Molecular mechanisms mediating contrasting flooding survival strategies in two *Rumex* species

Moleculaire mechanismes verantwoordelijk voor contrasterende overlevingsstrategieën tijdens overstroming in twee *Rumex* soorten
(*met een samenvatting in het Nederlands*)

Proefschrift

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Chapter 1

General introduction

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Growth strategies determine plant survival and the occupied niche in flood-prone environments

Terrestrial plant species face many challenges in an underwater environment. Primarily, the severely reduced gas exchange in the aqueous surroundings, ~10,000 times slower compared to air, can lead to a dramatic oxygen (O_2) shortage in mainly the belowground tissues. In the aboveground tissues, photosynthesis is severely reduced due to the poor exchange of carbon dioxide (CO_2) with the environment (Mommer and Visser 2005) and the low levels of light in turbid floodwaters (Vervuren et al., 2003). In the absence of O_2, mitochondrial respiration is arrested. Therefore, plants have to solely rely on glycolysis for energy production, leading to a mere 2 to 4 ATP produced per molecule hexose. It is essential that fermentation pathways are activated to regenerate NAD^+ to sustain the energy production through glycolysis (Bailey-Serres and Voesenek, 2008).

Interestingly, a large range of wild plant species experience partial to complete submergence during some stage of their life cycle. Indeed, a shift from an exclusive terrestrial to an amphibious lifestyle is estimated to have occurred more than 200 times throughout the diversification of angiosperms (Cook, 1999). A common response to submergence is the formation of new “aquatic” leaves, which have, compared to leaves that developed in aerial conditions, an increased specific leaf area (SLA), thinner epidermal cell walls and increased chloroplast orientation towards the leaf surface. These traits minimize the diffusion distance and thus resistance between the chloroplast and the environment and thereby significantly improve gas exchange underwater. This leaf plasticity is a widespread response that somewhat alleviates adverse underwater conditions (Sand-Jensen and Frost-Christensen 1999; Mommer and Visser, 2005; Mommer et al., 2007). Apart from this general response, two successful survival strategies to cope with complete submergence are quiescence and escape (Bailey-Serres and Voesenek, 2008).

The quiescence strategy aims to reduce energy expenditure by ceasing costly, less essential processes. Furthermore, growth and development of new tissues are reduced. In this way quiescent plants minimize oxygen consumption and preserve valuable carbohydrates for re-growth once the water level recedes. Indeed, species that successfully achieve such a quiescent state can survive prolonged periods of flooding and associated hypoxia, like for instance *Phalaris arundinacea* which has been reported to survive complete submergence for more than 100 days (Mommer et al., 2006a) and *Acorus calamus* where both the rhizomes and leaves are remarkably anoxia tolerant (Schlüter and Crawford, 2001). Furthermore, rice cultivars lacking underwater growth have significantly improved survival

during complete submergence compared to varieties that continue underwater elongation growth (Setter and Laureles, 1996; Ram et al., 2002).

Plants employing an escape strategy upon submergence show a rapid upward elongation of shoot tissue towards the water surface. The subsequently re-established air contact allows for resumption of aerial photosynthesis and re-aeration of underwater organs via aerenchyma tissue (Bailey-Serres and Voesenek, 2008).

Major costs are associated with the shoot elongation response. This was neatly demonstrated in quiescent and escaping rice varieties by pharmacological manipulation of hormone levels leading to the artificial activation or prevention of elongation growth (Setter and Laureles, 1996). Indeed, quiescent plants in which elongation was stimulated had an increased mortality when kept completely submerged. Similarly, the escaping plants in which elongation was inhibited had an increased survival when kept under water.

However, when elongation leads to successful emergence it greatly improves plant survival and reduces biomass loss, as has been demonstrated for *Rorippa amphibia* (Akman et al., 2012) and *Rumex palustris* (Pierik et al., 2009). Interestingly, establishment of air contact is by itself not always enough to improve plant performance. When *Rumex palustris*, which employs an escape strategy, regains aerial contact there is a significant increase in biomass. In contrast, when the close relative *Rumex acetosa,* which lacks the escape response, was artificially allowed to regain air contact, no such increase in biomass was observed. A key difference between both species is the petiole porosity, which is more than double in *R. palustris*, indicating that improved aeration is indeed a crucial aspect of the escape strategy (Pierik et al., 2009).

The nature of flooding events can differ dramatically, depending on the water supply, site elevation, water runoff and the soil drainage capabilities. This variation in flooding characteristics is the basis for a large amount of plant diversity in flood-prone areas (Silvertown et al., 1999). Indeed, a strong relationship exists between the flooding tolerance of a species and the frequency of flooding events (Van Eck et al., 2004). Given that the escape strategy is only beneficial if aerial contact can be made, one would expect this trait to be most prevalent on sites experiencing prolonged and relatively shallow floods. On the other hand, the quiescent strategy would be expected on sites subjected to deep or very short floods. Indeed, a study of the species distribution and their growth responses in the river Rhine floodplains demonstrated this relationship (Voesenek et al., 2004). Thus fine-tuning of the adaptation strategy to the environment is crucial for plant survival. In order to understand the development and evolution of the adaptive flooding survival strategies, it is essential to understand the underlying molecular and physiological mechanisms. Here we describe the current knowledge base related to the quiescence and escape strategy and introduce the experimental system and topics we address in the experimental chapters of this thesis.

Growth management: balancing energy generation and consumption

A consequence of adopting the quiescence strategy is that the plant still suffers from reduced photosynthesis and reduced oxygen levels. The belowground tissues rapidly

experience hypoxia upon flooding, with high levels of fermentation. The aboveground tissues usually retain oxygen levels ranging from 3 to 22 % depending on plant morphology, photosynthetic capacities and light conditions (Mommer et al., 2007). It is therefore essential for plants that adopt the quiescence strategy to keep energy production and consumption to a minimum to conserve valuable carbohydrates. In contrast, escaping plants require rapid mobilization of reserves to fuel expensive elongation growth (Bailey-Serres and Voesenek, 2008).

The reduced photosynthesis observed underwater means that most species will depend on reserve carbohydrates to supply energy for cellular maintenance. Indeed, amylases, glycolytic and other catabolic enzymes are invariably activated in various plant species upon exposure to flooding and low oxygen stress (Umeda and Uchimiya, 1994; Arpagaus and Braendle, 2000; Harada and Ishizawa, 2003; Mustroph and Albrecht, 2003). Interestingly, rice cultivars varying in the extent of underwater elongation showed a strong correlation between carbohydrate consumption and elongation growth (Das et al., 2005; Fukao et al., 2006). Furthermore, a mutant rice line that is unable to activate alpha-amylase failed to elongate underwater (Lee et al., 2009). Similarly, *Rumex palustris* lost its ability to elongate underwater when depleted of starch (Groeneveld and Voesenek, 2003). These studies experimentally demonstrate the importance of reserve mobilization in fuelling elongation. Indeed, *Potamogeton pectinatus*, that shows an escape response under anoxic conditions, display a characteristic Pasteur effect (Summers et al., 2000). Here the halt of respiratory activity restricts energy production to solely the glycolytic pathway coupled with fermentation, where the lower ATP yield per molecule hexose is compensated for by increasing the rate of glycolysis to fuel elongation growth. Glycolysis, coupled with ethanol fermentation has proven surprisingly efficient in generating energy for elongation, as a study on pondweed, arrowhead and rice shows that the adenylate energy status, during anoxic elongation, remains unchanged for rice and is even improved in pondweed and arrowhead (Ishizawa et al., 1999). Furthermore, a strong correlation between ethanol production and elongation is observed during the anoxic germination of coleoptiles from various rice cultivars (Magneschi et al. 2009a).

While for escaping plants the adverse conditions are only of temporary nature until aerial contact is made, during a quiescent strategy adverse conditions are not ameliorated. In this case, accessing reserve carbohydrates and energy production should preferably be kept to a minimum. The extent of reduced energy production in quiescent plants has not been studied in great detail. Nevertheless, long-term anoxia in rice coleoptiles is found to decrease the fermentation rate despite sufficient substrate availability without adverse effects on survival (Colmer et al. 2001; Zhang and Greenway 1994). It has also been proposed that plants can lower their respiration upon reduced oxygen availability, despite oxygen levels not dropping below the critical oxygen pressure for mitochondrial cytochrome C oxidase (Geigenberger 2003; Gupta et al. 2009; Zabalza et al. 2008), though oxygen dependent regulation of respiration is challenged by a mathematical model (Armstrong and Beckett, 2011). Downregulating respiration would lead to higher steady state oxygen levels, reduced carbon consumption and subsequently longer lasting carbon reserves. Overall this does suggest a possibility for delicate control of metabolism.

Where quiescent species could reduce energy expenditure to a bare minimum, escaping species do need to invest in elongation growth with the associated costs. A comparison of rice cultivars varying in elongation showed a strong correlation between anoxia-induced escape, ATP production and rate of protein synthesis (Edwards et al. 2012). Similarly, increased protein biosynthesis rates were also observed during the anoxia-induced escape response in *Sagittaria pygmaea*, arrowhead (Ishizawa et al., 1999). However, in maize seedling root tips, with reduced elongation, new protein biosynthesis rates were very low (Chang et al., 2000). Furthermore, in the model plant *Arabidopsis*, which lacks hypoxia-induced escape responses, there was a selective and significant repression (70%) of mRNA translation (Sachs et al., 1980; Branco-Price et al. 2008). Thus the repression of protein synthesis observed in maize and *Arabidopsis* suggests a major energy saving potential upon stressful conditions, whilst the continued protein synthesis in rice and arrowhead, despite being energetically expensive, is essential for sustaining the escape response.

Studies on *Arabidopsis* have identified a protein kinase complex, termed SnRK1, that plays a key role in regulating the transcriptome of more than a 1000 genes involved in metabolism (Baena-González et al., 2007). SnRK1 is activated upon low energy conditions, extended darkness and hypoxia, and alters cellular priorities by reducing biosynthesis and increasing catabolism. Recently, it was shown that Trehalose-6-Phosphate (T6P), an intermediate in the pathway of trehalose metabolism, could inhibit SnRK1 activity in *Arabidopsis* (Zhang et al., 2009). An across kingdom study on hypoxic trancriptomic responses revealed that the activation of T6P phosphatase, which breaks down T6P, is common amongst the plant kingdom. This could ultimately lead to reduced T6P and increased SnRK1 activity (Mustroph et al., 2010). With respect to the growth strategies, quiescent rice was also found to induce T6P phosphatase, whereas in the elongating variety this regulation was absent (Jung et al., 2010). Metabolic regulation via T6P could be an essential mechanism to match the energy production with the requirements of either quiescence or escape. Evidence is increasing for a strong energy and carbon management in submergence survival strategies, and these are different for escaping and quiescent plants.

Cell expansion and division as mechanisms of elongation

In *Rumex palustris* underwater shoot elongation occurs primarily via rapid elongation of mainly younger leaf petioles. In existing petioles it is exclusively mediated by cell elongation and occurs throughout the entire length of this organ (Voesenek et al., 1990). On the other hand, the underwater shoot elongation in rice occurs in the stem internodal regions which can be divided into three zones (Bleecker et al., 1986). In the basal zone of the internode both cell division and elongation occur. Submergence mainly induces growth in this segment (Kutschera and Kende, 1988) where division is stimulated independent of the increase in cell size (Lorbiecke and Sauter, 1998). In the second zone cells show an increased elongation, whereas in the next region cells differentiate. Here submergence also delays the rate of differentiation shifting the balance more towards elongation (Bleecker et al., 1986). In *Nymphoides peltata* (fringed waterlily) the balance between division and elongation during

underwater escape is age dependent (Ridge and Amarasinghe, 1984). The mature leaves show predominantly cell elongation of the petiole, whereas in young leaves the balance is shifted more towards cell division.

Cellular expansion

Cell elongation is a biophysical process, where through osmotic driven water uptake an outward driven turgor pressure is applied on cell walls. Depending on its extensibility, the cell wall yields to the turgor pressure facilitating cell growth. Therefore, osmoregulation and cell wall modifications are the main targets of growth control (Cosgrove, 2005; Sasidharan et al. 2011).

The osmolarity of deep-water rice only marginally increases upon submergence, whereas big changes in cell wall extensibility of internodes are observed (Kutschera and Kende 1988). Furthermore, expression of osmoregulatory aquaporins in elongating deepwater rice was dependent on the tissue water status, rather than the hormonal elongation cues (Malz and Sauter, 1999; Ridge and Osborne, 1989). Indeed, several studies support the notion of cell enlargement via the regulation of cell wall extensibility, whereas changes in turgor pressure rarely drive cell expansion (Cosgrove, 2005; Sasidharan et al., 2011).

The plant primary cell wall is composed of cellulose microfibrils that are interconnected by hemicellulose, a xyloglucan polymer, and surrounded by pectin (Sasidharan et al., 2011). Several enzymes can facilitate loosening of the cell wall by targeting the three main components, cellulose, hemicellulose and pectin. Expansins loosen the connections between cellulose microfibrils and hemicellulose (McQueen-Mason and Cosgrove, 1995), whereas XyloglucanEndotransglucosylase/Hydrolases (XTHs) can cleave or rejoin xyloglucan polymers (hemicellulose) (Rose et al., 2002). Pectin methylesterases (PMEs) catalyse the esterification of the pectin polysaccharides and can thereby alter the fluidity of pectin polysaccharide matrix (Micheli, 2001). As most of these enzymes have a low pH optimum, cell wall acidification is an essential aspect of cell wall loosening (Cosgrove, 2005).

Submerged plants adopting an escape strategy strongly upregulate cell wall modifying activity in the rapidly elongating organs. Indeed, upon submergence expansin expression and activity are increased in the elongating petioles of R. palustris (Vreeburg et al., 2005) and internodes of deepwater rice (Cho and Kende, 1997). The non-elongating Rumex acetosa and rice varieties did not show a similar induction upon submergence (Fukao et al., 2006; Vriezen et al., 2000). Interestingly, such a correlation between expansin expression and elongation was absent amongst anoxic coleoptile elongation of several rice varieties (Magneschi et al., 2009b), suggesting alternative modes of regulating cell wall loosening. For instance, a role of XTHs was identified in the underwater elongation of Sagittaria pygmaea (Arrowhead) (Ookawara et al., 2005). PMEs have not yet been associated with the underwater escape response. However, these enzymes are involved in growth regulatory processes upon different environmental cues, and a role in the escape strategy therefore seems likely.

During underwater elongation cell wall acidification, mediated by the proton ATPase, occurs in a wide range of escaping species such as Rumex palustris, Nymphoides peltata

and Potamogeton distinctus (Malone and Ridge, 1983; Vreeburg et al., 2005; Koizumi et al., 2011). Acidification of the apoplastic space is a potent mechanism to activate already present cell wall modifying enzymes allowing a rapid response upon submergence. Interestingly, apoplastic alkalinisation inhibits growth in root tissue (Staal et al. 2011). This inhibition can be relieved by the application of the fungal toxin fusicoccin that activates the proton-ATPase thereby acidifying the cell wall. Regulation of ATPase activity could therefore be instrumental in the regulation of cellular expansion and growth mediating the escape or quiescence strategy.

Cell division

The increased cell division observed in some submerged and elongating species requires proper control of cell cycle progression. However, the role of these processes in mediating underwater elongation has so far received little attention. Nevertheless, some work on deepwater rice has identified a set of genes that are regulated at various cell cycle stages: namely the mitotic (M), gap1 (G1), DNA synthesis (S) and gap2 (G2) phase. Cell cycle progression is regulated by the sequential expression of cyclin dependent kinases (CDKs). CDK activity can be modulated by binding of cyclins and requires phosphorylation by CDK activating kinases (CAKs).

At the basal zone of deepwater rice internodes, where cell division takes place, a strong induction of two cyclins, which mediate G2 to M phase transition, was observed upon gibberellin (GA) treatment (Sauter et al. 1995). Because of its dominant role in mediating escape, GA is often used to mimic flooding as it also induces internode elongation. However, it is not clear if the cyclin induction was the result of GA, or an indirect result of an increasing number of cells in the G2 phase compared to other mitotic states.

DNA synthesis (S phase) is clearly stimulated by submergence and GA, as was demonstrated by the cell cycle independent induction of a CDK gene, cdcOs-2 (Lorbiecke and Sauter, 1998). Additionally, a cyclin involved with G1 to S transition, cycA1;1, was transcriptionally activated (Fabian et al., 2000). Also CAK R2, another cell cycle regulatory component, was transcriptionally induced in a GA dependent manner (Sauter, 1997). R2 accelerates the progression through the S phase, and enhanced growth was observed when R2 was overexpressed in rice cell suspensions (Fabian-Marwedel et al., 2002). To complement the changes in cell cycle progression, factors involved in DNA replication, such as histone H3 and RPA1, show concomitant regulation (van der Knaap and Kende, 1995; van der Knaap et al., 1997).

In conclusion, a strong regulation of cell cycle progression is apparent during elongation of the deepwater rice escape strategy. The increased growth observed upon overexpression of R2 demonstrates the potential role of cell cycle regulation as a growth determinant during the escape strategy. Negative regulation of cell cycle control could be an alternative way of growth inhibition in species adopting a quiescence strategy, although there have so far been no investigations to support this hypothesis.

Interaction of hormones that regulate growth strategies

Upon complete submergence, quiescent and escaping species experience the same initial environmental and physiological cues. However, distinct signalling cascades are observed for different species and strategies that mediate either elongation growth or quiescence.

A range of amphibious plant species show a strong elongation response when supplied with exogenous ethylene. Indeed, this gaseous plant hormone has been suggested to be the driving force of underwater elongation (Ridge, 1987). The balance between biosynthesis and outward diffusion determines the internal ethylene concentration in plants, and both are affected by submergence. Methionine is the precursor of ethylene biosynthesis. After the formation of S-adenosyl-methionine (SAM), it is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS). Finally, ACC is converted to ethylene by ACC oxidase (ACO), in an oxygen requiring reaction (Bleecker and Kende, 2000).

The activity and transcription of both ACS and ACO are upregulated upon submergence in the escaping *Rumex palustris* and rice (Vriezen et al., 1999; van der Straeten et al., 2001; Rieu et al., 2005). Despite increases in gene expression, ethylene production was not increased in *R. palustris* (Banga et al., 1996; Vriezen et al., 1999). However, it is not the enhanced biosynthesis, but the severely reduced gas diffusion that results in the accumulation of extremely high internal levels of ethylene in a variety of different species, ranging from 1 to 12 μl l^{-1} (Métraux and Kende, 1983; Samarakoon and Horton, 1984; Jackson et al., 1987; Voesenek et al., 1993a; Banga et al., 1996; reviewed in Voesenek and Sasidharan, 2013).

Ethylene signalling is further modulated by changes in receptor abundance. Both in rice and *R. palustris* transcription of ethylene receptors is activated upon submergence (Vriezen et al., 1997; Watanabe et al., 2004). Receptors free of ethylene act as strong negative regulators of ethylene signalling, whereas for receptors bound by ethylene the suppression is released. However, with the saturating levels of ethylene typical of submergence the newly formed receptors will be bound by ethylene and not lead to a suppression of ethylene signalling. However, when plants emerge out of the water, the establishment of aerial contact leads to a rapid release of ethylene (Voesenek et al. 1993a) and a consequent dramatic suppression of ethylene signal transduction by the increased amount of receptors, providing a potential mechanism to quickly shut down elongation when aerial contact is made (Jackson, 2007).

Interestingly, both escaping and quiescent species accumulate high levels of ethylene, but respond to it in contrasting ways (Keith et al., 1986; Voesenek et al., 1993; Banga et al., 1996; Pierik et al., 2006). In both deepwater rice and *R. palustris,* ethylene accumulation led to an increase in GA levels, and inhibition of GA biosynthesis hampered elongation (Raskin and Kende, 1984; Benschop et al., 2006). Indeed, cell expansion and cell division in deepwater rice internodes is strongly regulated by GA. Application of GA to quiescent cultivars elicits an elongation response (Setter and Laureles, 1996), which means that quiescent plants are not resource limited and retain the capacity to elongate.

Interestingly, it was shown that underwater elongation in deep water rice is highly dependent on removal of ABA (Hoffmann-Benning and Kende, 1992). Plants treated with ABA were unable to elongate and only upon the addition of extra exogenous GA could an elongation response be restored. Indeed, a rapid drop of ABA was observed upon ethylene treatment. Not surprisingly, the ABA catabolic enzyme, *OsABA8ox1*, was strongly induced upon submergence in deepwater rice (Saika et al., 2006). Despite the importance of ABA catabolism for underwater elongation, a similar drop of ABA levels was also observed in non-elongating rice varieties (Ram et al., 2002; Fukao and Bailey-Serres, 2008; Hattori et al., 2009). This suggests that interactions between ethylene and ABA with GA are of primary importance for differences in the elongation responses of different rice varieties.

Also in *R. palustris* the accumulation of GA is dependent on an initial breakdown of ABA (Benschop et al., 2006). However, upon submergence or ethylene exposure, the non-elongating relative *R. acetosa* does not downregulate ABA levels (Benschop et al., 2005). The importance of internal ABA levels in regulating elongation responses in *Rumex* was further highlighted by a study on *R. palustris* ecotypes with different elongation rates. Ecotypes with the fastest elongation response showed the strongest reduction in ABA levels and concomitant expression of an ABA biosynthesis gene (*RpNCED1*, *9-cis-epoxycarotenoid dioxygenase*), whereas the slow elongating ecotypes had the smallest reduction in ABA levels and *RpNCED1* expression (Chen et al., 2010). In contrast to rice, the interaction of ethylene with ABA is most important in explaining variation between different *Rumex* species and genotypes. Interestingly, in the elongating monocot *Scirpus mucronatus* there was also a clear correlation between the degree of reduction in ABA levels and the extent of underwater elongation (Lee et al., 1996).

Apart from ABA and GA, auxin has been found to play an important role in the underwater elongation in some species. The most convincing work concerns *Ranunculus scleratus* where application of an auxin polar transport inhibitor prevented petiole extension, and where ethylene was found to induce polar auxin transport (Musgrave and Walters, 1973; Rijnders et al., 1996). Interestingly, a role of auxin in *R. palustris* has also been identified. Inhibiting polar auxin transport, chemically or by deblading the lamina, transiently reduced the underwater elongation response (Cox et al., 2006). Correspondingly, petiole auxin levels were highly dependent on the presence of the leaf blade. Furthermore, a rapid accumulation of auxin in the ab- and adaxial fragments of the elongating petioles was observed (Cox et al., 2004).

Despite the wealth of evidence for ethylene-induced elongation growth, in some species elongation growth under water is regulated by different cues. Furthermore, it remains unclear whether ethylene responses can explain the full underwater elongation or quiescent response. For instance, in rice coleoptiles, internodes and arrowhead tubers (*Sagittaria pygmaea*) optimal elongation was achieved by a combination of reduced O_2, and increased CO_2 and ethylene availability rather than an ethylene stimulus by itself (Ishizawa and Esashi, 1984; Métraux and Kende, 1984). Also in *R. palustris* a lowering of the oxygen levels increased ethylene-induced elongation, whereas it had the contrasting effect on the non-elongating *R. acetosa* (Voesenek et al., 1997). Under anoxic conditions rice germinates and elongates independently from ethylene (Alpi and Beevers, 1983). Additionally,

Potamogeton pectinatus showed a strong elongation response under pure anoxic conditions and reduced oxygen levels of 5 to 8 % (Summers and Jackson, 1994).

Clearly, a specific blend of O_2, CO_2 and ethylene is most effective in stimulating elongation, and maybe also in suppressing growth. An increase in ethylene levels to usually saturating levels upon submergence has been clearly established. However, the effects of submergence on the shoot O_2 and CO_2 levels are less robust. The internal levels of these gases are dependent on the balance between photosynthesis and respiration, and diffusive resistances. These vary greatly between flooding conditions such as turbidity, depth and light penetration, and anatomy of the tissue; all factors known to affect underwater plant survival (Ram et al., 2002; Mommer et al., 2006).

Group VII Ethylene Response Factors as regulators of rice flooding survival strategies and *Arabidopsis* hypoxic acclimation

Interestingly all species accumulate high internal levels of ethylene as the result of complete submergence (Voesenek and Sasidharan, 2013). Ethylene accumulation leads to an elongation response for some species whereas others adopt a quiescence strategy (Pierik et al., 2006). The contrasting growth strategies are the result of variation in hormonal interaction between ethylene and other hormones such as ABA, GA and auxin. However, there is an array of underlying molecular processes mediating these different hormonal interactions that vary between floodplain species giving rise to opposite growth responses. A first insight into the underlying molecular mechanism in how one signal leads to two contrasting responses comes from rice. Rice genetics has received much attention because of its agronomical importance. Interestingly, two mapping studies both identified candidates of the same transcription factor gene family that play an important role in mediating escape or quiescence in rice, the group VII Ethylene Response Factors (group VII ERFs) (Voesenek and Bailey-Serres, 2009).

A QTL was identified on chromosome 9 that explained 69% of the variation in submergence tolerance in rice (Xu and Mackill, 1996). The region responsible for this variation is a cluster of three genes, *SUB1A, SUB1B* and *SUB1C*, all encoding *group VII ERFs* that are induced upon submergence. Where *SUB1B* and *SUB1C* are present in all rice cultivars, *SUB1A* is present only in a select number of genotypes. Those with the *SUB1A-1* allele invariably show a high degree of tolerance to submergence (Xu et al., 2006). The introgression of the tolerant *SUB1A* gene cluster (encoding *SUB1A-1*) into an agronomically interesting rice variety revealed many novel functions. The *SUB1A* genotype showed a reduced elongation response underwater, with concomitant reduction of carbohydrate reserve consumption and cell wall loosening enzymes (Fukao et al., 2006). Furthermore, the *SUB1A* genotypes induced two suppressors of GA signalling upon submergence, Slender Rice-1 (SLR1) and SLR1 Like-1 (SLRL1), thereby decreasing the sensitivity to GA (Fukao and Bailey-Serres, 2008). Indeed, Sub1A confers tolerance to flooding by activating a quiescence strategy upon submergence.

Another study looking specifically at elongation responses of deepwater rice identified three other major QTLs, whose combined effect could close to completely restore

underwater elongation in the quiescent parental line (Hattori et al., 2009). The QTL on chromosome 12 encoded two group VII ERFs, *SNORKEL1* and *-2 (SK1, SK2)*. Interestingly, the *SK* genes were found to be present only in genotypes and wild relatives that showed a deepwater elongation response. SK1 and SK2 may act via GA biosynthesis to confer an escape strategy, as this was the main difference between the parental lines.

Thus, members of the same gene family (group VII ERFs) affect the hormone signalling responsible for the underwater growth strategies in opposite ways. Furthermore, the induction of *SK1* transcription is regulated directly downstream of ethylene signalling, demonstrated by binding of an ethylene signalling component (EIL1B) to the *SK* promoter, and a range of hormone treatments. Similarly, *SUB1A* transcriptional activation is regulated by ethylene, and in addition by reactive oxygen species and sucrose (Kudahettige et al., 2010; Fukao et al., 2011). However, *SUB1C* is activated by GA, and thus regulated further downstream of the flooding-induced hormonal signalling network.

Clearly, there is a lot of variation in group VII ERF behaviour among different rice genotypes, which has proven to be essential in adapting to a range of different flooding environments. The exploration of SUB1 loci in a range of wild rice species confirms the flooding tolerance mediated by SUB1A, in close relatives of *O. sativa*. Interestingly, very tolerant wild rice species lack the *SUB1A* locus, but do have *SUB1C* homologs, and therefore have a *SUB1A* independent mechanism conferring tolerance (Niroula et al., 2012).

Naturally, this specific family of transcription factors has sparked interest and detailed studies have also been done on related members in *Arabidopsis*. Here they function primarily in regulating hypoxia-induced expression of genes such *ALCOHOL DEHYDROGENASE* and *PYRUVATE DECARBOXYLASE* (Hinz et al., 2010; Licausi et al., 2010). Most interestingly, in *Arabidopsis* these transcription factors are subject to post-translational regulation depending on oxygen availability. Normal oxygen levels lead to specific N-terminal modifications that target the protein for proteolysis. However, under hypoxia these N-terminal modifications do not occur, thereby stabilizing these transcription factors and permitting the activation of their downstream target genes (Gibbs et al., 2011; Licausi et al., 2011). Interestingly, in rice no group VII ERFs have been detected yet with similar oxygen dependent proteolysis. It would be interesting to see if resistance to oxygen-dependent proteolysis of group VII ERFs is a more common response amongst wetland species and if these transcription factors also mediate the growth strategies in wetland species other than rice.

Rumex unlimited: Investigating the regulation of flooding survival strategies

Our understanding of the underlying mechanisms of plant adaptation via regulation of distinct flooding survival strategies remains poor. In this thesis plant adaptation to flooding was studied by using two wild species, *Rumex acetosa* and *Rumex palustris* (Polygonaceae) that occur in flood-prone areas along the river Rhine. *R. acetosa* occurs on the higher elevated grasslands, whilst *R. palustris* can be found on the lower located embankments (Fig. 1.1). Naturally these locations experience different flooding regimes,

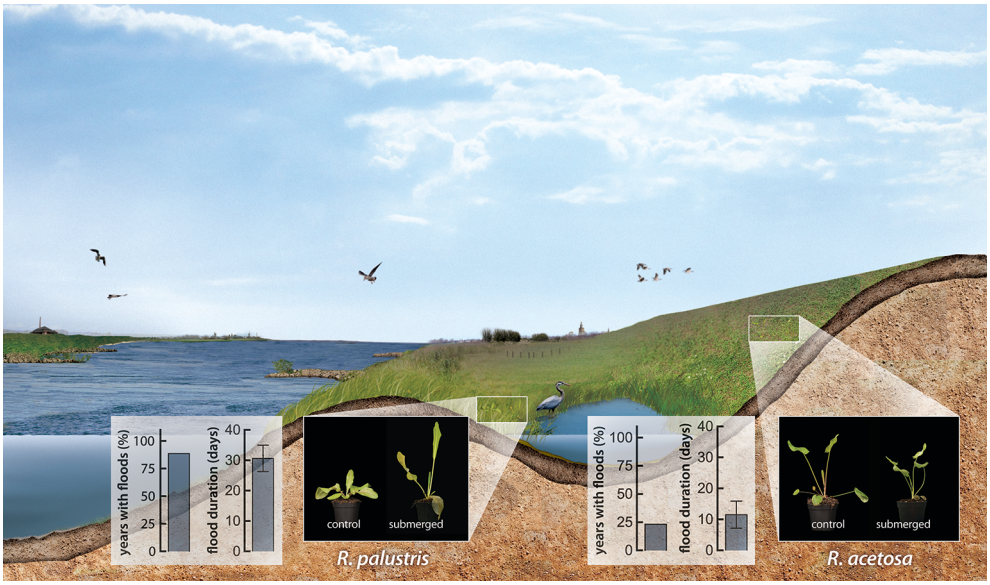

Figure 1.1 Artist impression of a river floodplain with typical habitats of *R. palustris* and *R. acetosa* indicated by rectangles. Insets represent growth responses of both species upon submergence and the flooding regimes in their habitats based on data from the Dutch Ministry of Infrastructure and the Environment over the period 1970-1995. Left bar-graph: the percentage of years with flood occurrence in the growth seasons (April-September). Right bar-graph: the average (± SEM) duration of the most extreme flooding event during the growth season of each year. Picture by Rob Mommer (www.epic.nl).

with highly frequent and long lasting flooding events for *R. palustris*, and less frequent short lasting floods for *R. acetosa* (Fig. 1.1). Concomitant with the flooding regimes, *R. palustris* escapes when submerged allowing it to benefit from the renewed aerial contact. The short flood period experienced by *R. acetosa* does not favour shoot elongation, and hence growth retardation is observed for this species upon submergence. These two related species thus represent a valuable source of tolerance mechanisms evolved in nature.

Flooding responses of *Rumex* have been studied quite extensively on a physiological and morphological level. The identification of the most responsive petioles, the involvement of cell wall loosening and the hormonal interactions have provided great insight into the precise temporal regulation of flooding-induced elongation growth (Voesenek, 1990; Banga, 1996; Vriezen, 2000; Rijnders, 2001; Cox, 2004; Vreeburg, 2004; Benschop, 2004; Chen, 2010). This extensive physiological basis now allowed for a focus on the molecular underlying players that explain the differences between the quiescence strategy of *R. acetosa* and the escape strategy of *R. palustris*. In thesis an unbiased transcriptomics approach and a targeted investigation into group VII ERFs have further increased our understanding of plant adaptation to the flooded environment.

Thesis outline

With the advent of next generation sequencing (NGS) technologies it has become feasible to characterize the transcriptomes of *R. acetosa* and *R. palustris*, and also identify

the changes in gene expression upon submergence. In **chapter 2** we describe how we obtained a high quality transcriptome and submergence-induced expression profiles; furthermore, the identified biological processes are discussed.

In **chapter 3** two potential mechanisms of growth regulation during submergence are explored. The whole transcriptome profiling identified metabolic changes specifically in the quiescent *R. acetosa*. Subsequent metabolite profiling and time course expression analysis led to a model of quiescence in *R. acetosa*. In *R. palustris* the regulation of crucial photomorphogenesis related genes is studied, showing that ethylene signalling converges on typically light regulated genes, leading to elongation growth underwater.

Global transcriptome profiling (chapter 2) also identified many genes for which no function was identified, but that did show a very species specific expression change upon submergence. In **chapter 4** we aim to assign a putative function to these unknown sequences by correlating their transcriptional profile to well-established tissue specific and time and hormone dependent processes of the underwater elongation response in *R. palustris*.

Despite the lack of hypoxia in submerged petioles of *R. palustris* and *R. acetosa*, core-hypoxia genes were regulated. **Chapter 5** shows how ethylene, as an early signal of flooding, can prepare (prime) *R. palustris*, but not *R. acetosa*, to future low oxygen conditions. We hypothesize that this is regulated via group VII ERFs.

Group VII ERFs have been identified as being important in adaptation to flooded conditions, in rice and *Arabidopsis*. In **chapter 6** the use of the two *Rumex* species and two other wetland *Rorippa* species (Brassicaceae) allowed for a comparison of group VII ERF regulation in different phylogenetic lineages. Together with an evolutionary analysis of group VII ERFs this chapter concludes a strong phylogenetic dependent role of group VII ERFs in adaptation to flooding.

In **chapter 7** the findings of the previous experimental chapters are briefly summarized and placed into wider context of plant adaptation to flooding. The use of conserved signalling modules by *R. palustris* and phylogenetically determined gene regulation are discussed. A case is made for the necessity of studying wild plant species that thrive in flood-prone areas to understand the mechanisms of plant adaptation.

Chapter 2

Transcriptome characterization of the submergence response of two *Rumex* species from contrasting hydrological niches

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Abstract

Flooding is lethal for most plant species since it restricts gas exchange and induces an energy and carbon crisis. Survival strategies to flooding stress have mainly been studied in *Oryza sativa* (rice), a cultivated monocot. However, our understanding of plant adaptation to natural flood-prone environments remains scant, even though wild plants represent a valuable resource of tolerance mechanisms that could be used to generate stress tolerant crops. Here we identify mechanisms that mediate the distinct flooding survival strategies of two related wild dicot species: *R. acetosa*, which suppresses growth to outlast the flood event, and *Rumex palustris*, which aims to escape the underwater environment via rapid shoot elongation. With a RNAseq approach transcriptomic changes upon submergence of both *R. acetosa* and *R. palustris* were quantitatively assessed. Gene orthology analysis allowed for a direct comparison between the two species. This yielded a molecular resolution of the contrasting growth responses of *R. acetosa* and *R. palustris*, revealing the distinctly regulated genes that constitute the main differences between the two species. In *R. acetosa* submergence-induced transcriptomic changes were mainly metabolism related and had a focus on ABA signalling genes, whereas for *R. palustris* a novel role for typically light-regulated genes was identified.

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Introduction

Flooding is a globally occurring abiotic stress and an integral part of many ecosystems. Flooding events, resulting in partial to complete inundation of plants, can have severe impacts on the abundance and distribution of wild plant species in natural ecosystems (Voesenek et al., 2004; Silvertown et al., 1999) and on the productivity of crops (Voesenek and Bailey-Serres, 2009). An aqueous environment is stressful to terrestrial plants due to 10^4-fold slower rates of gas diffusion compared to air. The consequent limited exchange of gases such as CO_2 and O_2 dramatically limits photosynthesis and respiration, respectively. Furthermore, muddy and turbid floodwaters reduce light intensity, thereby further limiting photosynthesis. Ultimately, an imbalance between the production and consumption of carbohydrates coupled with an accumulation of toxic metabolic end products proves fatal for most non-adapted terrestrial plants (Colmer and Voesenek, 2009).

Some plants have evolved traits to avoid and ameliorate the problems associated with complete submergence. Rapid acceleration of shoot elongation growth enables some plant species to outgrow floodwaters and thus maintain fast gas exchange and re-establish aerial photosynthesis (Bailey-Serres and Voesenek, 2008). Such an escape strategy is energetically expensive since it requires considerable amounts of carbohydrates to fuel the rapid growth towards the water surface (Setter and Laureles, 1996). Therefore, escape growth is beneficial only if the flooding event is not too deep to outgrow and if the growth investment is rewarded by restored gas exchange and aerial photosynthesis as the leaves emerge above the water surface (Pierik et al., 2009; Akman et al., 2012). If the water surface is not reached, survival of escape-driven plants is severely reduced. Deep or transient flood conditions favour species with growth suppressing behaviour upon submergence by limiting carbohydrate consumption and elongation growth, the so-called quiescent strategy (Fukao et al., 2006; Akman et al., 2012). Indeed, studies show that species with an escape strategy are prevalent on natural sites with frequent shallow and long-term flooding events, whereas those with a quiescent strategy are restricted to sites with deep or short-lasting floods (Voesenek et al., 2004).

Given the importance of employing a particular strategy, we investigated two wild species with a well characterized quiescence or escape strategy, *Rumex acetosa* and *R. palustris*, respectively. *R. acetosa* is rarely flooded in nature and these floods are generally short lasting, compared to *R. palustris,* which is frequently exposed to prolonged, but shallow floods (Voesenek et al., 2004). Upon submergence, *R. palustris* orientates its leaves from a horizontal to vertical position (hyponastic growth) in an ethylene-dependent manner, and subsequently enhances the elongation rate of mainly the youngest petioles (Cox et al., 2006). In *R. acetosa,* this petiole elongation is supressed by ethylene that accumulates during submergence (Voesenek et al., 1993a) and to survive floods it has to deal with limited gas exchange and must adjust its metabolism accordingly to limit energy and carbon use. To investigate the molecular processes underlying the adaptive growth differences between the two species, we used RNAseq technologies to quantitatively analyse genome-wide transcript changes upon submergence.

For each species, the transcriptome was analysed in the youngest, most responsive petiole when elongation differences due to submergence were most pronounced. This encompassed a gene orthology analysis of Polygonaceae species in relation to other angiosperms with de novo assembled reference transcriptomes of both Rumex species. Subsequently, quantitative RNAseq allowed direct comparison of ortholog expression between R. acetosa and R. palustris revealing a full suite of similarly and distinctly regulated transcripts upon submergence. This resulted in a molecular resolution of the flooding survival strategies of these two species occupying distinct hydrological niches.

Results and discussion

R. acetosa and *R. palustris* have contrasting underwater growth responses reflecting their field distribution

Both *R. acetosa* and *R. palustris* occur in the floodplains of major European rivers. The former inhabits the higher located grasslands, whereas *R. palustris* completes its life cycle in the lower riparian mudflats (Van Eck et al., 2004). Accordingly, both the number of flooding events, as well as their duration, is higher in habitats of *R. palustris* than of *R. acetosa* (Fig. 2.1a). Indeed, extended flooding periods have been shown to favour escaping species (Voesenek et al., 2004). Additionally, the long duration and high frequency of flooding stress experienced by *R. palustris* has led to a strong selection for increased low oxygen and flooding tolerance of *R. palustris* compared to *R. acetosa* (Voesenek et al., 1993b). Nevertheless, both *Rumex* species have been found to be well adapted to flood-prone environments with various reports of survival of more than three weeks for *R. acetosa* and even longer for *R. palustris* (Mommer et al., 2006a; Van Eck et al., 2004; Vervuren et al., 2003). A visual example of the contrasting survival strategies adopted in the *Rumex* species is shown in Fig. 2.1b. Detailed analysis of the growth kinetics of the youngest petiole identified important lag phases and circadian patterns in the growth suppression of *R. acetosa* and stimulation of *R. palustris* (Fig. 2.1c) and served as a guideline in determining the submergence duration to quantify global transcriptomic changes (Fig. 2.1d).

Transcriptome assembly and orthology analysis of two *Rumex* species

A two-step approach was taken to profile global transcriptome changes upon submergence in the two species. First, a *de novo* transcriptome was created using long-read (419 nt) sequencing, followed by identifying quantitative changes with short-read (78 nt) sequencing at the time point slightly prior to maximum growth differences (Fig. 2.1c-d). Using the youngest, most responsive petiole from control- and submergence-treated plants, the long-read sequencing resulted in *de novo* assembly of 32,022 *R. acetosa* and 49,070 *R. palustris* contigs (Table S2.1). The relatively low percentage of singletons (13%) indicated a well-assembled transcriptome (Ekblom and Galindo, 2010). Comparative gene family identification, using the OrthoMCL (OMCL) clustering algorithm (Enright, 2002; Li et al., 2003), amongst five angiosperms representing a wide range of phylogenetic lineages (*Arabidopsis thaliana*, *Medicago truncatula*, *Solanum lycopersicum*, *Beta vulgaris* and *Oryza sativa*) revealed 4,171 gene clusters unique to *Rumex*. Only 97 clusters were found in all

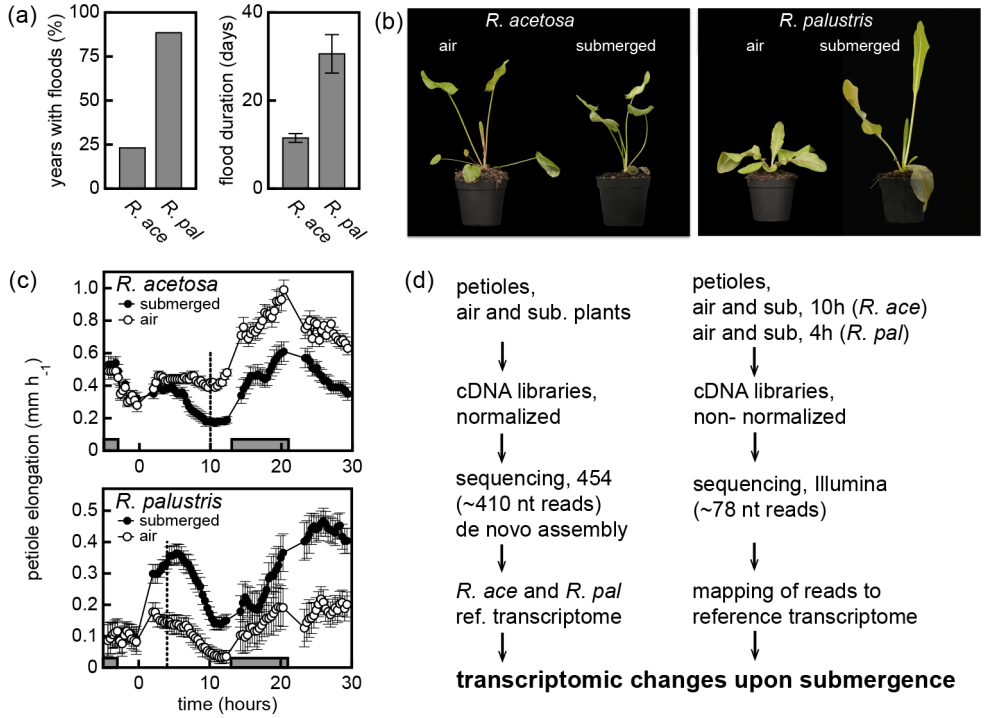

Figure 2.1 Growth strategies and habitat of two wild *Rumex* species, with the RNAseq experimental workflow.
(a) Flooding regimes based on data from the Dutch Ministry of Infrastructure and the Environment over the period 1970-1995 at elevation levels where *R. acetosa* (*R.ace*) or *R. palustris* (*R.pal*) are located. Left bar-graph: the percentage of years with flood occurrence in the growth season (April-September). Right bar-graph: the average (± sem) duration of the most extreme flooding event during the growth season of each year.
(b) Visual representation of the responses of *R. acetosa* and *R. palustris* after 10 days of complete submergence.
(c) Petiole elongation rate of the youngest developing leaf (third leaf), mean ± sem (n=11 for *R. acetosa*, n=18 for *R. palustris*). Grey bars represent the dark period and the vertical dashed line indicates the sampling time-point for Illumina sequencing. The submergence treatment started at time = 0.
(d) Experimental, analytical and bioinformatics workflow used to characterize and quantify transcriptomic changes upon submergence.

species but the two *Rumex* species, whereas 4,262 did include *Rumex*, indicating good sequence coverage and assembly using petiole mRNA (Fig. 2.2a). Identification of putative orthologs and putative paralogs specifically between *R. acetosa* and *R. palustris* yielded 16,476 and 16,371 clusters (OMCL families), in *R. acetosa* and *R. palustris,* respectively, of which 11,219 contained contigs that were present in both species (Fig. 2.2b). OMCL families were small and mostly contained only one or two contigs per species (Fig. 2.2c). The small OMCL families allowed us to circumvent the lack of genome information whilst maintaining a high resolution of sequence diversity, limiting the potential pooling of contigs with different physiological functions. Interestingly, more contigs were identified in *R. palustris* and the number of OMCL families with more members was higher, which could reflect its polyploid nature compared to the diploid nature of *R. acetosa* (Darlington and Wylie, 1955) or differences in genetic variation of the experimental populations used.

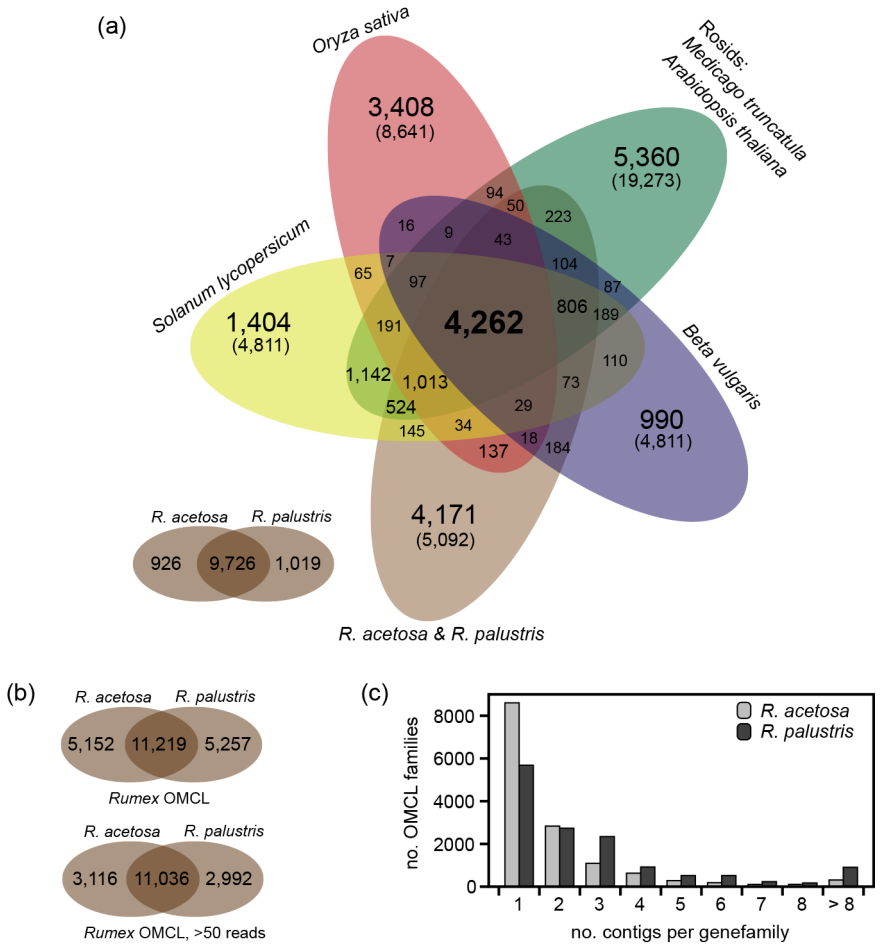

Figure 2.2 Gene orthology in *Rumex* and angiosperms

(a) Plant kingdom wide overlap in gene families as identified by OrthoMCL (inflation score of 1.1). Values in parentheses are number of genes not placed in an OMCL family. The Venn diagram in brown represents the distribution of OMCL families specifically between *R.* acetosa and *R. palustris* based on a plant kingdom-wide analysis. Size distribution of kingdom wide families can be found in figure S2.1.

(b) Overlap in OMCL families obtained from OMCL-family identification directly between *R. acetosa* and *R. palustris* (Inflation score of 3.0). Venn diagram with all identified OMCL families and those with more than 50 Illumina reads in at least one treatment. The latter was used for subsequent analysis.

(C) Size distribution of OMCL families with more than 50 sequence reads in at least one treatment.

Quantitative RNAseq identifies overlapping and distinct transcriptomic changes upon submergence

To identify molecular processes that mediate submergence-induced petiole growth stimulation and suppression, sampling for quantitative short-read RNAseq was based on time points just prior to the strongest effect of submergence on petiole elongation. This was after 10 h for *R. acetosa* and 4 h for *R. palustris* (Fig. 2.1c-d). Short-read RNAseq of air- or submergence-treated petiole tissue from these time points delivered approximately 30

million reads per sample. Out of these, 74% and 77% of the reads could be mapped to the reference transcriptome of *R. acetosa* and *R. palustris*, respectively (Table S2.1). By summing the reads of all OMCL family contig members, differential expression analysis based on a negative binomial model identified 469 Differentially Expressed OMCL Families (DEOF; $P_{adj.}<0.01$; Fig. 2.3 and S2.2). Quantitative RT-PCR on the same samples yielded results consistent with RNAseq (Fig. S2.3). Large-scale changes upon submergence were also characterized by analysing overrepresentation of Gene Ontology (GO) terms amongst OMCL families with estimated log_2FC exceeding |1| (Fig. 2.4). Distinct and overlapping transcriptomic responses, of both individual OMCL families and GO categories, between the two species were found. However, no case of contrasting regulation (i.e., up in one species whilst down in the other) was identified. Instead, different cohorts of DEOFs and GO categories are regulated in either species (Fig. 2.3a and 2.4). This suggests that at least the transcriptional regulation of growth stimulation and suppression are controlled by distinct sets of genes instead a single gene set that is contrastingly regulated. A full factorial generalized model identified 49 OMCL families with a significant species*treatment interaction term ($P_{adj.} < 0.01$; Fig. 2.3b). These are main candidate regulators of the distinct plasticity in growth of the two species during submergence.

Transcriptomic overlap between *R. acetosa* and *R. palustris* includes processes related to carbon starvation, toxins and ion homeostasis typical of underwater stress

The substantial overlap between *R. acetosa* and *R. palustris* in regulated OMCL families (Fig. 2.3a) and overrepresented GO terms (Fig. 2.4) are likely to be common responses of *Rumex* species to the physiological challenges of the underwater environment. This is demonstrated by, for instance, the induction of putative orthologs *of DARK INDUCIBLE 10* (fam02035, fam06971), which typically is induced upon carbon starvation in *Arabidopsis,* where they encode hydrolases that act on sugar polymers (Fujiki et al., 2000; 2001). Additionally, the up-regulation of *BETA AMYLASE* transcripts was observed (fam18839, fam08330). Overall changes identified by a GO analysis (Fig. 2.4) further supported general metabolic changes in both species as reflected by the overrepresentation of, for instance, carbohydrate metabolic process (GO:0005975), hydrolase activity (GO:0004553) and triglyceride catabolic process (GO:0019433). Additionally, an overrepresentation of GO terms associated with energy requiring processes such as DNA replication (GO:0006270, GO:0006268) and flavonoid biosynthesis (GO:0009813) amongst the down-regulated genes was identified. The physical challenge of reduced gas exchange underwater is reflected in these metabolic adjustments where both species appear to down-regulate energy consuming processes and activate reserve mobilisation. The impairment of photosynthesis underwater generally leads to metabolic imbalances and thus toxic end products and reactive oxygen species (ROS) formation. Concomitantly, commonly regulated OMCL families included two *LACTOYLGLUTATHIONE LYASE*s (fam15402, fam16911) and a *GLUTATHIONE-S-TRANSFERASE* (fam12707), which all function in detoxification processes (Moons, 2005). Furthermore, a drug transporter (fam20272) was strongly induced and there was a strong overrepresentation of the toxin catabolic GO term (GO:0009407; Fig. 2.4). Both *R. acetosa* and *R. palustris* induced some ROS-associated transcripts, such as a

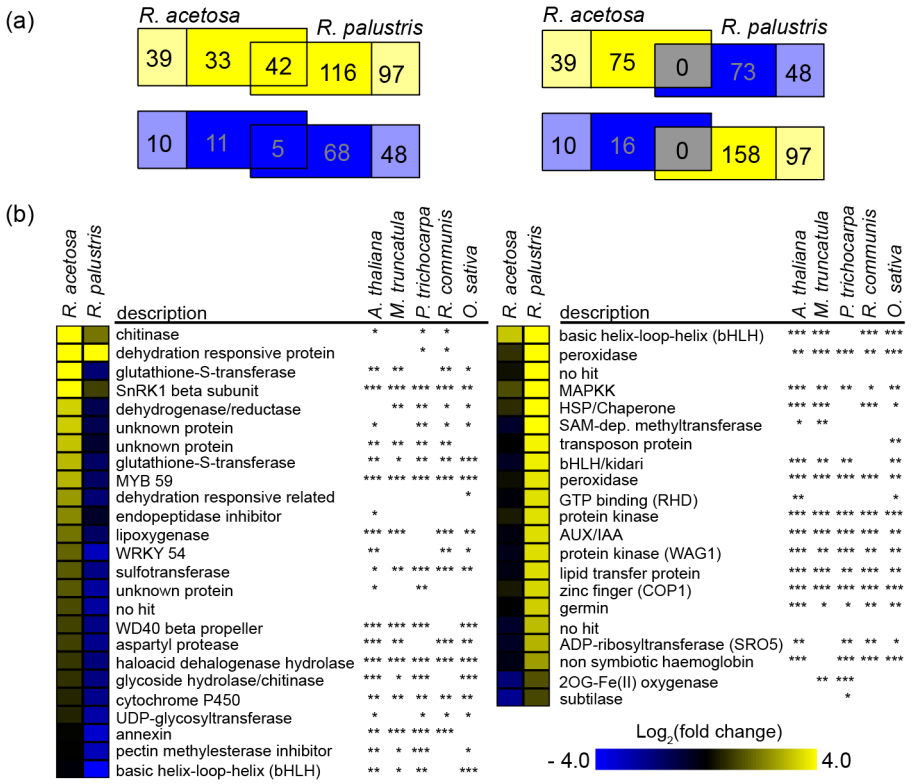

Figure 2.3 Differentially Expressed OMCL families

(a) Venn diagram of Differentially Expressed OMCL Families upon submergence, where yellow is up, blue is down and grey the overlap between up and down regulation ($P_{adj.} < 0.01$). The pale colours are OMCL families specific to either *R. acetosa* or *R. palustris* (*R.ace* or *R.pal*).

(b) OMCL families with a strong species*treatment interaction ($P_{adj.} < 0.01$). Annotation of the OMCL families was based on a discontiguous megablast against five other plant species (* Eval > 10^{-10}, ** 10^{-10} < Eval < 10^{-30}, *** Eval < 10^{-30}).

putative ortholog of the *Arabidopsis OXIDATIVE STRESS3* (fam21461) and a *PEROXIDASE* (fam15244). However, *R. palustris* activated an additional set of ROS-associated genes such as two more *PEROXIDASE*s (fam10911, fam19976) and glutathione biosynthetic enzymes (fam04087), suggesting enhanced ROS regulation. This might be an anticipatory response for the moment when re-aeration occurs when the water surface is reached via the elongation response. Alternatively, some photosynthetic capacity of submerged *R. palustris* has been reported (Mommer et al., 2006b), whilst *R. acetosa* does not benefit as much from underwater illumination (Mommer et al., 2006a). Therefore, the additional activation of ROS and redox players in *R. palustris* might be important to appropriately adjust redox regulation.

The GO analysis also indicated strong regulation of transcripts important for water and ion homeostasis (Fig. 2.4). This was also reflected in the shared activation of a proline dehydrogenase associated with osmotic stress (fam06756), and many transmembrane and ion transport terms were overrepresented in both species (GO:0015297, GO:0015238,

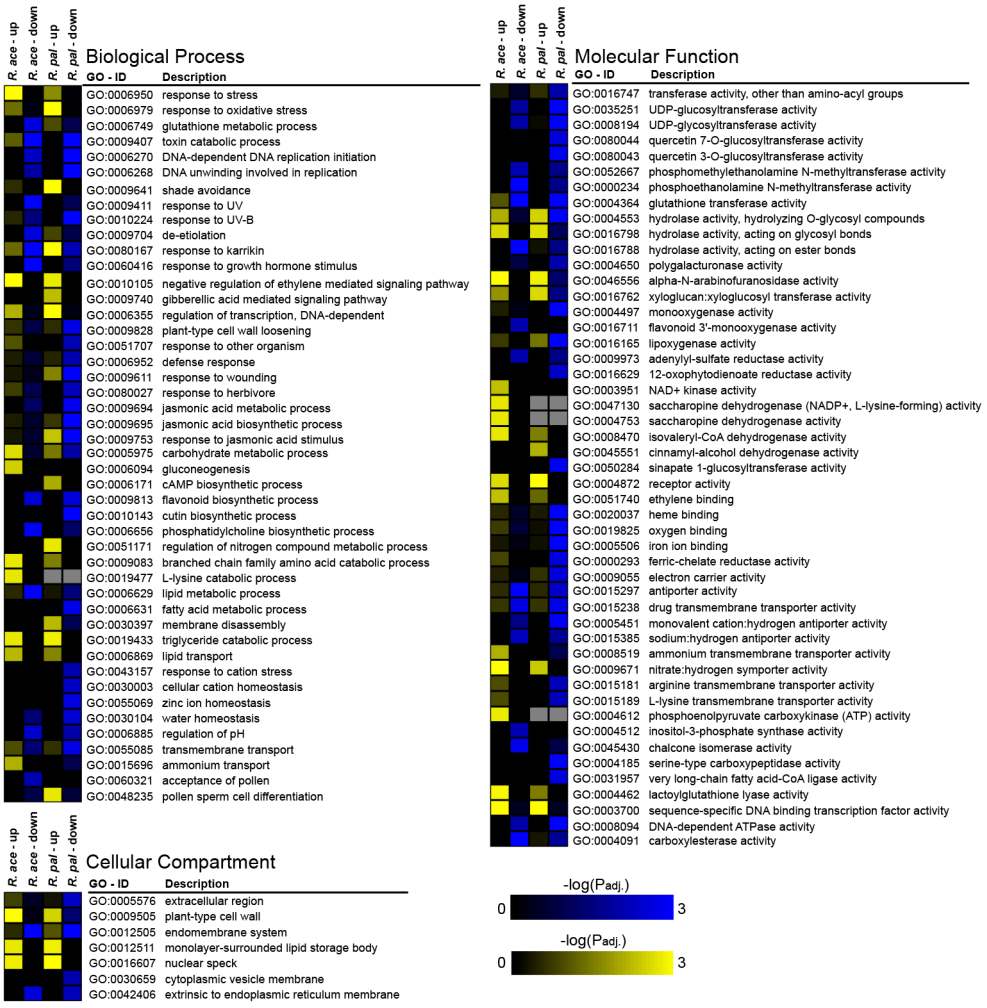

Figure 2.4 Overrepresented gene ontology (GO) terms amongst *Rumex* OMCL families which had a $\log_2FC > |1|$. Only GO terms are shown where $P_{adj.} < 0.01$ in at least one of the four gene sets (up or down in either *R. acetosa* or *R. palustris*). GO terms were assigned based on their closest *A. thaliana* relative (Eval < 10^{-10}).

GO:0005451, GO:0015385, GO:0030104, GO:0006885, GO:0055085). Under normal drained conditions, plants are supplied with water and nutrients via the root system, but during submergence water and nutrient uptake is severely reduced due to an impaired transpiration stream and severely impaired root functioning (Elzenga and van Veen, 2010). However, aquatic and submerged plants do retain some capacity for water transport (Pedersen, 1993). The observed changes in ion homeostasis in *Rumex* most likely reflect the need to adjust to changes in nutrient supply. Changes in ion regulation are thus far an underestimated aspect of flooding tolerance (Shabala, 2010). Together with detoxification and metabolic adjustments, these are typical responses that deal with physical limitations of the underwater environment encountered by both species.

Regulation of processes involved in petiole growth in *R. acetosa* and *R. palustris*

Previous work on the regulation of hyponastic growth and petiole elongation of *R. palustris* compared to the quiescence response of *R. acetosa* revealed a crucial role of an ethylene-driven signalling cascade. In *R. palustris,* ethylene accumulation reduced abscisic acid (ABA) levels, auxin redistribution within the plant and increased cell wall loosening (Cox et al., 2004; Benschop et al., 2005; Vreeburg et al., 2005). Continuation of elongation was shown to then require subsequent enhancement of gibberellic acid (GA) biosynthesis (Benschop et al., 2006). In contrast, submerged *R. acetosa* maintains ABA and GA at the same levels as control plants (Benschop et al., 2005). Additionally, upon submergence, GA sensitivity is reduced in *R. acetosa* but enhanced in *R. palustris,* accentuating the contrasting behaviour of these species (Rijnders et al., 1997). Accordingly, we investigated the underlying transcriptomic responses associated with these known hormonal changes (Fig. 2.5).

As expected, this transcriptome-wide analysis showed that *ACC OXIDASE*, a proxy for enhanced ethylene levels (Vriezen et al., 1999), accumulated in both species. A strong induction of an *EIN3 BINDING F-BOX* (*EBF*) OMCL family was observed in *R. palustris* only. The putative ortholog in rice is likely involved in ethylene-induced growth stimulation by preventing negative regulation of GA synthesis by ethylene (Kim et al., 2012).

Consistent with previous observations (Benschop et al., 2005), transcripts encoding the rate-limiting ABA biosynthesis enzyme 9-cis-epoxycarotenoid dioxygenase (NCED) were strongly down-regulated in *R. palustris* and not in *R. acetosa*. In line with the decrease of ABA levels in *R. palustris* (Benschop et al., 2005) the gene transcript that encodes an ABA breakdown enzyme ABA-8-hydroxylase (a cytochrome P450 monoxygenase) was induced. Surprisingly, this was also observed in *R. acetosa*, although a different OMCL family of ABA-8-hydroxylases showed this regulation. Furthermore, a strong induction of the ABA receptor OMCL family, *PYRABACTIN RESISTANCE/ PYR1-LIKE/ REGULATORY COMPONENT OF ABA RECEPTOR* (*PYR/PYL/RCAR)* was found in both species, whereas OMCL families encoding orthologs of two downstream components of ABA signalling (*ABRE BINDING FACTOR 2*, *ABF2*; *HOMEOBOX PROTEIN 33*, *HB33*) were induced only in *R. acetosa* (Kim et al., 2004; Wang et al., 2011). This indicates enhanced ABA signalling, possibly via the combination of maintained ABA levels and elevated receptor levels that could facilitate growth suppression in *R. acetosa*.

No strong changes were observed in either species in transcripts encoding GA biosynthetic enzymes, GA receptors or DELLA proteins. This is in line with previous physiological studies showing that GA is not an early signal in enhanced petiole elongation, as significant increases in GA_1 content in *R. palustris* were observed only after 6 h of submergence (Benschop et al., 2006). However, an overrepresentation was found for GA-mediated signalling in *R. palustris*, suggesting a sensitization to GA prior to the increase in actual GA levels. This early sensitization could potentially act via the observed changes in ABA signalling transcripts as an important antagonist to GA signalling in *Rumex* (Benschop et al., 2006; Chen et al., 2010).

Also, a large effect was seen on auxin transport modulating OMCL families. These included transcripts encoding putative orthologs of a *PINOID like* (*WAG1*) (Santner and

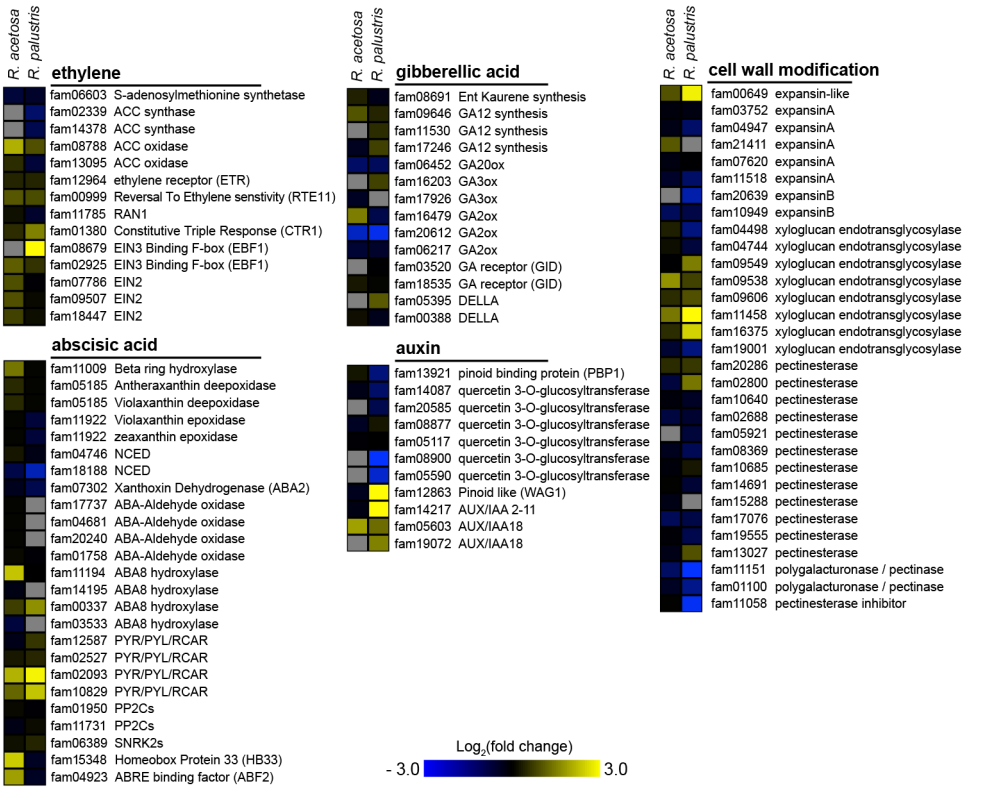

Figure 2.5 Differential expression (submergence vs. air) of *Rumex* OMCL families associated with signalling, metabolism and transport of the hormones ethylene, abscisic acid, gibberellic acid, auxin and cell wall loosening. Grey boxes represent the absence of OMCL family members.

Watson, 2006), *PINOID BINDING PROTEIN1* (Benjamins et al., 2003) and *QUERCETIN-O-GLUCOSYLTRANSFERASEs* (Jacobs and Rubery, 1988). Furthermore, transcripts of *AUX/IAA2-11* putative orthologs, a typical auxin responsive gene (Wyatt et al., 1993), were regulated only in *R. palustris*. These transcriptome changes are consistent with the previously identified role of auxin transport and abundance in the early hours of submergence-induced petiole elongation (Cox et al., 2006).

Putative downstream genes of these hormonal interactions, such as the cell wall modifying *EXPANSINS,* are induced in *R. palustris* and not in *R. acetosa* (Vreeburg et al., 2005). These include the previously identified *EXPANSIN (RpEXPA1*, fam00649*)* as well as *XYLOGLUCAN ENDOTRANSGLUCOSYLASE-HYDROLASEs* (*XTHs*), and *PECTINESTERASEs*, supporting the conclusion that submergence induces the full suite of cell wall modifying players (Sasidharan et al., 2011) in *R. palustris,* but not *R. acetosa*.

R. acetosa and *R. palustris* regulate distinct processes upon submergence

No contrastingly regulated OMCL families (Fig. 2.3a) or GO terms (Fig. 2.4) were identified, that is up in one species and down in the other. Nevertheless, OMCL families with a strong species*treatment interaction term highlighted the OMCL families differing the most

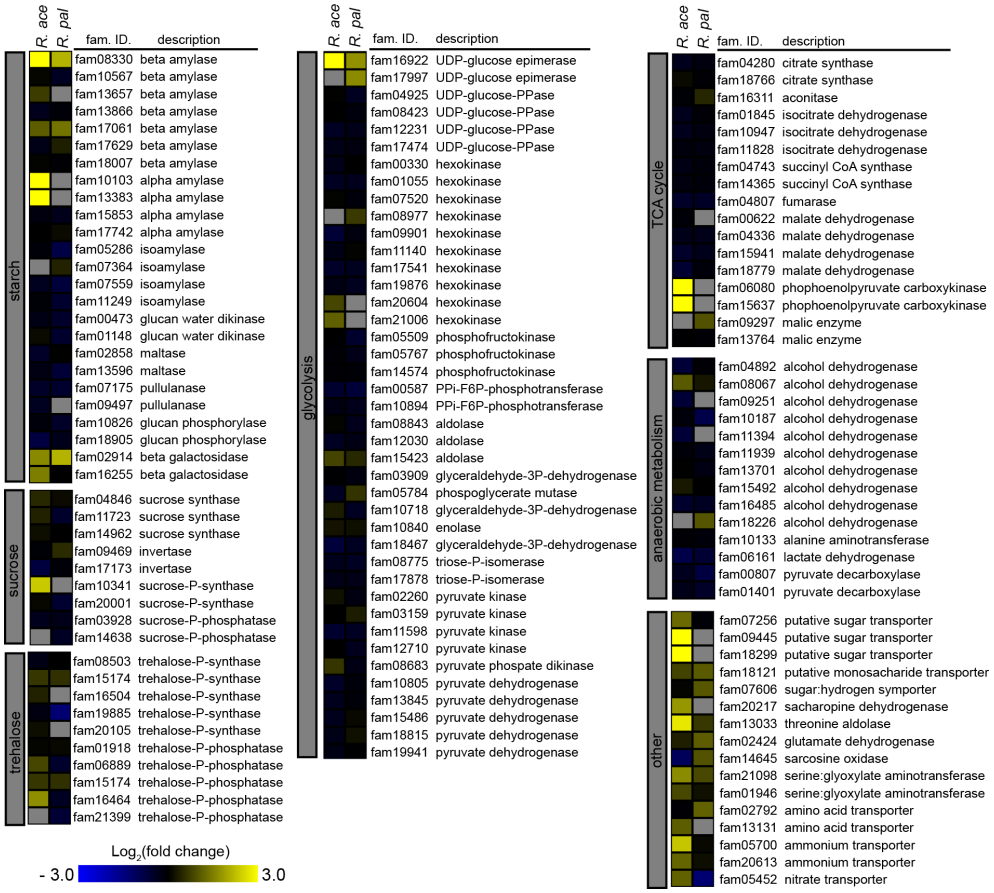

Figure 2.6 Transcriptional changes of *Rumex* OMCL families involved in metabolism.

between the two species (Fig 2.3b). Here some of the OMCL families appear to be contrastingly regulated but did not pass the significance threshold in one of the two species. Amongst the OMCL families with a strong interaction effect, there were several that had a low similarity with orthologs in other species, whilst for some OMCL families highly similar orthologs were identified but a clear biological function in a quiescence or escape strategy could not always be discerned. Nevertheless, we identified several OMCL families that could play an important role in escape and quiescence.

The beta subunit of the SnRK1 complex was strongly induced in *R. acetosa*, compared to *R. palustris* (Fig. 2.3b). SnRK1 (Snf1 Related Kinase) is considered a master regulatory protein whose activity results in energy conserving metabolic reprogramming (Baena-González et al., 2007; Baena-González and Sheen, 2008). SnRK1 is a hetero trimeric protein complex consisting of an α-, β-, and γ-subunit. An increase in expression of the β-subunit alters the function of SnRK1 towards a specific role in regulating nitrogen metabolism (Polge et al., 2008; Li et al., 2009). The strong transcriptional increase of the β-subunit of SnRK1 in submerged *R. acetosa*, therefore suggests specific metabolic

reprogramming. Accordingly, transcriptional changes of major metabolic pathways in both species were explored. This led to the identification of a range of OMCL families regulated during submergence by *R. acetosa* and not by *R. palustris*, such as α-*AMYLASES*, a *SUCROSE-P-SYNTHASE*, a *TREHALOSE-P-PHOSPHATASE*, *PHOSPHOENOLPYRUVATE CARBOXYKINASEs* and *AMMONIA TRANSPORTERS* (Fig. 2.6).

The lack of strong and distinct regulation of cell wall modifying enzymes by *R. acetosa,* together with the slow onset of growth retardation, uncharacteristic of cell wall modifications, suggests an alternative mechanism mediating quiescence. In line with this we do observe an array of specific metabolic processes (Fig 2.6) and two ABA signalling components (Fig. 2.5) specifically regulated by *R. acetosa*, suggesting that restriction of growth as observed for this species could involve these OMCL families.

Amongst the OMCL families only regulated in *R. palustris* were several genes associated with photomorphogenesis and shade avoidance (Fig. 2.3b). These include putative orthologs of *Arabidopsis AUX/IAA2-11* (Wyatt et al., 1993), zinc finger *CONSTITUVE PHOTOMORPHOGENIC 1* (*COP1*) and the basic Helix-Loop-Helix (bHLH) transcription factor gene *KIDARI/PRE6*. Both *COP1* and *KIDARI* are involved in light regulation of elongation growth via interaction with LONG HYPOCOTYL IN FAR-RED 1 (HFR1), an atypical bHLH that regulates shade avoidance. HFR1 can form heterodimers with PHYTOCHROME INTERACTING FACTORs (PIFs), thereby preventing their binding to promoters of target genes that are associated with enhanced shoot elongation during shade (Hornitschek et al., 2009; Galstyan et al., 2011). COP1 can function as an E3 ligase and targets a range of proteins involved in photoreceptor-mediated signal transduction for degradation, including HFR1 (Yang et al., 2005; Duek et al., 2004; Jang et al., 2005). KIDARI also negatively regulates HFR1 action, but does so by forming heterodimers and thereby releasing PIFs from HFR1 inhibition (Hyun and Lee, 2006; Lee et al., 2006). The PIFs directly interact with phytochrome photoreceptors and thus act downstream of red and far-red stimuli (Leivar et al., 2012). PIF4 and PIF5 in *Arabidopsis* also stimulate their direct negative regulator *HFR1*, thereby ensuring a fine-tuned elongation response during shading (Hornitschek et al., 2009; Lorrain et al., 2008). Other light signalling components were also

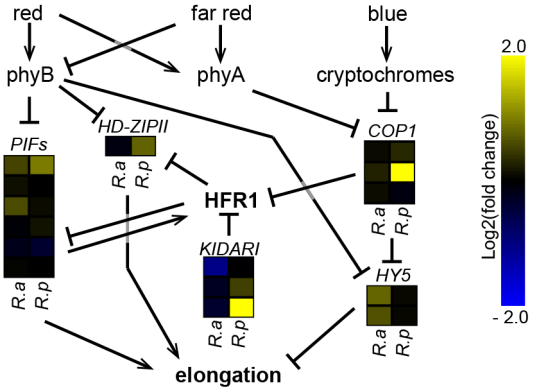

Figure 2.7 Model of interactions between molecular components and photoreceptors that regulate shade-induced elongation growth based on studies in *Arabidopsis*. Family IDs: *HD-ZIPII* - 20527; *PIF*s - 03405, 15415, 15827, 01516, 03135, 08791; *KIDARI* - 14376, 10925, 03530; *COP1* - 15699, 17228, 06784; HY5 - 09886, 05638. *R.a.* (*R. acetosa*), *R.p.* (*R. palustris*). Heat maps depict fold change values for indicated genes from the RNAseq dataset.

found to be regulated upon submergence, namely PIFs, the negative regulator of elongation growth *ELONGATED HYPOCOTYL* (*HY5*) (Osterlund et al., 2000; Li et al., 2010), and a *CLASS II HOMEODOMAIN-LEUCINE ZIPPER* (*HD-ZIPII*), a family of positive regulators of shade avoidance (Steindler et al., 1999). A model incorporating these known interactions from *Arabidopsis* with the expression changes found in *Rumex*, suggests that these changes result in enhanced elongation in *R. palustris* (Fig. 2.7).

Conclusions

Using an unbiased genome-scale approach with RNAseq technology we were able to characterize the transcriptomic submergence responses of the quiescent *R. acetosa* and escaping *R. palustris*. This added novel molecular players in the previously established hormonal interactions and cell wall loosening. The two species regulated distinct OMCL families, which are thus potentially associated with quiescence or escape growth. In *R. acetosa* these transcriptional reconfigurations encompassed mainly metabolic changes together with two ABA signalling components. In *R. palustris*, distinct changes in the expression of OMCL families associated with light signalling were found, implicating them in mediating the submergence escape strategy in this species.

Materials and methods

Plant growth and experimentation

R. acetosa and *R. palustris* seeds were germinated on floating polyethylene beads for 10 days (12h light, 25°C; 12h dark, 12°C). The seedlings were transplanted to a soil-sand mixture (2:1) supplied with 1 L nutrient solution (7.5 mmol NH_4SO_4, 15 mmol KNO_3, 15 mmol KH_2PO_4, 86.4 umol FeEDTA, 4.27 $MnSO_4$, 0.25 umol $ZnSO_4$, 4.23 nmol $CuSO_4$, 8.5 nmol H_3BO_3, 52.2 pmol Na_2MOO_4; divided over 42 pots of 75 mL). *R. acetosa* and *R. palustris* were grown for 16 days in climate-controlled walk-in growth chambers (160 µmol m^{-2} s^{-1} Photosynthetically Active Radiation; 16 h day, 8 h night; 20°C, 70% relative humidity). Treatment consisted of complete submergence (depth of 25 cm) in overnight acclimatized water. Petiole elongation was determined using linear displacement transducers (Benschop et al., 2005; Pierik et al., 2010).

RNA isolation and expression

RNA was extracted from the youngest petiole using the kiefer protocol (Kiefer et al., 2000), followed by DNAse treatment (Ambion, DNA-free). For qRT-PCR subsequent reverse transcription with random hexamers and including RNase inhibitor (Invitrogen) was done. qRT-PCR was performed using a 20-μL SYBR Green reaction mixture with gene-specific primers (Appendix, Table A1) and *TUBULIN* as reference.

454 sequencing and bioinformatics

RNA for 454 sequencing was derived from the youngest petiole over a 24-h time period from two independent experiments, which were pooled in equimolar amounts. Harvest timepoints were, for *R. palustris*: 0 h; air: 4 h; submerged: 1 h, 4 h and 12 h. For *R. acetosa*; 0 h; air: 10 h; submerged: 2 h, 10 h and 24 h. Normalized cDNA libraries were constructed

by Vertis Biotechnologie AG (Friesing, Germany) and sequenced by Macrogen Inc. (Seoul, South Korea). *De novo* reference transcriptome assembly was done with Roche GS FLX software (V2.3) using a minimum overlap length of 40 nt, seed length of 16 nt, minimum percent identity of 90, and alignment and difference score of 2 and -3, respectively. Gene family identification was done using OrthoMCL V1.4 (Enright, 2002; Li et al., 2003). Here Tribe Markov Clustering of the similarity matrix obtained by all-vs.-all blastN (Altschul et al., 1990) on transcript sequences used an inflation factor 1.1 for plant kingdom-wide analysis and 3.0 for the direct comparison between *R. acetosa* and *R. palustris*.

Illumina sequencing and bioinformatics

RNA for Illumina sequencing was pooled from two independent experiments consisting of ten replicates per treatment with ten individual youngest petioles each. Sequencing and library construction were performed by Macrogen Inc. (Seoul, South Korea). Read mapping against a reference transcriptome was done using MAQ0.7.1 software (max. mismatch of 2). Normalization, differential expression with Benjamini Hochberg correction and fold changes were based on a negative binomial model of Ortho Markov Clustering (OMCL) family read numbers, determined using the Bioconductor edgeR package (Robinson and Oshlack, 2010), and combined with the Limma package to implement a species*treatment model (Smyth, 2004). Common dispersion was estimated using 144 housekeeping genes based on *Arabidopsis thaliana* microarrays (Czechowski et al., 2005), which were assumed not to be differentially expressed. OMCL families with less than 50 reads in both air and submergence were excluded from analysis. A gene ontology (GO) analysis, with the Bioconductor GOseq package incorporating gene length bias correction (Young et al., 2010), was performed using OMCL families with |logFC|>1. A BLAST score of $E_{val}<10^{-10}$ was used for the annotation of these OMCL families.

Accession numbers

The raw sequencing files and contig assembly from the RNA sequencing are deposited at theSequence Read Archive (SRA), http://www.ebi.ac.uk/ena/data/view/ERP002093 and the Transcriptome Shotgun Assembly (TSA) Sequence Database under Accession range HAAK01000001- HAAK01031986 and HAAL01000001-HAAL01049016 for *R. acetosa* and *R. palustris,* respectively. OMCL family composition, Accession IDs, read counts, fold changes upon submergence and annotation can be found online at http://www.plantcell.org/content/25/11/4691/suppl/DC1.

Supporting information

Table S1. Sequencing output, assembly and mapping statistics of 454 and Illumina sequencing

454		*Rumex acetosa*	*Rumex palustris*
	no. Reads	771,079	829,811
	mean read length (nt)	410	409
	assembled reads	667,400	724,435
	no. contigs	32,022	49,070
	mean contig length (nt)	1,253	1,119

illumina	library	*R. acetosa* air	*R. acetosa* sub	*R. palustris* air	*R. palustris* sub
	no. reads	30,586,009	29,790,428	30,370,554	30,046,041
	read length (nt)	78	78	78	78
	mapped reads	23,664,225	23,161,619	22,440,624	22,185,347
	mean no. reads per contig	873	854	695	687
	mean fold coverage	51	50	29	28

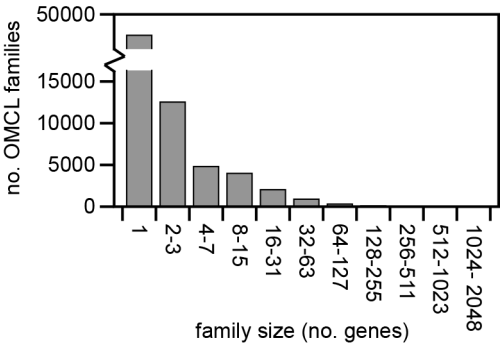

Figure S2.1 The size distribution of OMCL families identified by the plant kingdom-wide analysis shown in Fig. 2.2a.

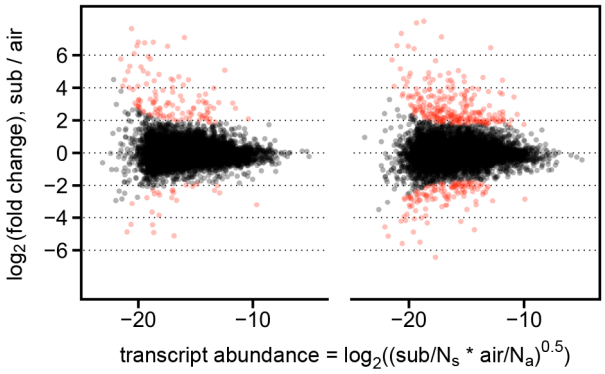

Figure S2.2 Smearplot of *Rumex* OMCL families with >50 reads in at least one treatment. Red dots are differentially expressed OMCL families, Padj. < 0.01. X-axis: submergence (sub) and air, reads in corresponding library; N_s, total submergence library size; N_a, total air library size.

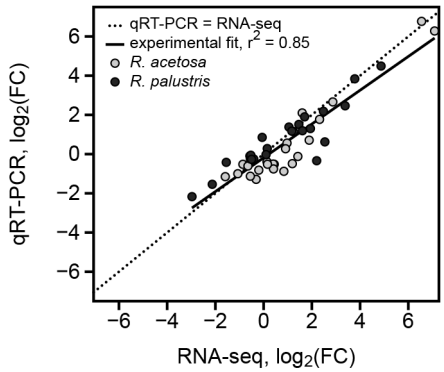

Figure S2.3 qRT-PCR validation of RNAseq estimated fold changes in expression of a selection of genes. Analysis was performed on an aliquot of the same RNA as used for Illumina sequencing.

Chapter 3

Adjustment of metabolism and regulation of photomorphogenesis are distinct processes underlying contrasting flooding survival strategies in *Rumex* species

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Abstract

Regulation of growth strategies is of major importance to plant species occupying flood-prone areas. An escape strategy, where rapid shoot elongation allows for aerial contact, is only beneficial during prolonged and shallow floods. Whereas quiescence, in which growth retardation prolongs survival of complete submergence, is advantageous only in short term and/or very deep floods. Here we investigated two distinct mechanisms that play a role in the opposite survival strategies of *Rumex acetosa* (quiescence) and *Rumex palustris* (escape) in response to complete submergence. In *R. acetosa* we have identified species specific transcriptional changes in metabolism upon submergence. These changes were further explored through metabolite profiling, and time course expression analysis providing a framework for regulating growth retardation. *R. palustris* was found to regulate genes involved in light dependent elongation. Here, the regulatory effects of the underwater light environment and hormonal changes on typical light signalling genes were examined. We conclude that independent of the light environment, ethylene accumulation in submerged *R. palustris* regulates photomorphogenesis-associated genes leading to an escape response.

This chapter is also ublished in:
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Introduction

Flooding events present a major abiotic stress for plant life (Colmer and Voesenek, 2009). Gas diffusion in the water environment is approximately 10^4 times slower than in air. Consequently, CO_2 uptake for photosynthesis and O_2 uptake for respiration are impaired. Two successful survival strategies have been identified in the plant kingdom to deal with adverse effects of complete submergence (Bailey-Serres and Voesenek, 2008). In the quiescence strategy plants aim to limit growth and energy consuming processes to conserve valuable carbohydrates and endure the flooding stress. During the escape strategy the plant regains aerial contact via a vigorous elongation of the shoot. If successful, leaf tips reach the water surface and aerial gas exchange is restored via longitudinally connected aerenchyma. As elongation is very costly (Setter and Laureles, 1996), this strategy is only beneficial when the elongation growth leads to restoration of contact with the atmosphere (Pierik et al., 2009; Akman et al., 2012). Therefore, plants that use elongation growth as an escape strategy are predominantly found in habitats with shallow and long-lasting floods (Voesenek et al., 2004).

Passive accumulation of ethylene is one of the main drivers of submergence survival strategies (Bailey-Serres and Voesenek, 2008). Some species respond to ethylene by an increased shoot elongation, whilst others suppress growth and elongation (Pierik et al., 2006). Main downstream processes of ethylene resulting in submergence-induced elongation are changes in hormone levels and sensitivities. Here abscisic acid (ABA) is a main suppressor of growth, whereas gibberellic acid (GA) a stimulator (Benschop et al., 2005; 2006; Hoffmann-Benning and Kende, 1992; Rijnders et al., 1997). Underlying molecular regulators have been identified in rice. Ethylene-mediated induction of *SNORKEL1* and *-2* is suggested to lead to an increased GA biosynthesis and shoot elongation (Hattori et al., 2009). SUB1A has been identified as a growth suppressor, by reducing the sensitivity to GA (Fukao and Bailey-Serres, 2008).

In chapter 2, global transcriptomic changes upon submergence were investigated in *R. acetosa* (quiescent) and *R. palustris* (escape), providing alternative mechanisms of regulating underwater survival strategies. Interestingly, here specific metabolic adjustments were identified as uniquely regulated in the quiescent *R. acetosa*. Thus far metabolic profiling of plant submergence responses has been restricted to soybean seedlings (Nakamura et al. 2012) and two rice cultivars, one with and one without the *SUB1A* locus (Barding et al., 2012; 2013). To further investigate the transcriptomic changes (Chapter 2, Fig. 2.6) and their link to metabolism, we profiled changes in metabolite levels in petioles of *R. acetosa* and *R. palustris* upon submergence.

On the other hand, the escaping *R. palustris* regulated a set of genes previously associated with photomorphogenesis (Chapter 2, Fig. 2.7). Responses to shade, and the associated changes in light quality, result in a strong elongation response to outcompete neighbours for sunlight (de Wit et al., 2012). Our transcriptomic study revealed that submerged *R. palustris* petioles activate genes known from this shade-regulated elongation response. Here we further explored the role of changes in the underwater light environment and the ability of typical submergence signals (e.g. ethylene and ABA) to converge on this signalling network that regulates elongation.

Results and discussion

Rumex species reconfigure primary metabolic pathways in response to submergence

Global transcriptome profiling suggested that changes in carbon and nitrogen metabolism occur especially in *R. acetosa* (Fig. 2.3, 2.6). This was reflected in the strong induction of OMCL families (group of highly similar sequences of *R. acetosa* and *R. palustris*; Fig. 2.2) encoding the nitrogen responsive β-subunit of the heterotrimeric SnRK1 complex (Fig. 2.3). SnRK1 (Snf1 Related Kinase) is considered a major regulatory protein that when active results in energy conserving metabolic reprogramming (Baena-González et al., 2007; Baena-González and Sheen, 2008). Additionally, a range of OMCL families (group of highly similar sequences of *R. acetosa* and *R. palustris*; Fig. 2.2) encoding metabolic enzymes, such as α-*AMYLASES*, a *SUCROSE-P-SYNTHASE* (*SPS*), a *TREHALOSE-P-PHOSPHATASE (TPP)*, *PHOSPHOENOLPYRUVATE CARBOXYKINASEs (PCK)* and *AMMONIA TRANSPORTERS* (Fig. 2.6) were regulated by *R. acetosa* only. Accordingly, the transcriptional profile over time for key transcripts, specific for *R. acetosa,* was investigated (Fig. 3.1a). Simultaneously, metabolic changes were profiled using GC-MS, UPLC-MS and biochemical methods (table 3.1, fig 3.1 and S3.1).

Gas exchange is restricted in underwater leaves and this compromises CO_2 availability and thus photosynthesis (Mommer and Visser, 2005). Under light conditions, as used during our experiments, this reduced availability of CO_2 could also promote photorespiration, as suggested by transcriptional activation of *SERINE:GLYOXYLATE AMINO TRANSFERASE* (*SGAT*) in both *Rumex* species (Fig. 3.1a). SGAT plays an essential role in photorespiration by recycling the formed 2-phosphoglycolate back to glycine and 3-phospho-glycerate for re-entry in the calvin cycle. These data strongly suggest that both species experience a strong carbon limitation. However, energy levels might not be compromised since shoots mostly remain normoxic when submergence occurs in the light (Mommer et al., 2007). Consistently, ATP levels and respiration were not negatively affected by submergence when compared to controls in both species (Fig. 3.1b-c). We also found that both species had increased lactate levels (Fig. 3.1b), suggesting that either fermentation occurs in submerged petioles or more likely that lactate produced in hypoxic roots is transported to the aerial tissues. Despite these observed similarities, metabolic requirements are expected to be distinct between the two species. While *R. palustris* requires resources to sustain elongation growth and quickly regain aerial contact, growth suppression upon flooding would require *R. acetos*a to adjust its metabolism to a situation where no aerial contact will be made. The two species were distinct in their composition of the measured metabolites (Fig. 3.2). Furthermore, species-specific trends in metabolite and transcript changes in response to submergence were observed (Fig. 3.1, Table 3.1).

While many reserve-mobilizing components were induced in both species, such as β-amylases and two sugar hydrolytic *DARK INDUCIBLE* OMCL families, *R. acetosa* activated an additional suite of catabolic reactions absent in *R. palustris*, such as gene transcripts associated with starch, lysine and threonine catabolism (Fig. 2.6 and 2.4). Correspondingly, a *TREHALOSE-6-PHOSPHATE PHOSPHATASE (TPP)* was induced throughout

Table 3.1. Identified metabolites and their behaviour during submergence (two-way ANOVA, * P<0.05, ** P<0.01, *** P<0.001, blank = no significant effect, sub = submergence main effect, time = time main effect, t*s = time*submergence interaction; dir. = direction of change, either down or up indicating lower or higher levels in submerged plants).

metabolite	*R. acetosa*				*R. palustris*			
	sub	time	t*s	dir.	sub	time	t*s	dir.
sucrose		**			**			down
glucose								
fructose								
6-phosphogluconate			*	down				
3-phosphoglycerate		**						
citrate								
α-ketoglutarate								
succinate		**						
malate								
oxalate		**						
glutamate					*			down
pyroglutamate								
aspartate		**						
glycine	*	*		up				
β-alanine	**			up				
lactate	**			up	*			up
myo-inositol								
ascorbate								
β-sitosterol	*			up				
campestrol	*			up				
shikimate		*						
tartarate		*						
propanoate		**						
erythronate	**	*		up				
total protein						***		
amino acids						*		
nitrate	***	**	***	up				
ammonia	**			up	***	***	*	up
starch			*	down	*	***		down
ATP	*	***		up				
ATP:ADP								

submergence only in *R. acetosa* (Fig. 3.1a). Its substrate, trehalose-6-phosphate (T6P), is a potent growth regulator that acts by inhibiting the activity of the SnRK1 heterotrimeric protein complex (Zhang et al., 2009). Assuming that *TPP* transcriptional elevation translates to higher TPP activity, the consequent drop in T6P and subsequent release of the inhibition on SnRK1 activity would lead to a shift from energy consuming anabolism towards energy producing catabolism (Baena-González et al., 2007). Furthermore, transcriptional induction specific to *R. acetosa* of *SPS* and *PCK*, the latter one of many targets transcriptionally activated by SnRK1 (Baena-Gonzalez and Sheen, 2008), indicates additional *R. acetosa*-specific metabolic changes directed to sucrose and activation of gluconeogenesis. Overall, the activation of a catabolic suite of genes in combination with a reduction in costly biosynthesis would lead to the maintenance of the major sucrose and hexose metabolite pools which is indeed observed in *R. acetosa* (Figure 3.1b and S3.1). A similar observation was made in rice, where a quiescent variety showed increased *TPP* transcript accumulation and a relatively high sugar status compared to a non-quiescent genotype (Jung et al., 2010;

Barding et al., 2012). Overall, this suggests an uncoupling of carbon status and trehalose metabolism in quiescent species, which under normal growth conditions are tightly linked. This would allow for the maintenance of high free sugar levels without engaging in expensive biosynthetic processes. Accordingly, submerged *R. acetosa* had significantly lower levels of 6-phosphogluconate when submerged (Fig. 3.1b). 6-phosphogluconate is an intermediate in the Oxidative Pentose Phosphate pathway (OPP) that supplies potent reducing power for biosynthesis. In contrast, *R. palustris*, which requires carbon building blocks for elongation growth suffered from a depleted sucrose pool and reduced starch levels (Fig. 3.1b).

The β-subunit of the SnRK1 complex, with a strong transcriptional induction in *R. acetosa* (Fig. 3.1a), has been shown to have a specific role in regulating nitrogen metabolism (Li et al., 2009). Combined with the simultaneous induction of *TPP,* that presumably relieves T6P inhibition on SnRK1, this would suggest SnRK1-mediated nitrogen metabolic reprogramming upon submergence in *R. acetosa*. Indeed, several GO terms associated with amino acid metabolism were overrepresented in *R. acetosa* only; e.g., arginine and lysine transmembrane transport, lysine catabolic process, regulation of nitrogen compound and ammonium transporters (GO:0015181, GO:0051589, GO:0019477, GO:0051171, GO:0015696; Fig. 2.4). Despite the catabolic nature of the transcriptional changes, the overall amino acid and protein levels did not change in either species. However, certain amino acid levels changed in a species-specific manner. Submergence increased glycine and alanine levels in *R. acetosa* and reduced glutamate levels in *R. palustris* (Fig. 3.1b). In *R. acetosa,* the strong glycine accumulation corresponded with an increase in *THREONINE ALDOLASE* expression, which converts threonine to glycine and acetaldehyde (Fig. 3.1a). Acetaldehyde could serve as a substrate for fermentation or be metabolized to acetyl-CoA. Interestingly, a significant increase of free ammonia was observed in submerged *R. acetosa* (Fig. 3.1b). So far, this has not been observed in submerged plants and a clear functional explanation remains absent. However, this could be the result of reduced biosynthesis or increased breakdown of protein and amino acids in this species, especially given that a minor change in protein content could already result in a dramatic increase in ammonia or nitrate.

Interestingly, two *PCK*s were among the most strongly induced OMCL families in *R. acetosa* (*PCK*; fam06080, fam15637; $\log_2$FC = 7.09 and 6.55). Indeed, real-time qPCR analyses revealed significant elevation of *PCK* transcripts over time in submergence-treated *R. acetosa* and not in *R. palustris* (Fig. 3.1a). Accordingly, *in vitro* PCK activity was increased in submerged *R. acetosa* (Fig. 3.1d). PCK was shown to be strongly induced by feeding certain amino acids, ammonia or nitrate (Delgado-Alvarado et al., 2007), and *PCK* knock out plants were shown to have altered amino acid profiles (Brown et al., 2010). PCKs take a central part in metabolism by converting oxaloacetate from the TCA cycle to phosphoenolpyruvate (PEP). This reaction is especially important in gluconeogenesis and the subsequently formed PEP acts as a potent inhibitor of phosphofructokinase activity, thus limiting glycolysis. However, we observed no reduction in levels of downstream components, such as TCA cycle intermediates (Table 3.1) or respiration (Figure 3.1c). However, PCK activation and ammonia transporters (Figure 3.1a) have been suggested to

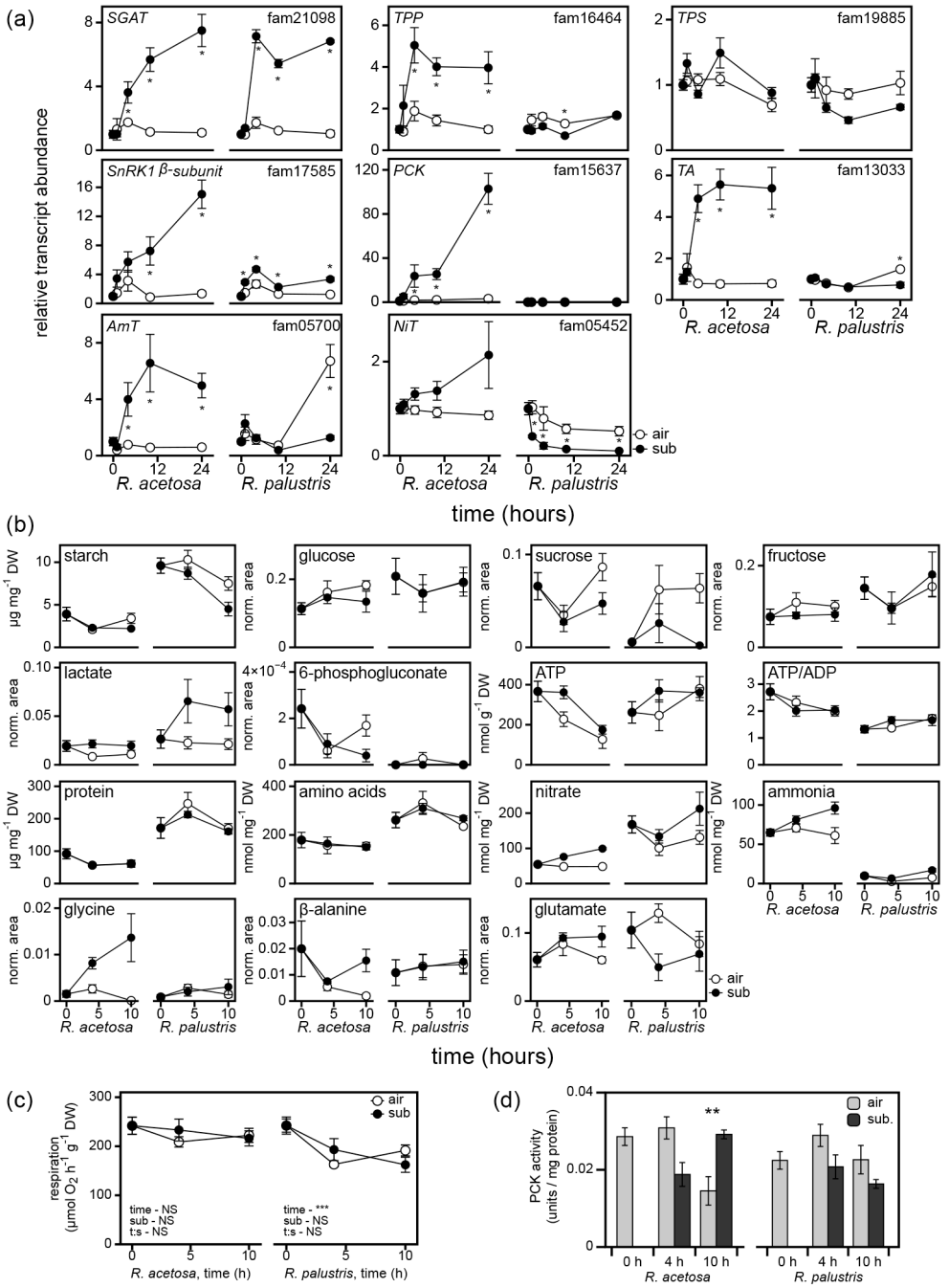


Figure 3.1 Changes in metabolism in two *Rumex* species upon submergence.

(a) Relative gene expression profiles in petioles from submerged plants (mean ± sem, n=6; * P<0.05, Tukey LSD). SGAT, *SERINE:GLYOXYLATE AMINOTRANSFERASE*; *TPP*, *TREHALOSE-6-PHOSPHATE PHOSPHATASE*; *TPS*, *TREHALOS-6-PHOSPHATE SYNTHASE*; *PCK*, *PEP CARBOXYKINASE*; *TA*, *THREONINE ALDOLASE*; *AmT, AMMONIUM TRANSPORTER*; *NiT*, *NITRATE TRANPSORTER*.

Fig. 3.1 continued
(b) Changes in levels of selected metabolites upon submergence in *Rumex* petioles (mean ± sem, n=5). Data for other measured metabolites can be found in Fig. S3.1. Significance levels for all metabolites can be found in Table 3.1.
(c) Respiratory rates, measured on excised *Rumex* petioles via consumption of oxygen in the dark (mean ± sem, n=4). Two-way ANOVA, *** P<0.001, NS = no significant effect, sub = submergence main effect, time = time main effect, t*s = time*submergence interaction
(d) PCK (PHOSHPOENOLPYRUVATE CARBOXYKINASE) activity of *Rumex* petioles from air or submerged plants (mean ± sem, n=5).

be required for removal of acids and ammonia following amino acid breakdown (Leegood and Walker, 2003).

In summary, our observations support distinct metabolic changes in response to submergence for *R. acetosa* compared to *R. palustris*. Directing resources towards appropriate processes and initiating the right metabolic adjustments is critical for survival of any stress. In *R. palustris,* metabolic adaptation is restricted to increased reserve mobilization, probably to fuel elongation growth. Given the slow onset of the inhibition of petiole elongation (Fig. 2.1c) and lack of strong and distinct regulation of cell wall modifying enzymes by *R. acetosa* (Fig. 2.5), a metabolic mechanism of growth retardation provides a fitting alternative. Additionally, two ABA signalling components were upregulated specifically by *R. acetosa* (Fig. 2.5). In *R. acetosa*, submergence induced transcriptional and metabolic signatures suggest that changes in carbon and nitrogen metabolism lead to a maintenance of the sucrose and starch pool and an arrest of biosynthetic processes. Overall, these processes could lead to a retardation of growth (Fig. 3.3).

Shade avoidance and photomorphogenesis genes are regulated by ethylene, not light, during submergence

In Chapter 2 regulation of OMCL families typically involved in photomorphogenesis were identified. We assume that, based on work on *Arabidopsis*, *COP1* (*CONSTITUVE PHOTOMORPHOGENIC 1*) and *KIDARI,* both specifically induced upon submergence by *R. palustris* only, act together to stabilize elongation-promoting PIFs (PHYTOCHROME INTERACTING FACTORs) via LONG HYPOCOTYL IN FAR-RED 1 (HFR1) (Yang et al.,

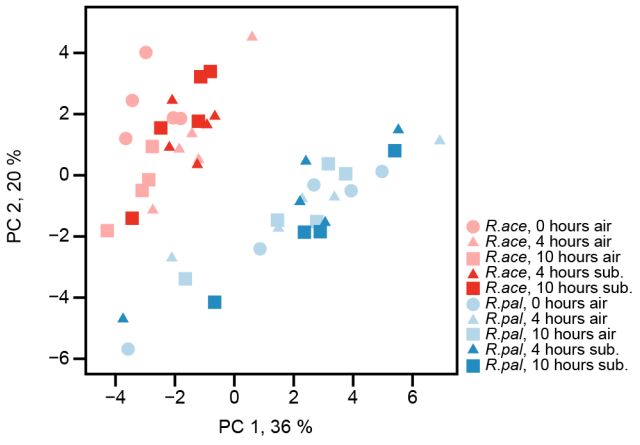


Figure 3.2 Principle Component Analysis of GC-MS and UPLC-MS identified metabolites separates into two groups, *R. acetosa (R. ace)* and *R. palustris (R. pal)*.

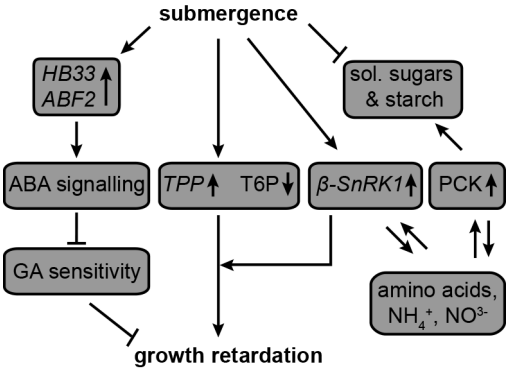


Figure 3.3 A schematic representation of the potential metabolic and signalling processes mediating the submergence-induced growth retardation in *R. acetosa*.
Submergence activates the expression of *HB33* and *ABF2* (*HOMEBOX PROTEIN 33*, *ABRE BINDING FACTOR 2*; Fig. 2.5), these are positive regulators of abscisic acid signalling and could thus act via gibberellic acid to retard growth. Simultaneously, submergence-induced *TPP (TREHALOSE-P-PHOSPHATASE)* expression could lead to a reduction in levels of its substrate T6P (Trehalose-6-phosphate). T6P inhibits activity of the growth regulating SnRK1 complex, whose specific activity is modulated by the β-SnRK1 subunit. Nitrogen metabolism, soluble sugars and starch metabolism, β-SnRK1 and PCK (PEP CARBOXYKINASE) activity are interconnected processes that are regulated during submergence likely resulting in a maintained carbon status.

2005; Duek et al., 2004; Jang et al., 2005; Hyun and Lee, 2006; Lee et al., 2006). Simultaneously, shade markers like the class II homeodomain-leucine zipper (*HD-ZIPII*), a family of positive regulators of shade avoidance (Steindler et al., 1999); the negative regulator of elongation growth *ELONGATED HYPOCOTYL* (*HY5*) (Osterlund et al., 2000; Li et al., 2010); and several PIFs appeared to have species specific regulation (Fig. 2.7). To verify the results of this global transcript profiling and exclude any time-dependent submergence effects, the submergence-induced expression of these OMCL families involved in photomorphogenesis were determined in a time-series (Fig. 3.4). This experiment identified a clear induction of *COP1*, *KIDARI*, *HD-ZIPII* and a *PIF* (fam03405). Based on knowledge from *Arabidopsis*, the combined and strong regulation of *KIDARI* and *COP1* transcripts would indirectly act via a release of PIF suppression, and the transcriptional activation of *PIF* and *HD-ZIPII.* These results suggest that photomorphogenesis-related OMCL families, typical of light dependent regulation of elongation, are also employed in submergence-induced elongation of petioles of *R. palustris*.

Directly upon submergence *R. palustris* orientates its leaves vertically (hyponasty) (Fig. 3.5a). This may result in horizontal far-red (FR) reflection from one leaf to another, much the same way as in dense canopies where this is an early signal for shade (de Wit et al., 2012). Measurements of the changes in red to far-red ratio (R:FR) between vertical leaves revealed that there was indeed a reduction in the R:FR, from 2.2 (standard growth chamber light quality) to 1.6 in *R. palustris*. Such a FR reflection was not observed in *R. acetosa* consistent with the lack of hyponasty in this species (Fig. 3.5a). However, experimentally reducing R:FR to 1.6 by supplying additional FR light was not sufficient to induce an elongation response or to induce the expression of a set of photomorphogenesis genes under non-submerged conditions in either species. Nonetheless, both *R. palustris* and *R. acetosa* were able to respond to a strong reduction in R:FR from 2.2 to 0.2 with enhanced petiole elongation and elevated marker gene expression (Fig. 3.5b-c). Furthermore, artificial

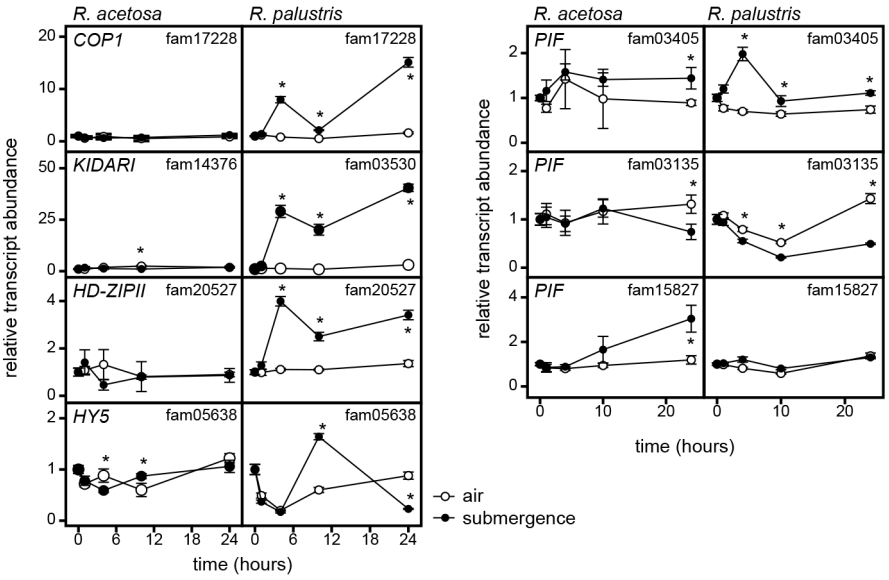


Figure 3.4 Transcript abundance of seven OMCL families over time under air or submerged conditions for *R. acetosa* and *R. palustris* (mean ± sem, n=5; * P<0.05, Tukey LSD).

filtering of FR wavelengths, resulting in high R:FR ratios underwater, did not alter the submergence-induced elongation response in *R. palustris* (Fig. S3.2). Overall, this suggests that the elongation response of *R. palustris* employs transcripts associated with photomorphogenesis and shade avoidance independent of the light environment.

Using pharmaceutical inhibitors, the role of the hormones ethylene and ABA in the regulation of these photomorphogenesis genes was explored. Indeed, the inhibition of ethylene perception by the ethylene receptor inhibitor 1-methylcyclopropene (1-MCP) demonstrated that during submergence, ethylene action is required for the activation of *COP1*, *KIDARI*, *HD-ZIPII and PIF* (Fig. 3.6a). The rapid accumulation of ethylene within submerged *R. palustris* leads to a fast decline in ABA content (Benschop et al., 2005). ABA acts as a major inhibitor of downstream elongation (Benschop et al., 2006; Cox et al., 2004). Addition of exogenous ABA was able to block the ethylene dependent induction of *COP1*, *KIDARI* and *HD-ZIPII* (Fig. 3.6b). Depletion of internal ABA levels by pre-treatment with the biosynthesis inhibitor fluridone appears to result in a higher expression, especially of *COP1*. The ABA requirements for the *PIF* expression underwater remain unclear. ABA content is not down-regulated in *R. acetosa* upon submergence (Benschop et al., 2005), potentially explaining the lack of regulation of photomorphogenesis genes in this species.

These results suggest that in *R. palustris,* shade cues and submergence converge on a similar signal transduction pathway (Pierik et al., 2010). A possible scenario for control of submergence-induced shoot elongation in *R. palustris* (Fig. 3.6c) would therefore be that ethylene accumulation, resulting in ABA down-regulation (Benschop et al., 2005), stimulates the expression of *KIDARI* and *COP1*. On top of ethylene induced *PIF* expression, KIDARI and COP1 would stimulate PIF action and therefore elongation growth. Simultaneously, *HD-ZIPII* also requires ethylene-mediated ABA degradation for its transcriptional activation,

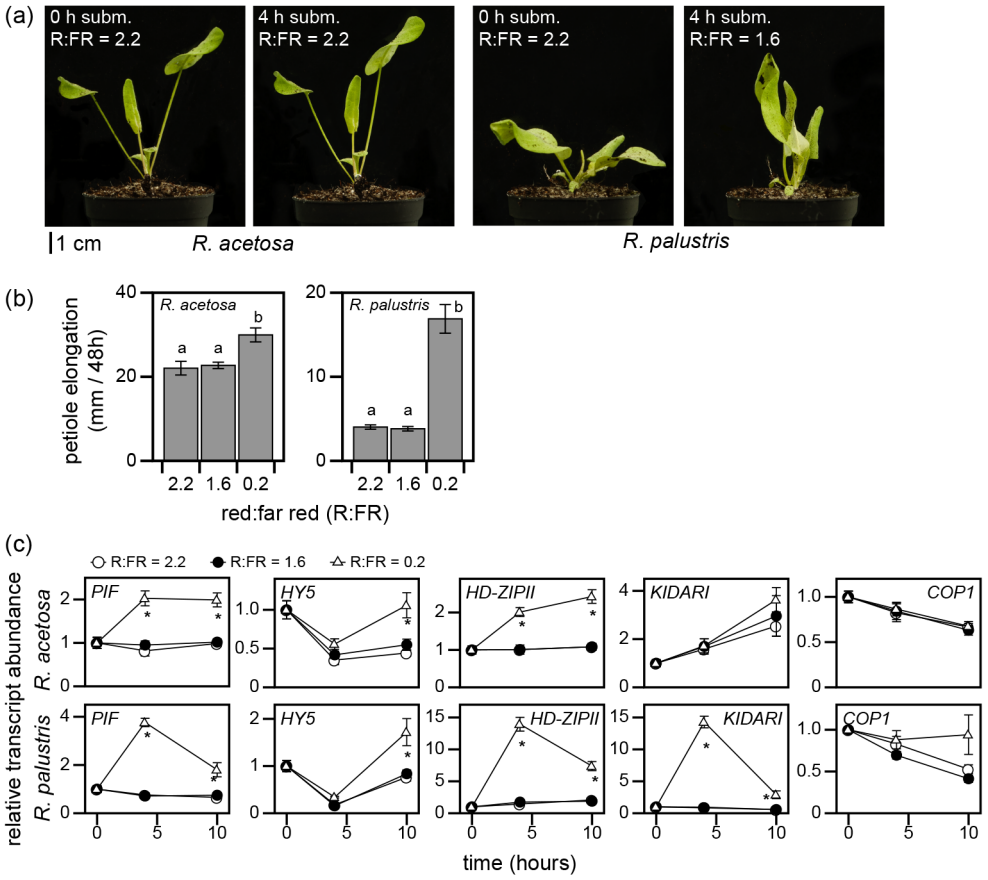


Figure 3.5 The role of light quality in underwater growth.
(a) Photographs demonstrating the typical hyponastic response of *R. palustris* upon submergence and the lack of this response in *R. acetosa*. R:FR (red : far-red light) is measured between hyponastic lamina or at the lamina surface under submerged or air conditions.
(b) Petiole elongation under various R:FR treatments (ambient (2.2), submergence mimic (1.6) and very low R:FR (0.2)) of the youngest leaf (third leaf) over a 48 hour period (mean ± sem, n=12; letters P<0.05, Tukey LSD).
(c) *Rumex* OMCL family transcript accumulation of key regulators of light modified elongation in petioles upon three R:FR levels (mean ± sem, n=5; *P<0.05 Tukey LSD). PIF is fam03405.

likely further enhancing elongation growth. Downstream of these modules several other regulators, such as GA, might be affected. Indeed GA levels start to increase after 6 hours of submergence in *R. palustris*, significantly after the initiation of elongation (Benschop et al., 2006). The ethylene-driven activation of photomorphogenesis and shade avoidance-associated genes appears not to occur during flooding-induced shoot elongation in lowland rice varieties, where amongst the differentially expressed genes from a transcriptome-wide analysis (Jung et al., 2010) no shade avoidance or photomorphogenesis genes were identified. With the strong induction of *KIDARI* and *COP1* in *R. palustris* and not in *R. acetosa*, and with their light independent regulation, we suggest that these genes are

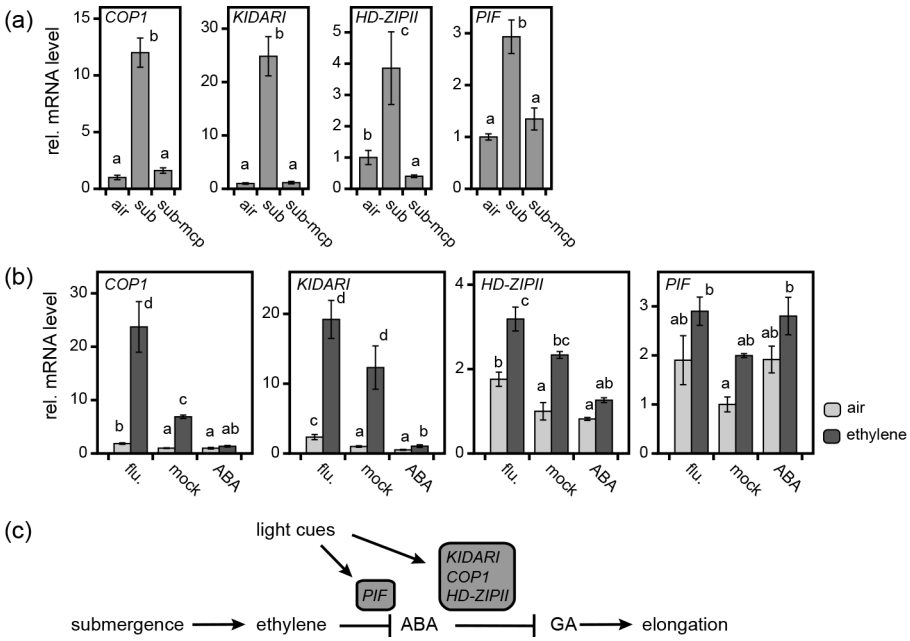


Figure 3.6 The role of hormones in photomorphogenic gene regulation.
(a) Effect of the ethylene action inhibitor 1-MCP during submergence (sub) on the expression of four light signaling genes in *R. palustris* after 4 h of treatment (mean ± sem, n=5; letters P<0.05, Tukey LSD).
(b) The effect of ethylene and abscisic acid (ABA) manipulations on the expression of four light signalling genes in *R. palustris* after 4 h of treatment (mean ± sem, n=4; letters P<0.05, Tukey LSD). Flu. is the ABA biosynthesis inhibitor fluridone.
(c) Schematic representation of the convergence between the submergence signals ethylene, ABA and gibberellic acid (GA), and light cues on photomorphogenesis genes.

regulators of the underwater elongation response using a conserved signal-transduction network that is also activated in response to light cues.

Materials and methods

Plant growth and experimentation.

R. acetosa and *R. palustris* seeds were germinated on floating polyethylene beads for 10 days (12h light, 25°C; 12h dark, 12°C). After potting (Chapter 2) plants were grown for 16 days in climate-controlled walk-in growth chambers (160 µmol m^{-2} s^{-1} Photosynthetically Active Radiation; 16 h day, 8 h night; 20°C, 70% relative humidity). Plants were completely submerged (depth of 25 cm) in overnight acclimatized water. 1-MCP pretreatment was applied in glass dessicators with 2 μL L^{-1} 1-MCP gas for 1 hour. Ethylene treatments were performed in humidified glass cuvettes with a continuous gas through-flow, with 5 μL L^{-1} ethylene-air gas mixture. Fluridone (10 ml 100 μM, 0.1% acetone; or mock, 0.1% acetone) was added to the soil 3 days prior the experiment and ABA (20 μM, 0.1% ethanol; or mock, 0.1% ethanol) sprayed on the plants 42, 18, 2 and 1 hour prior the experiment. Reduced

R:FR ratios were obtained through supplemental far red LED illumination (730 nm). Petiole elongation was determined by a digital calliper or based on photographs analysed with imageJ software.

RNA isolation and expression.

RNA was extracted from the youngest petiole using the kiefer protocol (Benschop et al., 2005; Pierik et al., 2010). This was followed by DNAse treatment (Ambion, DNA-free) and subsequent reverse transcription with random hexamers and including RNase inhibitor (Invitrogen). qRT-PCR was performed using a 20 μL SYBR Green reaction mixture with gene-specific primers (Appendix, Table A1) and *TUBULIN* as reference.

Metabolite profiling and enzyme activity.

Gas chromatography-mass spectrometry (GC-MS) and ultra performance liquid chromatography (UPLC)-MS metabolite profiling was performed as previously described (Barding et al., 2013). The metabolites 3-phosphoglycerate, 6-phosphoguconate, oxaloacetate, and α-ketoglutarate were analyzed by UPLC-MS and sucrose, glucose, citrate, succinate, malate, pyroglutamate, aspartate, glycine, myo-inositol, ascorbate, shikimate, glutamate, β-alanine, lactate, glycine, β-sitosterol, campesterol, tartrate, propanoate and erythronate were analyzed by GC-MS. For UPLC-MS measurements, WAX SPE cartridges were conditioned with methanol (MeOH) followed by basified water. Dried extracts were reconstituted in water, loaded onto the cartridge, washed with water and MeOH, eluted with 80/20 H_2O/MeOH containing 5% formic acid and dried by speed vacuum. Samples were reconstituted in 200 μL water and 20 μL was injected onto a 2.1 x 100 mm Acquity HSS T3 column. Solvent A was aqueous 10 mM dibutylamine buffer pH 7.4 and solvent B was 10 mM dibutylamine buffer in MeOH at a pH meter reading of 8.0. Mass spectrometry measurements used a Waters Electron spray ionization quadrupole time-of-flight (ESI qTOF) operated in negative mode. Metabolite identities were confirmed through a comparison of an in-house library generated from metabolite standards.

Adenylates, amino acids, nitrate and ammonia were identified from KOH (0.4 M) neutralized and bicine (1 M) buffered perchloric acid (0.83 N) extracts. The subsequent pellet was used for starch and protein determination after washing three times with ethanol and solubilisation with 0.2 M KOH (90ºC, 60 min). Adenylates were determined as previously described (Mustroph et al. 2006), as were ammonia (Novozamsy et al. 1974), nitrate (Cataldo et al. 1976), amino acids (Rosen, 1957), protein (Bradford et al., 1976) and starch (Mustroph et al. 2006). Phosphoenolpyruvate carboxykinase (PCK) activity was determined by assaying the activity in the reverse direction through monitoring the decline in NADH as previously described (Malone et al., 2007).

Respiration

Excised youngest petioles, one for *R. acetosa* and two for *R. palustris* per replicate, were fixed in 2 mL glass micro-respiration chambers filled with tap water, which was also used for submergence experiments. A decline in O_2 levels was determined with O_2 micro-electrodes (MicroResp, Unisense A/S, Denmark) at 20ºC in darkness (Brodersen et al., 2008).

Supporting Information

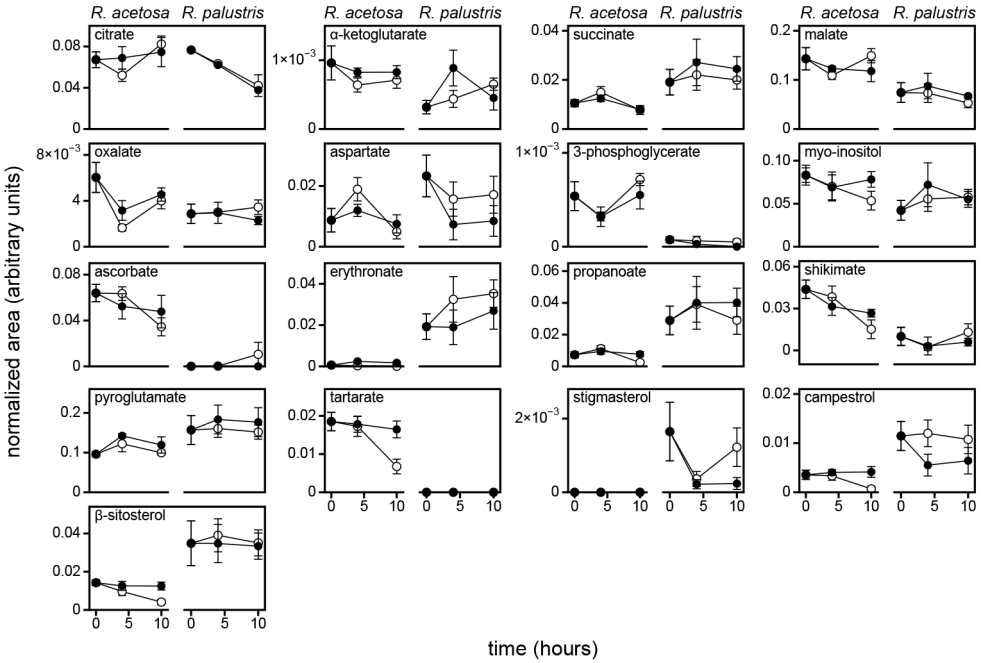
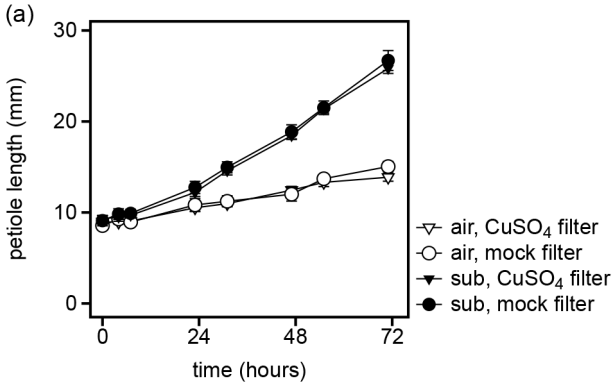


Figure S3.1 Selection of metabolites identified by GC-MS and UPLC-MS in two *Rumex* species (mean±sem, n=5). Remainder of metabolites and statistical results can be found in figure 3.1 and table 3.1.

(a)



(b)

red:far red	chamber 1		chamber 2
	mock filter	$CuSO_4$	no filter
horizontal leaf	1.6	13.4	2.2
hyponastic leaf	1.2	6.2	1.6

Figure S3.2 Effect of far red light removal on submergence-induced elongation growth of *R. palustris*.
(a) Petiole growth over time in the presence and absence of a far red light absorbing thin layer of $CuSO_4$ solution in a shallow perspex container (mean ± sem, n=6; sub = submergence).
(b) Red: far red (R:FR) in chamber 1 where light was selectively filtered and chamber 2 where plants were grown from seedling to a three leaf developmental age. The mock filter (perspex container with water) favours absorption of shorter wave lengths leading to a slight drop R:FR in chamber 1 compared to chamber 2. The CuSO4 drastically alters R:FR preventing the R:FR drop perceived by the leaf blade during the underwater hyponastic response.

Chapter 4

Novel candidate regulators of flooding escape in *Rumex palustris*

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Abstract

To overcome the adverse effects of the underwater environment *Rumex palustris* adopts an escape strategy. When submerged, vertical orientation of the leaf and rapid elongation of the petiole allow the plant to regain aerial contact, dramatically improving plant performance. In this study we have built on previous work on *R. palustris* as a model system to study flooding-induced shoot elongation. The existing physiological knowledge on hormonal interactions and cell wall loosening, combined with the here studied tissue specific gene regulation, provided a detailed framework of important processes during the submergence escape strategy. This encompasses an early downregulation of ABA throughout the entire plant; a rapid induction of cell wall loosening in the petiole; and activation of GA biosynthesis and novel molecular elongation regulators, specifically in the petiole. By associating the transcriptional behaviour of candidate genes with unknown roles, identified from global transcriptome profiling (Chapter 2), with the well-established processes (hormonal regulation and cell wall loosening), allowed elucidation of the potential functions of these uncharacterized and novel genes. This guilt by association approach provided three candidate genes for early regulation and seven for long-term regulation of the escape strategy, thus providing a firm basis for future molecular characterization of these novel genes and thus broadening our understanding of the submergence escape strategy.

Introduction

During seasonal flooding events many species adapt to complete submergence by a rapid elongation of the shoot to escape from the floodwater (Voesenek et al., 2004). Underwater gas exchange is severely limited as gas diffusion rate is 10^4 slower than in air, which is the underlying cause of plant mortality during flooding. Complete submergence normally leads to low oxygen availability in the root (Winkel et al., 2013; Vashisht et al., 2010), and depending on the light availability and the photosynthetic ability shoot oxygen levels can range from 3-24 kPa (Mommer et al., 2007). If the water is not too deep and shoot elongation sufficiently vigorous, aerial contact can be made via an escape strategy, overcoming limited gas exchange underwater.

Species that reclaim aerial contact via an escape strategy have improved O_2 levels within the entire plant (Colmer and Pedersen, 2008). This improved aeration throughout the submerged and belowground plant parts occurs via longitudinal connected airspaces, aerenchyma (Colmer, 2002), and even leads to increased biomass accumulation (Pierik et al., 2009; Akman et al., 2012). Given the effectiveness of the escape strategy in improving plant performance, understanding the processes that mediate this type of elongation is very important. To elucidate the underlying processes of underwater elongation growth, most work has used primarily deepwater rice and *Rumex palustris* as model species.

Here we made use of the previous physiological work and the recent whole transcriptome characterization of *R. palustris* petioles (Chapter 2) to further increase our understanding of the submergence escape strategy. When submerged, *R. palustris* rapidly accumulates ethylene within the entire plant (Banga et al., 1996; Voesenek and Sasidharan, 2013). This serves as an early signal initiating the escape response. Prior to petiole elongation in primarily the youngest leaves, leaf orientation changes from a horizontal to a vertical position (hyponasty). Once hyponastic, petiole elongation commences, which is approximately 2-3 hours after submergence (Cox, 2003).

A prerequisite of both hyponastic movement and petiole elongation is the reduction of endogenous abscisic acid (ABA) levels (Cox et al., 2004), which happens via a decline in ABA biosynthesis through reduced expression of *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE* (*NCED*) genes (Benschop et al., 2005) and increased ABA breakdown via induction of *ABA 8'-HYDROXYLASE* (*ABA8ox*) genes (Fig. 2.5). The decrease of ABA is fast and is initiated within 15 minutes of submergence and finished after 2 hours and is evident in all plant organs (Benschop et al., 2005). Subsequently the ABA inhibition on gibberellic acid (GA) biosynthesis is released in an ethylene dependent manner. GA biosynthesis is subsequently activated via an upregulation of *GA3 OXIDASE* (*GA3ox*) and results in increased GA_1 levels after 6 h of submergence (Benschop et al., 2006). Simultaneously, the sensitivity to GA is enhanced by ethylene, however, the exact timeframe in which this occurs has not been established yet (Rijnders et al., 1997). Chemically blocking either ABA breakdown or GA biosynthesis prevents the escape response of submerged *R. palustris* (Cox et al., 2004; Benschop et al., 2006; 2005).

Photomorphogenesis genes were recently identified as another important group of regulators of the escape response in *R. palustris* (Chapter 2 and 3). This involves a

signalling hub consisting of the submergence-induced orthologs of *KIDARI* and *COP1* (*CONSITITUTIVE PHOTOMORPHOGENIC 1*) in *Arabidopsis*, which act via stabilization of elongation stimulating PIFs (PHYTOCHROME INTERACTING FACTORs) (Leivar and Quail, 2011; Hyun and Lee, 2006). In addition, two positive regulators of shade avoidance, a *PIF/PIL* (*PIF LIKE*) and a *HD-ZIPII* (*CLASS II HOMEODOMAIN-LEUCINE ZIPPER*) (Steindler et al., 1999) were also upregulated upon submergence. These four genes were induced in *R. palustris* in an ethylene-dependent manner but not until 4 hours of submergence and were strongly suppressed by elevated ABA levels (Fig. 3.4 and 3.6).

Where the hormonal interactions and the submergence-induced photomorphogenesis genes provide a signalling framework, the physical elongation of submerged petioles is the result of cell elongation throughout the entire petiole (Voesenek et al., 1990). The main driver of this cellular expansion is loosening of the cell wall, which, in *R. palustris* occurs through a rapid apoplastic acidification, and the upregulation of cell wall modifying agents such as an expansin (*EXPA1*), (Vreeburg et al., 2005) xyloglucan endoTransglucosylase/Hydrolases (*XTH*s) and pectinesterases (Fig. 2.5).

Global trancriptome profiling also identified a large suite of additional regulated genes that were specifically regulated by the escaping *R. palustris*, and not by the close relative *R. acetosa* that doesn't elongate underwater (chapter 2). OMCL families (group of highly similar sequences of *R. acetosa* and *R. palustris*; Fig. 2.2) with a well-annotated function, such as the photomorphogenesis-genes, could with reasonable confidence be assigned a putative role during submergence responses, as similar homologs have been well described in other species. However, for many transcripts that were specifically regulated by *R. palustris*, a clear role in shoot elongation remains elusive, because it either had a very low homology to other gene sequences, or no characterization has been done in other species. Though specific to *R. palustris*, these genes are not necessarily involved in elongation, as *R. acetosa* and *R. palustris* differ in more ways than only their underwater elongation responses. Here the aim was to deduce a putative function for these unknown genes. To this end we investigated (i) the tissue specificity and temporal regulation of hormonal marker, cell wall modifying and photomorphogenesis OMCL families; and (ii) tissue specific expression, hormonal dependant transcriptional regulation and temporal expression patterns of novel candidate OMCL families. Correlating the transcriptional patterns of genes with unknown function in specific tissues, over time and in responses to hormones with the corresponding established process in shoot elongation allowed for the assignment of a putative function for these genes, thus providing a basis for future functional characterization.

Results

Regulation of hormone interactions, light signalling and cell wall loosening genes occurs in a temporal and tissue specific manner

Changes in ABA and GA metabolism, light signalling genes, and cell wall modification have been identified as important processes that mediate flooding escape in *R. palustris*. Here, the transcript levels of gene-families (OMCL families), as identified in chapter 2 via the

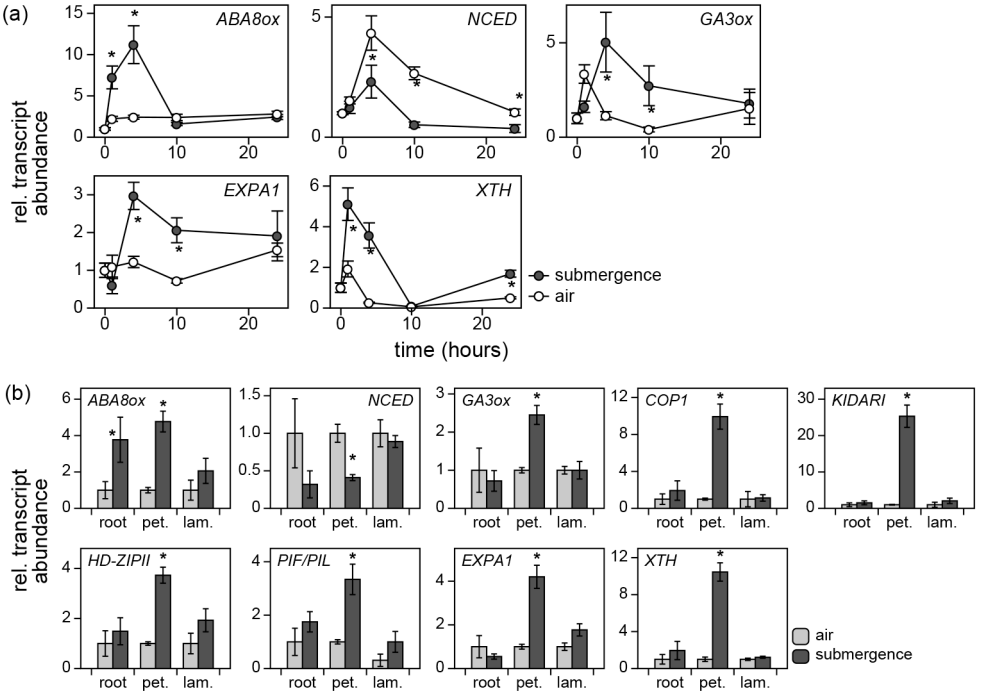


Figure 4.1 Transcriptional changes of important molecular components required for distinct aspects of the submergence escape response of *R. palustris*.

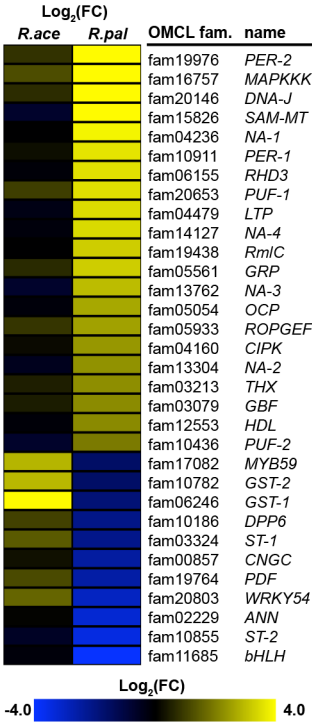
(a) Temporal changes of transcript abundance in the youngest petiole (n=5).

(b) The tissue specific transcript abundance after 4 hours of submergence or control conditions. Here root is the material from the entire root system, pet. and lam. are the petiole and lamina of the youngest developing leaf (n=4).

Value are mean ± sem, asterisk is $p<0.05$ (t-test). OMCL Family IDs: *EXP1* - fam00649; *XTH* - fam16375; *GA3ox* - fam16203; *ABA8ox* - fam00337; *NCED* - fam18188; *COP1* - fam17228; *KIDARI* - fam03530; *PIF/PIL* - fam03405; *HD-ZIPII* - fam20527.

OrthoMCL clustering algorithm (Enright, 2002; Li et al., 2003), were used as a proxy to establish and confirm the tissue specificity and temporal behaviour of these characteristic components of the submergence escape strategy. Indeed, *ABA8ox* (ABA catabolism) transcript levels increased already within 1 hour of submergence in the petiole. However, a downregulation of the ABA biosynthesis OMCL family *NCED,* and an upregulation of the GA biosynthesis via *GA3ox* was not observed until 4 hours after submergence. Transcript levels of the cell wall modifying OMCL families *XTH* and *EXPA1*, were induced after 1 and 4 hours, respectively (Fig. 4.1a). The light signalling genes are most likely involved in the molecular regulation of the submergence escape strategy in *R. palustris* (chapter 3). OMCL families of *COP1*, *KIDARI*, *HD-ZIPII* and a *PIF/PIL* are not induced until 4 hours after submergence (Fig. 3.4)

Apart from temporal variation, OMCL family transcript levels also varied between different plant organs after 4 h of submergence (Fig. 4.1b). *ABA8ox* was upregulated in both the submerged root and the petiole. Transcriptional changes for *NCED* were unclear for root tissue, but this gene was significantly downregulated in the petiole. Upregulation of *GA3ox*



OMCL fam.	name	AGI	Eval	description
fam19976	*PER-2*	AT5G05340	1.0E-19	Peroxidase
fam16757	*MAPKKK*	AT1G07150	1.0E-33	Mitogen-activated Protein Kinase Kinase Kinase
fam20146	*DNA-J*	AT3G14200	2.0E-44	mol. Chaperone, DNA-J domain
fam15826	*SAM-MT*	AT4G36470	2.0E-05	S-adenosyl-L-methionine-dependent methyltransferase
fam04236	*NA-1*	NA		no hit
fam10911	*PER-1*	AT5G42180	7.0E-85	Peroxidase
fam06155	*RHD3*	AT1G72960	2.0E-11	GTP binding, vessicle transport, cell enlargement
fam20653	*PUF-1*	AT2G23790	6.0E-43	Unknown Function
fam04479	*LTP*	AT4G12510	4.0E-41	Lipid transfer Protein; lipid transport and binding
fam14127	*NA-4*	NA		
fam19438	*RmlC*	AT5G39120	1.0E-31	RmlC-like cupin, a compound binding protein
fam05561	*GRP*	AT4G38680	5.0E-05	Glycine Rich Protein
fam13762	*NA-3*	NA		
fam05054	*OCP*	AT5G11270	3.1	Overexpressor of Cationic Peroxidase
fam05933	*ROPGEF*	AT1G52240	7.0E-69	Rho Guanyl-Nucleotide Exchange Factor, cytoskeleton organisation
fam04160	*CIPK*	AT5G01820	9.0E-19	CBL-interacting serine/threonine protein kinase
fam13304	*NA-2*	NA		
fam03213	*THX*	AT2G47870	4.0E-46	Thioredoxin
fam03079	*GBF*	AT2G46270	4.0E-40	a bZIP G-box binding
fam12553	*HDL*	AT3G10760	7.0E-39	Homeodomain-like, DNA binding
fam10436	*PUF-2*	AT2G28140	1.0E-13	Unknown Function
fam17082	*MYB59*	AT5G59780	1.0E-79	MYB-TF, cell cycle progression
fam10782	*GST-2*	AT3G09270	4.0E-23	Glutathione-S-Transferase
fam06246	*GST-1*	AT2G29490	6.0E-22	Glutathione-S-Transferase
fam10186	*DPP6*	AT1G21680	6.0E-134	DPP6 N-terminal domain, unclear function
fam03324	*ST-1*	AT5G07010	8.0E-10	Sulfotransferase
fam00857	*CNGC*	AT5G53130	6.0E-25	Cyclic Nucleotide Gated Channel 1
fam19764	*PDF*	AT2G42840	0.021	ProtoDermal Factor, L1 layer specific
fam20803	*WRKY54*	AT2G40750	3.0E-14	a WRKY-TF, DNA binding
fam02229	*ANN*	AT2G38750	3.0E-25	Annexin, Ca dependent membrane binding, golgi mediated secretion
fam10855	*ST-2*	AT2G03760	5.0E-05	Sulfotransferase
fam11685	*bHLH*	AT4G16430	2.0E-22	basic Helix-Loop-Helix

Figure 4.2 Heatmap of novel candidate OMCL families with an undetermined role that specifically were regulated in *R. palustris* during submergence responses, with details of their closest *A. thaliana* homologs.

and the cell wall modifiying *XTH* and *EXPA1* was petiole specific. The light signalling associated OMCL families *COP1*, *KIDARI*, *HD-ZIPII* and *PIF/PIL* were also transcriptionally upregulated only in the petioles.

Tissue specific, temporal and hormonal regulation of submergence-induced OMCL families identifies putative candidates associated with elongation

In chapter 2 a large range of OMCL families were identified that were regulated in response to submergence in *R. palustris*, but not in the non-escaping *R. acetosa*. For some conserved sequences, a clear role could be assigned based on functional characterization of orthologous sequences in other species, primarily *Arabidopsis*. However, such information was lacking for many of the identified OMCL families, either due to low similarity or lack of characterization of the putative homolog. We selected 32 OMCL families that were specifically regulated only in submerged *R. palustris* and were not previously functionally associated with submergence-induced shoot elongation (Fig. 4.2). The tissue specific expression pattern of these 32 OMCL families was investigated after 4 hours of submergence. The tissue specific expression provides the possibility to identify OMCL families associated with either the whole plant or specifically involved in the petiole submergence responses. Eleven gene transcripts were induced in the petiole only, and only

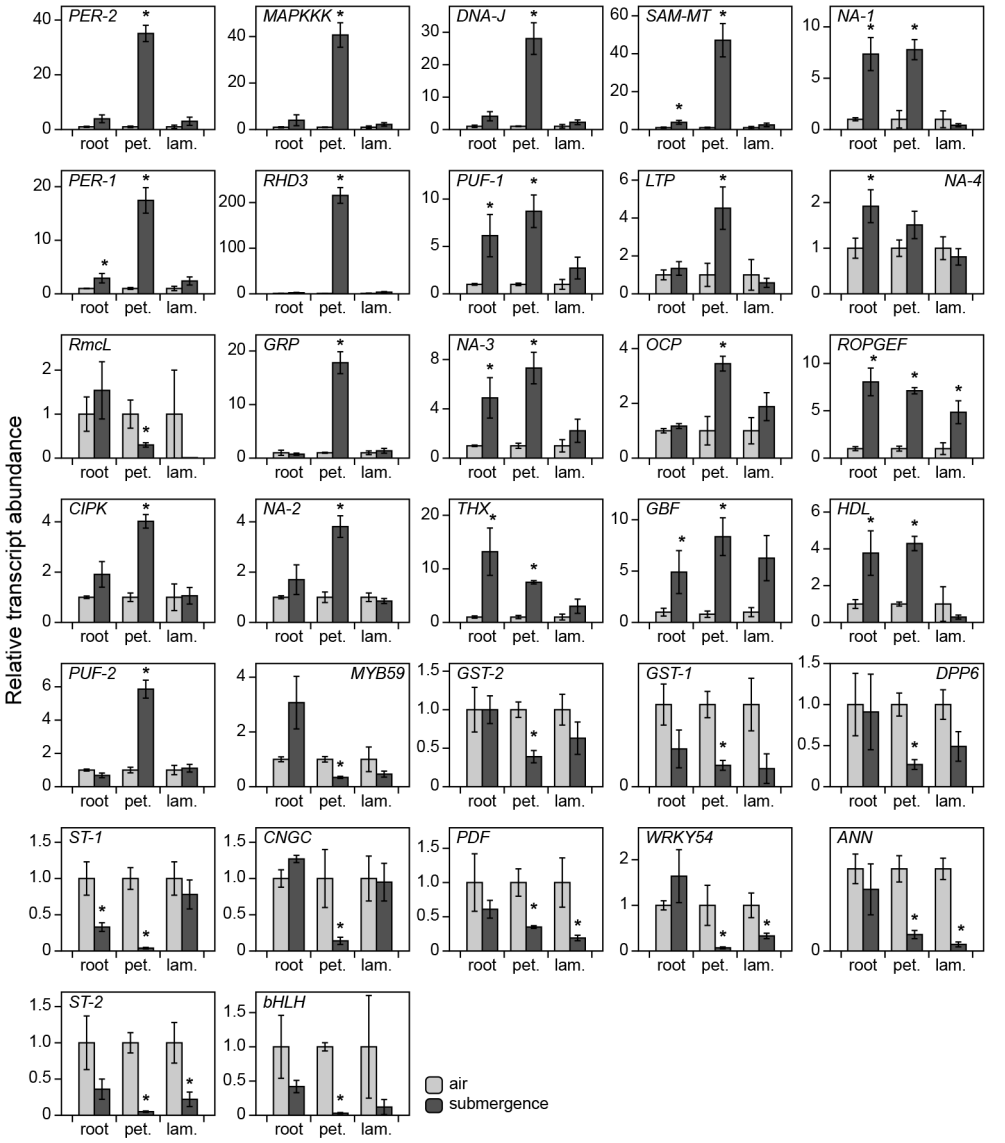


Figure 4.3 Submergence-induced changes in transcript abundance of novel candidate OMCL families in different plant tissues after 4 hours in *R. palustris*. Root is the material from the entire root system, pet. and lam. are the petiole and lamina of the youngest developing leaf (n=4). Values are mean ± sem, asterisk indicates significance between air and submergence for a specific tissue type ($p<0.05$, t-test).

one, *CNGC* (*CYCLIC NUCLEOTIDE GATED CHANNEL*) was specifically downregulated in the petiole (Fig. 4.3).

Petiole specific OMCL families were further investigated for their ethylene responsiveness, and their dependence on low ABA levels for upregulation. Also two OMCL families that were regulated throughout the plant (*PUF-1*, *NA-3*) were investigated for their

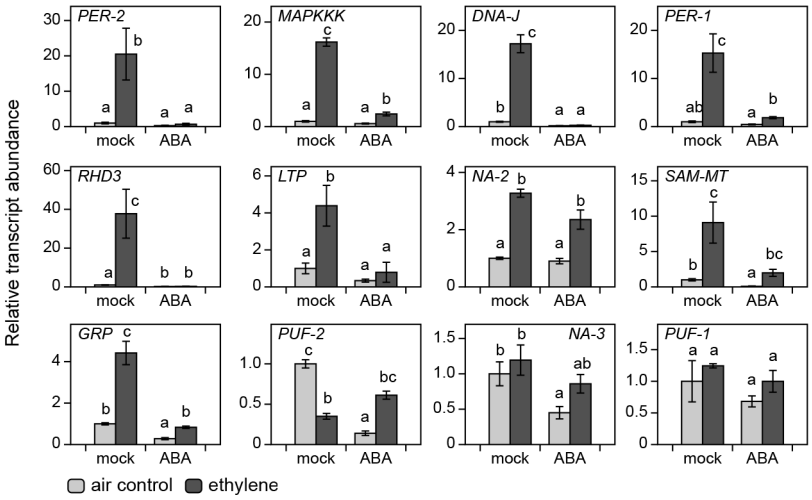


Figure 4.4 Ethylene (5 μL L^{-1}) and abscisic acid (ABA; 20 μM spraying pre-treatment) dependent regulation of OMCL families in petiole tissue of *R. palustris* after 4 hours of ethylene treatment. Values are mean ± sem, n=4, letters indicate significance at p<0.05 (Tukey LSD).

ethylene and ABA responsiveness, as representatives of whole plant regulated OMCL families. Nine out of ten of the tested petiole specific OMCL families were strongly upregulated by ethylene. For six of these the ethylene induction was negated by supplying exogenous ABA (Fig. 4.4). Despite petiole specific induction, *NA-2* ethylene-mediated induction was unaffected by exogenous ABA application. *GRP* and SAM-MT expression was seriously reduced by ABA, however, even after ABA treatment they retained the capacity to respond to ethylene. *PUF-1* and *NA-3*, that were not petiole specific (Fig. 4.3), did not respond to ethylene, nor did ABA treatment affect the ethylene responsiveness.

In line with the low ABA level requirement for activation by ethylene (Fig. 4.4), a temporal analysis during submergence showed that the expression of these petiole specific OMCL families was not significantly induced until at least four hours of submergence (Fig. 4.5). Indeed, at this point in the submergence response ABA levels are at their lowest level in the petiole (Benschop et al., 2005), thus allowing ethylene stimulation. Increased transcript levels of *GRP, NA-2* and *SAM-MT*, which retained ethylene induction independently of ABA levels, were observed already after 30 minutes of submergence. Indeed, these OMCL families appeared unhindered by the relatively high ABA levels during the very early stages of submergence.

Discussion

The current work combined with the past detailed studies on the *R. palustris* escape strategy, revealed a robust framework of regulatory processes. Following submergence, ABA is rapidly degraded primarily in the roots and petioles via up regulation of the catabolic *ABA8ox*, and a later reduction of the biosynthesis related *NCED*. The whole plant response, can therefore, on the basis of ABA catabolism, be separated into an early and late phase.

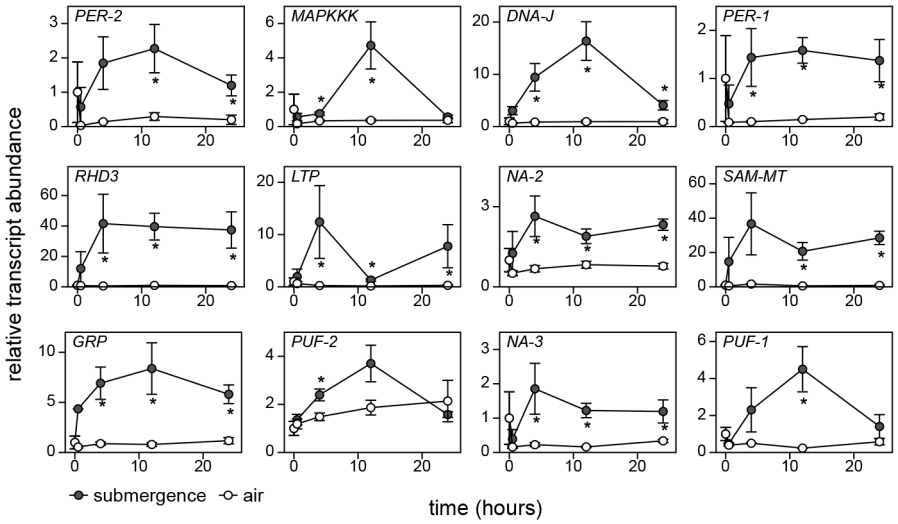


Figure 4.5 Change in transcript abundance in *R. palustris* petioles over time during submergence. Values are mean ± sem (n=4), asterisk indicates significant difference between air and submergence at a particular time point ($p<0.05$, Tukey LSD).

The early petiole specific transcriptional response is restricted to *XTH*, whilst late (four hours) petiole specific OMCL families are *GA3ox*, *COP1*, *KIDARI*, *HD-ZIPII*, *PIF/PIL* and *EXPA1*. Here only *EXPA1* induction does not require ABA removal (Vreeburg et al., 2005). Responses to the physiological stress of the underwater environment, such as carbon limitations, likely occur throughout the plant.

The transcriptional changes of OMCL families that previously were not associated with underwater elongation were investigated for their tissue specific, temporal and hormone controlled regulation. This allowed us to associate these novel candidate OMCL families with established processes that share the same temporal, spatial and hormonal specificity. A schematic representation of the previously described processes in underwater elongation in *R. palustris* and the involvement of new candidate OMCL families is depicted in figure 4.6.

Given that upon submergence, transcriptional regulation of GA biosynthesis, cell wall loosening and light signalling genes occur exclusively in the petiole, petiole specificity of the novel candidates suggests a role in these processes that mediate underwater elongation. Candidate genes whose expression is not limited to the petiole likely play a function in whole plant regulated processes such as ABA metabolism, and processes linked to adaptation to carbon limitation and stress imposed by submergence.

The requirement of reduced ABA levels and the late induction observed for the majority of petiole specific OMCL families (Fig. 4.4 and 4.5) indicates that these cannot be involved in very early processes such as the rapid induction of *XTH* (Fig. 4.1) or apoplastic acidification (Vreeburg et al., 2005). This is in contrast to relatively early induced OMCL families, where increased expression was independent of low ABA (*SAM-MT*, *NA-2* and *GRP*). A role in hyponasty, also an early response, seems unlikely for the early novel candidates given that hyponasty occurs via enhanced cell growth limited to a minor portion of cells at the abaxial

basal side of the petiole (Cox et al., 2004). Here the entire petiole was taken for gene expression measurements. It is questionable whether transcriptional changes of an entire petiole would bear any significance to the highly localized hyponastic process.

Amongst the novel candidate OMCL families, very few early genes were identified. These candidate OMCL families were identified from a global transcriptome study at four hours of submergence (Chapter 2). It is possible that a wave of transcriptional changes has already occurred prior to this point in time. Nevertheless, the typical early genes *ABA8ox* and *XTH* do persist in their high expression levels up to at least four hours of submergence. *GRP*, a *GLYCINE RICH PROTEIN*, was one of the few early genes. GRPs are a large family of proteins involved in a range of processes such as cell wall scaffolding and cold stress amongst others (Sachetto-Martins et al., 2000). However, the similarity of the *R. palustris* GRP to GRPs in other species is very low (Eval=5E-5). *SAM-MT* (*S-ADENOSYL-L-METHIONINE-DEPENDENT METHYLTRANSFERASE*) was also an early OMCL family without a low ABA requirement for induction. The homology of *SAM-MT* (Eval=2E-5) to a methyltransferase that is dependent on the ethylene biosynthesis precursor (SAM) is an interesting observation given the potential role of *SAM-MT* in ethylene-driven elongation. Like *GRP* and *SAM-MT*, also the *NA-2* ethylene induction was independent of ABA. The

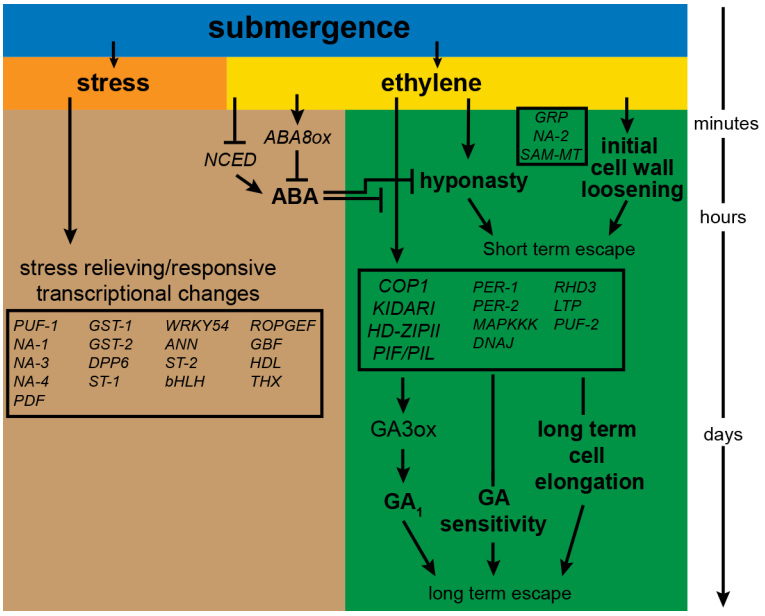


Figure 4.6 Novel transcripts in a tissue specific and temporal scheme of the submergence escape strategy in *R. palustris*. Here the green panel represents processes occurring only in the petiole, and the brown panel processes also in the root system and/or lamina. Submergence leads to ethylene accumulation and physiological stress. Typically stress responsive transcripts would be found throughout the plant. The ethylene accumulation leads to a breakdown of ABA (abscisic acid) in the entire plant. This relieves the block on ethylene-induced hyponasty, and induction of further downstream light signalling genes such as *COP1*, *KIDARI* and *HD-ZIPII*, all in the petiole. The initial cell wall loosening is independent of ABA, but mediated by ethylene. The novel candidates *GRP*, *NA-2* and *SAM-MT*, would likely play a role in the ABA independent early processes in the petiole. Other petiole specific candidates likely function in longer term processes such as GA biosynthesis, changes in GA (gibberellic acid) sensitivity and cell elongation.

early petiole specific and ABA independent regulation of *GRP*, *SAM-MT* and *NA-2* suggests a role in mediating early cell wall loosening responses, possibly in concert with *XTH*.

For the majority of identified petiole-specific genes, ethylene induction was dependent on ABA levels (Fig. 4.4). After the drop in ABA, a multitude of processes take place in the petioles, such as GA biosynthesis, increase in GA sensitivity and continued cell enlargement. Amongst candidates potentially involved in these processes (Fig. 4.6) some were found to have good homology with their *Arabidopsis* counterparts, namely the two peroxidases (*PER-1*, *PER-2*; Eval = 7E-85, 1E-19), *DNA-J* (Eval = 2E-44), *LTP* (4E-41) and *MAPKKK* (1E-33). Interestingly peroxidases have been implicated in cell wall loosening via production of oxygen radicals in the apoplast. These radicals can in turn, break down strong cell wall polymer structures in an unspecific manner (Passardi et al., 2004), and in *R. palustris* might be required for long-term cell enlargement. The Lipid Transfer Protein (LTP), is suggested to be important for binding lipids, lipid transport and endomembrane systems. An increase in cell size requires a corresponding increase in lipid membranes. Interestingly, LTPs have also been suggested to be involved in cell wall extension (Nieuwland et al., 2005). Cell enlargement of the elongating petiole during the escape strategy can involve a more than fourfold increase in cell length (Voesenek et al., 1990) and this size increase might be facilitated by LTP. *DNA-J* is a heat shock protein or molecular chaperone and *MAPKKK* is important for many signalling pathways (Rodriguez et al., 2010). Exactly what their role is in the elongating petiole remains unclear. *RHD3* has very low similarity to the *Arabidopsis* counterpart (Eval=2E-11), but in *Arabidopsis* this gene is important for cell enlargement induced by ethylene and auxin (Wang, 2002; Wang et al., 1997). Furthermore, it acts on tubular endoplasmatic reticulum development and golgi vesicle dynamics (Lee et al., 2013; Zhang et al., 2013).

It appears that after ABA removal (2h of submergence) a relatively large suite of genes is upregulated in addition to the rapidly activated (1 h after submergence) ones, with these latter early genes persisting throughout submergence. The dramatic increase in cell size clearly requires an additional set of processes. However, the possibility exists that only a hyponastic response and initial cell wall loosening leading to elongation growth, are sufficient for the leaf to restore aerial contact. Aerial contact allows for rapid release of the entrapped ethylene, and a subsequent loss of this driving signal for the escape strategy (Voesenek et al., 1993a). If aerial contact has been made, any further elongation would not be beneficial. However, when under a large water column *R. palustris* has the potential for impressive elongation via tremendous cell enlargement, which naturally cannot be achieved by cell wall loosening alone. The increase in GA and GA sensitivity are driving factors for this long-term response (Rijnders et al., 1997; Benschop et al., 2006). The late (4 h of submergence) petiole specific OMCL families identified here could be instrumental in initiating and facilitating long-term submergence-induced elongation growth.

Overall, the results of this chapter provide tissue specific resolution in addition to the detailed hormonal and molecular dynamics of important processes involved in the submergence escape strategy. This allowed for the association of novel candidate OMCL families with the established processes and thereby provided a basis for future molecular characterization of these candidate genes.

Materials and methods

Plant growth

R. acetosa and *R. palustris* seeds were germinated on floating polyethylene beads for 10 days (12h light, 25°C; 12h dark, 12°C). After potting (Chapter 2) plants were grown for 16 days in climate-controlled walk-in growth chambers (160 µmol m^{-2} s^{-1} Photosynthetically Active Radiation; 16 h day, 8 h night; 20°C, 70% relative humidity). When used for experiments plants were in the three leaf stage.

Plant treatments

Submergence treatment consisted of complete submergence of the plant (depth of 25 cm) in overnight acclimatized water. Ethylene treatments were performed in humidified glass cuvettes with a continuous gas through-flow, with 5 μL L^{-1} ethylene-air gas mixture. ABA (20μM, 0.1% ethanol) or mock (0.1 % ethanol) was sprayed at four time-points (30, 75, 110 and 165 minutes) prior to submergence (3 hours after the start of the photoperiod). 0.01% Triton X-100 was added as a detergent to facilitate uptake by the plant.

RNA isolation and gene expression

Biological replicates consisted of pooling material from three plants; the root system, and the petiole and lamina of the youngest leaf (third leaf). RNA was extracted using the kiefer protocol (Kiefer et al., 2000). This was followed by DNase treatment (DNA-free; Ambion) and subsequent reverse transcription with random hexamers including RNase inhibitor (Invitrogen). Quantitative RT-PCR was done with 5μl total volume powersybr reaction mix with gene-specific primers (Appendix, Table A1) and *TUBULIN* as reference.

Chapter 5

Ethylene primes *Rumex palustris*, but not *R. acetosa*, for future low oxygen conditions

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Abstract

Because gas diffusion is 10^4 times slower underwater than in air, flooded plants often experience reduced oxygen availability, especially in dark conditions and in the roots. Here we investigated the role of ethylene, which typically accumulates in all submerged tissues that produce this volatile plant hormone, in adjusting the responses of *Rumex acetosa* and *Rumex palustris* to anoxia (0% O_2). It was found that already under normoxic submerged conditions, typical hypoxic responsive genes were upregulated, especially in *R. palustris*, suggesting an adaptation to potential future low oxygen conditions. Indeed, pretreating plants with ethylene prior to anoxia improved the survival of *R. palustris*, but not *R. acetosa*. The improved tolerance induced by ethylene corresponded with a stronger induction of typical hypoxia responsive genes during anoxia, but much less so during the pretreatment itself. We hypothesize that a mechanism, where *Rumex* orthologs of the oxygen responsive *Arabidopsis* group VII Ethylene Response Factor (ERF) transcription factor family accumulate during ethylene pretreatment. Upon the subsequent anoxia, these group VII ERFs move to the nucleus and activate target gene expression. This might explain the ethylene-induced priming of anoxia tolerance and gene expression in *R. palustris* and the lack of this priming in *R. acetosa*.

This chapter is also published in:
van Veen H, Mustroph A, Barding GA, Vergeer-van Eijk MA, Welschen-Evertman RAM, Pedersen O, Visser EJW, Larive CK, Pierik R, Bailey-Serres J, Voesenek LACJ and Sasidharan R (2013) Two species from contrasting hydrological niches regulate flooding tolerance through distinct mechanisms. *The Plant Cell*, 25: 4691-4707

Introduction

A major challenge for plants in a flooded environment is to deal with the 10^4 lower rate of gas diffusion underwater compared to air. In dark conditions, or belowground organs, this results in reduced internal oxygen levels when submerged (Mommer et al., 2007; Colmer, 2002). These low oxygen levels in plant organs require reprogramming of many processes to adapt to these altered conditions. Well understood is the importance of activating fermentative pathways to recycle NADH to NAD^+ to sustain energy production via glycolysis (Greenway and Gibbs, 2003; Gibbs and Greenway, 2003). A large array of other transcriptomic changes have also been observed throughout the plant kingdom (Mustroph et al., 2010; 2009).

Recently, a mechanism with which plants are able to perceive low oxygen and relay this signal in transcriptional reconfiguration was identified. In *Arabidopsis* the protein RAP2.12 interacts with the membrane bound ACYL-CoA BINDING PROTEIN 1 (ACBP1). Upon hypoxia RAP2.12 moves to the nucleus where it activates the expression of typical hypoxia responsive genes such as *ADH1* (Licausi et al., 2011). RAP2.12 is part of the group VII Ethylene Response Factor (ERF) protein family of transcription factors. All five *Arabidopsis* members share a conserved N-terminal domain that makes them susceptible to the N-End Rule pathway of Proteolysis (NERP), leading to protein degradation in the presence of oxygen (Gibbs et al., 2011). Binding to ACBP1 protects RAP2.12 from degradation, but when unbound it is removed from the nucleus once re-aeration occurs. Overall this leads to an oxygen dependent signalling network. However, another signal of submergence, that in most conditions precedes hypoxia, is ethylene (Voesenek and Sasidharan 2013). This hormone accumulates within an hour to high levels (1-10 ppm) due the physical entrapment within the plant, both in roots and shoots (Banga et al., 1996; Visser et al., 1996a, 1996b; Voesenek and Sasidharan, 2013). Indeed, ethylene signalling has been shown to affect *ADH* gene expression (Peng et al., 2001), amongst many other adaptive responses to submergence, such as aerenchyma formation, development and outgrowth of adventitious roots and regulation of growth strategies (Evans, 2003; Voesenek et al., 2006).

In this study the impact of ethylene on anoxia signalling and tolerance in two species from different hydrological niches, *Rumex acetosa* and *Rumex palustris*, was investigated. Hypoxia treatment of root tips indicates that *R. palustris* has greater tolerance to low oxygen conditions than *R. acetosa* corresponding with their field distribution (Voesenek et al., 1993b). Here we present how global transcript profiling (Chapter 2) identified upregulation of typical hypoxia responsive genes, especially in the anoxia tolerant *R. palustris*, despite high oxygen levels. Furthermore, we demonstrate that *R. palustris* can use the early flooding signal ethylene to enhance expression of typical hypoxia responsive genes, and improve its performance during subsequent anoxia, whereas *R. acetosa* is unable to utilize the ethylene signal for this function.

Results & Discussion

In *Arabidopsis* global transcript profiling of seedlings identified a set of 49 genes that are upregulated upon hypoxia throughout all tissues (Mustroph et al., 2009). A cross kingdom

investigation identified that throughout the plant kingdom all identified orthologs of the *Arabidopsis* core hypoxia set of 49 genes were upregulated during hypoxia (Mustroph et al., 2010). A discontiguous megablast of the *Arabidopsis* core set against the shotgun assembled *Rumex* transcriptome (Chapter 2) revealed a *Rumex* core hypoxia gene set. *Rumex* OMCL families (group of highly similar sequences of *R. acetosa* and *R. palustris*; Fig. 2.2) for 30 of the 49 core hypoxia genes in *Arabidopsis* were identified (E_{val}<1.0E-10; Fig. S5.1). During submergence in light, four were upregulated in petioles of both *R. acetosa* and *R. palustris,* and an additional seven were specifically upregulated in *R. palustris* (Fig. 5.1a). Despite induction of numerous hypoxia core set OMCL families, oxygen levels inside petioles as measured by micro-electrodes did not drop below 17 kPa in either species during submergence in the light (Fig. 5.1b). Oxygen content in submerged tissue is typically light dependent (Mommer et al., 2007). Indeed, when submerged in the dark *Rumex* petioles showed a depletion of oxygen levels down to approximately 4 kPa (Fig. S5.2).

Based on this, we hypothesized that the induction of hypoxia responsive genes in the

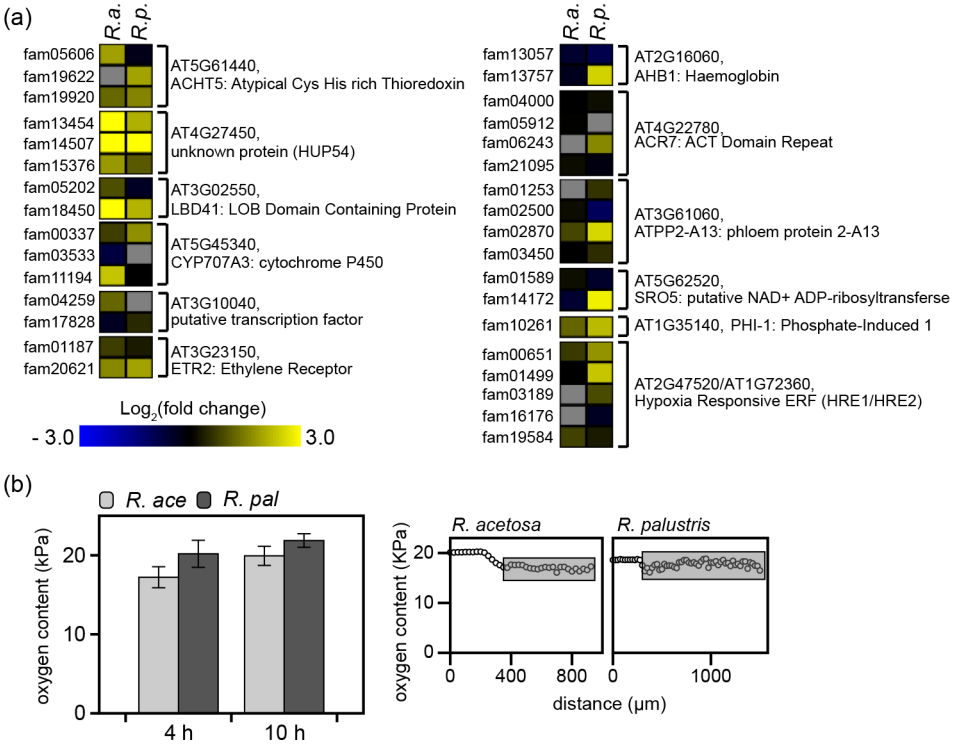

Figure 5.1 Regulation of typical hypoxia responsive OMCL families in two *Rumex* species despite high oxygen conditions.

(a) The *Rumex* OMCL families homologous to the *Arabidopsis* core hypoxia gene set that showed upregulation upon submergence in petioles. For most *Arabidopsis* genes several homologous OMCL families were found (discontiguous megablast, Eval<1.0E-10). (*R.a.* = *Rumex acetosa*; *R. p.* = *Rumex palustris*).

(b) Oxygen content in *Rumex* petioles upon complete submergence under light conditions in petioles (mean ± sem, n=3). Representative traces for both species are given, where the shaded areas mark measurements inside the petioles. (*R.ace* = *Rumex acetosa*; *R. pal* = *Rumex palustris*).

Rumex petioles during normoxia acts as a mechanism to prepare (prime) the plant to a potential imminent oxygen crisis associated with flooding. This can occur if aerial contact is not restored by escape of leaves above the water surface, if floodwaters are too turbid, or as a consequence of nightfall. Since ethylene entrapment is an inevitable consequence of submergence, we hypothesized that elevated ethylene might be responsible for the induction of these hypoxia-responsive genes. To test this, a 4 h ethylene pre-treatment was applied followed by complete anoxia in darkness for 18 to 27 h (Fig. 5.2a). After one week of recovery, both species were compromised by anoxia as reflected in their survival and biomass accumulation (Fig. 5.2b-c). For *R. acetosa* an ethylene pre-treatment did not affect tolerance to the subsequent anoxia. However, *R. palustris* significantly benefitted from the ethylene pretreatment to tolerate the following anoxia period (Fig. 5.2).

To probe a role for the hypoxia core set of genes, that are already regulated under normoxic submerged conditions, expression of a subset of these genes was determined after pre-treatment and during the subsequent anoxia period. Consistent with the observed survival differences between *R. acetosa* and *R. palustris*, none of the core hypoxia genes were induced by ethylene in *R. acetosa*, whereas in *R. palustris ADH* (*ALCOHOL DEHYDROGENASE*), *HB* (*HEMOGLOBIN*) and *ACR7* (*ACT DOMAIN REPEAT*) transcripts were elevated by ethylene pre-treatment (Fig. 5.3). Moreover, seven of the eight hypoxia core set OMCL families tested showed greater induction during anoxia in *R. palustris* pre-treated with ethylene compared to a pre-treatment with only air. In contrast, none of the investigated hypoxia core set OMCL families showed ethylene pre-treatment enhanced expression in *R. acetosa* during anoxia. Remarkably, the transcripts with an ethylene pre-treatment dependent anoxic expression level showed little or no change in abundance as a result of ethylene pre-treatment alone. Taken together, these data show that ethylene pre-treatment primes the expression of several hypoxia core set members in *R. palustris* only, providing a possible explanantion for the enhanced anoxia survival and biomass in ethylene pretreated *R. palustris*.

Despite that all the eight tested hypoxia core set members were induced by the anoxia treatment, little is known about their precise role under low oxygen stress. Nevertheless a wide variety of functions has been identified and suggested. The PHLOEM PROTEIN 2-A13 is associated with aquaporins and lectin binding (Dinant, 2003); ACR7 is possibly involved in amino acid binding (Hsieh, 2002); ACHT5 (ATYPICAL CYS HIS RICH THIOREDOXIN 5) is involved in regulating chloroplastic redox state (Dangoor et al., 2009); HB binds O_2 and plays an important role in NO scavenging and production of NO (Dordas, 2009); SRO5 (SIMILAR TO RCD ONE 5) is associated with mitochondrial located ROS amelioration (Borsani et al., 2005) and PHI (PHOPHATE INDUCED 1) is responsive to carbon and energy starvation (Schröder et al., 2011). The strong correlation observed between high core set expression and high tolerance does imply that these genes play an important role in adaptations to anoxia. However, *PHI* is a notable exception where anoxia induction is very small in *R. palustris* and high in *R. acetosa*.

The ACBP1 bound RAP2.12, that moves to the nucleus upon low oxygen levels to activate hypoxic transcription (Licausi et al., 2011) provides a possible mechanism via which such priming effects could occur. If during ethylene pre-treatment the abundance of group

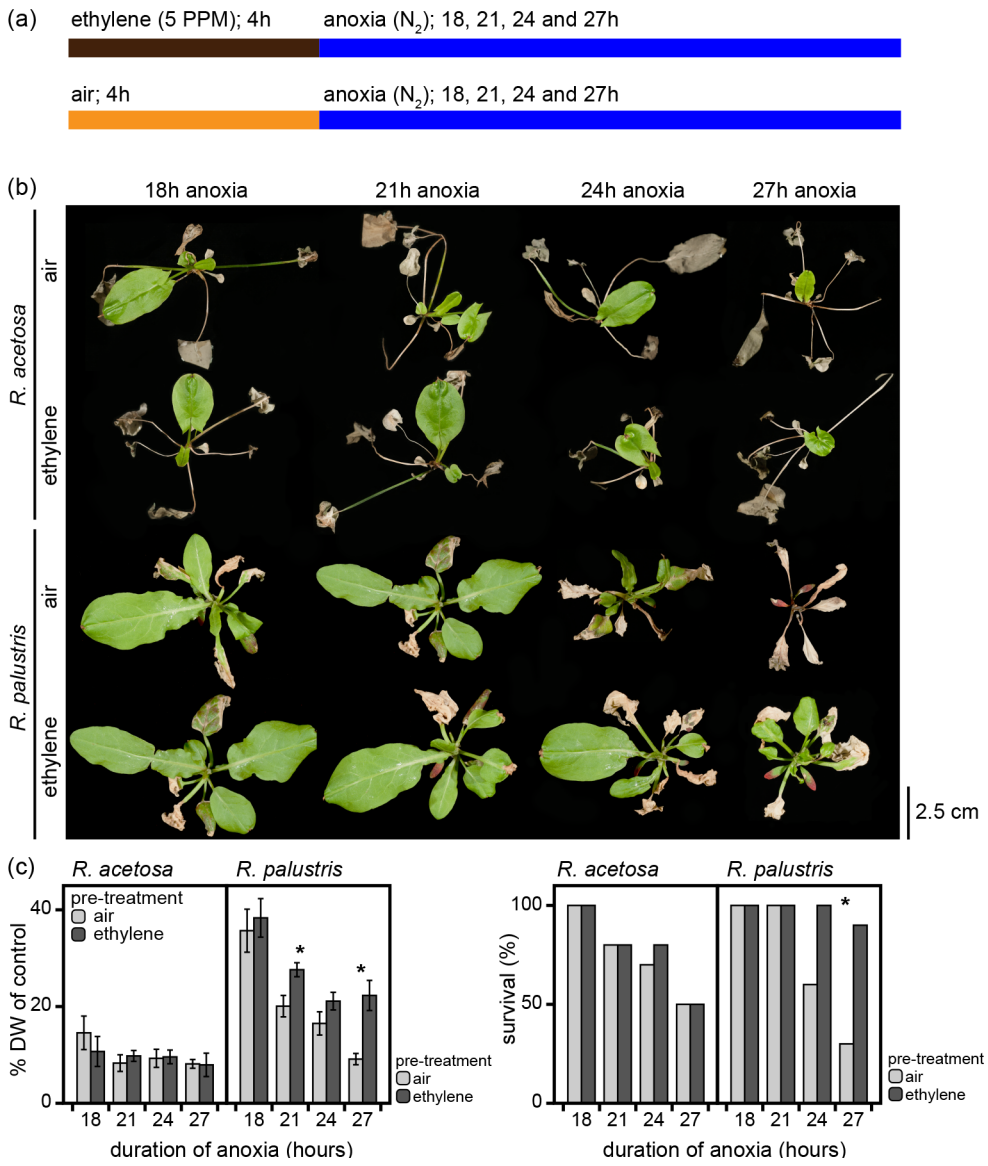

Figure 5.2 Ethylene primes *R. palustris* for upcoming anoxic conditions.

(a) Schematic representation of the experimental design, with two different pre-treatments (air or ethylene) in light conditions and subsequent anoxia in darkness.

(b) *Rumex* phenotypes after 4 h of normoxic pre-treatment with or without ethylene, followed by an anoxia (N_2) treatment in the dark (18-27 h) and 7 days of recovery under standard growth conditions (see methods).

(c) Effect of pre-treatment and anoxia on dry weight expressed as percentage of control plants (DW; mean ± sem, n=10; * P<0.05, Tukey LSD) and plant survival (n=10, * P<0.05, chi-square). The experiment was repeated three times independently with similar results (representative data are shown).

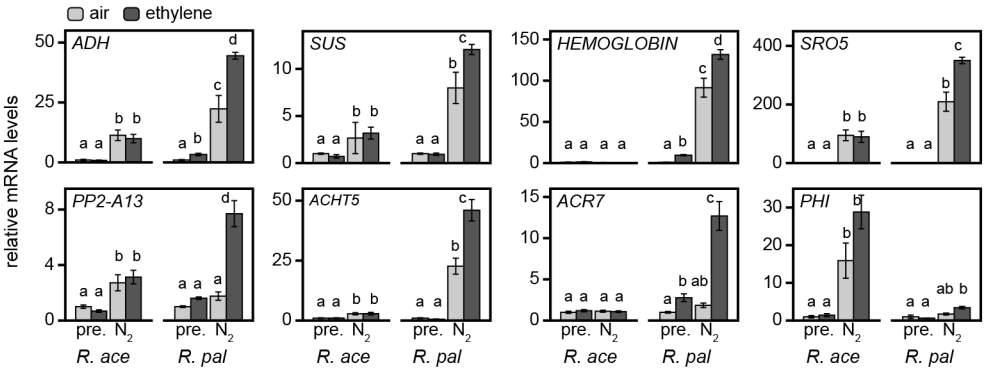

Figure 5.3 Expression of eight OMCL families in *R. acetosa* (*R.ace*) and *R. palustris* (*R.pal*) that were identified as core hypoxia responsive genes in *Arabidopsis* after either 4 h of pre-treatment (air or ethylene) or pre-treatment in combination with 4 h of anoxia (n=5, mean ± sem; letters P<0.05 Tukey LSD).

VII ERFs such as RAP2.12 anchored at the membrane would increase, this could lead to subsequently stronger activation of hypoxia target genes when RAP2.12 is released from ACBP1. Indeed, in *R. palustris* there was a modest up-regulation of the closest homolog to *AtRAP2.12* by ethylene and ethylene followed by anoxia, but not in *R. acetosa* (Fig. 5.4). These *Rumex* group VII ERFs also contain the characteristic N-end motif required for oxygen-dependent degradation. Overall this data supports the proposed priming mechanism. However, we cannot rule out that other mechanisms that lead to ethylene-induced accumulation of group VII ERF proteins might also facilitate priming.

The results presented here show that ethylene can act as an early signal in the transition to low oxygen conditions during a flooding event. The inability of *R. acetosa* to benefit from ethylene priming suggests this could be a trait typical of frequently flooded species. Nevertheless, *ADH1* induction during low oxygen conditions was also shown to require ethylene signalling in *Arabidopsis* (Peng et al., 2001). The effect of ethylene pre-treatment on anoxia tolerance in *R. palustris* shows a strong similarity to the effect of hypoxia pre-treatment (4-5 kPa O_2) on anoxia tolerance in several species, such as maize, *Vitis* and *Arabidopsis* (Saglio et al., 1988; Bouny and Saglio, 1996; Ismond, 2003; Mugnai et al., 2011). Hypoxia pre-treatment is suggested to improve adaptive protein synthesis already during hypoxia to subsequently enhance tolerance to anoxia (Chang et al., 2000). This matches with ethylene priming only for the ethylene increased expression levels of *ADH*, *HB* and *ACR7*, suggesting that for ethylene priming also expression and protein synthesis

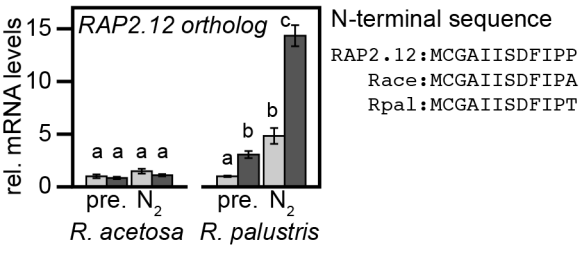

Figure 5.4 The relative transcript abundance of the *Rumex* orthologs to *RAP2.12* in *Arabidopsis* (letters P<0.05, Tukey LSD) upon ethylene (pre. = pre-treatment 4h) and subsequent anoxia (N_2, 4h). Also shown is the conserved N-terminus of *Arabidopsis* RAP2.12 and the *Rumex* orthologs that is essential for oxygen-dependent degradation of these proteins via the N-end rule of protein degradation.

during the anoxic phase is important for improved tolerance. Overall we suggest that ethylene-induced priming for future anoxia is a trait of wetland-adapted plants that are frequently at risk of severely impoverished oxygen conditions.

Material and methods

Plant growth and experimentation

R. acetosa and *R. palustris* seeds were germinated on floating polyethylene beads for 10 days (12h light, 25°C; 12h dark, 12°C). After potting (Chapter 2) plants were grown in climate-controlled walk-in growth chambers (160 µmol m^{-2} s^{-1} Photosynthetically Active Radiation; 16 h day, 8 h night; 20°C, 70% relative humidity). *R. acetosa* and *R. palustris* were grown for 21 and 27 days, respectively. Ethylene and anoxia treatments were performed in humidified glass cuvettes with a continuous gas through-flow, with 5 μL L^{-1} ethylene-air gas mixture or pure N_2 in darkness.

RNA isolation and expression

RNA was extracted from the youngest petiole using the kiefer protocol (Kiefer et al., 2000). This was followed by DNAse treatment (Ambion, DNA-free) and subsequent reverse transcription with random hexamers and including RNase inhibitor (Invitrogen). qRT-PCR was performed using a 20 μL SYBR Green reaction mixture with gene-specific primers (Appendix, Table A1) and *TUBULIN* as reference.

Internal oxygen concentrations

Profiles of internal O_2 partial pressure of the petiole were measured in light (175 μmol photons m^{-2} s^{-1}) and during darkness. O_2 and CO_2 of the submergence solution were kept at atmospheric equilibrium (20°C) by bubbling with air. A motorized micromanipulator (MC-232, Unisense A/S, Denmark) mounted with a microelectrode (tip diameter = 25 μm, OX25, Unisense A/S, Denmark) was used with a step size of 25 μm (Pedersen et al., 2006).

Supporting Information

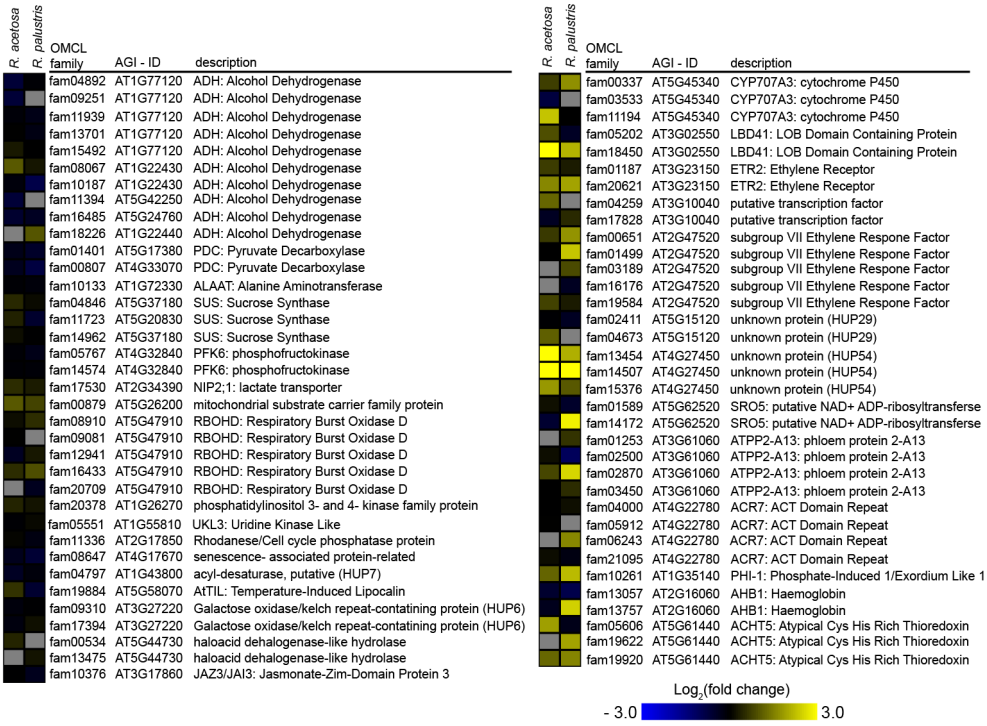

Figure S5.1 Regulation upon submergence of *Rumex* OMCL families orthologous to the *Arabidopsis* core set of hypoxia-responsive genes as identified through a discontiguous megablast (using a cutoff of Eval < 10^{-10}).

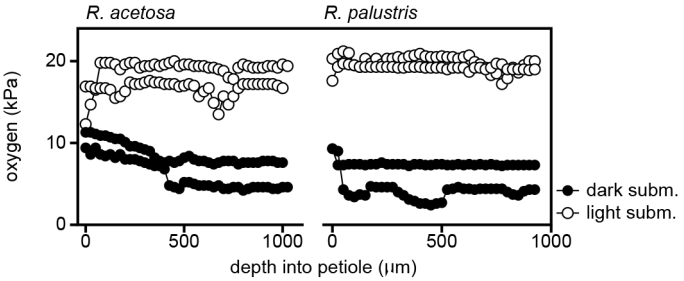

Figure S5.2 Microelectrode traces showing oxygen content in the petioles of *R. acetosa* or *R. palustris* during light and dark submerged (subm.) conditions.

Chapter 6

Group VII Ethylene Response Factor diversification and regulation in four species from flood-prone environments

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Abstract

Flooding events negatively affect plant performance and survival. Flooding gradients thereby determine dynamics in vegetation composition and species abundance. In adaptation to flooding, the group VII Ethylene Response Factor genes (*G7ERF*s) play pivotal roles in rice and *Arabidopsis* through regulation of anaerobic gene expression and survival strategies of rice. We investigated if *G7ERF*s have a similar role in mediating survival strategies in eudicot species from flood-prone environments. To this end, the evolutionary origin and regulation of *G7ERF*s, and the physiological responses in species from two genera of divergent taxonomic lineages (*Rumex* and *Rorippa*) were studied. Synteny analysis revealed that angiosperm *G7ERF*s arose from two ancestral loci and their diversification and subsequent duplications led to the present G7ERF variation. We propose that subtle variation in *G7ERF* regulation could potentially explain variation in tolerance amongst *Rorippa* species. In *Rumex*, the main difference in flood tolerance correlated with genetic variation in *G7ERF* genes. Large transcriptional differences were found by comparing the two genera: darkness and a dark submergence induced *Rumex* G7ERFs, whereas submergence induced *HRE2* expression in *Rorippa* roots. We conclude that the involvement of G7ERFs in flooding tolerance developed in a phylogenetic dependent manner, with subtle variations within taxonomic clades.

This chapter is also published in:
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Introduction

Flooding events occur worldwide, during which complete or partial submergence of plants has severe effects on plant functioning. Flooding in ecological and agronomical systems is a major determinant of species distribution and crop yields, respectively (Voesenek et al., 2004; Van Eck et al., 2004; Silvertown et al., 1999; Voesenek & Bailey-Serres, 2009). As gas diffusion in water is 10^4 slower than in air, the availability of external CO_2, for photosynthesis and O_2, for respiration, is severely limited. Belowground tissues typically become oxygen deprived, especially when the soil becomes anaerobic. Aboveground tissues can still obtain some oxygen from the water column, and under illuminated conditions produce O_2 during photosynthesis (Mommer et al., 2007). However, flooding events often coincide with severely reduced light levels due to turbid waters, which further limit underwater photosynthesis and thus O_2 production (Vervuren et al., 2003).

It is essential for the O_2-deprived tissues to induce fermentation to sustain glycolysis for some energy production. However, since ATP produced via glycolysis is significantly lower than aerobic respiration, consumption of carbohydrate reserves is faster and leads to starvation if submergence is prolonged (Bailey-Serres & Voesenek, 2008). Hence, some species adapt to excess water levels via the quiescence strategy aiming to limit expensive biosynthetic processes and restrict growth whilst the flood lasts. Other species display a vigorous elongation of shoot tissue, the escape strategy, and when the shoot breaches the water surface, aeration is restored throughout the plant via longitudinally interconnected aerenchyma tissue, subsequently improving survival (Colmer & Voesenek, 2009). Interestingly, studies on rice and *Arabidopsis* have identified important regulatory roles of a specific subfamily of transcription factors, GROUP VII ETHYLENE RESPONSE FACTORs (G7ERFs), in regulating the activation of genes involved in fermentation, reserve utilisation and regulating quiescence and escape strategies (Voesenek & Bailey-Serres, 2013).

G7ERFs are members of a large transcription factor family, characterized by a conserved DNA binding APETALA2 (AP2) domain (Nakano, 2006; Sakuma et al., 2002; Krizek, 2003). Though mostly unique to plants, it is suggested that plant ERF genes originated through horizontal gene transfer via homing endonucleases from bacteria and viruses (Magnani, 2004). The 122 identified ERFs in *Arabidopsis* can be subdivided into 12 subgroups based on AP2 domain similarity, whilst rice contains 139 ERFs with an additional 3 groups (Nakano, 2006). It is the group 7 (G7ERFs) that has been specifically shown to play an essential role in adaptations to flooding in both rice and *Arabidopsis*.

Genetic studies in deepwater rice identified SNORKEL1 and -2, both G7ERFs that are transcriptionally induced upon submergence and ethylene, as being responsible for a strong elongation response (Hattori et al., 2009). In contrast, another G7ERF, SUB1A, was found to confer a quiescence strategy to rice by limiting growth and inducing increased fermentation capacity (Fukao et al., 2006; Fukao & Bailey-Serres, 2008; Xu et al., 2006). Interestingly, throughout submergence treatment *SUB1A* is expressed specifically at the base of the leaf that elongates in the absence of this gene (Singh et al., 2010).

Arabidopsis does not show a clear quiescence or escape strategy when submerged. However, fermentative enzymes are induced (lee et al. 2011). Upon hypoxia, transcript

levels of two *G7ERF*s, namely *HYPOXIA RESPONSIVE ERF 1* and *-2* (*HRE1*, *HRE2*), are strongly increased. The double mutant *hre1hre2* is unable to induce *ADH* during long-term hypoxia (Licausi et al., 2010). Initial induction of *ADH* and probably also of *HRE1/2* occurs via RAP2.12, another G7ERF, through an oxygen sensing mechanism (Licausi et al., 2011; Gibbs et al., 2011; Sasidharan and Mustroph, 2012). *RAP2.12* is constitutively expressed and the protein is anchored to membrane bound ACYL-CoA BINDING PROTEIN 1 (ACBP1) under aerated conditions. Upon hypoxia, RAP2.12 is released and translocates to the nucleus to activate the expression of typical hypoxia responsive genes (Licausi et al., 2011). In the presence of oxygen, non-anchored RAP2.12 is degraded via the N-end Rule pathway of Proteolysis (NERP) (Graciet et al., 2010; Graciet and Wellmer, 2010). Most G7ERFs have a highly conserved Methionine-Cysteine N-terminal sequence, making them all potential NERP targets for degradation under aerobic conditions. All *Arabidopsis* G7ERFs are NERP susceptible, whereas for instance SUB1A in rice, despite a similar N-terminus, is not. This is likely due to its secondary structure and protein folding (Gibbs et al., 2011).

The amphibious lifestyle occurs widely throughout the plant kingdom (Cook, 1999). Furthermore, there is a wealth of wetland species with extreme tolerance to complete submergence. G7ERFs have been associated with escape, quiescence, carbon usage and fermentation based on studies in rice and *Arabidopsis*. Therefore, our main aim was to determine whether G7ERFs potentially have similar roles in mediating these traits in eudicot species from flood-prone environments such as river floodplains. To this end, we investigated (i) the evolutionary origin of G7ERFs in the angiosperm kingdom and their sequence similarities (ii) the G7ERF-mediated physiological processes in four eudicot wetland species, and (iii) the regulation of *G7ERF*s upon flooding stress in these four species.

For the purpose of this study, important wetland taxa from the two major evolutionary lineages of eudicots were selected; the Rosids and the Asterids. Namely, we studied

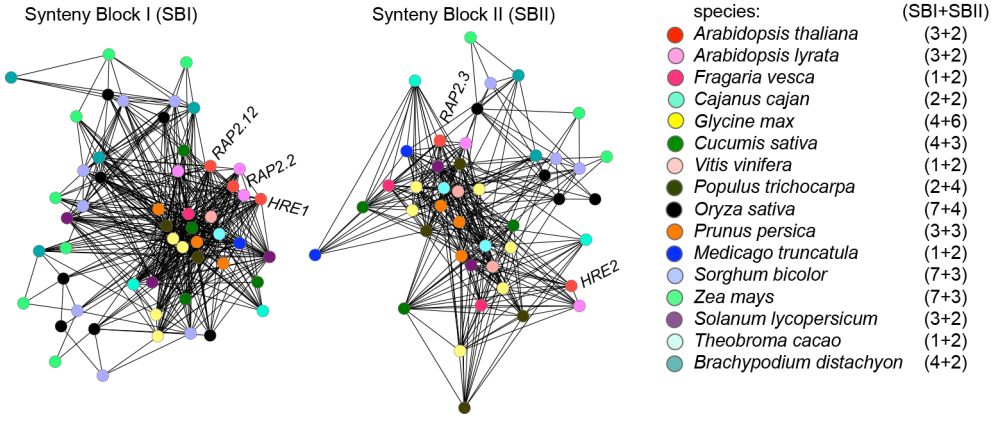

Figure 6.1 Syntenic relationships between *group VII ERF*s of 16 species. Nodes (coloured dots) represent individual *G7ERF*s whilst edges (connecting lines) indicate a synteny relationship. The length of the edges is indicative of the strength of a syntenic relationship; short edges have a relatively strong relationship.

Rorippa sylvestris and *Rorippa amphibia* of the Brasicaceae (Rosids), and *Rumex acetosa* and *Rumex palustris* of the Polygonaceae (Asterids). All four species complete their life cycle in flood-prone environments (Stift et al., 2008; Voesenek et al., 2004). *Ru. palustris* and *Ro. amphibia* have an escape response, and *Ru. acetosa* and *Ro. sylvestris* adopt a quiescence strategy under submergence (Voesenek and Blom, 1989; Akman et al., 2012). Based on our findings, we provide an overview of G7ERF conservation and diversification and its role in long term flooding stress in *Rumex* and *Rorippa*.

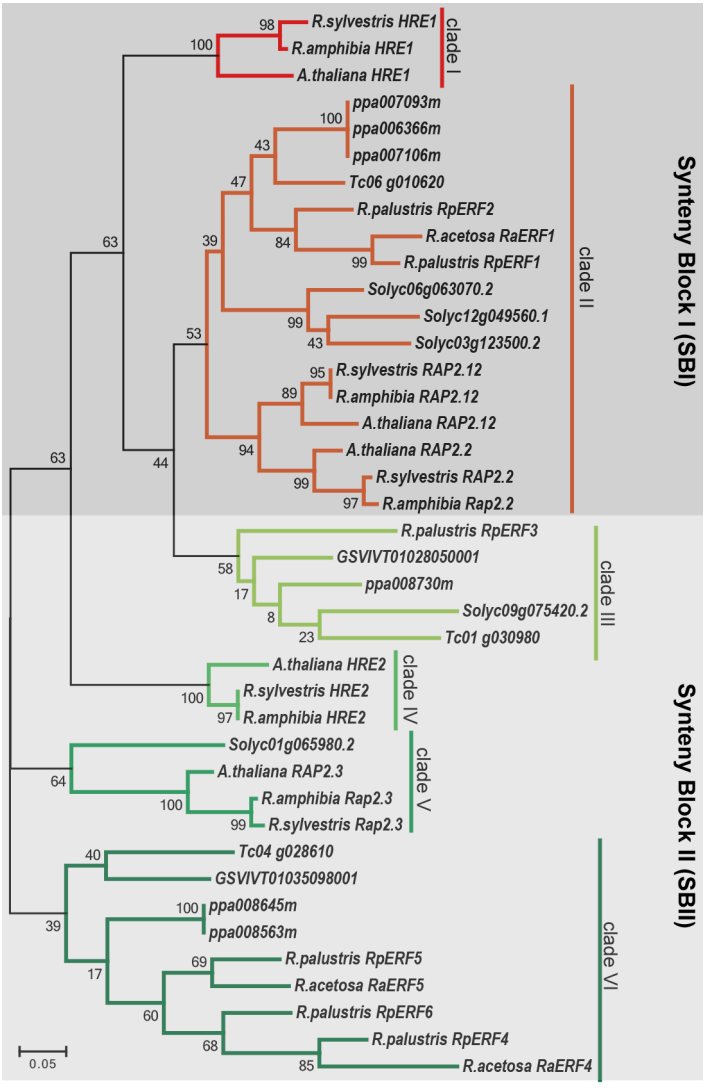

Figure 6.2 Phylogenetic tree of *group VII ERF*s from *Rorippa*, *Rumex*, *Arabidopsis*, *Solanum lycopersicum*, *Vitis vinifera*, *Theobroma cacao*. The tree is based on alignment of the AP2 domain and the 5' starting nucleotide sequences using a Neighbour joining method with a 1000 bootstraps in Mega 5.

Results

Angiosperm G7ERFs originate from two ancestral genes

Gene collinearity analysis of all *G7ERF*s and their surrounding genomic regions from 16 plant species established two separate syntenic clusters (Fig. 6.1). For *Arabidopsis*, *RAP2.12*, *RAP2.2* and *HRE1* share syntenic relationships, suggesting that these three genes evolved from a single ancestor through a series of genome duplications (Synteny Block I, SBI). *HRE2* and *RAP2.3* were contained within a second syntenic block indicating a second ancestral gene (Synteny Block II, SBII).

Within each of the two synteny blocks, the nodes representing *G7ERF*s appeared to cluster together for closely related species, due to extensive collinearity of the surrounding genomic regions (Fig. 6.1). A clear example, is between the sister-species *Arabidopsis thaliana* and *Arabidopsis lyrata*. *G7ERF*s from monocot grasses (*Brachypodium distachyon*, *Sorghum bicolor*, *Zea mays*, *Oryza sativa*) could generally be found on the outer area of the two synteny clusters, indicating a more distant evolutionary relationship with the eudicots analysed.

Given the established importance of G7ERFs in plant adaptation to flooding and hypoxia, we cloned *G7ERF*s from four species from flood-prone areas representing two major eudicot evolutionary lineages, namely, *Rorippa sylvestris* and *Rorippa amphibia* (Rosids), and *Rumex acetosa* and *Rumex palustris* (Asterids). Phylogenetic analysis was able to group together the cloned *G7ERF*s sequences of various plant species (AP2 domain and 5' conserved motifs) with high similarity (Fig. 6.2). In total six distinct clades were identified. However, due to the relatively short alignment length, uncertainty remains regarding relationships between the different clades, as also reflected in low bootstrap values for the basal branches of the tree. However, the results from both the synteny blocks and phylogenetic tree further resolves evolutionary relationships. The distinct synteny blocks and clades are largely supported by conserved motifs (CM) other than the AP2 domain and CM-I (Fig. 6.3), especially CM-III and CM-VI being present only in SBI. The clades I and IV, containing *HRE1* and *HRE2* respectively, do not include any other species outside the Brassicaceae. Using the synteny relationships as an evolutionary signature we are thus able to conclude that this gene family developed phylogenetically

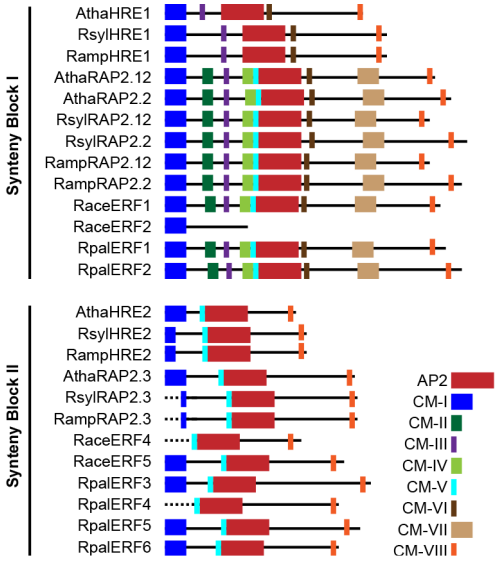

Figure 6.3 Conserved Motifs (CM) based on group VII ERF peptide alignments. Only regions conserved across Brassicaceae (*Arabidopsis* and *Rorippa*) and Polygonaceae (*Rumex*) are shown. Dotted lines represent unknown parts of the sequence.

specific clades in addition to a suite of conserved motifs from two common G7ERF ancestral genes.

The four species have high flooding tolerance and contrasting growth strategies

The main traits conferred by G7ERFs such as survival of flooding (Xu et al., 2006) and hypoxia (Hinz et al., 2010; Licausi et al., 2010), escape or quiescence strategies (Hattori et al., 2009; Fukao & Bailey-Serres, 2008), reserve utilization (Fukao et al., 2006; Kudahettige et al., 2010) and activation of fermentative metabolism (Fukao et al., 2006; Yang et al., 2011; Hinz et al., 2010; Licausi et al., 2010; 2011) were also investigated in the four species under study (Fig. 6.4).

The ability to survive long-term complete submergence in the dark (dark submergence), indicated by the survival parameter median lethal time-50 (LT_{50}), of *Ro. sylvestris*, *Ro. amphibia*, *Ru. acetosa* and *Ru. palustris* demonstrated high flooding tolerance, with the

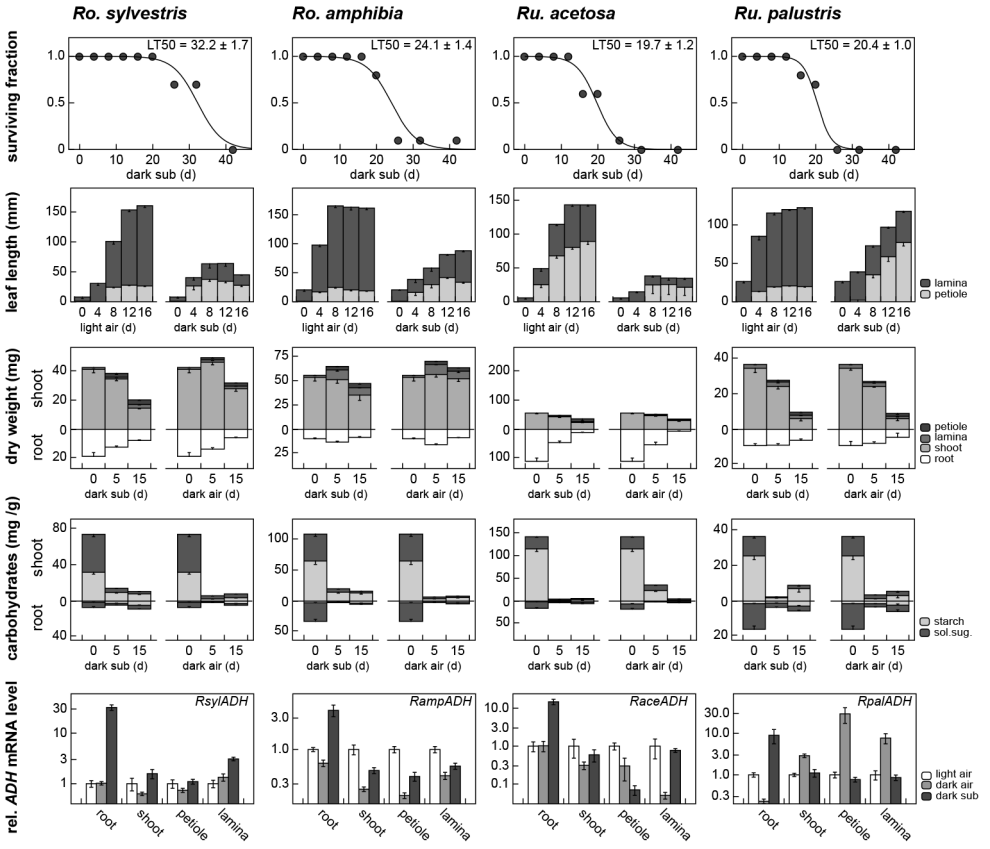

Figure 6.4 Responses of *Ro. sylvestris*, *Ro. amphibia*, *Ru. acetosa* and *Ru. palustris* to submergence and associated stresses. Survival (n=10), length of the youngest developing leaf (mean ± sem, n=10), dry weight (mean ± sem, n=5), carbohydrate content (mean ± sem, n=3) and *ADH* expression levels after five days of treatment (mean ± sem, n=5). Petiole and lamina are the corresponding parts of the youngest developing leaf, with shoot representing the remainder of the above ground biomass.

Rorippa genus being more tolerant than *Rumex*. *Ru. acetosa* (19.7±1.2 days) and *Ru. palustris* (20.4±1.0 days) did not differ in their flooding survival, whilst *Ro. sylvestris* (32.2±1.7 days) had significantly higher survival than *Ro. amphibia* (24.1±1.4 days).

The initial aboveground dry weight was similar in all four species. However, *Ro. sylvestris* and *Ru. acetosa* contained more initial belowground dry weight than their close relatives. In contrast to dry weight, the starting carbohydrate content varied between species. Within the *Rorippa* genus, *Ro. amphibia* had much higher starting sugar levels in belowground tissues. In the *Rumex* genus, *Ru. acetosa* had 3 to 4 times more starting carbohydrates in the shoot than *Ru. palustris*. Within five days of darkness in air (dark air) or dark submergence, all four species had utilized the vast majority of their carbohydrates. However, no mortality was observed until 16 days of dark submergence.

A gradual decline in dry weights over 15 days was observed in *Ro. sylvestris*, *Ru. acetosa* and *Ru. palustris*, but not in *Ro. amphibia*. However, *Ru. acetosa* only had a minor decline in shoot dry weight, compared to the serious reduction in root biomass throughout dark submergence and dark air. In contrast, *Ro. sylvestris* and *Rum palustris* were able to maintain some belowground biomass.

Ru. palustris showed a strong petiole length increase of the youngest developing leaf during flooding compared to the control (light air), a typical escape response. Such a response was absent in *Ru. acetosa* and *Ro. sylvestris*, and not so pronounced in *Ro. amphibia*.

During dark submergence, *ADH* was induced after 5 days in the roots of all species. However, regulation in aboveground tissue was more variable between species and treatments. During flooding root tissues rapidly become anoxic, especially in the anaerobic soil substrate. In contrast, shoot tissues are able to maintain reasonable amounts of oxygen via diffusion from the surrounding relatively O_2 rich floodwater (Mommer et al., 2006).

In summary, high flooding tolerance was observed in all the four species, with a clear escape response in *Ru. palustris*. In addition, there was a strong maintenance of aboveground dry weight in *Ru. acetosa* and *Ro. amphibia*, and belowground carbohydrates in *Ro. sylvestris* and as expected, *ADH* induction in the flooded roots of all species.

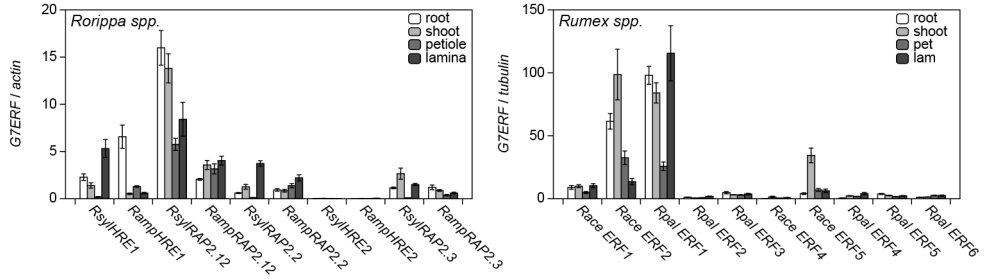

Figure 6.5 Constitutive *G7ERF* mRNA levels relative to a housekeeping gene. Petiole and lamina are the corresponding parts of the youngest developing leaf, with shoot representing the remainder of the above ground biomass. Values are mean ± sem (n=5).

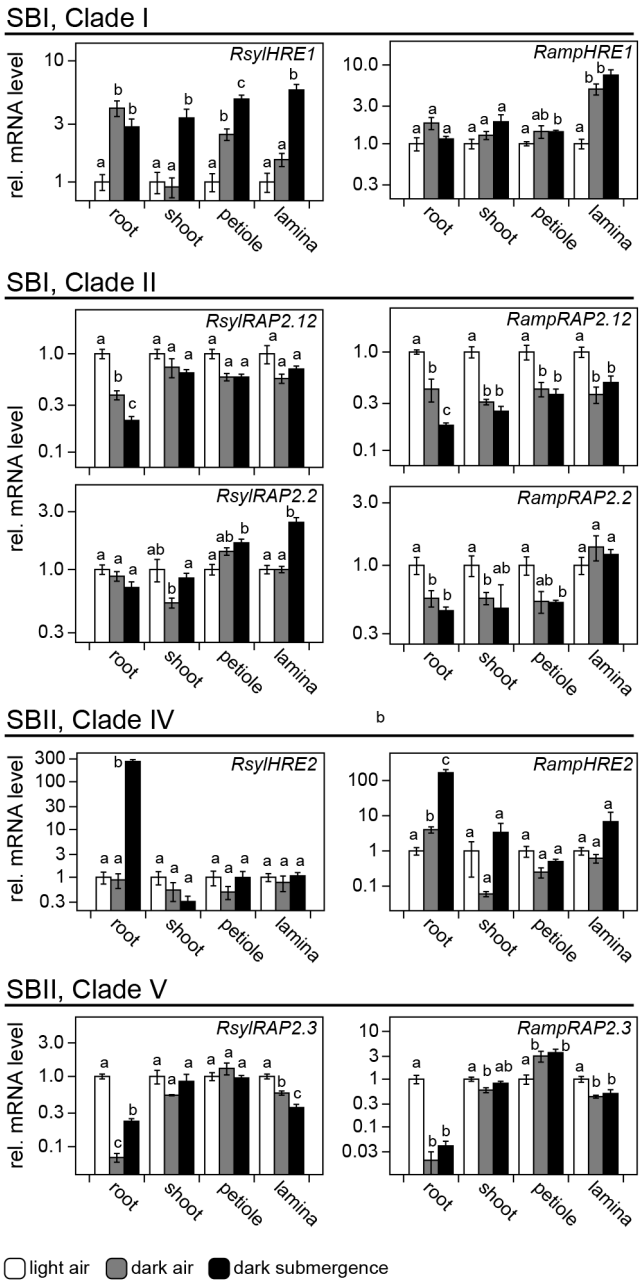

Figure 6.6 Transcriptional changes of *Rorippa G7ERF*s upon dark air and dark submergence ranked by synteny block (SB) and clade. Petiole and lamina are the corresponding parts of the youngest developing leaf, with shoot representing the remainder of the above ground biomass. Material was harvested after 5 days of treatment. Values are mean ± sem (n=5, letters P<0.05 Tukey LSD)

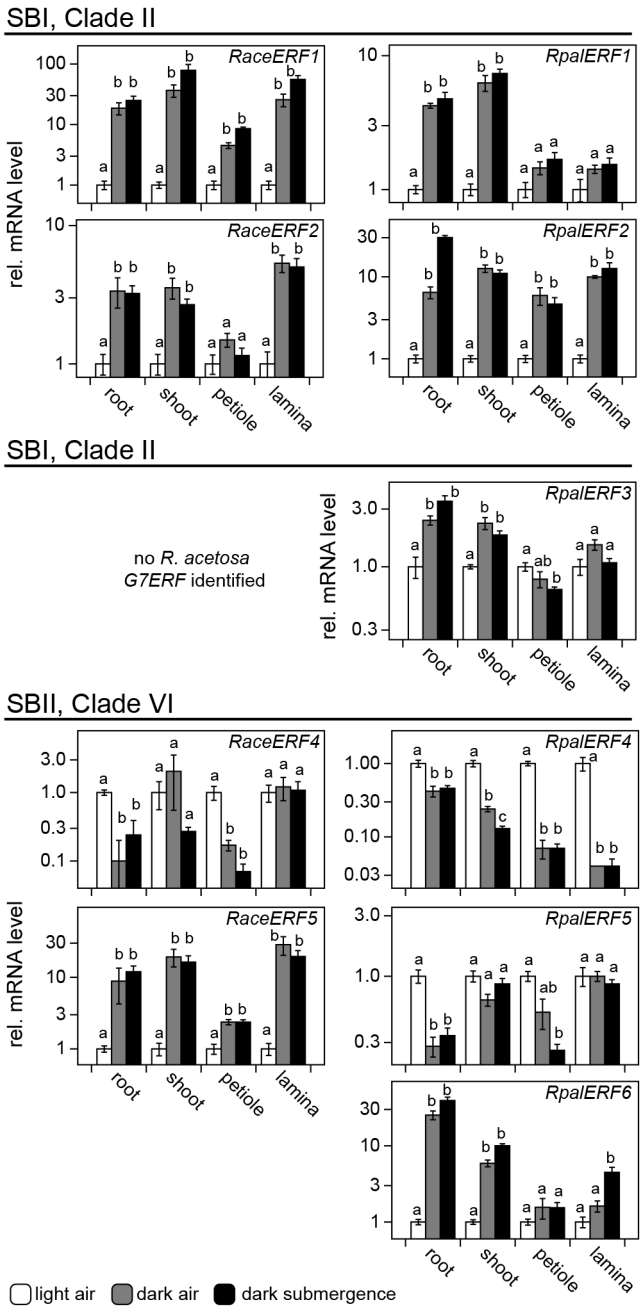

Figure 6.7 Transcriptional changes of *Rumex G7ERF*s upon dark air and dark submergence and ranked by synteny block (SB) and clade. Petiole and lamina are the corresponding parts of the youngest developing leaf, with shoot representing the remainder of the above ground biomass. Material was harvested after 5 days of treatment. Values are mean ± sem (n=5, letters P<0.05 Tukey LSD)

***Group VII ERF* regulation is related to their phylogenetic clades**

Transcriptional regulation of *G7ERF*s has been shown to be instrumental in the quiescence and escape flooding survival strategies in rice (Hattori et al., 2009; Fukao et al., 2006) and the induction of *ADH* (Licausi et al., 2010; Hinz et al., 2010; Yang et al., 2011). Accordingly, we investigated *G7ERF*s transcriptional behaviour in response to dark air and dark submergence treatment in different organs of the four species (Fig. 6.5, 6.6 and 6.7).

We found that the *G7ERF*s *RsylRAP2.12, RaceERF2* and *RpalERF1* had a relatively high constitutive expression level in light air conditions and also higher expression throughout all tissue types compared to other G7ERFs (Fig. 6.5). These genes are part of SBI and clade II (Fig. 6.2), which contain the G7ERFs implicated in the plant oxygen sensing mechanism in *Arabidopsis thaliana* (Licausi et al., 2011; Gibbs et al., 2011). These constitutively expressed *G7ERF*s showed very little regulation in both *Ro. amphibia* and *Ro. sylvestris* (Fig. 6.6). An exception was *HRE1*, especially in *Ro. sylvestris* where some induction was observed in dark submergence conditions. This was in strong contrast to *Rumex*, where in SBI G7ERFs
showed a clear induction upon dark air and dark submergence (Fig. 6.7). Only in the root for *RpalERF2* was there an additional effect of dark submergence compared to dark air.

For the SBII genes *RampHRE2* and *RsylHRE2*, only minor changes were found upon dark treatment. However, dark submergence dramatically induced these two ERFs only in the belowground tissues (Fig. 6.6). In *Rumex* no G7ERFs were identified with an *HRE2* like behaviour. Rather, the other *Rumex* G7ERFs in SBII were generally induced in all organs (*RaceERF5* and *RpalERF6*) or down-regulated (*RpalERF4, RpalERF5* and *RaceERF4*) upon dark air and dark submergence (Fig. 6.7). The other Brassicaceae specific phylogenetic group in SBII, clade V (Fig. 6.2), included *RAP2.3* for which no induction was observed upon treatment. However, a reduction in relative mRNA abundance was found especially in the roots during dark air and dark submergence conditions (Fig. 6.6).

On the whole, a similar *G7ERF* expression trend across the four species was found only for the SBI *G7ERF*s, which showed high constitutive expression. Within each genus there was minor variation in the transcriptional behaviour of *G7ERF*s between species, whereas a large contrast was found between the two genera.

Discussion

Angiosperm *group VII ERF*s are derived from two ancestral loci

In this study, we investigated the diversity and conservation of G7ERFs amongst angiosperms, and through synteny analysis widened our understanding of their evolution. The analysis of syntenic relationships within *G7ERF*s provides evidence that the *G7ERF*s of angiosperms originated from two ancestral genes (Fig. 6.1). The two ancestral genes maybe derived from the ancient polyploidy event (Whole Genome Duplication) that correlates with the origin of angiosperms approximately 192 mya (Jiao et al., 2011). We refer to the two ancestral genes and their derived syntenic clades as SBI and SBII. Through a series of subsequent lineage-specific paleopolyploidy events accompanied by chromosome

rearrangements and gene loss, we now can identify a large number and variation of *G7ERF*s members, with for instance fifteen in rice and five in *Arabidopsis* (Nakano, 2006).

Diversification has been such that only by a few strongly conserved elements, such as the AP2 domain and the 5' region, the G7ERFs can be identified. With such a limited amount of conservation, phylogenetic analysis gives a lack of resolution, particularly in resolving the very old SBI and SBII clades. However, the synteny analysis coupled with the phylogenetics allows us to resolve the groupings and changes more precisely. For example, for SBI clade II, four specific conserved motifs were identified (Fig. 6.3). Surprisingly, throughout the plant kingdom, roles are being suggested for this transcription factor family in adaptation to flooding and its associated stresses. Indeed, in addition to *Arabidopsis*, gene expression studies on waterlogging, submergence or hypoxia have invariably identified regulation of *G7ERF*s in a variety of species such as poplar (Kreuzwieser et al., 2009), cotton (Christianson et al., 2010), soybean (Nanjo et al., 2011) and rice (Jung et al., 2010; Lasanthi-Kudahettige et al., 2007) suggesting a function in flooding-induced adaptations (Fig. S6.2). Interestingly, major regulation under low oxygen related stresses (e.g. flooding, anoxia etc) was found in both SB I and II *G7ERF*s for Arabidopsis and rice. However, in poplar, soybean and cotton, only SBII *G7ERF*s are regulated during waterlogging (Fig. S6.2). Overall this suggests that despite diversification of *G7ERF*s from two ancestors, they have retained their involvement in adaptation to flooding.

Group VII ERF regulation in *Rorippa* is similar to *Arabidopsis*

Rorippa is a closely related genus to *Arabidopsis*, which is reflected in the presence of direct *Rorippa* orthologs for each *Arabidopsis* G7ERF (Fig. 6.2). Given the high similarity between these G7ERFs, it is likely that they share similar functions. However, flooding survival of *Rorippa* is four to five times longer than that of *Arabidopsis* (Vashisht et al., 2010). Therefore it is very surprising that similar expression patterns were observed between *Rorippa* and *Arabidopsis*.

Neither *RAP2.12* nor *RAP2.2* (SBI, clade II) showed much transcriptional regulation upon dark air or dark submergence conditions in *Ro. sylvestris* and *Ro. amphibia* (Fig. 6.6). This is similar to *Arabidopsis* where transcriptional regulation of these G7ERFs is of a small magnitude, with *RAP2.12* being reported to be only slightly induced by hypoxia (Licausi et al., 2011) and *RAP2.2* also by ethylene (Hinz et al., 2010). However both of these ERFs are also reported not to be induced upon hypoxia (Mustroph et al., 2009). Interestingly, constitutive expression is very high for *RsylRAP2.12* but not for the *Ro. amphiba* counterpart. *AtRAP2.12* is also constitutively expressed, but activates transcription of downstream target genes via movement from sequestration at the membrane to the nucleus upon hypoxia. Overexpression of *AtRAP2.12* improves hypoxia survival and results in a stronger induction of typical anaerobic genes in *Arabidopsis* (Licausi et al., 2011). *Ro. sylvestris* showed a much stronger *ADH* and *HRE2* induction than *Ro. amphibia*. It is tempting to speculate that constitutively high levels of *AtRAP2.12* ortholog are present in *Ro. sylvestris* leading to a stronger hypoxic response. Interestingly, root transcript profiling in *Ro. amphibia* and *Ro. sylvestris* showed that the quantitative transcriptional changes under submergence constitute the main difference between these two species and a potential

explanation for differences in flood tolerance (Sasidharan et al., 2013). The quantitative differences in *HRE2*, *RAP2.12* and *ADH* found here further support that hypothesis.

In both synteny blocks a Brassicaceae-specific clade was identified, namely *HRE1* (SBI, clade I) and *HRE2* (SBII, clade IV) (Fig. 6.2). Despite their independent origin, these genes act redundantly in *Arabidopsis* since the double mutant *hre1hre2* is unable to activate typical hypoxic genes such as *ADH*, whilst the single mutants *hre1* and *hre2* retain intact hypoxic signalling (Licausi et al., 2010). In *Arabidopsis HRE2* is strongly induced upon hypoxia in the shoot and the root, but *HRE1* only in root tissue (Licausi et al., 2010). In *Rorippa* transcriptional activation *of HRE2* was also found in the typically hypoxic roots. The exclusive role of *HRE2* and not *HRE1* in root tissue in *Rorippa*, compared to *HRE1* and *HRE2* involvement in *Arabidopsis* roots, does suggests that in *Rorippa* this works in a subtly different way. Nevertheless, the exclusivity of the *HRE*1 and *HRE2* clade to the Brassicaceae (Fig. 6.2), and their involvement in *Rorippa* and *Arabidopsis* does suggest that in this taxonomic lineage, an additional set of unique G7ERFs evolved to mediate long-term responses to flooding stress. The higher induction of *RsylHRE1* in the aboveground tissues in dark submergence compared to darkness does suggests an important role of this gene in regulating tolerance mechanisms such as the quiescence response, possibly by acting much like SUB1A.

The *Rumex Group VII ERF*s are genetically variable, but share transcriptional behaviour

Ru. acetosa and *Ru. palustris* appear to have equal tolerance to flooding. However, *Ru. acetosa* had a much larger amount of root biomass and much higher carbohydrate content than *Ru. palustris* prior to submergence. Additionally, an elongation response, like in *Ru. palustris*, is energetically expensive and is shown to have a negative impact on survival (Setter & Laureles, 1996). Despite these handicaps *Ru. palustris* still achieves a survival equal to *Ru. acetosa*, suggesting a genuinely high flood tolerance potential, as also supported by previous work (Voesenek et al., 1993b; Mommer et al. 2006).

*G7ERF*s were identified for *Rumex* in both synteny blocks. However, despite their close relatedness, considerable genetic differences were found within this genus. An additional two *G7ERF*s were identified in *Ru. palustris*, and for *RaceERF2* a stop codon was found before the AP2 domain. In SB II, no close *Ru. acetosa* ortholog was found for *RpalERF3.* The high number and strong expression of *Ru. palustris G7ERF*s could mean a high level of redundancy or a tight coordination of responses, and might be a reason for the high flooding tolerance found for *Ru. palustris*.

Despite these differences, there were some similarities in expression patterns between *Ru. acetosa* and *Ru. palustris*. This was especially clear for SBI members *RpalERF1*, *RpalERF2*, *RaceERF1* and *RaceERF2*, which invariably showed a clear induction upon dark air and dark submergence. Such behaviour was absent in the *Rorippa* counterparts. All four *Rumex* G7ERFs are putative targets for NERP given their N-terminal sequence (Fig. S6.1). The induction upon darkness could have a similar function as our earlier hypothesis where high *RAP2.12* expression leads to a larger population of anchored RAP2.12 orthologs, which would result in stronger hypoxia-gene induction once low oxygen conditions occur. It

is likely that all *Rorippa* G7ERFs are also NERP targets given the very high similarity to their *Arabidopsis* orthologs which have been shown to be targets for NERP (Gibbs et al., 2011). The *Rumex* orthologs and paralogs have a far more dissimilar sequence to their *Arabidopsis* counterparts. Like *SUB1A*, also a SBI G7ERF, the *Rumex* ERFs could have evolved to become NERP resistant. A clear role for *SUB1A* was identified in delaying dark-induced senescence (Fukao et al., 2012). This occurs mainly by suppressing salicylic acid and jasmonic acid-mediated senescence, whilst simultaneously reducing carbon reserve utilisation. Additionally, it has been shown that adding sugars artificially suppresses *SUB1A* expression, further highlighting its role in carbon and starvation metabolism (Kudahettige et al., 2010). Given the high sequence divergence and the dark regulation it is likely that the *Rumex* G7ERFs play a role in adaptation to carbon and energy starvation associated with darkness and flooding stress.

Is the dicot escape response regulated by ERFs, like in rice?

In deepwater rice the vigorous shoot elongation during the escape response is regulated by SK1 and SK2, both G7ERFs. This was characterized by a strong transcriptional induction throughout the elongation period (Hattori et al., 2009). The quiescence response is also regulated by a G7ERF, namely *SUB1A* (Fukao et al., 2006; Fukao and Bailey-Serres, 2008) which was also transcriptionally induced throughout the stress period. Interestingly, this induction was very tissue specific (Singh et al., 2010). If G7ERFs were to play a role in the escape response of the dicot species studied here transcriptional induction of a *G7ERF* would be expected specifically in the elongating tissue. However, we were unable to identify expression of a *G7ERF* specific to the elongating petiole (Fig. 6.6 and 6.7). This suggests that escape or quiescence in dicots is not regulated by a G7ERF. Genome wide transcript profiling in *Rumex* petioles has identified a novel role for shade responsive and photomorphogenic genes in mediating the escape response in *Ru. palustris* (Chapter 2 and 3). Nevertheless, we observed aboveground specific induction of *Ro. sylvestris HRE1*, which might explain the reduction in growth under submergence. The emerging picture appears to be that regulatory mechanisms of escape and quiescence strategies are most likely species specific and exists as a result of convergent evolution.

Conclusions

We conclude that the angiosperm G7ERFs originated from two ancestral genes. Our investigation on contrasting species pairs from two distant dicot lineages (Asterids and Rosids) reveals how these ancestral G7ERFs have developed to behave in varying ways, subtly within a genus, and with large differences across the dicots. Hereby we have gained insight in their potential to explain variation in the response and tolerance to flooding stress. Within *Rorippa* and the *Brassicaceae,* a variation in the magnitude of *G7ERF* regulation could potentially explain different flooding responses. In *Rumex* the genetic differences might be far more important. However, the largest contrast in G7ERFs is found between taxonomic entities, with an important role for *HRE1* and *HRE2* in *Rorippa* and the Brassicaceae, and a strong darkness and strong submergence regulation of SBI *G7ERF*s in *Rumex*.

Materials and methods

Plant Growth

Rumex palustris and *Rumex acetosa* seeds were germinated on floating polyethylene beads for 10 days (12h light, 25°C; 12h dark, 12°C). *Rorippa amphibia* and *Rorippa sylvestris* plants were propagated from rhizomes as previously described (Akman et al., 2012). Rhizomes were surface sterilized (8 min. 10% bleach) and washed (3 times sterile H_2O). Rhizome fragments (2-3 cm) were left to sprout on 0.5x MS, 0.8% agar, pH 5.7 (Duchefa, Haarlem, The Netherlands, Duchefa, Haarlem, The Netherlands) for 10 days with 16 h photoperiod at 20°C. Single plantlets were subsequently transferred to sand for roots development. *Rumex* seedlings and *Rorippa* plantlets were transplanted to a soil-sand mixture (2:1) supplied with nutrients (Chapter 2) and grown for 18 days in a climate chamber with 16 h photoperiod (160 µmol m^{-2} s^{-1}, PAR, 8 h dark 20°C; 70 % relative humidity).

Experimental conditions

Flooding treatments consisted of complete submergence in tubs, with sufficient depth to prevent plant emergence by escape growth (55X36 cm, depth 25 cm), in complete darkness and commenced 4 hours after the start of the photoperiod. Tubs filled with water the day prior to the experiments to allow pre-equilibration at 20°C in the dark in climate chambers. The dark treatments were performed in the same chamber. With this set-up sur

vival assays and shoot elongation measurements were performed in a single experiment and gene expression and carbohydrates were analysed in a second experiment. Petiole and lamina lengths were measured using a digital calliper, and plant survival was assessed after a recovery period of 15 days in identical conditions used for plant growth. Survival was determined by the presence of newly formed green tissues.

G7ERF identification in *Rorippa* and *Rumex*

Degenerate primers designed for conserved regions of *Arabidopsis* G7ERFs were used to sequence and clone *Rorippa* group VII genes from cDNAs with standard protocols (Sambrook & Russell, 2001). Based on sequences from at least ten independent clones for each gene per species, qRT-PCR primers were designed for conserved regions between species. *Rumex* G7ERFs were identified by using the BLAST algorhythm (Altschul et al., 1990) with known ERFs against the assembled *Rumex* transcriptome (Chapter 2). The identified sequences were confirmed by cloning from cDNA (Sambrook & Russell, 2001).

Gene expression

Rorippa RNA extraction and DNAse treatment was done with the RNeasy Mini Kit and RNase-Free DNase Set (Qiagen Benelux B.V., Venlo, The Netherlands). *Rumex* RNA was extracted using the Kiefer protocol (Kiefer et al., 2000) with subsequent DNA removal with Ambion DNA-free. The cDNA for all four species was synthesized with random hexamers and SuperScript III reverse transcriptase (Invitrogen, Bleiswijk, The Netherlands). Quantitative PCR was performed using Platinum SYBR green Supermix (Invitrogen) and with gene specific primers (Appendix, Table A1).

Carbohydrate determination

Freeze-dried samples were weighed in a balance for dry weight analyses. A ground subsample was used for the soluble carbohydrate and starch analyses as previously described (Vashisht et al., 2010). In short, 10 mg powdered material was treated with 80 % methanol (76 °C, 15 min.). After removal of methanol via freeze-drying the pellet was dissolved with milliQ. The supernatant was run on HPLC (Dionex, Carbopac PA1column, electrochemical detection). The remaining pellets were analysed for starch using a commercially available kit (Boehringer, Mannheim, Germany).

Group VII ERFs synteny analysis

The synteny relationships of all G7ERFs of *Arabidopsis thaliana* (Thale Cress), *Arabidopsis lyrata* (Lyrate Rockcress), *Vitis vinifera* (Grape vine), *Solanum lycopersicum* (Tomato), *Glycine max* (Soybean), *Populus trichocarpa* (Western Poplar*), Medicago truncatula* (Barrel Medic), *Theobroma cacao* (Cacao), *Prunus persica* (Peach), *Fragaria vesca* (Strawberry), *Cucumis sativa* (Cucumber), *Cajanus cajan* (Pigeon pea*), Brachypodium distachyon* (Purple False Brome*), Sorghum bicolor* (Sorghum), *Oryza sativa* (Rice) and *Zea mays* (Maize) were retrieved from Plant Genome Duplication Database (Tang et al., 2008). The number of homologous and collinear genes present between pairs of species was used as the measure of synteny. The Cytoscape 2.8.2 software (Shannon et al., 2003) was used for visualization of the syntenic networks using all pair-wise species comparisons. The number of genes in synteny blocks that are shared between two G7ERFs was log-transformed and used as length identifiers of the connector lines between the genes. Thus, a high number of shared genes in a synteny block is indicative of a small evolutionary distance between genes and their genomic regions.

Phylogenetic tree construction

Rorippa G7ERFs showed strong homology with *Arabidopsis* genes, thus they are represented in the same synteny blocks as the *Arabidopsis* orthologs. In order to resolve the evolutionary relationships of *Rumex* G7ERFs to other species, we analyzed the conserved motifs; the starting amino acids and the AP2 domain in a phylogenetic tree for *Arabidopsis*, peach, grape, *Rorippa*, tomato and cacao. In total we analyzed 244 nucleotide sequences by neighbor-joining method with a 1000 bootstraps in MEGA 5 (Tamura et al., 2011). We also BLASTed the *Rumex* genes to Plant Genome Duplication Database (PGDD) and investigated the genes showing the highest similarity in order to reveal the most likely relationship of *Rumex* genes to our two identified synteny blocks derived from other species.

Supporting Information

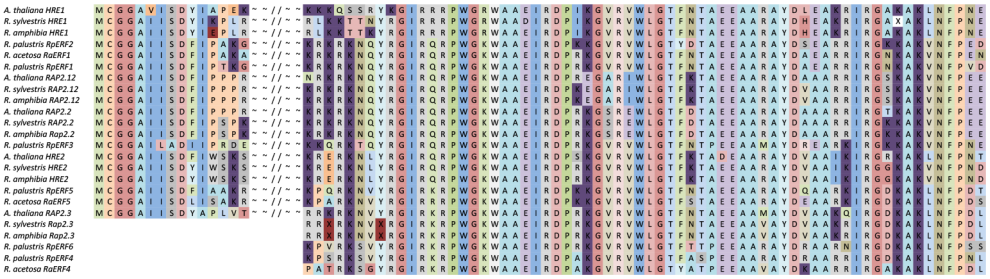

Figure S6.1 Protein alignments for 5' conserved motif and the AP2 domains for *Rumex* and *Rorippa* species.

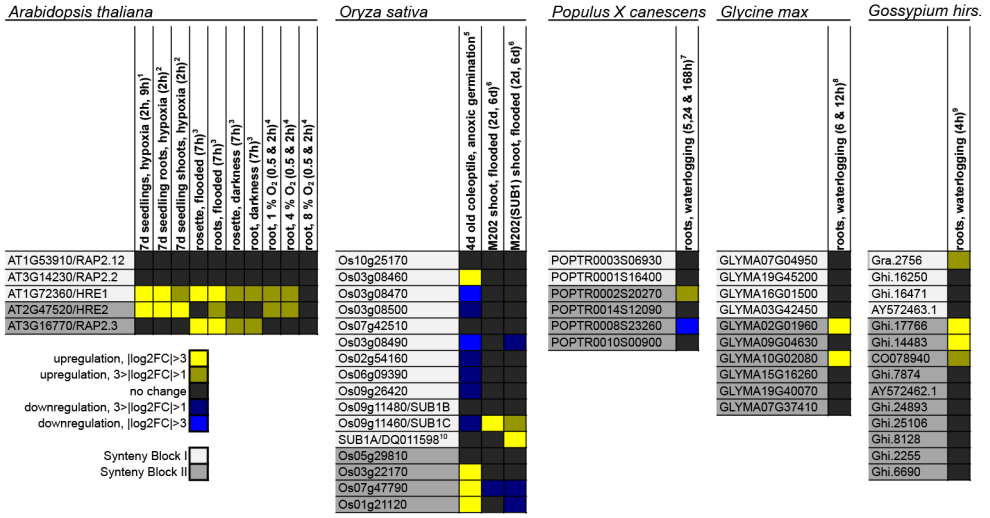

Figure S6.2 Expression of group VII ERFs in different microarray studies on *Arabidopsis*, *Oryza sativa*, *Populus X canescens*, *Glycine max* and *Gossypium hirsutum* (*hirs.*) under various flooding related stresses. [1]Branco-Price et al. 2008, [2]Mustroph et al. 2009, [3]Lee et al. 2011, [4]van dongen et al. 2009, [5]Lasanthi-Kudahettige et al. 2007, [6]Jung et al. 2010, [7]Kreuzwieser et al. 2009, [8]Nanjo et al. 2011, [9]Christianson et al. 2010, [10]Fukao et al. 2006.

Chapter 7

Summarizing discussion

Flooding tolerance is widespread throughout the plant kingdom. The source of this tolerance has largely been attributed to anatomical features, such as aerenchyma for improved internal aeration (Justin and Armstrong, 1987) and leaf anatomy (Mommer et al., 2006; 2007). However, in the ability of plants to deal with the physiological stress of flooding and poor gas exchange, a great deal of variation amongst plant species has been identified regardless of their anatomical features. This is reflected in the differences in tolerance to low oxygen conditions, an important aspect of submergence, in for instance *Rumex acetosa* and *R. palustris* (Voesenek et al., 1993), rice, wheat and maize (Mustroph and Albrecht, 2003; Menegus et al., 1991), and even amongst the semi-aquatic species *Trapa natans*, *Nuphar luteum* and *Scirpus mucronatus* (Menegus et al., 1992). Last, but not least important, having an appropriate growth strategy, quiescence or escape, during a certain flooding regime has shown to greatly improve plant performance (Setter and Laureles, 1996; Akman et al., 2012; Pierik et al., 2009), and is a key determinant of the hydrological niche occupied by species (Voesenek et al., 2004). This is the main reason why understanding of the underlying mechanisms of flooding survival strategies is important, and thus the main focus of this thesis.

Interestingly, many of the traits important for adaptation to life in flood-prone environments such as improved aeration, tolerance to low oxygen conditions and effective growth strategies, are absent in model organisms such as *Arabidopsis*. To really understand the underlying mechanisms of how plants have obtained and regulate their tolerance traits, it is thus essential to study species where these traits have evolved in nature and are an important determinant of their success in specific environments. Thus far this has led to predominately comparative physiological characterization of wetland and crop species. Attempts have been made to characterize, at a molecular level, the responses of wild species to flooding and the associated stresses. However, subtractive cDNA libraries of tolerant rice varieties or the wild *Potamogeton distinctus* could only poorly resolve transcriptional changes upon flooding or anoxia (Agarwal and Grover, 2005; Harada et al., 2007). However, targeted molecular approaches towards genes with a specific functions has provided great detail of physiologically established processes (e.g. Vriezen et al., 1999; Benschop et al., 2005; Vreeburg et al., 2005; Saika et al., 2006). Despite the disadvantages of working with genetically cumbersome non-model organisms, this thesis demonstrates that novel insights into plant adaptation at a molecular level can be obtained by making use of next generation sequencing technologies.

The unbiased investigation into the transcriptomic responses of the youngest petiole of *R. acetosa* and *R. palustris* of completely submerged plants (chapter 2) led to an overview of genes that are involved in adaptation to flooding stress. The ability to contrast the transcriptome response of the escaping *R. palustris* against that of the quiescent *R. acetosa*, and vice versa, was instrumental to develop hypotheses regarding general *Rumex* flooding responses and more specifically how different species respond to submergence.

The specific upregulation of genes encoding metabolic enzymes and regulators in the quiescent *R. acetosa*, but not *R. palustris*, pointed toward metabolic alterations to regulate quiescence. In chapter 3 the metabolic changes of both species upon submergence were further explored. This led to a model where enhanced sensitivity to abscisic acid (ABA), activation of trehalose-6-phophate breakdown, together with changes in nitrogen and carbon metabolism lead to maintained soluble sugar levels and growth retardation.

Additionally, a new role for several photomorphogenesis genes in underwater elongation was identified (Chapter 3). These genes, required for elongation during shading and low light conditions, were also activated upon submergence of *R. palustris* only. Not the changes in the light environment underwater, but the passive accumulation of the early flooding signal ethylene, together with the subsequent downregulation of abscisic acid (ABA) induces expression of the photomorphogenesis genes, and subsequently petiole elongation.

Where previously hypoxia pre-treatment was shown to improve anoxia tolerance (Saglio et al., 1988), here a similar role for ethylene was identified (chapter 4) in *R. palustris*, but not *R. acetosa*. Again making use of the global transcriptomic characterization, a set of typical hypoxia responsive genes, conserved throughout the plant kingdom (Mustroph et al., 2010; 2009), were activated despite normoxic conditions in shoots of *R. palustris*. Ethylene pre-treatment led to enhanced expression of these typical hypoxic responsive genes in *R. palustris*. However, *R. acetosa*, where a similar set of core hypoxia genes is present, is unable to use ethylene as a cue to activate these genes and increase tolerance to anoxia.

Interestingly, we were able to discern these important tolerance related processes (metabolism, light signalling and ethylene mediated anoxia tolerance) because the identified species-specifically regulated genes had a well described function in *Arabidopsis*. An emerging theme is the ability of *R. palustris* to use the ethylene signal to tap into the conserved light dependent growth machinery as an important part of the escape strategy, whilst simultaneously acting on plant kingdom wide hypoxia responsive genes to improve low oxygen tolerance (Mustroph et al., 2010; 2009). Apparently, a large part of adaptation to flood-prone areas requires the ability to use reliable flooding cues, such as ethylene (Voesenek and Sasidharan, 2013), to access commonly existing gene networks and processes.

By directly comparing *R. acetosa* to *R. palustris*, independent of angiosperm-wide derived gene families or Gene Ontology terms, it was possible to identify the interesting species-specifically regulated genes that are absent or not characterized in other species. Amongst the *Rumex* genes with species-specific regulation upon submergence, there were many for which either no or poor orthologs were identified in other species (Chapter 2). Unfortunately, functional characterization is a truly difficult endeavor in wild species. Many genetic resources required for this purpose, such as mutant collections, transformation protocols or the ability to make crosses, are absent in the *Rumex* system. In chapter 4 a first step to identify novel molecular components of flooding tolerance was initiated. By correlation to known processes, genetic components could be assigned a putative involvement with an aspect of submergence tolerance. Based on transcriptional responses to hormones and tissue specificity we suggest the involvement of a glycine rich protein, a

methyl transferase and an unknown protein in early cell wall loosening, but a role for peroxidases, a MAP kinase and molecular chaperones in long-term elongation.

In a targeted approach, the role of group VII ERFs (G7ERFs) was investigated for their role during flooding in four wild dicot species (chapter 6). G7ERFs have been identified as having a wide variety of functions in adaptation to flooding, during both quiescence and escape (Fukao et al., 2006; Hattori et al., 2009; Licausi et al., 2010). By studying evolutionary processes, gene composition and regulation of G7ERFs in two phylogenetic lineages with contrasting species from flood-prone sites allowed for a broad perspective on the role that these G7ERFs might play during adaptation to flooding. Within the two genera, subtle variation in the extent of expression (*Rorippa*) or differences in genetic variants (*Rumex*) provided clues that could potentially explain variation in tolerance. Interestingly, the phylogenetic lineage was more important in determining the composition and behaviour of G7ERFs than variation within the genera. This is in line with the evolutionary analysis that the G7ERF families diversified from two common angiosperm ancestors.

Thus two genera, both very tolerant and adapted to flooding, use a gene family involved throughout the plant kingdom in submergence tolerance in a very different way. This questions to what extent observations of one taxonomic group can be extrapolated to another. A similar observation is true for the submergence escape response. For instance, underwater petiole elongation in *Ranunculus scleratus* is mainly auxin driven, whilst petiole elongation in *Rumex palustris* is predominantly dependent on gibberellic acid (Rijnders et al., 1996). Additionally, a role for photomorphogenesis regulated elongation was not found in rice (Jung et al. 2010). It is suggested that an amphibious lifestyle evolved independently more than 200 times (Cook, 1999). Indeed it seems that several physiological solutions have been developed for the challenges of submergence, such as the variation in using G7ERFs and the different ways in how elongation can be regulated.

Concluding remarks

A true understanding of tolerance to flooding can only be gained from studying species that have proven to be successful in flood-prone environments. In this thesis it is shown that the ability to tap into genes and signalling modules that are ubiquitous throughout the plant kingdom, can lead to adaptive responses such as elongation and improved anoxia tolerance. This provides an opportunity to rewire crop plants to use reliable flooding cues to activate target gene networks and processes already present in these species, and so improve stress tolerance. For quiescence this is related to metabolism, as suggested in chapter 3 and ethylene-mediated gibberellin sensitivity (Rijnders et al., 1997; Fukao and Bailey-Serres, 2008). However, these mechanisms leading to flooding-induced growth retardation remain poorly understood. Again, we have seen that this, like G7ERFs, might be achieved in different manners throughout the plant kingdom. Investigating the physiology and underlying mechanisms of wild plant species will provide new insights into how plants have adapted to flooding and will allow for the assessment of potential opportunities for crop improvement. Indeed, with this thesis we have made a contribution to the understanding of plant adaptation to flood-prone environments.

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Appendix

Table A1 List of primers used throughout the thesis for qRT-PCR

species	OMCL family	description	forward	reverse
Rumex acetosa	fam18188	NCED	GGCGAGTCCAAATTCATTGT	TAAGCCATGAAACCCGTAGG
	fam11194	ABA8ox	GACCTCTCGGGTGATACA	TTTCCCAAACGGCATGTAC
	fam12964	ERS1	GGAGGTCAGATTTGGGTTGA	TGCTGAACAGCCAATGTCTC
	fam11458	XTH22	CACCAATGTGTTCAGCCAAG	CCGATCGATTCCATGTTCTT
	fam15637	PCK1	GTACGCAGGGGAGATGAAGA	AGGTACCGGATAGGCCAAAG
	fam06080	PCK2	TGATCCGTTGCTCAACTCTG	CAATGACGCACTGATGGACT
	fam19920	ACHT5	ACTCTCCTGCTTGTGGTGGTTGC	ACGCCCCTTTGAGCCCTTGT
	fam21095	ACR7	GGACGAGAAATCAGCCCATA	GAGGAGATAACGGCATGGAA
	fam13057	HB1	ATCCCTCATCACTCTCACCG	TTATGTCCATAATGGGCCGT
	fam02870	PP2-A13	GTAGGCTTCCCCTTCCCAGC	GCACTGGCTTAATGTCCCAT
	fam10261	PHI	TCACCAACCCCTTCAAGAAC	TTGTAGCTTCCTCCGCTTGT
	fam14172	SRO5	CTCTCCAAGCTATTGCCGTC	TGGATTCTTGGTTCTCCAGG
	fam11723	SUS	GTCGAATGGTACGGGAAGAA	GAATTGCCCGTTGAGATTGT
	fam20803	ADH	CACCATCAAAGGGAAGCCTA	CAAGAGGAGCAGAAGGGTTG
	fam14376	KIDARI	GGTAGATCTCGAGGCACGAA	CCTCGACCTCCTACTCGACA
	fam04892	ATHB2	GCACAACACTCTCAATCCCA	CGATTCTCCTCGGTTAGCTG
	fam20527	HY5	CTGCACAGCAAGCAAGAGAG	GATGCTTGAAGCGTTCACAA
	fam05638	PIF1	CAGGATCGGTGATTTGGACT	GCCTTGTCCGTCTTATTGGA
	fam03135	PIF2	GCTGCTGAAGTGCATAACCA	ATTGCTTCGTCGAGCATTG
	fam15827	PIF3	TCATGACTCCGTTTGCCATA	TTCTTTTACGCTCGGATTGG
	fam17228	COP1	GGGACAGCCCAACAATTATG	CTGCAGCATACGCATGTTTT
	fam17082	MYB111	GCCTCAAACGAGGAAAGATG	CCGTTCTTCCTGGCAATCTA
	fam04923	ABF4	TCCCAAGGATTGCTTCAAAC	TGACCAGCATCCCAGTATCA
	fam19406	bHLH93	GCCTCACCCAAGTTTGATGT	TTTCAAACCCCATTGCTTCT
	fam02303	SPL4	ACGCTAAAGCCCCTCTTGTT	CGGCAGCTTCTCTTGGTATC
	fam20803	WRKY70	CACCGTCGTCAATCAAACAC	GCCTGGCTCACGTTATTGTT
	fam11501	tubulin	AGCATCACTGGGTTGGAAAC	CACGAACTGCTTGATGTGCT
	fam00651	RAP2.12 ortholog	CGAGCAGTGTAATGCCAAGA	AGCATCAACAGCCCAGTTCT
	fam21098	SGAT	AGCCATACAGGGAGTTGGTG	GCGCTATTCCAGCTTGTACC
	fam16464	TPP	TCTGTTGTTCCGAAGCCTCT	TGGGGAAGAAGATCTCATGG
	fam19885	TPS	CGTGGCTGAAATTGTTCGTA	CCCGATATCTGATGGCAACT
	fam17585	SnRK1 beta	CTTCCTCCTTGTCGTCTTCG	CCGTTAGCTCCCTCCTCTCT
	fam15637	PCK	TTGGGGACTCAGTACGCAG	ATCTCCACGTTTGCCCATGT
	fam13033	TA	TGGAGTACCGGTTCATAGGC	CCTTTCTCGCCTTCAGACAG
	fam05700	AmT	GGAGAAGCTCCAGTTCGATG	GCTTGAATTGGCTCCGTAAA
	fam05452	NiT	CTCGTCCTCTTTTCCACAGG	CTAGAGATCGACGGGAGACG
	G7ERFs	RaERF1	CGAGCAGTGTAATGCCAAGA	AGCATCAACAGCCCAGTTCT
	G7ERFs	RaERF2	AGGATTCTGCCCCAGTTTCT	CCAGTCCTGAGAAGCTACCG
	G7ERFs	RaERF4	AGCAGCACGGAGAACAGTCT	GCTCCAAACCGACCTCAATA
	G7ERFs	RaERF5	TGGGACTGGAGGAAGAAGTG	GTCCAAACACCACAAATCCA
Rumex palustris	fam18188	NCED	GTCCACTCTCTGCTCGGAAC	CACTGTGCACTCCTTCTTGC
	fam00337	ABA8ox	GCGGTGCTTTCAATCTTAGG	TGTTCCAGGGAGGTTAATCG
	fam04892	ADH	AAGGGCCAAACACCTCTTTT	ACACATGGTCTCCTGGCTTC
	fam11723	SUS	TGACTGTCGTCGAGGCTATG	ATGTGGAAGCCTGATTGACC
	fam19622	ACHT5	GGCCAAGTGTGCAGCTTTAG	CCAAAAACAAGGTCGGATTG
	fam06243	ACR7	AGACCGTCCATGCTTATTGG	GCAGCTCCTGGACTTTCAAC
	fam13757	HB	ATATGGAGTTGCCGATGAGC	TATTGGGGCCTAAGAAGCCT
	fam02870	PP2-A13	GGACATCAAGCCTGTGGAGT	GTGCAGTCTATCTGGGCCAT
	fam10261	PHI	ATCATCGTGTTCCTCTCGCT	GATTTTGGGAGCTGTGGGTA
	fam01589	SRO5	CTCCAGATTCCAGGGTCAAA	AATTGCCGATGATTTCTTGC
	fam03530	KIDARI	TTGCCCTTGTCTCCAAACTC	TATTTGCTGATGCCTTGTCG
	fam05638	HY5	CAAAATGCTAGAGCAAGCGA	CGCTCTCCATTCCTTCTTTG
	fam03135	PIF1	CGCAAAATTCTCAACTGCAA	AAACGTAAAGCCAGTGCGAT
	fam03405	PIF2	TATCTGAAAGGAGGCGGAGA	CATCGAGCATTGACGCTTTA
	fam17228	PIF3	ACTCCGTTTGCCACAGTAGG	CGCTCGGACTGGTTATGAAT
	fam17228	COP1	GGATGAAGCCGATCAGGATA	TCAGGTGGACAGCGTGAGTA
	fam20527	ATHB2	CAATCATGGCAGCAAAGCTA	GAACACCGAAGGCATGAGAT
	fam17082	MYB59	GCCTGTGTGTGAGGAAACAA	TCACCATCATCCATCTTCCA
	fam04923	ABF4	CCTGCAAAGACAGGGTTCAT	AGCCATGTCTCTCCACACCT
	fam19406	bHLH93	ATCCAACGCCATATCCAGAG	GCTGTCCTTCAACCCTCCTT

Table A1 continued

R. palustris continued	fam09638	SPL5	CCAAGGCTCCTGTCGTAGTC	CAGCTCCTTTTCACCTCGTC
	fam20803	WRKY70	TCCCATCCTCATCTCCTGAC	GGAAGAAGGGGTTGTTGTGA
	fam11501	tubulin	TGGACCTTGCATTACGATCA	GTTTCCAACCCAGTGATGCT
	fam00651	RAP2.12 ortholog	GCAAATGCAAAGACAAACCA	TATGGGTTCCACCTTTCCAA
	fam21098	SGAT	AAAACTCCTGCAAAGGCTGA	GGTGAAGCAAAGTGGAGGAG
	fam16464	TPP	CCATGTCCTTCTCCCACAAG	TGCCATCCAAGAAGAGAGGT
	fam19885	TPS	AAGACGACGGTCCAAGAGAA	AGAGTGTACCTTGCGCTCGT
	fam17585	SnRK1 beta	GGGGTTCGAAATTTTGGAGT	CCGTTAGCTCCCTCCTCTCT
	fam15637	PCK	TTGGGGACTCAGTACGCAG	ATCTCCACGTTTGCCCATGT
	fam13033	TA	TTAGGCGGCGGTATGAGGCAT	CTTTGATTTGGTTGAGTCCATCTG
	fam05700	AmT	GTTGGACACTGGAACGTCCT	GAGGCAACGAAACCACAGAT
	fam05452	NiT	GTTCAAGTGCTAGGGCGAAG	CCGAGAGGTCATTACCCAAA
	fam11685	bHLH	GACGTGGAGGTGAGGTTTGT	TCGAAATCGTAGCATGGTGA
	fam16757	MAPKKK	CGGTGGAGAGGATTGGTAAA	CCGAAACATCCCTAACCTCA
	fam20146	DNA-J	GGAGGAAATGGCGACTATGA	TTTGGTGTCGTTTGATTGGA
	fam10911	PER-1	CCTTTGCTGCACAACTGAGA	CCCCTGGAGAAGAAACTTGA
	fam06155	RHD3	AATGGAGGCAGCAAAAGAGA	CCAACACCAGAAAGTCAGCA
	fam20653	PUF-1	TGGCGTTCAAGAAGACAATG	TGAATGAGGGGAGGTTTGAG
	fam04479	LTP	CCTTGTTGTCGGAACTCCTC	GGGAACATTCTTGCCACAGT
	fam19438	RmlC	ACAGCCGACGACTTCCTCTA	TGACAGGTGTCACGATGGAT
	fam05933	ROPGEF	TGAAAATGCAGATCCAAGCA	TACACACTGCCATCCACCTC
	fam04160	CIPK	GAAGAGGAGCCGGAGAAGAT	GTTTACCCCCACAACAATCG
	fam03213	THX	CATGGAGAGGGTCAAAGAGC	CGTGGAAAAGTGTCGTGATG
	fam02229	Annexin	GTCTTTGCTTCATCGCCTTC	GCCATTTTCCGAGTGTTGTT
	fam03079	GBF	TGAAGCCTTGACTGCTGAGA	CGGTCTTGTTTGGACTCGTT
	fam12553	HDL	GACCCTTTGTTTGCATCCAC	TAGGCATGTGCATCACCATT
	fam03324	ST-1	TGCGCTTTGTAGCTTTCAGA	AACAAGCTCTCGTTCCCAAA
	fam04236	NA-1	ACTCAGTTTCCCCCAAATCC	CAATCAAGGCGACATCTTCA
	fam05054	OCP	AGGAAGAGCAGGAAGGGAAG	TAGCCGTGCCATTATCATCA
	fam20803	WRKY54	GTCCATCAAACGGGAACAAC	TAATCGGACTGAGGGGATTG
	fam00857	CNGC	ATGCTCTGGCAAAAGGAGAA	AAACGGTCTTGGCTTGCTTA
	fam10186	DPP6	CCAACACCTCTTCCTCGAAA	GGTTCCCCTGAAAACCTCTC
	fam06246	GST-1	CTGGACGTTCTGGAAGAAGC	GGCGGCATTGTCTTCTGTAT
	fam10782	GST-2	TCGCTGAATCACTCGTCATC	TCATAAGGATGGGACGGAAG
	fam10855	ST-2	AAGAGGTTGGCGGATTTTCT	CATGTTCTGGAAGCTGCAAA
	fam19976	PER-2	TCAGGCAATACAGCAACTCG	TGAATGACGTGGATCGGTTA
	fam05561	GRP	ACAAGGAGGCAGCAACAG	AGTTTGGGAGGGCTACGA
	fam10436	PUF-2	GGAGCAATGCAGTACTCCT	TTCAAGCTCTGATGTGGTCTG
	fam13304	NA-2	AAACCAGGTGAAGAAGAGAACC	TGGCCCATCCTTCTTTGC
	fam13762	NA-3	ATGAATTTGAGCATCAGGTGG	AATGGAAGTGCTTGCCGA
	fam14127	NA-4	CAACACGAGAAATGCGTGG	TTCATCTGAAAGGCCGTGTC
	fam15826	SAM-MT	GAACCCTCCATTTCGTCCA	CCTCGCTCAATCCTTTGCT
	fam17082	MYB59	GGAAACAAGCACTCACTTCTC	TTCACCATCATCCATCTTCCA
	fam19764	PDF	ACCCTACAAGCCTGACGT	CCGAGTTCAGGAAGGAAGC
	G7ERFs	RpERF1	GCAAATGCAAAGACAAACCA	TATGGGTTCCACCTTTCCAA
	G7ERFs	RpERF2	TGAGGAAGAAGAGGCTCAGG	ACTGCCCAATCCTCATCAAG
	G7ERFs	RpERF3	TCCTCAATCCTCCTCCTCCT	CTAATCGCGGCTTCAGAGTC
	G7ERFs	RpERF4	AGAGTTTGCTGGGTCTGGAA	GGGAACTGAAACTCGTCCAA
	G7ERFs	RpERF5	CCACTCTGTTGCTGTGAACG	CTTGTCCCCACTTCTTCGTC
	G7ERFs	RpERF6	TTCTTTGTCCGGGTCAACTC	ACTCCCCTCCTCAACCTCAT
Rorippa sylvestris and *Rorippa amphibia*	G7ERFs	HRE1	TGATTTCTTGTGGGAGGAGAA	CAAGAAGCTCTTCTGAAAGCAA
	G7ERFs	HRE2	TCAGAGGAGCTCATGGCTTT	AAATGTCCACAGATTTAGGTCGAG
	G7ERFs	RAP2.2	AGCCAAGAAGCTCAAAACCA	CTTCCTGAGTCACTGCACCA
	G7ERFs	RAP2.3	AAGAAGCTCTGCGTTTCGTC	ATCGAGTTGACTCGGTTGCT
	G7ERFs	RAP2.12	CATTGATTTCGAGGCACCTTA	AAGACTCCTCCAATCATGGAA
	NA	Actin	TTTGTGGGAATGGAAGCTG	GTGGTGCAACGACCTTAATC

Nederlandse samenvatting

Planten die normaal op het land groeien komen in moeilijkheden als ze onderwater komen te staan tijdens een overstroming. Gasdiffusie is namelijk ~10,000 keer langzamer in water dan in lucht. Hierdoor ontstaat er een tekort aan zuurstof (O_2) en een gebrek aan koolstofdioxide (CO_2). Zonder zuurstof kan er geen mitochondriale respiratie plaatsvinden en dus kunnen planten enkel energie verkrijgen via de glycolyse, met slechts een productie van 2 tot 4 ATP per glucose molecuul in vergelijking met ~36 ATP tijdens normale respiratie. Om NAD^+ te regenereren, essentieel voor het in stand houden van de glycolyse, moet de plant fermentatie reacties activeren. CO_2 is noodzakelijk voor fotosynthese in the bovengrondse plantdelen. Als gevolg van overstroming ontstaat er dus een algehele energie- en koolstofcrisis wat uiteindelijk leidt tot sterfte van de plant.

In het plantenrijk zijn twee succesvolle strategieën geïdentificeerd om met overstroming om te gaan, ontsnappen en uitzitten. Bij plantensoorten die voorkomen in gebieden met langdurige en relatief ondiepe overstroming vindt een ontsnappingsreactie plaats waarbij de spruit sterke strekkingsgroei vertoont. Door deze verticale verlenging kan contact met het wateroppervlak worden gemaakt. Via aerenchyma (luchtkanalen) kan er nu relatief snelle gasuitwisseling plaatsvinden, ook met de delen van de plant die nog onderwater blijven; het is dus vergelijkbaar met snorkelen. In gebieden waar de overstromingen van korte duur of erg diep zijn, strekken plantensoorten niet onderwater, maar proberen ze zoveel mogelijk de groei en andere energie vragende processen te minimaliseren om zo overstroming overleven.

De regulatie van deze strekkingsgroei en het overleven van overstroming is bestudeerd in veel verschillende plantensoorten. Echter, de meeste details zijn bekend in rijst met grote variatie in de strekkingsreacties; in *Rumex palustris* (moeraszuring) met een sterke strekkingsreactie en *Rumex acetosa* (veldzuring) dat zonder strekkingsreactie de overstroming uitzit. Tijdens een overstroming vindt er binnen de plant passieve ophoping plaats van het gasvormige plantenhormoon ethyleen. Dit is voor sommige soorten een signaal voor de activering van de strekkingsreactie, maar voor anderen juist om groei te vertragen en de overstroming uit te zitten. Ten gevolge van ethyleenophoping vinden er veranderingen plaats in de hormonale samenstelling en gevoeligheid hiervoor; dit zijn voornamelijk abscissinezuur (ABA; remt strekking) en gibberellinezuur (GA; activeert strekking). Daarnaast is er in rijst een belangrijke rol geïdentificeerd voor een specifieke familie van transcriptie factoren, de zogenaamde groep VII Ethyleen Response Factoren (G7ERF), in de regulatie van ofwel strekking, dan wel het uitzitten van overstroming.

In dit proefschrift is voortgebouwd op de gedetailleerde fysiologische kennis van het ontsnappen en uitzitten van overstroming door respectievelijk *R. palustris* en *R. acetosa* en is zonder vooroordeel (“unbiased”) gezocht naar de onderliggende processen die verantwoordelijk zijn voor de verschillende responsen. Daarnaast is er doelgericht gekeken naar de rol van G7ERFs als potentiële kandidaten voor verschillen in overstromingsreacties tussen planten.

In **hoodstuk 2** is door middel van krachtige DNA sequentie technieken de samenstelling van de genen en de verandering in expressie ervan tijdens overstroming in *Rumex acetosa* en *Rumex palustris* bepaald. Nadat gelijksoortige genen tussen twee soorten waren bepaald kon een directe vergelijking tussen de twee soorten gemaakt worden. Hierbij zijn, op RNA niveau, de verschillen die potentieel verantwoordelijk kunnen zijn voor de ontsnappings- en uitzitreactie van de twee *Rumex* soorten in beeld gebracht.

Veranderingen in genexpressie specifiek voor *R. acetosa* waren voornamelijk gerelateerd aan metabolisme en de activatie van twee ABA signaleringscomponenten. Veranderingen in metabolieten en genexpressie profielen gedurende overstroming resulteerde in **hoodstuk 3** tot de vorming van een model van groeiremming voor *R. acetosa* wanneer deze wordt overstroomd. Hierbij wordt ABA-signalering geactiveerd dat groei zou kunnen remmen via een reductie in GA gevoeligheid. Tegelijkertijd vind er een toename in de expressie van de afbraak van trehalose-6-phophate plaats. Hierdoor zal de activiteit van een centrale regulator van groei (SnRK1) afnemen. Bovendien wordt de specificiteit hiervan sterk gereguleerd tezamen met stikstof metabolisme en triose-synthese (PEP Carboxykinase activiteit) wat resulteert in instandhouding van het suikerniveau, terwijl tegelijkertijd groei afneemt.

De specifieke veranderingen in genexpressie tijdens overstroming in *R. palustris* waren voornamelijk gerelateerd aan lichtsignalering. Tijdens laag licht, of bij veranderingen in lichtkwaliteit (schaduw) vertoont een plant ook een sterke strekkingsgroei. De genen die hiervoor verantwoordelijk zijn bleken ook sterk gereguleerd te zijn in *R. palustris* onderwater. **Hoofdstuk 3** laat zien dat niet de verandering in lichtkwaliteit onderwater belangrijk is, maar dat de passieve accumulatie van ethyleen deze typische schaduwgenen aanzet via een serie van hormonale interacties. Hier wordt dus een bestaande signaleringsroute gebruikt voor het ontsnappen aan overstroming door deze te koppelen aan ethyleen. Een vergelijkbaar mechanisme om onder water te strekken is in rijst niet geïdentificeerd. Het lijkt er dus op dat in rijst de strekkingsgroei op een andere wijze wordt gereguleerd.

Voor veel genen die specifiek gereguleerd worden in *R. palustris* is geen bestaande functie bekend, maar is de regulatie hiervan waarschijnlijk erg belangrijk voor het overleven tijdens overstroming (we noemen dit kandidaat-genen). Doordat hormonale interacties en strekkingsprocessen erg precies in de tijd en in specifieke organen gereguleerd zijn is in **hoofdstuk 4** een correlatie gemaakt tussen expressie van mogelijke kandidaat-genen en de reeds bekende en precies gereguleerde processen. Hierdoor konden deze kandidaat-genen met een functie worden geassocieerd en dit vormt de basis voor het ontrafelen van nieuwe aspecten van de regulatie van strekkingsgroei onder water.

R. acetosa en *R. palustris* ondervinden in de bovengrondse delen tijdens overstroming geen zuurstofgebrek. Dit wordt veroorzaakt door de nog steeds aanwezige fotosynthese. Het was echter verbazingwekkend dat er wel genen gereguleerd worden die kemerkend zijn voor lage zuurstofgehaltes. Dit werd voornamelijk in *R. palustris* geconstateerd. **Hoodstuk 5** laat zien hoe *R. palustris* beter bestand is tegen toekomstige lage zuurstofgehaltes als deze worden voorafgegaan door ethyleen, een belangrijk signaal voor overstroming. *R. acetosa* was niet instaat het ethyleen signaal te gebruiken om de tolerantie ten aanzien van lage zuurstofconcentraties te verbeteren. In lijn met deze observatie werden ook door *R. palustris*

genen die typisch zijn voor zuurstofgesprek sterker gereguleerd als de lage zuurstofconcentratie werd voorafgegaan door ethyleen; ook dit effect was afwezig in *R. acetosa*. Een mogelijk verklaring van het mechanisme is een model waarbij door ethyleen een of meerdere G7ERF eiwitten sterker accumuleren om vervolgens actief te worden tijdens lage zuurstofcondities, hoewel alternatieve verklaringen niet zijn uit te sluiten.

G7ERFs zijn erg belangrijk bevonden in *Arabidopsis* en rijst voor aanpassingen aan overstroming. In **hoofdstuk 6** werd de compositie en transcriptionele regulatie van de G7ERFs vergeleken in vier plantensoorten die voorkomen in overstromingsgebieden, maar verschilden in overstromingstolerantie en mate van strekkingsgroei onder water. Dit waren twee contrasterende *Rumex* soorten en twee *Rorippa* soorten. Sterke regulatie van G7ERFs is in beide taxonomische groepen geïdentificeerd, echter de compositie en expressie patronen waren sterk afhankelijk van de taxonomische achtergrond en in veel mindere mate van de verschillende overlevingsstrategieën. De subtiele verschillen in expressie tussen de *Rorippa* soorten en verschillen in G7ERF compositie tussen de *Rumex* soorten waren wellicht bepalend voor de fysiologische verschillen tijdens overstroming binnen de taxonomische groepen.

Een goed begrip van overstromingstolerantie kan alleen worden verkregen door planten te bestuderen die zijn aangepast aan frequent overstroomde gebieden. Het aanspreken van geconserveerde signaleringsroutes, zoals schaduw signalering en typische laag zuurstof genen, door de betrouwbare overstromingsindicator ethyleen blijken belangrijke mechanismen te zijn tijdens aanpassingen aan overstroming. Dit biedt in principe de mogelijkheid om al bestaande signaleringsroutes in landbouwgewassen te gebruiken om overstromingstolerantie te vergroten. Echter, er is veel variatie in aanpassingen aan overstroming en in het plantenrijk zijn er meerdere oplossingen voor hetzelfde probleem. Een voorbeeld is de strekking van rijst en *R. palustris* onderwater. Tevens identificeren we taxonomisch gerelateerde verschillen in het gebruik van G7ERFs tijdens overstromingen en een scala van genen met onbekende functie. Voor het verkennen van de mogelijkheden voor gewasverbetering en begrip van plantadaptatie aan ongunstige omstandigheden zal het van belang blijven om wilde plantensoorten te bestuderen. In dit proefschrift is hieraan een bijdrage geleverd.

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Curricullum Vitae

Hans van Veen was born on the 22nd June 1985 in Coevorden. He obtained his atheneum diploma in 2003 from the Esdal College in Emmen. Hans continued by studying biology at the Rijksuniversiteit Groningen (RUG) where he got his BSc in 2006. Followed was work on seed ion fluxes with Marten Staal in the laboratory of plant physiology of Theo Elzenga (RUG); investigation of the ecophysiological responses and antioxidants in contrasting *Eucalyptus* species at the school of forestry and ecosystem science in Creswick (University of Melbourne) with Michael Tausz and Luit de Kok; and a brief stay at the plant ecophysiology group of Rens Voesenek (Utrecht University), where shade induced expression changes of cell wall modifying enzymes were studied with Rashmi Sasidharan. At the end of 2008, Hans acquired his MSc from the Rijksuniversiteit Groningen. In 2009 an "ALW open programma" grant application was awarded by the Dutch Scientific Organisation, which allowed Hans the opportunity the complete a PhD at the Utrecht University. This thesis is the result of that work.

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