Root transcript profiling of two Rorippa species reveals gene clusters associated with extreme submergence tolerance


Published in:
Plant Physiology

DOI:
10.1104/pp.113.222588

Citation for published version (APA):

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Complete submergence represses photosynthesis and aerobic respiration, causing rapid mortality in most terrestrial plants. However, some plants have evolved traits allowing them to survive prolonged flooding, such as species of the genus *Rorippa*, close relatives of *Arabidopsis* (*Arabidopsis thaliana*). We studied plant survival, changes in carbohydrate and metabolite concentrations, and transcriptome responses to submergence of two species, *Rorippa sylvestris* and *Rorippa amphibia*. We exploited the close relationship between *Rorippa* species and the model species *Arabidopsis* by using *Arabidopsis* GeneChip microarrays for whole-genome transcript profiling of roots of young plants exposed to a 24-h submergence treatment or air. A probe mask was used based on hybridization of genomic DNA of both species to the arrays, so that weak probe signals due to the close relationship between *Rorippa* species and *Arabidopsis* mismatches were removed. Furthermore, we compared *Rorippa* species microarray results with those obtained for roots of submerged *Arabidopsis* plants. Both *Rorippa* species could tolerate deep submergence, with *R. sylvestris* surviving much longer than *R. amphibia*. Submergence resulted in the induction of genes involved in glycolysis and fermentation and the repression of many energy-consuming pathways, similar to the low-oxygen and submergence response of *Arabidopsis* and rice (*Oryza sativa*). The qualitative responses of both *Rorippa* species to submergence appeared roughly similar but differed quantitatively. Notably, glycolysis and fermentation genes and a gene encoding sucrose synthase were more strongly induced in the less tolerant *R. amphibia* than in *R. sylvestris*. A comparison with *Arabidopsis* microarray studies on submerged roots revealed some interesting differences and potential tolerance-related genes in *Rorippa* species.

Submergence as a result of flooding is very harmful to most plants. Restricted gas diffusion under water leads to the repression of photosynthesis and aerobic respiration, resulting in an energy and carbohydrate crisis (Drew, 1997; Voesenek et al., 2006; Bailey-Serres and Voesenek, 2008, 2010). Excessive water, either during prolonged waterlogging or complete submergence, will kill most terrestrial plants (Setter and Laureles, 1996; Drew, 1997).

Nevertheless, some specialized plant species from flood-prone areas may survive complete submergence for many months (van Eck et al., 2004; Mommer et al., 2006). Two different strategies to cope with submergence have been proposed: (1) escape from submergence by restoring air contact, characterized by rapid shoot elongation and the formation of longitudinal air channels called aerenchyma; and (2) quiescence, meaning that growth is restricted and carbohydrate reserves are preserved that can be utilized for recovery after the flood waters have subsided (Bailey-Serres and Voesenek, 2008). Even though a wide variety of plant responses have been described that can increase the survival of submergence stress, the mechanisms underlying such extreme submergence tolerance are still poorly understood, especially at the molecular level (Bailey-Serres and Voesenek, 2008).

Even many rice (*Oryza sativa*) varieties can be severely damaged or killed within a few weeks of complete submergence (Setter and Laureles, 1996). Lowland rice can germinate in a layer of water and flourish as long as the shoot is in contact with air.
Deepwater rice, as long as it keeps up its shoot growth so that leaves remain in contact with air, is tolerant to meters of water. Recently, the genetic determinants of submergence tolerance in lowland and deepwater rice have been identified as SUB1 (Xu et al., 2006) and SNORKEL1 and SNORKEL2 (Hattori et al., 2009), respectively. These genes belong to the group VII Ethylene Response Factor (ERF) family in rice but control opposite submergence responses. The presence of the SUB1A-1 allele will suppress the growth of rice when flooded (Xu et al., 2006). Quiescence of this kind is demonstrably advantageous when paddy rice is flooded for a few weeks and the flood water is too deep to be outgrown (Fukao et al., 2006). The presence of the SNORKEL genes in deepwater rice promotes shoot elongation (Hattori et al., 2009). Recent studies in Arabidopsis (Arabidopsis thaliana) have shown that certain members of the group VII ERFs are degraded in an oxygen-dependent manner by the N-end rule pathway of protein degradation. It is suggested that this pathway functions as an oxygen-sensing mechanism in higher plants (Gibbs et al., 2011; Licausi et al., 2011). Interestingly, overexpression of one of these genes, RAP2.12, made Arabidopsis more flood tolerant (Licausi et al., 2011).

An important question that remains is what determines extreme submergence tolerance at the molecular level. Detailed understanding of the response to submergence at the level of gene expression and metabolism is mostly based on studies using maize (Zea mays), rice, and the model species Arabidopsis exposed to low oxygen levels (Sachs et al., 1996; Bailey-Serres and Voesenek, 2008). Genome-wide transcriptome studies of Arabidopsis, rice, and poplar (Populus spp.) subjected to low oxygen levels have shown that a large number of genes are differentially expressed in response to this stress, including the repression of genes involved in energy-consuming processes such as cell wall biosynthesis and the induction of genes in the glycolysis and fermentation pathways (Klok et al., 2002; Branco-Price et al., 2005, 2008; Liu et al., 2005; Loreti et al., 2005; Lasanthi-Kudahettige et al., 2007; Kreuzwieser et al., 2009; Mustroph et al., 2009; van Dongen et al., 2009). Transcriptomic studies of Arabidopsis in the past have mostly used seedlings or cell cultures exposed to low oxygen levels. However, recent studies have investigated both the molecular response and natural variation between Arabidopsis accessions to complete submergence (Lee et al., 2011; Vashisht et al., 2011) or the molecular response of Arabidopsis to root flooding (Hsu et al., 2011).

The study of wild relatives of Arabidopsis provides exciting research opportunities because it allows the use of molecular tools developed for this model and greatly extends the range of natural variation that can be studied (Mitchell-Olds, 2001; Schranz et al., 2007). Among Arabidopsis wild relatives, the genus Rorippa (yellow cress) includes the polyplod, hybridizing species Rorippa amphibia and Rorippa sylvestris, which inhabit periodically flooded river banks throughout Europe (Bleecker and Hurka, 2001; Stift et al., 2008). We have recently shown different morphological responses to submergence in R. amphibia and R. sylvestris (Stift et al., 2008; Akman et al., 2012). As will be shown here, R. amphibia is less tolerant to complete submergence than R. sylvestris, which survived over 3 months of submergence under natural light conditions. Therefore, these species provide an excellent system to address whether such variation in tolerance is associated with distinct transcriptome changes in response to submergence. In general, such effects of environmental variation on transcript profiles of contrasting genotypes or genotype-environment interactions are of particular interest in ecological genomics research (Filatov et al., 2006; Nettleton, 2006; Kammenga et al., 2007). The close relatedness of Rorippa species to Arabidopsis allowed us to use DNA microarrays developed for this model species using a genomic DNA (gDNA)-based probe selection method, shown previously to work with other members of the Brassicaceae family (Hammond et al., 2006; Broadley et al., 2008).

While underwater photosynthesis may produce some oxygen in shoots of submerged plants when light is available, oxygen is more rapidly depleted in non-photosynthetic tissues (Voesenek and Sasidharan, 2013). Furthermore, previous molecular comparisons of submergence tolerance have been performed only on shoot tissues, related to the elongation or quiescence strategies of shoots (Xu et al., 2006; Hattori et al., 2009; Jung et al., 2010; Mustroph et al., 2010). However, very few studies have investigated root responses in relation to flooding tolerance; mostly, they investigate the effect of only hypoxia, a component of flooding stress (Klok et al., 2002; Mustroph et al., 2009; van Dongen et al., 2009). In the experimental setup used in this study, the flooding was too deep to be outgrown and the roots would experience severe hypoxia, making them important determinants of submergence tolerance. Therefore, we focused on roots to characterize the effect of submergence on whole-genome transcript profiles. Furthermore, we took advantage of recently published microarray data from roots of completely submerged Arabidopsis plants to make a direct comparison of submergence-induced transcript profiles of the extremely submergence-tolerant Rorippa species and the much less tolerant Arabidopsis.

RESULTS

R. sylvestris Has Higher Submergence Tolerance Than R. amphibia

In order to confirm the submergence tolerance of Rorippa species, 52-d-old plants were subjected to complete submergence in a normal day/night rhythm. After 95 d of complete submergence in a basin, all R. sylvestris plants had survived the stress (Fig. 1). In contrast, only 30% of R. amphibia had survived at this point, with its survival gradually decreasing across the entire time course studied here. At the
final time point (109 d after submergence), *R. sylvestris* had also suffered mortality, but the large difference between the two genotypes was maintained. Under normal growth conditions, *R. amphibia* produces a stem that can reach more than 1 m in length, whereas *R. sylvestris* does not form such a stem and reaches 0.4 m maximally (Jonsell, 1968). In accordance with previous reports, *R. amphibia* elongated clearly more than *R. sylvestris* when submerged (data not shown; Akman et al., 2012). The plants could not reach the water surface at any time because a cloth was draped over the top of the water basins. Toward the end of the experiment, most of the shoot and especially the leaves had died in both genotypes. Survival was scored as the ability to form new leaves. Asterisks denote significant differences at that time point between the two species (*P* < 0.05, Fischer’s exact test).

Submergence Reduced Root Sugar Content within Days

During submergence, plants rapidly have to cope with an energy crisis, because photosynthesis under water occurs at a much lower rate than above water, and glycolysis needs to be enhanced because of the low ATP yield of fermentation in comparison with oxidative respiration (Bailey-Serres and Voesenek, 2008). The adaptive responses to submergence stress that determine survival in the long run, therefore, must include efficient carbohydrate usage and energy conservation. To test the carbohydrate usage of *Rorippa* species, roots of young (28 d) plants submerged for up to 7 d were analyzed for sugar and starch contents (Fig. 2). Submergence did not have a significant effect on starch levels in roots of either species after 1 d of treatment (*P* > 0.05, Student’s *t* test). However, while root starch content increased in controls thereafter, it remained low in submerged roots. After 7 d of submergence, starch levels were not significantly different between the two species. The three main sugars detected were Glc, Suc, and Fru (Fig. 2). In both *Rorippa* species, 1 d of submergence already significantly

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**Figure 1.** Survival upon submergence in mature *Rorippa* species plants. Seven-week-old *R. amphibia* and *R. sylvestris* plants were completely submerged in shaded, 1-m-deep outdoor basins. Ten individuals per genotype were taken out of the treatment at the indicated time points, and survival was scored after a 2-week recovery period as the ability to form new leaves. Asterisks denote significant differences at that time point between the two species (*P* < 0.05, Fischer’s exact test).

**Figure 2.** The effect of submergence on starch and sugar concentrations in the roots of *R. amphibia* and *R. sylvestris* plants. Values are means ± se of four to five biological replicates. DW, Dry weight.
reduced the concentration of these sugars, compared with controls in air. After 7 d, submerged R. sylvestris roots had significantly higher levels of total soluble sugars and Glc compared with R. amphibia (P < 0.05, Student’s t test; Fig. 2).

**gDNA-Based Probe Selection Using Affymetrix Arabidopsis ATH1 GeneChip Arrays**

In order to determine the molecular basis of submergence tolerance of *Rorippa* species, the Arabidopsis ATH1 GeneChip arrays were used for microarray experiments. To reduce possible bias in the transcriptome data due to sequence differences between *Rorippa* species and Arabidopsis, a probe selection method was employed based on the hybridization of gDNA of *R. amphibia* or *R. sylvestris* to the arrays. Because replicate arrays would be interrogating the same gDNA, amounting to technical but not biological replication, we analyzed the gDNA of each species once. Arabidopsis ecotype Columbia-0 gDNA was also analyzed in the same way for comparison, since it should theoretically hybridize to all perfect-match probes on the array. The control mismatch probes on this array were discarded from the analysis altogether, as they are uninformative in cross-species hybridizations. Sequence differences between *Rorippa* species and Arabidopsis are expected to result in reduced hybridization and, hence, a low signal of the corresponding perfect-match probes. Because every gene is interrogated by a probe set of 11 perfect-match probes on the array, discarding up to 10 poorly performing probes based on gDNA hybridization still allows measurement of the expression of the associated gene (Hammond et al., 2005, 2006). By applying increasing thresholds of gDNA hybridization signal, decreasing numbers of individual probes were retained in the resulting probe mask file, while the number of probe sets retained was reduced at a lower rate (Supplemental Fig. S1). This is consistent with previous studies using this probe selection method (Hammond et al., 2005, 2006; Broadley et al., 2008). More probes were retained at any given threshold for the Arabidopsis gDNA data set than for the *Rorippa* species sets, as expected. The two *Rorippa* species showed a similar decline in numbers of probes and probe sets with increasing thresholds. An optimal threshold was empirically determined at which the number of completely removed probe sets was minimized in order to avoid the loss of all information regarding the corresponding gene transcript. At a threshold level of the gDNA hybridization signal of 80, almost all probe sets (97%) were retained, and the number of probes was reduced by approximately two-thirds (Supplemental Fig. S1). Most probe sets contained three probes when this probe mask was used (Supplemental Fig. S2). The gDNA hybridization signal did not appear to be correlated with the number of probes per probe set, indicating that no bias had occurred in the probe selection (Supplemental Fig. S3).

**Submergence-Induced Large-Scale Changes in Gene Expression**

Affymetrix GeneChip arrays were used to analyze root transcript profiles of the two *Rorippa* species genotypes exposed to 24 h of complete submergence. Approximately 400 genes were induced and approximately 470 genes were repressed by the submergence treatment in both *R. amphibia* and *R. sylvestris* (Fig. 3A). When no probe mask was applied, less than one-half the number of significantly regulated genes were detected (data not shown), confirming previous findings that the probe filtering enhanced the sensitivity to detect changes in transcript levels (Hammond et al., 2005, 2006). About 60% of induced genes were shared between both *Rorippa* species, while only about 50% of reduced genes were found to be changed in both species (Fig. 3A). The commonly induced genes included well-known hypoxic genes involved in glycolysis and fermentation (alcohol dehydrogenase [ADH1], pyruvate decarboxylase [PDC1], phosphofructokinase [PFK6], and sucrose synthase [SUS4]) and transcription factors LATERAL ORGAN BOUNDARIES DOMAIN-CONTAINING protein (LBD41). A principal component analysis (PCA) of transcript levels of all interrogated genes clearly separated treated from control samples along the first and second components, explaining 94% and 3% of total variation, respectively (Fig. 3B). As expected, for each of the genotypes, the three independent replicates clustered together (Fig. 3B).

**Comparison of Submergence-Induced Root Transcript Profiles between *R. amphibia* and *R. sylvestris**

In order to compare the two *Rorippa* species with varying tolerance to submergence, we first compared both lists of submergence-regulated genes (Fig. 3A). About 40% of the up-regulated genes were specific for either species. In order to get more insight into the gene expression differences, we also performed a pairwise comparison between species. Second, a complex pairwise comparison was performed to find differentially regulated genes (Supplemental Table S1). Both methods revealed some differences in submergence response between the two species. About 16% of induced genes were more strongly induced in *R. amphibia* as compared with *R. sylvestris* and vice versa. Genes with a higher induction in *R. amphibia* included genes involved in photosynthesis, while *R. sylvestris* had more genes induced that were involved in defense response. Among the most differentially induced genes in *R. amphibia*, there was POLYGALACTURONASE-INHIBITING PROTEIN2 (At5g06870), a WRKY transcription factor (At5g13080, AtWRKY75), and an F-box family protein (At4g29360). In *R. sylvestris*, the more induced genes included a nodulin MtN21 family protein (At4g28040), an unknown protein (At5g19030), and CATALASE1 (At1g20630). The PCA further emphasized that the two species were not dramatically different from each other under control and submerged conditions (Fig. 3B).
Submergence Led to the Repression of Energy-Consuming Processes and the Induction of Photosynthesis, Glycolysis, and Fermentation Genes

In order to get an overview of the transcriptome changes induced by the submergence treatment, overrepresentation analysis of gene categories based on the Gene Ontology (GO) database was performed using the GOHyperGAll function (Fig. 4; Horan et al., 2008). In both genotypes, this analysis showed an overrepresentation in down-regulated genes of various energy-demanding pathways, namely cell wall precursor synthesis, fatty acid biosynthesis, and amino acid synthesis (Supplemental Table S2). Glycolysis and fermentation pathways were more overrepresented in the data set of submergence up-regulated genes of *R. amphibia* than in *R. sylvestris* (i.e. GO:0006006, Glc metabolic process; adjusted P value = 4.51E-05 and 9.22E-03, respectively; Supplemental Table S2). Surprisingly, genes involved in the light reactions of photosynthesis were clearly overrepresented in the set of up-regulated genes of *R. amphibia* than in *R. sylvestris* (Fig. 4). Hence, the qualitative response to submergence appeared roughly similar, but genotypes did show quantitative differences.

Submergence Caused Stronger Induction of SUS4 and Glycolysis and Fermentation Genes in *R. amphibia* Than in *R. sylvestris*

Closer inspection of genes involved in carbohydrate catabolism revealed that several were expressed at a significantly higher level under submerged conditions in *R. amphibia* than in *R. sylvestris* (Table I). Cloning and expression analyses of selected genes using quantitative reverse transcription (qRT)-PCR confirmed that transcript levels of the homolog of the Arabidopsis gene SUS4 as well as of the genes involved in the final reactions of the fermentation pathway, PDC2 and ADH1, were higher in submerged *R. amphibia* roots (Fig. 5, C, D, and F). Accordingly, submerged (24 h) roots of *R. amphibia* also had higher ADH activity than submerged roots of *R. sylvestris* relative to their respective air controls (Fig. 5B). Surprisingly, submerged *R. amphibia* roots recorded lower ethanol content than corresponding air controls after 7 d (Supplemental Fig. S4). At other days, the values did not differ significantly. It is likely, however, that most ethanol was released into the root medium during the submergence period, as shown previously (Mustroph and Albrecht, 2007). In accordance with the expression and activity of ADH, *R. amphibia* roots contained more ethanol than *R. sylvestris* roots (Supplemental Fig. S4).

An exception to the differential induction of fermentation genes between the two species was LACTATE DEHYDROGENASE1 (*LDH1*), which was similarly up-regulated upon submergence in both species (Fig. 5E). Lactate levels in submerged roots of both species, however, were not significantly higher than the respective controls, but in general, more lactate was present in roots of *R. amphibia* in comparison with *R. sylvestris* (Supplemental Fig. S4). Other carbohydrate catabolism genes showing a stronger induction in *R. amphibia* than *R. sylvestris* included SUS1 and various glycolysis genes such as PGIC, pyruvate kinase, and aldolase (Table I).

We were further interested in changes of metabolite contents during the submergence treatment and...
analyzed selected metabolites of primary metabolism. Interestingly, after 24 h of submergence, \textit{R. sylvestris} accumulated significantly more sugar phosphates (Glc-6-P and Fru-6-P) than \textit{R. amphibia} (Fig. 6). Considering that there were no significant differences in starch content of submerged roots after 24 h relative to controls (Fig. 2), an accumulation of these glycolytic intermediates likely indicates a restriction of glycolysis in \textit{R. sylvestris} (Fig. 6, A and B). The levels of phosphoenolpyruvate and pyruvate were also slightly, but not significantly, higher in the more tolerant \textit{R. sylvestris} (Fig. 6, C and D). These data suggested a slower glycolytic process in \textit{R. sylvestris}, but despite this assumption, ATP levels did not decrease in \textit{R. sylvestris} but instead dropped significantly by 30% in \textit{R. amphibia} during 24 h of submergence (Fig. 6E). The ratio of ATP to ADP, however, remained unchanged in both species and treatments (Supplemental Fig. S5).

**Comparison of Tolerant \textit{Rorippa} Species with the Related But Submergence-Intolerant \textit{Arabidopsis}**

The use of Affymetrix GeneChips to monitor \textit{Rorippa} species root transcriptomes allowed us to compare our data set with that of Lee et al. (2011), who studied roots and shoots of \textit{Arabidopsis} plants also using a 24-h submergence treatment, although that experiment was performed in complete darkness. The gene expression data of \textit{Rorippa} species were compared with root transcript profiles of submerged \textit{Arabidopsis} plants, which are relatively flooding intolerant (Lee et al., 2011; Vashisht et al., 2011). This comparison was based on the Affymetrix identifiers and included a cluster analysis of the mean expression values of each sample. This analysis revealed a large variation in gene expression between the submergence-induced transcriptome profiles of \textit{Rorippa} species versus \textit{Arabidopsis} (Fig. 7A). Only some genes were induced in all three species at all times (cluster 10 of Supplemental Table S3), including 21 of the previously identified “core hypoxic response” genes (Mustroph et al., 2009). Among these genes were known hypoxic genes involved in primary metabolism (\textit{SUS1}, \textit{SUS4}, \textit{ADH1}, and \textit{PDC1}) and other hypoxic genes (wound-responsive protein, hemoglobin, and stearoyl-acyl-carrier-protein desaturase family protein). Many genes responded in different ways in \textit{Arabidopsis} and \textit{Rorippa} species. For example, genes of clusters 2 and 17 were more strongly induced in \textit{Rorippa} species than in \textit{Arabidopsis}. Interestingly, genes of cluster 2 were regulated preferentially by darkness in \textit{Arabidopsis}. Therefore, the induction of these genes in \textit{Rorippa} species could be attributed to the...
change in light level rather than to a submergence-associated change in oxygen concentration. Although the submergence treatment of Rorippa species plants was performed in the light at approximately 200 μmol m⁻² s⁻¹, light quantity and quality can vary in a water column, possibly leading to the observed changes in the expression of light-regulated genes, even in roots. Therefore, the relatively large number of transcriptional regulators found in cluster 2 (e.g. a basic helix loop helix transcription factor [At2g43140], WOX14 [At1g20700], and a calcium-binding elongation factor hand family protein [At5g51430]) are most likely not involved in mediating hypoxia tolerance in Rorippa species.

Genes of cluster 17 were preferentially submergence induced in Rorippa species. While these genes were not submergence induced in Arabidopsis under darkness, some of them were induced by hypoxic treatment of Arabidopsis in light [SRO5 (At5g62520), PDC family protein (At5g01320), and 2OG-Fe(II) oxygenase family (At1g20630, CATALASE1)]. This cluster also contained genes that were related to amino acids (At2g40420, amino acid transporter family protein; At2g38400, Ala:glyoxylate transaminase). In contrast, clusters 23 and 7 contained genes that were more induced in submerged Arabidopsis roots: surprisingly, the previously identified hypoxia-responsive ERF HRE1 (Licauzi et al., 2010) and a phosphate-related protein (PHII, At1g35140). A fraction of these genes, mostly from cluster 23, were induced after 7 h, rather than after 24 h, of submergence in Arabidopsis, and this could explain the lack of induction in Rorippa species after 24 h. However, we cannot exclude that orthologs of these genes in Rorippa species can be regulated by submergence but are not represented on the ATH1 microarray.

In order to confirm this contrasting regulation between Rorippa species and Arabidopsis, the expression of a selection of these genes was measured in roots of Arabidopsis plants using qRT-PCR. Orthologs of these genes were also cloned and their expression measured in both Rorippa species. The submergence treatment for all three species (24 h) was identical to conditions used for the microarray studies on Rorippa species, including the use of similar light levels, thereby allowing a direct comparison, since the Arabidopsis microarray data were obtained from plants submerged in the dark (Lee et al., 2011). Twenty-four-hour submerged roots of both Arabidopsis and Rorippa species experienced hypoxic stress, as evidenced by a significant up-regulation of ADH1 (Fig. 7, C–E). Most of the 10 genes tested in Arabidopsis showed either no significant change (At2g43610, chitinase family protein; At4g04040,
PFPB2) or significant down-regulation (At1g76550, PFPA2; At4g32840, PFK6; At3g44880, PAO, phophorbid e a oxygenase family protein; At1g03475, HEMF1, coproporphyrinogen III oxidase) of transcripts due to submergence (Fig. 7B), while orthologs of eight of these 10 genes showed significant up-regulation in both Rorippa species in accordance with the microarray results. Our expression analyses also confirmed the overall trends revealed by the cluster analyses. For example, roots of 24-h submerged Rorippa species plants had significantly higher transcript levels of PFPA2 (cluster 17) compared with air controls. In contrast, roots of Arabidopsis plants submerged under identical conditions had significantly lower PFPA2 transcript abundance relative to their nonsubmerged controls. The cluster 22 genes PAO (R. amphibia versus R. sylvestris [log fold change 1.7 versus 1.5]) and PFPB2 (R. amphibia versus R. sylvestris [log fold change 1.36 versus 0.8]) were submergence inducible only in Rorippa species, with a higher fold induction in R. amphibia.

In Arabidopsis, transcripts for CXXS1 (At1g11530), Ala:glyoxylate aminotransferase3 (At2g38400), and PPDK (At4g15530) were significantly up-regulated by submergence stress, in contrast with the results of Lee et al. (2011). Notably, that study (Lee et al., 2011) was done under dark submerged conditions and compared with dark controls, while the light control at the start of the treatment showed much lower expression values for these three genes (Supplemental Table S3), pointing to the fact that these genes are induced by a decrease in light intensity, independent of a decrease in oxygen concentrations. It can be speculated that transcript profiles during submergence, and thereby tolerance, can be considerably affected by light conditions during submergence.

**DISCUSSION**

The results presented here demonstrate the successful use of Arabidopsis gene chips in profiling the flooding-induced transcriptome of closely related Rorippa species. The difference between R. amphibia and R. sylvestris in the degree of submergence tolerance (Fig. 1) could be explained by some quantitative differences in gene
expression in the roots after short-term submergence. Furthermore, a direct comparison of these extremely submergence-tolerant *Rorippa* species with the relatively intolerant Arabidopsis allowed the identification of potential tolerance-related genes.

### Submergence-Regulated Genes in *Rorippa* Species Roots

**Included Many Known Hypoxia-Regulated Genes**

An overview of gene regulation showed some similarity between our results obtained for *Rorippa* species and those found for Arabidopsis and rice (Klok et al., 2002; Branco-Price et al., 2005, 2008; Liu et al., 2005; Loreti et al., 2005; Lasanthi-Kudahettige et al., 2007; Mustroph et al., 2009; van Dongen et al., 2009). Notably, genes involved in energy-demanding processes such as cell wall precursor or fatty acid synthesis were repressed upon submergence, whereas glycolysis and fermentation genes were induced (Supplemental Tables S1 and S2). Furthermore, transcript abundance and activity of the hypoxia marker gene *ADH1* were also clearly induced by the 24-h submergence treatment (Table I; Fig. 5). These data suggest that (1) submergence treatment resulted in hypoxia in the roots of the *Rorippa* species genotypes and (2) species widely differing in submergence tolerance up-regulate a common set of core hypoxia-responsive genes, as demonstrated previously (Christianson et al., 2010; Mustroph et al., 2010; Narsai et al., 2011). The observation that many of these genes were induced similarly in all three species despite differing tolerance (cluster 10 of Supplemental Table S3) demonstrates that the core hypoxic response, although required for plant survival, does not determine the specific tolerance of *Rorippa* species.

### Regulation of Carbohydrate Respiration Might Underlie the Tolerance Differences between *R. amphibia* and *R. sylvestris*

Carbohydrate reserves are important for survival when plants experience an energy crisis that ensues from complete submergence, particularly when escape from (deep) floodwater is impossible and the rates of carbohydrate production under water are low, as was the case here.

Transcript levels of *SUS1* and *SUS4* were significantly increased after 24 h of submergence in both species (Table I; Fig. 5), and the level of gene induction was greater in *R. amphibia* than in *R. sylvestris*. It is known that *SUS* genes are induced by low oxygen levels in many plant species (Bailey-Serres and Voesenek, 2008), and out of the six *SUS* genes in Arabidopsis, it was also *SUS1* and *SUS4* that were found to be induced by low oxygen stress (Baud et al., 2004). Moreover, mutant studies have demonstrated that *SUS* genes are necessary for tolerance to soil flooding in Arabidopsis (Bieniawska et al., 2007) and tolerance...
to hypoxia in potato (*Solanum tuberosum*; Biemelt et al., 1999). The Suc degradation pathway mediated by SUS and UDP-Glc pyrophosphorylase is less energy consuming than the invertase-mediated Suc degradation pathway (Drew, 1997; Bailey-Serres and Voesenek, 2008; Huang et al., 2008). Notably, a putative invertase inhibitor (At5g62350) was more induced in *R. sylvestris* than in *R. amphibia* (Supplemental Table S1). Greater induction of SUS gene shoots into tolerant varieties was associated with faster elongation and carbohydrate depletion, probably causing its more rapid mortality (Fukao et al., 2006). Similarly, it is possible that in *R. amphibia*, which also shows strong underwater shoot elongation (Akman et al., 2012), the greater mortality is associated with more carbohydrate use through the SUS and INVERTASE pathways. After 1 week of submergence, significantly higher amounts of total soluble sugar remained in *R. sylvestris* roots (Fig. 2). Greater depletion of root carbohydrates in *R. amphibia* could also indicate a remobilization of these reserves to fuel the shoot elongation in this escaping species. Stem growth during anaerobic conditions in pondweed (*Potamogeton distinctus*) was associated with enhanced starch consumption in underground turions (Harada and Ishizawa, 2003). Thus, carbohydrate translocation to stems could provide the required substrates for energy production to mediate shoot extension growth. In pondweed, elongation under anoxic conditions was accompanied by a stimulation of glycolysis (Sato et al., 2002). Genes involved in starch breakdown were also

![Figure 7. Comparison of the submergence response of tolerant *Rorippa* species and sensitive Arabidopsis.](image)
compared with the more tolerant SUB1 rice (Fukao et al., 2006), but we did not observe any significant treatment effects on amylase genes in our study (Supplemental Table S1). Accordingly, submergence did not affect root starch content after 24 h in either species, and both recorded similar starch contents after 7 d of submergence (Fig. 2). Under low-oxygen conditions, the breakdown products of starch and/or Suc provide substrates for glycolysis and fermentation.

Our analysis further showed that, besides carbohydrate cleavage enzymes, fermentative enzymes (ADH1 and PDC2) were also more strongly induced in R. amphibia than in R. sylvestris (Fig. 5; Table I). Accordingly, higher ADH activity was recorded in submerged R. amphibia roots than in R. sylvestris (Fig. 5B). Although levels of the fermentation end products ethanol and lactate were not higher in submerged R. amphibia (Supplemental Fig. S4), the loss of these metabolites into the root medium is very likely (Mustroph and Albrecht, 2007). Although mutant and overexpression studies have shown that PDC and ADH genes are important for low-oxygen stress tolerance (Ismond et al., 2003), the level of induction of these genes is not directly correlated with tolerance. Rather, it has been suggested that tolerance to low oxygen levels requires a tight balance between energy use for growth and other processes, on the one hand, and the generation of ATP through glycolysis and fermentation, on the other.

The measurements of glycolytic intermediates showed accumulations of Glic-6-P and Fru-6-P in roots of R. sylvestris after 24 h of submergence treatment, suggesting a possible inhibition of subsequent consumption processes. Although the accumulation of these metabolites could also be attributed to increased starch breakdown, this can be ruled out, considering that there were no significant changes in starch content after 24 h (Fig. 2). The intermediates phosphoenolpyruvate and pyruvate showed a similar, but not significant, trend. Remarkably, the ATP content was less reduced in R. sylvestris than in R. amphibia under submergence (Fig. 6E), despite the slower metabolism of the first species. This might indicate the higher energy demand of elongation growth in R. amphibia in comparison with R. sylvestris and, therefore, hints at the benefit for the quiescence strategy if elongation growth does not reach the oxygen-rich air zone.

Tolerant SUB1 rice, using the quiescence strategy, showed higher mRNA levels and enzyme activities of PDC and ADH during submergence than intolerant rice without SUB1, but remarkably, the level of ethanol production was lower in SUB1 rice (Fukao et al., 2006), possibly mediated by the lower induction of Suc synthases and amylases and other unknown factors. These data illustrate that mRNA levels and in vitro enzyme activities are not necessarily correlated with the rate of an enzymatic process in planta. The contrasting observations in fermentative enzyme induction of quiescent SUB1 rice and quiescent R. sylvestris could be due either to species differences and/or to different organs analyzed in the two studies. Indeed, shoots were analyzed from rice, while our study on Rorippa species was focused on roots. Both organs could have very different responses to submergence, as already suggested before for Arabidopsis (Ellis et al., 1999; Mustroph et al., 2009). Therefore, while the expression differences between the two Rorippa species, especially of SUS4, could perhaps be explained in terms of contrasting escape and quiescence strategies, it is premature to link this to differences in submergence tolerance. In addition, it is also not known how the different induction of glycolysis and fermentation genes in the two Rorippa species is regulated at the molecular level.

Submergence-Induced Induction of Photosynthesis Genes in Rorippa Species Roots

One remarkable observation was the strong induction of genes associated with the light-harvesting complexes, particularly in R. amphibia (Fig. 4), which could imply the presence and/or formation of chloroplasts in belowground tissues. Both Rorippa species are root sprouters, and in older plants, the rhizomes contain numerous buds competent to form green shoots (see “Materials and Methods”). Perhaps these tissues already possess chloroplasts, although in our experimental setup, no root sprouting was observed. It is known that several aerenchymatous species, including rice, form functional chloroplasts in the roots after prolonged illumination (Armstrong and Armstrong, 1994; Jackson and Armstrong, 1999; Rich et al., 2011). Furthermore, Arabidopsis roots contain chloroplasts and express photosynthetic genes in the root cortex (Dinneny et al., 2008). Roots may have been illuminated in our experimental setup as well, particularly near the surface of the pots, but this can be neglected because this exposure to light would be similar in both air and submerged conditions. In Arabidopsis, photomorphogenesis in roots is normally repressed, but in certain photomorphogenesis mutants, roots appear green because chloroplasts have developed (Chory et al., 1989; Oyama et al., 1997). What caused the strong induction of these photosynthesis genes after 24 h of submergence is not immediately clear. One possibility is sugar starvation, a well-known signal stimulating photosynthesis genes (Sheen, 1990; Baena-González et al., 2007). If so, this would suggest that sugar starvation was more severe in R. amphibia than in R. sylvestris. However, marker genes for energy or sugar starvation were not more strongly induced in R. amphibia than in R. sylvestris.

Comparison with Submergence-Intolerant Arabidopsis Reveals a Unique Complement of Submergence-Induced Genes in Roots

The close relatedness of Rorippa species to Arabidopsis allowed a direct comparison of root transcriptome profiles for these highly submergence-tolerant species with Arabidopsis, which is relatively sensitive to complete
submergence. Arabidopsis accessions show a considerable variation in their tolerance to submergence (Vashisht et al., 2011), but even the most tolerant accession (C24) was found to have an median lethal time of only 20 d (submerged in the light). This is in contrast with median lethal time values of 75 d for R. amphibia and more than 110 d for R. sylvestris under similar conditions of submergence. Gene clusters that are differentially regulated between these two species, therefore, are potential tolerance-related genes. Here, cluster 22 and cluster 1 contain potential candidates for species differences within the Rorippa genus, while cluster 17 has genes differentially expressed between Rorippa species and Arabidopsis (Supplemental Table S3). Interestingly, these clusters contain a number of genes that are associated with the inorganic pyrophosphate (PPi)-dependent alternative pathways of phosphorylation reactions in primary metabolism (PPFA2 and PFPB2). These were discussed previously as having a potential benefit for energy metabolism in plants under low-oxygen stress (Mustroph et al., 2005; Bailey-Serres and Voesenek, 2008; Huang et al., 2008). Furthermore, rice genotypes differing in submergence tolerance [M202 versus M202(SUB1)] also express PFP genes at different levels under submerged conditions (Mustroph et al., 2010). Other differentially expressed genes are associated with photosynthesis or amino acid metabolism, but their role in low-oxygen survival remains unclear so far. Interestingly, transcription factors of the ERF group VII family that have been associated with submergence tolerance in rice (Xu et al., 2006; Hattori et al., 2009) and that are involved in low-oxygen signaling (Gibbs et al., 2011; Licausi et al., 2011) had very low expression in this data set under all conditions and were also not induced by submergence (Supplemental Table S1). However, one cannot rule out orthologs that could not be detected with the Arabidopsis microarray GeneChip and differences in posttranslational regulation. Indeed, RAP2.12 was removed from the data set during the probe selection process.

The observed differences between Rorippa species and Arabidopsis could be caused by a number of factors. First, they could be caused by species-specific adaptational response differences, the most interesting factor, which are most likely different from the core hypoxic response concept mentioned before. This could involve, for example, an energy-saving quiescence strategy, including the use of PPi-using enzymes and adaptations of the photosynthetic apparatus. Second, technical problems due to variation in Rorippa species gene sequences in comparison with Arabidopsis cannot be excluded completely and could be relevant for some genes that are hardly expressed in Rorippa species as measured by Arabidopsis ATH1 chips. Third, the expression differences of some genes in the two genera could be also attributed to differences in oxygen concentrations in the roots during the submergence treatment, which is dependent on temperature and light, on the duration of the treatment, or on variation in root and shoot anatomy (e.g. aerenchyma). Therefore, it is essential to be aware that transcriptome profiles can be influenced by factors such as the conditions of submergence (dark versus light) and the developmental stage at which plants are submerged. Since the Arabidopsis microarray data set used for comparisons here were from plants submerged in the dark, we repeated submergence experiments on Arabidopsis plants with conditions similar to those used for the Rorippa species microarrays. Gene expression measurements of selected genes from the differentially expressed cluster (Fig. 7) showed that even in the roots, light conditions during submergence affected transcript profiles to some extent. For example, the gene for PPDK, identified as being induced only in submerged Rorippa species roots, was highly induced in Arabidopsis roots as well when illuminated control conditions were used as a reference. However, these experiments confirmed that some genes (PFPA2, PFPB2, PAO, and HEMF1) that are strongly induced under submergence in Rorippa species are not induced in Arabidopsis, making them interesting candidates for future studies.

CONCLUSION

In this study, we have successfully exploited the close relatedness of wild Rorippa species with Arabidopsis in order to utilize ATH1 chips for profiling the submergence-induced transcriptome of these two species. Our results suggest that differences in submergence tolerance between R. amphibia and R. sylvestris, adapting an escape and a quiescent survival strategy, respectively, could be related to differences in the fermentation capacity and carbohydrate consumption in the roots. We also compared the molecular responses to submergence of Rorippa species with that of Arabidopsis. The commonalities in short-term gene expression responses between Rorippa species, and their common differences from the flooding-intolerant Arabidopsis, suggest important tolerance genes and pathways, such as the use of alternative PPi-dependent pathways. Future studies of gene expression under prolonged submergence of roots and shoots and the development and analyses of transgenic plants expressing candidate genes from Rorippa species will further unravel the molecular basis of the contrasting tolerance responses between these two genera.

MATERIALS AND METHODS

Plant Material

Rorippa amphibia and Rorippa sylvestris are clonal root sprouters that can be propagated using rhizome fragments. The origin of the tetraploid genotypes used in this study has been described previously (Stift et al., 2008). An R. amphibia rhizome was collected along the Yssel River near Doesburg, The Netherlands (N: 52°11'25", E: 06°08'42"), and an R. sylvestris rhizome was collected along the Rhine River near Millingerwaard, The Netherlands (N: 51°52'02", E: 06°08'42"), in 2003 and maintained in a greenhouse. Rhizome fragments of each genotype were surface sterilized by soaking in 10% (v/v) commercial bleach for 5 min, rinsed twice in deionized water, and transferred to petri dishes containing...
Plants were fully submerged in outdoor concrete basins of length 1. Pots remained in a layer of 1 g L⁻¹ nutrient solution (Peat Lite Special, Peter's Professional; Scotts Europe) for 4 d, after which they were placed in a layer of rainwater. After 16 d, when approximately six leaves were visible, a uniform selection of 18 plants of each genotype was made, and stored at −70°C until further use. A total of 12 arrays were used to interrogate the transcriptome of roots from R. amphibia and R. sylvestris plants submerged for 24 h or kept in air as a control. RNA extracted from these samples was used for qRT-PCR and microarray hybridizations. Arabidopsis plants grown according to the method of Vashisht et al. (2011) were used for submergence experiments in conditions similar to those described above. After 24 h of submergence, plants were taken out of the water and roots were quickly cleaned, separated from the shoot, and flash frozen in liquid N₂.

**RNA and gDNA Isolation and Microarray Hybridization**

gDNA was isolated from 100 mg of fresh leaf material using the DNeasy Plant Mini Kit (Qiagen). gDNA (800 ng) was labeled and amplified by using the Bioprime DNA labeling kit (Invitrogen) and analyzed with a Nanodrop spectrophotometer and Agilent Bioanalyzer, after which 6.9 pg of labeled gDNA probe of each species was hybridized to an ATH1-121501 GeneChip (Affymetrix). Complete information about this platform can be found at the Gene Expression Omnibus Web site (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL198). Total RNA was extracted from 100 mg of tissue using the RNeasy Plant Mini Kit (Qiagen). RNA quality was assessed by electrophoresis on agarose gels, Nanodrop spectrometry, and Agilent Bioanalyzer. Complementary RNA probe generation, microarray hybridization, washing, and scanning were performed as described by the manufacturer’s protocols (Affymetrix). The data discussed in this publication have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus (Edgar et al., 2002) and are accessible through Gene Expression Omnibus Series accession number GSE13641 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13641).

**gDNA-Based Probe Filtering and Microarray Data Analysis**

The CEL files from hybridizations with gDNA were used to create custom CDF files for the different species, based on the original Arabidopsis ATH1 CDF file. According to the procedure described by Hammond et al. (2005, 2006), probe masks were created with the easy_script.pl file obtained from http://affymeetrix.arabidopsis.info/species/, testing different thresholds. In this manner, individual probes were removed that had lower gDNA hybridization intensity than the threshold. Out of 11 probes in a probe set interrogating each gene, up to 10 probes could be removed, or the entire set was removed when all 11 probes fell below the threshold. Using these data, plots were made showing the number of probes and probe sets retained at different gDNA hybridization thresholds (Supplemental Fig. S1). Based on these plots,
a threshold was empirically determined at which the number of removed entire probe sets was minimized (because in those cases, the corresponding genes can no longer be interrogated) but the number of removed individual probes was maximized. Based on this evaluation, we decided to use a threshold of 0.8.

For the final CDF files for Rorippa species, the original Arabidopsis CDF file was first masked with the hybridized R. amphibia gDNA CEL file and subsequently masked with the R. sylvestris gDNA CEL file. The custom CDF file was then used to produce a R-visible library with the “makedcfln” package in R. Subsequently, CEL files from the mRNA hybridization Affymetrix chips were processed by use of the R program and Bioconductor packages (Gentleman et al., 2004) and the custom-made CDF library. Robust multichip average (RMA) normalization was performed using the default settings of the corresponding R function (Irizarry et al., 2003). Analysis of differentially expressed genes between different conditions was presented with the Limma package using RMA-normalized expression values (Smyth, 2004). The Benjamini and Hochberg method was selected to adjust p values for multiple testing and to determine false discovery rates (FDRs; Benjamini and Hochberg, 1995). As a confidence threshold, an FDR of less than 0.05 and a fold change cutoff of 2.0 was used.

Furthermore, the Rorippa species expression data were compared with expression data from submerged Arabidopsis plants (Lee et al., 2011). To avoid systematic errors, we treated the original Arabidopsis microarray data exactly as the Rorippa species data by first creating a custom CDF file with the help of a CEL file derived from Arabidopsis gDNA and then following the exact same procedure as for the Rorippa species data set. To more easily compare the response to submergence between species, k-means clustering was performed with all genes that showed differential expression due to submergence as determined by the Limma analysis described above. Unlogged RMA-normalized expression values (means of replicates) were first transformed by mean centering and subsequent division by the sum of the expression values separately for each genus. These data were then used for clustering in R with the function “pam.” After testing several numbers of clusters, we decided to use 26 clusters for the data set.

Overrepresentation analysis of GO categories was performed for specific gene lists obtained by the Limma and k-mean clustering analyses as described by Horan et al. (2008). In summary, the Arabidopsis gene-to-GO mappings from those databases were corrected against fluorescent in situ hybridization against Arabidopsis and Rorippa species gDNA hybridization-based probe selection of ATH1 GeneChips.

Cloning and qRT-PCR
Cloning was performed using standard protocols (Sambrook and Russell, 2001). Partial genomic sequences of orthologs of Arabidopsis genes were obtained from R. amphibia and R. sylvestris using primers designed based on the Arabidopsis sequences. Complementary DNA (cDNA) was used to PCR amplify the respective Rorippa species genes, and PCR products were directly sequenced. Since the Rorippa species genotypes used in this study are tetraploid, up to four alleles may be present. Based on the obtained sequences, primers were designed that detected all present alleles in both species and amplified approximately 150 bp for use in qRT-PCR with primer3 software (http://primer3.ut.ee). Primer sequences for the Arabidopsis and Rorippa species genes studied using real-time reverse transcription-PCR are given in Supplemental Table S5. RNA samples were treated with DNase (DNAfree; Ambion) to remove any gDNA, and cDNA was synthesized from 500 ng of RNA using 50 ng of random hexamers (Invitrogen) and 100 units of SuperScript III reverse transcriptase (Invitrogen) in a 20-μL reaction according to the manufacturer’s instructions. Quantitative PCR was performed using an ABI 7500 in a reaction mixture (20 μL) containing 10 μL of 2× SYBR Green (Platinum SYBR Green Supermix, Invitrogen), 0.3 μL of 0.1M of each primer, 0.04 μL of a one-tenth dilution of 50× ROX reference dye, and 10 ng of cDNA. Relative transcript levels were corrected against ACTIN2 transcript levels (for Rorippa species genes) and AtUBQ10 (for Arabidopsis) and calculated using the ΔΔCt method (Livak and Schmittgen, 2001). Measurement of Metabolite Content
Rorippa species plants were grown as described above, and roots were harvested after 24 h of submergence treatment. Metabolites were extracted with perchloric acid and subsequently neutralized with KOH as described previously (Murstroph et al., 2006). Adenylate concentrations in the extracts were determined with the luciferase system as described by Wulff and Döppen (1988) for ATP and by Hampp (1985) for ADP. Other metabolites were determined after Burrell et al. (1994) with modifications. Glc-6-P and Fru-6-P were measured in 100 mM HEPES, pH 7.5, with the addition of 1 mM MgCl2 and 0.4 mM NADP. The reaction was started by adding 10 units of Glucose-6-phosphate dehydrogenase for Glc-6-P and 7 units of phosphoglucone isomerase for Fru-6-P. Pyruvate and phosphoenolpyruvate were measured in 100 mM HEPES, pH 7.5, with the addition of 2 mM MgCl2, 0.2 mM ADP, and 0.1 mM NADH. The reaction was started with 10 units of LDH for pyruvate and 1 unit of pyruvate kinase for phosphoenolpyruvate. Measurements were done with eight replicates per species and treatment.

Sequence data from this article can be found in the GenBank database under the following accession numbers: for R. amphibia, HB1 (KF042825), ADH1 (KJ828280), GH (KJ042927), SUS4 (KJ042929), PAO (KJ042933), HEMF (KF042833), LDH1 (KJ042836), PDC2 (KF042838), PFPB2 (KF042842), PPFA1 (KF042844), PPFA2 (KF042846), PFK6 (KF042848), and PDPD (KF042850); for R. sylvestris, HB1 (KJ042826), ADH1 (KJ828280), GH (KJ042827), SUS4 (KJ042828), PAO (KJ042832), HEMF (KF042834), CXXS1 (KF042835), LDH1 (KJ042837), PDC2 (KF042839), PFPB2 (KF042843), PPFA1 (KF042845), PPFA2 (KF042847), PFK6 (KF042848), and PDPD (KF042851).

Supplemental Data
The following materials are available in the online version of this article.
Supplemental Figure S1. Rorippa species gDNA hybridization-based probe selection of ATH1 GeneChips.
Supplemental Figure S2. Histogram of the number of probes per probe set.
Supplemental Figure S3. Box plots showing the average R. amphibia and R. sylvestris gDNA hybridization intensity by the number of probes per probe set.
Supplemental Figure S4. Submergence-induced changes in fermentation end products in Rorippa species roots.
Supplemental Figure S5. Submergence-induced changes in the ATP-ADP ratio in Rorippa species.
Supplemental Figure S6. Enzyme activities in roots of Rorippa species or Arabidopsis plants that were treated with submergence for 24 h or kept in aerated soil.
Supplemental Table S1. Microarray data for Rorippa species genes hybridized to Arabidopsis ATH1 microarray chips.
Supplemental Table S2. GO term enrichment analysis of significantly enriched gene groups identified by pairwise comparisons from Supplemental Table S1.
Supplemental Table S3. K-means cluster analysis of microarray data from Rorippa species and Arabidopsis.
Supplemental Table S4. Confirmation of microarray expression data of selected Rorippa species genes by qRT-PCR.
Supplemental Table S5. Sequences of primer combinations (5′–3′) for the Arabidopsis and Rorippa species genes studied using real-time reverse transcription-PCR.

ACKNOWLEDGMENTS
We gratefully acknowledge Neil Graham and Sean May (Nottingham Arabidopsis Stock Centre) for assistance with the probe filtering method and comments on the manuscript. We thank Han Rauwerda (Microarray Department Amsterdam) for providing Supplemental Figures S2 and S3 and for

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useful discussions. We thank Floyd Wittink and other members of the Microarray Department Amsterdam for performing the microarray hybridizations. Rob Bregman, Peter Kuperus, Amit V. Bhikarie, and Ludek Tikkovsky (University of Amsterdam, The Netherlands) provided excellent technical assistance.

Received June 3, 2013; accepted September 26, 2013; published September 30, 2013.

LITERATURE CITED


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