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

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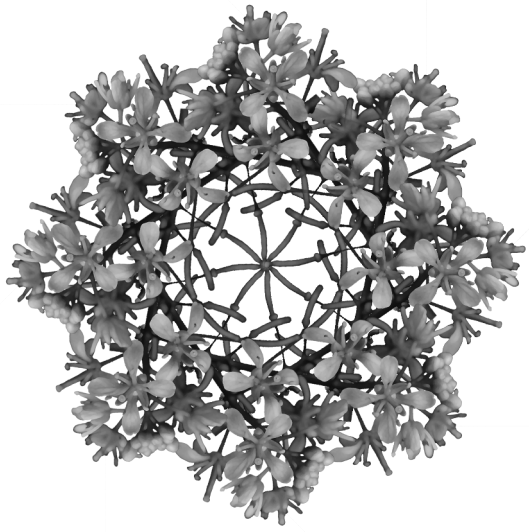

CHAPTER 5

Transcriptome profiling of two Arabidopsis accessions Col (*gl1*) and Kas-1 with different submergence tolerance

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SUMMARY

Background and Aims Arabidopsis accessions Col (*gl1*) and Kas-1 differ in their submergence tolerance, which could be explained by a quantitative trait locus, *Come Quick Drowning 1* (*CQD1*) on chromosome 5 increasing tolerance in Kas-1. Our aim with this study was to identify using RNA-seq (i) common submergence responses of these two accessions, (ii) differentially regulated genes between the accessions and (iii) differentially regulated genes between the accessions specifically in *CQD1*.

Methods We profiled the transcriptomes of the two accessions by RNA-seq on an Illumina Solexa platform. Col (*gl1*) and Kas-1 plants were completely submerged in dark and sampled 4 hours after treatments started. We used two controls; plants in only darkness and plants in light. Gene ontology (GO) analysis was performed to detect common differentially regulated pathways for submergence treatment for the two accessions. We also investigated differentially regulated genes between the accessions for submergence. A detailed analysis on the *CQD1* locus was performed to select candidate genes for this previously described QTL.

Key Results Alterations in transcriptomes under submergence stress give clues about acclimation strategies even after 4 h. Many GO categories commonly regulated in both accessions included low oxygen, carbohydrate and anaerobic metabolism related groups. Several candidate genes potentially involved in differential submergence tolerance were selected both in the whole genome and specifically in the QTL region.

Conclusions Genes or gene groups both commonly and differentially regulated for and between the accessions were similar implying the importance of these groups in submergence survival. Our study also shows that RNA-seq can be used effectively in research on natural variation as a source to understand adaptations to several environments.

INTRODUCTION

Seasonal floods act as a strong selection force on plant communities in flood-prone environments (Blom, 1999; Van Eck *et al.*, 2004; Mommer *et al.*, 2006a) since prolonged submergence leads to severe tissue damage and mortality in many plant species. Fast depletion of oxygen and carbon dioxide, as a result of slower gas diffusion under water, hamper crucial biological processes such as photosynthesis and respiration (Armstrong, 1980). Additionally, floods can be accompanied by turbid waters, that due to low light availabilities limit photosynthesis (Vervuren, 2003; Parolin, 2009). Low photosynthetic rates underwater and low rates of respiration lead to an energy crisis and eventually high mortality. Furthermore, accumulation of reactive oxygen species increases mortality even if the waters subside quickly (Bailey-Serres & Voesenek, 2008; van Dongen *et al.*, 2009). Hence, plant populations varying in flooding tolerance form a distribution gradient in flood-exposed ecosystems determined by the duration and depth of these floods (Vervuren, 2003).

In order to overcome the lethal effects of various flooding regimes, plants have evolved different strategies to survive (Chen *et al.*, 2011; Akman *et al.*, 2012; Bailey-Serres *et al.*, 2012). A *low-oxygen escape* strategy enables deep-water rice cultivars, *Rumex palustris* and *Rorippa amphibia* to survive shallower, but prolonged floods by re-establishing air contact via elongated leaves/stems that protrude above the water surface (Hattori, 2007; Pierik *et al.*, 2009; Akman *et al.*, 2012). In contrast, lowland rice cultivars and *Rorippa sylvestris* achieve a higher survival by limiting growth and conserving carbohydrates, the so called *quiescence* strategy (Xu, 2006; Akman *et al.*, 2012).

The molecular basis of these two strategies in rice are well studied and revealed that *SUB1A* and *SNORKEL* genes, both members of group VII ethylene response factors (ERFs) are important in regulating these responses (Fukao *et al.*, 2006; Xu *et al.*, 2006; Hattori, 2008; Hattori *et al.*, 2009). Lowland rice cultivars with the quiescence strategy carry the ethylene inducible *SUB1A* gene that limits shoot elongation and carbohydrate consumption by inducing *Slender Rice-1* (*SLR1*) and *SLR1 Like-1* (*SLRL1*), inhibitors of GA activity. In deep-water rice, *SNORKEL* genes (*SK1* & *SK2*) are potential positive regulators of GA action and thus shoot elongation (Fukao & Bailey-Serres, 2008). Arabidopsis orthologs of the same ERF subfamily (group VII) were shown to improve hypoxia tolerance (Hinz *et al.*, 2010; Licausi *et al.*, 2010) and were recently identified as transcription factors involved in oxygen sensing (Gibbs *et al.*, 2011; Licausi *et al.*, 2011).

Unbiased whole transcriptome profiling studies are important to resolve other, still unknown components and regulators of flooding adaptive traits. Genome-wide transcriptome

alterations upon anoxia/hypoxia stress have been studied extensively using Arabidopsis microarrays. These studies have revealed many anoxia/hypoxia regulated gene families (Klok *et al.*, 2002; Branco-Price *et al.*, 2005; Liu *et al.*, 2005; Loreti *et al.*, 2005; van Dongen *et al.*, 2009). Nevertheless, most of these transcriptome profiling studies mentioned above are based on the Arabidopsis accession Col-0 and its mutants for genes of interest (*hre1*, *hre2*, *rap2.2*, *rap2.12*). So far there has been no study focusing on the transcriptome profiling of different Arabidopsis accessions that might shed light on variation in submergence tolerance among Arabidopsis accessions. Accordingly, Vashisht *et al.* (2011) showed that there is a considerable natural variation in submergence tolerance among 86 Arabidopsis accessions.

Most whole transcriptome studies on flooding tolerance have so far focused only on hypoxia/anoxia treatments, i.e. one component of the compound stress of submergence. Recently, Lee et al., (2011) used submergence treatments to uncover the molecular regulation of flooding tolerance in the Arabidopsis accession Col-0. (Lee *et al.*, 2011) This study focused on two time points, 7 and 24 h of submergence in dark, to capture early and later submergence response genes. This study revealed that there was a significant overlap between hypoxia and submergence regulated genes. Nevertheless, it was also shown that there was a considerable number of genes differentially regulated solely under submergence.

In the previous chapter of this thesis, we showed that Col (*gl1*) and Kas-1 accessions had different submergence tolerances. This difference in tolerance is partially due to a quantitative trait locus (QTL), *Come Quick Drowning 1* (*CQD1*) on the lower arm of chromosome 5. Since this locus is a part of a large chromosomal region, a detailed transcriptome analysis might assist in pinpointing the candidate gene(s) contributing to the submergence variation in these accessions. With this study, we aim to identify (i) gene categories that respond similarly in these two accessions Col (*gl1*) and Kas-1, as global submergence responses, (ii) genes differentially regulated between these accessions, and finally (iii) genes that are differentially regulated between these accessions specifically within the *CQD1* QTL region. For this purpose we performed a genome-wide transcriptome analysis using the RNA-seq platform in order to avoid cross-hybridization discrepancies that might arise by using microarrays specific for the standard lab accession Col-0 and also to detect low abundance and rare transcripts that are likely to be overlooked in a microarray analyses. Plants from both accessions were submerged completely in darkness and two controls (light and dark) were used to detect alterations in the transcriptome at an early time point. Our results indicate that even after 4 h of submergence, many gene groups are differentially regulated both in darkness and submergence (in darkness) in both accessions. The two accessions also showed variation in some gene groups (such as ERFs or pyrophosphate related genes) that might determine the differential submergence tolerance.

MATERIALS AND METHODS

Plant material

Seeds of Col (*gl1*) and Kas-1 accessions were obtained from the Nottingham Arabidopsis Stock Centre (NASC, UK) and sown densely on a soil:perlite (1:2) mixture in pots (9 x 9 x 9.5 cm^3). After sowing, they were transferred to 4°C for stratification for 4 days and later to a growth chamber at 20°C with 9 hours photoperiod, 200 µmol m^{-2} s^{-1} active radiation and 70% relative humidity. After germination, individual seedlings were transferred to single pots (70 ml) with the same soil:perlite mixture supplemented with 0.14 mg MgOCaO (17%; Vitasol BV, Stolwijk, the Netherlands) and 0.14 mg of slow release fertilizer (Osmocote 'plus mini'; Scotts Europe B.V., Heerlen, the Netherlands) per pot. Forty pots were placed in a tray supplemented with 1 l of nutrient solution as described before (Millenaar *et al.*, 2005). We covered the surface of the pots with black mesh cloths with a small hole in the middle for a seedling to be transplanted in, to prevent soil to float when submerged as described before (Vashisht *et al.*, 2011). Pots were put back in growth chambers. When all plants reached similar developmental stage of 8-9 leaves, a homogeneous subset was selected for experiments.

Experimental setup

We grew 200 plants per accession from which 105 per accession were selected for homogeneity in size for the experiment one day before the treatments started. We used disinfected plastic tubs (60 x 40 x 27 cm^3) filled with tap water (18-20 °C) for submergence and placed them in growth chambers one day before the treatments started. Two hours after the photoperiod began, 35 plants were submerged in prefilled tubs (submergence in dark), 35 were put in similar unfilled tubs in the same chamber (dark controls) and 35 were left in the growth chambers with the normal day/night regime (light controls). The chambers used for submerged plants and dark controls had the same conditions as the light control chambers except that all the lights were switched off (Fig. 1). We selected a four-hour time point since oxygen concentrations drop and stabilize in both petioles and roots and changes in oxygen concentrations alter the transcriptome significantly even after two hours (van Dongen *et al.*, 2009; Lee *et al.*, 2011). After 4 hours of treatments, 5 plants from each treatment were pooled to form one of the five replicates. Sampling was done simultaneously in dark and light chambers and was completed within 20 minutes for all the treatments. Root and shoot tissues were sampled separately, immediately frozen in liquid nitrogen and stored at -80°C until RNA isolations.

The remaining 10 plants for each accession in each treatment were used for petiole measurements. The youngest leaf of these plants was marked before the treatments started, and after 3 days of treatments (light and dark controls and submergence in dark), petiole elongation of the marked leaf was measured using a digital caliper. The whole procedure explained above was then repeated, yielding a second set of samples consisting of five pooled replicates of 5 plants per accession and treatment. No petiole measurements were taken in this second experiment.

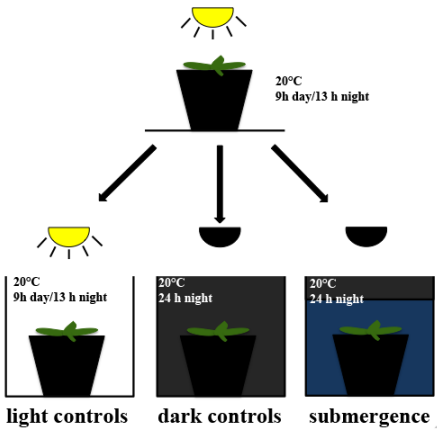

Fig. 1 Representation of experimental set-up.

RNA isolations and sample pooling

RNA isolations and DNase treatments were done with RNeasy Mini Kit and RNase-Free DNase Set (Qiagen Benelux B.V., Venlo, The Netherlands) according to manufacturer's instructions. RNA quantity and quality was assessed by using a Nanodrop and RNA intactness was checked on an agarose gel. As a control, we performed qRT-PCRs to test if anoxia/hypoxia marker genes (*ADH1;* At1g77120, *HB1; At2g16060*, *HRE2; At1g72360*) were up-regulated and if all the replicates behaved similarly. Out of five, four replicates per accession, treatment and tissue type from each experiment were used in cDNA synthesis with 500 ng RNA, 50 ng random hexamers and 200 U SuperScript III reverse transcriptase (Invitrogen, Bleiswijk, The Netherlands). The remaining replicate was kept at -80 to be used if any of the analyzed samples was not suitable for the RNA-seq analysis. Primer sequences for the qRT-PCRs were (5'-3')

ADH1 Forward: GAATCGCTGGTGCTTCTAGG
ADH1 Reverse: TGGCACTGTGTGAGTGATGA
HB1 Forward: GGCTCTTGTAGTGAAGTCTTGGA
HB1 Reverse: TAATGGCAGCAACAAGGTGA
HRE2 Forward: GGCCTCTGCCTTATCCCTCTGT
HRE2 Reverse: GCGTAAACCCGTCTCAGTGAGTG

Quantitative RT-PCR reaction mixtures included 2X SYBR green (Platinum SYBR green Supermix qPCR UDG; Invitrogen, Bleiswijk, The Netherlands), 3 µM of each primer and 125 ng cDNA 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 *TUBULIN* transcript levels. Results of qRT-PCR were used to select the replicates to be pooled for RNA-seq samples in order to test the consistency between the replicated experiments.

RNA-seq

We pooled all the four replicates analyzed in the qRT-PCR, since they showed similar expression patterns for tested genes. From each replicate 2 and 8 ng of RNA were pooled for roots and shoots, respectively. These samples were commercially sequenced by Macrogen Inc. (Korea) using the Illumina/Solexa sequencing platform. In total, twelve samples were sequenced for two accessions, two tissue types and three treatments. Sequencing of the twelve samples was done on four lanes by running three samples per lane to get 50 base pair reads.

Data analyses

Sequence read alignments were done with Bowtie 2 (Langmead *et al.*, 2009) by using local alignments of 35 base pairs, allowing two mismatches and four multiple alignments for each read. We used Col-0 as a reference genome, downloaded from TAIR 10 database. Differential expression analysis was performed with the bioconductor package EdgeR (Robinson *et al.*, 2010). Normalization coefficients for library size varied between 0.97-1.02 for roots and 0.96-1.03 for shoots. We treated the two accessions as replicates to test the global expression patterns of each treatment at species level. We compared light controls to dark controls to test effects of dark treatment, dark controls to submergence in dark to test effects of submergence only and finally light controls to submergence in dark to test combined effects of submergence and darkness. We used general linear models to fit our data and to test differentially expressed genes (DEGs) and the interactions by using bioconducter package limma (Smyth, 2005). The dispersion values calculated and used in DEG analysis varied between 0.07-0.09 for roots and 0.14-0.21 for shoots. We used a cut-off of adjusted p-value <0.05 for selecting DEGs for treatment effects. Venn diagrams were constructed for DEGs for all pairwise treatment comparisons for up- and down-regulated genes and for the two tissue types (Fig. 4). For identification of genes differentially regulated for each treatment between species (interaction effects in our model) for the whole genome region, we used a cut-off of p-value<0.001. For detailed analysis of the differentially regulated genes between the accessions in the *CQD1* QTL region, we used a cut-off of p-value<0.05.

GO analyses

Gene ontology (GO) analysis was performed with bioconductor package GOSEQ (Young *et al.*, 2010). In the GO analysis we used the DEGs between light controls vs. submergence in dark and dark controls vs. submergence in dark by excluding the DEGs of light control vs. dark controls comparison (dashed section in Fig. 4a). For instance, the example labeled with the treatment names in Fig. 4a represents a gene that was up regulated only in dark controls. Since it showed no up-regulation in combined effect of darkness and submergence, it is not included in the GO analysis for up-regulated genes. Nevertheless, the same gene is also represented in the down-regulated genes on the far right section. This time this gene is included in the GO analysis of down-regulated genes because although it was up-regulated only in darkness, the combined effect of submergence and darkness showed no change in expression compared to light controls. This implies that this gene would have been down-regulated if submergence was performed in light. Thus, this group of genes was included in GO analysis of down-regulated genes. The intersection of all three comparisons represents the genes that are significantly up-regulated in darkness compared to light controls and even more up-regulated in submergence compared to darkness. By using the genes in dashed sections, we performed four GO analyses for two tissue types and for up- and down-regulated genes separately in order to capture effects of submergence. For the over-represented GO categories we used a cut-off of p-value<0.01.

Statistical analyses

We performed ANOVA analyses and Tukey's b post-hoc tests (Tukey's b) to test differences in petiole growth in dark and air controls and submergence. The qPCR data was analyzed also with ANOVA post-hoc tests each species and gene individually. All analyses were performed with SPSS 16.0 for Mac (SPSS Incorporated, Chicago, USA).

RESULTS

Consistent anoxia/hypoxia marker gene regulation in both experiments

We measured petiole elongation of the youngest leaf for submergence in darkness and the two controls after 3 days of the treatments (Fig. 2). Petiole growth was suppressed in both accessions in submerged conditions and in dark controls but more so in the latter.

Using qRT-PCR, we first tested regulation of three anoxia/hypoxia marker genes (*ADH1*, *HB1* and *HRE2*) that are known to be up-regulated upon anoxia/hypoxia and submergence

in Arabidopsis. In both experiments there was a consistent up-regulation of all three genes under submergence compared to darkness in both qRT-PCR and the RNA-seq count data for roots of both accessions (Fig. 3). All three genes showed a down-regulation in darkness.

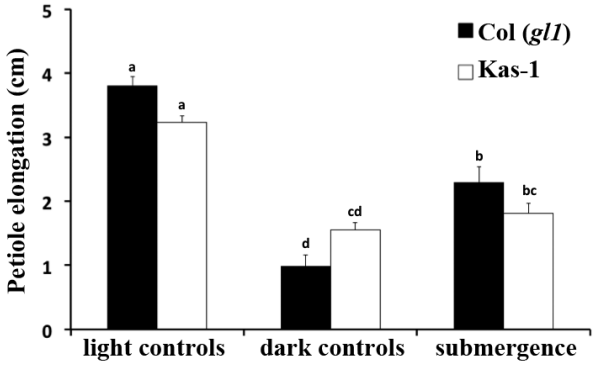

Fig. 2 Petiole elongation of Col (gl1) and Kas-1 in light and dark controls and submergence in dark. ANOVA post-hoc results are indicated as letters as significant differences at P<0.05.

Quality of alignments

The reads generated from the sequencing of the EST/cDNA libraries were aligned to the genome of the Col-0 accession with allowing two mismatches that improved the alignment rate by three percent (data not shown) compared to alignments with no mismatches allowed. The percentage of aligned reads for Col (*gl1*) was 2.2% higher for roots and 1.3% for shoots compared to Kas-1 (Table 1). For both accessions, shoots gave a better alignment score than root tissues. An average of 3% of reads were aligned to multiple genes for both species (Table 1).

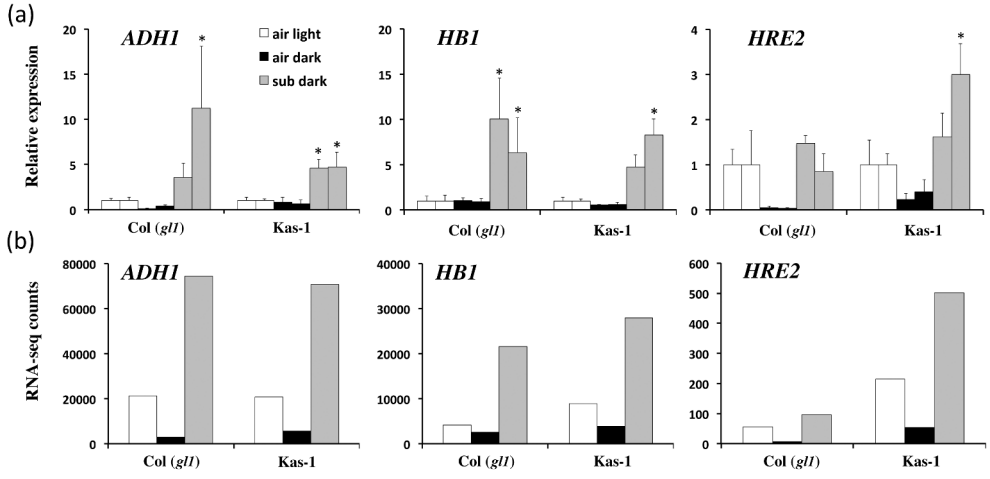

Fig. 3 Root transcript abundance of submergence marker genes *ADH1*, *HB1* and *HRE2* as measured using (a) qRT-PCR (as relative expression); ANOVA post-hoc test results (P<0.05) are indicated for significantly different groups and (b) RNA-seq (as count data) experiments.

Table 1 Bowtie2 alignment results for all libraries sequenced

Library name	Library size (# reads)	% aligned reads (once)	% aligned reads (more than once)	% Total aligned reads	% non-aligned reads
Col (gl1) light controls root	38884713	92.5	3.3	95.8	4.2
Col (gl1) dark controls root	42921704	91.4	2.9	94.3	5.7
Col (gl1) submerged root	40543049	91.5	2.9	94.4	5.6
Col (gl1) light controls shoot	27320214	92.9	3.4	96.3	3.7
Col (gl1) dark controls shoot	29262982	92.9	3.3	96.2	3.8
Col (gl1) submerged shoot	21655964	93.1	3.1	96.2	3.8
Kas-1 light controls root	36783160	89.8	3.1	92.9	7.1
Kas-1 dark controls root	37226377	89.8	2.7	92.5	7.5
Kas-1 submerged root	33411646	89.8	2.8	92.6	7.4
Kas-1 light controls shoot	33008003	91.9	3.3	95.2	4.8
Kas-1 dark controls shoot	34885304	91.7	3.1	94.8	5.2
Kas-1 submerged shoot	34318638	91.6	3.0	94.7	5.3

Differentially regulated genes due to darkness

In order to detect responses of Arabidopsis to the treatments that were common to both accessions, we treated Col (*gl1*) and Kas-1 as replicates in our analysis. We compared number of up and down-regulated genes in Venn diagrams (Fig. 4). Switching from light to dark and submergence in dark promoted alterations of a considerable number of genes in both roots and shoots (Fig. 4 b&c). These two comparisons revealed 345 and 440 overlapping genes in roots for up- and down-regulation, respectively. In order to eliminate effects of only darkness, we also performed comparisons of dark controls and submerged plants in darkness. The number of genes differentially regulated between these comparisons was significantly lower than the other two comparisons for both root and shoot tissues.

Comparisons of light controls and submergence in dark revealed more regulated genes than

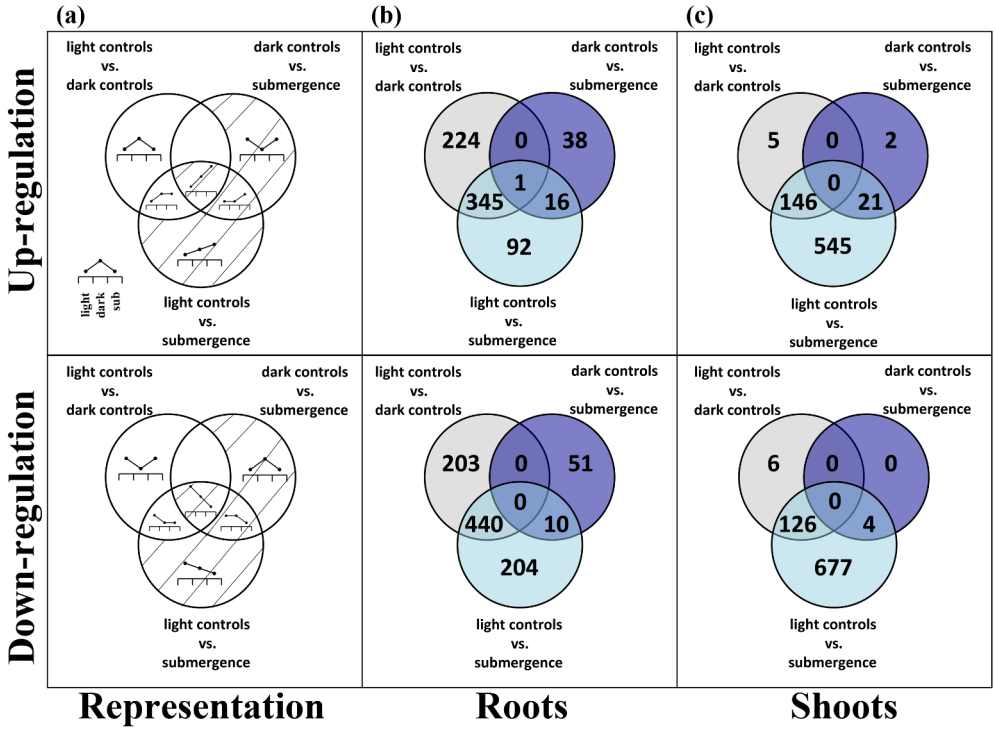

Fig. 4 Venn diagrams showing number of up and down-regulated DEGs in (b) roots and (c) shoots. (a) represents of type of gene regulation for different treatments (see materials and methods, GO analysis section). The dashed categories represent the genes used in GO analysis for characterization of up and down-regulated gene ontologies.

any other comparison in shoots. We observed 712 and 807 genes up and down-regulated respectively when light controls were compared to submergence in darkness in shoots. Furthermore, there was a large overlap between light controls vs. dark controls and light controls vs. submergence treatment in shoots (146 and 126 genes for up and down-regulation, respectively), similar to the root tissues.

Numerous gene ontology categories related to oxygen stress in roots

In order to separate the effects of darkness from submergence in regulated genes, we performed a gene ontology (GO) analysis for genes regulated between dark controls vs. submergence treatment and light controls vs. submergence treatment by excluding the commonly regulated genes between light controls vs. dark controls and light controls vs. submergence in dark (as indicated with dashes in Fig. 4a).

Table 2 GO categories over-represented for up-regulated genes in submergence in roots

GO category identifier	Over-representation p-value	GO category description
GO:0030976	0.000	thiamine pyrophosphate binding
GO:0004737	0.000	pyruvate decarboxylase activity
GO:0016831	0.000	carboxy-lyase activity
GO:0034059	0.000	response to anoxia
GO:0009061	0.000	anaerobic respiration
GO:0009815	0.000	1-aminocyclopropane-1-carboxylate oxidase activity
GO:0080031	0.000	methyl salicylate esterase activity
GO:0001666	0.001	response to hypoxia
GO:0071398	0.001	cellular response to fatty acid
GO:0080032	0.001	methyl jasmonate esterase activity
GO:0080030	0.001	methyl indole-3-acetate esterase activity
GO:0009696	0.001	salicylic acid metabolic process
GO:0047800	0.002	cysteamine dioxygenase activity
GO:0009611	0.002	response to wounding
GO:0015035	0.004	protein disulfide oxidoreductase activity
GO:0016157	0.004	sucrose synthase activity
GO:0009873	0.005	ethylene mediated signaling pathway
GO:0045454	0.006	cell redox homeostasis
GO:0017153	0.007	sodium:dicarboxylate symporter activity
GO:0051453	0.007	regulation of intracellular pH

For the GO analysis of roots we used a total of 16,848 genes for up-regulation and 17,123 for down-regulation categories out of which 147 and 265 were up- or down-regulated, respectively. Twenty GO categories were significantly up-regulated in roots (Table 2, p-value < 0.01). More than 50% of up-regulated GO categories that were significantly over-represented in roots include known anoxia/hypoxia responsive and carbohydrate metabolism related groups. These include sucrose synthase activity, thiamine pyrophosphate (a co-enzyme of decarboxylases) binding, pyruvate decarboxylase activity, sodium:dicarboxylate symporter activity and anaerobic respiration categories. Two GO categories related to ethylene biosynthesis (1-aminocyclopropane-1-carboxylate oxidase activity) and ethylene-mediated signaling were also up-regulated.

Table 3 GO categories over-represented for down-regulated genes in roots

GO category identifier	Over-representation p-value	GO category description
GO:0048046	0.000	Apoplast
GO:0010266	0.000	response to vitamin B1
GO:0004805	0.001	trehalose-phosphatase activity
GO:0052622	0.001	ATP dimethylallyltransferase activity
GO:0052623	0.001	ADP dimethylallyltransferase activity
GO:0004497	0.001	monooxygenase activity
GO:0019825	0.001	oxygen binding
GO:0009824	0.002	AMP dimethylallyltransferase activity
GO:0009573	0.002	chloroplast ribulose bisphosphate carboxylase complex
GO:0016165	0.002	lipoxygenase activity
GO:0009695	0.002	jasmonic acid biosynthetic process
GO:0009055	0.003	electron carrier activity
GO:0015976	0.003	carbon utilization
GO:0016765	0.003	transferase activity, transferring alkyl or aryl groups
GO:0009579	0.003	Thylakoid
GO:0009627	0.004	systemic acquired resistance
GO:0015977	0.005	carbon fixation
GO:0005506	0.005	iron ion binding
GO:0005992	0.006	trehalose biosynthetic process
GO:0006817	0.007	phosphate ion transport
GO:0042579	0.007	Microbody
GO:0030504	0.007	inorganic diphosphate transmembrane transporter activity
GO:0030505	0.007	inorganic diphosphate transport
GO:0016045	0.007	detection of bacterium
GO:0009673	0.007	low affinity phosphate transmembrane transporter activity
GO:0010478	0.007	chlororespiration
GO:0010299	0.007	detoxification of cobalt ion
GO:0042391	0.007	regulation of membrane potential
GO:0009691	0.008	cytokinin biosynthetic process
GO:0008271	0.009	secondary active sulfate transmembrane transporter activity
GO:0008266	<0.010	poly(U) RNA binding
GO:0010218	<0.010	response to far red light
GO:0009637	<0.010	response to blue light

Other related groups were salicylic acid metabolic process, methyl salicylate and methyl jasmonate esterase activity categories known to be important in disease responses. Methyl indole-3-acetate esterase activity, which is involved in activation of Me-IAA by converting it to indole-3-acetate (IAA, auxin) (Yang *et al*., 2008), was also significantly up-regulated. The other two categories up-regulated were related to cell redox homeostasis and regulation of intracellular pH.

Table 4 GO categories over-represented for up-regulated genes in shoots

GO category identifier	Over-representation p-value	GO category description
GO:0016798	0.000	hydrolase activity, acting on glycosyl bonds
GO:0080039	0.000	xyloglucan endotransglucosylase activity
GO:0050832	0.000	defense response to fungus
GO:0051740	0.000	ethylene binding
GO:0034605	0.000	cellular response to heat
GO:0009741	0.000	response to brassinosteroid stimulus
GO:0010105	0.000	negative regulation of ethylene mediated signaling pathway
GO:0010411	0.000	xyloglucan metabolic process
GO:0009646	0.001	response to absence of light
GO:0010200	0.001	response to chitin
GO:0016762	0.001	xyloglucan:xyloglucosyl transferase activity
GO:0004673	0.001	protein histidine kinase activity
GO:0004872	0.001	receptor activity
GO:0008289	0.001	lipid binding
GO:0010136	0.001	ureide catabolic process
GO:0010468	0.003	regulation of gene expression
GO:0071497	0.003	cellular response to freezing
GO:0048046	0.003	Apoplast
GO:0004353	0.004	glutamate dehydrogenase [NAD(P)+] activity
GO:0046658	0.004	anchored to plasma membrane
GO:0043043	0.004	peptide biosynthetic process
GO:0080022	0.004	primary root development
GO:0051707	0.006	response to other organism
GO:0010167	0.007	response to nitrate
GO:0008194	0.007	UDP-glycosyltransferase activity
GO:0016847	0.008	1-aminocyclopropane-1-carboxylate synthase activity
GO:0042218	0.008	1-aminocyclopropane-1-carboxylate biosynthetic process
GO:0009693	0.009	ethylene biosynthetic process
GO:0070370	0.009	cellular heat acclimation

GO categories significantly down-regulated in roots (Table 3) included processes related to chloroplast and ATP/ADP/AMP dimethyl transferase activity coupled with phosphate ion transport. A GO category related to cytokinin biosynthetic process and oxygen binding was also down-regulated. Additionally, a trehalose biosynthesis processes and poly(U) RNA binding categories were among this list. Two categories for pyrophosphate transport were significantly down-regulated together with low affinity phosphate transmembrane transporter activity.

GO categories related to cell wall loosening and ethylene in shoots

In the GO analysis of shoot tissues, 568 up-regulated genes out of 14,085 and 781 down-regulated genes out of 14,461 were used. Shoot tissues showed a different profile than roots; there were less anoxia/hypoxia related categories. Ethylene related categories were again up-regulated (1-aminocyclopropane-1-carboxylate synthase activity and biosynthetic activity, ethylene binding, negative regulation of ethylene-mediated signaling pathway) and also many categories were related to sugar metabolism such as glutamate dehydrogenase [NAD(P)+] activity, UDP-glycosyltransferase activity, hydrolase activity (acting on glycosyl bonds). Xyloglucan endotransglucosylase activity, xyloglucan metabolic process, xyloglucan:xyloglucosyl transferase activity categories related to cell wall loosening were over-represented in up-regulated genes. We observed several up-regulated categories related to diverse stresses such as freezing, fungus, heat and heat acclimation. Response to absence of light and regulation of gene expression were also over-represented in shoots. Shoot tissues show down-regulation in similar categories as roots related to oxygen and iron binding, monooxygenase and electron carrier activity. Some categories in the up-regulated genes were also found in down-regulated categories such as response to heat and fungus in shoots. In addition salinity, cold, desiccation and water deprivation, glucosinolate biosynthetic process and jasmonic acid stimulus related categories were down-regulated in shoots. One abscisic acid (ABA) stimulus responsive category and one indole-acetic acid (IAA, auxin) biosynthesis process was also down-regulated together with lipid metabolism, binding and transport categories. Interestingly, response to oxidative stress was one of the over-represented categories in down-regulated genes.

Genome-wide differentially regulated genes between accessions

We investigated gene-wise differences of the responses between the two accessions for the treatments. We found fifty genes differentially regulated under submergence (both compared to light and dark controls) between accessions in root tissues (Fig. 5) and 20 genes in shoot tissues (Fig. 6), some of which are members of gene groups that have similar functions.

Table 5 GO categories over-represented for down-regulated genes in shoots

GO category identifier	Over-representation p-value	GO category description
GO:0019825	0.000	oxygen binding
GO:0012505	0.000	endomembrane system
GO:0004497	0.000	monooxygenase activity
GO:0005506	0.000	iron ion binding
GO:0080167	0.000	response to karrikin
GO:0004091	0.000	carboxylesterase activity
GO:0009055	0.000	electron carrier activity
GO:0020037	0.000	heme binding
GO:0016788	0.000	hydrolase activity, acting on ester bonds
GO:0009414	0.000	response to water deprivation
GO:0042538	0.000	hyperosmotic salinity response
GO:0009409	0.000	response to cold
GO:0005788	0.000	endoplasmic reticulum lumen
GO:0009631	0.000	cold acclimation
GO:0009269	0.000	response to desiccation
GO:0048046	0.001	apoplast
GO:0008194	0.001	UDP-glycosyltransferase activity
GO:0005576	0.001	extracellular region
GO:0008289	0.001	lipid binding
GO:0009753	0.001	response to jasmonic acid stimulus
GO:0006730	0.001	one-carbon metabolic process
GO:0006629	0.001	lipid metabolic process
GO:0009408	0.002	response to heat
GO:0019761	0.002	glucosinolate biosynthetic process
GO:0031012	0.002	extracellular matrix
GO:0009620	0.003	response to fungus
GO:0016207	0.003	4-coumarate-CoA ligase activity
GO:0009737	0.004	response to abscisic acid stimulus
GO:0004190	0.004	aspartic-type endopeptidase activity
GO:0004837	0.004	tyrosine decarboxylase activity
GO:0010017	0.005	red or far-red light signaling pathway
GO:0080043	0.005	quercetin 3-O-glucosyltransferase activity
GO:0006979	0.005	response to oxidative stress
GO:0009411	0.005	response to UV
GO:0031407	0.006	oxylipin metabolic process
GO:0009807	0.007	lignan biosynthetic process
GO:0006188	0.008	IMP biosynthetic process
GO:0006869	0.008	lipid transport
GO:0010439	0.008	regulation of glucosinolate biosynthetic process
GO:0005544	0.009	calcium-dependent phospholipid binding
GO:0009684	0.009	indoleacetic acid biosynthetic process
GO:0009698	0.009	phenylpropanoid metabolic process

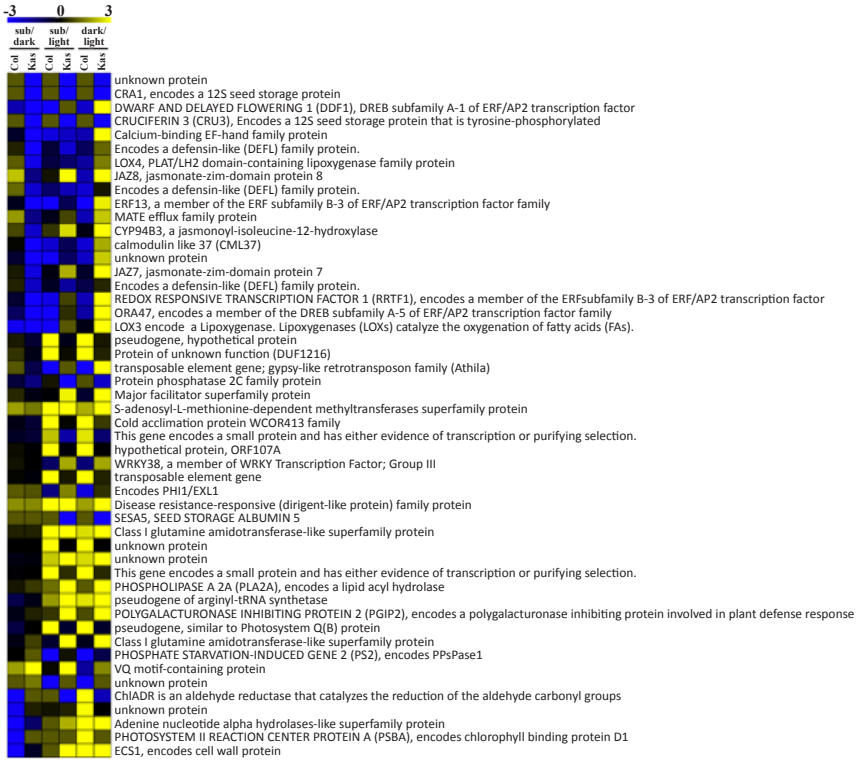

Fig. 5 Differentially regulated genes between roots of accessions Col (*gl1*) and Kas-1 (p-value<0.001), log-fold change between treatments are represented in the heatmaps. For instance, the first column represents the log fold change in gene expression from dark control to submergence in dark.

In roots, four DEGs belong to the ERF/AP2 transcription factor family (ERF13; AT2G44840, RRTF1; AT4G34410, ORA47; AT1G74930, DDF1; AT1G12610) and two belong to lipoxygenase family protein (LOX3; AT1G17420, LOX4; AT1G72520). There were three genes encoding defensin-like (DEFL) family proteins (AT1G34047, AT5G33355, AT2G36255). Two class-I glutamine amidotransferase-like superfamily proteins (AT1G15040, AT1G66860), two jasmonate-zim-domain proteins (*JAZ7*; AT2G34600, JAZ8; AT1G30135) and two cruciferin-seed storage genes (*CRA1*; AT5G44120, CRU3; AT4G28520) were also differentially regulated between the accessions in roots. There were eight genes with unknown functions. Some of the most interesting genes differentially regulated between the accessions were the phosphate starvation-induced gene 2 (*PS2*; AT1G73010), a cell wall protein (*ECS1;* AT1G31580), and a calcium binding protein (AT3G01830) since all of these genes might contribute to submergence tolerance. Additionally, *EXORDIUM-LIKE 1* (*EXL1*)

was up-regulated in Kas-1 roots under both darkness and submergence but not in Col (*gl1*). There were 20 genes differentially regulated between accessions in shoot tissues (Fig. 6). Four of these include methyltransferases; two S-adenosyl-L-methionine-dependent methyltransferases superfamily proteins (AT3G44870 and AT3G44840), one gene encoding a farnesoic acid carboxyl-O-methyltransferase (AT3G44860) and a member of the Arabidopsis SABATH methyltransferase gene family (*PXMT1*, AT1G66700). There was also one ERF/AP2 transcription factor family gene (*DREB1A*, AT4G25480). *BIP3* (AT1G09080), an ATP binding protein and three genes with unknown functions were included in the list of genes differentially regulated between the accessions in shoots.

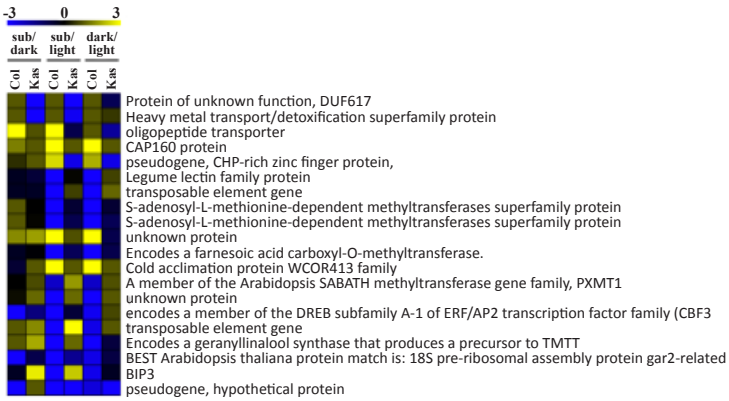

Fig. 6 Differentially regulated genes between shoots of accessions Col (*gl1*) and Kas-1 (p-value<0.001), log-fold change between treatments are represented in the heatmaps.

Differentially regulated genes on *Come Quick Drowning 1* (*CQD1*) locus

For detection of differentially regulated genes between Col (*gl1*) and Kas-1 in the QTL region previously detected (containing approximately 600 genes), we report DEGs using a less stringent cut-off p-value<0.05. Out of 32 differentially regulated genes between accessions within the QTL region in roots (Fig. 7), there were four ERF/AP2 transcription factor family genes (*ERF2*; AT5G47220, *ERF5*; AT5G47230, *CBF4*; AT5G51990 and *TINY*; AT5G52020). There was one gene (AT5G46845) encoding a microRNA, targeting several auxin responsive factors. A heat shock protein (HSP20-like chaperons superfamily protein; AT5G51440) and a transcription activator promoting tolerance to salt, osmotic stress and drought (AT5G49450) were amongst genes, which might have important role in modulating responses to submergence stress.

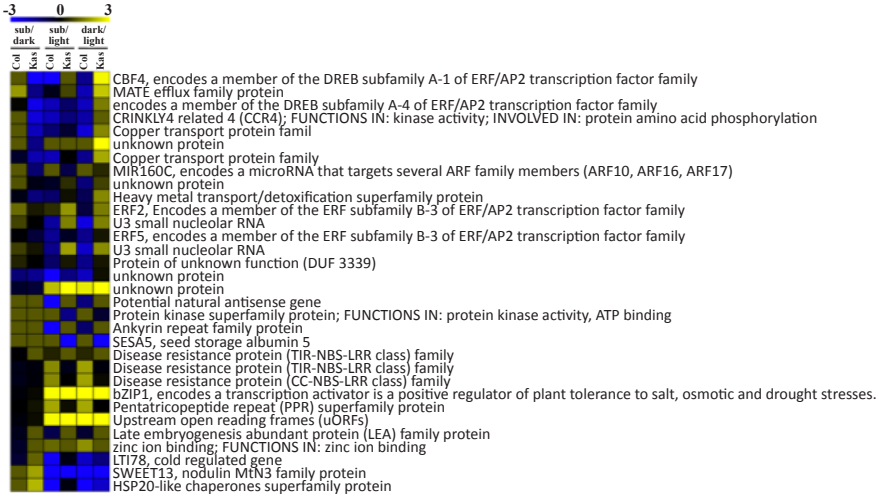

Fig. 7 Differentially regulated genes between roots of accessions Col (*gl1*) and Kas-1 in the QTL, *CQD1* region (p-value<0.05), log-fold change between treatments are represented in the heatmaps.

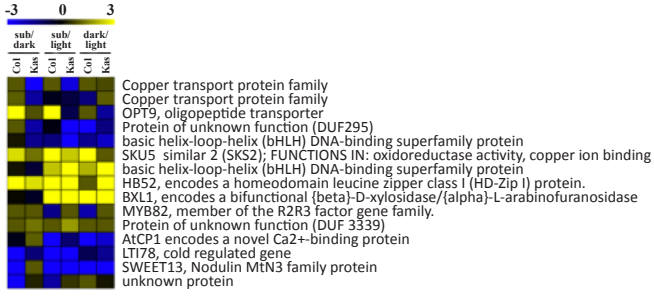

Fig. 8 Differentially regulated genes between shoots of accessions Col (*gl1*) and Kas-1 in the QTL, *CQD1* region (p-value<0.05), log-fold change between treatments are represented in the heatmaps.

In shoot tissues, there were 15 genes that show variation between accessions in their responses to different treatments (Fig. 8). There was one SAUR-like-auxin responsive protein family gene (AT5G50800) and a Ca^{+2}- binding protein (AT5G49480). There was also one copper transport protein (AT5G52760) and a copper ion binding protein (SKU5; AT5G51790). *SWEET13* (AT5G50800), a sugar transport family protein was differentially regulated between the accessions both in roots and shoots.To summarize, for both tissue types there were many genes differentially regulated for switching to darkness and submergence in darkness. These two treatments had a large overlap in differentially regulated genes. Our results indicated that even after 4 hours of submergence, many GO categories related to low oxygen conditions were regulated in roots which were common for the two accessions analyzed. GO categories up-regulated in shoots were different from roots and included groups related to ethylene

and cell wall loosening. We also observed several differentially regulated genes between the accessions both genome-wide and specifically in the *CQD1* QTL.

DISCUSSION

Using RNAseq, we identified the early common transcriptional submergence responses of two *Arabidopsis thaliana* accessions Col (*gl1*) and Kas-1. These were the parental accessions for the RIL population that was used for detecting submergence tolerance genes using a QTL mapping approach (Chapter 4 of this thesis). We then specifically focused on the differences between these two accessions since they differ in their submergence tolerance, Kas-1 being more tolerant than Col (*gl1*) (Akman, *et al.*, this thesis Chapter 4). We also analyzed the DEGs in the previously identified QTL region, *CQD1*. Both darkness and submergence in darkness altered the transcriptome significantly in both accessions and many genes were commonly regulated under these two stresses. GO categories related to carbohydrate metabolism and low oxygen were up-regulated in roots that are anoxic due to submergence (Lee *et al.*, 2011; Vashisht *et al.*, 2011). Being the primary low-oxygen accumulating hormone, ethylene related pathways were also significantly altered. We also observed changes in both biotic and abiotic stress related categories. Differentially regulated genes between the accessions included gene groups with similar functions such as ERFs and *LOX* genes, confirming their functional importance in differential submergence tolerance of these accessions. We also found several potential candidates such as auxin response factor regulating microRNAs, several ERFs and a Ca^{+2} binding protein in the *CQD1* locus.

RNA-seq as a platform for detecting molecular stress responses of different Arabidopsis accessions

One of the aims of using the RNA-seq platform was to avoid discrepancies that might arise from the gene sequence differences between the two accessions that might be excluded in a microarray study. Compared to microarrays that do not cover the entire Arabidopsis transcriptome, RNA-Seq has proven to be less biased with no cross-hybridization and have a greater dynamic range (Shendure, 2008). Furthermore, completion of the Arabidopsis 1001 genomes project will increase the specificity of this platform by supplying more reference genomes and better enabling comparisons of different accessions that show variation in their responses to diverse stresses. These advances will promote studies using natural variation as a source to understand adaptations to several environments. Col-0 was the first Arabidopsis accession to be sequenced, whereas reference genomic data for Kas-1 has not yet been released. Thus, the alignment scores for all Col (*gl1*) RNA-seq libraries were higher in our analyses. Nevertheless, we were able to increase the number of alignments for Kas-1 by 3 %

by allowing two mismatches. More sequences were aligned successfully in shoot tissues for both accessions, possibly because the RNA isolated from soil fauna also contributed to the total RNA content in each library and was sequenced. Nevertheless, we observed consistent results with qRT-PCRs and RNA-seq count data for the genes we investigated; *ADH1, HB1* and *HRE2* were up-regulated in submergence treatments consistent with previous studies (Licausi *et al.*, 2010; Lee *et al.*, 2011). These results indicate the suitability of the RNA-seq platform for studies involving different Arabidopsis accessions and complex stresses such as submergence.

Different physiological and molecular responses to darkness and submergence

Although petiole growth was hampered in both accessions in darkness and submergence treated plants, submerged Col (*gl1*) plants showed a higher petiole elongation than dark controls. This suggests that when submergence is coupled with dark stress, Col (gl1) accession do not limit its growth as much as it does under only darkness and still show a higher petiole elongation. On the other hand Kas-1 did not show a significant difference in growth between darkness and submergence treated plants. Although growth is lower than in air controls, these patterns resemble a lighter version of the escape and quiescence strategies of *Rorippa* and *Rumex* as shown in Chapter 3 of this thesis. These differences in growth might be important in different submergence tolerances in these accessions.

Both darkness and submergence induced significant alterations in gene expression. We observed fewer DEGs compared to previously published data on submergence response of Col-0 accession that analyzed transcriptome alterations after 7 h of submergence stress (Lee *et al.*, 2011). It has been shown that differentially expressed genes vary in numbers as anoxia/hypoxia or waterlogging prolongs in poplar (Kreuzwieser *et al.*, 2009), in Arabidopsis (Klok *et al.*, 2002; Liu *et al.*, 2005; van Dongen *et al.*, 2009) and in rice (Narsai *et al.*, 2009). We selected a 4 h time-point since roots become anoxic after 2 h and oxygen levels in petioles drop to 6% after 3 h of submergence (Lee *et al.*, 2011) and earlier responses such as acclimation initiation might be a determining factor of survival ability.

Consistent with results of Lee *et al*. (2011) we observed that there were more genes up-regulated by switching from light to darkness (effects of only darkness) compared to switching from darkness to submergence in dark (effects of only submergence). This might be due to the fact that both darkness and submergence in darkness lead to high carbohydrate consumption since photosynthesis is inhibited and there is a high demand for soluble carbohydrates, which results in regulation of similar genes. Nevertheless, we observed that there was a significant number of genes only regulated in combined effects of submergence

and darkness, and especially in shoot tissues. Although darkness alters the transcriptome for a wide range of genes, submergence causes numerous additional changes.

GO categories related to carbohydrates and anaerobic metabolism are up-regulated mostly in roots

We observed many GO categories in up-regulated genes in root tissues composed of groups related to carbohydrate and anaerobic metabolism. One of the most common alterations is within the aerobic respiration since oxygen becomes limiting. Anaerobic metabolism becomes the major source of NAD^+ that is necessary to sustain glycolysis that is essential for ATP production. (Bailey-Serres & Voesenek, 2008; Bailey-Serres *et al.*, 2012). Anaerobic metabolism produces less ATP than aerobic respiration; therefore conservation of ATP becomes crucial during low oxygen conditions. As an example the *INVERTASE* pathway for the breakdown of sucrose, which is an ATP demanding process, is down-regulated and replaced by *SUCROSE SYNTHASE* (*SUS*) pathway as an alternative glucose 1-phosphate source (Liu *et al.*, 2005; Mustroph *et al.*, 2010). Accordingly, we showed that in our experiments a *SUS* activity GO category was up-regulated in roots. *SUS* activity is pyrophosphate dependent and we also found several GO categories related to pyrophosphate and phosphate transport to be down-regulated. This might be a way to keep pyrophosphate (PPi) in intracellular spaces for increased *SUS* activity in order to decrease ATP demands. Since oxygen levels drop quicker in roots compared to shoot tissues, there is a higher demand for ATP (supplied by glycolysis) and NAD^+ (supplied by fermentation for glycolysis). This loop of high demand may drive plants to an energy crisis, severe damage and eventually mortality but is also necessary for acclimations to low oxygen conditions. So there is a trade-off between regulating energy demanding processes for acclimating to new conditions and consuming carbohydrates during the process. Plant strategies are usually a compromise between these trade-offs depending on flooding lengths and durations (Vervuren, 2003; Akman *et al.*, 2012).

Trehalose is a sugar known to be a tolerance enhancer factor to many abiotic stresses (Chen & Murata, 2002; Garg *et al.*, 2002). Furthermore, its precursor trehalose-6-phosphate which controls sugar influx into glycolysis in yeast (Thevelein & Hohmann, 1995), was proposed as a sugar metabolism regulator in plants (Eastmond *et al.*, 2003). Trehalose-6-phosphate synthase is up-regulated in submerged *SUB1* rice (Mustroph *et al.*, 2010). In our experiments a trehalose biosynthetic process category was down-regulated and might have an effect on regulation of influxes in carbohydrate metabolism under low oxygen conditions.

Alterations in growth related hormonal pathways

Ethylene and auxin biosynthesis related categories are significantly up-regulated in roots possibly inhibiting root growth since these hormones were shown to act reciprocally or independently on root growth inhibition in Arabidopsis (Stepanova *et al.*, 2007). On the other hand, these hormones promoted adventitious root formation in waterlogged roots of tomato and enabled replacement of damaged roots by healthy new root systems (Vidoz *et al.*, 2010). Thus, both ethylene and auxin might act on alterations on root profiles (adventitious roots and/or primary root formation) in an attempt to increase survival also in Arabidopsis. Cytokinin biosynthesis processes were also among down-regulated clusters. It has been shown that cytokinin over-expresser Arabidopsis mutants cannot synthesize cytokinins under submerged conditions, but after de-submergence, synthesis is up-regulated and helps over-expresser lines to recover faster (Huynh *et al.*, 2005). In our experiments, down-regulation might be an early energy conserving strategy which might be turned on after de-submergence.

Responses vary between different tissue types

Sugar metabolism and ethylene-related GO categories were up-regulated in shoots similarly to roots and thus constitute global submergence responses throughout the plant tissues and accessions. We observed several up-regulated cell wall related categories in only shoots such as cell wall loosening, which might explain growth of submerged petioles more than in dark only. Although not a true escape strategy, Arabidopsis might also harbor mechanisms involved in petiole elongation under submergence with a decreased functional ability as a result of being not naturally flooded. We also observed a down-regulation of an auxin stimulus responsive category in shoots, which might be promoted by ethylene biosynthesis resulting in petiole growth.

Cell homeostasis and reactive oxygen species related GO categories

We observed GO categories related to regulation of intracellular pH and cell redox homeostasis. During submergence, cytosolic pH changes dramatically as a result of accumulation of glycolysis products and lactic acid in earlier stages of stress and these modifications in pH may lead to a faster mortality (Bailey-Serres & Voesenek, 2008). Accumulation of reactive oxygen species (ROS) can become very harmful for plants upon changes in oxygen concentration and ROS-related genes were upregulated, albeit usually at later stages or during the recovery from the oxygen stress (Bailey-Serres & Voesenek, 2008; van Dongen *et al.*, 2009). Furthermore, *SUB1A-1*, induced by ethylene, was also shown to be involved in ROS

amelioration. Nevertheless, we observed a ROS related GO category (response to oxidative stress) in down regulated genes in shoots. It is possible that ROS were not yet accumulating in cells since we capture very early stages in the stress and thus ROS responsive genes were down-regulated to limit any process that might be unnecessarily energy demanding.

Regulation of GO categories related to other stresses

Arabidopsis plants pre-treated with high temperatures survive longer during subsequent anoxia, and heat shock proteins are up-regulated under anoxic conditions (Banti *et al.*, 2008). Heat and heat acclimation related categories up-regulated in our experiments also support a cross-adaptation between these stresses as proposed by Banti *et al*., 2008. The evolutionary basis of the variation in submergence tolerance in these accessions might be due to interchangeable adaptations for different and/or related stresses plants encounter in their diverse habitats. We found several categories down-regulated both in roots and shoots related to other stresses (salinity, desiccation, glucosinolate and jasmonic acid biosynthesis). This might represent a strategy to save resources by decreasing demand for unnecessary processes.

Differential regulation of genes between accessions

Four ERF genes were differentially regulated between accessions in roots, all of which were down-regulated in submergence compared to darkness in both accessions but up-regulated in submergence and darkness compared to light controls only in Kas-1. Ethylene response factor genes are known for their abilities to increase low oxygen tolerance by initiating transcription of several other genes in rice and Arabidopsis (Bailey-Serres *et al.*, 2012). *SUB1A* locus controlling the quiescence strategy and *SNORKEL* genes controlling the escape strategy in rice are also ERFs and they contribute to higher survival in different flooding regimes. These ERF genes might also increase survival of Kas-1 under submergence.

Another group of genes differentially regulated between these accessions includes lipoxygenase proteins which have regulatory roles in defense mechanisms for herbivory and pathogens (Bannenberg *et al.*, 2009). Up-regulation of *LOX4* in Col (*gl1*) might be expensive for a plant under a heavy stress and might lead to faster mortality. This hypothesis might also apply to defensin-like antimicrobial proteins and cold acclimation protein (WCOR413 family) down-regulated in Kas-1 and up-regulated in Col (*gl1*). *The PHOSPHATE STARVATION-INDUCED GENE 2* (*PS2*) has been shown to be important in catalysis of PPi (May *et al.*, 2011) and is crucial for regulating PPi levels. Induction of this enzyme only in Kas-1 makes this gene a good candidate for being responsible for different submergence

tolerances. Another gene, *EXORDIUM-LIKE 1* (*EXL1*) up-regulated in Kas-1 roots under both darkness and submergence but not in Col (*gl1*) might also enhance survival in Kas-1 since this gene is induced in C-starvation and knock-out mutants show decreased survival under anoxia (Schroder *et al.*, 2011)

Candidate genes in *CQD1* QTL

In the genome-wide scan, highly differentially regulated genes between the two accessions were not identified in the QTL region, *CQD1*. The gene(s) underlying the *CQD1* locus could be regulated at the protein level, or be a major regulatory gene for which even minor changes in gene expression could have profound down-stream effects. We investigated genes differentially regulated between the accessions in this interval with a less stringent cut-off value ($p<0.05$), as the risk of false positives in a small region is lower. Differentially expressed genes in the roots included several ERFs (two of which also show amino acid variations between the accessions) showing a down-regulation only in Kas-1, a microRNA targeting several auxin responsive factors again down-regulated only in Kas-1 and a heat shock protein (showing variation in amino acid sequences between the accessions) up-regulated more in Kas-1. All of these genes could have a profound effect on submergence tolerance differences between the two accessions. In shoots, the Ca^{+2} binding protein (with four amino acid substitutions between the accessions) could cause a differential response since it is only up-regulated in Kas-1 and Ca^{+2} might be an indirect oxygen deprivation sensing molecule (Bailey-Serres *et al.*, 2012) enabling Kas-1 to react faster. *SWEET13*, a sugar transporter, is differentially regulated both in roots and shoots of the accessions. This gene is down-regulated by both darkness and combined darkness and submergence treatments. Nevertheless, it shows an up-regulation when dark controls and submergence are compared for both accessions in roots and up-regulation only in Kas-1 in shoots. *SWEET11* and *SWEET12* were shown to be involved in sucrose loading to phloem cells (Chen *et al.*, 2012) and *SWEET13* might also act similarly in Kas-1 preventing a more severe carbon starvation. All above mentioned genes are candidates as regulators of differential survival in these two accessions and should be further investigated also in other accessions showing variation in their submergence tolerance. Accordingly, we are also studying the transcriptomes of six more accessions with different submergence survival (Vashisht *et al.*, 2011) using RNA-seq to test if these accessions share common mechanisms that explain differences in submergence survival.

In conclusion, major transcriptome alterations occur even within 4 h of submergence, particularly in GO categories related to carbohydrate metabolism, anaerobic fermentation, PPi dependent processes, ethylene biosynthesis and auxin related pathways. Two accessions having different submergence tolerance show similar expression patterns for these global

anoxia/hypoxia/submergence-induced responses. However, they also show different patterns in genes that might constitute the basis of their differential submergence tolerance such as a PPi starvation gene, ethylene responsive transcription factors and auxin responsive genes. The similarities of commonly and differentially regulated processes between the two Arabidopsis accessions imply that these processes are the main mechanisms that affect survival both in species and accession level. Although transcriptome analysis reveals some important aspects of the responses of these two accessions, a more detailed analysis with knock-out mutants and over-expression lines would draw a clearer picture about the functionality of these genes under submerged conditions. The difference in submergence tolerance explained by *CQD1* locus could also be a simple amino acid variation within genes similarly induced, and these RNA-seq results should not be the only determinant of candidate gene selection. Since submergence tolerance is a process that is affected by several factors during a longer time scale, we were not able to capture later responses that might also contribute to higher survival of Kas-1. Further transcriptome analysis experiments with longer timescales and more extreme accessions might also be useful in order to detect potential regulators of submergence tolerance in later stages of the stress.

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