Sink or swim: submergence tolerance and survival strategies in Rorippa and Arabidopsis

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CHAPTER 3

Submergence tolerance in *Rorippa* and *Rumex* and the evolution of group VII Ethylene Response Factors

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SUMMARY

**Background and Aims** The response to submergence and hypoxia have been extensively studied in rice (moderately flooding tolerant) and Arabidopsis (flooding sensitive), in combination with the regulatory role of group VII ethylene response factors (ERFs). Our aim was to comparatively study flooding tolerances in *Rumex* and *Rorippa* species and investigate the evolutionary relationships among their group VII ERFs with those in other angiosperms.

**Methods** We studied changes in carbohydrates and growth in four species with different strategies to cope with submergence: *Rumex palustris* and *Rorippa amphibia* with a strong shoot elongation response to re-establish air contact (*escape*), and *Rumex acetosa* and *Rorippa sylvestris* with a limited growth response underwater (*quiescence*). We also analyzed the gene expression and syntenic and phylogenetic relationships of group VII ERFs in *Rorippa* and *Rumex* with orthologs in other angiosperms.

**Key Results** We found that there are similar escape and quiescence strategies in *Rumex* and *Rorippa*. We also showed that angiosperm group VII ERFs can clearly be split into two clades based on synteny and phylogenetic analysis (syntenic block I and block II). Constitutively expressed syntenic block I genes are good candidates as oxygen sensors increasing survival. There are no obvious conserved patterns of group VII ERFs in *Rorippa* and *Rumex* that could explain the parallel evolution of escape and quiescence strategies.

**Conclusions** Submergence tolerance is orchestrated by a complex network of factors such as carbohydrate availability, molecular modifications and morphological function. Similar strategies are present in *Rorippa* and *Rumex*, and although group VII ERFs are involved in both genera, we did not find support for convergent evolution and similar regulation patterns of these genes.
INTRODUCTION

Flooding is a recurring event in many ecosystems and plays an important role in shaping vegetation composition and dynamics in flood-prone areas, due to the dramatic impact of submergence on plant performance and survival (Silvertown et al., 1999; Vervuren, 2003; Voesenek et al., 2004). Agricultural fields are also increasingly exposed to flooding, resulting in severely reduced yields in many crops (Bailey-Serres & Voesenek, 2008). Considering the prediction that due to the changing climate flooding events will become more frequent, widespread and severe (Milly et al., 2002; Durack et al., 2012), a better understanding of how plants cope with flooding may help to increase crop tolerance and yields in flood-prone areas.

Submergence is a compound stress involving many factors. Underwater gas diffusion is 10,000 times slower compared to air (Armstrong, 1980). The subsequent reduction of CO$_2$ and O$_2$ exchange with the environment hampers plant metabolism, leading to an energy/carbon crisis as well as the accumulation of toxic by-products (Colmer & Voesenek, 2009). Furthermore, water turbidity frequently leads to extreme low-light conditions, further limiting photosynthesis in submerged plants (Vervuren, 2003; Parolin, 2009). Thus, extended flooding events are fatal for most plant species, despite the fact that modern terrestrial plants evolved from aquatic ancestors. Interestingly, terrestrial plants have re-adopted an aquatic lifestyle more than 200 times during the evolutionary course of the angiosperms (Cook, 1999), and diverse adaptations have evolved for surviving submergence stress. These include development of aerenchyma tissue, development of specific leaf morphologies, formation of gas films around leaves to facilitate gas diffusion, shifting to anaerobic metabolism in the absence of oxygen, and the ability to deal with toxic by-products of an altered metabolism (Smirnoff & Crawford, 1983; Colmer & Pedersen, 2007).

More in general, two main strategies have been identified in higher plants in response to flooding; escape and quiescence (Bailey-Serres & Voesenek, 2008). The escape strategy consists of traits that facilitate a faster exchange of gases between the environment and the plant (shoot elongation, aerenchyma)(Voesenek & Blom, 1989; Hattori et al., 2009; Manzur et al., 2009; Akman et al., 2012). In contrast, in a quiescence strategy, which is beneficial in deeper floods, valuable carbohydrates are conserved via traits that suppress growth and energy expenditure, in order to rapidly resume growth after the flood water recedes (Xu & Mackill, 1996; Akman et al., 2012).

Extensive research in rice cultivars has revealed a key role for a specific group of the Ethylene Response Factors (ERFs) for the onset of quiescence and escape strategies. (Fukao et al.,
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2006; Xu, 2006; Hattori, 2007; Fukao & Bailey-Serres, 2008; Hattori, 2008; Hattori *et al.*, 2009; Bailey-Serres *et al.*, 2012). One of these transcription factors, *SUB1A* was identified as part of a cluster of three group VII ERFs essential for flooding tolerance in lowland rice (Xu, 2006). Transcriptional activation of *SUB1A* upon flooding increased fermentation capacity, but inhibited shoot elongation, carbohydrate reserve breakdown and gibberellin sensitivity (Fukao *et al.*, 2006; Fukao & Bailey-Serres, 2008). Other rice group VII ERFs, *SNORKEL1* and *SNORKEL2*, activate rapid shoot elongation in deep water rice, conferring an escape strategy, presumably by a GA mediated mechanism (Hattori *et al.*, 2009; Nagai *et al.*, 2010).

Unlike rice, *Arabidopsis* lacks the characteristic elongation or quiescence responses. Nevertheless, group VII ERFs are also regulated upon hypoxia, and enhance submergence and hypoxia tolerance in *Arabidopsis* (Hinz *et al.*, 2010; Licausi *et al.*, 2010). This has been suggested to occur via the observed effect of group VII ERFs on the activation of fermentation and sucrose synthase expression. Recently, members of group VII ERFs were identified as low-oxygen sensors in Arabidopsis (Gibbs *et al.*, 2011; Licausi *et al.*, 2011), transported from the cell membrane to the nucleus under low oxygen and thereby activating transcription of hypoxia related genes. It was speculated that the constitutively present *Arabidopsis* group VII ERFs, *RAP2.12* and *RAP2.2*, switch on a cascade of early hypoxia responsive genes including other group VII ERFs (Sasidharan & Mustroph, 2011).

Despite the extensive work on association of group VII ERFs with flooding and hypoxia, it is still unknown if and how they are involved in differences in flooding tolerance and shoot elongation in other more flooding tolerant plant species. Furthermore, the evolutionary history of these genes is unclear. Therefore, our aim with this study is to clarify the evolutionary relationships of group VII ERFs and their role in conferring growth strategies and tolerance by studying species that show distinct adaptive growth strategies upon submergence.

We first investigated the physiological changes in four species from two genera under submergence. Two of these species, *Rumex palustris* and *Rorippa amphibia* showed an elongation response (escape), and two species, *Rumex acetosa* and *Rorippa sylvestris* showed a quiescence response (Rijnders *et al.*, 1997; Vriezen *et al.*, 2000; Stift *et al.*, 2008; Akman *et al.*, 2012). All four species commonly occur in European floodplains, but have their own characteristic flooding-regimes (Jonsell, 1968; Voesenek *et al.*, 2004). By analyzing carbohydrate changes and growth patterns, we aimed to reveal if and how the functioning of the two survival strategies present in rice are comparable between these two distinct lineages (Asterids - *Rumex* and Rosids - *Rorippa*). Additionally, we studied the diversity and expression patterns of group VII ERFs of *Rorippa* and *Rumex*, as the most likely regulators of these strategies. By coupling tissue-specific ERF expression with physiological alterations,
our aim was to reveal the ecological and evolutionary significance of the group VII ERFs in mediating the adaptive growth strategies during submergence. In order to compare the evolutionary and regulatory patterns of *Rorippa* and *Rumex* group VII ERFs with other species, we analyzed the synteny relationships in several angiosperms. We also compared our results with previously published expression patterns of *Arabidopsis*, rice, poplar, soybean and cotton.

**MATERIALS AND METHODS**

**Plant material and experimental set up**

*Rumex palustris* and *Rumex acetosa* seeds were germinated on floating polyethylene beads for 10 days (12 h light at 25° C and 12 h dark at 12° C). *Rorippa amphibia* and *Rorippa sylvestris* plants were propagated from rhizomes collected and surface sterilized by using 10% (v v<sup>-1</sup>) bleach solution for 8 minutes and washed 3 times with deionized water. The rhizomes were later cut in 2-3 cm fragments and placed on 0.8% agar (Duchefa, Haarlem, The Netherlands) and 0.5X MS (Duchefa, Haarlem, The Netherlands) media (pH 5.7). Rhizomes were allowed to sprout in a germination cabinet (Sanyo MLR-350; Sanyo, Etten-Leur, The Netherlands) for 10 days with 16 h photoperiod at 20°C. Single plantlets were transferred to sand until they developed roots for 10 days. *Rumex* seedlings and *Rorippa* plantlets were transplanted to a soil and sand mixture (2:1) supplied with nutrients (7.5 mmol NH<sub>4</sub>SO<sub>4</sub>, 15 mmol KNO<sub>3</sub>, 15 mmol KH<sub>2</sub>PO<sub>4</sub>, 86.4 umol FeEDTA, 4.27 MnSO<sub>4</sub>, 0.25 umol ZnSO<sub>4</sub>, 4.23 nmol CuSO<sub>4</sub>, 8.5 nmol H<sub>3</sub>BO<sub>3</sub>, 52.2 pmol Na<sub>2</sub>MOO<sub>4</sub> divided over 42 pots) and grown for 18 days in a climate chamber with 16 h photoperiod (160 μmol m<sup>-2</sup> s<sup>-1</sup>, PAR, 8 h dark 20° C; 70 % relative humidity). Initial plant size, at the start of the experiment, was standardized between species, and for each species a homogenous set of a similar developmental stage was selected. The tubs (55X36 cm, depth 25 cm) were filled with water one day before the experiments started. One day later and four hours after the photoperiod started (in the light chambers), the plants used for submergence treatment were placed in these tubs in darkness. Water depth (25 cm) was sufficient to prevent plants from reaching the surface for the duration of the submergence treatment. For dark controls, plants were put in the same chamber used in submergence treatments, but in tubs regularly watered to prevent drying out. The climate chamber used for submergence and dark treatments had the same conditions as the chambers used for rearing the plants, except that the lights were switched off during the experiment. For air light controls the same number of plants as used in submergence and dark treatments were left in the growing chamber without changing the conditions. Two experiments were performed with the same set-up; one for survival assays and shoot elongation measurements and the second for gene expression analyses and carbohydrate measurements.
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**Submergence survival and shoot elongation measurements**

We grew 200 plants for each species and selected a homogenous subset of 80 plants per species to be used in survival assays. At the start of the experiment roots and shoots of ten plants per species were harvested separately for dry weight and carbohydrate measurements. The plants were submerged as mentioned above, and at predetermined time points (4, 8, 12, 16, 20, 25, 30, 40 days) ten plants per species were taken out of water. The petiole and lamina length of the youngest leaf with a visible lamina at the start of the experiment was measured at 4, 8, 12, 16 days using a digital caliper. Plants were put in the growth chamber for a recovery period of 15 days. The youngest leaves of ten air light controls were also measured at each time point for a comparison. After the recovery period submerged plants were assessed as dead or alive by examining the newly growing green parts as an indication of living meristems. Lethal median time, LT$_{50}$ values were calculated by fitting a Weibull function (Hosmer & Lemeshow, 1999) to the data.

**Gene expression and carbohydrate analysis**

For gene expression analyses a similar experiment was performed. Different from the survival assay, dark controls were also included for both carbohydrate and gene expression analyses. In total 450 plants were grown for each species and a homogenous subset of similar developmental stage was either submerged in dark, put in darkness only or left in the growth chambers as air light controls as mentioned above. After 5 days of the treatments, petioles and laminas of the newly growing leaves, remaining shoot material and roots were sampled separately and snap-frozen in liquid nitrogen. Three individuals were pooled together per replicate for either carbohydrate analyses (3 replicates per species, treatment and tissue type) or gene expression (5 replicates per species, treatment and tissue type). Samples were kept in -80°C until RNA isolation or freeze-drying for carbohydrate analysis.

**RNA isolations**

RNA isolations and DNase treatments for *Rorippa* samples were done with RNeasy Mini Kit and RNase-Free DNase Set (Qiagen Benelux B.V., Venlo, The Netherlands) according to manufacturer’s instructions. RNA quantity and quality were assessed by using Nanodrop and RNA intactness was checked on an agarose gel. RNA from *Rumex* tissues were extracted with a modified version of the Kiefer Protocol (Kiefer et al., 2000), an additional ethanol cleaning step of the RNA was implemented. DNase treatment was done with the “Ambion DNA-free” kit, according to manufacturer’s instructions.
Gene cloning and qRT-PCR

Degenerate primers designed for conserved regions of Arabidopsis group VII ERFs were used to sequence and clone *Rorippa* group VII ERFs 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. The qRT-PCR primer sequences for *Rorippa* orthologs were (5’-3’):

- **HRE1**
  - Forward: TGATTCTTGTTGGAGGAGAA
  - Reverse: CAAGAAGCTCTTTCTGAAAGCAA
- **HRE2**
  - Forward: TCAAGAGGAGCTCATGGCTTT
  - Reverse: AAATGTCCACAGATTTAGGTCGAG
- **RAP2.2**
  - Forward: AGCCAAAGAGCTCAGAAAACCA
  - Reverse: CTTCTGAGTCACTGCAACCA
- **RAP2.3**
  - Forward: AAGGAGCTCTGGGTTTCGTC
  - Reverse: ATCGAGTTGACTCGGTTGCT
- **RAP2.12**
  - Forward: CATTGATTTCGAGGCAACCTTA
  - Reverse: AAGACTCCTCCAATCATGGA

*Rumex* group VII ERFs were identified by 454 pyrosequencing of *Rumex acetosa* and *Rumex palustris* petiole tissue under submerged and air conditions. The *de novo* assembly of individual reads led to a high quality transcriptome (Hans van Veen *et al.*, unpublished). Using reciprocal BLAST analysis, group VII ERF sequences were identified. The qRT-PCR primer sequences for *Rumex* are (5’-3’):

- **RpERF1**
  - Forward: GCAAAATGCAAGACAAAACCA
  - Reverse: TATGGTTTACCTCTTTCCAA
- **RpERF2**
  - Forward: TAGGAAGAAGAGGCTCAGG
  - Reverse: ACTGCAATACCTCATCAAG
- **RpERF3**
  - Forward: TCCTCAATCCCCTCTCTCT
  - Reverse: CTATCGCGAATTCAGAGTC
- **RpERF4**
  - Forward: GAGGTTGCTGGGTGAAGTTT
  - Reverse: ACGACTGCGTGGTCTGGAA
- **RpERF5**
  - Forward: CCACCTGTTTGCTGTGAAACG
  - Reverse: CTTGTCCCAACTTCTCTCGTC
- **RpERF6**
  - Forward: TTTTGTCCGCGTGTTCAACTC
  - Reverse: ACTCCCTTCCTCAACCTCAT
- **RaERF1**
  - Forward: CGACAGTGAATGCTCAAGAG
Complementary DNA was synthesized according to manufacturer’s instructions with 500 ng RNA, 50 ng random hexamers (Invitrogen, Bleiswijk, The Netherlands) and 100 U SuperScript III reverse transcriptase (Invitrogen, Bleiswijk, The Netherlands). Quantitative PCR reaction mixtures included 2X SYBR green (Platinum SYBR green Supermix qPCR UDG; Invitrogen, Bleiswijk, The Netherlands), 0.4 µl 10 µM of each primer, 0.08 µl 1/10 dilution 50× ROX reference dye and 25 ng cDNA in a total volume of 20 µl. The reactions were performed with a real-time PCR system (Applied Biosystems, CA, USA) and relative expression levels were calculated using the ΔΔCt method (Livak & Schmittgen, 2001) and corrected for TUBULIN gene for Rumex and ACT2 gene for Rorippa transcript levels. Similarly, absolute expression in control conditions was calculated with ΔCt method. The fold changes and the constitutive expression were visualized with MeV software (Saeed et al., 2003).

Carbohydrate analysis

Freeze-dried samples were weighed in a balance for dry weight analyses. A ground subsample was used in the soluble carbohydrate and starch analyses according to Vashist et al. (2011). 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 PA1 column, electrochemical detection). The remaining pellets were analyzed for starch using a commercially available kit (Boehringer, Mannheim, Germany).

Group VII ERFs synteny analysis

The synteny relationships of all group VII ERFs 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 (Corn) 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 syntenic blocks were log-transformed and used as length identifiers of the connector lines between the genes.

**Phylogenetic tree construction**

In order to resolve the relationships of *Rumex* group VII ERFs with the syntenic blocks they belong to, we analyzed the conserved motifs; the starting amino acids and the Apetala 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 PGDD (Plant Genome Duplication Database) and investigated the genes showing the highest similarity in order to reveal the most likely relationship of *Rumex* genes to our two identified syntenic blocks from other species.

**Group VII ERF expression data analysis**

Based on previous microarray studies, an inventory was made for group VII ERF expression upon hypoxia, flooding and waterlogging in *Arabidopsis thaliana* (Branco-Price et al., 2008; Mustroph et al., 2009; van Dongen et al., 2009; Lee et al., 2011), *Oryza sativa* (Lasanthi-Kudahettige et al., 2007; Jung et al., 2010), *Populus trichocarpa* (Kreuzwieser et al., 2009), *Glycine max* (Nanjo et al., 2011) and *Gossypium hirsutum* (Christianson et al., 2010). Two levels of regulation upon treatments were used based on fold change, $|2\log FC|>1$, or $|2\log FC|>3$, both with a $P_{\text{adj}}<0.05$. In the case of multiple time-points an average fold change was taken.

**Statistical Analyses**

We performed ANOVA analyses and post-hoc tests (Tukey’s b) to test differences in initial carbohydrate levels and dry weight for both submergence experiments and for leaf elongation. Six pairwise contrast analyses were performed for testing similarities between reduction in both dry weight and carbohydrates between day 5 and 15 for all four species for each treatment. All analyses were performed with SPSS 16.0 for Mac (SPSS Incorporated, Chicago, USA).
RESULTS

*Rorippa* species are more tolerant to submergence

All species showed high survival rates under dark submerged conditions, *Rorippa sylvestris* being the most tolerant of all with a median lethal time, $LT_{50}$, of 32 days (Fig. 1). *Rorippa* species survived submergence longer than both *Rumex* species that showed similar submergence tolerances. At the start of the survival experiment, plants did not show a significant difference in their dry weights (data not shown). All species had more starch and more total carbohydrates in their shoot tissues compared to roots at the start of the experiments (Fig. 2). Shoot carbohydrate contents varied significantly between the species; *Rumex* species had less carbohydrates, *Rumex palustris* having the lowest amounts (Fig. 2a). Although, there was a variation between different carbohydrates in the roots, the total content did not show a significant difference between the species (Fig. 2b).

![Fig. 1 Survival of *Rorippa* and *Rumex* species under complete submergence. Median lethal time, $LT_{50}$ values and standard errors are indicated for all species.](image)

*Rorippa amphibia* and *Rumex palustris* show escape strategy

Total leaf elongation was similar in air controls in the two *Rorippa* species and was mostly due to a larger growth of the lamina (Fig. 3a). Both *Rumex* species showed a smaller growth in air, *Rumex palustris* forming the smallest leaves. *Rumex palustris* showed most of the growth
in the lamina similar to the *Rorippa* species, whereas *Rumex acetosa* mostly elongated its petiole. All species also retained some leaf growth under submerged conditions (Fig. 3b). Leaf elongation of the youngest leaf in *Rorippa amphibia* and *Rumex palustris* exceeded that of *Rorippa sylvestris* and *Rumex acetosa*. *Rorippa sylvestris* and *Rumex acetosa* ceased leaf elongation under submerged conditions at an earlier stage (after eight days), while *Rorippa amphibia* and *Rumex palustris* still continued their leaf elongation. *Rumex palustris* showed a substantial elongation of the petiole upon submergence, in contrast to the normal pattern in air. *Rorippa amphibia* had mostly lamina elongation under submergence.

Fig. 2 Shoot and root soluble carbohydrate and starch content at the beginning of the survival experiments for *Rorippa* and *Rumex* species. ANOVA post-hoc (Tukey’s b test) results are indicated on the graphs, different letters meaning a significant difference at $P<0.05$. Roots did not show a significant variation among species. Bars indicate standard errors.

**Dry weight and carbohydrates are reduced under submergence**

All species showed a marked decline in dry weight upon submergence between day 5 and 15 (Fig. 4). The difference between air light controls (data not shown) and either submergence in dark or dark controls were approximately five-fold for *Rorippa amphibia* and *Rumex acetosa* and three fold for *Rorippa sylvestris* and *Rumex palustris* after 5 days of treatments. After 15 days of submergence *Rumex acetosa* showed a greater decline in root tissues (Supplementary Table). Submergence had a significant effect on carbohydrate contents and all species showed a decline in total carbohydrate content after 15 days (Fig. 5). Nevertheless, carbohydrates were not completely depleted in any of the species. In contrast to the other species, *Rumex acetosa* also showed a very rapid depletion of both starch and soluble carbohydrates in roots between 5 and 15 days, similarly to the decline in dry weight (Supplementary Table). The newly growing leaf tissue contributed largely to the carbohydrate pool of *Rorippa amphibia* after 5 days of submergence, unlike the pattern in *Rorippa sylvestris*. *Rorippa amphibia* also showed the highest amount of carbohydrates in laminas amongst all species, confirming its escape strategy for investing in new leaves.
Submergence tolerance in *Rorippa* and *Rumex*

Fig. 3 Leaf elongation in *Rorippa* and *Rumex* species throughout 16 days of submergence and their corresponding light controls in air. Petiole and lamina lengths are stacked to represent the whole leaf length. ANOVA post-hoc results for total leaf elongation at day 16 are indicated on the graphs, different letters meaning a significant difference at P<0.05 of petioles or lamina. Bars indicate standard errors.

Fig. 4 Dry weights of root, shoot, petiole and lamina tissues of *Rorippa* and *Rumex* plants after 5 and 15 days of dark treatments (dark controls) and submergence (submerged). Bars indicate standard errors. Pair-wise ANOVA contrast analyses results for dry weight reduction (between 5 and 15 days) between species is indicated in Supplementary Table.
Identification of *Rorippa* and *Rumex* group VII Ethylene Response Factors and synteny analysis

We cloned the *Rorippa* orthologs of the annotated Arabidopsis group VII ERFs. Six *Rumex palustris* (*RpERF1, RpERF2, RpERF3, RpERF4, RpERF5, RpERF6*) and three *Rumex acetosa* (*RaERF1, RaERF4, RaERF5*) genes were identified in the 454 RNA-seq analyses of petioles. These gene sequences showed strong similarity with group VII ERFs of Arabidopsis as well as other species such as cacao, tomato, grape and peach. By analyzing the starting motif and the Apetala domain (AP2) within these genes, we found several gene clusters within the two genera (Fig 6a). However, resolution of the gene family tree was limited, due to the short length of the compared sequence and deep evolutionary relationships. Thus, we sought other methods to unravel the evolutionary history and relationships within this gene family.
Submergence tolerance in *Rorippa* and *Rumex*

Fig. 6 (a) Gene family tree of group VII ERFs of Arabidopsis, peach, cacao, tomato, grape vine, *Rorippa* and *Rumex* species based on alignment of the starting motifs and the AP2 domain (b) Synteny relationships of group VII ERFs. Names of Arabidopsis genes are indicated, numbers in parenthesis after the species names represent number of genes in synteny block I and II, respectively.

The analysis of colinearity and synteny using genomic data showed a much clearer picture. Group VII ERF genes of both dicots (Arabidopsis, lyrate rockcress, grape vine, tomato, soybean, western poplar, barrel medic, cacao, peach, strawberry, cucumber, pigeon pea) and monocots (purple false brome, sorghum, rice and corn) clearly clustered into two separate synteny blocks (Fig. 6b). The comparison of genes from all species showed that they all had representatives in both blocks, and both groups showed similar levels of diversity and several further duplication events. Arabidopsis genes *HRE1*, *RAP2.12* and *RAP2.2* were in the same synteny block I (SBI). The other Arabidopsis genes *HRE2* and *RAP2.3* were contained in the second synteny block (SBII). *SUB1* genes could not be extracted from the Plant Genome Duplication Database. Nevertheless, homology analyses of these genes indicated synteny with SBI.
In both syntenic block clusters, there were *Rorippa* orthologs of the known Arabidopsis genes and at least one gene from each *Rumex* species (cf. Fig. 6a, b). The analysis of sequence homology of the conserved starting amino acids and the AP2 domain as shown on the phylogenetic tree also supported the distinction of genes falling into these two synteny blocks for Arabidopsis, tomato, peach, grape vine and cacao (cf. Fig. 6a, b). *Rorippa* genes RoRAP2.2, RoRAP2.12 and RoHRE1, and *Rumex* genes RpERF1, RpERF2 and RaERF1 are in SBI, whereas SBII contained *Rorippa* genes RoRAP2.3 and RoHRE2, and *Rumex* genes RpERF3, RpERF4, RpERF5, RpERF6, as well as RaERF4 and RaERF5.

### Synteny block II group VII ERFs are regulated under low oxygen stress

Transcriptional regulation data of group VII ERFs upon flooding and hypoxia stress was retrieved from publically available microarray database (Fig. 7). These included five species (Arabidopsis, rice, poplar, soybean & cotton) and a range of treatments and developmental stages. The Arabidopsis ERFs *RAP2.12* and *RAP2.2* show no change in expression in any tissues under any of the treatments (Fig. 7). *HRE2* is induced only under low oxygen treatments and root tissue of flooded *Arabidopsis* plants. *HRE1*, on the other hand, is up-

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**Fig. 7 Regulation of group VII ERFs in Arabidopsis under hypoxia, submergence and darkness; rice under anoxia and submergence; poplar, soybean and cotton under waterlogging.**

In both syntenic block clusters, there were *Rorippa* orthologs of the known Arabidopsis genes and at least one gene from each *Rumex* species (cf. Fig. 6a, b). The analysis of sequence homology of the conserved starting amino acids and the AP2 domain as shown on the phylogenetic tree also supported the distinction of genes falling into these two synteny blocks for Arabidopsis, tomato, peach, grape vine and cacao (cf. Fig. 6a, b). *Rorippa* genes *RoRAP2.2, RoRAP2.12* and *RoHRE1*, and *Rumex* genes *RpERF1, RpERF2* and *RaERF1* are in SBI, whereas SBII contained *Rorippa* genes *RoRAP2.3* and *RoHRE2*, and *Rumex* genes *RpERF3, RpERF4, RpERF5, RpERF6*, as well as *RaERF4* and *RaERF5*.
regulated in all tissue types under low oxygen and flooding as well as in darkness. In contrast, *RAP2.3* shows induction only under submergence and dark treatments. Up regulation of both *HRE1* and *RAP2.3* is higher upon submergence than in the dark.

The rice genome contains many more group VII ERFs than Arabidopsis. Furthermore, there is considerable variation in the regulation of these genes. Anoxic coleoptiles (*O. sativa* ‘Nipponbare’) show down regulation for most of the group VII ERFs (7 out of 11) belonging to synteny block I and genes in synteny block II are mostly up-regulated (3 out of 4). However, there is no consistency between flooded fully-grown plants and anoxic coleoptiles. Upon submergence, in fully-grown plants (*O. sativa* ‘Japonica’ cv M202) only block I genes *SUB1A* and *SUB1C* are up regulated. Synteny block II gene *Os07g47790* shows down regulation in submerged shoot tissues. Additionally synteny block I gene *Os03g08490* and synteny block II gene *Os01g21120* are down regulated in submerged shoots in the rice variety with *SUB1A* insertion.

Two out of six group VII ERFs of poplar are up regulated, both belonging to synteny block II. Soybean contains a large number of group VII ERFs, but only two, again synteny block II members, are up regulated after waterlogging. Waterlogged root tissues of cotton shows up-regulation in one gene from synteny block I and three genes from synteny block II.

**Synteny block I genes are constitutively expressed in *Rorippa* and *Rumex***

We analyzed regulation of the *ERF* genes during submergence in darkness by using two controls; air in light and air in dark after five days of the treatments. We compared submergence in darkness to the dark controls to capture sole effects of submergence. We also compared dark controls to light controls to detect expression patterns changing only in darkness (Fig. 8).

To avoid confusion of gene names, we will refer to the *Rorippa* orthologs of Arabidopsis genes (*RAP2.2, RAP2.12, RAP2.3, HRE1* and *HRE2*) with initials *Ra* (*Rorippa amphibia*) and *Rs* (*Rorippa sylvestris*) further in the text. Of the synteny block I genes, *RaHRE1* and *RsHRE1* were up regulated both under darkness and submergence in both *Rorippa* species, similar to the Arabidopsis ortholog. *RaRAP2.12* and *RsRAP2.12* showed higher constitutive expression levels under normal conditions compared to other genes, especially so in *Rorippa sylvestris*. This gene showed a down-regulation under darkness in all tissues and an additional down-regulation under submergence in roots. *Rorippa RaRAP2.2* and *RsRAP2.2* genes were down-regulated in darkness and only *Rorippa sylvestris* showed a slight induction under submergence in shoot tissues and lamina of the growing leaf. *Rumex palustris* SBI gene
RpERF1 showed high constitutive expression plus an up-regulation in darkness, but did not show and additional up-regulation under submergence in darkness. Although it was not highly expressed in normal conditions, Rumex acetosa gene RaERF1 was up-regulated in darkness in all tissues and showed and additional up-regulation in aboveground tissues under submergence. The Rumex palustris homolog of this gene RpERF1 showed similar patterns in normal and dark conditions but had an additional induction in roots of submerged plants.

**Synteny block II genes are regulated under submergence in Rorippa but not in Rumex**

Synteny block II genes of Rorippa were not constitutively expressed as some SBI genes were (Fig. 8). The Rorippa SBII genes RaHRE2 and RsHRE2 were highly induced in submerged roots (>200 fold) similar to Arabidopsis. Additionally, this gene also showed an induction in aboveground tissues of Rorippa amphibia, unlike Rorippa sylvestris. Although Arabidopsis RAP2.3 is darkness regulated, this gene was down-regulated in Rorippa in most tissues in darkness. However, there was an induction under submergence, especially in roots.

In contrast to Rorippa ERF genes, Rumex orthologs in synteny block II were expressed in normal conditions, except for Rumex acetosa gene RaERF4. Rumex acetosa gene RaERF5 and Rumex palustris RpERF6 were darkness regulated but did not show an additional induction under submergence. Although they were the closest homologs, Rumex ERF5 genes showed a different pattern of regulation in the two species. The Rumex palustris gene was down regulated both in darkness and submergence whereas Rumex acetosa gene was up-regulated in both darkness and submergence.

**High ADH1 expression in roots**

ADH1 was up-regulated in roots of all the species under submergence (Fig. 8). This gene is also up-regulated in all aboveground tissues of Rorippa species. Rumex acetosa showed an up regulation in shoots and lamina but not the petioles. In addition, Rumex palustris did not show an additional induction of ADH1 in submerged aboveground tissues as this gene was already up-regulated in dark controls.
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**DISCUSSION**

**Growth strategies in *Rumex* and *Rorippa* under submergence**

We found that *Rorippa amphibia* and *Rumex palustris* showed an escape strategy by elongating their leaves, whereas *Rorippa sylvestris* and *Rumex acetosa* mostly showed a quiescence strategy with a reduction of growth under submergence. In the previous chapter, we already showed that *Rorippa sylvestris* survived complete submergence longer than *Rorippa amphibia* when plants were submerged at a later stage; a stem was formed in both
species and was the elongating organ to establish air contact in *Rorippa amphibia* (Akman et al., 2012).

*Rumex* species did not show a similar pattern in their survival as *Rorippa* species; although *Rumex acetosa* adopted a quiescence strategy when submerged, survival was similar to that of *Rumex palustris*. *Rumex acetosa* inhabits sites that are not frequently flooded and populations encounter 10-14 days of flooding a year; however, exposure of *Rumex palustris* to flooding is much higher, 25-35 days (Nabben et al., 1999). Although *Rumex acetosa* displayed a quiescence strategy, submergence coping mechanisms common in more frequently flooded plant species might be absent in this species, leading to a faster mortality even though growth is reduced. In comparison, the other quiescent plant *Rorippa sylvestris* inhabits sites that are more frequently flooded and may have evolved adaptations that are lacking in *Rumex acetosa*.

Although both escaping species elongate their shoots, they showed a different pattern in leaf growth; elongation in *Rorippa amphibia* was mainly in the lamina, whereas in *Rumex palustris*, the petiole was the primary growing organ under submergence. In the previous chapter, we showed that there was an increase in carbohydrates in *Rorippa* species under submergence in the light. This indicates that these species can profit from underwater photosynthesis, further enhancing their tolerance to flooding (Stift et al., 2008; Akman et al., 2012). The stronger elongation and expansion of leaves in *Rorippa amphibia* might be a strategy to increase the underwater photosynthesis level, as the leaf grows closer to the water surface, the better lit zone of the water column. Moreover, *Rorippa amphibia* in particular can also form a long and slender stem under submergence. The newly growing leaves of the other species with an escape strategy, *Rumex palustris*, were smaller compared to *Rorippa amphibia*, and the leaf growth is mostly in the petioles. Despite observed underwater photosynthesis (Mommer & Visser, 2005), the main function of elongation in *Rumex palustris* is thought to be establishment of air contact, allowing re-aeration throughout the plant into the root tissue (Colmer & Voesenek, 2009). Re-aeration of the roots might be less important for *Rorippa amphibia*, given the observation that shoots that become detached from the slender stem can form a new root system by clonal growth, and establish as new plants on the embankment.

**Carbohydrate availability does not determine submergence tolerance**

Both submergence and darkness treatments substantially lowered the soluble carbohydrate and starch concentrations in all four species suggesting strong alterations in carbon metabolism and consumption induced by the underwater environment. Furthermore, a dramatic reduction of total plant dry weight was also observed. These demonstrate the severe effect of flooding
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on plant functioning.

The carbohydrate content at the start of the experiments could explain the survival differences between the two genera; the more tolerant *Rorippa* species had more carbohydrates compared to *Rumex*. Nevertheless, carbohydrate content did not correlate with survival for species within the same genus. *Rorippa sylvestris* had less total carbohydrate content compared to *Rorippa amphibia* both at the start of the experiments and also after 15 days of submergence, counterintuitive to its higher survival under submergence. The carbohydrate content in the two *Rumex* species also indicates that survival is not correlated to the carbohydrate availability. Indeed, *Rumex acetosa* showed higher mortality after 15 days of submergence compared to *Rumex palustris*, whilst the carbohydrate availability of *Rumex palustris* remains considerably lower throughout the experiment. These results indicate that solely carbohydrate availability does not determine submergence tolerance.

**Conserved ADH1 induction in submerged roots**

In low oxygen conditions, anaerobic metabolism becomes the major source of NAD$^+$ necessary for glycolysis and indeed fermentative routes are induced in many species under low oxygen (Bailey-Serres & Voesenek, 2008). We tested a key gene *ADH1* in the ethanolic fermentation pathway in order to confirm effects of submergence on *Rorippa* and *Rumex* species. In roots, where the lowest oxygen levels are expected, *ADH1* is induced in all the four species under submergence. It has been shown that *SUB1A* induced ethanolic fermentation and similarly in Arabidopsis *HRE1* and *HRE2* knockout mutants cannot induce *ADH1* under low oxygen conditions. *Rorippa* *HRE2* orthologs are also highly induced in roots and might be involved in *ADH1* induction in these tissues. *Rorippa* species also showed an induction of *ADH1* in all aboveground tissues. Nevertheless, *RsHRE2* as a possible inducer of low oxygen responsive genes, is not up-regulated in shoots of *Rorippa sylvestris*, whereas *RsHRE1* is. Thus, both orthologs of *HRE1* and *HRE2* in *Rorippa* might be responsible for orchestrating induction of *ADH1* as is in Arabidopsis. In contrast, the *Rumex* species do not show a common pattern that would explain regulation of *ADH1* expression. Interestingly, we also did not identify any root specific ERF induction in *Rumex*, which in combination with aboveground expression patterns, indicate a complex mechanism of hypoxic gene regulation.

**Evolutionary relationship of group VII ERFs**

It has been hypothesized that ERFs and other AP2 domain containing transcription factors evolved by transfer of AP2 domain containing HNH endonucleases of cyanobacteria (by endosymbiosis) or viruses into earlier plants, which then was transposed and spread
in the genome (Magnani et al., 2004). This theory explains why AP2 domain containing transcription factors are only plant-specific in eukaryotes. This ancestral gene then gave rise to a larger gene family, which has taken important roles in crucial aspects of plant development and environmental adaptations. The phylogenetic relationships of ERFs have been studied extensively in Arabidopsis, rice and soybean (Nakano et al., 2006; Zhang et al., 2008). None of these studies investigated the genome evolution and synteny relationships of these genes. The synteny analyses can resolve the evolutionary relationships of group VII ERFs since evolutionary relationships studied by sequence homology can be uninformative or even misleading when analyzing shorter fragments of conserved regions as in these gene groups. In all the twenty plant species from across the angiosperms we analyzed, including our study objects Rorippa and Rumex, we found at least three members for group VII ERFs for each species. The two conserved synteny blocks suggested that all group VII ERFs potentially evolved from two ancestral genes formed by a duplication event early in angiosperm evolution.

Expression and functional divergence of group VII ERFs

Differential expression patterns of the two synteny blocks indicated functional variation and distinct regulation mechanisms. In our analysis of microarray data for Arabidopsis, rice, poplar, soybean and cotton, we found that most genes in SBI are not regulated under low oxygen stress, whereas numerous SBII genes are induced. Arabidopsis HRE1 gene is an exception, as it is highly induced by low oxygen although it belongs to SBI. The other genes in this synteny block, RAP2.2 and RAP2.12 are duplicates that presumably evolved as a result of a more recent duplication event (the α-event) (Tang et al., 2008) since the direct synteny between them is still conserved and they share high sequence homology. HRE1, on the other hand, most likely evolved by the older γ or β-event and lost its direct synteny with the other two genes as a result of numerous chromosomal rearrangements. This gene then might have gone through a loss/gain of function and a switch in its regulatory mechanisms, resembling that of the other SBII genes, HRE2 and RAP2.3.

In rice the regulation of the escape and quiescence strategies have been ascribed to the group VII ERFs. Transcriptional activation of SUB1A mediates a quiescence strategy, whereas SNORKEL1 and SNORKEL2 are highly induced and activate underwater shoot elongation (Fukao et al., 2006; Hattori et al., 2009). Indeed, specific regulation is observed in the primary elongating internodal part (Singh et al., 2009). Although not expressed in normal conditions, synteny block II genes RaHRE2 and RsHRE2 were induced in submerged roots of both Rorippa species. This gene was also up-regulated in shoots and lamina of Rorippa amphibia but not in aboveground tissues of Rorippa sylvestris. This difference in expression
makes *Rorippa HRE2* orthologs candidates for regulating the difference between the *Rorippa* species, as especially lamina growth is the main form of growth in the youngest leaf.

Recently, group VII ERFs of Arabidopsis have been shown to act as low oxygen sensors initiating transcription of hypoxia induced genes and ceasing activity under normal oxygen conditions through degradation by the N-end rule (Gibbs *et al.*, 2011; Licausi *et al.*, 2011). Nevertheless this mechanism is absent in rice for *SUB1A* locus but still might be present in *Rorippa* and *Rumex* species. It has been hypothesized that higher constitutive expression of oxygen sensing group VII ERF genes might be indicative of higher submergence and/or low oxygen tolerance (Sasidharan & Mustroph, 2011). *Rumex palustris RpERF1* and *Rorippa sylvestris RsRAP2.12* ortholog were highly expressed constitutively in all tissue types and are good candidates as low oxygen sensors. *RAP2.12* orthologs are also expressed in normal conditions in *Rorippa*, especially in *Rorippa sylvestris*. The lower levels of this hypothetical oxygen sensor combined with consumption of carbohydrates for growth might be leading to lower survival in *Rorippa amphibia* compared to *Rorippa sylvestris*. Higher expression of *RpERF1* might be one of the factors that increase survival of *Rumex palustris*, although it is an escaping species and consumes carbohydrates for growth. These genes might be responsible for initiating expression of submergence-induced genes that are important in submergence acclimations and enhance survival as shown in Arabidopsis (Gibbs *et al.*, 2011; Licausi *et al.*, 2011).

*Rumex acetosa* might not be as responsive to low oxygen as the other species since we did not observe a high constitutive expression of any synteny block I genes; *RaERF1* was only slightly expressed in normal conditions. This might lead to a lower ability to rapidly activate the hypoxia-induced genes such as the genes of anaerobic metabolism. Indeed, root tips of *R. acetosa* were found to suffer more from hypoxia than *R. palustris* (Voesenek *et al.*, 1993). A lack of a strong selection pressure in the less frequently flooded habitats of *Rumex acetosa* might explain the absence of a sensitive sensing mechanism to low oxygen, which leads to higher mortality in submerged environments.

**Rumex acetosa** has fewer regulated group VII ERFs

Interestingly all the synteny block I genes of *Rumex* are darkness induced in roots and shoots, two of which (*RpERF2* and *RaERF1*) also show induction in the growing leaf. These genes, showing no additional induction under submergence, might be regulated by the decreased carbohydrate concentrations after photosynthesis is arrested in the dark. The low levels of carbohydrates in darkness might also explain the absence of a further induction in these genes under submergence as carbohydrate levels are reduced under both stresses.
Additionally, in *Rumex* we did not find any up regulation resembling that of *HRE2* orthologs in *Rorippa* roots. So the *Rumex* genes were not extremely up-regulated, or missing in our analysis. The gene sequences for group VII ERFs of *Rumex* were extracted from 454-sequencing of transcriptomes of submerged and control petiole tissues and considering that *HRE2* orthologs expressed in root tissues, rather than in the petiole might not have been picked up. The lower number of group VII ERFs in *Rumex acetosa* also indicates that either fewer genes are induced under submergence or that these genes are lacking or lost in this species. Though we cannot exclude a role of unknown root specific group VII ERFs, our data suggest that, similarly to rice, the *Rumex* genus does not have the drastic HRE-like induction of group VII ERFs in roots.

In conclusion, submergence tolerance is not determined by a single factor such as carbohydrate availability, molecular modifications or morphological function but rather orchestrated by a more complex network of these factors. We showed that there is no conserved tissue-specific expression of a group VII ERF that would be a likely regulator of escape and quiescence strategies, indicating that elongation growth is regulated differently in these two dicot genera compared to rice. Rather, we suggest that the observed regulation of group VII ERFs is more related to phylogenetic lineage as demonstrated by the genus specific expression patterns. Although similar strategies are present in *Rorippa* and *Rumex*, their onset and their advancement might be regulated and achieved by different mechanisms. However, the high constitutive expression of some group VII ERFs in synteny block I, which could potentially act as oxygen sensors, likely play a role in the relatively high survival of both *Rorippa sylvestris* and *Rumex palustris*. The four species studied represent a variety of strategies and evolutionary lineages of flooding selection pressures. Therefore further evolutionary and functional analysis of group VII ERFs in these two genera will be essential to reveal their importance and significance in mediating adaptation to submergence.

**ACKNOWLEDGEMENTS**

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**SUPPLEMENTARY INFORMATION**

Supplementary Table. Contrast results comparing the reduction between species in 5 and 15 days of the treatments.

<table>
<thead>
<tr>
<th>Contrast comparison</th>
<th>Submergence treatment</th>
<th>Darkness treatment</th>
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<tr>
<td></td>
<td>Root DW</td>
<td>Root soluble carbs</td>
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<td><em>R. amphibia</em> vs. <em>R. sylvestris</em></td>
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<tr>
<td><em>R. palustris</em> vs. <em>R. acetosa</em></td>
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DW: dry weight; carbs: carbohydrates.