Research

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 aerobe organisms, plants depend on atmospheric oxygen (O_2) 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 O_2 and CO_2, as a combined result of replacing air-filled (soil) spaces with water and restricted gas diffusion in water (Jackson,

1985). When O_2 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; Vashisht *et al.*, 2011; van Veen *et al.*, 2013). During daytime, underwater photosynthesis can provide some of the required O_2 for respiration, but this process is restricted by low light and a slow CO_2 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 auto-tetraploid 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 (t_0) and after submergence (t_x) and growth increase was calculated as $t_x - t_0$. 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 breath adapter directional sequencing (BrAD-seq) protocol for non-strand-specific libraries (Townsley *et al.*, 2015). Briefly, 200 μl of lysates was used for messenger RNA (mRNA) isolation, following the manufacturer's 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 KALLISTO (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 combined 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 with the EDGER and LIMMA BIOCONDUCTOR packages in R (McCarthy *et al.*, 2012). Here the log$_2$ fold changes (log$_2$ 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 ($P_{adj} < 0.001$) 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 O$_2$ status and dark respiration measurements

Tissue O$_2$ status was measured following the approach of Herzog & Pedersen (2014). Respiration was measured as O$_2$ 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 TRIsure (Bioline, Luckenwalde, Germany) followed by a DNAse treatment using DNaseI (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 SsoAdvancedTM 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 $2^{-\Delta C_T} \times 1000$ 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, Taufkirchen, Germany), GA$_3$ (Duchefa), paclobutrazol (PAC; Duchefa), and abscinazole-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). Abscinazole-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 $10\,\mu M$ GA$_3$. Ethylene gassing was applied in 10 l flow-through cuvettes at a concentration of 2–4 ppm. Aminoethoxyvinylglycine (AVG; (S)-trans-2-amino-4-(2-aminoethoxy)-3-butenoic acid hydrochloride; Sigma Aldrich) was dissolved in distilled water to a concentration of $50\,\mu M$, 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 for 1 h without 1-MCP. ACC was applied as $100\,\mu M$ solutions by spraying the aboveground tissues at the start of the experiment.

Statistical analyses

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

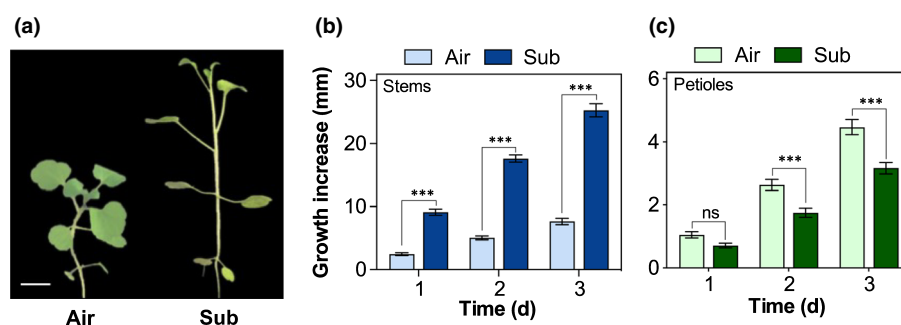### Submergence triggers stem elongation and petiole growth suppression in watercress

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.
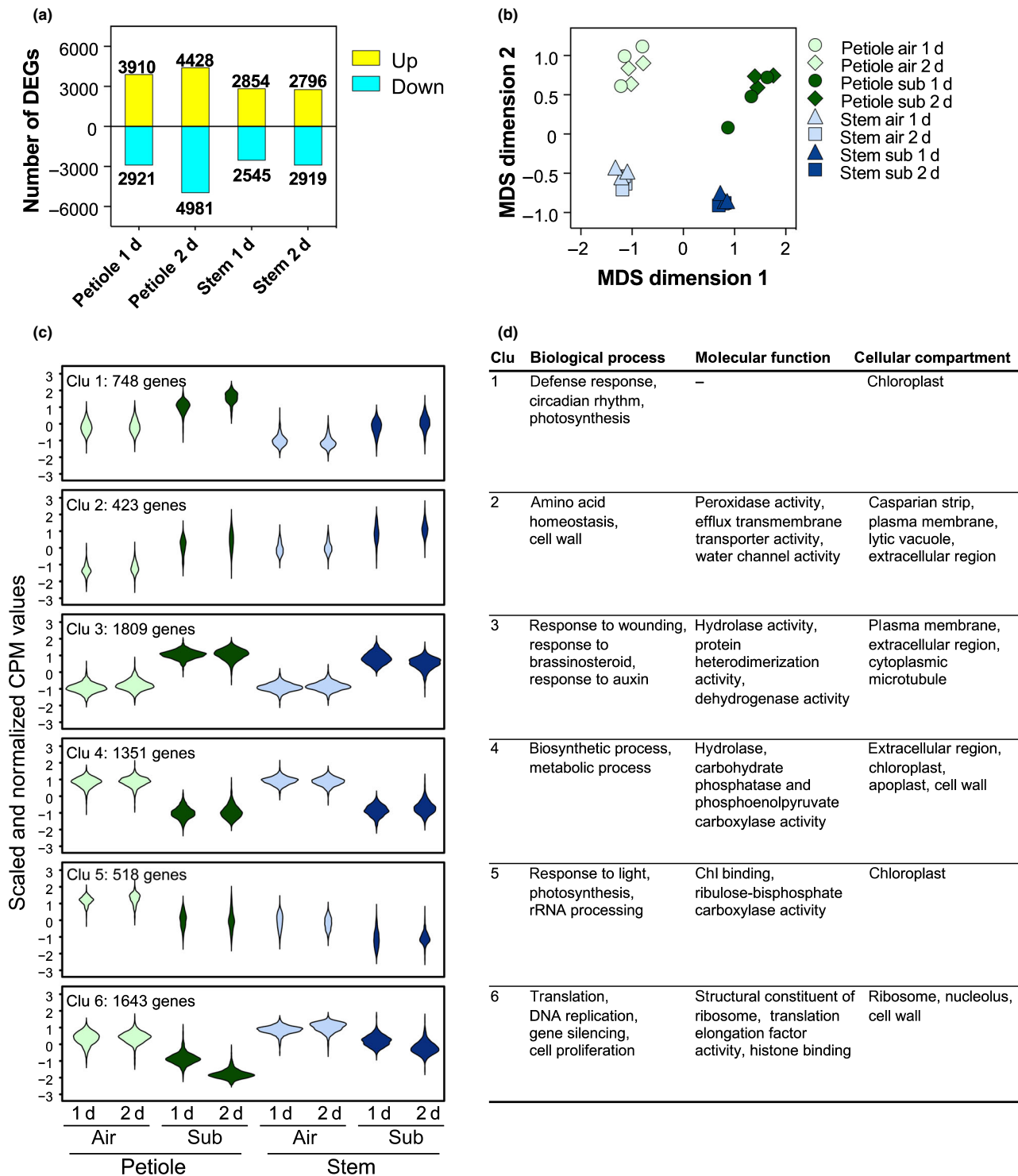
Transcriptome profiling revealed many core submergence transcripts and few contrasting or tissue-specific transcripts

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 ($P_{adj.} < 0.05$, log$_2$ FC > 1 or log$_2$ 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

Fig. 1 Submergence leads to stem elongation and petiole growth suppression in watercress. (a) Representative picture of an air (left) and submerged (sub, right) plant after 5 d of treatment. The air plant was fixed vertically with a string for imaging only and the submerged plant was under water when pictures were taken. Bar, 1 cm. (b) Plants were either submerged (sub) or kept in air under short-day conditions. The length of the entire stem (left) and the youngest petiole (right) was measured before and after treatment, and growth was calculated as the difference with respect to before treatment. Data are the mean ± SE of five independent experiments ($n = 34$). Significant differences between air and submergence for each time point are indicated: ***, $P < 0.001$ (two-way ANOVA, Sidak's multiple comparisons test).

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: $\log_2$ FC > 1 or $\log_2$ 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* (*PEPCK*, AT5G65690), *PYRUVATE ORTHOPHOSPHATE DIKINASE* (*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), *ISOPROPYLMALATE DEHYDROGENASE 1* (*IMD1*, AT5G14200), *ISOPROPYLMALATE 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 protein*s (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 term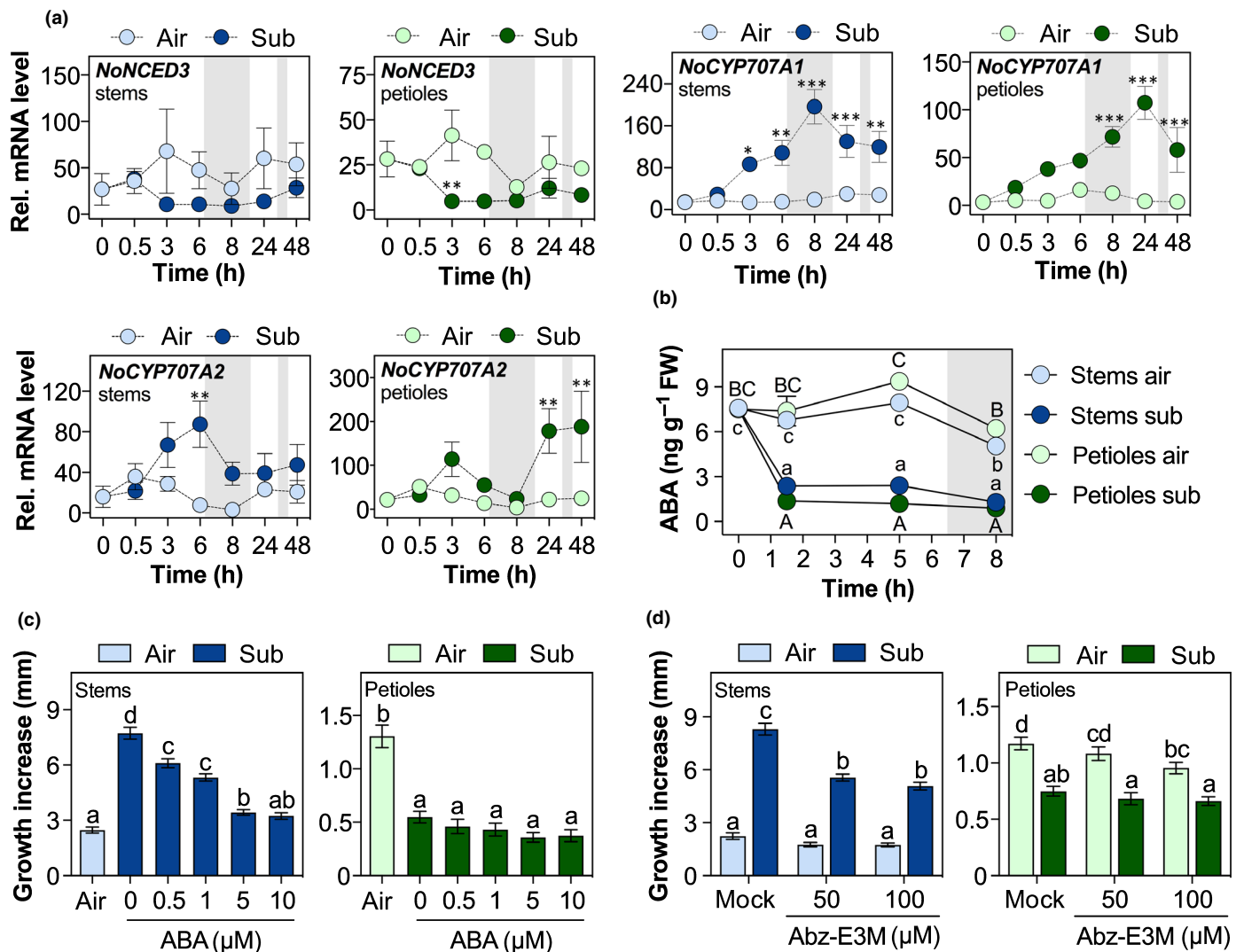s included processes involved in light, photosynthesis, and ribosomal RNA processing. Genes with a stronger downregulation in petioles were assigned to cluster 6 (1643 genes). These genes included, for example, *CELL DIVISION CONTROL 6* (AT2G29680) and *WUSCHEL RELATED HOMEOBOX 4* (*WOX4*, AT1G46480). 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 & Voesenek, 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*-epoxycarotenoid dioxygenase 3 (*NCED3*, AT3G14440) and ABA-aldehyde oxidase (AT5G20960, AT2G27150) and the catabolism enzymes ABA 8′-hydroxylases (*CYP707A*s; 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

Fig. 3 Submergence-induced stem elongation of watercress requires abscisic acid (ABA) depletion. (a) Time-dependent expression analysis of ABA biosynthesis (*NoNCED3*) 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).

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.

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 GA2oxidase (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, *NoGA20ox2* (AT5G51810) showed a significant induction over time in the petioles and after

48 h in the stems (Fig. 5a). In both tissues, *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.
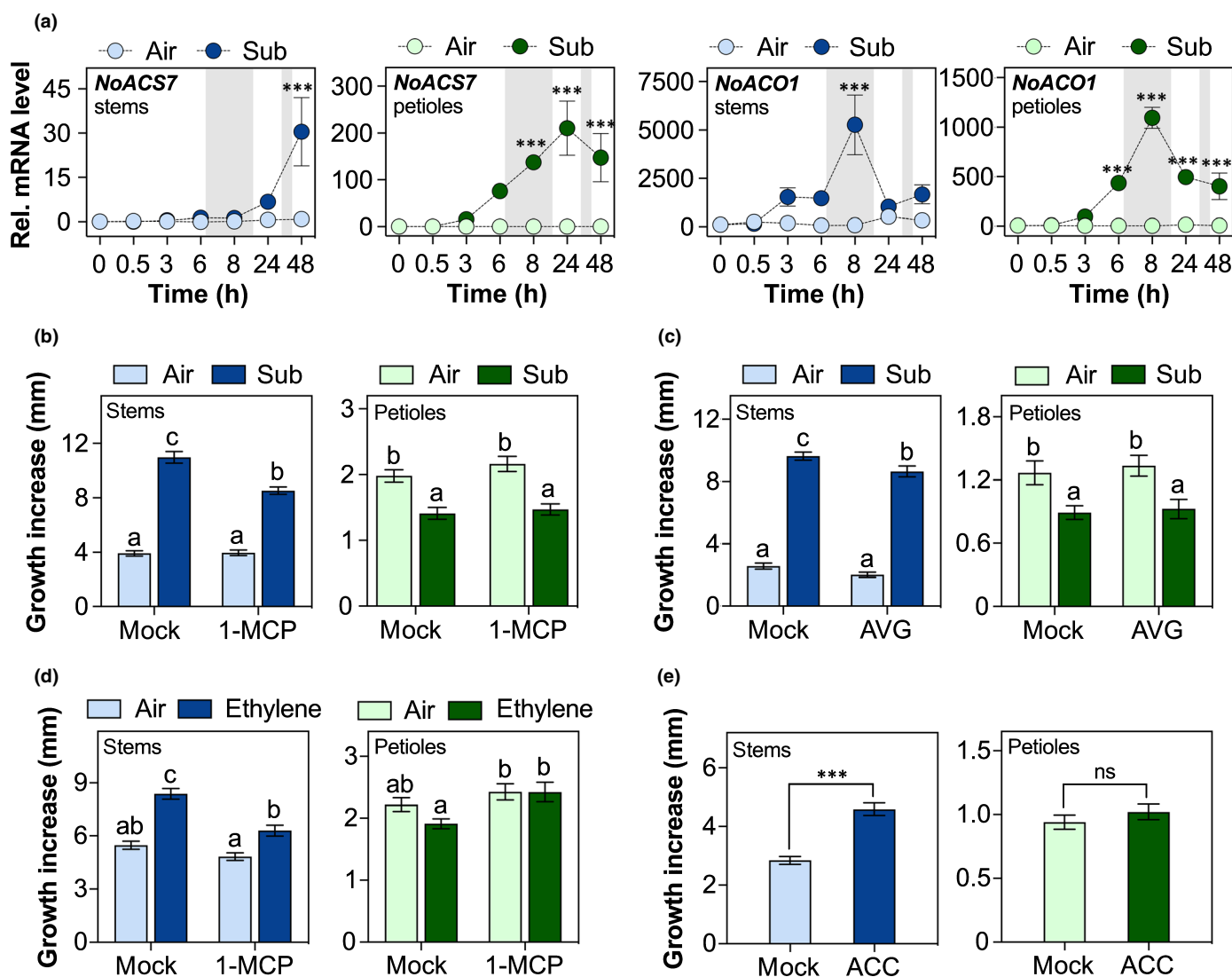
Fast underwater stem elongation during the night is not hypoxia induced

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 O_2 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 O_2 status (Fig. S10a–e). Indeed, we found a decrease in internal O_2 in both tissues only during dark submergence (Fig. 6b), with the stem O_2 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 hyperoxic 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 (Mustroph *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.
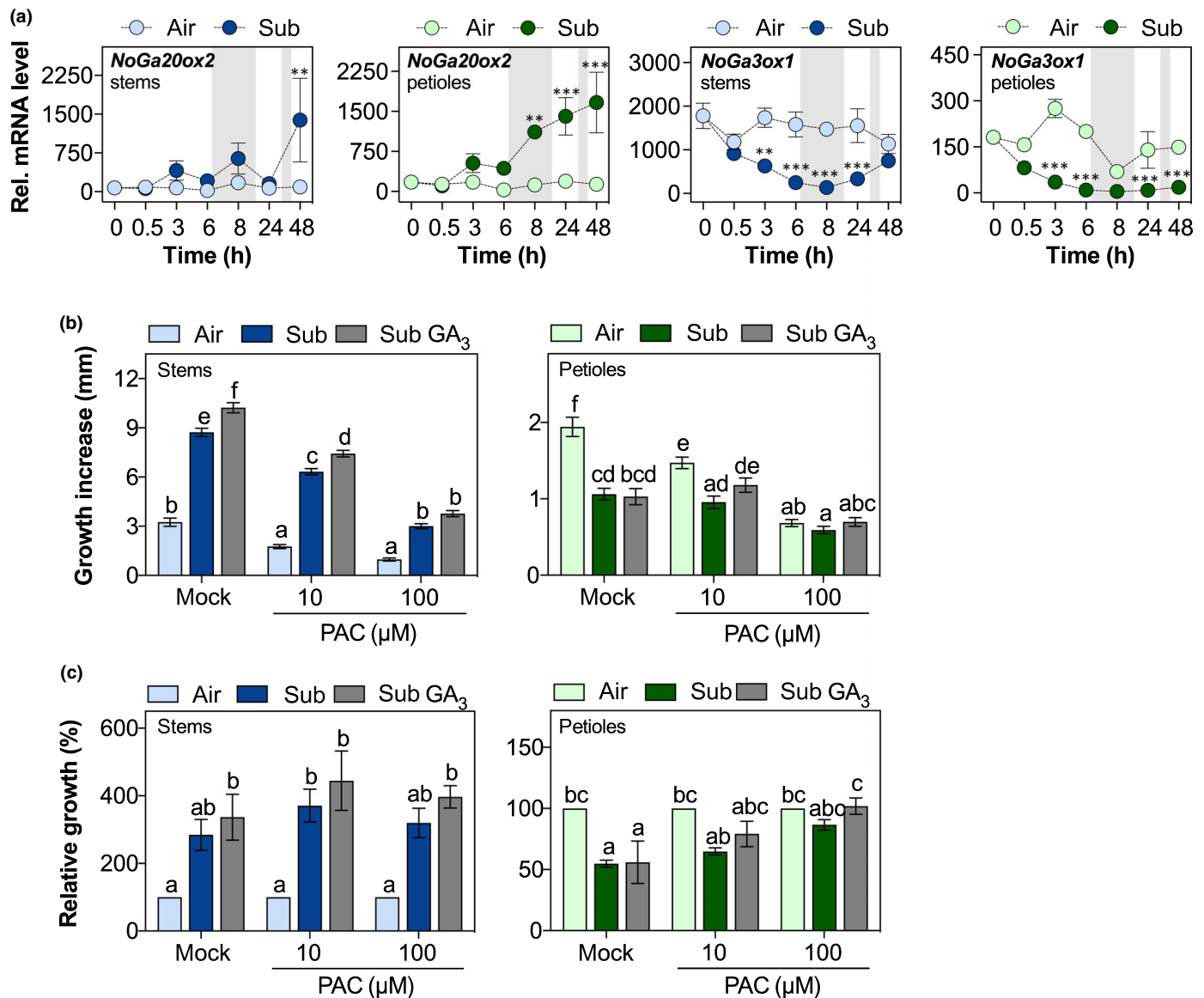
Fig. 4 Effect of ethylene on stem and petiole elongation of watercress in response to submergence. (a) Time-dependent expression analysis of ethylene biosynthesis genes (*NoACS7*, *NoACO1*). Plants of the five to six-leaf stage were either submerged (sub) or kept in air under short-day conditions (8 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 represent mean ± SE of four biological replicates (*n* = 4). Significant differences between air and submergence for each time point are indicated: ***, $P < 0.001$ (two-way ANOVA, Sidak's multiple comparisons test). Inhibition of (b) ethylene signaling or (c) biosynthesis only partially inhibits stem elongation. Plants were pretreated with (b) 10 ppm 1-methylcyclopropene (1-MCP) for 1 h or (c) with 50 μM aminoethoxyvinylglycine (AVG) 18 and 1 h before submergence. Plants were either submerged (sub) or kept in air. (d) Ethylene and (e) 1-aminocyclopropane-1-carboxylic acid (ACC) induce stem elongation but do not influence growth in the petioles. Plants were (d) pretreated with 10 ppm 1-MCP for 1 h before 21% oxygen gassing ± ethylene (≥ 2 ppm) for 24 h or (e) sprayed with mock solutions or 100 μM ACC and kept in air. (b–e) The length of the entire stem and the fifth petiole was measured before and after 24 h of treatment, and growth was calculated as the difference. Data are the mean ± SE of three biological replicates with (b) *n* = 40, (c, d) *n* = 30, (e) *n* = 45. Significant differences are indicated: (e) ***, $P < 0.001$ (Student's *t* test) or (b–d) different letters at $P < 0.05$ (two-way ANOVA, Tukey's honestly significant difference test). ns, not significant.

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
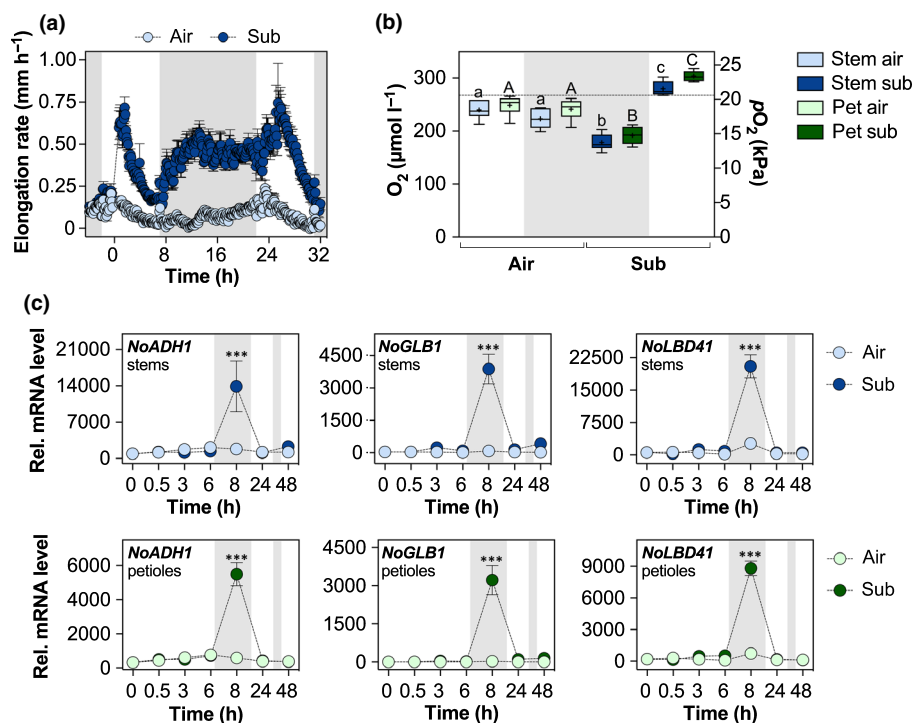
Fig. 5 Inhibition of GA biosynthesis does not repress stem elongation of watercress under submergence. (a) Time-dependent expression analysis of GA biosynthesis genes (*NoGa20ox2*, *NoGa3ox1*). Plants of the five to six-leaf stage were either submerged (sub) or kept in air under short-day conditions (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: **, $P < 0.01$; ***, $P < 0.001$ (two-way ANOVA, Sidak's multiple comparisons test). (b) Plants were watered with 5 ml of 10 μM or 100 μM paclobutrazol (PAC) or mock solution (0.1% ethanol) 17 h before submergence. Two hours after the start of the photoperiod, plants were then either submerged in water containing 0.01% ethanol or 10 μM GA$_3$ or kept in air. The length of the entire stem and the fifth petiole was measured before and after 24 h of treatment and growth was calculated as the difference. Data represent mean ± SEM of three biological replicates ($n = 30$). Different letters indicate values that vary significantly at $P < 0.05$ (two-way ANOVA, Tukey's honestly significant difference (HSD) test). (c) Relative data to data shown in (b). Data are mean ± SE of three biological replicates ($n = 3$). Different letters indicate values that vary significantly at $P < 0.05$ (two-way ANOVA, Tukey's HSD test).

(Bailey-Serres & Voesenek, 2008). 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 (PP$_i$)-dependent enzymes (Bailey-Serres & Voesenek, 2008; Bailey-Serres *et al.*, 2012a). The PP$_i$-utilizing enzyme PPDK and the gluconeogenic enzyme PEPCK were highly induced during submergence in watercress. Their

Fig. 6 Hypoxic conditions in watercress stems and petioles only occur during dark submergence. (a) Elongation rate in watercress stems measured using linear displacement transducers. Plants were grown under short-day conditions (9 h photoperiod) until the five to six-leaf stage. Two hours after the start of the photoperiod (0 h), plants were either submerged (sub) or kept in air. Growth rate is shown for the oldest internode and the hypocotyl, since linear displacement transducers were attached at the leaf stem junction of the oldest leaves. Gray areas indicate the night period. Data are the mean ± SE of five plants per treatment (n = 5). (b) Stem or petiole tissue atmospheric oxygen (O$_2$) with time as a response to light–dark switches with the shoot in air or the shoot submerged. The O$_2$ microsensor was positioned 550–600 μm into the stem and 200–225 μm into the petiole. Summary of six replicates in a box–whisker plot (horizontal line indicates the median; + indicates the mean; box and error bars indicate min–max). Different letters indicate statistically significant differences at $P < 0.05$ (two-way ANOVA, Sidak's multiple comparisons test), with lowercase letters representing stem tissues and uppercase letters representing petiole tissues. The dotted horizontal line indicates air equilibrium of O$_2$ at 23°C. (c) Time-dependent expression analysis of core hypoxia genes (*NoADH1*, *NoGLB1*, *NoLBD41*). 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 with 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: ***, $P < 0.001$ (two-way ANOVA, Sidak's multiple comparisons test).

gluconeogenic role in mediating submergence tolerance has already been discussed in rice, *R. acetosa*, *R. sylvestris*, and *A. thaliana* accessions (Moons *et al.*, 1998; Sasidharan *et al.*, 2013; van Veen *et al.*, 2013, 2016). Additionally, higher availability of pyruvate for fermentation could be achieved by simultaneously reducing branched-chain amino acid (BCAA) biosynthesis and inducing BCAA catabolism. Indeed, genes involved in BCAA turnover, like *IPMI*, *IMD*, and *BCAT* (Binder, 2010), were strongly regulated in watercress. Taken together, these results imply tissue-independent metabolic adjustments to arrest biosynthetic processes and increase the mobilization of alternative C resources during submergence in watercress.

The petiole growth suppression is associated with cell cycle arrest

The transcriptome analysis revealed a higher number of DEGs in the petioles and, concomitantly, more transcripts allocated to the petiole-specific clusters (Fig. 2), implying that 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 S5, S6). *WOX4*, which was only downregulated in petioles, promotes vascular cell division, and *A. thaliana 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, *NAD(P)-binding Rossmann-fold*

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 abscinazole-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 *NoGLB1* were strongly induced only during dark submergence, when the internal O_2 concentration declined significantly (Fig. 6b,c). In watercress, the internal O_2 concentration was even higher than in air when switched from dark to light submergence (Fig. 6b). These hyperoxic conditions are the result of O_2 production via underwater photosynthesis and the slow diffusion rate of O_2 from the plant to the environment (Pedersen *et al.*, 2006). A decline in internal O_2 only during darkness and an increase in internal O_2 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 O_2. 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. sceleratus*, 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 O_2 (Sasidharan *et al.*, 2018). Also, submergence-induced elongation in *Echinochloa 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).

Author contributions

JTM, HvV, RS and AM designed research; JTM, HvV, MMB, MA, OP, PS and RCS performed experiments; JTM, HvV, ARW and AM analyzed data; JT and YT provided material; JTM, HvV, RS and AM wrote the manuscript. All authors read and commented on the manuscript.

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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 O_2 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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