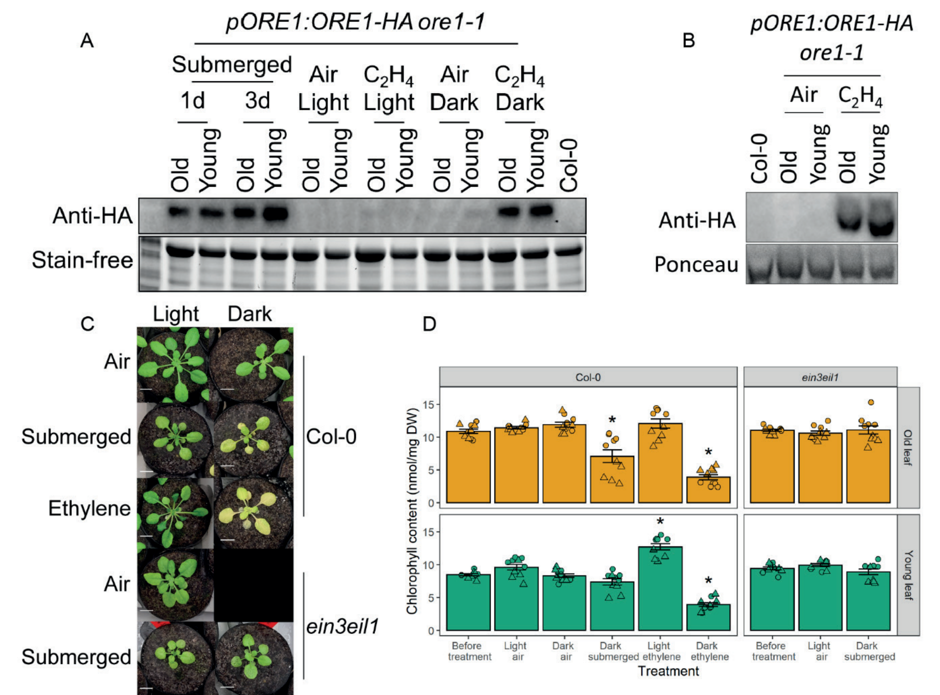


Figure 4 | ORE1 accumulation and phosphorylation are controlled by ethylene signaling

A) Immunoblots showing ORE1 accumulation in old and young leaves after submergence or one day of ethylene
treatment in darkness. Five old or young leaves from different plants were pooled together per *pORE1:ORE1-HA ore1-1* sample. The Col-0 sample was from one whole rosette. Stain-free imaging of the protein gel was used as a loading control.

B) ORE1-HA from old leaves treated with ethylene in darkness for one day moves slower through a PhosTag gel than ORE1-HA from young leaves. Samples are the same as the ones run on the non-PhosTag gel in panel A. Ponceau staining of the large subunit of Rubisco was used as a loading control.

C) Shoot phenotypes in response to submergence or ethylene in light or dark conditions. Representative images
of Col-0 and *ein3eil1* plants immediately after five days of the indicated treatments are shown. Scale bars indicate 1cm.

D) Chlorophyll content of old and young leaves following treatments with different combinations of ethylene and
darkness. Asterisks indicate significant differences from chlorophyll levels before the treatment (One-way ANOVA + Dunnett's test), error bars indicate SEM. n=10 per sample from two independent experiments, circles
and triangles indicate experimental replicates.

ORE1 has been shown to be phosphorylated by CPK1 in vivo (Durian et al., 2020). *CPK1* mRNA levels showed a leaf age-dependent increase in submerged plants, although the absolute changes in expression were small (Figure 5A). *CPK1* also possessed an EIN3 binding site in its promoter (Figure 5B). We thus investigated it as a candidate kinase that phosphorylates and activates ORE1 downstream of ethylene. However, *CPK1* expression did not change in response to ethylene treatment in either old or young leaves (Figure 5C). Consistent with this, the chlorophyll content and visual phenotypes of *cpk1-1* mutants were not different from Col-0 after either submergence or ethylene treatment (Figure 5D-G). A comparison of Col-0, *ore1-1*, and *cpk1-1* plants revealed age-dependent leaf death in all genotypes upon submergence. This was significantly delayed in *ore1-1* compared to Col-0 but not in *cpk1-1* (Figure 3H). Thus, although ethylene exposure selectively induces senescence in old leaves via the age-dependent phosphorylation of ORE1, this does not seem to depend on *CPK1*.

Summarizing, these results provide a mechanism by which plants ensure that leaf senescence follows an age-dependent gradient during flooding stress. Such a mechanism might safeguard a total overall collapse of the plant due to high ethylene accumulation during flooding. Interestingly it is still unclear what prevents ethylene-mediated activation in young leaves. Although flooding stress induces systemic ethylene signaling and ORE1 accumulation, the age-dependent phosphorylation of ORE1 ensures that it can only activate its downstream targets in older tissues (Figure 7). The accumulation of ORE1 in young leaves can prepare them to rapidly transition into senescence if the submergence duration is long enough.

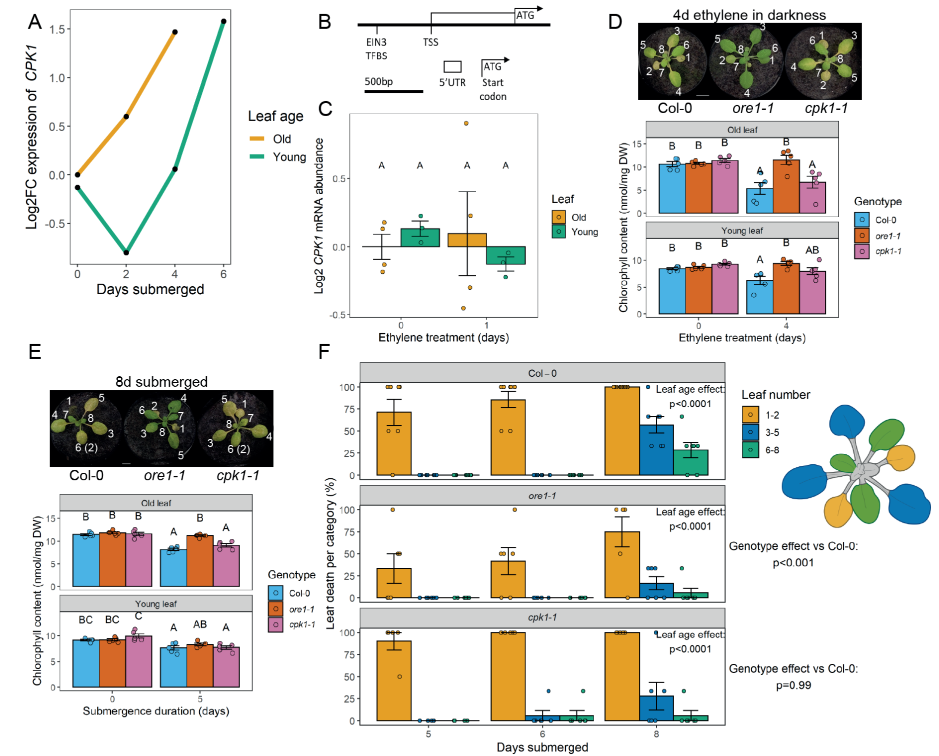


Figure 5 | Ethylene-mediated ORE1 phosphorylation does not require *CPK1*
A) Expression of *CPK1* during submergence in old and young leaves, based on mRNAseq data. Expression was calculated relative to that of non-submerged old leaves.
B) Schematic overview of the EIN3 binding site (AYGWAYCT) in the promoter of *CPK1*, 443bp from its transcriptional start site (TSS).
C) Expression of *CPK1* in response to ethylene in darkness, as determined by qPCR. Different letters indicate significant differences between groups (two-way ANOVA + Tukey's post-hoc test). n=4 for old leaves, n=3 for young leaves. Two leaves were pooled together per sample.
D) Representative images and chlorophyll content of Col-0, *ore1-1*, and *cpk1-1* plants after four days of ethylene treatment in darkness. Different letters indicate significant differences between groups (two-way ANOVA + Tukey's post-hoc test), n=5 per sample.
E) Representative images and chlorophyll content of Col-0, *ore1-1*, and *cpk1-1* plants after eight (images) or five (chlorophyll measurements) days of submergence. Different letters indicate significant differences between groups (two-way ANOVA + Tukey's post-hoc test), n=5 per sample.
F) Col-0, *ore1-1*, and *cpk1-1* all show age-dependent leaf death. This process was slowed down in *ore1-1* mutants but not in *cpk1-1* mutants. The schematic on the right shows the distribution of the three leaf age categories throughout an Arabidopsis plant. Differences between genotypes were determined by one-way ANOVA followed by Tukey's post-hoc test. Scale bars indicate 1cm

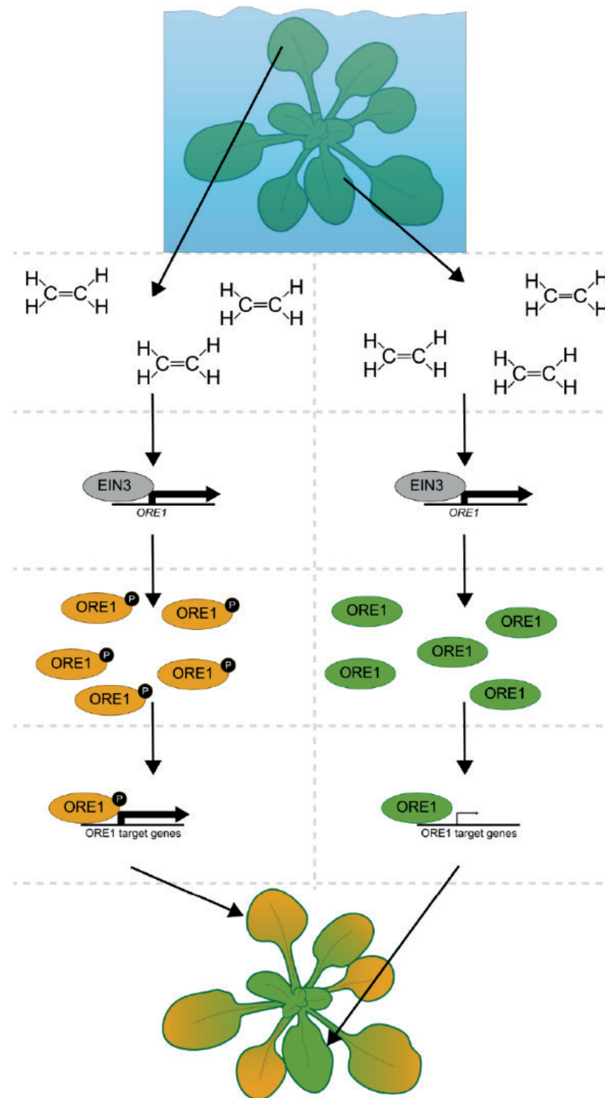


Figure 7 | A model for ethylene-mediated sequential leaf death in flooded plants. Upon submergence, ethylene rapidly accumulates throughout a plant. This age-independent accumulation of ethylene induces the age-independent accumulation of *ORE1* mRNA and protein via EIN3 stabilization. Leaf-age dependent senescence is triggered by ethylene via ORE1 phosphorylation and activation specifically in old leaves, via an unknown mechanism. This age-dependent phosphorylation of ORE1 ensures that it induces senescence in old leaves, which results in the oldest leaves being broken down first and the youngest leaves and meristem last.

Discussion

Our results demonstrate a mechanism whereby plant responses to a systemic stress cue are locally determined via post-translational modification of a transcription factor. Submergence of Arabidopsis rosettes leads to ethylene accumulation throughout the plant, activating ethylene signaling in all leaves, and yet initiates senescence in a specific leaf-age dependent pattern. Leaf senescence upon submergence starts in the oldest leaves, but eventually spreads down the age gradient to younger leaves. Ethylene accumulation and signaling throughout the plant causes this age gradient, whereby the transcription factor ORE1 is essential to rapidly start the de-greening process preferentially in older leaves. Though ethylene leads to ORE1 protein accumulation independent of age, ORE1 activation via phosphorylation is ethylene-driven specifically in the older leaves. Such a mechanism ensures ORE1 target activation and senescence only in older leaves. Although ORE1 protein was already produced in young leaves within one day of submergence, its effects on the transcriptome were minimal during 4 days of submergence. The premature production of ORE1 in young leaves means that during prolonged submergence, when energy levels are low, senescence can be induced without the need to make new ORE1 protein. In this case ORE1 only needs to be phosphorylated to induce the transcription of its downstream targets.

ORE1 is arguably one of the best-studied transcription factors controlling leaf senescence in Arabidopsis. Transcription of *ORE1* is, besides EIN3, directly induced by ATAF1, ATAF2, PIF4, PIF5, ABI5, EEL, PRR9, WRKY71, GI, and ARF2 (Garapati et al., 2015; Nagahage et al., 2018; Sakuraba et al., 2014; Kim et al., 2018; Yu et al., 2021; Kim et al., 2020; Xue et al., 2022). *ORE1* mRNA levels are regulated posttranscriptionally by miR164 (Kim et al., 2009), and ORE1 protein levels are controlled by NLA, PHO2, UBP12, and UBP13 via ubiquitination and deubiquitination (Park et al., 2018, 2019). Lastly, the transactivation activity of ORE1 is requires its phosphorylation by CPK1 (Durian et al., 2020). Despite the plethora of regulators that affect the abundance of *ORE1* mRNA and protein, we found that ORE1 abundance does not explain the difference in ORE1 target gene activation between old and young leaves during submergence. Rather, this difference was controlled by ethylene-induced post-translational modification of ORE1, which limits its activity to old leaves. ORE1 induces the transcription of its targets via an interaction with the positively charged C-terminus of Mediator complex subunit 19a (MED19a), which recruits RNA Polymerase II to target genes (Cheng et al., 2022). Phosphorylation of a protein typically reduces its charge, and the phosphorylation of ORE1 could potentially facilitate its binding to MED19a. This is also consistent with the impaired transactivation activity of ORE1Δ17, although it

still exhibits DNA binding activity (Durian et al., 2020).

This proposed mechanism of a phosphorylation-dependent interaction between ORE1 and the Mediator complex is similar to the functioning of the metazoan transcription factor ELK1. ELK1 is constitutively bound to the promoters of its target genes (Wang et al., 2005). External stimuli can trigger the phosphorylation of ELK1 in its C-terminal transactivation domain, which facilitates its interaction with the Mediator complex subunit MED23 and the recruitment of RNA Polymerase II to induce transcription of its downstream targets (Stevens et al., 2002; Wang et al., 2005). Like ORE1, ELK1 can be phosphorylated on multiple amino acid residues. The first phosphorylation events on ELK1 rapidly induce the interaction between ELK1 and MED23 whereas later phosphorylation events dampen this interaction (Mylona et al., 2016). Understanding the exact roles of the different phosphorylation sites on ORE1 will help in determining how the activity of ORE1 is controlled in different tissues under different environmental conditions.

Our results show that ethylene-induced leaf senescence requires darkness. It is currently unclear if this is an effect of light signaling or of darkness-induced carbon starvation, as both of these interact with ethylene signaling (Yanagisawa et al., 2003; Zhong et al., 2012; Shi et al., 2016; Kim et al., 2017). In addition to ethylene accumulation, impaired gas diffusion also leads to a decline in oxygen levels in flooded plants. Hypoxia is also considered an important regulatory signal mediating flood survival responses. Hypoxia by itself does not impose a gradient of age-dependent leaf yellowing, and mutants with impaired N-degron-mediated hypoxia sensing still showed age-dependent leaf death during flooding stress (Figure 1D-F). Furthermore, core hypoxia genes were not affected by the loss of *ORE1*, showing that *ORE1* is not upstream of hypoxia signaling (Figure S4D). Lastly, a survey of published transcriptomes of hypoxia-treated plants revealed that the expression of *ORE1* and its targets was generally not induced in response to hypoxia (Figure S1B). During senescence, the catabolism of macromolecules fuels the survival of other tissues. Since the efficiency of respiration is greatly reduced under hypoxic conditions, it might be more favourable to enter a state of metabolic quiescence rather than to break down molecules. This would explain why hypoxic conditions do not induce physiological or transcriptional signs of senescence. Based on these results we conclude that the sequential leaf death described here does not appear to involve oxygen sensing and signaling via the N-degron pathway. Arabidopsis ORE1 and rice SUB1A are both important regulators of the response to submergence, but both of them are primarily controlled by ethylene rather than hypoxia (Gibbs et al., 2011; Lin et al., 2019). This likely stems from the prevalence of hypoxia in normal plant development and the variation in oxygen concentrations among submerged plant tissues (Weits et al., 2019; Sasidharan et al., 2018).

The mechanism of age-dependent phosphorylation of ORE1 is an example of post-translational control of leaf senescence during flooding. Several mechanisms of this type have previously been shown to play a role in the response to flooding stress (Licausi et al., 2011; Gibbs et al., 2011; Liu et al., 2021a). Interestingly, the role of the ERFVII transcription factors in the response to hypoxia depends on the developmental stage of a plant (Giuntoli et al., 2017). In early plant development, ERFVIIs are essential to induce the expression of genes associated with mitochondrial dysregulation. In older plants, this requirement is partially lost as other unidentified regulators can take over this function of ERFVIIs (Giuntoli et al., 2017). The age-dependent functioning of ERFVIIs is independent of their posttranslational regulation via the N-degron pathway, although other posttranslational mechanisms could be involved.

Ethylene accumulation upon submergence induced senescence of old leaves via the age-dependent phosphorylation of ORE1 (Figure 7). Our results suggest that this phosphorylation was independent of CPK1, which is known to phosphorylate ORE1 in vivo (Durian et al., 2020). Future research should focus on how exactly the age-independent accumulation of ethylene leads to the age-dependent phosphorylation of ORE1 via an unidentified kinase. Kinases themselves are often controlled post-translationally and the interactions between kinases and their targets can be highly context-specific (Simeunovic et al., 2016), potentially complicating the identification of the kinase regulating ORE1 during submergence.

Materials and methods

Plant growth conditions and treatments

Plants were grown and treated with submergence as described in chapter 2. Ethylene treatments were done as described in chapter 4.

Chlorophyll quantification

Chlorophyll was quantified as described in chapter 4.

Gene expression

RNA was extracted from the indicated tissues using the Qiagen plant RNeasy mini kit, including an on-column DNAse treatment, according to the manufacturer's

instructions. Extracted RNA was converted into cDNA using RevertAid H Minus Reverse Transcriptase (Thermo Scientific). For qPCR 20ng of cDNA was used per 5µl reaction, using SYBR Green master mix (BioRad) and the primers indicated in Supplemental Table 2.

RNA sequencing

Between 8 and 16 young and old leaves of Col-0 and *ore1-1* were harvested before submergence, after four days of dark submergence, and after six hours of post-submergence recovery in the light. RNA extraction, library preparation, sequencing, quality control, and read alignment was done as described in chapter 2. Genes which showed a genotype-dependent response to the treatment in either young or old leaves were determined using EdgeR and Limma, genes were determined as differentially expressed when FDR<0.05 and $|\log_2 FC|>1$. Reads have been deposited at the European Nucleotide Archive under accession number PRJEB57289. Lists of differentially expressed genes can be found at https://www.biorxiv.org/content/10.1101/2022.11.23.517613v1.

ORE1 binding site density

To determine the density of ORE1 binding sites in the promoters of putative target genes, genes were selected from the mRNAseq dataset that are induced significantly stronger in Col-0 old leaves than *ore1*-1 old leaves after four days of dark submergence. Promoters (1kb upstream and 100bp downstream of the transcriptional start site) of these 287 genes were extracted from the TAIR9 genome sequence using the GenomicRanges R package (Lawrence et al., 2013). These promoters were scanned for the occurrences of the ORE1 motifs $VMGTR_{N5-6}YACR$ and TDRCGTRHD, allowing one mismatch (Matallana-Ramirez et al., 2013; Olsen et al., 2005). The density of motif centers along the promoter sequences was corrected for the amount of scanned promoters and plotted using ggplot2.

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as previously described by (Wu et al., 2012). ORE1-GST protein was purified as previously described by (Durian et al., 2020). Binding reactions were performed using the Odyssey infrared EMSA kit (LI-COR) following the manufacturer's instructions. DNA-protein complexes were separated in a 6% (w/v) retardation gel (EC6365BOX, Invitrogen). DY682 signal was detected using the Odyssey infrared imaging system from LI-COR.

ChIP-qPCR

For ChIP, 10-leaf stage Col-0 and *pORE1:ORE1-HA ore1-1* plants were submerged for one day in darkness to induce ORE1-HA protein accumulation. Chromatin was extracted from 1.5g of whole-rosette tissue for each replicate. Protein-DNA complexes were immunoprecipitated using anti-HA antibodies (Miltenyi Biotec) (Kaufmann et al., 2010). After reversion of the cross-linking, DNA was purified with the QIAquick PCR Purification Kit (Qiagen) and was analyzed by qPCR using the primers in Supplemental Table 2. Enrichment of ORE1 at the target promoters was calculated relative to Col-0, significance was determined by comparing the enrichment at each of the target loci to that of the negative control (AT2G22180).

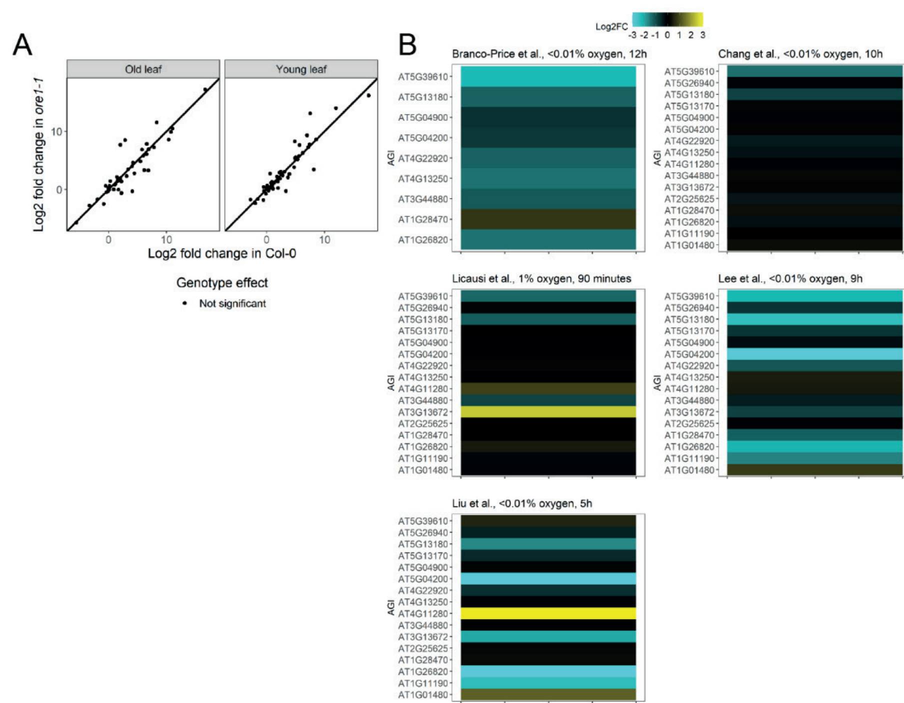
Western blotting

Regular western blots were performed as described in chapter 4. To identify phosphorylated proteins, 50µg of protein extract was precipitated by incubating the sample with 4x the volume of the protein sample 100% ice-cold acetone for one hour at -20°C. After precipitation the samples were centrifuged for 10 minutes at 13,000g at 4°C and the supernatant was removed. The pellet of precipitated proteins was resuspended in 12µl water and 3µl 5x sample loading buffer (250mM Tris (pH 6.8) 25% glycerol, 10% SDS, 0.05% bromophenol blue) containing 5% beta-mercaptoethanol. Samples were boiled for five minutes at 95°C to denature the proteins and were separated via electrophoresis on a SuperSep Phos-Tag 7.5% gel with 50µmol/l Phos-Tag (198-17981, Fujifilm Wako, Japan). The gel was run at a stable 20mA for 2.5 hours. After electrophoresis, the gel was washed twice in running buffer containing 10mM EDTA for ten minutes and once in running buffer without EDTA. Transferring the proteins, blocking the membrane, antibody incubation, and imaging were done identically to non-PhosTag ORE1-HA Western blots.

Statistical analysis

All statistical tests were done in R version 3.6.1 using the indicated statistical tests, differences were deemed significant at $p<0.05$.

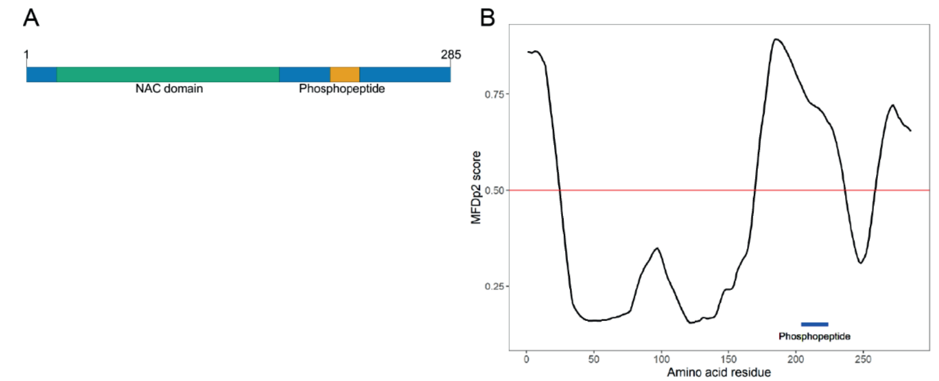
Supplemental Figures



Supplemental Figure 1

A) The expression of core hypoxia genes (Mustroph et al., 2009) after four days of dark submergence was not different between Col-0 and *ore1-1* plants, in either old or young leaves.

B) The expression of *ORE1* (AT5G39610) and its *in vivo*-verified targets (Supplemental Table 1) from various published transcriptome surveys of hypoxia-treated plants. Expression is calculated relative to samples in normoxia. Not all targets were detected in each dataset, only the detected ones are shown.



Supplemental Figure 2

A) Overview of the *ORE1* protein, including its NAC domain and phosphopeptide.

B) Predicted disorder along the protein sequence of *ORE1*, using MFDp2 (Mizianty et al., 2013). The red line is the threshold score for considering residues as disordered (0.5).

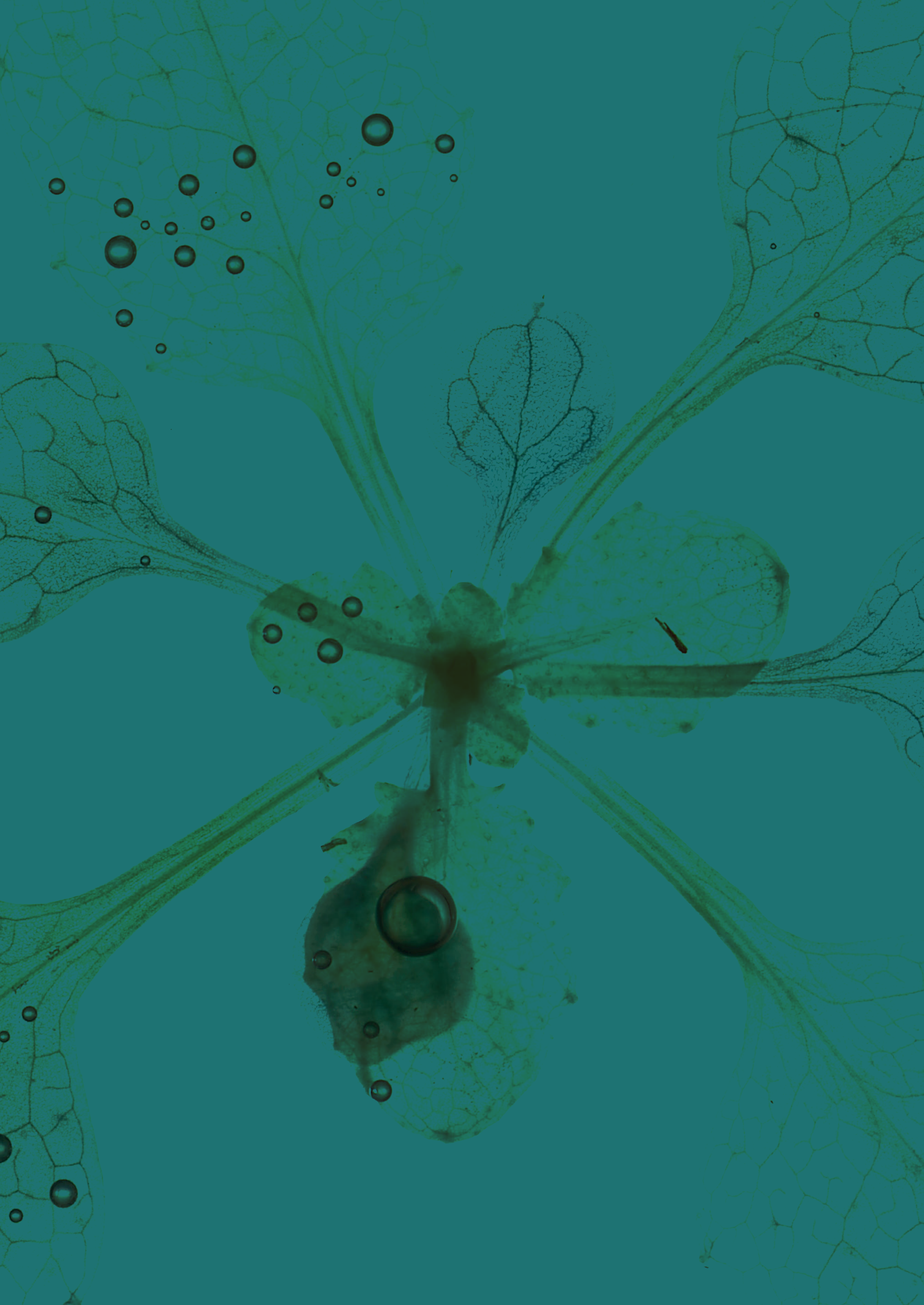
Supplemental Table 1 | ORE1 and its targets

AGI codes for *ORE1* and 15 target genes that have been verified *in vivo*, used in Figure 2C.

AGI	Name	Reference
AT1G01480	*ACS2*	Qiu et al., 2015
AT1G11190	*BFN1*	Matallana-Ramirez et al., 2013
AT1G26820	*RNS3*	Rauf et al., 2013
AT1G28470	*ANAC010*	This study
AT2G25625	*CV*	This study
AT3G13672	*SINA1*	Rauf et al., 2013
AT3G44880	*PAO*	Qiu et al., 2015
AT4G11280	*ACS6*	Zhang et al., 2021
AT4G13250	*NYC1*	Qiu et al., 2015
AT4G22920	*NYE1*	Qiu et al., 2015
AT5G04200	*MC9*	This study
AT5G04900	*NOL*	Qiu et al., 2015
AT5G13170	*SWEET15*	Matallana-Ramirez et al., 2013
AT5G13180	*VNI2*	Rauf et al., 2013
AT5G26940	*DPD1*	This study
AT5G39610	*ORE1*	-

Supplemental Table 2 | Primers used in this chapter

Primer name	Sequence (5' to 3')	Purpose	AGI
ACT F	TTCGTGGTGGTGAGTTTGTT	qPCR	AT3G18780
ACT R	GCATCATCACAAGCATCCTAA	qPCR	AT3G18780
ORE1 F	TCTGCTACTGCCATTGGTGAAGT	qPCR	AT5G39610
ORE1 R	TCGGGTATTTCCGGTCTCTCAC	qPCR	AT5G39610
BFN1 F	CAGCCATCTCCATTACATCG	qPCR	AT1G11190
BFN1 R	CCGTAATGCTGAAGCTGAGA	qPCR	AT1G11190
MC9 F	CGTGAAAAACACGTGGACTC	qPCR	AT5G04200
MC9 R	TTTGAATCGCGTTGCTAAAC	qPCR	AT5G04200
ANAC010 F	ACTCCATCTCTCGTCACCTTTC	ChIP-qPCR	AT1G28470
ANAC010 R	CCAACCACACTTAGGAAAACCC	ChIP-qPCR	AT1G28470
CV F	CGACAAGAACCTTGTTGACGTG	ChIP-qPCR	AT2G25625
CV R	CGTGAATCTGTTTGCTACGTCTG	ChIP-qPCR	AT2G25625
MC9 F	TGAACTTCACTACACGAAACCTC	ChIP-qPCR	AT5G04200
MC9 R	TGGGGTTTCTTGTGTACGTG	ChIP-qPCR	AT5G04200
DPD1 F	ACGTAGATCATCACTGCGAGAC	ChIP-qPCR	AT5G26940
DPD1 R	AATGTTGCACTCCGAGAAGC	ChIP-qPCR	AT5G26940
AT2G22180 F	GTTGCGTATGAAGATGACGTGG	ChIP-qPCR	AT2G22180
AT2G22180 R	GCCGAGTTAACGACGTGCTTAT	ChIP-qPCR	AT2G22180
BFN1 F	CATGACACATGGATGATGCTGT	ChIP-qPCR	AT1G11190
BFN1 R	CATTGCGAGTCTCATACGTTCC	ChIP-qPCR	AT1G11190



Chapter 6

Summarizing discussion

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6

Increasing the tolerance of crops to abiotic stresses like flooding, will be essential in reducing yield losses caused by climate change. Abiotic stress resilience often varies depending on plant or organ age. However, the mechanisms underlying this are poorly understood (Rankenberg et al., 2021). This thesis explores the gradient in flooding tolerance with leaf age in the model plant species *Arabidopsis thaliana*. When submerged, the older leaves of an Arabidopsis rosette are the first to die whereas the younger leaves have a higher resilience to flooding. As flooding is a compound stress consisting of several external signals converging on a plant, both simultaneously and sequentially (Sasidharan et al., 2018), resilience involves the activation of multiple distinct signaling pathways (Yuan et al.).

We used an unbiased mRNAseq approach to identify molecular processes that could explain the age-dependent stress resilience to submergence and recovery. Both submergence and recovery triggered a massive reprogramming of the transcriptomes of old and young Arabidopsis leaves. Approximately half of the genes detected in the dataset were significantly differentially expressed relative to control at some point (Chapter 2, Figure 2B). This was consistent with other transcriptome datasets that have also reported several thousands of genes being differentially expressed upon submergence (van Veen et al., 2016; Meng et al., 2020). The mRNAseq dataset generated here revealed the induction or repression of several signaling pathways, but in many cases differences were mostly in the strength of this response between old and young leaves during the submergence and post-submergence phases. The induction of defense-related processes upon submergence, for example, has previously been described in Arabidopsis (Hsu et al., 2013). Here we found that these processes were induced stronger in young leaves than in old leaves (Chapter 2, Figure 4). Defense responses are often described to vary with leaf age (Hu and Yang, 2019). It could be that there is variation with leaf age in how a submergence treatment affects the immune response of a plant. Two other processes of note that were induced in an age-dependent manner were the stronger induction in old leaves of senescence-associated processes during submergence and dehydration-associated processes during recovery (Chapter 2, Figures 4 & 7).

Post-submergence water loss is age-dependent

A stronger induction of dehydration-associated genes during post-submergence recovery in old leaves (Chapter 2) was consistent with greater proportion of old leaves desiccating during post-submergence recovery compared to young leaves (Chapter 3, Figure 1). The stomata in old leaves were found to have a lower ABA-responsiveness after a submergence treatment (Chapter 3, Figure 7A). This reduced response to ABA was not reflected in the transcriptional response of guard cell-specific genes, as these had a stronger induction during submergence and recovery in old leaves (Chapter 3, Figure 7B). This suggests that there could be unidentified regulators mediating the inhibition of stomatal closure in old leaves despite their active transcriptional response to ABA. A good candidate for ABA-mediated stomatal closure is the protein phosphatase 2C SAG113. The expression of *SAG113* increases gradually with leaf age (Zhang and Gan, 2012) and its induction during the post-submergence phase in the flooding-sensitive Arabidopsis accession Bay-0 requires both ABA and ethylene signaling (Yeung et al., 2018). Elevated *SAG113* expression has been shown to contribute to increased water loss in the post-submergence phase (Yeung et al., 2018), and it could also be responsible for the dehydration phenotype observed in old leaves. To test this, the water loss of old and young leaves of *sag113* mutants during the post-submergence phase could be compared to that of wild-type Arabidopsis plants. If the age-dependent dehydration phenotype of wild-type plants is lost in *sag113* mutants, this fits with the proposed role of *SAG113* in controlling age-dependent dehydration in the post-submergence phase.

Ethylene signaling controls age-dependent flooding resilience

Ethylene controls several aspects of beneficial plant responses to flooding stress, like metabolic adaptations (Hartman et al., 2021) and shoot and petiole elongation (Sasidharan and Voesenek, 2015). However, ethylene accumulation during flooding can also induce leaf senescence (Ella et al., 2003). The age-dependent induction of senescence-associated genes is often seen during abiotic stresses (Rankenberg et al., 2021). Chapter 4 describes how the age-dependent induction of senescence-associated genes is controlled by ethylene-mediated induction of the transcription factor *ORE1*. Ethylene-insensitive plants no longer showed the pattern of age-dependent senescence that was seen in wild-type plants (Chapter 4, Figure 1). The phenotype of delayed leaf death in old leaves of ethylene-insensitive plants could be explained by the reduced induction of ORE1-mediated senescence (Chapter 4, Figures 3-6). However, this does not explain the increased leaf death of young leaves of ethylene-insensitive mutants. This could be caused by the role of ethylene in ameliorating ROS damage upon reoxygenation (Liu et al., 2022). Whereas ethylene-mediated senescence leads to the death of old leaves, it could be that other age-dependent effects of ethylene led to the survival of young leaves. Knocking out *ORE1* reduced ethylene-mediated senescence, but *ore1* mutants still exhibited age-dependent leaf death (Chapter 4, Figures 4A & S1A; Chapter 5, Figure 5H). This suggests that ethylene signaling had other age-dependent effects beyond inducing ORE1-mediated senescence in old leaves. The loss of the age-dependent gradient in leaf death during submergence in ethylene-insensitive mutants also suggests that ethylene signaling was essential to establish this gradient.

Another potential role of ethylene signaling in age-dependent stress resilience is in controlling the effect of ABA on stomatal closure. ABA signaling in young leaves induces stomatal closure, but inhibits it in old leaves via *SAG113* (Zhang et al., 2012). The induction of *SAG113* in the post-submergence phase requires both ethylene and ABA signaling, as inhibition of either of these hormones reduced *SAG113* expression in the Arabidopsis accession Bay-0 (Yeung et al., 2018). Although *SAG113* plays an important role in promoting water loss, potentially in an age-dependent manner, *sag113* mutants still showed a visual gradient with leaf age in flooding resilience (Yeung et al., 2018). If *SAG113* controls water loss of old leaves, this makes it similar to the role of *ORE1* in leaf senescence: loss of function abolishes their effects in old leaves but the gradient with leaf age is still present in both *ore1* and *sag113* mutants. Senescence and dehydration are just two processes important for flooding resilience. Comparing the age-dependent transcriptomes of wild-type and ethylene-insensitive mutants could highlight which other signaling pathways play a role in flooding-induced sequential leaf death. As leaf age-dependent stress resilience is also described for other stresses beyond flooding (Rankenberg et al., 2021), investigating whether ethylene also plays an essential role here is a promising avenue for future research.

EIN3 – a principal transcription factor mediating ethylene signaling, was stabilized during submergence in both old and young leaves, and the expression of its targets was also induced age-independently (Chapter 4, Figure 1C; Chapter 5, Figure 1E). However, there were still several EIN3 targets with an age-dependent induction under submerged conditions. The genes induced by EIN3 vary depending on environmental conditions (Harkey et al., 2019; Binder, 2020), and it could be that leaf age plays a role here. A deeper analysis of these genes could shed light on how ethylene signaling establishes a gradient with leaf age in flooding tolerance. Age-dependent specificity in the induction of EIN3 targets could be controlled by a dimerization partner, or by chromatin accessibility, both of which affect the activity of EIN3 (Liu et al., 2017; Zemlyanskaya et al., 2017).

Age-specific activity of ORE1 is controlled by ethylene-induced phosphorylation

Although ethylene accumulation and subsequent ORE1 increase were both systemic (Chapter 4), the activity of ORE1 was limited to the old leaves (Chapter 5). This age-dependent activity, leading to age-dependent senescence, was controlled by ORE1 phosphorylation in old leaves (Chapter 5, Figure 3). Ethylene treatment in darkness was sufficient to mimic the effect of a submergence treatment on both ORE1 protein levels and ORE1 phosphorylation (Chapter 5, Figure 4). The age-dependent phosphorylation of ORE1 after ethylene treatment could be an effect of the ethylene treatment, but it could also be that the kinase responsible for ORE1 phosphorylation is already active in old leaves under control conditions and that ethylene-induced ORE1 accumulation merely presents it with a substrate. The role of ethylene signaling in inducing ORE1 phosphorylation could be assayed by using a line expressing *ORE1* under a synthetic estradiol-inducible promoter (Matallana-Ramirez et al., 2013; Gao et al., 2018). If ORE1 is specifically phosphorylated in old leaves upon its induction by estradiol, this would suggest that ethylene merely controls the accumulation of ORE1, and that its age-dependent phosphorylation is regulated by other processes.

There are several possible functions of the premature production of ORE1 protein in young leaves, which is kept inactive for longer than ORE1 in old leaves. Having ORE1 already present in young leaves before it is active could facilitate the later onset of senescence, as young leaves also start to senesce after prolonged submergence treatments (Chapter 2, Figure 1A). Overall protein biosynthesis is strongly reduced during submergence stress (Cho et al., 2022; Branco-Price et al., 2005), and only needing to phosphorylate already-existing ORE1 proteins is an efficient way to induce the transcription of senescence-associated genes once it is necessary. Another mechanism in which unphosphorylated ORE1 could play a role is by competing with phosphorylated ORE1 proteins or other NAC domain transcription factors with similar binding motifs. Senescence of a leaf is a gradual process, chloroplasts are broken down first and nuclei are broken down last (Woo et al., 2016). The requirement of phosphorylation for the transactivation activity of ORE1 allows a plant to first build up a pool of ORE1 proteins, and then gradually increase their activity by increasing the proportion of phosphorylated ORE1. As the ratio of phosphorylated to unphosphorylated ORE1 proteins increases, the transcription of ORE1 targets will gradually increase. The presence of inactive ORE1 at the promoters of senescence-associated genes could potentially prevent the binding of other senescence-regulating NAC domain transcription factors, as there some similarity between the binding sites of these transcription factors (Lindemose et al., 2014; Chun et al., 2022). ORE1 can be phosphorylated up to six times in a stretch of 17 amino acids (Durian et al., 2020). The exact amino acid residues that are phosphorylated have not been identified, and it is unclear what the function of each individual phosphorylation is. There are examples of multi-site phosphorylation contributing to the gradual activation of a transcription factor (Mylona et al., 2016). It could be that there is both a gradual activation of ORE1 by more residues on individual proteins being phosphorylated, as well as by more ORE1 proteins having phosphorylated residues.

ORE1 has been shown to be phosphorylated by CPK1 (Durian et al., 2020). We did not observe any differences between *cpk1* mutants and wild-type plants in ethylene- or submergence-induced senescence (Chapter 5, Figure 5). This suggests that there are other kinases that can phosphorylate ORE1. Several kinases have been implicated in leaf senescence in Arabidopsis (Zhou et al., 2009; Matsuoka et al., 2015). Since the regulation of protein kinases upon environmental changes often occurs at the posttranslational level, identifying which kinase controls the age-dependent phosphorylation of ORE1 can be complicated (Simeunovic et al., 2016; Vu et al., 2018).

The importance of posttranslational modifications in regulating the age-dependent activity of transcription factors is not only highlighted by the results on ORE1, but also by the expression of the targets of ABF transcription factors (Chapter 3). Whereas the expression of *ABF* transcription factors themselves showed little variation between old and young leaves and few changes during the post-submergence phase, (Chapter 3, Figure S1), their targets showed a strong age-dependent induction in this phase (Chapter 3, Figure 3). Signaling cascades involving multiple posttranslational modification events, like those of ABA and ethylene signaling, often do no manifest themselves in transcriptomic datasets by changes in expression of the upstream regulators and receptors, but rather by changes in expression of downstream target genes. Determining the expression of these downstream target genes can be more informative in identifying which pathways are activated than simply assaying the transcript levels of the upstream regulators.

Plants often induce premature leaf senescence under adverse environmental conditions (Sade et al., 2018). The breakdown of old leaves allows them to funnel nutrients and energy towards growing tissues and fuel their survival (Yu et al., 2015). Although stress-induced senescence is a commonly observed phenomenon, genetically reducing its severity or the timing of its onset has been shown to improve the performance of multiple crop species (Gregersen et al., 2013). This leaves the question of why plants go into senescence during stress periods, when it negatively affects their performance. One potential explanation for this is that responses that are evolutionarily favorable are not necessarily the same responses that would be classified as beneficial for stress resilience. The premature sacrifice of some tissues could improve the chance of survival of the whole plant, but if this comes at the expense of agriculturally relevant parts it is not desirable from a human perspective. In such instances it might be more attractive to have a plant that uses an all-or-nothing strategy where the plant remains in metabolic quiescence for as long as possible until ultimately the entire plant collapses.

The role of ORE1-mediated senescence in other species and other stresses

The induction of leaf senescence upon submergence stress has been observed in many species, across both monocots and dicots (Alpuerto et al., 2016; Krishnan et al., 1999; Yeung et al., 2018; Campbell et al., 2015; Buraschi et al., 2020). Furthermore, the role of NAC domain transcription factors in inducing leaf senescence is also highly conserved across plant species (Podzimska-Sroka et al., 2015). It could be that the mechanism of age-dependent senescence via phosphorylation-mediated activation of NAC domain transcription factors exists in other species beyond Arabidopsis. The NAC domain proteins in rice with the highest similarity to Arabidopsis ORE1 are OsNAC1 and OsNAC2 (Kang et al., 2019). Like ORE1, OsNAC2 has a disordered C-terminal region containing multiple serines (encoded by Os04g0460600, UniProt accession Q7XUV6). OsNAC2 induces leaf senescence in rice and binds to the promoters of *OsSGR* and *OsNYC3*, two genes involved in chlorophyll catabolism (Mao et al., 2017). The Arabidopsis homologs of these genes are directly induced by ORE1 (Qiu et al., 2015). Rice NAC domain transcription factors with high similarity to ORE1, like OsNAC2, would be prime targets to explore the regulation of senescence during flooding in rice

Leaf senescence is not only induced by flooding stress, but also by a wide range of other stresses (Sade et al., 2018). ORE1 has been shown to be involved in senescence in response to salt stress, darkness, high temperature, and pathogen infection (Balazadeh et al., 2010; Sakuraba et al., 2014; Kim et al., 2020; Zhang et al., 2021). It would be interesting to see if ORE1 is also activated via phosphorylation in an age-dependent manner in response to these stresses or if this is specific to flooding stress.

Mechanisms increasing flooding resilience of young leaves

The experimental approach in this thesis has often focused on the specific responses of an old leaf: for example why a submergence treatment induces its senescence or renders its stomata less sensitive to ABA. Another approach would be to focus on why these changes do not happen in young leaves. Mechanisms to prevent the onset of specific responses in young leaves could be complementary to those that ensure their start in old leaves, adding another layer of regulation. A lack of a response can be triggered by an insensitivity to external signals that induce that response, but can also be caused by the activation of a repressor. The latter is the case in the mechanism of *SUB1A*-mediated repression of shoot elongation, where the induction of *SUB1A* upon submergence leads to a state of quiescence. The lack of detrimental physiological changes in a young during flooding stress can in part be attributed to pre-existing differences from an old leaf. For example, the greater specific leaf

area of an old leaf likely makes it more susceptible to dehydration. Age-dependent stress responses could also be prevented using genetic approaches. For example, a potential mechanism in preventing the premature induction of senescence in young leaves is via BALANCE OF CHLOROPHYLL METABOLISM1 (BCM1) and BCM2. These two proteins have recently been found to inhibit chlorophyll catabolism by reducing the stability of NYE1 while increasing the activity of the chlorophyll biosynthesis enzyme GENOMES UNCOUPLED4 (GUN4) in both Arabidopsis and lettuce (Wang et al., 2020; Yamatani et al., 2022). An age-dependent dimension to mechanisms like this one could prevent the premature onset of senescence in young leaves.

Conclusion

The resilience of a plant to environmental stresses is the result of its developmental state, its response to changes in the environment, and the interaction between those two. To complicate things, plants never stay in a single, static developmental state, but are a combination of different organs all undergoing developmental progression. For a leaf, such developmental changes are linked to distinct physical and biochemical properties like leaf shape or antioxidant concentration, that affect its stress resilience. These properties can be difficult to change from a breeding perspective, as they are also present in non-stressed plants. Improving environmental resilience is a major target of current crop breeding programs. These have largely targeted generic stress resilience mechanisms. For example, in the case of *SUB1A* rice, introgression of a gene from flooding-tolerant rice enhanced the tolerance of commercial varieties. However, age dependent resilience mechanisms might also offer novel tolerance traits. For example, results from this thesis demonstrate how manipulation of senescence regulators that are more active in old leaves can boost tolerance. Similar approaches could be made for other age-dependent responses to flooding stress. As flooding is a compound stress, there are likely many ways to improve flooding stress resilience.

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Layman's summary

Plants form new leaves throughout their development, and above-ground parts thus consist of leaves of different ages. There can be considerable variation between these leaves in their stress resilience, often decreasing with age. This is also the case for flooded plants. Flooding events are increasing in frequency due to climate change, making it a frequently encountered abiotic stress for plants. Most terrestrial plants are sensitive to flooding.

Photosynthesis is severely hampered during a flooding event due to limitations in light availability and gas exchange. The reduction in gas exchange also leads to a decrease in oxygen within the plant. Oxygen limitation restricts a plant's efficiency in converting existing carbon reserves into energy. The resulting energy shortage can lead to the death of a plant during prolonged flooding. The subsiding of floodwaters does not mark the end of the stressful period for a plant. Following desubmergence, plants are suddenly exposed to air and oxygen, causing the formation of harmful reactive oxygen species. Additionally, damaged roots are often unable to compensate for water lost through the leaves, leading to rapid dehydration even though the soil is still wet.

The goal of this thesis was to determine the role of leaf age in resilience to flooding stress. This knowledge can help in developing new crop varieties that are more flooding-resilient, by potentially selecting for the best aspects of both old and young leaves. The research described here was done using the model organism Arabidopsis thaliana (thale cress).

Chapter 2 describes how old Arabidopsis leaves are less flooding-tolerant than young leaves. The transcriptome of old and young leaves before, during, and after a flooding event was mapped to determine which molecular processes contribute to observed age-dependent differences. Genes involved in chlorophyll breakdown were induced faster in old leaves, consistent with their faster yellowing during flooding. Old leaves also showed a stronger induction of genes involved in the response to water loss during recovery from flooding.

The greater water loss of old leaves during recovery from flooding was further investigated in Chapter 3. During this phase, old leaves showed a stronger induction of genes associated with the hormone abscisic acid (ABA). ABA typically induces stomatal closure during drought. Despite the strong induction of these genes during flooding recovery, the stomata of old leaves were less responsive to ABA after a flooding event. This loss of ABA sensitivity in old leaves might be caused by an interaction between the responses to ABA and to ethylene, another plant hormone.

Flooding speeds up the leaf aging, this effect being stronger in old leaves than in young leaves. This is visible in the yellowing of leaves caused by chlorophyll degradation, but also involves the breakdown of other macromolecules like proteins and RNA. Chapter 4 describes how this process is controlled by ethylene. As it is a gas, ethylene cannot escape from a flooded plant and therefore rapidly accumulates. Ethylene signaling is known to help a plant survive flooding events via several mechanisms, but it can also induce leaf aging. The leaf-age dependent death of leaves during flooding was lost in ethylene-insensitive plants. Upon ethylene exposure plants induced the transcription of ORESARA1 (ORE1), which is an important regulator of leaf aging. The rapid leaf aging induced by flooding was reduced in ore1 mutants, correlating with increased flooding tolerance. Although the effect of losing ORE1 function was mostly visible in the old leaves, ORE1 protein was found to accumulate in both old and young leaves during flooding stress.

Chapter 5 describes how ORE1 accumulation of in all leaves only led to an activation of genes related to aging in old leaves. Analysis of the transcriptomes of wild-type and ore1 mutants showed that the loss of ORE1 had a much greater effect in old leaves than in young leaves. Many genes involved in aging were only induced in old leaves of wild-type plants but not in ore1 mutants or in young leaves. We found that the age-dependent activity of ORE1 required a small region of the protein in which it can be phosphorylated. ORE1 was also found to be phosphorylated to a greater extent in old leaves than in young leaves. A brief treatment with ethylene in darkness already sufficed to induce the age-dependent phosphorylation of ORE1, which suggests that ethylene is the main signal during a flooding event that leads to the activation of ORE1.

The results described here show how several processes in a plant are regulated in an age-dependent manner during a flooding event. These differences ultimately lead to variation with leaf age in flooding resilience.

Samenvatting

Omdat planten tijdens hun ontwikkeling continu nieuwe bladeren maken, bestaan hun bovengrondse delen uit een mix van zowel oudere als jongere bladeren. Tussen deze bladeren is er een grote variatie in hun stressbestendigheid, die vaak afneemt als een blad ouder wordt. Hier is ook sprake van tijdens overstromingsstress. Overstromingen komen steeds vaker voor als gevolg van klimaatverandering, en kunnen leiden tot grote oogstverliezen.

Tijdens een overstroming is de fotosyntheses van een plant gereduceerd door een gebrek aan licht en een sterk verminderde gaswisseling. De gereduceerde gaswisseling leidt ook tot een tekort aan zuurstof in de plant. Dit zuurstoftekort zorgt ervoor dat de plant zijn bestaande koolstofreserves minder efficiënt kan omzetten in energie. Uiteindelijk leidt dit energietekort tot de dood van de plant tijdens een langere overstroming. Als waterniveaus dalen en een overstroming is afgelopen, is dit niet het einde van de stress voor een plant. Tijdens het herstel van een overstroming worden planten plotseling weer blootgesteld aan licht en zuurstof, wat leidt tot de productie van zuurstofradicalen. Hiernaast zijn de beschadigde wortels na een overstroming niet langer in staat om voldoende water op te nemen om te compenseren voor de verdamping van water uit de bladeren. Dit leidt vaak tot een snelle uitdroging van de bladeren, ondanks dat er voldoende water in de grond aanwezig is.

Het doel van dit proefschrift was om te bepalen hoe de leeftijd van een blad van invloed is op zijn overstromingstolerantie. Deze informatie kan helpen in het ontwikkelen van nieuwe gewasvariëteiten die stressbestendiger zijn, door de sterke kanten van zowel oude als jonge bladeren te combineren. In dit onderzoek is het modelorganisme *Arabidopsis thaliana* (zandraket) gebruikt.

Hoofdstuk 2 beschrijft dat oude bladeren van *Arabidopsis* minder overstromingstolerant zijn dan jonge bladeren. Het transcriptoom van oude en jonge bladeren voor, tijdens, en na een overstroming is in kaart gebracht om te bepalen welke moleculaire processen bijdragen aan het verschil tussen deze bladeren. We vonden dat genen betrokken bij de afbraak van chlorofyl sneller werden geactiveerd in oude bladeren dan jonge bladeren, wat overeenkomt met de snellere vergeling in oude bladeren. Hiernaast vertoonden oude bladeren een sterkere inductie van genen betrokken bij uitdroging tijdens het herstel van een overstroming.

In **Hoofdstuk 3** wordt het grotere waterverlies in oude ten opzichte van jonge bladeren tijdens het herstel van een overstroming onderzocht. Oude bladeren vertoonden in deze fase een sterkere inductie van genen die geassocieerd worden met het hormoon abscisinezuur (ABA) dat normaal gesproken de sluiting van huidmondjes induceert om waterverlies te voorkomen. Ondanks de sterkere inductie van deze genen bleek dat de huidmondjes van oude bladeren na een overstroming minder gevoelig waren voor ABA dan die van jonge bladeren. Dit verlies van ABA-gevoeligheid van de stomata van oude bladeren komt mogelijk door een interactie tussen de reacties op ABA en die op ethyleen, een ander plantenhormoon.

Een overstroming versnelt het verouderingsproces van bladeren sneller in oude bladeren dan in jonge bladeren. Dit is goed zichtbaar in de snellere afbraak van chlorofyl, maar omvat ook de afbraak van andere macromoleculen zoals eiwitten en RNA. **Hoofdstuk 4** beschrijft hoe dit proces wordt gereguleerd door ethyleen. Tijdens een overstroming kan ethyleen niet meer ontsnappen uit een plant en hoopt het snel op. Ethyleen induceert verschillende reacties die helpen bij het overleven van een overstroming, maar het kan ook de veroudering van bladeren versnellen. In ethyleen-ongevoelige planten was de leeftijdsafhankelijke gradiënt waarin dit gebeurt verloren, en stierven oude en jonge bladeren ongeveer even snel. Ethyleen induceerde de transcriptie van *ORESARA1* (*ORE1*), een transcriptiefactor die een belangrijke regulator van veroudering is. In *ore1* mutanten bleven oude bladeren langer groen dan in wild-type planten, dit zorgde uiteindelijk voor een beter herstel van overstromingsstress. Ondanks dat het effect van de *ore1* mutatie voornamelijk zichtbaar was in de oude bladeren, bleek dat ORE1 eiwit accumuleerde in zowel de oude als de jonge bladeren tijdens een overstroming.

Hoofdstuk 5 beschrijft hoe de systemische accumulatie van ORE1 alleen leidt tot de activatie van verouderingsgenen in oude bladeren. Uit analyses van de transcriptomen van wild-type en *ore1* mutanten tijdens een overstroming bleek dat het effect van het verlies van *ORE1* veel groter was in oude bladeren dan in jonge bladeren. Veel genen betrokken bij veroudering werden alleen geïnduceerd in oude bladeren van wild-type planten maar niet in *ore1* mutanten of in jonge bladeren. De activiteit van ORE1 bleek afhankelijk te zijn van een kleine regio van het eiwit waarin het gefosforyleerd kan worden. Bovendien werd ORE1 voornamelijk gefosforyleerd in oude bladeren en minder snel in jonge bladeren. Een korte behandeling met ethyleen in het donker was al voldoende om de leeftijdsafhankelijke fosforylatie van ORE1 te induceren, wat er op wijst dat tijdens een overstroming ethyleen het signaal is dat leidt tot de activatie van ORE1.

De hier beschreven resultaten laten zien hoe meerdere processen tijdens een overstroming op een manier worden gereguleerd die afhankelijk is van de leeftijd van een blad. Deze verschillen zorgen uiteindelijk voor een leeftijdsafhankelijke stresstolerantie van bladeren.

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Curriculum Vitae

Tom Rankenberg was born on February 12th in 1994, in Wageningen. After obtaining his gymnasium diploma from RSG Pantarijn he moved to Utrecht University to study Biology. In 2015 he finished his bachelor's degree and started the Environmental Biology MSc program, also at Utrecht University. During this program Tom did a nine-month internship in the Molecular Plant Physiology group at UU under the supervision of Dr. Marcel Proveniers and Dr. Savani Silva, studying the mechanisms of shoot elongation in Arabidopsis thaliana. In addition to this project, he did a six-month internship at the University of Copenhagen in Denmark. Here, he studied the transcription of the chloroplast genome, supervised by Dr. Sebastian Marquardt and Dr. Peter Kindgren. After completing his MSc studies in December 2017, Tom started his PhD at Utrecht University in January 2018. In this project, funded by NWO and supervised by Prof. Rens Voesenek and Prof. Rashmi Sasidharan, the mechanisms controlling the variation with leaf age in flooding resilience were explored. The results of this research are presented in this PhD thesis.

List of publications

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