Plant Signaling & Behavior 9, e27847; February; © 2014 Landes Bioscience

Extreme flooding tolerance in *Rorippa*

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Keywords: *Rorippa*, *Arabidopsis*, fermentation, flooding tolerance, hypoxia, root, submergence

Low oxygen stress imposed by floods creates a strong selection force shaping plant ecosystems in flood-prone areas. Plants inhabiting these environments adopt various adaptations and survival strategies to cope with increasing water depths. Two *Rorippa* species, *R. sylvestris* and *R. amphibia* that grow in naturally flooded areas, have high submergence tolerance achieved by the so-called quiescence and escape strategies, respectively. In order to dissect the molecular mechanisms involved in these strategies, we investigated submergence-induced changes in gene expression in flooded roots of *Rorippa* species. There was a higher induction of glycolysis and fermentation genes and faster carbohydrate reduction in *R. amphibia*, indicating a higher demand for energy potentially leading to faster mortality by starvation. Moreover, *R. sylvestris* showed induction of genes improving submergence tolerance, potentially enhancing survival in prolonged floods. Additionally, we compared transcript profiles of these 2 tolerant species to relatively intolerant *Arabidopsis* and found that only *Rorippa* species induced various inorganic pyrophosphate dependent genes, alternatives to ATP demanding pathways, thereby conserving energy, and potentially explaining the difference in flooding survival between *Rorippa* and *Arabidopsis*.

Flooding has detrimental effects on plants as a result of decreased underwater gas diffusion leading to rapid depletion of oxygen and a halt in aerobic respiration.[1,2] A common plant response to low oxygen is increased glycolysis and a switch to anaerobic metabolism for supplying the plant with the necessary ATP, which help them to survive short periods of submergence. However, this leads to a faster consumption of carbohydrates since this ATP gain is much lower than that obtained via aerobic respiration.[3] If submergence is prolonged, mortality is inevitable in this energy crisis. Thus flooding acts as a strong selection force on plant communities inhabiting flood-prone areas and as a result shapes typical adaptations in flood-exposed plant species.[4,5] Understanding how these adaptations facilitate survival in submergence tolerant plants is very valuable for crop improvements as well as for management programs in flood-prone areas. We studied the responses of 2 submergence tolerant *Rorippa* species that inhabit naturally flooded sites and adopt different strategies to overcome lethal effects of the associated low oxygen stress.[6] The oxygen content in the roots and shoots of flooded plants can be very different. Submerged shoots can often maintain close to normoxic levels of oxygen, especially when light levels are sufficient. In contrast, roots surrounded by an oxygen depleted soil often experience severe hypoxia. We performed global transcriptome profiling, carbohydrate, and metabolite analyses on the roots of *Rorippa* plants subjected to 24 h of complete submergence. Additionally, we compared the submergence responses in the roots of the relatively flood intolerant *Rorippa* relative, *Arabidopsis*, to potentially identify candidate genes or processes that could explain the highly effective adaptive mechanisms evolved in *Rorippa*.

Different strategies for different flooding regimes

Rorippa amphibia and *Rorippa sylvestris* both grow in frequently flooded habitats, the former occurring in sites with more stable and shallow water tables and the latter with prolonged deep floods and summer dry-outs. Hence, *R. sylvestris* can survive much longer than *R. amphibia* under complete submergence and shows no mortality for up to 100 d while around 70% mortality is observed in *R. amphibia* under similar conditions.[6,7] Adaptations evolved as a response to flooding gradients determine species distributions in flood prone environments.[8] In accordance with the flooding regimes at their natural habitats, the survival difference in these *Rorippa* species can be explained by their contrasting strategies (**Fig. 1**). When completely submerged, *R. sylvestris* displays a quiescence strategy characterized by limited shoot growth while *R. amphibia* shows enhanced shoot elongation, typical for the escape strategy.

The escape strategy, typified by *R. amphibia,* requires a considerable investment of carbohydrate and energy reserves in the submerged environment where photosynthesis and respiration is already restricted. Higher levels of glycolysis and fermentation related gene expression and reduction in ATP levels after 24 h of submergence in *R. amphibia* roots suggest that there is a higher demand for energy, possibly to supply for the aboveground growth. This coupled with higher induction of SUCROSE SYNTHASE and INVERTASE pathways in *R. amphibia* roots leads to reduction

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Submitted: 01/08/2014; Accepted: 01/14/2014; Published Online: 02/13/2014
Citation: Akman M, Bhikharie A, Mustroph A, Sasidharan R. Extreme flooding tolerance in Rorippa. Plant Signaling & Behavior 2014; 9:e27847; PMID: 24525961; http://dx.doi.org/10.4161/psb.27847

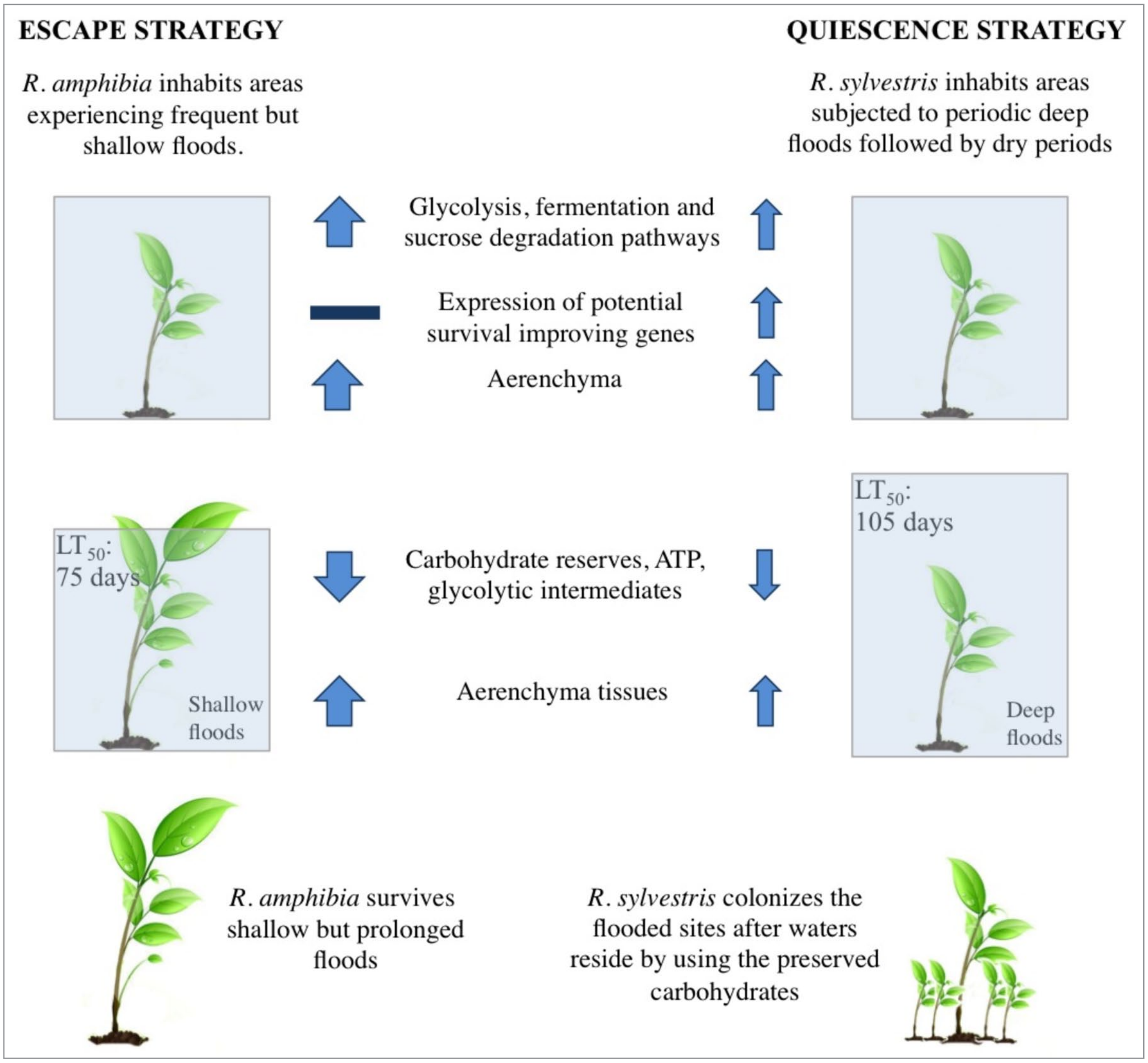

Figure 1. Submergence tolerance strategies in *Rorippa*. LT_{50} = Median lethal time (Number of days after which 50% of the population is dead due to stress). Arrows indicate submergence-induced increase (up) or decrease (down). Thickness of arrows indicates the magnitude of the effect.

of soluble carbohydrates at earlier phases of submergence[6] and later to a severe reduction in root starch reserves.[7] Root carbohydrates are most likely channeled to the elongating shoot as evidenced by increased aboveground biomass in *R. amphibia* after 2 wk of submergence at the expense of root biomass.[9]

Although energetically expensive, the escape strategy pays off when the shoot outgrows shallow floodwaters. In *R. amphibia*, emergence of the shoot tip leads to an obvious increase in plant biomass.[7] This is likely due to the improved aeration of the plant via the emergent shoot tip that is aerenchyma rich (high porosity in *R. amphibia* petioles). We also showed that when air contact was established after 3 d of submergence, *ADH1* was downregulated dramatically within 2 h in the shoot tips above water, and to a lower extent in the submerged parts of these *R. amphibia* plants (**Fig. 2**). This rapid change indicates a fast recovery from low oxygen stress and that oxygen can be efficiently transported via aerenchyma tissues to submerged parts of the plant indicating the vital role of aerenchyma in the escape strategy. The quiescent *R. sylvestris* having much lower petiole porosity does not benefit from partial shoot emergence as much as *R. amphibia,* both in terms of biomass accumulation[7] and reduction in *ADH1* expression.

Establishing aerial contact not only supplies oxygen but also the carbon dioxide necessary for underwater photosynthesis, which is an important factor affecting submergence tolerance.[10,11] Floods are usually coupled with turbid waters, thus light intensity decreases with water depth.[12] Elongating new leaves of submerged *R. amphibia* show higher specific leaf area (data not shown) in order to increase underwater gas diffusion and potentially to capture more light as the photosynthetic tissues get close to the surface and hence higher intensities of light. Moreover, underwater shoot elongation might also be a plastic response enhanced by light availability and carbohydrate levels, since submergence in darkness obscures the elongation differences between *R. amphibia* and *R. sylvestris* (data not shown).

R. sylvestris inhabits sites with shorter and deeper floods, where an escape strategy would not be useful. This species displays a more conservative use of ATP and carbohydrates and the accumulation of glycolytic intermediates all pointing toward a restriction of energetically expensive processes and economization of reserves typical of the quiescence strategy. This conservative strategy might enable fast colonization of previously flooded sites after waters reside.[13] However, this requires adaptation to submergence and associated stresses for longer periods and genes expressed at higher levels specifically in *R. sylvestris* might potentially explain the difference in survival between the 2 *Rorippa* species. These include *CATALASE1*, an antioxidant gene, and a WRKY transcription factor (AtWRKY75), member of a gene family recently shown to be important in submergence tolerance in *Arabidopsis*.[14] The F1 hybrid of these *Rorippa* species,[7] displays an escape strategy similar to *R. amphibia*. However, the higher growth did not lead to a lower survival like that of *R. amphibia*, but was rather intermediate. We also studied the root transcript profiles of the hybrid and found that although the hybrid is phenotypically more similar to *R. amphibia* than to *R. sylvestris* based on survival strategies, this conclusion does not hold when gene expression patterns are considered (data not shown). It is highly likely that expression of the above-mentioned potential survival improving genes of *R. sylvestris* also facilitate survival in the hybrid, despite the higher growth rate.

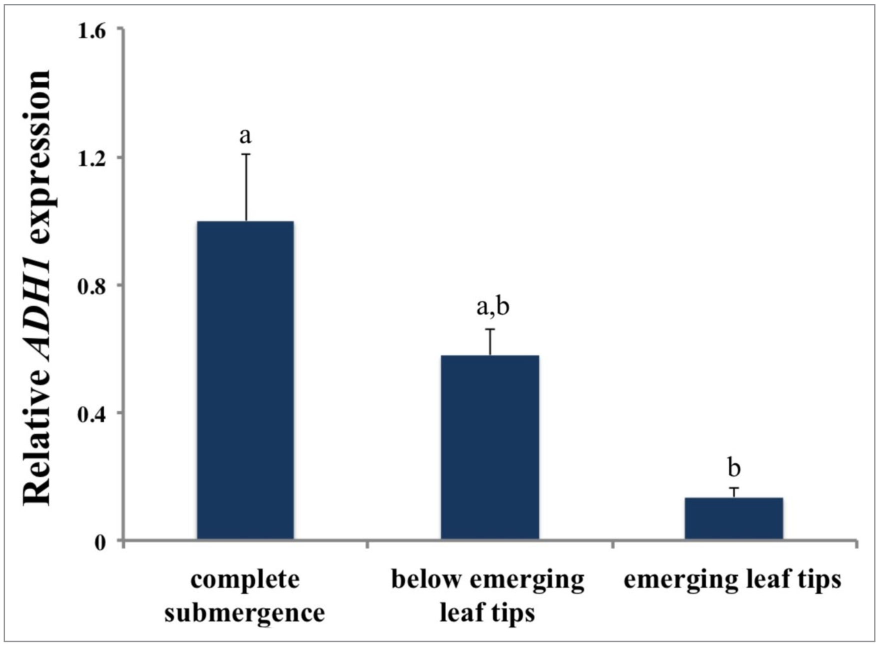

Figure 2. Relative *ADH1* expression in completely submerged shoots, submerged shoots below emerging leaves and emerging leaf tips of *R. amphibia*. Data points represent means ± standard errors ($n = 4$). ANOVA Tukey test HSD results are indicated with letters at $P < 0.05$. Submergence started 4 h after the onset of the photoperiod and experiments were continued in 9/15 h day/night regime. Gene expression was measured in 2 sets of plants: 1) completely submerged for 74 h, and 2) plants that established air contact for 2 h after 72 h of complete submergence (emerging leaf tips and below emerging leaf tips). 18S was used as an internal reference gene. Primer sequences for *ADH1* and *18S* rRNA genes were same as in Akman et al. 2012.

PPi-dependent alternative pathways: potential tolerance associated genes enhancing survival in Rorippa

The significant relatedness of *Rorippa* and *Arabidopsis* allows a more direct comparison of their submergence responses and reveals potential mechanisms leading to the survival difference between these 2 closely related genera. There is a significant variation in submergence tolerance even in naturally non-flooded *Arabidopsis* accessions.[15] However, even the most tolerant accession, C24, has a lethal median time of 20 d, much lower than 75 d for *R. amphibia* and 105 d for *R. sylvestris*. This substantial difference could be as a result of various mechanisms that evolved in *Rorippa* as a consequence of inhabiting flood-prone environments. One of these mechanisms is proposed to be the conservation of ATP via utilization of PPi-dependent pathways.[16,17] A preference for these alternative PPi-dependent over ATP consuming pathways in *Rorippa* would conserve ATP pools and might also contribute to the higher tolerance in these 2 species compared with their relative *Arabidopsis*.

The study of wild species like *Rorippa* that have naturally evolved to survive in flood prone niches is important to understand the mechanisms mediating distinct flooding survival strategies. Exploring not only molecular and physiological changes but also the ecology of flood tolerant species and the mechanisms of adaptation to their habitats serves as a strong potential for understanding and improving plant reactions to increasing water depths.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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