Sink or swim: submergence tolerance and survival strategies in Rorippa and Arabidopsis
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CHAPTER 2

Wait or Escape? Contrasting submergence tolerance strategies of *Rorippa amphibia*, *Rorippa sylvestris* and their hybrid

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SUMMARY

Background and Aims Differential responses of closely related species to submergence can provide insights into the evolution and mechanisms of submergence tolerance. Several traits of two wetland species from habitats with contrasting flooding regimes, *Rorippa amphibia* and *Rorippa sylvestris*, as well as F₁-hybrid *Rorippa x aniceps* were analyzed to unravel mechanisms underlying submergence tolerance.

Methods In a submergence experiment (lasting 20 days) we analyzed biomass, stem elongation and carbohydrate content and in a second submergence experiment (lasting 3 months) we analyzed survival and the effect of re-establishment of air contact on biomass and carbohydrate content. In a separate experiment we analyzed expression of two carbohydrate catabolism genes *ADH1* and *SUS1*, upon re-establishment of air contact following submergence.

Key Results All plants had low mortality even after three months of submergence. *Rorippa sylvestris* was characterized by 100% survival and higher carbohydrate levels coupled with lower *ADH1* gene expression as well as reduced growth compared to *R. amphibia*. *Rorippa amphibia* and the hybrid elongated their stems but this did not pay-off in higher survival when plants remained submerged. Only *R. amphibia* and the hybrid benefited in terms of increased biomass and carbohydrate accumulation upon re-establishing air contact.

Conclusions Results demonstrate contrasting “escape” and “quiescence” strategies between *Rorippa* species. Being a close relative of *Arabidopsis*, *Rorippa* is an excellent model for future studies on the molecular mechanism(s) controlling these strategies.
INTRODUCTION

The frequency of major floods has increased significantly in the last century and this trend is predicted to continue due to climate change (Milly et al., 2002). Although modern terrestrial plants evolved from aquatic ancestors, very few angiosperm species can cope with the severe effects of flooding (Voesenek et al., 2006), which can lead to crop failure and demise of natural plant populations (Silvertown et al., 1999; Normile, 2008b). The combined effects of limited underwater gas diffusion (Jackson, 1985) and low light levels in turbid waters (Vervuren, 2003) lead to mortality typically within just a few days of submergence. Thus, submergence acts as a strong selection force controlling the distribution of plant species in flooded areas (Blom, 1999; Van Eck et al., 2004; Mommer et al., 2006a).

Plants that inhabit frequently flooded areas have evolved traits to overcome the lethal effects of submergence. Two strategies have been proposed to cope with flooding, the low oxygen escape strategy and the low oxygen quiescence strategy. In the low oxygen escape strategy, the plant grows and/or elongates its shoot in an attempt to reach the surface and restore air contact (Bailey-Serres & Voesenek, 2008). Plants adopting this strategy often have more internal aerenchyma tissue for efficient gas transport, especially to the belowground parts where the effects of low oxygen stress are most severe (Sauter, 2000; Voesenek et al., 2003). This strategy is beneficial only in shallow and prolonged floods, when plants are able to reach the surface before the stress becomes lethal. Since low levels of oxygen upon submergence can limit aerobic respiration, plants switch to anaerobic lactic acid and ethanol fermentation as a source of NAD\(^+\) and ATP and carbohydrate catabolism genes such as ADH, PDC and SUS are up-regulated (Bailey-Serres & Voesenek, 2008). Compared to aerobic respiration, fermentation is far less efficient in terms of carbohydrate consumption. Plants can temporarily withstand submerged conditions, however if air contact can not be re-established, high carbohydrate consumption to accommodate stem elongation and cell division will lead to an energy deficit, severe tissue damage and mortality (Pierik et al., 2009; Chen et al., 2011). In contrast to the escape strategy, the low oxygen quiescence strategy is defined by reduced levels of growth, conservation of energy reserves and hence a delay of the energy crisis (Setter & Laureles, 1996; Sauter, 2000; Bailey-Serres & Voesenek, 2008). This strategy is particularly beneficial when floods are deep but transient. In these conditions, rapid growth by the plant would be futile and likely lead to stem breakage when water levels drop (Chen et al., 2011).

The escape and quiescence strategies have been well described in varieties of rice cultivated in different habitats. Deep-water rice can grow up to four meters when flooded (Kende et al., 1998) in contrast to lowland rice that almost completely ceases its growth upon flooding.
and survives longer by conserving carbohydrates (Ram et al., 2002). Molecular studies have shown that ethylene response factor (ERF) genes SNORKEL and SUB1A control escape and quiescence strategies respectively (Fukao et al., 2006; Xu, 2006; Hattori, 2007; Hattori, 2008; Hattori et al., 2009). Rice and Arabidopsis studies have increased our fundamental molecular knowledge about submergence tolerance; however, neither species exhibits an extreme flooding tolerance phenotype. Submergence tolerant lowland cultivars of rice and Arabidopsis thaliana accessions can survive only up to 10-14 days of submergence (Jackson & Ram, 2003; Vashisht et al., 2011). Introgression of the SUB1 locus that improves the survival by only a few days to rice cultivars already increased yields significantly after flooding (Singh et al., 2009), so identifying the molecular basis of extreme tolerance would be of great interest for agriculture.

Rice varieties displaying these strategies are artificially selected genotypes and there is little evidence that these cost-benefit patterns also exist as a result of natural selection for different flooding regimes. Rumex species show variation in ethylene induced petiole elongation depending on the type of floods they encounter in their natural habitats (Voesenek et al., 1996). One of these species, Rumex palustris elongates its leaves under submerged conditions, and there also exists variation in elongation ability among different populations (Chen et al., 2009). Petiole elongation and reaching water surface have benefits in biomass recovery (Pierik et al., 2009; Chen et al., 2011) but no evidence has been reported for effects on survival.

Studies on tolerant Rumex species have led to the discovery of fundamental knowledge about hormonal regulation pathways of elongation and consequences of submergence (Voesenek et al., 1991; Voesenek et al., 2003; Benschop et al., 2005; Vreeburg et al., 2005; Mommer et al., 2006a; Chen et al., 2009; Chen et al., 2011); however, the lack of molecular resources makes it hard to reveal the genetic mechanisms behind these responses in the detail possible for model organisms. Applicability of molecular tools developed for Arabidopsis to its wild relatives (Mitchell-Olds, 2001; Schranz et al., 2007) provides greater possibilities for unraveling the genetic basis of extreme submergence tolerance. In this study we use three wild relatives of Arabidopsis belonging to the same lineage in the Brassicaceae (Al-Shehbaz et al., 2006), Rorippa amphibia, Rorippa sylvestris and their hybrid as models to study mechanisms of extreme submergence tolerance. The cytotypes studied were tetraploids with 32 chromosomes, most likely with an autoploid origin (Stift et al., 2010). These species are clonal wetland perennials, inhabiting major river plains in Europe. Rorippa amphibia usually occurs at stable water tables, with waterlogged belowground plant parts, whereas R. sylvestris occupies river beaches and habitats that undergo periodic deep floods and dry-outs (Jonsell, 1968; Blom, 1999).
Natural interspecific hybrids can be found in intermediate habitats along major rivers that experience periodic flooding, such as the Danube and Elbe. A previous study with these species found differential responses to waterlogging and submergence (Stift et al., 2008). Stift et al. (2008) showed that *R. amphibia* was better able to cope with waterlogging. When completely submerged for 2 weeks, aboveground biomass of *R. amphibia* increased at the expense of decreased belowground biomass. This aboveground and belowground growth tradeoff was not observed in *R. sylvestris*. This study suggested that these two species evolved different responses due to the different flooding regimes they encounter in their natural habitats. But are these differences shaped by natural selection? And, if so, what are the components that enhance or decrease submergence survival? To answer these questions we investigated the submergence responses and strategies of *R. amphibia*, *R. sylvestris* and their artificial F1-hybrid, by measuring changes in growth, biomass, carbohydrate metabolism, regulation of genes related to carbohydrate catabolism (*ADH1* and *SUS1*) and their effects on survival. In order to understand the genotypic influence on the trait phenotypes, we have tested if the hybrid behaved as either of the parents and if so how these were reflected in survival.

**MATERIALS AND METHODS**

**Plant material**

The genotypes of *Rorippa amphibia*, *Rorippa sylvestris* and their artificial hybrid *Rorippa x anceps* used in this study were previously described in Stift et al. (2008). *Rorippa amphibia* and *R. sylvestris* rhizomes were collected from IJssel River, Doesburg, The Netherlands (N:52°01’25”E:06°08’42” and Rhine River, Millingerwaard, The Netherlands (N:51°52’02”E:05°59’18”) respectively. All plants used were tetraploids (4n=32). The F1 hybrids were derived by hand-pollination using the *R. amphibia* plant as the pollen donor and the *R. sylvestris* as the maternal plant. Five random seeds were selected from the crosses and were germinated on sterile filter paper with 2 ml of 3 M gibberellic acid solution. Seedlings were transferred to soil and grown for three months. Intermediate morphology of the progeny confirms that the crosses were successful and hybrids were created [Supplementary Information – Fig. S5].

One random hybrid genotype was selected and together with the parents propagated by rhizome cuttings for two years. Replicate plants used in all experiments were genetically identical clones propagated from rhizomes. The rhizomes were collected and surface sterilized by using 10% (v v⁻¹) 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
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(Hispanagar, Burgos, Spain) and 0.5X MS (Duchefa, Haarlem, The Netherlands) media. Rhizomes were initially grown in a growth cabinet (Sanyo MLR-350; Sanyo, Etten-Leur, The Netherlands) for 10 days with 16 hour light at 20°C and 8 hour dark at 16°C. Subsequently, individual plantlets were transferred onto 55 mm diameter mesh pots with sterile sand (0.5-1.0 mm grain size; Filcom BV, Papendrecht, The Netherlands) supplied with nutrient solution (0.1 g l⁻¹ Peters Professional 20:10:20 General purpose, Scotts Europe BV, Heerlen, The Netherlands). The plants were grown in a greenhouse under natural light supplemented with 600W SON-T lamps (Philips, Eindhoven, The Netherlands) when necessary. The temperature of the greenhouse was 20°C (± 2°C) with a 16 hour photoperiod. After 2 weeks plants were transferred to 3 l pots containing 1.5 g (per pot) controlled slow-release fertilizer (Osmocote Plus 15+11+13+2MgO+Trace Elements; Peters Professional, Scotts Europe BV, Heerlen, The Netherlands).

**First submergence experiment**

This experiment was conducted in May-June 2008. After 50 days of pre-growth, the stem lengths of 36 plants per genotype used in the experiment were measured. Plants were placed in four outdoor cement basins on 36 randomly assigned positions in each basin (length x width x depth = 400 cm x 100 cm x 100 cm). The plants were either submerged completely or left in empty basins as air controls. All the basins were covered with shade cloth to prevent plants from emerging and to mimic deep flooding light conditions. Light intensity was measured with a light data logger (LI-1400, Licor, Lincoln, NE) and light under the shade cloth was found to be 65% of the normal light intensity. At the start of the experiment and 7, 14, 20 days after the treatment started, stem lengths were measured and aboveground and belowground tissues of six plants from each genotype and treatment were washed and sampled separately. Dry biomass was measured after freeze-drying and a subsample was used for carbohydrate analysis.

**Second submergence experiment**

In the summer of 2009 a similar but longer-term submergence experiment was performed. After 51 days of pre-growth 117 plants per genotype were randomly placed in six cement basins. At the start of the treatment, aboveground and belowground tissues of nine plants of each genotype were washed and sampled separately for carbohydrate analyses. All the plants were submerged completely and shade cloth was used to cover the basins. After 37 days of complete submergence, a further nine plants per genotype were sampled (for carbohydrate analysis) and the water level in half of the basins (three basins) was lowered to half of the original height to let all genotypes gain contact with the atmosphere (semi-submergence
treatment). Nine completely submerged and nine semi-submerged plants were harvested for dry weight and carbohydrate analyses, 21 and 42 days after the water level decrease. Forty-two days after reaching the surface, some plants were lethally damaged, nevertheless we were able to sample both aboveground and belowground tissues. For all samples dry biomass was measured after freeze-drying and a sub-sample was used in carbohydrate analyses.

For survival assays, separate sets of 12 plants from each genotype were taken out of the water after 37, 58 and 79 days of complete submergence, as well as plants submerged for 37 days followed by 21 and 42 days of semi-submergence. Survival was scored both immediately and after a recovery period of 15 days, based on absence or presence of green parts above ground. In the survival data only immediate survival was included for \textit{R. amphibia} and the hybrid since the stems were very weak and unable to support the large healthy shoot tissues formed when the water was removed.

\textbf{Gene expression experiment}

After 30 days of pre-growth, 48 plants from each genotype were randomly assigned to 16 L plastic buckets either completely filled with rainwater one day before the start of the experiment (submergence treatment) or with 1 cm of water for air controls. This experiment was conducted in a greenhouse with same conditions as growth period. After three days of complete submergence, the water level was reduced in half of the buckets from the submergence treatment so that tips of the leaves were emerging. Four plants were harvested for each genotype and treatment 2, 26, 74 hours after water level decrease. For air controls and submergence treatments the plants were quickly rinsed in water, roots and shoots were sampled separately. For semi-submerged samples roots, leaves above water level and shoots under water were sampled separately. All the samples were quickly frozen in liquid N\textsubscript{2} for RNA isolations and expression analysis.

\textbf{Carbohydrate analysis}

A sub-sample of 10-150 mg of ground tissue (sampled in the first and second experiments for carbohydrate analysis) was suspended in 1.0 ml 70\% MeOH in water (v:v), vortexed and boiled for 5 minutes. After placing the tubes in an ultrasonic bath for 15 minutes, samples were centrifuged (10 min at 10000 rpm) and the supernatants were transferred to new tubes. Pellets were extracted once more, excluding the boiling step. Supernatants of each sample were combined and 70\% MeOH was used to bring the final volume to 2 ml. For HPLC quantification, 10 µl of extract was diluted in 990 µl of MilliQ water and measurements and data analysis were performed as described previously (Van Leur \textit{et al.}, 2008). For starch
measurements, the pellets were mixed with 1 ml of water and incubated at 65 °C for 30 min. The supernatants were transferred to new tubes after centrifugation at 12000 rpm for 5 min and 1 ml water was added to the pellets once more. The supernatants were combined with the previous fractions. The pellets were then boiled in a water bath for 10 min. After cooling to room temperature, 500 µl of 0.2 M Na acetate (pH 5.5), 7 units amyloglucosidase and 0.7 units α-amylase were added to the pellets and incubated at 37 °C for 4 hours. After centrifugation at 13000 rpm for 5 min the supernatants were transferred to new tubes for starch analysis. The hexose concentration was measured by a modified version of the anthrone method by using fixed glucose standards (Smith & Zeeman, 2006).

**Gene cloning and expression analysis**

DNA was isolated with a DNeasy Plant mini kit (Qiagen, Leusden, The Netherlands) from *R. amphibia* and *R. sylvestris*. Primers for *ADH1* and *SUS1* were designed by Primer3 software ([http://frodo.wi.mit.edu/primer3/](http://frodo.wi.mit.edu/primer3/)) based on Arabidopsis sequences obtained from TAIR. Amplified fragments with these primers from gDNA of *R. amphibia* and *R. sylvestris* were cloned with standard protocols (Sambrook & Russell, 2001). Based on sequences from at least ten independent clones for each gene per species, qPCR primers were designed for conserved regions among species. Primers were blasted to search for similar genes in *A. thaliana* to confirm specificity. Primers for 18S rRNA, used as a reference gene for qRT-PCR, were designed based solely on Arabidopsis sequences. The primers used in qRT-PCR were (5’-3’)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA-forward</td>
<td>AAACGGCTACCACATCAAGAG</td>
</tr>
<tr>
<td>18S rRNA-reverse</td>
<td>ACTCGAAAGAGCCCGGTATT</td>
</tr>
<tr>
<td>ADH1-forward</td>
<td>GGACTTGGTGCTGTTGGTTAG</td>
</tr>
<tr>
<td>ADH1-reverse</td>
<td>CTGGTTTGTCTGCTCTCTCG</td>
</tr>
<tr>
<td>SUS1-forward</td>
<td>GGAGAGTTTGCTTCCATTGC</td>
</tr>
<tr>
<td>SUS1-reverse</td>
<td>TCCGCTTTCTCAAGATGTG</td>
</tr>
</tbody>
</table>

Primer specificity was assessed by melting and dilution curve analysis. Each primer set amplified only one product [Supplementary information – Fig. S4]. RNA isolations were done with a modified version of the hot phenol method (Slater, 1984). Genomic DNA was digested with DNase (DNA-free, Ambion, Nieuwerkerk aan de IJssel, The Netherlands) and cDNA was synthesized with 500 ng RNA, 50 ng random hexamers (Invitrogen, Bleiswijk, The Netherlands) and 100 U SuperScript III reverse transcriptase (Invitrogen, Bleiswijk, The Netherlands) according to manufacturer’s instructions. Quantitative PCR reaction mixtures included 2X SYBR green (Platinum SYBR green Supermix qPCR UDG; Invitrogen, Bleiswijk, The Netherlands).
The Netherlands), 0.3 µl 10 µM of each primer, 0.04 µl 1/10 dilution 50× ROX reference dye and 10 ng cDNA, except 0.001 ng cDNA was used for 18S rRNA in a total volume of 20 µl. The reaction was 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 18S rRNA transcript levels. The data were log₂ transformed and then used in the contrast analysis.

**Porosity measurements**

Porosity was measured as volume air space as a percentage of total tissue volume according to Raskin (1983) and using calculations as adapted by Thomson et al. (1990).

**Statistical Analyses**

All analyses were performed with SPSS 16.0 for Mac (SPSS Incorporated, Chicago, USA). We performed ANOVA analysis to test treatment, time-point and species effects for both submergence experiments. Three contrast analyses were performed for both first and second experiments. The difference between starting values (day 0) and submerged values (20 days for first and 37 days for second experiments) were contrasted with an ANOVA test among *R. amphibia* and *R. sylvestris*, *R. amphibia* and the hybrid and lastly *R. sylvestris* and the hybrid. The trait was considered additive when there was a difference in the parental lines and the hybrid was intermediate and differed from both (A>H>S or S>H>A). When the hybrid was different from one parent only, the trait was considered dominant. If the hybrid exceeded any of the parental lines, trait was evaluated as overdominant.

For the gene expression experiment, three contrasts were performed to test if the pattern of gene expression was different among species. In the contrast analysis, all time-points were included and values for one species at a time point was contrasted against the same time point for other genotypes. The contrasts for following comparisons were done: air controls vs. submerged samples, air controls vs. semi-submerged samples and submerged vs. semi-submerged samples.
RESULTS

*Rorippa sylvestris* displayed a quiescence strategy, whereas *Rorippa amphibia* and the hybrid displayed an escape strategy upon submergence

All genotypes exhibited at least some stem elongation under submerged conditions (Fig. 1a). Compared to air controls, submerged *R. amphibia* showed increased stem elongation, whereas no difference was found between treatments in *R. sylvestris*. The hybrid had the tallest stems under both normal and submerged conditions, reaching more than twice the height of both of the parental lines in air controls. However, the relative treatment effect was not as strong as it was in *R. amphibia*: submerged *R. amphibia* plants showed 82 % greater stem elongation (length submerged/ length in air) compared with 24 % in the hybrid after 20 days of submergence.

Aboveground biomass was reduced in all species after submergence (Fig. 1b). The reduction of biomass in *R. amphibia* and the hybrid was comparable to that in *R. sylvestris* (Table 1). Growth of belowground tissue in all the genotypes ceased upon submergence, and there were no significant differences in belowground biomass after 20 days of submergence compared to starting values [Supplementary Information - Table S1].

![Fig. 1](image_url)

Fig. 1 (a) Stem elongation of *Rorippa amphibia*, *Rorippa sylvestris* and their hybrid, and (b) aboveground and belowground dry weight of control and submerged plants after 7, 14 and 20 days of treatment (n=6; error bars indicate SEs).
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Fig. 2 Aerenchyma in petioles of 30 days old control non-submerged plants of (a) *Rorippa amphibia*, (b) the hybrid and (c) *Rorippa sylvestris*, and (d) porosity content of 21 day old petioles. All three genotypes differ in their porosity levels significantly (ANOVA, LSD tests) [Supplementary Information - Table S3-4] (n=4 or 5; error bars indicate SEs).

*Rorippa amphibia* had a higher petiole porosity compared to *R. sylvestris*, indicating more aerenchyma tissue, whereas the hybrid showed intermediate levels (Fig. 2, [Supplementary Information - Table S3-4]). More aerenchyma tissue coupled with stem elongation enables greater internal gas diffusion rates as soon as plants re-establish contact with the atmosphere. The growth and morphology upon submergence thus suggest that *R. amphibia* and the hybrid display escape strategies, while *R. sylvestris* has a quiescence strategy.

**Aboveground carbohydrates decreased more in *R. amphibia* upon submergence**

Total aboveground carbohydrate content decreased in all the genotypes upon submergence relative to air controls (Fig. 3a, [Supplementary Information - Table S2]), probably as a result of energy deficit caused by oxygen deficiency and a shift from aerobic respiration to anaerobic metabolism. This reduction was strongest in *R. amphibia*; the aboveground starch concentration was reduced to zero after 20 days of submergence. Although there also was a decrease in carbohydrates in *R. sylvestris* compared to air controls, total carbohydrate content was maintained close to starting levels. This also applied to the hybrid (Table 1). Submerged *R. amphibia* had higher glucose and fructose concentrations compared to air controls, while sucrose concentration was reduced (Fig. 3a, [Supplementary Information – Fig. S1a]). This trend was not found in *R. sylvestris*.

**All genotypes showed a rapid reduction in total belowground carbohydrates in the first week of submergence**

In the first week of submergence there was an initial reduction in belowground carbohydrates in all the genotypes, with *R. amphibia* showing the greatest reduction (Fig. 3b). Carbohydrate levels subsequently increased again in all genotypes later on. The hybrid showed the smallest reduction in soluble carbohydrates and displayed a faster recovery after 14 days...
of submergence when compared to either of the parental lines (Table 1). At the end of the treatment total belowground carbohydrates were therefore highest in the hybrid, followed by *R. sylvestris*.

Table 1 Summary of pair-wise comparisons of the response to 20 days of submergence (first experiment) of the parental lines and their hybrid, using ANOVA contrasts. For instance, the increase in stem length from day 0 till day 20 (of submergence) differs among all three groups (cf. the slopes in Figure 1a).

<table>
<thead>
<tr>
<th>Response variable</th>
<th>F-value contrast</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stem length (mm)</td>
<td>21.33*** 16.12*** 74.52***</td>
<td>H&gt;A&gt;S A + overdominance</td>
</tr>
<tr>
<td>BG DW (g)</td>
<td>0.03 0.65 0.39</td>
<td>H=A=S Inconclusive</td>
</tr>
<tr>
<td>AG DW (g)</td>
<td>0.91 2.91 0.56</td>
<td>H=A=S Inconclusive</td>
</tr>
<tr>
<td>AG glucose (mg g⁻¹DW)</td>
<td>0.54 0.37 1.72</td>
<td>H=A=S Inconclusive</td>
</tr>
<tr>
<td>AG fructose (mg g⁻¹DW)</td>
<td>17.58*** 5.68* 3.27</td>
<td>H=S&lt;A S dominance</td>
</tr>
<tr>
<td>AG sucrose (mg g⁻¹DW)</td>
<td>11.00*** 4.32* 1.53</td>
<td>H=S&gt;A S dominance</td>
</tr>
<tr>
<td>AG starch (mg g⁻¹DW)</td>
<td>15.81*** 25.92*** 1.24</td>
<td>H=S&gt;A S dominance</td>
</tr>
<tr>
<td>BG glucose (mg g⁻¹DW)</td>
<td>1.57 12.83*** 23.38***</td>
<td>H&gt;S=A Overdominance</td>
</tr>
<tr>
<td>BG fructose (mg g⁻¹DW)</td>
<td>0.04 20.15*** 22.02***</td>
<td>H&gt;S=A Overdominance</td>
</tr>
<tr>
<td>BG sucrose (mg g⁻¹DW)</td>
<td>0.04 6.56* 5.61*</td>
<td>H&gt;A=S Overdominance</td>
</tr>
<tr>
<td>BG starch (mg g⁻¹DW)</td>
<td>3.87 3.65 0.00</td>
<td>H=A=S Inconclusive</td>
</tr>
<tr>
<td>BG total carbs. (mg g⁻¹DW)</td>
<td>3.18 8.26* 1.19</td>
<td>H≥S=A Overdominance</td>
</tr>
<tr>
<td>AG total carbs. (mg g⁻¹DW)</td>
<td>9.19* 26.76*** 3.41</td>
<td>H&gt;S&gt;A S dominance</td>
</tr>
</tbody>
</table>

BG, belowground; AG, aboveground; DW, dry weight; A, *Rorippa amphibia*, S, *Rorippa sylvestris*, H, hybrid; significance levels, * P<0.05, ** P<0.005, ***P<0.0005.
Carbohydrate catabolism genes are differentially regulated

Partial sequences of ADH1 and SUS1 Rorippa homologs (genebank no: JQ582800, JQ582801, JQ582802, JQ582803) constituted 377 and 297 amino acids, respectively. Homology between A. thaliana ADH1 and the most common allele of Rorippa was 94%, and 99% for SUS1 [Supplementary Information – Fig. S6-7]. After three and six days of complete submergence, R. amphibia displayed high ADH1 transcript levels similar to air controls (Fig. 4a). However, R. sylvestris showed a stronger down regulation, in contrast to hybrids, which showed a similar pattern to that of R. amphibia (Table 2). Three days after plants were allowed to establish air contact (74 hrs), ADH1 levels were similar to air controls in R. amphibia and the hybrid, however this effect was less apparent in R. sylvestris. There was no differential regulation for SUS1 among species (Fig. 4b), as all genotypes showed a down-regulation in submerged conditions and an up-regulation upon reaching water surface. Transcript levels of SUS1 and ADH1 showed a good correlation ($R^2=0.4385$) supporting similar regulation mechanisms [Supplementary Information – Fig. S3]. The effects of treatments and genotypes were not as pronounced in aboveground tissues (data not shown).
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Fig. 4 (a) $ADH1$ and (b) $SUS1$ expression in *Rorippa amphibia*, *Rorippa sylvestris* and the hybrid roots following 2, 26, 74 hours of complete and semi-submergence after 3 days of complete submergence (n=4; error bars indicate SEs). *Rorippa amphibia* air control at 2 hours was selected as the reference ($\log_2=0$) for both genes.

Table 2 Summary of pair-wise differences for contrast analysis between parental lines and the hybrid for $ADH1$ expression levels in the belowground tissues (expression experiment)

<table>
<thead>
<tr>
<th>Relative $ADH1$ expression</th>
<th>$R. amphibia$ vs $R. sylvestris$</th>
<th>$R. amphibia$ vs hybrid</th>
<th>$R. sylvestris$ vs hybrid</th>
</tr>
</thead>
<tbody>
<tr>
<td>air vs. submerged</td>
<td>2.68*</td>
<td>2.05</td>
<td>2.93*</td>
</tr>
<tr>
<td>submerged vs. semi-submerged</td>
<td>0.89</td>
<td>4.37*</td>
<td>1.93</td>
</tr>
<tr>
<td>air vs. semi-submerged</td>
<td>0.49</td>
<td>1.44</td>
<td>2.69*</td>
</tr>
</tbody>
</table>

* P<0.055.

Escapers benefited from elongation only if they reached the water surface

All three species had extreme tolerance to submergence, as evident from their survival in the long-term experiment. *Rorippa sylvestris* showed 100% survival after up to 100 days of complete and semi-submergence (Fig. 4a). No mortality was observed in either *R. amphibia* or the hybrid after 58 days, and only after 79 days the effects of complete submergence
without air contact became evident, with *R. amphibia* being the least tolerant of the three genotypes.

The restoration of shoot air contact after the period of complete submergence led to a lower mortality in both escapers. *Rorippa amphibia* still had high levels of mortality after 42 days of semi-submergence following the 37 days of complete submergence. The hybrid was more tolerant than *R. amphibia*; mortality was lower both in complete and semi-submergence treatments.

![Fig. 5](image)

**Fig. 5** (a) Schematic view of experimental timeline (b) Survival and (c) aboveground and belowground dry weight of plants at start and after 37 days of complete submergence followed by controlled air contact for 21 and 42 days for *Rorippa amphibia*, *Rorippa sylvestris* and their hybrid (n=12 for survival, n=9 for dry weight; error bars indicate SEs).

**Only escapers benefited in terms of biomass accumulation when reaching the water surface**

There was a significant reduction in mainly aboveground biomass in all genotypes after 37 days of complete submergence (Fig. 4b). After restoring air contact (semi-submergence), biomass increased significantly in the escaper species. Reaching the surface mostly led to aboveground biomass accumulation, although the effect was less apparent in belowground tissues. Remarkably, reaching the surface had almost no effect on quiescent *R. sylvestris* in terms of biomass recovery.
No increase in aboveground carbohydrate content occurred upon reaching the water surface

After 37 days of complete submergence, total aboveground carbohydrates were reduced in all genotypes and again more so in *R. amphibia* (Fig. 5a, Table 3) similarly to the first experiment (Fig. 3a). Aboveground glucose and fructose content [Supplementary Information Fig. S2a] was reduced in all genotypes and most dramatically in *R. sylvestris* after 79 days of complete submergence. The hybrid showed a similar trend to *R. sylvestris* in glucose and fructose reduction (Table 3). Sucrose levels significantly increased in aboveground tissues of all genotypes, but more so in *R. sylvestris* (Table 3). The reduction in starch levels was strongest in *R. amphibia*. Following 37 days of complete submergence, an increase in carbohydrates was observed only in *R. amphibia* after 21 days of air contact. However, after 42 days of air contact, there was no clear benefit in terms of carbohydrate levels from reaching the surface in any of the species. Nevertheless, the biomass increase upon air contact establishment led to a higher total amount of carbohydrates in the escapers; although the carbohydrate concentrations did not change (as shown in Fig. 5a), the total amount within the plant increased as a result of increased biomass. Sucrose levels increased further during the first 21 days of air contact in *R. amphibia*, whereas *R. sylvestris* and the hybrid showed a continuous increase in sucrose levels regardless of being submerged or semi-submerged [Supplementary Information – Fig. S2a-b]. We also observed increased trehalose levels in roots of all genotypes after 79 days of complete submergence [Supplementary Information – Fig. S2b].

Quiescent *R. sylvestris* showed little reduction of belowground carbohydrate levels after prolonged submergence

Carbohydrate levels were significantly reduced in belowground tissues of all the genotypes after 37 days of complete submergence, but this was particularly striking in *R. amphibia* (Fig. 5b, Table 3). At longer periods of complete submergence, further reductions in belowground carbohydrate levels occurred in both *R. amphibia* and the hybrid. *Rorippa sylvestris* maintained more of its belowground carbohydrates and showed less than 30% reduction after 100 days of submergence, compared to 78% and 95% reductions in *R. amphibia* and the hybrid, respectively. After 21 days of air contact, carbohydrates had increased in all the genotypes, but more significantly in the escaper genotypes. However, the effects were no longer evident after 42 days of air contact.

Similar to aboveground tissue, belowground sucrose levels increased in all species upon submergence. After 37 days, *R. sylvestris* roots had more sucrose than any of the other
Fig. 6 Soluble carbohydrate and starch content of (a) aboveground and (b) belowground tissues at start and after 37 days of complete submergence followed by controlled air contact for 21 and 42 days for *Rorippa amphibia*, *Rorippa sylvestris* and their hybrid (n=9); sub: submerged, semi: semi-submerged.

Sucrose levels were higher after 21 days of air contact in the escapers, but after 42 days of air contact, levels were comparable to submerged plants. *Rorippa sylvestris* maintained higher underground starch levels regardless of being fully or semi-submerged.

**DISCUSSION**

Flooding can shape plant species distributions along a submergence gradient and can act as a strong selective pressure for key adaptations, potentially leading to speciation (Keddy, 1984; Silvertown *et al.*, 1999; Vervuren, 2003; Van Eck *et al.*, 2004; Lenssen & De Kroon, 2005; Voesenek *et al.*, 2006). We have shown that two wetland species *Rorippa amphibia* and *Rorippa sylvestris* from habitats with different flooding regimes, display different tolerances and survival strategies for submergence stress: *R. amphibia* has adopted the escape strategy and *R. sylvestris* the quiescence strategy.
Table 3 Summary of the contrast analyses between parental genotypes and the hybrid for the change from starting values (day 0) to 37 days of submergence (second experiment). For instance, the decrease in aboveground DW from day 0 till day 37 (of submergence) does not differ among all three groups (cf. the slopes in Figure 5a)

<table>
<thead>
<tr>
<th>Response variable</th>
<th>R. amphibia vs R. sylvestris</th>
<th>R. amphibia vs hybrid</th>
<th>R. sylvestris vs hybrid</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>BG DW (g)</td>
<td>0.49</td>
<td>0.69</td>
<td>0.02</td>
<td>H=S=A</td>
</tr>
<tr>
<td>AG DW (g)</td>
<td>0.26</td>
<td>0.09</td>
<td>0.04</td>
<td>H=S=A</td>
</tr>
<tr>
<td>AG glucose (mgg-1DW)</td>
<td>4.33*</td>
<td>10.91**</td>
<td>1.50</td>
<td>H=S&lt;A S dominance</td>
</tr>
<tr>
<td>AG fructose (mgg-1DW)</td>
<td>16.14***</td>
<td>11.59**</td>
<td>0.38</td>
<td>H=S&lt;A S dominance</td>
</tr>
<tr>
<td>AG sucrose (mgg-1DW)</td>
<td>46.95***</td>
<td>9.15**</td>
<td>14.64***</td>
<td>S&gt;H&gt;A Additivity</td>
</tr>
<tr>
<td>AG starch (mgg-1DW)</td>
<td>10.58**</td>
<td>10.71**</td>
<td>0.00</td>
<td>H=S&gt;A S dominance</td>
</tr>
<tr>
<td>BG glucose (mgg-1DW)</td>
<td>1.06</td>
<td>1.87</td>
<td>5.75*</td>
<td>H≥A=S Overdominance</td>
</tr>
<tr>
<td>BG fructose (mgg-1DW)</td>
<td>0.35</td>
<td>6.86*</td>
<td>10.33**</td>
<td>H&gt;S=A Overdominance</td>
</tr>
<tr>
<td>BG sucrose (mgg-1DW)</td>
<td>2.27</td>
<td>1.05</td>
<td>0.23</td>
<td>H=S=A Inconclusive</td>
</tr>
<tr>
<td>BG starch (mgg-1DW)</td>
<td>8.39**</td>
<td>7.16*</td>
<td>0.10</td>
<td>H=S&gt;A S dominance</td>
</tr>
<tr>
<td>BG total carbs. (mgg-1DW)</td>
<td>8.47**</td>
<td>4.47*</td>
<td>0.82</td>
<td>H=S&gt;A S dominance</td>
</tr>
<tr>
<td>AG total carbs. (mgg-1DW)</td>
<td>7.08*</td>
<td>5.24*</td>
<td>0.14</td>
<td>H=S&gt;A S dominance</td>
</tr>
</tbody>
</table>

BG, belowground; AG, aboveground; DW, dry weight; A, *Rorippa amphibia*; S, *Rorippa sylvestris*; H, hybrid; carbs, carbohydrates; significance levels, * P<0.05, ** P<0.005, ***P<0.0005.
Yellowcress species have contrasting strategies to cope with flooding

When submerged, *R. amphibia* displays an escape strategy defined by prolonged stem elongation in an attempt to reach the surface and supply the submerged tissues with oxygen necessary for respiration. Supported by aerenchyma tissue, this strategy could be very beneficial when floods are long lasting and relatively shallow, allowing plants to restore air contact and thus internal aeration (Bailey-Serres & Voosenk, 2008). In contrast, *R. sylvestris* displays a quiescence strategy by minimizing its growth and conserving carbohydrate reserves. Since *R. sylvestris* occupies habitats with deeper and transient floods (Jonsell, 1968; Blom, 1999), the quiescence strategy can increase the fitness of the plant by avoiding consumption of carbohydrates in growth processes. The high levels of carbohydrates we measured in this species even after 79 days of complete submergence suggest that underwater photosynthesis may also play an important role in survival of *R. sylvestris* (Mommer & Visser, 2005; Mommer et al., 2006b; Stift et al., 2008). We observed increasing levels of sucrose in belowground tissues of all the genotypes under submerged conditions but more in *R. sylvestris*. Sucrose plays an important role in the onset of the quiescence strategy in rice (Kudahettige et al., 2011) and in submergence tolerance of *Arabidopsis thaliana* (Loreti et al., 2005) and might also be crucial for the extreme submergence tolerance in Rorippa. Increasing sucrose levels in shoots might be a result of efficient underwater photosynthesis, which would enhance tolerance.

After five weeks of complete submergence *R. sylvestris* had larger starch reserves than either of the other genotypes, both above and belowground. A previous study of *R. amphibia* and *R. sylvestris* roots using Arabidopsis GeneChip microarrays showed that genes related to carbohydrate catabolism (*SUS1, ADH1, PDC1*) are up-regulated in all genotypes upon 24 hours of submergence, but to a greater extent in *R. amphibia* (Boonman et al, unpublished data). In addition to this initial up-regulation, we showed *ADH1* and *SUS1* are down-regulated at later stages of complete submergence. It has also been shown that in *A. thaliana, ADH* and *PDC1* are initially up-regulated under anoxia but levels decline at later stages (Loreti et al., 2005). However, in *R. amphibia, ADH1* and *SUS1* transcript levels were higher even after 6 days of complete submergence in contrast to the low levels in *R. sylvestris*. This might explain the lower consumption of carbohydrates and a higher survival in *R. sylvestris*. The quiescence strategy controlled by *SUB1A-1* in rice is also defined by lower growth rates and conservation of carbohydrates in spite of induction of fermentation genes (Fukao et al., 2006). The high correlation in transcript levels of *ADH1* and *SUS1* in different treatments and genotypes supports that these pathways are co-regulated in *Rorippa*.
Stem elongation can be an efficient process to reach the surface. Nevertheless, growing tissues need energy, which would lead to a faster depletion of carbohydrates by anaerobic metabolism under low oxygen (Bailey-Serres & Voesenek, 2008; Bailey-Serres & Voesenek, 2010). Complete depletion of aboveground starch in *R. amphibia* after 20 days of submergence and higher *ADH1* and *SUS1* transcript levels support the fact that elongation demands high levels of energy (Groeneveld & Voesenek, 2003). Glucose and fructose levels were also higher in *R. amphibia*, possibly because of the breakdown of starch reserves for glycolysis and anaerobic metabolism to supply ATP for growth (Perata & Alpi, 1993; Guglielminetti *et al*., 1995; Perata *et al*., 1996).

In all the genotypes, a rapid reduction of carbohydrates was observed within the first week of submergence, followed by increasing or stable levels in the later weeks. Consumed carbohydrates might be supplied to organs that undergo acclimations for submergence which can include newly formed leaves with more aerenchyma tissue and/or higher underwater photosynthesis ability and tissues more resistant to reactive oxygen species (Voesenek *et al*., 2006). The increase in carbohydrates after the second week also indicates that the plants acclimated to the submergence stress. As a result, plants mostly had healthy and green leaves even after three weeks of complete submergence, although there were significant effects of submergence on biomass and carbohydrate content in all the genotypes.

Although starch was completely depleted in 20 days, the carbohydrate levels recovered at a later stage (37 days of complete submergence) in *R. amphibia*. This may be the result of most of the stem elongation, taking place in the first two weeks of submergence. After stem growth ceased, the plant might have accumulated more carbohydrates by increased underwater photosynthesis of the stem and its small leaves (Raskin & Kende, 1984; Beckett *et al*., 1988). Additionally, observed increases in trehalose levels might also have been a factor increasing submergence tolerance since this carbohydrate appears to improve tolerance to several abiotic stresses (Chen & Murata, 2002; Garg *et al*., 2002).

**Hybrids are mostly escapers, and are able to elongate a stem while conserving carbohydrates**

In growth and morphology, the hybrid displayed an escape strategy, although the effect of the treatment on stem elongation was not as strong as in *R. amphibia*. Surprisingly, the carbohydrate levels were overall comparably high to those in *R. sylvestris*. The hybrid had a similar level of aboveground carbohydrate reduction to *R. sylvestris* in the first experiment (after 20 days of submergence) and similar patterns of both above and belowground carbohydrate concentrations in the long-term experiment (after 37 days of submergence).
This explains the higher survival of the hybrid compared to *R. amphibia*, and could be due to the presence of the mechanisms of the *R. sylvestris* parent for conserving carbohydrates, or due to more efficient underwater photosynthesis. Potentially, this could also lead to higher fitness of hybrids than their parents under certain field conditions. Hybrids are often found in flood plains where both parents are present, occupying intermediate locations (Bleeker, 2004; Bleeker, 2007). The intermediate levels of the hybrid in various traits suggest that neither of the parental strategies is dominant over the other, in agreement with such a distribution pattern in the wild (Bleeker & Hurka, 2001).

**Quiescent *R. sylvestris* lacks the recovery mechanisms of the escape strategy after reaching the water surface**

Although *R. amphibia* inhabits sites that can be completely submerged, it has a lower survival rate compared to *R. sylvestris* since the latter shows no mortality even after more than three months of submergence. The depth of submergence determines the stem length necessary to reach the surface and hence the carbohydrate demands for this elongation. It has been showed that reaching the water surface favors fitness related traits (Pierik *et al.*, 2009; Chen *et al.*, 2011) but the effect on survival has never been shown before. If plants are able to reach the surface, survival is improved significantly in *R. amphibia* and the hybrid. Some plants showed mortality even after air contact was established; possibly because of the existing damage caused by the long complete submergence period or the sudden switch to normoxia, resulting in oxidative damage. Upon establishing air contact, surviving plants of both *R. amphibia* and the hybrid grew extensively above the water level. This phenotype was almost completely absent in *R. sylvestris* after partial air contact establishment. This suggests that a quiescent period in this species extends to a semi-submerged state, and that vigorous growth is resumed only after a further drop in the water table, and restoration of normoxic conditions belowground. The lower porosity in *R. sylvestris* might explain the absence of a recovery after air restoration. Since the stem above the water surface cannot function as an efficient snorkel when sufficient aerenchyma is absent, the parts under water might remain anoxic for a longer time. We also observed that as soon as *R. sylvestris* was de-submerged completely, clones were emerging from rhizomes, forming new healthy plants within weeks (personal observation), possibly enhanced by the higher carbohydrate levels in belowground tissues.

**Submergence tolerance strategies and clonal growth**

The escape and quiescent strategies of the species are correlated with other aspects of clonal growth. Although both of the species are rhizome sprouters (Jonsell, 1968), clonal growth from rhizomes is more vigorous in *R. sylvestris*, possibly stimulated by the high
levels of carbohydrates in belowground tissues. This trend is also observed under submerged conditions and can increase plant survival by means of rhizome sprouts after a prolonged flood even if aboveground tissues completely die. It has been shown that adventitious bud formation after a heavy injury increases fitness in *Rorippa palustris* (Klicesová & Klimes, 2007; Klimesova et al., 2008). The higher abundance of rhizome sprouting clonal species in wetlands (Sosnova et al., 2010) might be due to higher survival achieved by this strategy. During semi-submergence, we observed that the growing aboveground parts of the escapers were forming adventitious roots in stems and leaves, and were becoming detached from the slender underwater stem. This was also observed in naturally flooded areas, where emergent *R. amphibia* plants can become detached from the underwater stems, float around and settle at a different location (Jonsell, 1968). This slender and deteriorating stem might explain the low recovery in biomass of the root system compared to the vigorous aboveground tissues, due to inefficient transport of resources to belowground tissues.

In conclusion, *R. amphibia*, *R. sylvestris* and their hybrid all exhibit extreme submergence tolerance, which is achieved by different strategies selected by their natural habitats. Escaper *R. amphibia* invests in elongating its stem and consumes its carbohydrate reserves in order to reach the floodwater surface and if this is not established, plants from this species die sooner than those of their quiescent relative *R. sylvestris*, which shows a higher survival by limiting growth and conserving resources. Their hybrid also displays an escaper strategy but at the same time conserves its carbohydrates better than *R. amphibia* and thus has lower mortality. Being close relatives of the model plant Arabidopsis, *Rorippa* species constitute a good model for studying the molecular basis of extreme submergence tolerance with their escape and quiescence strategies. Although the mechanisms of these responses are largely unknown, the opportunities of using information from Arabidopsis will accelerate the research to unravel the genetics underlying these strategies. Many advantages such as ease of cloning genes by using available Arabidopsis sequence data and applicability of gene expression assays designed for Arabidopsis increases the potential of *Rorippa* flooding research.

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SUPPLEMENTARY INFORMATION

Detailed carbohydrate content figures for both experiments, correlation graphs for ADHI and SUS1 expression, melting and dilution curves for RT-qPCR results, gene alignments, plant morphology pictures and statistical analyses are supplied in the online version of this manuscript as supplementary information.