Transcriptomes of eight *Arabidopsis thaliana* accessions reveal core conserved, genotype- and organ-specific responses to flooding stress

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Summary: A study of eight Arabidopsis accessions reveals novel insights into early transcriptional and post-transcriptional responses to starvation and flooding stress.

Footnotes:

Author contributions: HvV, DV, MA, JB-S, MES, PvT, LACJV and RS conceived the research plans. JB-S, MES, PvT, LACJV and RS supervised the experiments. HvV, DV, MA did most of the experiments. ER and SH provided technical assistance. HvV, DV, MA, TG and RS analyzed the data. RS and LACJV conceived the project and HvV and RS wrote the article with
contributions of all the authors. MA, JB-S, MES, PvT, LACJV supervised and complemented the writing.

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Abstract
Climate change has increased the frequency and severity of flooding events with significant negative impact on agricultural productivity. These events often submerge plant aerial organs and roots, limiting growth and survival due to a severe reduction in light reactions and gas exchange necessary for photosynthesis and respiration, respectively. To distinguish molecular responses to the compound stress imposed by submergence, we investigated transcriptomic adjustments to darkness in air and under submerged conditions using eight Arabidopsis thaliana accessions differing significantly in sensitivity to submergence. Evaluation of root and rosette transcriptomes revealed an early transcriptional and post-transcriptional response signature that was primarily conserved across genotypes, although flooding susceptibility-associated and genotype-specific responses were also uncovered. Post-transcriptional regulation encompassed darkness- and submergence-induced alternative splicing of transcripts from pathways involved in alternative mobilization of energy reserves. The organ-specific transcriptome adjustments reflected the distinct physiological status of roots and shoots. Root-specific transcriptome changes included marked upregulation of chloroplast-encoded photosynthesis and redox-related genes, whereas those of the rosette were related to regulation of development and growth processes. We identified a novel set of ‘tolerance-genes’, recognized mainly by quantitative differences. These included a transcriptome signature of more pronounced gluconeogenesis in tolerant accessions, a response that included stress-induced alternative splicing. This study provides organ-specific molecular resolution of genetic variation in submergence responses involving interactions between darkness and low oxygen constraints of flooding stress and demonstrates that early transcriptome plasticity including alternative splicing is associated with the ability to cope with a compound environmental stress.
Introduction

The environment that surrounds a plant changes constantly, often imposing constraints on metabolism that modify vegetative and reproductive development. Flooding can have a dramatic impact on plant performance; and while it occurs regularly in some natural ecosystems, it is usually disastrous in controlled agricultural environments. Flooding restricts gas diffusion between submerged organs and the surrounding aquatic environment. The limited exchange of oxygen (O₂) and carbon dioxide (CO₂) slows down aerobic respiration and photosynthesis (Mommer and Visser, 2005; Zabalza et al., 2008). Turbid and muddy floodwaters restrict light penetration, further compromising photoautotrophic generation of critical carbohydrates (Vervuren et al., 2003). Finally, O₂ deficient flooded soils often have a severely reduced redox potential and accumulate toxic compounds, which limit root growth (Armstrong and Armstrong, 2001).

Flooding is therefore a compound stress, imposing multiple constraints on submerged plants. Despite this, marshes and river floodplains support a rich diversity of plant life that display a gradient of flood tolerance traits and responses (Van Eck et al., 2004; Voesenek et al., 2004). Studies on rice and several wild species have identified two antithetical survival strategies, dependent on the selection pressure of their natural flooding regime. An escape response involving rapid shoot elongation allows plants to regain air contact by forming a snorkel during shallow and prolonged floods (Voesenek and Bailey-Serres, 2015). Deep or very short floods require a quiescent strategy where a restriction of growth combined with conservation of energy expenditure and reserve utilization promotes survival until the floods recede (van Veen et al., 2014b). Fundamental knowledge of the genetic, physiological and molecular regulation of these traits is not only of general interest, but essential to improve the tolerance of many economically relevant crops, most of which are very sensitive to floods (Voesenek et al., 2014). The genetic and molecular regulation of flood adaptive strategies has been most extensively studied in semi-aquatic flood tolerant species of the genera Oryza, Rorippa and Rumex (Fukao et al., 2006; Hattori et al., 2009; Lee et al., 2009; van Veen et al., 2013; Sasidharan et al., 2013; van Veen et al., 2014a; Narsai et al., 2015).
The understanding of the flooding-induced low O$_2$ and low energy signaling networks, has also greatly benefited from studies on flood sensitive Arabidopsis thaliana. These investigations have identified the main players in energy and carbon signaling (Smeekens et al., 2010; Ljung et al., 2015) and revealed whole plant and cell-type-specific transcriptional and translational adjustments induced by low O$_2$ stress (Mastroph et al., 2009; Juntawong et al., 2014). Importantly, O$_2$ dependent degradation of the group VII family of ethylene response factors (ERF-VIIs) via the N-end rule pathway of protein degradation has been identified as a molecular mechanism that translates O$_2$ availability into transcriptional reprogramming (Licausi et al., 2011; Gibbs et al., 2011; Weits et al., 2014). Recent studies have also revealed how this molecular hypoxic response is highly regulated and fine tuned to maintain cellular homeostasis during low O$_2$ conditions (Gibbs et al., 2014; Giuntoli et al., 2014; Gonzali et al., 2015).

Despite the progress in our understanding of flooding-induced signaling pathways, much remains to be discovered regarding the molecular mechanisms that cause variation in flooding tolerance across and within species (Voesenek and Bailey-Serres, 2015). Variation in flooding responses amongst natural plant populations is an important tool to identify the underlying causal genes and processes (Xu et al., 2006; Magneschi et al., 2009; Chen et al., 2010; Cambell et al., 2015). Despite their relative intolerance to flooding stress, Arabidopsis accessions show considerable variation in their tolerance to complete submergence (Vashisht et al., 2011). Remarkably, this variation is not linked to differences in internal O$_2$ content or initial carbohydrate reserves, the two parameters generally considered to be essential for surviving flooding events.

The majority of studies investigating molecular regulation of transcriptional reprogramming in response to changes in O$_2$ availability in Arabidopsis have relied on hypoxia and/or used agar-based seedling assays (Baena-González et al., 2007; Branco-Price et al., 2008; Mustroph et al., 2009; Dennis et al., 2009; Christianson et al., 2009; Banti et al., 2010). However, in natural conditions, flooding results in a gradual decline in O$_2$ levels and is often accompanied by other physiological changes, such as a rapid build-up of the gaseous hormone ethylene (Voesenek and Sasidharan, 2013). Furthermore,
flooding imposes distinct environmental constraints on the root and the shoot, and thereby also elicits different physiological responses. Accordingly, an exploration of shoot and root responses of flooded, soil-grown plants is more relevant for understanding flooding stress as experienced in the field.

Here, we characterized the early molecular response to darkness and flooding acclimation in eight different *Arabidopsis* genetic backgrounds (Supplemental Table S1), varying in their tolerance to complete submergence, using poly(A)$^+$ mRNA-sequencing (mRNAseq). The use of soil-grown plants subjected to submergence (in the dark), mimicked naturally flooded conditions in a highly controlled way, and the inclusion of a darkness only (without submergence) treatment, allowed us to simultaneously disentangle dark effects from submergence effects (Lee et al., 2011; Vashisht et al., 2011).

Given the distinct carbohydrate and O$_2$ status of the root and shoot (rosette) tissues under these two stress conditions, these organs were analyzed separately, and then each organ response was compared to the other. This was performed for all eight accessions. Our data suggests an important role for gluconeogenesis in short-term stress acclimation, which includes alternative splicing of transcripts encoding key regulatory enzymes and quantitative transcriptional differences between tolerant and intolerant accessions. A conservative mode of energy and resource utilization via metabolic reprogramming and constrained growth contributes towards prolonged survival underwater. Shoot specific flooding-induced transcriptional reprogramming was primarily growth related, whereas in the root mainly plastidial and developmental processes changed. Our results provide insight into the interactive and additive effects of the different elements of flooding stress, present a detailed picture of early molecular events mediating stress acclimation, and identify putative novel aspects of flooding tolerance.

**Results**

**Early transcriptomic responses to flooding and darkness are largely conserved amongst accessions**

To identify early transcriptome modifications upon flooding and dark-induced starvation, eight *Arabidopsis* genotypes (Cvi-0, Bay-0, Ita-0, Col(gl1),
Kas-1, Lp2-6, Ws-2, C24) (Supplemental Table S1) were exposed to light (air + light; AL), dark (air + darkness; AD) or complete submergence (submerged + darkness; SD) for 4 hours (Figure 1A). At this time point, the decline in O$_2$ levels caused by submergence have stabilized in both the root (<0.5 kPa) and petiole (~6 kPa) as shown by O$_2$ micro-electrode measurements in submerged Arabidopsis (Col-0) plants (Lee et al., 2011).

The mapping of mRNAseq reads to the Col-0 genome was successful with 90.7 to 94.9 % of the reads mapping to only a single genomic location. The number of mapped single-hit reads ranged from 20.5 to 43.9 million per library (Supplemental Table S2). Genes of very low abundance were removed from the analysis leaving a final number of 21,940 genes. Multi-Dimensional Scaling (MDS) of the samples demonstrated a strong difference between root and shoot transcriptomes (Figure 1B). A separate MDS analysis solely on shoot transcriptomes separated all three treatments over the x-axis and the eight accessions over the y-axis. A similar result was found for the root, but here for each accession the AD and SD samples clustered together, suggesting similarity between the dark and submergence transcriptional response.

By comparing the transcriptomes of SD to AL we identified genes that respond to the compound stress. Here the compound stress is the effect of a combination of complete submergence and darkness, as often experienced by plants under naturally flooded conditions. The darkness response was teased apart from the compound stress using the AD to AL comparison. Finally, comparing SD to AD revealed a darkness-independent submergence response (Figure 1A). Depending on the accession, 2356 to 3102 genes in the shoot were identified as being differentially expressed ($P_{adj} < 0.05$) in response to the compound stress (Figure 1C). Fewer differentially expressed genes (DEGs) were identified for the darkness response (975 to 1481) and an even lower number were significantly regulated solely by submergence (84 to 581). Compared to the shoots, the compound response in the roots was of a lesser magnitude (782 to 1,133 DEGs), and a similar magnitude was found for darkness (415 to 1,325 DEGs). Furthermore, consistent with the MDS results (Figure 1B) in the roots, there was hardly any effect of submergence only (26 to 76 DEGs). To investigate the overlap in the response amongst accessions
for all identified DEGs, the accession-specific fold-changes were plotted against the average fold-change of all eight accessions (Supplemental Data Sheet C and D). The strong correlations showed that the transcriptional adjustments of all accessions were very similar, especially for the compound and darkness effects (Figure 1D, Supplemental Table S3). However, for the submergence effect, there was more variation among accessions, especially for the roots where there was substantial scatter around a mean response of zero. For the shoot, two accessions, Cvi-0 and Ws-2 clearly showed a deviation from the average submergence response, as reflected in the much lower slopes (Cvi-0: 0.755; Ws-2: 0.759; average: 1.040) (Supplemental Table S3). These also showed significantly overlapping responses to dark and compound stress in shoots (Supplemental Figure S1) and the fewest shoot submergence DEGs of the accessions (Figure 1C).

The natural variation in transcriptome responses was further investigated by identifying genes that responded in an accession-dependent manner (P_{accession*treatment, adj.} < 0.05, Supplemental Data Sheet E). In the root, 196, 288 and 137 genes were identified as significantly varying among accessions in their response to compound, darkness and submergence respectively, whereas in the shoot 562, 311, 181 genes were identified (Supplemental Figure S2). To further investigate the conserved and accession specific responses to compound, darkness and submergence and highlight the implicit important processes and players, a gene ontology (GO) overrepresentation analysis was used.

**Darkness leads to metabolic and resource adjustments; submergence is characterized by regulation of hormonal processes**

To identify the overall nature of the conserved transcriptomic responses, enrichment of GO-terms was investigated amongst genes that behaved similarly in all eight accessions (P_{accession*treatment, adj.} > 0.1) and also showed a considerable response to the imposed stresses (P_{mean response, adj.} < 0.01, and log₂FC|1.6|) (Figure 2).

Typical overrepresented processes for conserved darkness and compound effects in all accessions were related to downregulation of energetically expensive cell wall construction, sulphur metabolism, starch biosynthesis
(shoots only) and secondary metabolism. Interestingly, sucrose and fructose responses and trehalose phosphate synthase activity terms were overrepresented amongst upregulated genes in both the root and shoot. Not surprisingly, the ‘response to absence of light’ category was also overrepresented amongst the upregulated genes in both shoot and root in dark and compound stress. We also identified ‘cellular response to iron ion’ overrepresentation amongst downregulated genes in the root in response to the compound stress. The GO analyses further revealed changes associated with nitrogen metabolism. This included the ‘nitrate transport’, ‘amino acid transport’ and ‘leucine catabolic process’ terms that were overrepresented amongst upregulated genes in the shoot. Together these results suggested a fundamental change in the metabolic network in response to the applied stresses.

Compared to the darkness and compound effects, a lower number of significantly enriched GO terms were identified for submergence stress only. The upregulated genes, as expected, included anaerobic metabolism, hypoxic response and sucrose synthase activity. Other uniquely submergence responsive GO categories were hormone related (ethylene, auxin, abscisic acid (ABA)), indicating transcriptional regulation associated with these hormonal cascades that is not activated by the more metabolically determined darkness effects.

To characterize the accession-dependent responses, GO overrepresentation analysis was also performed on genes that varied in their treatment response (P\textsubscript{accession*treatment, adj.} < 0.05) (Supplemental Figure S3). The GO terms enriched amongst these genes encompassed a wide range of categories. These were mostly associated with photosynthesis and metabolism (lipids, amino acids and sulphur) and biotic defense. There was some overlap in the GO enrichment categories between the conserved and accession-dependent responses. This indicated a strong regulation of the related processes but in varying levels of conservation amongst accessions.

The compound stress response is an amplified darkness response in the shoot
In nature, severe flooding often consists of submergence coupled with very low light intensities. Here we investigated the relative contribution of darkness and submergence towards the final compound flooding stress response. In the shoot, the direct comparison of the compound and darkness response showed a strong positive correlation (Figure 3A). The steep slope suggested that the compound response was similar to the dark response but was enhanced by the addition of submergence. A similar comparison of the compound versus darkness response of the root also showed a strong correlation. However, no additional effect of submergence on the global transcriptomic response was observed, i.e. the compound and darkness effect were of a similar magnitude (Figure 3A).

To further characterize the gene categories constituting the relationships identified above, we grouped genes that were co-expressed, and are therefore potentially members of same or similarly regulated gene pathways. We used Weighted Gene Co-expression Network Analysis, WGCNA; (Langfelder and Horvath, 2008) to perform a comparative analysis of gene networks between the three conditions (AL, AD, SD). Fifteen and eight co-expression modules were identified for the shoot and root, respectively, where each module consists of genes that show largely similar expression patterns across the different accessions and conditions (Figure 3B, Supplemental Figure S4). GO term enrichment was subsequently investigated for the identified modules (Supplemental Data, Sheet F and G).

For both the root and the shoot, two very large gene co-expression modules were identified, namely R01, R02, S01 and S02 (Figure 3B). The R01 and R02 module both showed a consistent change in expression upon darkness (either an increase or decrease) in all accessions, but no change upon submergence. However, R01 and R02 differed in the constitutive expression levels of the accessions (Figure 3C). These were enriched in GO terms related to metabolism such as glycolysis/gluconeogenesis, fatty acid breakdown, acetylCoA and secondary metabolism (Glucosinolates and isopentenyl pyrophosphate (IPP)/methylerythritol (MEP) pathway), but also included sugar transport and signaling. Enrichment terms also indicated a role for jasmonic acid and brassinosteroids in the root upon darkness and compound stress (Figure 3C).
By comparison, the genes in module S01 were expressed similarly in all accessions and only had a darkness response and no additional submergence effect (Figure 3D). This module was enriched for GO categories related to the photoperiod, lipid breakdown (in the peroxisome), protein transport (required for peroxisome function), and sugar-mediated signaling (Figure 3D, Supplemental Data, Sheet G). Gene expression patterns in the other large shoot module, S02, demonstrated the amplified dark response by submergence for the compound stress. Enriched GO terms included starch and secondary metabolism. Furthermore, enrichment was found for the processes of cell division and meristem function. No clear submergence-specific module was identified in the shoot or the root (Supplemental Figure S4), likely because of the relatively small number of genes affected by submergence only (Figure 1C).

Root- and shoot-specific treatment-responsive genes are associated with photosynthesis and growth regulation

Since the organ specific responses to the treatments were more distinct than the response across accessions (Figure 1B), these differences were further explored. First, DEGs that were dependent on the organ, i.e., genes with a significant organ*treatment interaction, were identified (P_{organ*treatment, adj.} < 0.05, Supplemental Figure S5A, Supplemental Data Sheet H). These organ-dependent treatment responses were largely conserved across accessions (Supplemental Figure S5B). Genes with an organ*treatment interaction (P_{organ*treatment, adj.} < 0.05) and a significant treatment effect (P_{adj.} < 0.05) in only one organ for six or more accessions were identified and designated as either root- or shoot-specific response genes (Figure 4A, red and blue dots, respectively). The number of shoot-specific genes identified for the compound, darkness and submergence effect were 340, 33 and 13, respectively. Fewer root-specific genes were found: 59 and 48 for compound and darkness, respectively. There were no root specific genes for the submergence response. Clustering of the organ specific genes identified a strong overlap between the three treatments (Figure 4B). The compound response of the root-specific genes mirrored the darkness response, whereas
shoot-specific genes of the compound response also illustrated the amplification of the darkness response by submergence.

There was a very strong overlap in shoot specific-genes between the compound, darkness and submergence responses (Figure 4B). Among these shoot specific genes were those involved in hormonal metabolism and signaling, cell growth and cell wall modification (Supplemental Data, Sheet H). For example, the mRNA levels of *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 4*, catalyzing a crucial enzymatic step in ABA biosynthesis, was downregulated, whereas ethylene (1-AMINO-CYCLOPROpane-1-CARboxylate SYNTHASE (ACS) and 1-AMINOCYCLOpropane-1-CARboxylate OXIDASE (ACO)), gibberellin (GA20oxidase and GA2oxidase6) and cytokinin (CYTOKININ OXIDASE 3) metabolism enzymes were upregulated. Downstream signaling components typical for auxin and brassinosteroids were among the upregulated shoot-specific genes (*SMALL AUXIN UPREGULATED* (SAUR) and *SAUR-LIKE* genes, and *AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE* (ARGOS); *BXR1-SUPPRESSOR1* (BZS1), *BR ENHANCED EXPRESSION 1* and the BZR1-interacting *GENERAL REGULATORY FACTOR 8* (GRF8)). Downstream effector genes such as cell wall modifying enzymes with shoot-specific regulation (in both directions) included six genes involved in pectin esterification, two cell wall loosening EXPANSINs and eight *XYLOGLUCAN ENDOTRANsGLUCOSYLASe/HYDROLASEs*.

Several plant developmental control and light signaling genes were also amongst the regulated shoot specific genes (Supplemental Data, Sheet H). These included the genes *SQUAMOSA PROMOTER-LIKE 11* responsible for seedling to juvenile to adult stage transitions (Huijser and Schmid, 2011), but also *CLAVATA3/ESR-RELATED 16* (CLE16), *CLE6* and *CLAVATA2*, that are regulatory factors in shoot apical meristem activity (Gaillochet et al., 2015). Amongst the light signaling genes were the negative regulators of photomorphogenesis *B-BOX DOMAIN PROTEIN 18*, *SPA1-RELATED 3* and *FAR-RED Elongated Hypocotyl1* required for phyA signaling (Jigang Li, 2011). The photoperiod-related gene *FLOWERING bHLH 3* and circadian clock gene *PSEUDO-RESPONSE REGULATOR 9* were also amongst the compound shoot specific DEGs.
In the root, the compound and dark responses were identical, and no submergence root-specific genes were identified (Figure 4A and Figure 4B). Interestingly, the root-specific upregulated genes consisted mainly of chloroplast localized and photosynthesis related genes (Supplemental Data, Sheet H). This included at least seven genes involved in photosystem biosynthesis and maintenance, five additional proteins localized to the chloroplast, one essential for chlorophyll biosynthesis and two involved in photorespiration. Only a few root-specific downregulated mRNA were identified, which included two nitrate transporters, and a MATE efflux protein. In summary, mostly growth, developmental and hormonal regulatory gene transcripts were stress-induced in the shoot, whilst chloroplast encoded and photosynthesis associated genes dominated the root specific DEGs.

**Induction of the core hypoxia gene set is organ independent only when the darkness component is excluded**

Previous studies identified 51 genes that were upregulated in Arabidopsis seedlings upon hypoxic stress, regardless of organ or cell type (Mustroph et al., 2009), and which are frequently used as core hypoxia response markers. In soil grown plants, roots and shoots have distinct O$_2$ profiles under both control and submerged conditions. Soil grown roots of Arabidopsis are constitutively hypoxic and upon submergence, internal O$_2$ levels drop further from 6 % to ~0 % pO$_2$ KPa within 3 hours (Lee et al., 2011). Although the O$_2$ dynamics of Arabidopsis leaf blades is unknown, the petiole goes from 17 % to 6 % pO$_2$ KPa upon submergence in the same time span. We investigated the expression pattern of the 51 cell type-independent hypoxia-responsive genes in the context of the severe and mild low O$_2$ levels in the submerged root and shoot, respectively (Figure 4A, green dots; Figure 4C).

A majority of core hypoxia genes were regulated in both shoots and roots upon compound, darkness or submergence. However an organ-independent hypoxia signature response, involving upregulation of most of the 51 genes, was only observed for the submergence response (when the effects of darkness were excluded) (Figure 4C). This submergence response was also very similar in magnitude in the roots and shoots. In contrast, for the compound response, 18 out of the 51 core hypoxia genes were classified as
shoot specifically regulated ($P_{\text{organ*treatment, adj.}} < 0.05$ in six or more accessions). Only few of the hypoxia marker genes were classified as root or shoot-specific upon darkness. However, during darkness, the root had a predominant down regulation of most core hypoxia genes, and in the shoot several were dark upregulated in Cvi-0 and Ws-2 (Figure 4C). Interestingly, a small subset was induced upon darkness in both organs (At4g27450, At1g33055, At1g19530, At4g39675, At5g61440, At3g61060). These were previously identified as induced by C-starvation (Usadel et al., 2008) and include EXORDIUM LIKE-1 (Schröder et al., 2011). In conclusion, it is clear, that for the compound response, the behavior towards darkness is an important determinant of the difference between the shoot and root for these cell-type independent hypoxia marker genes (Figure 4A and Figure 4C).

Conserved alternative splicing events indicate an additional layer of regulation in the adaptation to compound, darkness and submergence stresses.

By using mRNAseq as a platform we were able to investigate transcriptome reconfiguration at the mRNA isoform level corresponding to variations in mRNA splicing events. These events can include exon skipping, mutually exclusive (alternative) exon usage and alternative donor and acceptor splice sites that can alter protein coding and untranslated regions, all of which are generally termed as alternative splicing (AS). Another event is intron retention (IR), which involves the retention of introns in the mature mRNA. IR events that result in an open reading frame that is upstream of an intron junction typically target transcripts for nonsense mediated decay and are therefore unstable mRNA isoforms (Kazan, 2003). We focused on splice site selection and IR variants similar to the method described in Chang et al. (2014), by characterizing the relative increase or decrease in specific variants between samples. More specifically, for each accession, we characterized AS and IR induced by the three treatments (compound, darkness, submergence); and for each of the three conditions (AL, AD, SD) we characterized the relative variant usage between the eight accessions.

A considerable level of both IR and AS events was identified between the 8 accessions, which was largely independent of the treatment (Supplemental
Figure S6A and S6C). 1819 and 1014 IR events were treatment independent
(|log_{2}FC| > 1, P_{adj.} < 0.01) in the root and shoot respectively (Supplemental
Figure S6B). For AS, 2061 and 1798 treatment independent root and shoot
events (|log_{2}FC| > 1, P_{adj.} < 0.01) were identified (Supplemental Figure S6D).
The consistency and strong overlap in AS and IR across the three conditions
(AL, AD and SD) indicated that these are robust differences between the
accessions. However, with respect to the acclimative responses to darkness,
compound and submergence stress, the treatment-induced splicing events
were of more interest (Figure 5). While 1214 and 2122 genes with IR events
(|log_{2}FC| > 1, P_{adj.} < 0.01, in at least one accessions) were found in the root
and shoot, corresponding numbers for treatment induced AS events (|log_{2}FC|
> 1, P_{adj.} < 0.01, in at least one accessions) were 210 (root) and 2471 genes
(shoot). For both AS and IR events, the overlap between accessions was
minimal (Figure 5B and Figure 5D). However, the genes with conserved
splicing behaviour across the accessions were of interest as robust examples
of darkness and submergence-induced post-transcriptional regulation. Indeed,
the 167 and 63 genes in the shoot and root, that showed IR in 5 or more
accessions, showed consistent behavior across the accessions and,
depending on the gene, IR was favoured either upon the stress condition (AD
and SD) or under air light conditions (Supplemental Figure S7 and
Supplemental Figure S8).

Compared to IR, fewer treatment-dependent conserved AS events were
identified, with 15 and 31 genes displaying AS in five or more accessions for
roots and shoots, respectively (Figure 6). For these genes this additional
aspect of transcriptome reconfiguration in response to compound, darkness or
submergence stress would not only affect mRNA stability, as is the case for
IR, but could potentially also lead to altered protein function, localization or
influence other post-transcriptional processes such as translational efficiency.
These regulatory processes would be in addition to the differences we already
observed in the total transcript abundance of these genes between the
treatments (Supplemental Figure S9). To verify the validity of the observed AS
patterns, independent qRT-PCR analyses using Cvi-0 and C24 accessions as
representatives was done. We tested 6 genes that in addition to showing
distinct AS patterns, also showed strong transcriptional regulation. All the six
genes tested, confirmed the mRNAseq-based evidence of AS (Figure 7). This also revealed that AS began within a few hours of stress and persisted at elevated levels over 48 h of compound, darkness and submergence treatments (Figure 7). For most genes tested, the increase in splice variant isoform(s) occurred rapidly and then declined somewhat (e.g., ROPGEF11, At1g52240; Figure 7). Several of the 46 genes with conserved AS events could have an important role in acclimation to the imposed stress.

For instance in the root, ROP (RHO OF PLANTS) GUANINE NUCLEOTIDE EXCHANGE FACTOR 11 (ROPGEF11, At1g52240) preferentially produced a short transcript over a longer transcript isoform under darkness and compound stress, with total transcript abundance elevated by both stresses (Figure 6 and 7). The shorter isoform lacks the Rop Nucleotidyl Exchanger domain (PRONE, PF03759), which for ROPGEF11 is implicated in phytochrome interactions in the regulation of root development (Shin et al., 2010), and instead contains a dynein light chain domain (PF01221) (Figure 8A). Another interesting gene was ARABIDOPSIS RESPONSE REGULATOR 1 (ARR1), a cytokinin receptor (Kieber and Schaller, 2014) that was darkness-induced and accumulated an alternatively spliced variant in the shoot and root (Figure 6 and Figure 8B). Although AS of the pre-mRNA of ARR1 results in transcript isoforms that encode two distinct proteins, both contain all known conserved domains of the receptor, but differ in their carboxy termini. The isoform variant of ARR1 that is elevated in darkness also has a shorter and distinct 3’ untranslated region, which could influence interaction with RNA binding proteins that alter mRNA stability or translation.

Among the genes displaying AS was a relatively large group of genes associated with metabolic functions (Figure 6). Many of these were differentially regulated by either darkness or submergence. These included two FRUCTOSE BISPHOSPHATE ALDOLASEs (FBA1 and FBA5), a SUCROSE 6-PHOSPHATE PHOSPHORYLASE and GLUTAMATE DEHYDROGENASE 2. Also of relevance was PEROXISOMAL NAD-MALATE DEHYDROGENASE 2 (PMDH2), which is required for redox balance during fatty acid breakdown in the peroxisome (Pracharoenwattana et al., 2007). PMDH2 AS upon darkness and submergence favored an enzyme form with increased activity (Figure 8C). Of particular interest were the AS patterns of
PYRUVATE ORTHOPHOSPHATE DIKINASE (PPDK, At4g15530) and LYSINE-KETOGlutARATE REDUCTASE/SACCHAROPINE DEHYDROGENASE (LKR/SDH, At4g33150). PPDK is a single-copy gene that is induced by low O₂ in a variety of species (Huang et al., 2008). Of the five PPDK transcript isoforms, the shortest transcript (At4g15530.2) preferably and progressively accumulated upon darkness and submergence (Figure 7 and Figure 8D). This transcript encodes the cytosol-localized form of PPDK (Parsley and Hibberd, 2006) that is suggested to be important during amino acid remobilization and senescence (Taylor et al., 2010), and for gluconeogenesis (Eastmond et al., 2015). Interestingly, several other genes involved in gluconeogenesis were quantitatively up or downregulated in response to the compound and darkness treatments (Supplemental Figure S10). Of these, only PPDK showed pronounced upregulation across accessions under submergence.

LKR/SDH (At4g33150) encodes a bifunctional enzyme catalyzing the first two steps of lysine catabolism. While the LKR component of the protein works in the lysine catalytic direction, the SDH component has bidirectional enzymatic activity (Zhu et al., 2002). Besides being strongly upregulated by darkness and compound stress in roots and shoots (Supplemental Figure S9), AS of LKR/SDH was evident in the root (Figure 6, Figure 7 and Figure 8E), with the longer transcript being favored under both conditions (Figure 7E). The long transcript results in a protein with both LKR and SDH activity, whereas the short transcript only has SDH activity (Zhu et al., 2002).

In the shoot, a relatively large amount of AS occurred in transcripts related to photorespiration (GLYCERATE KINASE, GLYCOLATE OXIDASE 1), light capture (PHOTOSYSTEM 1 LIGHT HARVESTING COMPLEX GENE 1, a putative cytochrome b6f complex subunit, NON PHOTOCHEMICAL QUENCHING 1 and 4 (NPQ1 and 4)), CO₂ sensing (BETA CARBONIC ANHYDRASE 1 AND 4 (BCA1 and BCA4)) and plastid development (F-box family protein, PLASTID REDOX INSENSITIVE 2 (PRIN2), TRANSLOCON AT THE OUTER ENVELOPE MEMBRANE OF CHLOROPLAST). These AS variants do not necessarily lead to distinctions in the encoded protein, but modify untranslated regions of the mRNA (Figure 8F and Figure 8G), and hence could influence other post-transcriptional processes. Intriguingly, both
beta carbonic anhydrase loci (*BCA1* and *BCA4*) displayed differences in the 5' UTR between the treatments (Figure 8H and Figure 8I). The isoforms preferentially accumulating in darkness and submergence encode N-terminally truncated proteins that retain enzymatic activity but lack the sequences responsible for specific subcellular targeting (*BCA1* to the chloroplast and *BCA4* to the plasma membrane) (Fabre et al., 2007). Altogether these data demonstrate that AS can serve as a post-transcriptional control point that impacts the accumulation, location and activity of a number of proteins that regulate carbon flux.

**Natural variation in submergence tolerance is associated with relatively minor transcriptomic differences in a group of putative “tolerance genes”**

A previous study that used an identical experimental set up showed considerable variation in the tolerance to complete submergence in the dark (compound stress) among 86 Arabidopsis accessions (Vashisht et al., 2011). This variation could not be ascribed to difference in anatomy, decline in organ O$_2$ content, or initial carbon resources. Based on that study, three accessions profiled here were classified as submergence sensitive (Cvi-0, Bay-0 and Ita-0), and three as tolerant (Lp2-6, Ws-2, C24). The tolerant accessions also performed better when their survival under submerged conditions (SD) was compared to their survival under darkness (AD) (submergence stress). Based on this prior study, genes that responded differently to compound and submergence stress were identified by comparing the tolerant with the sensitive accessions (P$_{tolerance*treatment}$, adj., < 0.05, Supplemental Data Sheet K). In this way, 33 and five potential ‘tolerance genes’ were identified in the shoot for compound and submergence stress, respectively (Figure 9A, Supplemental Data Sheet K). A larger number of potential tolerance genes were identified for the root (47 compound and 43 submergence). Although this relatively large number of potential tolerance genes could be responsible for differential tolerance among these accessions, the magnitude of the difference was not always large (Figure 9B).

Interesting shoot ‘potential tolerance genes’ with a stronger overall increase in expression in tolerant genotypes included the PPi utilizing and
gluconeogenic enzyme PPDK (AT4G15530) and a gene encoding a natural antisense RNA (AT4G20362) to a RAB GTPase homolog (AT4G20360). Also predominantly upregulated in tolerant genotypes was plant DEFENSIN 1.2b (AT2G26020). Additionally the shoot potential tolerance-genes included several growth- and cell wall-associated genes such as XTH4, an auxin responsive GH3 family protein and EXPANSIN A16, which were more induced in the sensitive accessions (Supplemental data, Sheet K). This suggested a more conserved growth response in the tolerant accessions in our treatment conditions. To assess whether this was indeed true, petiole elongation rates of a sensitive (Cvi-0) and tolerant (C24) accession were measured as a marker for shoot growth (Supplemental Figure S11). The tolerant C24 had a greater reduction in petiole elongation rates (relative to AL) compared to Cvi-0 in both light and dark submerged conditions. In the dark, Cvi-0 petiole elongation rates were similar to control plants. In contrast, in C24, petiole elongation rates were reduced to less than 33% of control (AL) rates.

The ‘potential tolerance genes’ from the root were of a different nature compared to the shoot, and no overlap in gene composition was found between the two organs. For instance FERRIC REDUCTION OXIDASE 4 and 5 (FRO4 and FRO5, AT5G23980 and AT5G23990), which play an important role in the uptake of iron and copper from the soil (Jain et al., 2014; Bernal et al., 2012), were identified. Additionally a vacuolar iron transporter and a metal transporter (At3g25190, VACUOLAR IRON TRANSPORTER-LIKE 5; AT5G59520, ZRT/IRT-LIKE PROTEIN 2) were classified as ‘potential tolerance genes’. All of these genes had stronger downregulation in the tolerant accessions, especially upon submergence. Another root potential tolerance gene, LOW PHOSPHATE ROOT1 (AT1G23010) is involved in sensing and signaling of low Pi availability in the root in an iron dependent manner (Svistoonoff et al., 2007; Müller et al., 2015), and was upregulated in the sensitive and downregulated in the tolerant accessions (Supplemental Data, sheet K).

Interestingly, several of the ‘potential tolerance genes’ identified here have been previously identified as commonly hypoxia regulated through the plant kingdom (Mustroph et al., 2010). This was especially the case in the root, with
tolerance group dependent regulation upon the compound stress for HYPOXIA UNKNOWN PROTEIN 37 (HUP37, AT2G41730), SIMILAR TO RCD ONE 5 (SRO5, AT5G62520), an unknown protein (AT3G23170) and CALMODULIN-LIKE 38 (AT1G76650), recently shown to be a calcium-regulated cytosolic RNA binding protein (Lokdarshi et al., 2016). A close homolog of SRO5, namely SRO4 (AT3G47720) was also identified as a ‘tolerance gene’ in the root. For the shoot compound stress this category of genes included PPDK (AT4G15530) and PYRUVATE DECARBOXYLASE 1 (AT4G33070).

Discussion

Anatomical and physiological features contribute little towards the large variation in the tolerance to darkness and submergence observed in A. thaliana accessions (Vashisht et al., 2011). For this reason, the short-term transcriptomic acclimation was investigated in a selection of eight accessions under highly controlled conditions that closely mimic natural flooding events in the field. Furthermore, the experimental design made it possible to disentangle the effects of darkness from the responses caused by reduced gas diffusion (i.e., O2, CO2 and ethylene) in the underwater environment. This allowed us to identify conserved processes in relation to the naturally occurring stress, whilst simultaneously identifying accession and tolerance specific processes. The systematic comparison of the shoot and root transcriptomic adjustments of eight accessions to flooding and starvation stress revealed robust conservation in a response, which encompasses specific transcript isoform production, but also contained subtle and possibly significant distinctions between flooding tolerant and intolerant accessions. Our results emphasize that understanding plant adaptation to flooding requires consideration of its compound nature, which often includes reduction in light or even complete darkness in combination with a severe reduction of gas exchange.

Alternative metabolic reserve mobilization as a coordinated transcriptional response to darkness and submergence
Darkness and submergence both cause similar physiological changes in affected plants, including carbohydrate depletion, utilization of alternative carbon sources and chlorophyll degradation. However, *Arabidopsis* has an amazing capacity to buffer its metabolism to unexpected darkness (Graf et al., 2010). An unexpected early night leads to appropriate reductions in the rate of starch breakdown. This adjustment allows the available carbohydrate reserves to last throughout the longer-than-expected night and thus prevents starvation-related transcriptional responses. However, our dataset revealed transcriptome reconfigurations typical of starvation responses already within 4 hours. This suggests that imposing darkness or submergence stress during approximately the first half of the light period under short day conditions is taxing on the buffering capacity of *Arabidopsis*. The metabolic buffering capacity has been shown to require the circadian clock and targets starch breakdown (Graf et al., 2010). Consistently, in our analysis starch breakdown and biosynthesis were predominately down regulated upon darkness, an effect that was even stronger when coupled with submergence. However, while the clock and circadian machinery were also affected by darkness, there was no additional effect of submergence, in line with the large contribution of light cues in entraining the circadian rhythm (Hsu and Harmer, 2014).

Instead of starch, the transcriptional activation of fatty acid and amino acid breakdown was observed, indicating the occurrence of autophagy (intracellular degradation and recycling of cellular components). *Arabidopsis* mutants defective in autophagy are highly sensitive to submergence (Chen et al. 2015). Several of these genes involved in fatty acid and amino acid breakdown are crucial to maintain energy status and performance during stress conditions or in non-photosynthetic developmental stages. This was shown in mutant studies on *LKR/SDH* (lysine breakdown) and *PMDH2* (fatty acid beta oxidation) in heterotrophic germinating seeds (Pracharoenwattana et al., 2007; Angelovici et al., 2010). Interestingly, for both these genes, specific transcript isoforms were preferentially induced upon compound, darkness and submergence stress. For *LKR/SDH*, the preferentially induced longer transcript favors lysine breakdown (Tang et al., 2002), and for PMDH2, isoforms with the intact enzymatic domain preferentially accumulated, suggesting increased enzymatic activity upon the imposed stresses. Several
other genes encoding key metabolic enzymes of the amino acid and fatty acid breakdown pathways, displayed AS upon compound, darkness and submergence (Supplemental Figure S12). This suggests that AS provides an additional layer of regulation that could have a significant impact on metabolite fluxes during these stress conditions.

A strong upregulation of transcripts of glyoxylate pathway enzymes was also observed. The glyoxylate pathway shortcuts a part of the TCA cycle (from isocitrate to succinate) thereby preventing the loss of two CO₂ molecules and thus preserving fixed carbon. This suggests that protein and fatty acid breakdown is not necessarily being utilized in respiration and energy production, but also is used for sugar biosynthesis. The activation of key steps of the gluconeogenic pathway, involving PEP CARBOXYKINASE (PCK) and PPDK, upon compound, darkness and submergence further points towards the utilization and mobilization of alternative carbon resources (Supplemental Figure S12). PPDK regulation was of special interest since the cytosolic transcript isoform that was preferentially upregulated has been shown to increase nitrogen mobilization when overexpressed (Taylor et al., 2010). Furthermore, PPDK was more strongly upregulated upon compound stress in the tolerant accession C24 than the sensitive Cvi-0, a pattern that persisted over time (Figure 7). Besides their function in C4 photosynthesis, PCK and PPDK have been studied primarily in the context of their gluconeogenic role in reserve mobilization in germinating seeds (Penfield et al., 2004; Delgado-Alvarado et al., 2007; Malone et al., 2007; Eastmond et al., 2015), and around vein tissue where they also display high activity (Hibberd and Quick, 2002; Brown et al., 2010). Furthermore, PPDK and PCK are upregulated in submerged Rumex acetosa (van Veen et al., 2013), anoxic rice coleoptiles (Narsai et al., 2009) and waterlogged Arabidopsis roots (Hsu et al., 2011).

In line with previously identified roles and functions we hypothesize that under compound, darkness and submergence stress, the enzymes PCK and PPDK could occupy a key function in fuelling starving plant organs with reserves alternative to starch, possibly by redirecting energy rich metabolites from source leaves to sink meristems and roots. An additional adaptive benefit of utilizing alternative resources to starch is that the maintenance and upkeep associated with high protein levels and organelles (Amthor, 2000) can
be minimized. Thus sacrificing older leaves, minimizing the requirements of young leaves and concentrating resources in the meristems might provide a useful strategy to persist under adverse flooded conditions.

A common observation in studies profiling metabolic changes upon flooding is an increase in the levels of certain amino acids. This has been documented for instance, in submerged rice shoots (Barding et al., 2013), anoxic rice coleoptiles (Narsai et al., 2009) and waterlogged poplar and *Lotus japonicus* (Kreuzwieser et al., 2009; Rocha et al., 2010). Indeed, in senescing leaves, increased protein breakdown and amino acid catabolism coincides with increased amino acid content (Hildebrandt et al., 2015; Watanabe et al., 2013). Similarly, in petioles of submerged *R. acetosa* plants a large increase in free ammonia was observed upon submergence, suggesting an increased amino acid breakdown (van Veen et al., 2013). Further metabolic evidence for amino acid catabolism comes from *Arabidopsis* mutants defective in energy starvation signaling. In these lines, a similar suite of catabolic and gluconeogenic genes were regulated as observed here, including PPDK, and a subsequent altered metabolic profile was observed (Hartmann et al., 2015).

However, it is difficult to discern metabolic fluxes from transcriptomic and metabolomic data except when start or end products of specific routes are quantified. Indeed, Rocha et al. (2010) and Antonio et al. (2015) provide a model based on isotope flux determination, specific to low O2 availability induced by waterlogging, where the intertwining of nitrogen metabolism and the TCA cycle potentially doubles ATP production relative to glycolysis alone, when the mitochondrial electron transport chain is compromised. This requires pyruvate to be funneled to alanine, to prevent pyruvate-induced respiration, the blocking of TCA cycle at succinate dehydrogenase (also downregulated in our study (Supplemental Figure S12), and activation of the GABA shunt. These, however could be fundamentally different processes than what is observed under our experimental conditions, given the bulk of the transcriptomic changes we observed occurred in response to darkness in air-grown plants, where the advantages of these adaptations to low O2 would be less apparent.

Although we assign a significant role to PPDK in resource mobilization, its relevance during hypoxic conditions has been previously attributed to its role...
in the low O$_2$-induced switch to PPI dependent glycolysis (Huang et al. 2008; Mustroph et al., 2014b). The primary advantage of PPI utilizing enzymes such as PPDK is the conservation of ATPs and the yield of additional ATPs for each sugar molecule going through glycolysis. This could be essential for survival during low O$_2$ conditions when the electron transport chain, which provides the bulk of ATP, is hampered. However, the activation of PPDK already under darkness strongly favors a role in gluconeogenesis, since under these conditions the ATP gain is almost negligible. Although PPI utilizing enzymes do provide a more energetically favorable route during anaerobic metabolism, whether these pathways are preferred during low O$_2$ conditions is now under scrutiny. Analyses of sucrose synthase mutants under hypoxic conditions suggest that, despite hampered performance under flooding stress, a major portion of the carbon provided for glycolysis is still generated by the ATP-dependent invertase and not via the PPI-linked sucrose synthase route, at least in Arabidopsis thaliana (Santaniello et al. 2014). In the context of the role of PPDK as well, a reassessment might be required for its precise role during hypoxic glycolysis.

The observation that, upon submergence the shoot shows an amplification of the darkness response, underscores the knowledge that plants tightly adjust their metabolism to suit their environmental conditions. Additionally, we observed that the transcriptional changes identified here are typical of carbon and energy starvation, which requires resource mobilizations. In addition to this, differential regulation of translation is another regulatory control point under hypoxia and in darkness (Branco-Price et al., 2008; Pal et al., 2013; Juntawong et al., 2014). Our studies also suggest that AS might play an important role in this response as an additional layer of regulation in the coordinated mobilization of existing and alternative reserves to endure starvation conditions and prolong underwater survival (Supplemental Figure S10).

Organ specific transcriptome reconfiguration and O$_2$ dependent responses

Previous studies have shown that following low O$_2$ stress and submergence, the root and shoot transcriptomes are reconfigured in a distinct manner
underscoring variation between these organs in cues and protective
mechanisms (Ellis et al., 1999; Mustroph et al., 2009; Lee et al., 2011;
Mustroph et al., 2014a). The differences between root and shoot in their
transcriptome responses to darkness and submergence signals can be
attributed to several factors, including the autotrophic and heterotrophic
nature of the shoot and root, respectively, different cellular identities and
composition, distinct physiological functions and varying O2 profiles.
Endogenous O2 levels are primarily determined by a balance between the
internal production/consumption and the rate of inward diffusion from the
surrounding environment (soil, air or water). In Arabidopsis roots, O2 levels
drop from 6% to 0 % and in the petiole from 17 % to 6 % pO2 KPa (Lee et al.,
2011) within 3 hours (O2 levels in the lamina are unknown). The
transcriptomic profiling of both organs using mRNAseq allowed for a detailed
investigation of organ-specific responses to darkness, submergence and the
role of O2 herein.

The large-scale differences between the organs were typified by a higher
number of DEGs and stronger gene expression fold-changes in the shoot,
than the root. A possible explanation for the greater responsiveness of the
shoot is that roots continuously habituate a dark environment under control
(AL) conditions, meaning that the transition to dark treatment would have had
a relatively smaller impact. The fact that Arabidopsis roots are non-
photosynthetic and constitutively a sink may also contribute to their less
dramatic responsiveness. More striking, however, was the lack of an
amplification of the darkness responses in the compound transcriptome
behavior in the root. This could also reflect the existing sink-source
relationship between the root and shoots; wherein roots typically dependent
on the shoot for carbon resources were already maximally starved after the
dark treatment, whereas the shoot had more reserves to buffer the response.

Despite the fewer transcriptional changes observed in the root, several
processes were identified as root-specific. Plants have several sensing and
signaling mechanisms to detect changes in redox and maintain redox
homeostasis, which is important in all aspects of plant growth and
development. Interestingly, genes of this category were prevalent among the
root specific genes such as thioredoxins and rubredoxin. Furthermore, many
photosynthesis related genes were amongst the root-specific genes (e.g., photosystem I and II proteins). The increased expression of these genes and several other chloroplast-associated genes in the roots indicates the presence of chloroplasts in this organ, likely in the cortex (Dinneny et al., 2008). While root greening has been described before, it is known to occur only in the presence of a light signal (Usami et al., 2004; Kobayashi et al., 2012). Elevated transcripts encoding photosynthesis-chloroplast associated proteins in roots was reported previously in hypoxic/flooded seedlings of Arabidopsis and Rorippa (Sasidharan et al., 2013; Chang et al., 2012). In contrast to this work, the roots in these studies were at some point exposed to light signals. Sugar starvation and salt stress are also reported to induce photosynthesis-associated genes in roots (Sheen, 1990; Baena-González et al., 2007; Dinneny et al., 2008). It has been speculated that this might be triggered by reactive oxygen species (ROS) generated during the stress and with a potential role in ROS amelioration (Dinneny et al., 2008). The relevance of the expression of these genes in a root specific manner upon darkness and compound stress in the absence of a light signal remains intriguing. The regulation of ROS production is likely a relevant function in stressed roots. However, whether this is the case here and what the underlying mechanism is, remains to be determined.

Unlike the root, the shoot responded differently to darkness and submergence. Shoot-specific genes upregulated by these conditions across the accessions were associated with growth, senescence and oxidative stress, all elements of the underwater response. Although there was an upregulation of transcripts of genes associated with growth and growth-associated hormonal signaling (GA, ABA), in the shoot, the petiole elongation response to dark and compound stress was varied across accessions (Supplemental Figure S11) (Vashisht et al., 2011). Considering that whole shoots were sampled here, the involvement of these shoot-specific genes in mediating changes in leaf expansion or perhaps hyponasty cannot be ruled out. Nevertheless, the shoot core gene set reflects growth regulation and extensive regulation of cell wall modifying proteins and growth regulatory hormones. This likely is reflective of specific growth strategies that are an
important mechanism to deal with both flooding (van Veen et al., 2014b) and
low light conditions (Gommers et al., 2013). Interestingly, amongst the compound shoot-specific genes, was a subset of
core hypoxia genes. Although previous studies have established their cell-
type independent hypoxia upregulation, the shoot-specific regulation here was
not surprising (Mustroph et al., 2009). Oxygen measurements on soil-grown
plants in an identical set-up have revealed that despite being in well-aerated
soils, these soil-grown roots were already hypoxic (~6% pO2). Considering the
already hypoxic conditions of roots under control conditions, it can be
speculated that constitutive expression of these genes is associated with
acclimation to hypoxic conditions. Unlike seedlings grown on vertical agar
plates that experience a normoxic to hypoxic transition, in our system,
submerged roots transition from hypoxic to severely hypoxic conditions (Lee
et al., 2011). Interestingly, it has been shown that low O2 in the root tip only is
sufficient to activate low O2 responsive genes throughout the entire root
(Mugnai et al., 2012). In active meristems, hypoxia is a common event during
periods of high activity (Van Dongen and Licausi, 2015). Interestingly,
darkness caused a significant repression of the core hypoxia genes in the
root. This suggests that although similar physiological responses are triggered
during submergence and darkness, associated with starvation conditions,
transcriptomic responses are prioritized to adapt to starvation in the presence
of O2. The core hypoxia signature including the inefficient fermentative mode
of energy generation would then be a wasteful mode of energy generation
under carbon limiting conditions.

Clearly, in the final compound response the behaviour in response to
darkness largely determines the difference between the shoot and root for
these cell type-independent hypoxia-responsive genes (Figure 4C). However,
overall a shift to severe or mild low O2 levels did not lead to a different
response when the effects of darkness were disregarded and only
submergence-induced hypoxia was considered. Even the magnitude of the
core hypoxia gene upregulation was similar for shoots and roots (Figure 4A
and Figure 4C). Remarkably, these distinct transcriptomic reconfigurations of
the shoot and root systems were highly conserved across the eight
accessions of Arabidopsis.
Natural variation in submergence tolerance

Natural variation in stress responses can be exploited to identify molecular processes and components that regulate stress responses in a differential way and therefore determine tolerance. Previous studies established significant variation in flooding tolerance of six accessions used in this study at the level of whole plant survival (Vashisht et al., 2011). This allowed us to classify the accessions into two tolerance groups and correlativey identify potential tolerance genes based on altered transcript accumulation or distinctions in AS. Interestingly, the potential tolerance genes identified in the root had no overlap with those from the shoot, further underscoring the distinct physiological states and functions of these two organs. Despite the lack of overlap between root and shoot potential tolerance genes, both included members of the core hypoxia responsive gene set.

Interesting shoot potential tolerance genes that have previously not been implicated in flooding survival included the gluconeogenic enzyme PPDK, which was highly upregulated in the tolerant accessions. Targeted qPCR analyses confirmed this trend over a 48h period (Figure 7) and revealed a much stronger upregulation in the tolerant (C24) accession. Previous studies have suggested a role for the PPI utilizing PPDK in mobilizing protein stores (Huang et al., 2008) and in facilitating nitrogen remobilization in senescing leaves (Taylor et al., 2010). Furthermore, it has a key position in a metabolic network we identified as being important during starvation and submergence (Supplemental Figure S12). It further stresses the importance of efficient alternative reserve mobilization during energy limiting conditions. Future biochemical and metabolic studies are necessary to determine if upregulation of a cytosolic PPDK enhances the utilization of non-carbohydrate stores to enhance energy production and long-term survival.

Another interesting shoot-specific potential tolerance gene was an antisense to a RAB GTPase homolog (AT4G20360) with organellar translation elongation factor (EF) activity. Previous studies have shown that Arabidopsis seedlings exposed to hypoxia drastically limit translation as a means to curb energy expenditure (Branco-Price et al., 2008; Juntawong et al., 2014; Sorenson and Bailey-Serres, 2014). The upregulation of this antisense RNA
in tolerant accessions could serve to function to limit EF-Tu synthesis, thereby limiting overall levels of plastid or mitochondrial mRNA translation.

Several shoot potential tolerance genes that were induced only in the sensitive accessions had functions in growth- and cell wall-remodeling, implying more dampened growth responses in the tolerant genotypes. Consistently, we found that petiole elongation rates were significantly lower in submerged plants (relative to control (AL) grown plants) of a tolerant accession (C24). In contrast, a sensitive accession (Cvi-0) maintained control petiole growth rates under compound stress (SD) conditions (Supplemental Figure S11). Taken together, this suggests that tolerance in Arabidopsis can be attributed to a conservative mode of energy utilization and efficient carbohydrate management resulting in prolonged underwater survival.

Tolerance is a complex phenomenon, especially in the case of a compound stress like flooding. Several aspects come into play, such as the environmental conditions and the physiological state of the plant before, during and after submergence. Our data suggests that tolerant Arabidopsis accessions have restricted shoot growth and exhibit conservative and alternative resource utilization, involving specific stress-induced metabolic readjustments. This is likely an important factor influencing tolerance in the shoot, whilst in the roots tolerance appears to involve genes related to hypoxia and development. This suggests that for achieving tolerance, different alterations may be required in the root and shoot.

The tolerance to starvation stress is another factor that interacts with low O2 stress to influence the final outcome of tolerance. As observed here, some accessions (i.e., Ws-2 and Cvi-0) showed largely overlapping dark and submergence transcriptomes. This suggests that the tolerance of Ws-2 could partly be due to its ability to withstand starvation stress. Similarly, the sensitivity of Cvi-0 is likely linked to its poor performance under dark conditions (Vashisht et al., 2011). Accordingly, Cvi-0 petiole growth rates in the dark (AD) equaled control (AL) rates. This would likely result in a faster depletion of existing energy and carbohydrates reserves under stress conditions and hasten plant demise.

**Conclusions**
The current upsurge in the number of global flooding events underscores the importance of understanding tolerance mechanisms and plant responses to flooding stress. Knowledge of the basis of variation in stress tolerance is also critical for developing more stress resistant crops for environments experiencing unexpected floods. This work details transcript abundance and AS alterations in soil-grown vegetative-stage Arabidopsis rosettes that was surprisingly conserved across a set of eight diverse genotypic backgrounds. Contrasting this conservation was the distinct transcriptomic reconfiguration of the shoot and root across the accessions, reflecting each organ’s anatomical and physiological identity and highlighted unique metabolic and developmental plasticity as a result of the stress. We showed that alternative selection of splice sites provides an additional layer of molecular regulation to fine tune the response to flooding and starvation stress. Our study also reveals that tolerance in Arabidopsis is related to the ability to restrict shoot growth and exhibit conservative and alternative resource utilization, involving specific stress-induced metabolic readjustments.

Materials and Methods

Plant material and growth conditions. Seeds of the studied accessions (Bay-0: N22633, C24: N22620, Col(gl1): N3879, Cvi-0: N22614, Ita-0: N1244, Kas1: N22638, Lp2-6: N22595,Ws-2: N1601) were obtained from Nottingham Arabidopsis Stock Centre (NASC, UK). They were sown in a soil : perlite (1 : 2) mixture and cold (4°C) stratified in the dark for 4 days. Germination occurred at 20°C, 160 μmol m⁻² s⁻¹ photosynthetically active radiation (PAR) (9 h photoperiod) and 70% relative humidity. At the two-leaf stage seedlings were transplanted, one seedling per pot (70 ml) with the soil : perlite (1 : 2) mixture enriched with 0.14 mg MgOCaO (17%; Vitasol BV, Stolwijk, the Netherlands) and 0.14 mg of slow-release fertilizer (Osmocote ‘plus mini’; Scotts Europe bv, Heerlen, the Netherlands) per pot. Each pot received 25 ml of nutrient solution of the composition described by (Vashisht et al., 2011). The soil was covered with black mesh with a small hole for the seedling to grow through. The mesh prevented floating of soil material during submergence experiments. Plants were grown in climate-controlled chambers (20°C, 160 μmol m⁻² s⁻¹ PAR (9 h photoperiod) and 70% relative humidity).
Plants were automatically watered each day at the start of the photoperiod. Submergence experiments were performed after plants reached a developmental stage of 10 leaves.

**Experimental conditions.** Plants were completely submerged in plastic tubs (60 x 40 x 27 cm) filled to the brim with tap water and allowed to acclimatize overnight. Both darkness only and darkness and submergence treatment took place in the same conditions as plant growth, but with the lights off (in complete darkness). Experiments were started two hours after the start of the photoperiod. Tissue harvest was done with a low intensity green safe light.

Samples for air light, air darkness and submergence darkness were harvested after 4 hours of treatment. Roots and shoots were harvested separately and the hypocotyl region (region between shoot base and at the beginning of first lateral root) was left out. The experiment was performed individually three times. Each time, five biological replicates, each including a pool of five individual plants were sampled and the tissues were flash frozen in liquid nitrogen. All samplings were completed within 30 minutes minimizing effects of circadian rhythms, and plants for each treatment were harvested simultaneously at the two chambers (light and dark chamber).

**Petiole elongation measurements.** Plants of Cvi-0 and C24 were grown as described above and when they reach the 10 leaf developmental stage subjected the same experimental conditions. From a homogenous set of plants the leaf blades of the 7th developed leaf were marked with a pink dye. Petiole lengths were measured using a digital caliper before and after 72h of treatment on the same petiole.

**RNA extraction and sequencing.** Plant tissue was ground with a mortar and pestle, after which the RNA was extracted using the RNeasy plant RNA isolation kit (Qiagen). DNA was removed via on-column DNAase digestion using the RNAase-Free DNase kit (Qiagen). For RNA sequencing, for each treatment, RNA samples consisted of RNA pooled from biological replicates that showed consistent results in terms of marker gene expression and petiole elongation response to submergence. Library preparation and sequencing (on a HiSeq 2000) was done commercially (Macrogen (www.macrogen.com)). All treatments for each accession per organ type were bar coded in the same
sequencing reaction to allow multiplexing of three samples per lane. Single end reads of 50 bp length were obtained.

**Quality control and read mapping.** All the sequenced libraries had the Phred quality score ranging between 30-40 indicating 99.9% of base call accuracy. Therefore all reads were mapped to the TAIR10 Arabidopsis Columbia-0 genome by using tophat2 with bowtie2 (Kim et al., 2013) and allowing two mismatches. Only single hits were used for further analysis. The number of reads mapping to the exons, introns, and splice variant identifying gene regions (VIGRs; see MM splicing) were determined with the R packages genomic ranges (Lawrence et al., 2013), Rsamtools (Morgan et al., 2013). For the exons, only reads that had no overlap with non-exonic regions (i.e. introns or intergenic; IntersectionStrict) were counted, whereas for intron and VIGRs counts, overlap with neighboring genomic regions was permitted (IntersectionNotEmpty).

**Differential expression analysis.** Differential expression analysis was done with generalized linear modeling approaches of the R package edgeR (Robinson and Oshlack, 2010). Where no degrees of freedom were available a common dispersion of 0.08 was used, which is realistic for controlled experiments with genetically identical organisms and is conservative compared to common dispersion estimates that were assessed by known housekeeping genes in the dataset (0.02-0.06). Additionally, only genes with more than 1 RPKM in at least one sample were included. Differential expression upon treatment for each accession and organ was done with a model including all three conditions. Genes that responded differently in the shoot compared to the root were assessed in a full factorial model for each accession and treatment. The overall response across accessions (mean response) to the treatments was assessed in a paired design correcting for baseline differences amongst genotypes (i.e. an additive model with no interaction), which was done for each treatment and organ separately using tagwise dispersions. An analogous approach was taken for the organ dependent mean response, but with the added factor of the organ*treatment interaction.

Genotype dependent treatment responses were determined with a full factorial model and with testing for accession*treatment effects. Here also
organs and treatments were analyzed separately, and with the manual
common dispersion parameter. Genes showing differentiation between
tolerant and intolerant accessions were identified by contrasting the sensitive
genotypes (Cvi-0, Bay-0, Ita-0) against the tolerant genotypes (Lp2-6, Ws-2,
C24). Here the overall response of tolerant genotypes was tested for
significant difference from the overall response of the sensitive genotypes.
Where the overall responses of each group were determined by a paired
design without an interaction term, subsequently tagwise dispersion was
used.
Gene ontology overrepresentation was assessed with the GOseq Rpackage
(Young et al., 2010), which incorporates genelength biases. Multi-Dimensional
Scaling was done with the edgeR package (Robinson and Oshlack, 2010).
Weighted Gene Co-expression Network Analysis (WGCNA). WGCNA was
used to calculate co-expressed gene modules (Langfelder and Horvath,
2008). Since gene expression shows distinct patterns in roots and shoots,
they were analyzed separately in the clustering analysis. The raw count data
were filtered with RPKM method yielding 17525 and 15550 genes for roots
and shoots, respectively. Library size normalization was done with the edgeR
package (Robinson and Oshlack, 2010) and the data was transformed in
limma package with “voom” function (Ritchie et al., 2015) in order to enable
usage of WGCNA package designed for microarrays. The clustering was
performed with default settings and soft thresholds of 4 and 9 were used for
roots and shoots, respectively. As a representative of each module, an Eigen
gene was calculated as the first PC axis of the gene expression patterns in
that module. For each gene within a module, a module membership score
was computed based on the similarity of the gene to this Eigen gene.
Alternative Splicing. Estimation of alternative splicing (AS) and intron
retention (IR) due to treatment or genotype effects was based on existing
TAIR10 Col-0 annotation, using a method analogous to (Chang et al., 2014).
Genomic regions which provide information regarding variant use, i.e.
genomic regions which are transcribed in one variant but not in the alternative
variant, were identified with the R package GenomicRanges (Lawrence et al.,
2013). Reads mapping to these variant identifying gene regions (VIGRs) were
counted allowing for overlap with neighboring genomic regions
(IntersectionNotEmpty). Similarly, reads mapping to unambiguous intron regions were counted to assess IR.

Expected reads for each intron and VIGR where determined assuming that splice variant use and intron use does not change upon treatment or between genotypes. For instance, the expected reads for a treatment in a particular genotype would be $(VIGR_{AL} + VIGR_{AD} + VIGR_{SD}) / (Exon_{AL} + Exon_{AD} + Exon_{SD}) * Exon_{SD}$. In case of multiple VIGRs in a single gene, they were calculated independently, whereas introns were grouped as one unit. Only introns and VIGRs with an average read count of more than 12 were included.

The magnitude of AS and IR was determined by the ratio of the observed and expected reads, and subsequently log2 transformed so that AS and IR equals 0 when observed and expected reads have equal values. Significance was estimated with a chi square test ($\sum((O-E)^2/E)$) and Benjamini Hochberg corrected for multiple testing. Treatment dependent AS and IR was assessed separately for genotypes and organs. Genotype dependent AS and IR was assessed separately for each condition and organ. The maximum difference in splice variant usage is the highest log2(obs/exp) minus the lowest log2(obs/exp).

**RT-qPCR.** From RNA extracted with the RNeasy plant RNA isolation (Qiagen) kit and treated with DNase (Qiagen), cDNA was made by reverse transcription (SuperScript® III Reverse Transcriptase, invitrogen) with random hexamers and including RNase inhibitor (ThermoScientific). qRT-PCR was performed in a Viia7™ Real/Time PCR system (ThermoScientific) using iTaq universal SYBR Green Supermix (Bio-Rad) in 5 µL reaction mixtures with gene-specific primers and five reference genes (Supplemental Table S4).

**Accession numbers**

The raw sequencing files from RNA sequencing are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-4730.

**Supplemental Material.** The following supplemental data accompanies this manuscript.
Supplemental Figure S1. Compound and darkness responses of the eight accessions in root and shoot tissues.

Supplemental Figure S2. Number of genes with an accession dependent treatment response.

Supplemental Figure S3. GO overrepresentation of genes that vary in their response across accessions.

Supplemental Figure S4. Weighted Gene Co-expression Network Analysis (WGCNA) of shoots and roots.

Supplemental Figure S5. Genes with an organ dependent response to the treatments.

Supplemental Figure S6. Overview of intron retention (IR) and alternative splicing (AS) across genotypes and upon treatments.

Supplemental Figure S7. The conserved responses in IR upon treatment in the root.

Supplemental Figure S8. The conserved responses in IR upon treatment in the shoot.

Supplemental Figure S9. Change in total transcript abundance of genes with evidence of alternative splicing upon compound, darkness and submergence.

Supplemental Figure S10. Genes encoding important enzymatic steps of gluconeogenesis and the glyoxylate pathway.

Supplemental Figure S11. Change in petiole growth rate upon different combination of darkness and submergence.

Supplemental Figure S12. Schematic simplification of pathways transcriptionally regulated by compound, darkness and submergence.

Supplemental Table S1. Variation in tolerance to complete submergence in the dark of the 8 accessions used in this study.

Supplemental Table S2. Summary statistics of Illumina sequencing of the mRNAseq libraries and subsequent mapping to the TAIR10 *Arabidopsis thaliana* genome.
Supplemental Table S3. Correlation statistics of the response of an individual genotype compared to the mean responses of all eight genotypes.

Supplemental Table S4. Primers used for qRT-PCR analyses of transcript abundance.

Supplemental Data Set: Containing differential expression data from the RNAseq dataset for all the different comparisons investigated in the current study. (SupplementalData.xlsx)
Acknowledgements

We would like to acknowledge Johanna Kociemba, Ankie Ammerlaan, Rob Welschen and Judith Koerselman for practical assistance; Reed Sorenson and Marcel van Verk for advice on mRNAseq data analyses.

FIGURE LEGENDS

Figure 1. Transcriptional responses to compound, darkness and submergence stress in eight Arabidopsis accessions.

A. Schematic representation of the experimental setup, light cycle and treatments used. Arabidopsis seedlings were grown until the 10-leaf stage (9h photoperiod (Zeitgeber time (ZT): ZT0-ZT9). Plants then remained in control (air light; AL) conditions, or were transferred to submerged (dark) (SD) or dark (air dark; AD) conditions 2 h after photoperiod initiation (ZT2). Shoot and root material from Arabidopsis seedlings exposed to AL, AD and SD conditions for 4h were harvested at ZT6, and used for mRNAseq. Black bars indicate darkness. Double ended arrows indicate datasets compared to deduce the compound stress (AL vs. SD), darkness (AL vs. AD) and submergence (AD vs. SD) differentially expressed genes.

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Figure 2. Gene Ontology terms overrepresented in the conserved compound, darkness and submergence responses of the eight accessions.
Overrepresentation was determined for genes where the average response across accessions was $|\text{mean Log}_2\text{FC}| > 1.6$ and $P_{\text{adj.}} < 0.01$, and where variation in the response between accessions was absent ($P_{\text{accession*treatment, adj.}} > 0.1$). GO terms with a $P_{\text{adj.}} < 0.01$ are shown.

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B. Gene co-expression modules and their sizes as identified by a Weighted Gene Co-expression Network Analysis (WGCNA). Gene-modules show similar expression patterns across the three treatments and eight accessions. Roots (R) and shoots (S) were analyzed separately. Not all genes included in the analysis could be placed in a module of co-expressed genes and these were unplaced.

C and D. Mean and variation centered RPKM values of the largest two root (C) and shoot (D) co-expression modules identified by WGCNA. Top 12% of the genes with the highest module-membership-score are shown. The blue and red lines reflect the trends of the gene with the strongest positive and negative correlation to the mean module behavior. The remaining modules are visualized in Supplemental Figure S4. To the right of each module are representative terms related to the identified enriched GO terms. The complete GO analysis is in the Supplemental Data Sheet F and G.

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A. Histograms that shows the magnitude of IR (Calculated as the maximum difference in IR) upon the treatments for each accession.

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Figure 7. RT-qPCR validation of a selection of genes alternatively spliced upon compound, darkness and submergence.

Fold change of transcript abundance in the accessions Cvi-0 and C24 upon compound, darkness and submergence of six genes that were identified as alternatively spliced upon the treatment in a conserved manner across accessions. Primers were used that either amplified all transcript variants (red lines), or selectively amplify only specific variants (blue and green lines). Root or shoot tissue was analyzed depending on the organ in which alternative splicing was identified. Details for each gene are as follows. LYSINE-KETOGLUTARATE REDUCTASE/SACCHAROPINE DEHYDROGENASE (AT4G33150, LKR/SDH, root): blue line is AT4G33150.1 and AT4G33150.2; RHO GUANYL-NUCLEOTIDE EXCHANGE FACTOR 11 (AT1G52240, ROPGEF11, root): blue line is AT1G52240.1; GLUTAMATE DEHYDROGENASE 2 (AT5G07440, GDH2, root): blue line is AT5G07440.1 and AT5G07440.2 and green line is AT5G07440.1 and AT5G07440.3; ERYTHRONATE-4-P DEHYDROGENASE (AT1G75180, E4PDH, shoot): blue line is AT1G75180.2 and AT1G75180.3; FRUCTOSE-BISPHOSPHATE ALDOLASE 1 (AT2G21330, FBA1, shoot): blue line is specific for AT2G21330.2; PYRUVATE ORTHOPHOSPHATE DIKINASE (AT4G15530, PPDK, shoot): blue line represents all transcripts, excluding AT4G15530.2

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D. PPDK, PYRUVATE ORTHOPHOSPHATE DIKINASE, AT4G15530, in Bay-0

E. LKR/SDH, LYSINE-KETOGLUTARATE REDUCTASE/SACCHAROPINE DEHYDROGENASE, AT4G33150, in Bay-0. Red domain – LKR activity, green domain – SDH activity (Zhu et al. 2002)

F. NPQ1, NON-PHOTOCHEMICAL QUENCHING 1, AT1G08550, in Bay-0.

G. PRIN2, PLASTID REDOX INSENSITIVE 2, AT1G10522 in Bay-0.

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