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Keeping the shoot above water – submergence triggers antithetical growth responses in stems and petioles of watercress (*Nasturtium officinale*)

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**Summary**

- The molecular mechanisms controlling underwater elongation are based extensively on studies on internode elongation in the monocot rice (*Oryza sativa*) and petiole elongation in *Rumex* rosette species. Here, we characterize underwater growth in the dicot *Nasturtium officinale* (watercress), a wild species of the Brassicaceae family, in which submergence enhances stem elongation and suppresses petiole growth.
- We used a genome-wide transcriptome analysis to identify the molecular mechanisms underlying the observed antithetical growth responses. Though submergence caused a substantial reconfiguration of the petiole and stem transcriptome, only little qualitative differences were observed between both tissues. A core submergence response included hormonal regulation and metabolic readjustment for energy conservation, whereas tissue-specific responses were associated with defense, photosynthesis, and cell wall polysaccharides.
- Transcriptomic and physiological characterization suggested that the established ethylene, abscisic acid (ABA), and GA growth regulatory module for underwater elongation could not fully explain underwater growth in watercress.
- Petiole growth suppression is likely attributed to a cell cycle arrest. Underwater stem elongation is driven by an early decline in ABA and is not primarily mediated by ethylene or GA. An enhanced stem elongation observed in the night period was not linked to hypoxia and suggests an involvement of circadian regulation.

**Introduction**

The frequency and severity of extreme weather events, such as floods and droughts, have increased globally in recent decades due to climate change (Hirabayashi et al., 2008, 2013; Alfieri et al., 2018). Flooding stress—submergence or waterlogging—is a major environmental threat for many terrestrial plants (Bailey-Serres & Voesenek, 2008; Bailey-Serres et al., 2012b). As aeroeae organisms, plants depend on atmospheric oxygen (*O₂*) to sustain mitochondrial respiration (Fukao & Bailey-Serres, 2004). Flooding is a compound stress, during which the plant is confronted with reduced light levels due to turbid floodwaters (Vervuren et al., 2003) and a shortage in *O₂* and *CO₂*, as a combined result of replacing air-filled (soil) spaces with water and restricted gas diffusion in water (Jackson, 1985). When *O₂* becomes limited (hypoxia), mitochondrial respiration is compromised due to the loss of a functional electron transport chain, resulting in an energy crisis. Therefore, plants need to rely exclusively on glycolysis and fermentation as the energy source by utilizing soluble sugars and starch (Fukao & Bailey-Serres, 2004). Whereas roots experience hypoxia more often, even under well-drained conditions (van Veen et al., 2016), shoots only suffer from hypoxia during the night when completely submerged unless floodwaters are turbid and severely reduce access to light (Mommer et al., 2007; Vashishth et al., 2011; van Veen et al., 2013). During daytime, underwater photosynthesis can provide some of the required *O₂* for respiration, but this process is restricted by low light and a slow *CO₂* entry into the leaves (Mommer & Visser, 2005; Pedersen et al., 2013).
The detrimental effects of flooding stress vary between plant species. Whereas crops (e.g. *Zea mays*, *Brassica napus*, *Glycine max*) are rather sensitive to flooding, wild species (e.g. from the genera *Oryza*, *Rumex*, and *Rorippa*) from flood-prone areas are well adapted to excess water (Bailey-Serres & Voesenek, 2008; Mustroph, 2018a). Morphological acclimations like adventitious roots and aerenchyma formation or the ability to elongate above-ground organs allow these plants to improve internal aeration and thrive in water. The key regulator of these flooding-induced acclimations is ethylene, which, owing to its gaseous nature, rapidly accumulates in flooded tissues independent of light conditions. Ethylene, therefore, represents a robust and reliable signal to perceive flooding events (Sasidharan et al., 2018; Hartman et al., 2019).

Flooding-tolerant species have evolved two contrasting survival strategies to withstand flooding: the suppression (quiescence) and the promotion (escape) of growth (Bailey-Serres & Voesenek, 2008). Lowland rice varieties (e.g. FR13A), *Rumex acetosa*, and *Rorippa sylvestris* show a quiescence strategy (Singh et al., 2001; Akman et al., 2012; van Veen et al., 2013). Quiescent plants restrict energy-demanding processes (e.g. elongation) and preserve carbohydrates until the flood recedes, which is beneficial to facilitate regrowth in the post-submergence phase (Fukao & Bailey-Serres, 2008; Yeung et al., 2019). Plants with an escape strategy (e.g. *Rumex palustris*, the deep-water rice variety C9285, and *Ranunculus sceleratus*) rapidly elongate stems, leaves, or petioles to reach the water surface and restore air contact (Rijnders et al., 1996; He et al., 1999; Hattori et al., 2009; van Veen et al., 2013). At the same time, underwater growth is beneficial only if there is emergence and enhanced internal aeration; the latter is enabled by high tissue porosity (Pierik et al., 2009; Akman et al., 2012). In rice and *Rumex*, both survival strategies involve interactions between ethylene, ABA, and GA. Ethylene accumulation upon submergence results in ABA breakdown, which allows GA-mediated elongation in deepwater rice (Hoffmann-Benning & Kende, 1992) and *R. palustris* (Benschop et al., 2005, 2006). By contrast, ethylene accumulation in *R. acetosa* does not induce ABA depletion, explaining the adoption of the quiescence strategy (Benschop et al., 2005). In rice, ethylene response factors control the contrasting strategies, with *SNORKEL1* and *SNORKEL2* stimulating GA biosynthesis and/or sensitivity to promote elongation, and *SUB1A* suppressing GA biosynthesis and responsiveness by inducing negative regulators for GA signaling to dampen growth (Fukao & Bailey-Serres, 2008; Hattori et al., 2009; Kuroha et al., 2018). Interestingly, ethylene application is sufficient to mimic the submergence-induced elongation response in deepwater rice and *R. palustris* (Kende et al., 1998; Benschop et al., 2005).

Submergence promotes internode elongation in watercress, *Nasturtium officinale* (Ridge, 1987; Mustroph, 2018b), an autotetraploid and non-rosette-growing Brassicaceae species with a natural habitat near rivers and streams (Howard & Lyon, 1952; Bleeker et al., 1999). Current mechanistic knowledge on internode elongation is strongly biased towards the monocot rice, whereas research on internode elongation in dicots remains limited to the flower stalk of the rosette-growing species *Rorippa amphibia* (Akman et al., 2012).

In this study, we elucidate the internode elongation response in a non-rosette-growing Brassicaceae species at a molecular and physiological level. Watercress exhibits antithetical, tissue-specific growth responses during submergence: growth promotion in stems vs growth suppression in petioles. These observations offer the unique opportunity to use *N. officinale* as a dicot model system to study contrasting growth strategies within one single plant species. Here, we used an RNA-sequencing (RNA-seq)-based transcriptomics approach to unravel the molecular mechanisms underlying these antithetical growth responses. Our results indicate that the molecular processes regulating underwater growth in watercress deviate from the established ethylene–ABA–GA regulatory growth module. Whereas ABA depletion was a prerequisite for enhanced stem growth, it was not linked to hypoxia, ethylene, and GA. In the petioles, growth suppression was associated with active downregulation of cell cycle genes. However, it remains elusive what signal causes the initial ABA depletion required for stem elongation.

Materials and Methods

Plant growth and submergence treatment

Watercress (*N. officinale*) seeds were obtained from Saatgut-Vielfalt (Weilheim, Germany). For this ecotype, we observed self-compatibility, no requirement for vernalization, and the behavior as a long-day plant. For germination, several seeds were sown in the same pot on sterilized soil. After 6–7 d, germinated seedlings were transplanted to single pots and grown as previously described (Benschop et al., 2005). All experiments were carried out under short-day conditions: 20–23°C, 8 h : 16 h or 9 h : 15 h, light : dark cycle, and 100–150 μmol photons m⁻² s⁻¹. Plants were grown until the five or six leaf stage and selected for developmental homogeneity before the start of the experiments. Two hours after the start of the photoperiod, plants were either completely submerged in plastic tubs filled with temperature-adjusted water or kept in air in similar plastic tubs. Sampling for metabolites, ABA, and RNA was performed in independent cultivation rounds. The entire stem and petioles were measured with a digital caliper before (*t₀*) and after submergence (*tₙ*) and growth increase was calculated as *tₙ* − *t₀*. The stem elongation rate of the oldest internode and the hypocotyl was determined by using linear displacement transducers according to Benschop et al. (2005).

RNA-sequencing library construction

The entire stem (all internodes excluding the hypocotyl) and all petioles of air and submerged plants (five plants pooled per sample) were harvested separately after 1 and 2 d. Frozen samples of three replicates were ground for 3 min in 2 ml tubes with zirconia/silica beads using a Mini-Beadbeater (BioSpec Products, Bartlesville, OK, USA) in plates frozen with liquid nitrogen. After grinding, 1 ml lysis binding buffer (Dynabeads mRNA
Direct Kit; Thermo Fisher Scientific, Waltham, MA, USA) was added and samples were homogenized with this buffer for an additional 2 min in the Beadbeater. Homogenized samples were centrifuged at 12 000 g for 3 min and loaded onto a homogenizer spin column (Omega Bio-Tek, Norcross, GA, USA) and centrifuged to remove excess tissue. Lysates were transferred to a 96-well plate and stored at −80°C until library preparations. RNA-seq libraries were constructed using breast adapter directional sequencing (BrAD-seq) protocol for non-strand-specific libraries. Detailed instructions (Dynabeads mRNA Direct Kit; Thermo Fisher Scientific). Isolated mRNA was fragmented by reverse transcription (RT) buffer for 90 s at 94°C (RevertAid RT enzyme; Thermo Fisher Scientific), and complementary DNA (cDNA) synthesis was done on the fragmented mRNA. Second-strand synthesis was done according to BrAD-seq protocol and was followed by adapter ligation. PCR enrichment was performed using unique indexed oligos for multiplexing. After purification, sample concentration was measured on a plate reader (with SYBR Green I Nucleic Acid Gel Stain; Invitrogen, Carlsbad, CA, USA) and sample concentrations for multiplexing were adjusted accordingly. Multiplexing within the plate was done in a completely randomized design. Multiplexed samples were analyzed on a bioanalyzer for contamination and size confirmation. The size of the fragments varied between 200 and 500 bp. Samples were sequenced on a Hiseq4000 with 100 bp paired-end mode.

RNA-sequencing bioinformatics

Adapters were trimmed from the FASTQ files using the BBduk algorithm. Quantification of aligned reads to the published transcriptome by Voutsina et al. (2016) was performed using KALISTO (Bray et al., 2016), and 80 741 transcripts had reads mapping to them. The number of mapped reads ranged from 17.6 to 48.9 million and the mapping ratio was 83–85% per cDNA library (Supporting Information Table S1). Sequence similarities to Arabidopsis thaliana Col-0 transcriptome (obtained from araport.org) were calculated by a nucleotide blast (BLASTN). Transcripts that shared high similarity to the same A. thaliana transcripts (BLASTN E-value < 0, Fig. S3b see later) were aligned and their read counts summed. Lowly expressed transcripts were filtered based on a threshold (transcript per million > 6, based on the sum of all libraries), leading to 41 880 remaining transcripts. Differential expression analysis on these 41 880 transcripts was performed using the EDGER and limma BIOCONDUCTOR packages in R (McCarthy et al., 2012). Here the log2 fold changes (log2 FC) and Benjamini–Hochberg-corrected P-values were calculated fitting a negative binomial generalized log-linear model with a full factorial design matrix of treatment and tissue (Table S3, see later). Multidimensional scaling was obtained by using the plotMDS function within the EDGER package in R. Counts per million values of all transcripts were scaled and normalized. Highly significant (Padj < 0.001) differentially expressed genes (DEGs) were clustered with fuzzy K-means clustering based on Euclidean distances and membership exponent of 1.2 (Rlibrary ‘cluster’). Gene Ontology (GO) analysis was performed on gene clusters using the GOSEQ BIOCONDUCTOR package (Young et al., 2010).

Tissue O2 status and dark respiration measurements

Tissue O2 status was measured following the approach of Herzog & Pedersen (2014). Respiration was measured as O2 uptake by excised stem or petiole segments and followed the procedure described in Colmer & Pedersen (2008a). For more details, see Methods S1 and S2.

Metabolite and ABA quantification

Entire stems and all petioles of air and submerged plants were harvested at the indicated time points and the FW was measured. Metabolites were extracted and measured as previously described (Riber et al., 2015). For the extraction, determination, and quantification of ABA we used LC–MS as described by Floková et al. (2014) with modifications, listed in Methods S3.

Reverse transcription quantitative PCR analysis

RNA of the oldest internode (stem segment from cotyledons until the first true leaves) or all petioles was extracted using TRIzol (Bioline, Luckenwalde, Germany) followed by a DNase treatment using DNase1 (Thermo Fisher Scientific, Karlsruhe, Germany). cDNA synthesis through RevertAid Reverse Transcriptase (Thermo Fisher Scientific) was performed according to the manufacturers’ instructions. RT-quantitative PCR (qPCR) was performed using a 10 µl SsoAdvanced™ Universal SYBR® Green Supermix reaction and the CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Feldkirchen, Germany) with gene-specific primers listed in Table S2. The relative mRNA levels were determined by the 2−ΔCt × 1000 method and normalized to the average of watercress reference transcripts, homologous to CAP-binding protein 20 (CBP20, AT5G44200) and Ribosomal protein L13e family protein (RPL13e, AT5G23900) for stems, and to Cyclophilin 57 (CYP57, AT4G33060) and uncharacterized protein family (UPF0041, AT4G22310) for petioles.

Application of phytohormones and phytohormone inhibitors

The solvent concentration (ethanol or dimethyl sulfoxide (DMSO)) in the mock solutions (spraying or submergence) did not exceed 0.1% (v/v). ABA (Duchefa, Haarlem, the Netherlands), 1-aminocyclopropane-1-carboxylic acid (ACC; Sigma-Aldrich, Taukirchen, Germany), GA3 (Duchefa), paclobutrazol (PAC; Duchefa), and abscisazole-E3M (Takeuchi et al., 2016) were dissolved in ethanol or DMSO to a stock solution of 100 mM. ABA was added to the floodwater at varying concentrations (0, 0.5, 1, 5, or 10 µM). Abscisazole-E3M was applied as 50 or 100 µM solution by spraying the aboveground tissues 18 h and 1 h before submergence. Plants were pretreated once by watering each pot with 5 ml of 10 or 100 µM PAC 18 h before
submergence in water containing 10 μM GA₃. Ethylene gassing was applied in 101 flow-through cuvettes at a concentration of 2–4 ppm. Aminoethoxyvinylglycine (AVG; (S)-trans-2-amino-4-(2-aminoethoxy)-3-butenolic acid hydrochloride; Sigma Aldrich) was dissolved in distilled water to a concentration of 50 μM, which was used to spray the aboveground tissues 18 h and 1 h before submergence. Application of 1-methylcyclopropene (1-MCP) was at a concentration of 10 ppm for 1 h in an airtight glass desiccator before submergence or ethylene treatment. To control for humidity effects, control plants were placed in a separate glass desiccator before submergence or ethylene treatment. ACC was applied as control for humidity effects, control plants were placed in a separate glass desiccator before submergence or ethylene treatment. To control for humidity effects, control plants were placed in a separate glass desiccator before submergence.

Data were plotted using Prism 8 (GraphPad Software, San Diego, CA, USA) and R. One-way and two-way ANOVA and Tukey’s honestly significant difference test were performed using R. Two-way ANOVA with Sidak’s multiple comparison test and Student’s t-test were performed using Prism 8.

Accession numbers
Sequence data are stored at the Gene Expression Omnibus database under accession no. GSE138020. Watercress transcript codes and the homologous AGI codes for A. thaliana genes are listed in Table S3.

Results
Submergence triggers stem elongation and petiole growth suppression in watercress
Watercress responded to complete submergence under short-day conditions with increased stem internode elongation (Fig. 1a), accelerated leaf senescence, and delayed leaf formation compared with air-grown plants (Fig. S1a,b). Already after 1 d, submerged plants had a significantly higher stem elongation than controls, which was maintained at the later time points (Fig. 1b). Plants submerged for several weeks eventually outgrew the water surface (data not shown). Interestingly, submergence significantly suppressed petiole growth not only in the youngest (Fig. 1b), but also in the second and third youngest petioles (Fig. S1c).

We determined the sugar status of petioles and stems at several time points, since elongation is an energy-requiring process and a low sugar availability or usability could restrict elongation. As expected, in air-grown plants, sugar and starch content increased with illumination time and decreased during the night (Fig. S2). Both tissues consumed more than 50% of their stored carbohydrates already within the first 3 h of submergence. Although initial sugar levels were higher in stems (3.3 μmol glucose equivalents g⁻¹ FW) compared with petioles (2.1 μmol g⁻¹ FW), there were no significant differences between the tissues at each time point tested. This suggests a similar carbohydrate availability in both tissues, and no correlation with the contrasting growth responses. After 24 h of submergence, the sugar levels dropped to 20% of the starting level, indicating sugar starvation in both tissues.

Transcriptome profiling revealed many core submergence transcripts and few contrasting or tissue-specific transcripts
We performed RNA-seq on stems and petioles exposed to 1 and 2 d of submergence, where we observed strong tissue-specific growth differences between treatments (Figs 1b, S3a). The number of DEGs (Padj < 0.05, log₂ FC > 1 or log₂ FC < −1) was higher in petioles than in stems at both time points (Fig. 2a). In petioles, after 1 d of submergence there were 3910 transcripts upregulated and 2921 downregulated, and after 2 d of submergence 4428 transcripts were upregulated and 4981 downregulated. In stems, after 1 d of submergence there were 2854 transcripts upregulated and 2545 downregulated, and after 2 d of submergence 2796 transcripts were upregulated and 2919 downregulated. Multidimensional scaling showed a separation by tissue and treatment, but no difference in biological replicates or time points (Fig. 2b). In addition, more than 1000 transcripts were commonly up or downregulated in both tissues at both time points.
Transcriptome profiling of watercress revealed a common submergence core response but also tissue-specific responses. RNA-sequencing was performed on entire stems (internodes minus hypocotyl) and all petioles of submerged (sub) and air-grown plants after 1 and 2 d under short-day conditions. (a) The number of differentially expressed genes (DEGs) is higher in the petioles than in the stems. DEGs were selected based on the following criteria: log2 FC > 1 or log2 FC < -1 and P_adj < 0.05. Yellow represents up regulated genes and cyan downregulated genes. (b) Multidimensional scaling (MDS) plot shows separation by tissue and treatment. The distance between the different samples was calculated based on the top 4000 pairwise contrasting genes. (c) Violin plots illustrating the fuzzy K-means clustering of similarly regulated transcripts. The x-axis shows the different samples and the y-axis the scaled and normalized counts per million (CPM) values of highly significant (P_adj < 0.001) DEGs. Clusters (Clu) 3 and 4 represent the common response and clusters 1, 2, 5 and 6 the tissue-specific responses. The width of each violin represents the number of genes at that expression level. (d) Selected Gene Ontology (GO) categories on gene clusters. Selected examples of the top enriched (based on the P-value) GO terms for biological process, molecular function, and cellular compartment are shown. See Supporting Information Table S4 for complete GO analysis.

**Fig. 2** Transcriptome profiling of watercress revealed a common submergence core response but also tissue-specific responses. RNA-sequencing was performed on entire stems (internodes minus hypocotyl) and all petioles of submerged (sub) and air-grown plants after 1 and 2 d under short-day conditions. (a) The number of differentially expressed genes (DEGs) is higher in the petioles than in the stems. DEGs were selected based on the following criteria: log2 FC > 1 or log2 FC < -1 and P_adj < 0.05. Yellow represents up regulated genes and cyan downregulated genes. (b) Multidimensional scaling (MDS) plot shows separation by tissue and treatment. The distance between the different samples was calculated based on the top 4000 pairwise contrasting genes. (c) Violin plots illustrating the fuzzy K-means clustering of similarly regulated transcripts. The x-axis shows the different samples and the y-axis the scaled and normalized counts per million (CPM) values of highly significant (P_adj < 0.001) DEGs. Clusters (Clu) 3 and 4 represent the common response and clusters 1, 2, 5 and 6 the tissue-specific responses. The width of each violin represents the number of genes at that expression level. (d) Selected Gene Ontology (GO) categories on gene clusters. Selected examples of the top enriched (based on the P-value) GO terms for biological process, molecular function, and cellular compartment are shown. See Supporting Information Table S4 for complete GO analysis.
points (Fig. S3c,d), representing an induction and repression of submergence core transcripts. To identify the molecular pathways during submergence, we clustered all genes that responded to submergence in at least one of the tissues or time points ($P_{adj} < 0.001$; Fig. 2c) and performed a GO enrichment analysis on the resulting clusters of similarly regulated genes (Fig. 2d, Table S4). From this point on, we use AGI gene codes from *A. thaliana* to describe the homologous watercress transcripts. Watercress gene codes are listed in Table S3.

The 1809 transcripts of cluster 3 that were highly induced in both tissues included hypoxia and starvation-induced genes, for example the watercress homologues for *BRANCHED-CHAIN AMINO ACID TRANSAMINASE 2 (BCAT-2, AT1G10070), MYO-INOSITOL OXYGENASE 2 (MIOX2, AT2G19800), PHOSPHOENOLPYRUVATE CARBOXYKINASE 2 (PEPKC, AT5G65690), PYRUVATE ORTHOPHOSPHATE DIKINASE 2 (PPDK, AT4G15530)*, but also transcription factors (AT1G68320, AT2G43140) and defensins (AT3G63360, AT5G33355). Yet, the GO analysis of these commonly induced genes revealed an enrichment in responses to wounding, brassinosteroid, and auxin. Among the 1351 commonly repressed genes in cluster 4 were metabolism-related genes like *INORGANIC PYROPHOSPHATASE 1 (AT1G73010), GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE 2 (AT1G02390), ISOPROPYLALDEHYDE DEHYDROGENASE 1 (IMDH1, AT5G41200), ISOPROPYLALDEHYDE ISOMERASE 1 and 2 (IPMI1/2, AT3G58990, AT2G43100), and BCAT-4 (AT3G19710). The strong GO enrichment of biosynthetic and metabolic processes in cluster 4 supports the importance of downregulating energy-demanding processes in both tissues during submergence.

Interestingly, no gene cluster was identified that included genes that were upregulated in one tissue but downregulated in the other. In fact, only *P-glycoprotein 14 (AT1G28010) and NAD (P)-binding Rossmann-fold superfamily protein (AT5G02540)* were clearly induced in petioles and repressed in stems, whereas no unique stem-specific gene was found. Nevertheless, we discovered tissue-specific differences in expression level during submergence. Cluster 1 represents 748 genes with a higher overall expression and induction in petioles than in stems. These genes included homologues for the *HEAT SHOCK TRANSCRIPTION FACTOR A4A (AT4G18880)* and *protein kinase superfamily proteins (AT1G56720, AT1G03740, AT1G74330)*. Among the overrepresented GO terms were defense response and photosynthesis-related processes. Genes with a higher expression and induction in stems were assigned to cluster 2 (423 genes) and were associated with amino acid homeostasis and metabolic processes of cell wall polysaccharides, suggesting a structural rearrangement of the cell wall during elongation. However, genes encoding cell wall loosening enzymes, such as expansins, xyloglucan endotransglucosylase/hydrolases (XTHs), and pectin methylesterases (PMEs), were similarly expressed in both tissues (Fig. S4).

Cluster 5 (518 genes) represents genes with a lower expression level in stems than in petioles. The corresponding GO terms included processes involved in light, photosynthesis, and ribosomal RNA processing. Genes with a stronger downregulation in petioles were assigned to cluster 6 (1643 genes). These genes included, for example, *CELL DIVISION CONTROL 6 (AT2G29680) and WUSCHEL RELATED HOMEBOX 4 (WOX4, AT1G46480)*. The GO enrichment analysis of cluster 6 revealed a clear set of processes involved in translation, DNA replication, and cell proliferation. Indeed, cell cycle and DNA replication-related genes were remarkably repressed in petioles and much less downregulated in stems (Figs S5, S6). Furthermore, the cell cycle inhibitor *ICK1* (Wang et al., 2000) was significantly induced in the petioles after 2 d, raising the possibility of cell cycle arrest during submergence.

In rice and *Rumex*, growth promotion and suppression are predominantly linked to the hormonal trinity of ethylene, ABA, and GA (Sasidharan & Voeseneck, 2015). Unexpectedly, in none of the clusters tested were ethylene, ABA, or GA-related GO terms among the top enriched GO categories. Nonetheless, we proceeded with a biased approach to determine the role of these phytohormones in the antithetical growth responses in watercress.

Submergence-induced stem elongation requires ABA depletion

The absence or presence of ABA is the key switch that ultimately determines growth promotion in *R. palustris* or growth suppression in *R. acetosa* (Benschop et al., 2005). To find out if this is also the case in watercress, we first investigated the expression level of ABA biosynthesis and catabolism genes in petioles and stems during submergence. Homologous transcripts encoding the ABA biosynthesis enzymes 9-cis-epoxy-carotenoid dioxygenase 3 (*NCED3*, AT3G14440) and ABA-aldehyde oxidase (*AT5G20960, AT2G27150*) and the catabolism enzymes ABA 8′-hydroxylases (*CYP707A4; AT4G19230, AT2G29090, AT5G45340*) suggested downregulation of ABA biosynthesis and induction of ABA catabolism processes in response to submergence (Fig. S7a). This effect was present in both tissues after 1 and 2 d, suggesting a decline in ABA content during submergence. We therefore monitored the transcript levels of the biosynthesis gene *NoNCED3* and the catabolism genes *NoCYP707A1 (AT4G19230)* and *NoCYP707A2 (AT2G29090)* in a time-dependent manner (Fig. 3a). In the petioles, *NoNCED3* transcripts levels were significantly reduced after 3 h submergence, whereas it was slightly, but not significantly, downregulated in the stems. Moreover, there was a time-dependent upregulation of the ABA catabolism genes in both tissues, with the highest expression level of *NoCYP707A1* after 8 h in the stems and 24 h in the petioles. The transcript level of *NoCYP707A2* reached its maximum in the stems right before the night period (6 h) and in the petioles after 24 and 48 h. Interestingly, when we determined ABA levels during early submergence, we observed a decrease already after 1.5 h, and it remained low in both tissues during the rest of the experiment (Fig. 3b).

We submerged the plants in increasing ABA concentrations to further characterize the involvement of ABA on the observed growth responses (Fig. 3c). This resulted in a dose-dependent...
suppression of underwater stem elongation, and plants submerged with 10 µM ABA had growth responses similar to air-grown plants, demonstrating that ABA inhibits underwater elongation. Unlike the strong effect observed in the stems, the repression of petiole growth upon submergence was unaffected by exogenously applied ABA. To test whether submergence-induced stem elongation requires ABA catabolism, plants were treated with abscinazole-E3M, a selective inhibitor of CYP707As action (Takeuchi et al., 2016). As expected, abscinazole-E3M-treated plants were unable to elongate to the same extent as mock-treated plants, but not to the level of control growth conditions (Fig. 3d). Together, these results demonstrate that submergence-induced ABA depletion stimulates underwater stem elongation but does not affect petiole responses.

Fig. 3 Submergence-induced stem elongation of watercress requires abscisic acid (ABA) depletion. (a) Time-dependent expression analysis of ABA biosynthesis (NoNeced3) and catabolism genes (NoCYP707A1, NoCYP707A2). Plants of the five to six-leaf stage were either submerged (sub) or kept in air under short-day conditions (8 h photoperiod). At each time point the oldest internode (stem segment from cotyledons until the first true leaves) and all petioles of five plants were harvested separately for one sample. Relative messenger RNA (mRNA) level was measured using reverse transcription quantitative PCR and normalized to NoCBP20 and NoRPL13e for stems and to NoCYP57 and NoUPF0041 for petioles. Gray areas indicate the night period; x-axis is not to scale. Data are the mean ± SE of four biological replicates (n = 4). Significant differences between air and submergence for each time point are indicated: *, P < 0.05; **, P < 0.01; ***, P < 0.001 (two-way ANOVA, Sidak’s multiple comparisons test). (b) ABA content declines in response to submergence in both tissues. ABA quantification of submerged (sub) or air plants. At each time point entire stems and all petioles of five plants were harvested separately for one sample. Data are the mean ± SE of three biological replicates (n = 3). Different letters indicate statistically significant differences at P < 0.05 (two-way ANOVA, Tukey’s honestly significant difference (HSD) test) with lowercase letters representing stem tissues and uppercase letters representing petiole tissues. (c) ABA inhibits submergence-induced stem elongation completely. Plants were either submerged in water containing different ABA concentrations and 0.01% ethanol or kept in air. (d) Chemical inhibition of ABA breakdown enzymes partially inhibits stem elongation. Plants were sprayed with 50 and 100 µM abscinazole-E3M (Abz-E3M) or mock solution (0.1% dimethyl sulfoxide) 18 h and 1 h before submergence. (c, d) Plants were either submerged (sub) or kept in air. The length of the entire stem and the youngest petiole was measured before and after 24 h of treatment, and growth was calculated as the difference with respect to before treatment. Data are the mean ± SE of three biological replicates (n = 30). Different letters indicate values that vary significantly at P < 0.05 (two-way ANOVA, Tukey’s HSD test).
Next, we investigated whether the observed ABA depletion is regulated by ethylene. Treatment with the ethylene precursor ACC did not lead to a significant downregulation of NoNCED3 or induction of ABA 8’-hydroxylase expression (Fig. S8), indicating that the submergence-induced ABA decline is unlikely to be triggered by ethylene.

Ethylene only plays a minor role in the submergence-induced growth responses

We investigated whether ethylene affects the underwater growth responses in watercress at all since ethylene probably does not induce ABA degradation. Expression analysis of homologous transcripts encoding the ethylene biosynthesis enzymes ACC synthase (ACS; AT2G22810, AT4G26200, AT4G08040, AT5G51690) and ACC oxidase (ACO; AT2G19590, AT1G62380, AT1G77330) showed an elevated expression in both tissues after 1 and 2 d of submergence (Fig. S7b), indicating enhanced ethylene biosynthesis. We identified a time-dependent induction of NoACS7 (AT4G26200) in the petioles upon submergence, with the highest expression at 24 h (Fig. 4a). The expression level in the stems only increased after 48 h submergence, which was five-fold lower than in the petioles. The transcript level of NoACO1 (AT2G19590) increased significantly in both tissues with time, especially during the night (8 h).

To further investigate the role of ethylene on stem and petiole growth during submergence, plants were pretreated with the ethylene perception inhibitor 1-MCP (Fig. 4b) and the ethylene biosynthesis inhibitor AVG (Fig. 4c). Inhibition of biosynthesis and signaling only partially blocked underwater stem elongation and had no effect on petiole growth suppression. Ethylene (Fig. 4d) or ACC (Fig. 4e) treatment in air induced a significant growth increase in the stems, but not to the full extent of the submergence response (Fig. 4b, c mock). This suggested that ethylene only plays a minor role in the opposite growth responses.

The opposite growth responses in petioles and stems are not GA-mediated

We compared the expression levels of GA biosynthesis and catabolism genes in watercress as GA has been implicated in promoting underwater elongation, and submergence increased GA biosynthesis in the elongating R. palustris but not in the quiescent R. acetosa (Rijnders et al., 1997). Homologous transcripts encoding the GA biosynthesis enzyme GA20oxidase (AT4G25420, AT5G51810) and the GA inactivation enzyme GA3oxidase (AT1G30040) significantly accumulated, whereas transcripts encoding the GA biosynthesis enzyme GA3oxidase (AT1G15550, AT1G80340) were strongly downregulated (Fig. S7c). These results did not suggest an important role for GA in the tissue-specific growth responses during submergence. To verify this, we studied the involvement of GA by monitoring the expression of GA biosynthesis genes and the growth responses in GA-depleted plants during submergence (Fig. 5). Consistent with the transcriptome analysis, NoGA3ox1 (AT1G15550) was significantly downregulated, again indicating no increased synthesis of bioactive GAs during submergence.

Next, we treated the plants with 10 and 100 μM PAC to inhibit GA biosynthesis and deplete the plants of GAs before submergence (Fig. 5b, S9). Even though plants displayed a typical GA-deficient dwarf phenotype, they were still able to elongate during submergence. The relative elongation ability of PAC-treated plants was even similar to mock-treated plants (Fig. 5c). Additionally, the growth suppression in the petioles was unchanged upon PAC pretreatment, and even exogenous GA3 did not enhance petiole growth during submergence. Thus, we conclude that the submergence-induced tissue-specific growth responses are not mediated by GA.

Fast underwater stem elongation during the night is not hypoxia induced

Since we observed an ABA decline very early during submergence, we monitored the stem elongation in higher resolution over time by using linear displacement transducers (Fig. 6a). Using this technique, we detected differences already at 2 h following submergence, where underwater stem elongation rates were more than four times higher relative to controls. Elongation rates declined gradually until the end of the day to that of control plants. During the night, the elongation rate in submerged stems increased again and remained seven-fold faster than that of control plants.

We furthermore measured the tissue O2 status in stems and petioles with respect to dependence of light, darkness, air, and submergence to test whether the fast stem elongation during the night coincides with changes in the O2 status (Fig. S10a–e). Indeed, we found a decrease in internal O2 in both tissues only during dark submergence (Fig. 6b), with the stem O2 being slightly, but not significantly, lower than in petioles. Additionally, dark respiration rates were significantly higher in stems than in petioles (Fig. S10f). When switched to light submergence, plants were even hypoxic and again grew faster than control plants (Fig. 6a,b), indicating an improved energy status after re-exposure to light. This was consistent with the transcriptome analysis, where only a few core hypoxia genes (Moustroph et al., 2009), like ACO1, ETR2 (AT3G23150), ATPP2-A13 (AT3G61060) and unknown proteins (AT5G10040, AT1G19530, AT4G27450) were induced during submergence in the light (Fig. S11). By analyzing the expression level of three core hypoxia marker genes, NoADH1 (AT1G77120), NoGLB1 (AT2G16060), and NoLBD41 (AT3G02550), in a time-dependent manner we were able to confirm that watercress only experiences hypoxic conditions during dark submergence (Fig. 6c).

Finally, to find out whether the fast elongation during the night/dark submergence was indeed connected to hypoxia, we compared the stem elongation of plants submerged under short-day and under continuous light conditions (Fig. S10g). In the latter case, plant tissues would not experience hypoxia. Despite the absence of a night period, there was no significant difference in elongation, indicating that the underwater stem elongation does not require hypoxia.
Discussion

Submergence core transcripts are involved in responses to carbon starvation and metabolic processes

Submergence of watercress resulted in tissue-specific contrasting growth responses: stem elongation was enhanced, and petiole growth was suppressed (Fig. 1). This provided the unique opportunity to study antithetical growth responses during submergence within one plant species. Despite strong contrasting growth responses, a genome-wide transcriptome analysis revealed no qualitative tissue-specific differences. We found a high degree of overlap in transcriptional changes between stems and petioles. These core submergence transcripts reveal generic submergence-triggered pathways that are likely independent of growth regulation. Submergence deprives plants of essential carbohydrates.
Accordingly, both tissues were almost completely depleted of their stored carbohydrates after 1 d of submergence (Fig. S2), which was reflected in the similar expression of carbon (C)-starvation marker genes such as MIOX2 or BCAT-2 (Schuster & Binder, 2005; Osuna et al., 2007). Those genes are also highly expressed in an extended night (Table S3; Usadel et al., 2008). This response fits with the observation in Arabidopsis that submergence-induced transcriptional changes resemble starvation (Lee et al., 2011; van Veen et al., 2016).

Besides glycolysis and fermentation, plants use other alternative pathways to cope with this energy crisis; for example, the use of inorganic pyrophosphate (PPi)-dependent enzymes (Bailey-Serres & Voesenek, 2008; Bailey-Serres et al., 2012a). The PPi-utilizing enzyme PPDK and the gluconeogenic enzyme PEPCK were highly induced during submergence in watercress. Their

![Fig. 5](https://www.newphytologist.com/2021/229/140-155)
The petiole growth suppression is associated with cell cycle arrest

The petiole growth suppression is more transcriptionally regulated than growth promotion. Interestingly, processes related to translation, cell proliferation, and cell cycle were more strongly downregulated in petioles than in stems (Figs 5, 6). WOX4, which was only downregulated in petioles, promotes vascular cell division, and Arabidopsis wox4 RNA interference mutants had a small plant phenotype (Ji et al., 2010). So far, the role of negative regulation of cell division in growth inhibition upon submergence (e.g. quiescence strategy) has not been intensively studied. SUB1A represses genes responsible for cell elongation in rice (Fukao et al., 2006), and ethylene can cause cell cycle arrest during environmental stress (Skirycz et al., 2011). However, since neither ethylene nor ACC treatment alone negatively affected growth in the petioles (Fig. 4), it is unlikely that ethylene triggers the possible cell cycle arrest in watercress. Other factors, such as sugar or energy status, light signals, or a change in redox status, could cause this response, leading to a change in calcium signatures or a phosphorylation/dephosphorylation response to modulate gene expression. Accordingly, \( \text{NAD(P)} \)-binding Rossmann-fold domain-containing protein (RDRP) has been implicated in the control of cell cycle arrest in Arabidopsis (Park et al., 2013).
superfamily protein, which was induced in petioles and repressed in stems, has a high sequence similarity to *Pisum sativum* Tic32, which is thought to be a redox sensor (Hörmann et al., 2004; Tonfack et al., 2011). Furthermore, upon stress, cyclin-dependent kinase (CDK)/cyclin complexes are repressed by CDK inhibitor proteins (e.g. ICK1; Wang et al., 2000; Vandepoele et al., 2002). Since ICK1 was significantly induced, we suspect that submergence triggers cell cycle arrest in watercress petioles.

The key factor that mediates underwater stem elongation is ABA depletion, not ethylene or GA

In watercress, submergence-induced expression of ABA catabolism genes corresponded with a rapid ABA decline in both tissues (Fig. 3a,b). Submergence-mediated ABA decline is a key factor promoting flood escape in several species. For example, submergence triggered ABA breakdown in the escaping species *R. palustris* but not in the quiescent species *R. acetosa* (Benschop et al., 2005). Additionally, the rate of underwater petiole elongation in different *R. palustris* accessions was determined by the natural variation of endogenous ABA concentrations (Chen et al., 2010). Also, flooding-induced internode elongation of deepwater rice requires ABA removal via 8'-hydroxylation (Hoffmann-Benning & Kende, 1992; Saika et al., 2007). Interestingly, artificial ABA depletion enhanced petiole elongation in *R. acetosa* (Benschop et al., 2005), unlike watercress, where ABA depletion did not enhance petiole elongation (Fig. 3). However, ABA depletion does not always trigger elongation, since a rapid ABA catabolism upon submergence has also been reported in nonelongating rice (Ram et al., 2002; Fukao & Bailey-Serres, 2008; Hattori et al., 2009).

Exogenous ABA in the floodwater did not affect the petiole response, but an increasing ABA concentration dramatically decreased stem elongation in a dose-dependent manner (Fig. 3c). Moreover, inhibition of ABA 8'-hydroxylation by abscisazole-E3M significantly reduced underwater stem elongation, but not to the level of air-grown plants (Fig. 3d). Although 8'-hydroxylation is considered as the major pathway of ABA catabolism, ABA can also be inactivated by conjugation to ABA-glucosyl ester via uridine diphosphate glycosyltransferases (e.g. UGT71B6–B8 and UGT71C5; Kushiro et al., 2004; Dong et al., 2014; Liu et al., 2015). Therefore, the remaining elongation ability could be due to the activity of alternative ABA catabolic processes, as it has already been conjectured in *R. palustris* (Benschop et al., 2005).

Whereas the submergence-induced ABA decline in *R. palustris* and rice is linked to ethylene accumulation (Benschop et al., 2005; Saika et al., 2007), ABA breakdown in watercress is not likely mediated by ethylene, since ACC application did not alter *NoNCED3* or *NoCYP707A1/A2* gene expression (Fig. S8). Ethylene application strongly stimulated elongation in deepwater rice and *R. palustris*, similar to or even up to twice as much as submergence did (Kende et al., 1998; Cox et al., 2006). We observed a three-fold growth increase during submergence but only a 1.5-fold increase by ethylene or ACC treatment. Ethylene promoted elongation in watercress cuttings, but this has only been demonstrated for newly developing lateral shoots, not for internodes (Schwegler & Brändle, 1991). In our study, inhibition of ethylene signaling or biosynthesis only partially blocked stem elongation, implying that the underwater elongation cannot be fully attributed to ethylene (Fig. 4). Overall our results indicate regulation by submergence signals other than ethylene. A similar observation has been made in *Solanum dulcamara*, where an ABA decline is necessary for adventitious root formation during flooding, but flooding-induced induction of ABA 8'-hydroxylase could not be mimicked by ethylene treatment (Dawood et al., 2016).

Reduced ABA levels resulted in GA-mediated elongation by an increase in GA biosynthesis or sensitivity in other species (Hoffmann-Benning & Kende, 1992; Benschop et al., 2006). By contrast, submergence did not induce GA biosynthesis genes in watercress (Fig. 5a). Additionally, depleting the plants of GA was not sufficient to inhibit stem elongation (Fig. 5b,c). Taken together, these results demonstrate that underwater stem elongation requires ABA depletion, but it is only marginally mediated by ethylene and not by GA.

Underwater stem elongation is not hypoxia induced

The underwater stem elongation can be divided into an early phase, which initiates the ABA degradation, and a late phase, where rapid elongation occurs during the night (Fig. 6a). We hypothesized that this fast elongation could be mediated by dark-submergence-induced hypoxia. This idea was supported by numerous reports of hypoxia as a driver of elongation growth in many species and under various conditions (Métraux & Kende, 1984; Summers & Jackson, 1994; Voesenek et al., 1997; Sasayama et al., 2018). Indeed, the core hypoxia genes *NoADH1*, *NoLBD41*, and *NoGLI1* were strongly induced only during dark submergence, when the internal O2 concentration declined significantly (Fig. 6b,c). In watercress, the internal O2 concentration was even higher than in air when switched from dark to light submergence (Fig. 6b). These hypoxic conditions are the result of O2 production via underwater photosynthesis and the slow diffusion rate of O2 from the plant to the environment (Pedersen et al., 2006). A decline in internal O2 only during darkness and an increase in internal O2 in the light is a common phenomenon in submerged plants (Rijnders et al., 2000; Colmer & Pedersen, 2008b; Lee et al., 2011; Vashisht et al., 2011). The fact that elongation was not compromised under continuous light indicates that the internode elongations do not require low O2. Rhythmic growth patterns are ubiquitous in nature and frequently ascribed to the circadian clock, especially when maintained under continuous light conditions (Farré, 2012; Dornbusch et al., 2014). We therefore hypothesize some level of gating in the elongation response.

What other factors could contribute to the antithetical growth responses during submergence?

The requirement of low ABA for underwater elongation appears to be almost ubiquitous in amphibious species. These ABA declines are often coupled with a growth-stimulating hormone, most commonly GA (Benschop et al., 2006; Hattori et al., 2009), but...
also auxin, as in Hydrocharis morsus-ranae, Regnellidium diphyllum, R. scleratus, and R. palustris (Cookson & Osborne, 1978; Horton & Samarakoon, 1982; Cox et al., 2004, 2006). Conversely, a role for brassinosteroid in mediating SUB1A-regulated growth suppression in rice has been identified (Schmitz et al., 2013). In our data, petioles and stems had a similar auxin and brassinosteroid response (Fig. 2d), and we found no role for GA in mediating elongation (Fig. 5).

Specific cell wall enzymes have often been associated with elongation (Kim et al., 2000; Vriezen et al., 2000; Ookawara et al., 2005; Minami et al., 2018). In watercress, the expression of expansins, XTHs, and PMEs during submergence was tissue independent (Fig. S4). No correlation between expansin expression and elongation was observed in rice coleoptiles either (Magneschi et al., 2009). However, the activity of cell-wall-modifying enzymes is pH dependent (Cosgrove, 2000; Micheli, 2001), implying that the antithetical growth responses could be regulated at the protein level or require tissue-specific apoplastic acidification, like in R. palustris, Nymphoides peltata, and Potamogeton distinctus (Malone & Ridge, 1983; Vreeburg et al., 2005; Koizumi et al., 2011).

The aforementioned aspects concern the more downstream responses mediating elongation, but it remains unclear which signal lowers the ABA levels in watercress. Our work suggests no involvement of the main signals, ethylene and O2 (Sasidharan et al., 2018). Also, submergence-induced elongation in Echinoclora oryzoides could not be reconstituted by hypoxia or ethylene (Pearce & Jackson, 1991). Interestingly, some species already elongate when partially submerged, such as deepwater rice and Lotus tenuis (Hattori et al., 2009; Manzur et al., 2009).

Partial submergence of these species likely results in reduced ethylene accumulation and limited hypoxia, implying additional flood-specific sensing mechanisms. Rachis elongation in R. diphyllum and N. peltata was only stimulated when ethylene was applied in combination with physical tension to mimic the buoyant tension of submergence (Musgrave & Walters, 1974; Ridge & Amarasinghe, 1984). How watercress perceives flooding remains unknown.

Conclusion and outlook

Many economically relevant crops are negatively impacted by submergence. By contrast, plants that inhabit flood-risk areas have evolved specific mechanisms to overcome the major challenges in an excess water environment. This study highlights the diversity in mechanisms in seemingly similar behaviors, which emphasizes the importance of studying flood-adaptive traits in a wide range of species. Transferring our understanding of flood-adaptive traits in wild species to crops will be the key to improve their productivity during flooding. Here, we have presented N. officinale as a novel dicot plant system to study flood-adaptive traits. We believe that this data set will serve as a base for future studies to identify as yet unknown flood-adaptive genes and processes. These studies could include evaluation of tissue-specific anatomy and hormone signaling pathways, as well as natural variation of the elongation response within existing ecotypes of N. officinale (Voutsina et al., 2016).

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Growth analyses of watercress stems and petioles under control conditions and submergence.

Fig. S2 Submergence induces sugar starvation in both tissues.

Fig. S3 Overview of the experimental setup and Venn-Diagrams for up- and downregulated DEGs.

Fig. S4 Expression pattern of genes encoding cell wall modifying proteins in petioles and stems of watercress.

Fig. S5 Expression pattern of core cell cycle genes in petioles and stems of watercress.

Fig. S6 Expression pattern of core DNA replication genes in petioles and stems of watercress.

Fig. S7 Expression pattern of ABA, ethylene and GA related genes in response to submergence in petioles and stems of watercress.

Fig. S8 ACC treatment does not influence NoNCED3 and ABA-8’hydroxylase (NoCYP707A1, NoCYP707A2) gene expression.

Fig. S9 Dose-dependent influence of GA on the antithetical growth responses.

Fig. S10 Measurement of the oxygen status in stem and petiole.

Fig. S11 Organ-specific regulation of the low oxygen response upon submergence.

Methods S1 Tissue O₂ status with shoot in air or during complete submergence.

Methods S2 Respiration rates of stem or petiole tissues.

Methods S3 ABA quantification.

Table S1 Summary statistics of the Illumina sequencing of all libraries.

Table S2 List of primers used in this paper.

Table S3 Expression values and comparisons of the Illumina sequencing.

Table S4 GO term enrichment analysis of differentially expressed transcripts.

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