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Xylem cells rapidly sense and respond to nitrate to promote expansion

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Abstract

Nitrate, beyond being an important plant nutrient, serves as a signaling molecule. During vascular development, nitrate stimulates xylem cell expansion and suppresses xylem maturation, but the underlying signaling has remained unclear. In this study, we explored nitrate signaling during vascular development by monitoring transcriptomic changes in developing xylem tissues in a time series after administering potassium nitrate directly to the xylem sap in hybrid aspen (*Populus tremula* x *P. tremuloides*) trees. Gene expression analysis revealed a dynamic response, with gene expression being transiently activated at two hours post-application for genes related to xylem expansion and at four to eight hours for genes related to xylem maturation. The two-hour timepoint was therefore selected to examine nitrate mediated cell expansion in expanding xylem elements isolated from the surface of the woody tissue. Using microfluidics coupled with single cell RNA-sequencing (scRNA-seq), we found that majority of the xylem cells could sense and respond to nitrate, and that the cells having the strongest response included both xylem fibers and vessel elements at the earliest stages of cell expansion. Among the candidate transcription factors mediating the nitrate-driven xylem cell expansion we discovered the *Populus CYTOKININ RESPONSE FACTOR 4 (CRF4)*. Overexpression of the *P. tremula CRF4* in hybrid aspen mimicked the stimulatory effect of

nitrate on xylem cell expansion, supporting the function of CRF4 in nitrate-mediated xylem cell expansion. Taken together, we identified a coordinated, system-wide response to nitrate whereby nitrate is rapidly and transiently sensed in most of the xylem elements and provide evidence for nitrate being taken up from the xylem sap into the ray parenchyma and regulate early xylem development through transcriptional circuits unique to xylem cells.

Introduction

Nitrate is critical as a nutrient, being a major form of nitrogen utilized by plants (Crawford and Forde, 2002). Nitrate availability often varies in the soil and can be a limiting factor to plant growth and development. Depending on this, plants have adopted several mechanisms to balance nitrate uptake and distribution and ensure energy-efficient nutrient utilization. In tree species, such as *Populus*, coordination of these processes is challenging due to the sheer size of the organism. In addition to the apical growth, lateral growth needs to be sustained for optimal growth of the tree. Surprisingly little is known about nitrate uptake and metabolism taking place in the tree stem. It is known that nitrate can be taken up by the roots and transported into the shoot of trees for assimilation (Black et al., 2002), but the extent and the molecular players of nitrate uptake from the xylem sap along its transport route are not known.

Nitrate is distinct from other nitrogen sources as it also acts as a signaling molecule and can rapidly induce gene expression changes after its perception. The machinery of nitrate acquisition, sensing, and signaling is well documented, including NRT1.1/NPF6.3 as the nitrate transceptor, NIN-like protein (NLP) gene family members as sensors and several other transcriptional regulators of the nitrate response (Ho et al., 2009; Rubin et al., 2009; Varala et al., 2018; Fredes et al., 2019; Liu et al., 2022). Nitrate-elicited molecular events are time dependent as the expression change for different sets of genes can be observed within a few minutes to several days after its application (Scheible et al., 2004; Krouk et al., 2010; Krapp et al., 2011; Alvarez et al., 2012). Nitrate rapidly triggers genes responsible for its own transport and assimilation, and influences several other genes considered vital in signaling and primary nitrate response in *Arabidopsis thaliana* (from now on referred to as *Arabidopsis*) (Krouk et al., 2010; Bouguyon et al., 2015; Liu et al., 2017; Varala et al., 2018; Alvarez et al., 2020).

Another enticing fact is that nitrate uptake and response is not just systemic communication across different organs but also comprises of a local signal that acts at the cellular level (Delgado et al., 2024). Different cell types with their specialized functions can respond in a unique manner to the external stimulus, allowing plants to tweak their responses

and optimize resource use. Nitrate has been shown to induce coordinated yet distinct cellular responses in different cell types of Arabidopsis roots. Cortex and pericycle cells subjected to 48h ammonium nitrate treatment showed specific changes in gene expression that were, however, governed by a common set of transcription factors (Walker et al., 2017). More recently, nitrate-responsive transcriptomic changes were reported as early as 12 minutes in the outermost cells of the root, followed by spreading of the response into all other cell types of roots (Contreras-López et al., 2022). These changes align well with real-time *in vivo* monitoring of nitrate levels using a genetically encoded biosensor, which demonstrated the entrance of exogenous nitrate into most of the Arabidopsis root cells within 5 minutes (Chen et al., 2022). Together, these studies reveal spatiotemporal specificity in nitrate sensing, uptake and assimilation, likely serving efficient adaptation towards prevailing nitrate conditions in the soil.

Much of the research on nitrate signalling has focused on roots, while less is known about cellular responses to nitrate in the shoot. Trees are interesting in this respect since their shoot systems provide majority of the total biomass on earth. It has been reported in *Populus* trees that nitrate stimulates the activity of the apical and the vascular meristems, and influences differentiation of xylem elements in the wood (Poovaiah et al., 2019; Renström et al., 2024). In particular, nitrate stimulates the expansion of xylem elements in trees (Renström et al., 2024; Xu et al., 2024). However, these studies have captured only a snapshot of a long-term exposure of nitrate in bulk samples and do therefore not give information on temporal or spatial regulation of xylem cell expansion. In this study, we aimed to provide molecular-level insights into the early events of xylem development after nitrate application by dissecting the transcriptional response with an unprecedented spatial and temporal resolution in woody tissues. To narrow down the observed nitrate responses exclusively in wood, we designed a strategy to bypass the root-to-shoot communication of the nitrate signal by applying nitrate directly into the xylem sap of hybrid aspen (*P. tremula* x *P. tremuloides*) trees. We performed a time-series analysis of gene expression from 2h up to 48h after nitrate treatment to follow the progression of nitrate response with time and reveal the molecular players underlying nitrate mediated xylem development. Genes associated with xylem cell expansion were induced as early as 2h after nitrate application, which led us to profile the xylem transcriptome on a cellular level at this time point. scRNA-seq analysis revealed strongest response to nitrate in the xylem rays as well as fibers and vessels at their earliest developmental stage. Combination of the temporal and the spatial data revealed transcriptional networks unique to the different types of xylem elements. Based on these networks, we propose a role for a number of factors, including

the *CYTOKININ RESPONSE FACTOR 4 (CRF4)*, in mediating xylem cell expansion in response to nitrate.

Results

The timing of nitrate response is dynamic in woody tissues

Nitrate elicits fast metabolic and developmental changes in plants. To determine the timing of nitrate response exclusively in woody tissues, we injected KNO_3 directly into the xylem sap of nitrogen starved hybrid aspen (*Populus tremula* x *P. tremuloides*) trees (Figure 1), and followed transcriptomic changes in the developing xylem tissue along a time-course series from 2h up to 48h (Figure 1A-B). Parallel experiments with injection of acid fuchsin stain demonstrated that the whole woody tissue was likely to receive the injected solution at a distance of 60 cm from the injection point within 2h (Figure 1C-D). Samples for transcriptional analysis were therefore collected 40-60 cm from the injection point.

Principal component analysis (PCA) displayed clustering of transcriptome profiles mainly according to the different time points after nitrate application (Figure 2A). The transcriptomes corresponding to timepoints between 2h and 12h, for both the control (KCl) and nitrate (KNO_3) treatments, differed from the 0h timepoint, while the transcriptomes for the timepoints of 24h and 48h were similar to the 0h timepoint. The nitrate treatment seemed to influence transcriptome profiles only in the early timepoints (Figure 2A). Differential gene expression analysis revealed accordingly that 142, 372, 98, and 2 unique genes (DEGs) responded to nitrate after 2h, 4h, 8h, and 12h of nitrate treatment, respectively (Figure 2B, Table S1A-D), while there was no significant change in gene expression later on. The GO analysis of the DEGs revealed significant enrichment of terms related to *nitrate transmembrane transporter activity* and *nitrogen cycle metabolic process* (Figure 2C). Accordingly, several nitrogen uptake and metabolism related genes, including the homologs of the *NRT1.1/NPF6.3* and *NLP7*, were differentially regulated by nitrate.

All 29,591 genes expressed in RNA-seq data from the time-course experiment were used to create a whole-transcriptome co-expression network with 10 clusters and 28,821 nodes (genes) (Table S1F-G). A sub-network was created with all the *Populus* transcription factors (TFs) and DEGs that were first-degree neighbors of TFs (Table S1H). Seven TFs with a significant nitrogen-specificity index (see Materials and Methods) were identified ($P < 0.05$; Table S1H, Figure 3). All of these except *LBD19* have already been linked to nitrate responses

in either poplar or Arabidopsis (references in Table S3), demonstrating at least partial evolutionary conservation in nitrate responsiveness across these species.

Cell expansion related transcriptome responds rapidly to nitrate

To clarify the dynamicity of nitrate response in the different stages of vascular development, the DEGs were organized into ‘time bins’ based on their first appearance during the time-course experiment. The genes in the ‘time bins’ were grouped based on their $\log_2(\text{TPM}+1)$ -transformed Transcripts Per Kilobase Million (TPM) values in the Aspwood database (Sundell et al., 2017) which consists of high-resolution, spatial expression data in woody tissues of *Populus* stem (Figures 2E and S1, Table S1E). This analysis revealed the influence of 2h nitrate treatment predominantly on genes with preferential expression during xylem expansion (EX) according to the AspWood data. At 4h, the process of secondary cell wall formation appeared to be suppressed by nitrate based on the time bin analysis of the DEGs in the AspWood database (Figure 2E) as well as the enrichment of GO terms related to *cell wall organization or biogenesis* among the genes downregulated after 4h of nitrate treatment (Figure 2C). 4h timepoint also marked an increase in the numbers of time bin DEGs with the expression at a later stage of xylem maturation (CD) (Figure 2E). Taken together, the results demonstrate that the 2h timepoint captured the early effects of nitrate signaling especially on genes related to xylem cell expansion and that the signaling effect disappeared after 8h, indicating that the effect of nitrate was short-lived or "transient" in the current experimental setup.

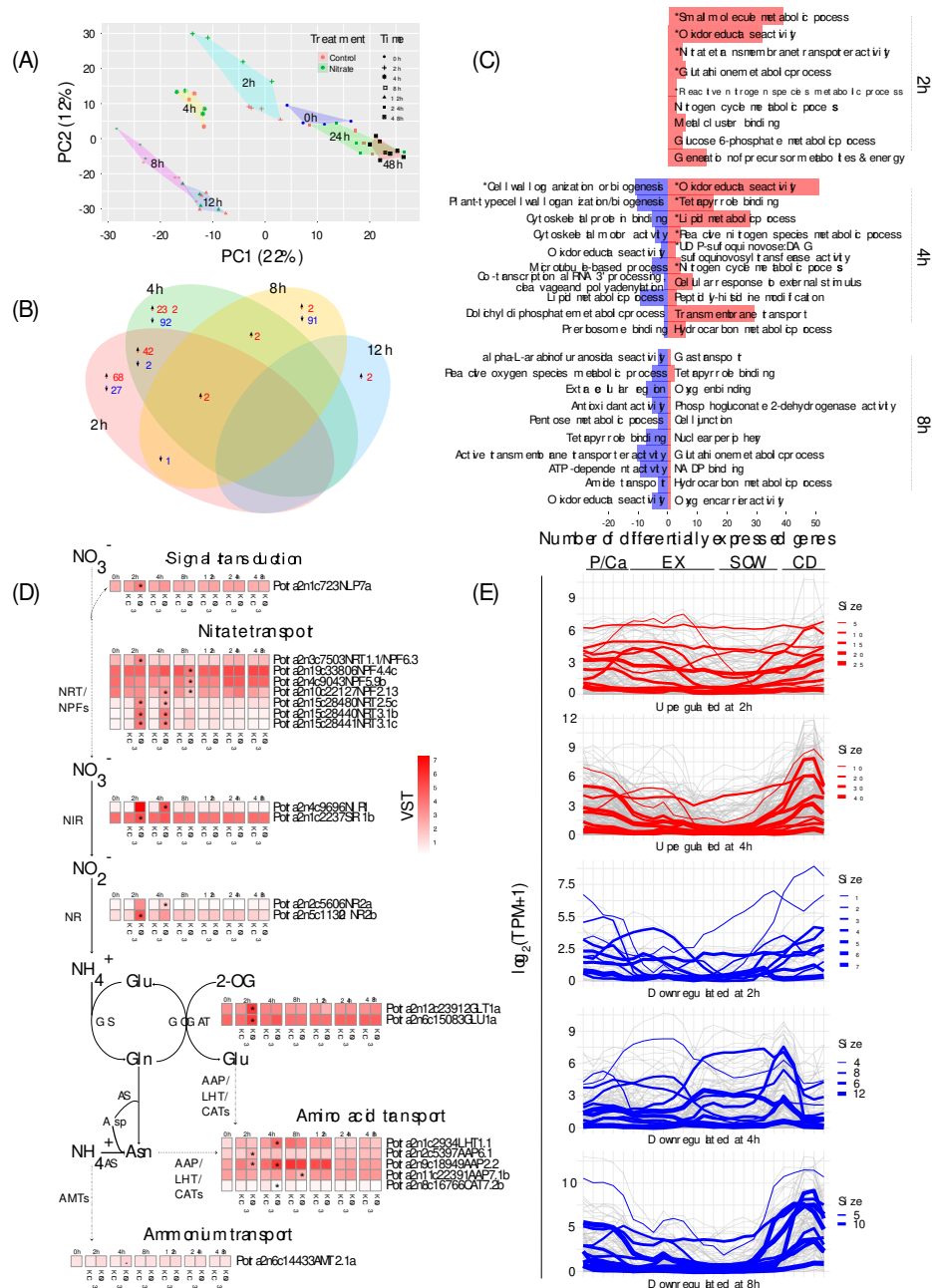


Figure 2 Time-course analysis of gene expression after nitrate treatment in developing xylem tissues of hybrid aspen. (A) Principal component analysis of data from RNA-seq of bulk developing xylem tissues. Samples from the developing xylem tissues were collected at 0h, 2h, 4h, 8h, 12h, 24h, and 48h after injection with nitrate (KNO₃) or control (KCl) solution. (B) Differentially expressed genes after nitrate treatment. The number of differentially expressed genes (DEGs) with absolute log₂ fold change (LFC) >1 and false discovery rate (FDR) <0.05 are shown in pairwise comparisons between the control and the nitrate-treated samples at a specific time point after nitrate injection. See Table S1A-D and Figure S1 for details. (C) Top ten most significant gene ontology (GO) terms for the DEGs. Asterisks (*) indicate significant terms. Gene annotations are based on the closest Arabidopsis homolog in TAIR. (D) Expression of nitrogen transport, metabolism and assimilation genes in response to nitrate treatment in developing xylem tissues of hybrid aspen trees. The heatmap is based on variance stabilized values (VST) from RNA-seq of xylem tissues 0, 2, 4, 8, 12, 24 and 48 h after injection of nitrate (KNO₃) or control (KCl) solutions to stems. A significant change in gene expression (absolute LFC >1 and FDR <0.05) after nitrate treatment versus control at a specific time point is marked with an asterisk (*). *P. tremula* gene annotations are based on Zhang et al. (2025). (E) Cluster analysis of time-specific genes. DEGs were grouped into 'time bins' based on their first appearance in the time series after nitrate treatment (Table S1E). Hierarchical clustering was performed using log₂(TPM+1)-transformed expression values obtained from the AspWood database. Up- and down-regulation in panels B, C and E are indicated by red and blue, respectively.

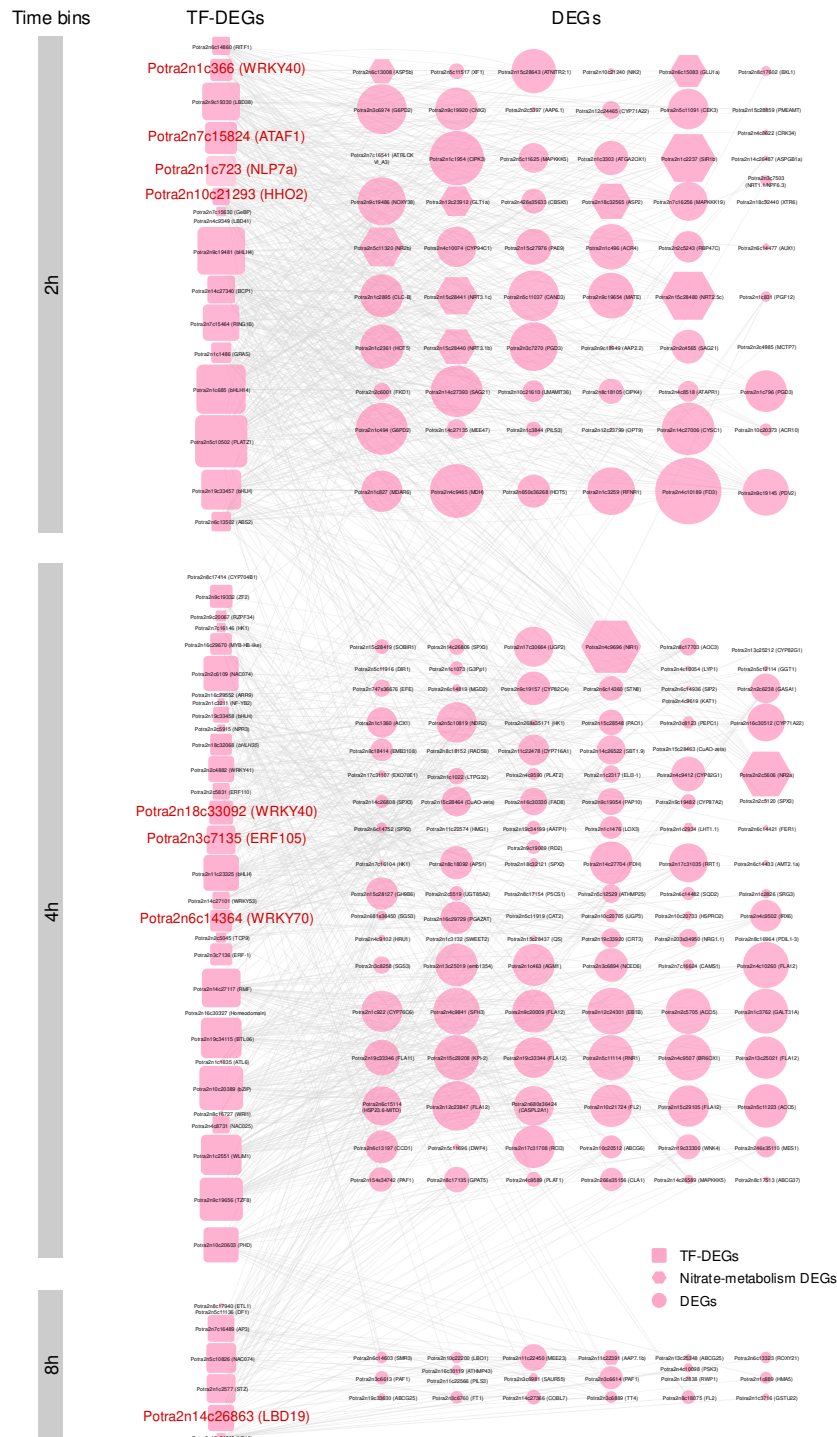


Figure 3 A transcription factor co-expression network in the developing xylem tissues of hybrid aspen. The network is based on differentially expressed transcription factors (denoted as TF-DEGs) and other DEGs that were first-degree neighbors of the TF-DEGs. Only edges between TF-DEGs and other DEGs are shown. The size of the node is directly proportional to its degree i.e., the number of directly connected genes. Seven TF-DEGs with a significant nitrogen-specificity index ($P < 0.05$) are marked in red. See Table S1H for details.

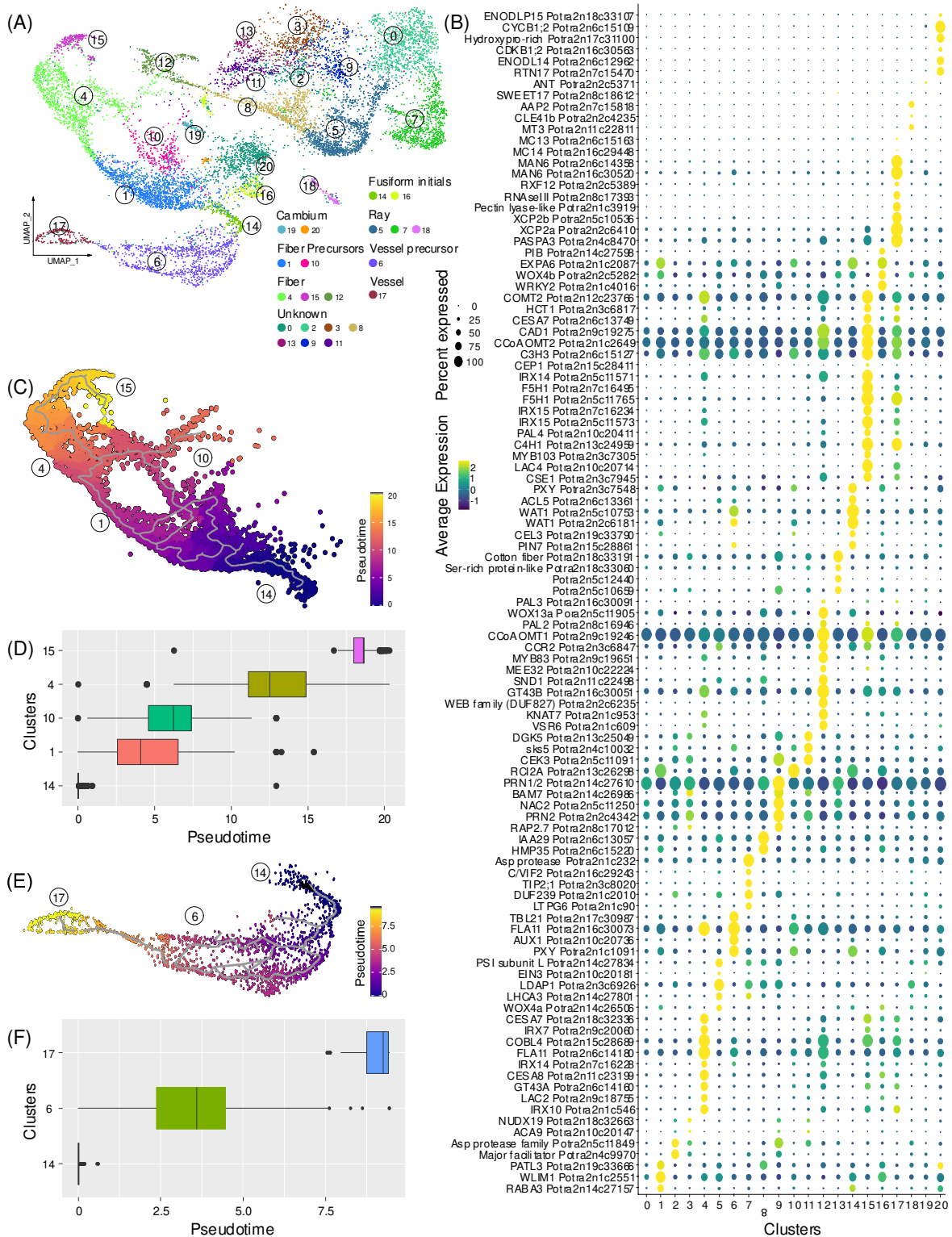
Most xylem cell types respond to the nitrate signal

Single cell RNA-sequencing (scRNA-seq) was performed to explore the nitrate-mediated regulation of xylem cell expansion on a cellular level. scRNA-seq libraries were prepared from

protoplasts isolated from the surface of the woody tissue that was injected with KNO₃ or KCl (control) and incubated for 2h in the same way as for the time-course experiment.

The transcriptome profiles from 20,566 protoplasts were clustered using a graph-based approach, yielding 21 clusters (Figure 4A). Clusters were identified using both previously characterized marker genes (Figure 4B) in *Populus* as well as genes enriched in different clusters based on the Wilcox test (Table S2A-B). References to literature for the cluster-specific genes in Figure 4B are given in Table S2B. We also performed transcriptomic correlations with existing gene expression datasets in woody tissues of *Populus* (Figure S2), including the high-resolution RNA-seq data from AspWood database (Sundell et al., 2017), spatial profiles (Du et al., 2023), single nuclei profiles (Conde et al., 2022) and laser capture microdissected (LCM) cells (Shi et al., 2017; Tung et al., 2023) to define our scRNA-seq cluster identities (Figure S2). Briefly, clusters 5, 7 and 18 were annotated as rays based on the enrichment of photosynthesis-related genes and correlation with LCM data (Tung et al., 2023). Various secondary cell wall associated cellulose synthases (*CESA*) were among the highly expressed genes in clusters 4, 12, 15 and 17, xylan biosynthetic *IRREGULAR XYLEM* (*IRX9*, *IRX14*, *IRX15*) and glycosyltransferase (*GT43A*) genes in clusters 4 and 15, and core lignin-biosynthetic genes and laccases (*LAC*) in clusters 12, 15 and 17 (Figure 4B). The transcriptomes of these four clusters (4, 12, 15 and 17) correlated with the AspWood sections from the zone of early xylem maturation (Figure S2A), which suggests that these clusters are likely to contain vessels and fibers at different stages of differentiation. Correlation of the clusters 4, 12 and 15 with the LCM fiber transcriptomes (Tung et al., 2023) (Figure S2B), suggest that these three clusters contain fibers, while correlation of cluster 17 with the LCM vessel transcriptome (Tung et al., 2023) and the expression of known vessel-specific genes, such as xylem cysteine protease (*XCP2a* and *XCP2b*), ribonuclease (*RNAseIII*) and endo-beta-mannanase (*MAN6*) specifically

197 in cluster 17 suggests that this cluster contains vessels (Figure 4B). Clusters 14, 16 were



198

199 **Figure 4 Single cell transcriptome profiling in developing xylem of hybrid aspen.** (A) UMAP visualization of
200 cell clusters in the scRNA-Seq data. (B) Marker genes enriched in each cluster that were selected based on Wilcox
201 test (Table S2A) in Seurat and from literature (Table S2B). For cluster annotations, see Figure S2 for more
202 information. Gene annotations are based on the closest Arabidopsis homologs. (C-F) Pseudotime developmental
203 trajectory of xylem fibers and vessels showing differentiation from fusiform initials (cluster 14) to fibers (C-D)
204 and vessels (E-F).

identified as fusiform (vessel and fiber) initials, cluster 6 as a vessel precursor and clusters 1 and 10 as fiber precursors as they correlated with the early expanding samples of the AspWood database (Figure S2A, C). These annotations were supported by the analysis of enriched GO terms (Table S2C) as well as developmental trajectories which demonstrated a bifurcated pseudotime backbone from the fusiform initial cluster 14 towards either the vessel (6 and 14) or the fiber clusters (1, 10, 4 and 15). (Figures 4C-F and S4A-D). Evidence for the presence of cambial cells was obtained through identification of 100 cells expressing the stem cell marker *AINTEGUMENTA* (*ANT*) in cluster 19 and cell division genes, *CYCLIN* (*CYCBI*;2) and *Cyclin-dependent kinase* (*CDKBI*;2) in cluster 20. Clusters 0, 2, 3, 8, 9, 11 and 13 did not clearly correlate with the earlier published datasets and were left without annotation (Figures 4A and S2). However, transcriptome closeness to the ray clusters (Figure 4A), correlation with the LCM ray sample (Tung et al., 2023) and early AspWood cambial samples (Figure S2A, B) suggest identity for 3, 8, 11 and 13 as early rays, but some of the clusters could even represent unique cell types. For instance, cells in cluster 9 showed high expression of *PIRIN2* (Figure 4B) which is expressed in specific ray cells next to vessel elements (Zhang et al., 2020).

Next, we investigated the nature of the cell types responding to the nitrate signal. First, a Canonical Correlation Analysis (CCA) analysis was used to integrate the two scRNA-seq libraries from the nitrate vs control treated samples (Figure 5A, Table S2D). Pre-processing at cell and gene levels resulted in 11,514 and 9,052 high-quality cells with a median UMI count of 2,062 and 2,608 and median gene count of 1,390 and 1,702 detected per cell, for control and nitrate-treated samples, respectively. A total of 28,217 genes were detected from the scRNA-seq covering 95% of the genes detected by the bulk RNA-seq of the xylem tissues in the time series. Differential expression analysis between nitrate treatment versus control identified 1,358 DEGs (Table S2E). The fact that DEGs were observed in every cluster revealed that all the xylem cell types could sense and respond to the nitrate signal (Figure S3). For instance, the early nitrate marker *NIR1* responded to the external nitrate supply in all xylem cell types, except for the small number of cells putatively involved in cell division in cluster 20 (Figure 5B). However, clear differences in nitrate responsiveness were present between the different cell types (Figure 5B). Differential enrichment of GO terms demonstrated that *cell wall organization or biogenesis* was significantly upregulated in clusters 4 and 6, *nitrogen cycle metabolic process* was upregulated in cluster 18, and *cell cycle* was downregulated in cluster 4 (Table S2F). The clusters containing the most nitrate responsive cell populations in the scRNA-seq data were the ray clusters 5, 7 and 18 (Figure 5C).

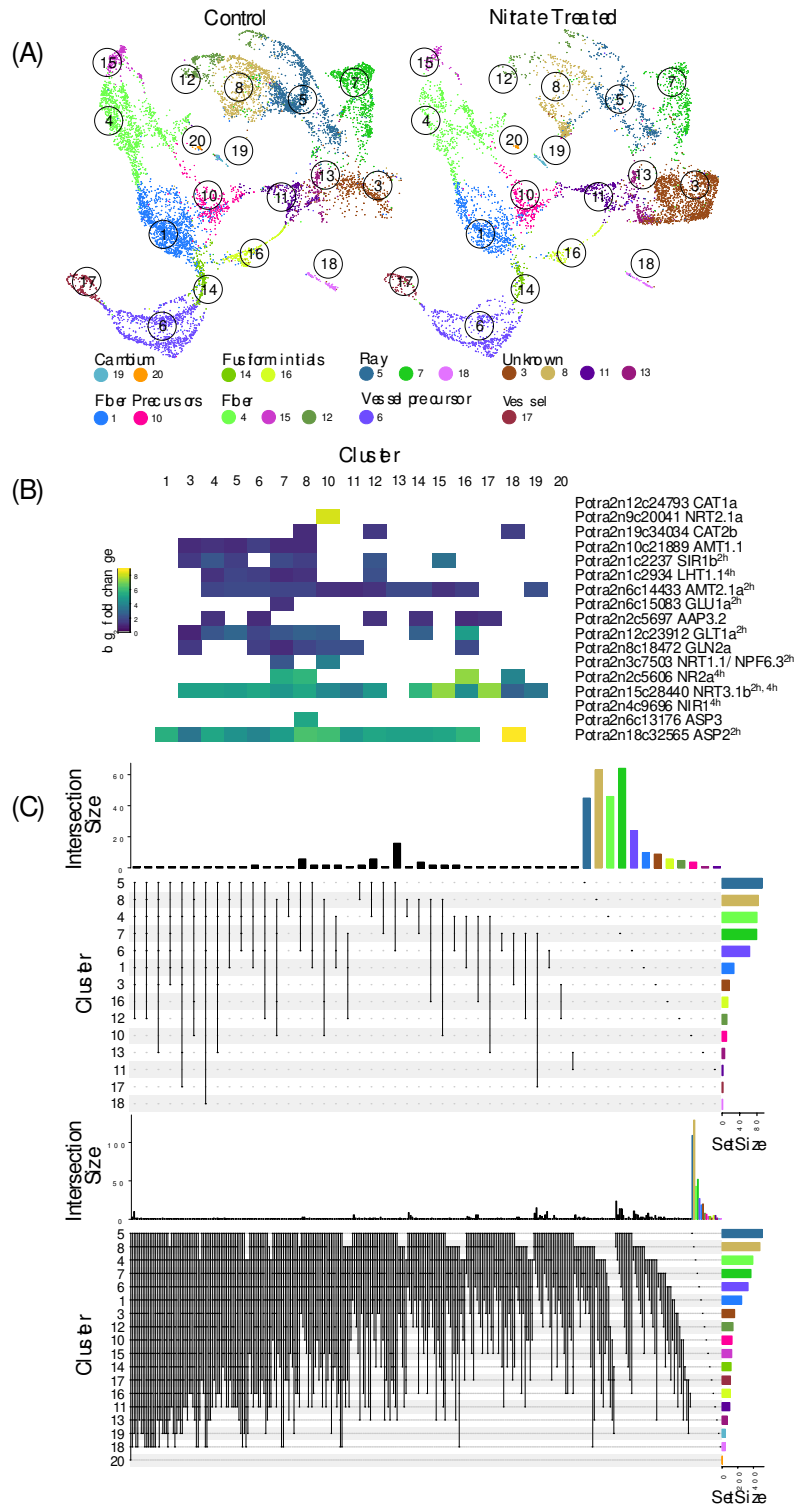


Figure 5 Gene expression analysis after nitrate treatment in different xylem cell types of hybrid aspen. (A) UMAP visualization after reclustering of the cells from control and nitrate treated samples. Distinct colors depict unique cell clusters. Clusters 0, 2 and 9 that either lacked a marker gene or did not show any correlation with the previous spatial or cellular *Populus* transcriptomes were excluded from this analysis. (B) Cluster-wise expression profile of DEGs related to nitrogen uptake and metabolism in the scRNA-seq data. The superscript on the gene labels indicates that the gene was a DEG in the time-series study. (C) UpSet plot showing the unique and shared number of upregulated (left panel) and downregulated (right panel) genes identified in each cluster for nitrate versus control treatment.

Nitrate stimulates the expression of cell wall modifying proteins in the fusiform cells

In addition to rays, fibers and vessels at the early stages of development (scRNA-seq clusters 1, 4 and 6) responded strongly to nitrate (Figures 5C, S3 and S4E-F). These cells are likely to undergo xylem cell expansion on the basis of the vessel and fibers trajectories (Figure 4C, E). Nitrate regulated several members of the gene families such as pectin methylesterase inhibitor (*PME1*, *PMEI-51*, *PME34*), pectin acetylerase (*PAE3*, *PAE10*, *PAE11*), xyloglucan endotransglucosylase/hydrolase (*XTR6*, *XTH16*), cellulose synthase (*CESA2*, *CES-like*), and expansins (*EXP6*, *EXP4*) in clusters 1, 4 and 6 (Table S2, Figure S3) that are all in different ways involved in xylem cell expansion (Scheible et al., 2004; Hacke et al., 2010; Plavcová and Hacke, 2012). Interestingly, we found that many of the lignin-biosynthetic genes (*CCoAOMT1*, *CAD1*, *C4H1*, *F5H1*) were suppressed in the fiber and vessel clusters 4, 12 and 17 after nitrate application (Table S2E, Figure S3).

To elucidate novel molecular factors for transcriptional regulation of the processes underlying nitrate-mediated xylem cell expansion, we explored TFs responding to nitrate in the different xylem cell types (Table S3). Out of these, *WRKY40*, *ASR3*, *ATAF1*, *HHO5* and *LBD38* are known nitrate-responders (see references and gene models in Table S3). Novel types of TFs in nitrate signaling included *ERF1B*, *ERF4*, *ERF110*, *HB-8*, *FBH4*, *HAT22*, bZIP TF, *NERD*, *WRKY48* and *CBF1*. We further refined our search to identify xylem-expansion related TF-DEGs on the basis 1) upregulation in response to nitrate in the scRNA-seq data, 2) specific expression in the expanding xylem according to the AspWood dataset, and 3) expression in the fusiform trajectories that included clusters 1, 4, 6, 10, 15 and 17 (Figure 6 A-C). There were 15 TF genes fulfilling the criteria, among which we identified the *Populus* transcription factor *CYTOKININ RESPONSE FACTOR 4* (*CRF4*, Potra2n15c29002, aka *ETHYLENE RESPONSE FACTOR 85*) that was already previously linked to xylem cell differentiation (Seyfferth et al., 2021). *CRF4* expression increased in response to nitrate in all cell clusters, but significantly in clusters corresponding to fiber precursors (1), fibers (4), vessel precursors (6) and rays (5, 7, 8) (Figure 6D). Taken together, the evidence supports a function for *CRF4* in xylem cell expansion. Furthermore, *CRF4* was co-expressed in the fibers with *NRT1.1/NPF6.3*, *ETHYLENE RESPONSE FACTOR 4* (*ERF4*), and *DEFECTIVE REGION OF POLLEN 1* (*LRL1*) (Figure 6E), pointing to a putative mode of action for the *CRF4*-mediated cell expansion.

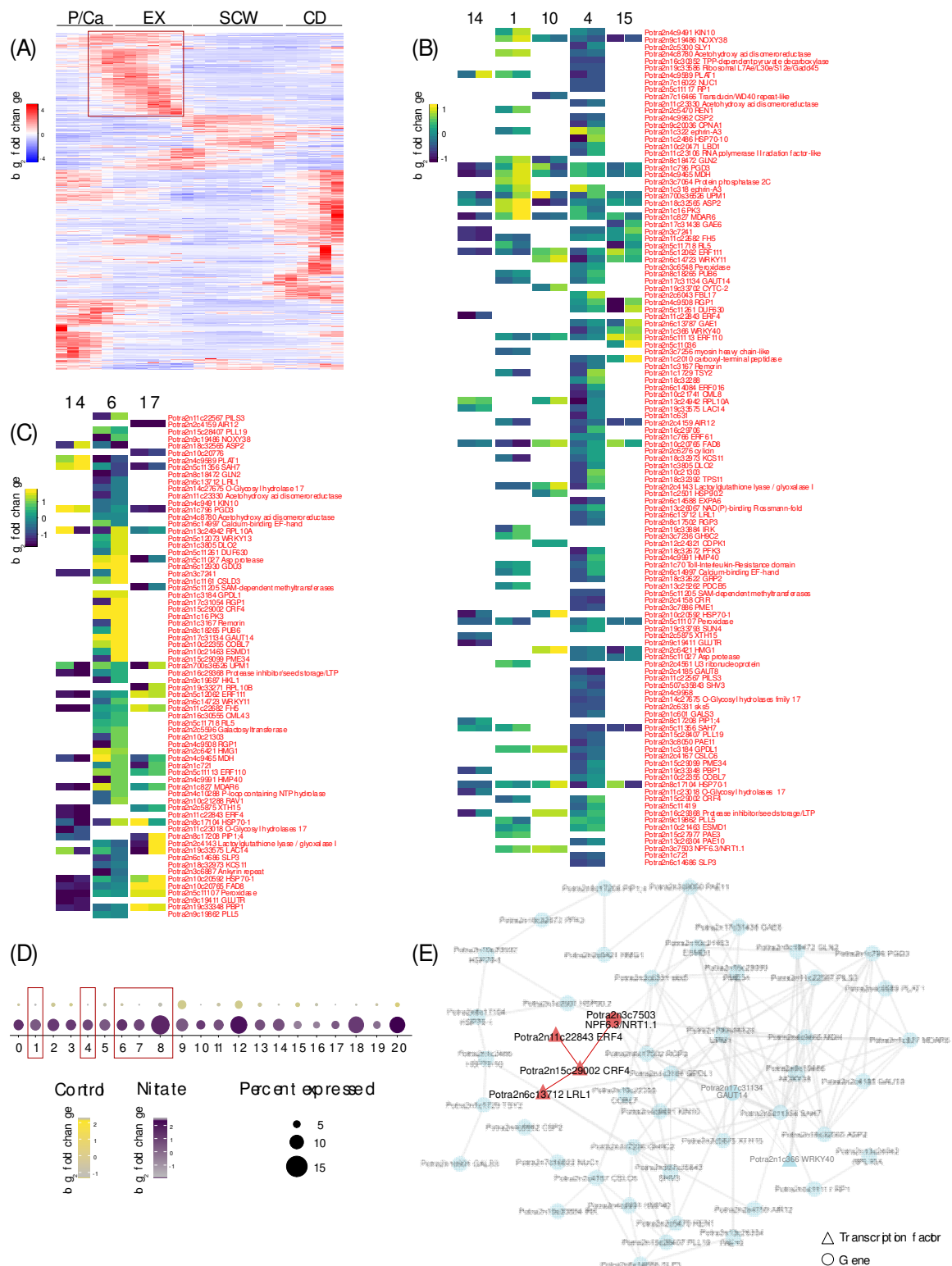


Figure 6 Gene expression circuits in the vessel and fiber trajectories. (A) Heatmap of significantly upregulated genes from scRNA-seq data according to their $\log_2(\text{TPM}+1)$ -transformed expression values (scaled for each gene) during cambial growth and wood development mapped to Tree1 in the AspWood database. P/Ca, phloem/cambium; EX, expanding xylem; SCW, secondary cell wall formation; CD, cell death. The red box marks the expression of selectively up-regulated genes with comparatively high expression in the expanding xylem region. (B-C) The expression of DEGs obtained from (A) in the fiber trajectory following a continuum of clusters 14, 1, 10, 4 and 15 (B) and in the vessel trajectory of clusters 14, 6, and 17 (C). (D) Cluster-wise *CRF4* expression after nitrate treatment. The red boxes show clusters where expression changes are significant (absolute $\log_2$ fold change >1 and false discovery rate <0.01). (E) A sub-network of fiber-cell expansion. The sub-network was

extracted from the whole-transcriptome co-expression network, constructed based on the data in the RNAseq of the time series. The sub-network contains TF-DEGs and their first-degree DEG neighbors that were specifically expressed in the phase of xylem expansion in the AspWood database and present in the scRNAseq fiber trajectory. Genes co-expressed with *CRF4* are highlighted in red.

To investigate if *CRF4* is involved in nitrate-mediated cell expansion, we analyzed the growth parameters (Figure 7A-B) and xylem cell morphology (Figure 7C) in transgenic *P. tremula* x *P. tremuloides* plants overexpressing the *P. tremula* *CRF4*. Overexpression of *CRF4* resulted in an increased diameter of fibers (Figure 7D) and a trend of increased diameter of the vessel elements (Figure 7E). Interestingly, similar to the effect of *CRF4* overexpression, increasing availability of nitrate was earlier shown to stimulate diameters of both the fibers and the vessel elements in *Populus* trees (Renström et al., 2024). The overexpression of *CRF4* has therefore a similar effect on xylem cell morphology as nitrate application. The fact that *CRF4* was co-regulated in fibers with *NRT1.1/NPF6.3* raised the question whether *CRF4* stimulates xylem cell expansion through increased availability of nitrate in the stem tissues. However, no differences were observed in nitrogen content of the stem (Figure 7F) or nitrogen uptake capacity of the roots (Figure 7G) between the trees overexpressing *CRF4* and the wild type.

Discussion

Response to nitrate in developing xylem is short-lived

Nitrate rapidly induced transcriptomic changes in the developing xylem tissue of hybrid aspen trees (Figure 2), demonstrating not only uptake of nitrate along its transport route but also sensing of nitrate to coordinate xylem development. We propose on the basis of these results that xylem cells constantly monitor the levels of nitrate in the transpiration stream and very quickly modify processes such as cell expansion and secondary cell wall formation according to the levels of nitrate in the xylem sap. This idea is supported by the transient nature of the nitrate response observed in our study (Figure 2) as well as in *Arabidopsis* roots (Krouk et al., 2010; Contreras-López et al., 2022), allowing rapid switches in developmental programs according to the prevailing nitrate levels. We propose further that the fast and transient nitrate responses provide plasticity in hydraulic conductivity of the stem by influencing the dimensions of the water-transporting xylem vessels according to the changes in the soil water content.

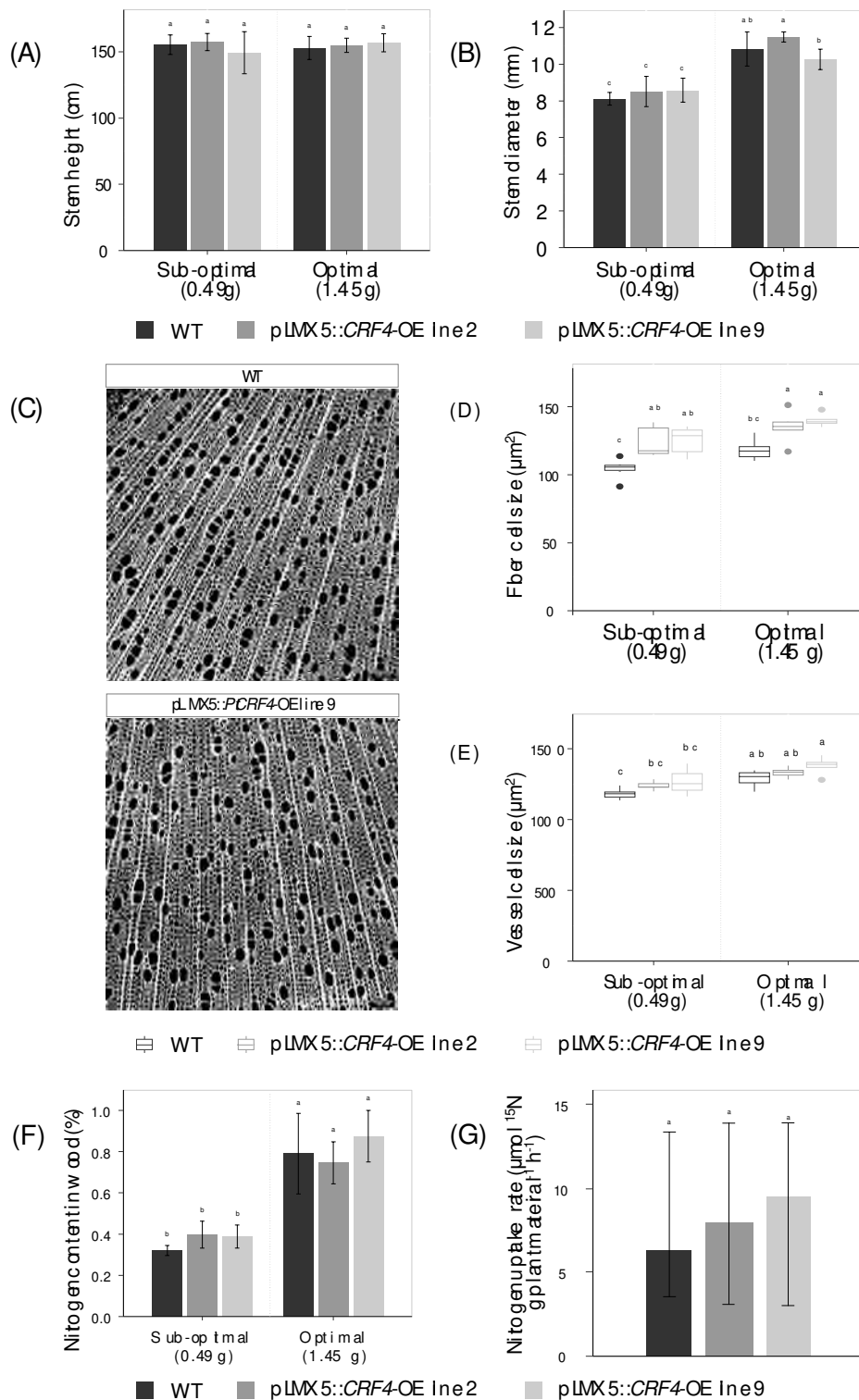


Figure 7 *CRF4* overexpressing (*CRF4*-OE) trees mimic the effect of nitrate on xylem cell expansion. (A-B) Overall growth of the *CRF4*-OE trees. Stem height (A) and stem diameter (B) in two transgenic *CRF4*-OE lines and wild-type trees grown for 10 weeks in two different fertilization regimes corresponding to a total amount of 0.49 g or 1.45 g nitrogen. (C-E) Wood anatomy in the *CRF4*-OE trees. Representative images of wood in *CRF4*-OE and wild-type trees. Bright-field images were made binary in ImageJ. Size of the fiber (D) and vessel elements (E) of *CRF4*-OE and WT trees in sections collected 20-25 cm from the base of the stem. N=5-7. (F) Nitrogen content in the woody tissues of *CRF4*-OE versus WT trees. N=3. (G) The rate of $K^{15}NO_3$ uptake in roots of the *CRF4*-OE lines versus WT trees. N=8-19. Statistical significance, comparing WT and *CRF4*-OE trees as independent groups, is tested with two-way ANOVA and Tukey HSD test for D-E. Means not sharing any letter are significantly different at 5% level of significance. Error bars indicate mean $\pm$ SD.

Xylem cell expansion is rapidly modified by nitrate

Cellular profiling is crucial for elucidating the regulatory networks that underlie cell-specific responses to nitrogen (Contreras-López et al., 2022). Our scRNA-seq analysis revealed that all xylem cell types responded to nitrate, but significant differences were observed in nitrate responsiveness of the different cell types (Figure 5B, Table S2E). For instance, the clusters corresponding to precursors of vessels and fibers were among those having the highest numbers of DEGs (Figures 5C, S3). Early xylem development was also the developmental stage that responded fastest to nitrate application in the time-series analysis (Figure 2).

The rapid responses in the early developing xylem elements suggest involvement of nitrate in xylem cell expansion (Figures 2, S5), which aligns well with our previous data on the positive effect of long-term nitrate application on the dimensions of the xylem elements (Renström et al., 2024). A question arises whether nitrate stimulates xylem cell expansion directly or indirectly by modulating the processes of cell division or secondary cell wall formation. In *Arabidopsis*, nitrate stimulates organ size preferably through cell expansion rather than division (Moreno et al., 2020; Abualia et al., 2022; Pereyra et al., 2023). However, we observed a significant enrichment of GO terms related to *cell cycle* in genes downregulated in response to nitrate in developing xylem (Table S2F), suggesting that nitrate might regulate xylem cell expansion by suppressing cambial cell division and thereby promote transition to xylem expansion. In addition, our results suggested that the effect of nitrate on xylem cell expansion is influenced at least to some parts through suppression of secondary cell wall formation on the basis of the time bin and the enriched GO term analyses of gene expression (Figure 2C, E). In particular, the expression of the lignin-biosynthetic genes was suppressed specifically in the fibers and vessels in response to nitrate (Table S2E). On the other hand, nitrate regulated the expression of a large number of genes that have preferential expression in the developmental phase of xylem expansion in *Populus* stem (Figures 2E and 6A) as well as the expression of genes in families, such as XTH, EXP, PAE, and PME, known to be central in xylem cell expansion (Siedlecka et al., 2008; Nishikubo et al., 2011), supporting also a direct effect of nitrate on xylem cell expansion.

CRF4 is involved in xylem cell expansion

Although substantial information exists regarding the molecular machinery that drives cell expansion, alternative pathways are likely to operate in response to nitrate in specific cell types, such as xylem elements (Pereyra et al., 2023). To understand this phenomenon, we explored

these cell types for TF genes induced by nitrate and observed some common players in Arabidopsis roots and xylem tissues in *Populus* (Figure 3, Table S3). A genome-wide analysis revealed that, even though common aspects exist, xylem elements respond in a multitude of ways that have not been reported in other tissues or cell types. Among the candidates leading to xylem cell expansion downstream of nitrate we found the *CRF4* transcription factor that was already earlier implicated in regulation of xylem development in *Populus* trees (Seyfferth et al., 2021). Interestingly, the *Populus CRF4* is a homolog of Arabidopsis *CRF4* which was earlier identified as a potential link in nitrate signaling and responsiveness (Varala et al., 2018). We show here that overexpression of the *Populus CRF4* increases xylem cell sizes in a manner that mimics the effect of nitrate (Renström et al., 2024), supporting the function of *CRF4* in nitrate-mediated xylem cell expansion. Different from *CRF4* overexpression in Arabidopsis, overexpression of the *Populus CRF4* did not influence nitrate uptake of the roots, and the exact mechanism remains to be elucidated. Interestingly, *CRF4* was co-expressed in our analyses with *ERF4* (Figure 6E) which has been shown to control cell size in Arabidopsis leaves through regulation of endoreduplication (Ding et al., 2021; Ding et al., 2022). Endoreplication has also been implicated in xylem development (Wendrich et al., 2020), although the underlying mechanisms remain unclear. It is tempting to speculate on the basis of our results that *ERF4*, perhaps together with *CRF4*, promotes transition from cell division to xylem cell expansion by stimulating endoreplication.

Materials and methods

Plant material, cultivation and nitrate application

Hybrid aspen (*P. tremula* L. x *P. tremuloides*, Michx, clone T89) rooted cuttings were cultivated for 12 weeks in the greenhouse in low nitrogen conditions followed by two weeks of nitrogen starvation prior to nitrate applications. Trees were grown in peat-based substrate with low nitrogen content (Solmull, Hasselfors Garden, Sweden). The substrate was pre-treated with 2 g lime per liter of peat to increase pH to approximately 5-6. Greenhouse and light conditions were kept the same as Renström et al. (2024). Trees were treated with 50 ml RIKAS (SW HORTO AB) based fertilizer once a week. Nitrogen fertilization started with a concentration of 6 mM ammonium nitrate (NH_4NO_3) per liter in week one and was increased to 40 mM NH_4NO_3 per liter by the last week, resulting in a total of approximately 0.38 mg of nitrate supplied to the trees by the end of week 12 (Figure 1A). After 12 weeks, the leaves were

removed from the lowest part of the stem, and the trees were nitrogen-depleted (no nitrogen added) for an additional two weeks. Subsequently, after the nitrogen-depletion period, 5 mM of either potassium nitrate (KNO_3) or potassium chloride (KCl, as a control) was injected into the xylem sap at the base of the stem using two 1-ml syringes (Injekt®-F Solo) that were connected to 1.2 mm Ø Sterican® needles as adapted from previous protocols (Sano et al., 2005; Umebayashi et al., 2007). A total of ~0.14 mg of nitrogen was injected in each tree. The piston of the syringe was removed to allow the solution to be drawn out of the syringe by the tension of the xylem sap, and a drop of the solution was placed on the stem to prevent air from entering the needle. The two syringes were placed on opposite sides of the stem (Figure 1B). Four biological replicates for each treatment (KNO_3 or KCl) were collected at 0h, 2h, 4h, 8h, 12h, 24h, 48h after the injection. Stem pieces, collected between 40-60 cm from the injection point, were frozen in liquid nitrogen for bulk tissue RNA-seq analysis and those between 20-60 cm were incubated in enzymatic solution for protoplast isolation for the scRNA-seq experiment.

The efficiency of the injection method was evaluated by following the fate of a stain in parallel experiments. 0.375% (w/w) acid fuchsin was injected into the base of hybrid aspen trees following the same procedure as for KNO_3 and KCl. Two hours after injection, stem pieces were collected in 10 cm intervals up to 60 cm from the injection point in six replicate trees. Hand sections were taken and imaged by a stereomicroscope Leica M205 FA (Leica Microsystems, Wetzlar, Germany). The images were analysed for the extent of positive staining by ImageJ. The positively stained area was calculated as a percentage of the total wood area in a transverse section of the stem. This was repeated for every 10 cm of the stem up to 60 cm from the dye injection point (Figure 1C-D).

RNA extraction of developing xylem for bulk RNA-sequencing (RNA-seq)

A frozen, 10 cm stem piece, collected 40-60 cm from the injection point, was debarked, and the developing xylem was scraped from the surface of the woody tissues with a scalpel and homogenized by mortar and pestle to a fine powder. RNA was extracted according to a protocol combining CTAB extraction protocol (Chang et al., 1993) and the RNeasy Plant Mini kit (©QIAGEN). Briefly, 800 µl of pre-warmed (65°C) CTAB solution [2% CTAB, 2% PVP, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2.0 M NaCl] containing 18 µl β-mercaptoethanol, was added to approximately 100 mg of frozen sample, vortexed for 25 sec and incubated at 65°C for 1 minute. Samples were extracted with 800 µl of chloroform-isoamylalcohol (24:1) twice,

mixed gently, and centrifuged at 3000 rpm for 10 minutes at room temperature. RNA was precipitated using 2.5 M LiCl (at a final concentration) from the upper phase by keeping on ice for one hour, followed by centrifugation at 13,000 rpm for 20 minutes at 4°C. The pellet was dissolved carefully in 400 µl of RLT buffer (©QIAGEN). RNA was extracted using RNeasy Plant Mini kit as per manufacturer's instructions except an additional purification step with DNase I (Invitrogen™) after first wash with 350 µl of RW1 buffer. Finally, RNA was eluted twice with 33 µl of RNase-free milliQ water. RNA was quantified with a Nanodrop 1000 Spectrophotometer (Thermo Scientific), and the quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The RIN values ranged from 8.6 to 9.7.

RNA-sequencing (RNA-seq) of developing xylem and differential gene expression analysis

52 samples, including four replicates at each of the seven timepoints (0, 2, 4, 8, 12, 24, and 48h), after nitrate treatment and controls were sequenced on one lane of the Illumina NovaSeq X platform with paired-end setup (300 bp). RNA-seq data analysis was performed using nf-core pipeline (Ewels et al., 2020). Briefly, raw reads were trimmed to remove low-quality bases (Quality Phred score <20) and sequencing adapters using Trim Galore v0.6.7. The pre-processed reads were quantified with Salmon v1.10.0 using the transcriptome index of *P. tremula* v2.2. Differential gene expression analysis between nitrate versus control was done using DESeq2 v1.44.0 and differentially expressed genes (DEGs) fulfilling the selection criteria of log₂ fold change (LFC) >1 and false discovery rate (FDR) <0.05 were reported significant. The VST of the read count data was utilized for heatmaps, which were generated using gplots package v3.1.3.1. The absolute TPM values, gene ontology (GO) enrichments and the data for the gene co-expression networks were obtained from <https://plantgenie.org/>. The genes expressed at VST>0 in at least two replicates of a timepoint were taken as background for GO enrichment analysis. The hierarchical or k-means clustering was performed on the log₂(TPM+1)-transformed expression data with the hclust function of R v4.2.2 assuming number of clusters (k) as 10 and visualized with ggplot2 v3.5.1.

Gene co-expression network

Seidr v0.14.2 was used to compute different network inference algorithms including, ARACNE, CLR, GENIE3, linear SVM (LLR- ensemble), mutual information, Narromi, partial correlation, Pearson correlation, Spearman, TIGRESS, and partial least squares feature

selection method (Schiffthaler et al., 2023) on all genes expressed at VST>0 in the time series study. Inverse rank product was calculated to aggregate the networks obtained from different methods and the edges were filtered with a noise-corrected backbone at a sigma of 1.34. Network clustering was performed following leiden algorithm using igraph v2.1.4 and graphs were visualized in cytoscape v3.10.3.

Populus TF genes were extracted from <https://plantgenie.org/> (Sundell et al., 2015) and used to construct a sub-network containing all TFs and their first-degree DEG neighbors. The nitrogen-specificity index for the differentially expressed TFs (TF-DEGs) was calculated as reported earlier (Varala et al., 2018). First, the best Arabidopsis homologs were identified for all the DEGs present in the subnetwork. The genome-wide binding data was retrieved for 11 out of 60 TF-DEGs in our study from the Arabidopsis DAP-Seq database (O'Malley et al., 2016; Ecker, 2017) and compared to their genome-wide targets among rest of the DEGs. TF-DEGs with significantly higher number of targets among the DEGs relative to their genome-wide distribution tested using one-tailed Fisher test ($P<0.05$) were considered specific to nitrate response (Table S1H). As mentioned earlier (Varala et al., 2018), this calculation although applicable to any TF with known genome-wide targets might be limited due to lack of data availability for Arabidopsis (O'Malley et al., 2016) as well as missing homologs for *Populus* TFs in Arabidopsis.

A sub-network for fiber-cell expansion was extracted from the whole-transcriptome co-expression network for the TF-DEGs and their first-degree DEG neighbors. This network consisted of genes upregulated in the fiber trajectory (clusters 1, 4, 10, 14 and 15) from the scRNA-seq dataset that were specifically expressed during xylem expansion according to the AspWood dataset.

Single-cell RNA-seq (scRNA-seq) of developing xylem and differential gene expression analysis

Protoplast isolation and single-cell RNA-seq (scRNA-seq)

After two hours of nitrate treatment, a stem piece was harvested 20-60 cm from the injection point for scRNA-seq. To account for the inherent variation between individual plants, five plants were harvested for protoplast isolation and pooled for scRNA-seq of nitrate treatment and control. Protoplasts were isolated according to an earlier protocol (Lin et al., 2014) with some modifications. Briefly, the internode segments were debarked and subjected to cell wall hydrolysis with a solution comprising of 1.5% (w/v) cellulase R-10, 0.4% (w/v) macerozyme

R-10, 0.5 M mannitol, 20 mM KCl, 10 mM calcium chloride (CaCl₂), 20 mM 4-Morpholineethanesulfonic acid (MES; pH5.7), and 0.1% (w/v) bovine serum albumin (BSA) for 2h in dark at room temperature. The digested tissue was then transferred to a wash solution containing 5 mM glucose, 125 mM CaCl₂ 154 mM NaCl, 4 mM MES (pH 5.7) and 5 mM KCl for 1-2 minutes on ice and gently inverted for 30 seconds to allow the release of protoplasts from digested tissue. The protoplasts were filtered with a 40 µm cell strainer to remove any clumps and washed three times with the wash solution using Allegra X-I5R centrifuge (Beckman Coulter,) at 100×g (at an acceleration of 8 and deceleration of 4) for 5 minutes at 4°C. The protoplasts were finally resuspended in 500 µl of solution containing 0.5 M mannitol, 20 mM KCl, 20 mM MES (pH5.7), and 0.1% (w/v) BSA. Protoplast concentration and viability was estimated by trypan blue staining.

The high-throughput scRNA-seq was performed using the 10x Genomics Chromium technology. The sequencing libraries were prepared according to the Chromium Next GEM Single Cell 3' Reagent Kits v3.1 User Guide CG000315 (Rev E) for targeting 10000 cells. The quality of DNA libraries was analyzed using an Agilent 2100 Bioanalyzer and paired-end reads were generated on an Illumina NovaSeq 6000 SP-100 with custom read setup of 28-10-10-90.

Clustering and annotations of the cell types

The raw scRNA-seq reads were aligned to the *P. tremula* reference genome v2.2 using the 10x Genomics Cell Ranger v7.0.0 pipeline (Zheng et al., 2017) that also performs barcode processing and cell calling with the intron mode option. The resulting expression matrices from Cell Ranger pipeline were processed with Seurat v4.3.0.1 (Hao et al., 2021). The Read10X_h5() function in Seurat returned a unique molecular identifier (UMI) count matrix comprising of number of molecules for each feature (i.e., gene in rows) that are detected in each cell (columns). The UMI count matrix was used to create a Seurat object with cell-level filtering criteria as follows: (i) number of UMI > 500; (ii) number of genes ranging from 200-7,000; (iii) log₁₀GenesPerUMI > 0.9; (iv) percentage mitochondrial count <5%; and (v) percentage chloroplast content <0.5%. The gene-level filtering was set to keep only the genes detected in at least three cells. The droplets potentially containing more than one cell were removed with doubletFinder v2.0.3 (McGinnis et al., 2019) utilizing the first 20 principal components (PCs). The optimal neighborhood size (pK) was calculated using paramSweep_v3. Based on the doublet formation rate of 8.0%, the number of expected real doublets (nExp) value was estimated by integrating artificial doublets at a defined proportion (pN). In brief, the

values of nExp, pN, and pK were set to 0.25, 0.26, 4046; and 0.25, 0.3, 3879, for control and nitrate datasets, respectively. Following this, only ‘singlets’ were kept for further analysis. The cell cycle scores were calculated by the CellCycleScoring() and PercentageFeatureSet() functions in Seurat, based on the *P. tremula* homologs for the genes defined in Arabidopsis (Gutierrez, 2009; Zhang et al., 2021). Normalization by the regularized negative binomial regression method (Hafemeister and Satija, 2019) was done by the SCTransform() function of Seurat with 3000 highly variable features and regressing out the variation due to cell cycle.

Cluster-specific markers were determined using Wilcox test in the FindAllMarkers() function in Seurat with genes expressed in at least 25% of all cells in a cluster and LFC>0.25. Cluster wise GO enrichment was achieved using topGO() function in R. The *P*-values obtained from the GO test were adjusted by Benjamini-Hochberg method for multiple testing correction (*P*-adj <0.01). The genes expressed in at least three cells of either of the samples were taken as background for GO enrichment.

Developmental trajectories for fiber and vessels were created using Monocle3 v1.4.26. A subset of data from specific cell clusters was extracted and cells were re-clustered at a resolution of 0.001. Graphs were constructed using learn_graph() followed by inference of pseudotime trajectory using order_cells() and plotted using plot_cells() function. The dynamic expression of genes along pseudotime were visualized by ggplot2 v3.5.2.

Differential gene expression

The CCA method was used to integrate the scRNA-seq datasets from control and nitrate treatment, which is highly recommended as it helps to match the shared cell types across conditions, boosts the statistical power, and facilitates the comparative analysis (Butler et al., 2018). After scaling the integrated data by the VST method based on the top 2000 highly variable genes and linear dimensionality reduction by PCA, the first 50 PCs were used in a graph-based approach to identify cell clusters that were visualized by Uniform Manifold Approximation and Projection (UMAP). Only 18 clusters showing some degree of correlation to the previous spatial or cellular *Populus* transcriptomes or marker genes were kept for differential gene expression analysis. The gene expression variability in different cell types due to nitrate treatment was inferred using Wilcox test in the FindMarkers() function of Seurat with LFC>1.0 and FDR< 0.01 in at least 10% of all the cells in a cluster. Significantly enriched GO terms under nitrate treatment were examined as described earlier.

Analyses of the *Populus CRF4* overexpression lines

Plant material and cultivation

The setup for the experiment was the same as in (Renström et al., 2024). In short, hybrid aspen cuttings, *P. tremula* L. x *P. tremuloides*, Michx, clone T89, and pro*LMX5::CRF4* lines 2 and 9, described earlier (Vahala et al., 2013; Seyfferth et al., 2021), were cultivated for 10 weeks in greenhouse conditions. Rooted cuttings generated *in vitro* were transplanted into 3-liter pots with a soil mixture of peat:perlite (10:1), mixed with 2 g lime per liter. The pot substrate (Solmull, Hasselfors Garden, Sweden) was treated with 40 mg nitrogen per liter, using RIKAS (SW HORTO AB, Sweden; 7-1-5 N:P:K) based fertilizer. Trees were subjected to 18:6 h day/night cycles, with a daily temperature of ~22°C and 50-60% relative humidity. Ambient light was supplemented with Fionia FL300 Sunlight LED lamps (Senmatic A/S, Denmark) with a maximal irradiance of 150-250 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Trees were fertilized at one- or two-day intervals, with RIKAS-based nutrient solution and NH_4NO_3 as nitrogen source, the total nitrogen amount added during the whole experiment reaching either 0.49 g or 1.45 g.

Analysis of stem nitrogen content

Nitrogen content in wood was measured by mass spectrometry (EA-IRMS). The instrument setup consisted of an elemental analyzer (Flash EA 2000, Thermo Fisher Scientific, Bremen, Germany) connected to a continuous flow isotope ratio mass spectrometer (DeltaV, Thermo Fisher Scientific, Bremen, Germany). The lignified part of wood, 10-20 cm from the base of the stem, was used for the analysis. The stem piece was freeze dried and milled after removal of the pith and initial leaves were homogenized to a fine powder.

Wood anatomy analysis

Wood anatomy samples were collected at a distance of 20-25 cm from the base of the stem. Radial sections were cut with Vibratome VT100 (Leica Microsystems, Wetzlar, Germany) at a thickness of 30 μm , always right above the node. Eight pictures per section were taken with a Leica Dmi8 microscope with color Leica DFC7000T color camera and LasX software (Leica Microsystems, Wetzlar, Germany), avoiding the areas closest to the pith and the developing xylem. For vessel measurements, sections were stained with 0.05% Toluidine blue O and imaged in bright field mode. For fibers and cell wall area analysis, autofluorescence of wood was used, and pictures were captured (Ex:490 nm, DC: 510 nm EM: LP515 nm) with Leica DFC9000GT monochromatic camera (Leica Microsystems, Wetzlar, Germany), 10x objective.

Images were then processed in ImageJ by the ‘analyze particle’ function counting numbers of vessels, fibers, and their lumen area.

Nitrate uptake

Uptake experiments were adapted from (Filleur et al., 2001). The cuttings were cultivated in low nitrogen media (½ MS medium with 1 mM KNO₃ as the sole source of nitrogen, pH 5.6) until rooted. The rooted cuttings were washed thrice in 0.1 mM CaSO₄ before being transferred from the growth media containing 0.2 mM K¹⁵NO₃ for 35 minutes. The roots were washed thrice to remove excess isotope-labeled nitrate, dried in an oven at 65°C and homogenized to a fine powder in a bead mill. Samples were weighed and analyzed by EA/IRMS, as described for the analysis of stem nitrogen content.

Figure legends

Figure legends appear in-line with text under figures (above).

Availability of data and materials

The raw reads from the time series RNA-seq experiment are available under the EBI project ID# PRJEB78718. The single cell RNA-seq data and clustered Seurat object are accessible under the NCBI GEO series ID# GSE304804. The code for data analysis from preprocessing of RNA-seq data to the gene co-expression network analysis can be found at <https://github.com/nicolasDelhomme/ShortTermNitrateTimeSeries-SNRIV> and for scRNA-seq data analysis at <https://github.com/shruti281989/SNRIII>.

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Author contributions

AR, SC and HT conceived the project. SC performed RNA-Seq data analysis, extracted protoplast suspension and performed scRNA-seq library prep and data analysis. AR performed the plant experiments, collected and prepared plant material for RNA-seq, and wood histology.

PM helped with RNA extraction and histology. TK contributed to the scRNA-seq data analysis. Both EP and TK supervised the construction of the gene co-expression network. SJ and HT supervised the work. JH assisted with the scRNA-seq experiments. SC wrote the first draft of the manuscript, and all authors contributed to the writing.

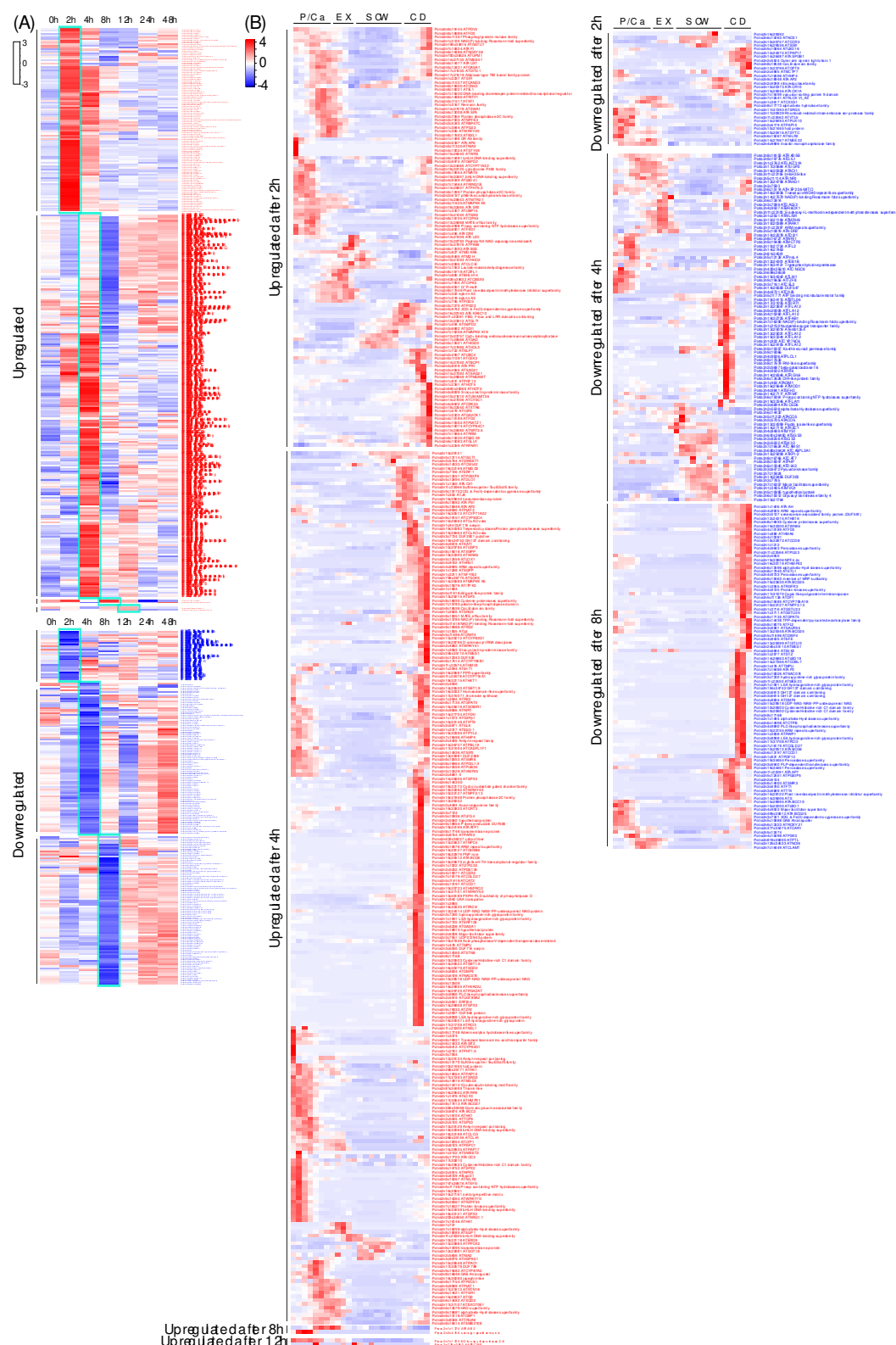
Supplementary data

Additional file 1: Table S1 Supplementary data from the time course RNA-seq experiment.

Additional file 2: Table S2 Supplementary data from the scRNA-seq experiment.

Additional file 3: Table S3 Differentially expressed genes (DEGs) of this study that were reported nitrate responsive in earlier studies in literature.

Additional file 4: Figure S1-S5 Figure legends appear under the supplementary figures (below).



814

815 **Figure S1 Time bin analysis where DEGs were grouped based on their first appearance after nitrate**
 816 **treatment.** (A) Heatmaps for genes of each time bin according to their variance stabilized expression values
 817 (scaled for each gene) obtained from RNA-seq. (B) Heatmaps for genes of each time bin according to their
 818 $\log_2(\text{TPM}+1)$ -transformed expression values (scaled for each gene) during cambial growth and wood development
 819 in Tree1 of the AspWood database (Sundell et al., 2017). P/Ca, phloem/cambium; EX, expanding xylem; SCW,
 820 secondary cell wall formation; CD, cell death. Gene annotations are based on the closest Arabidopsis gene from
 821 TAIR. See Table S1E for details.

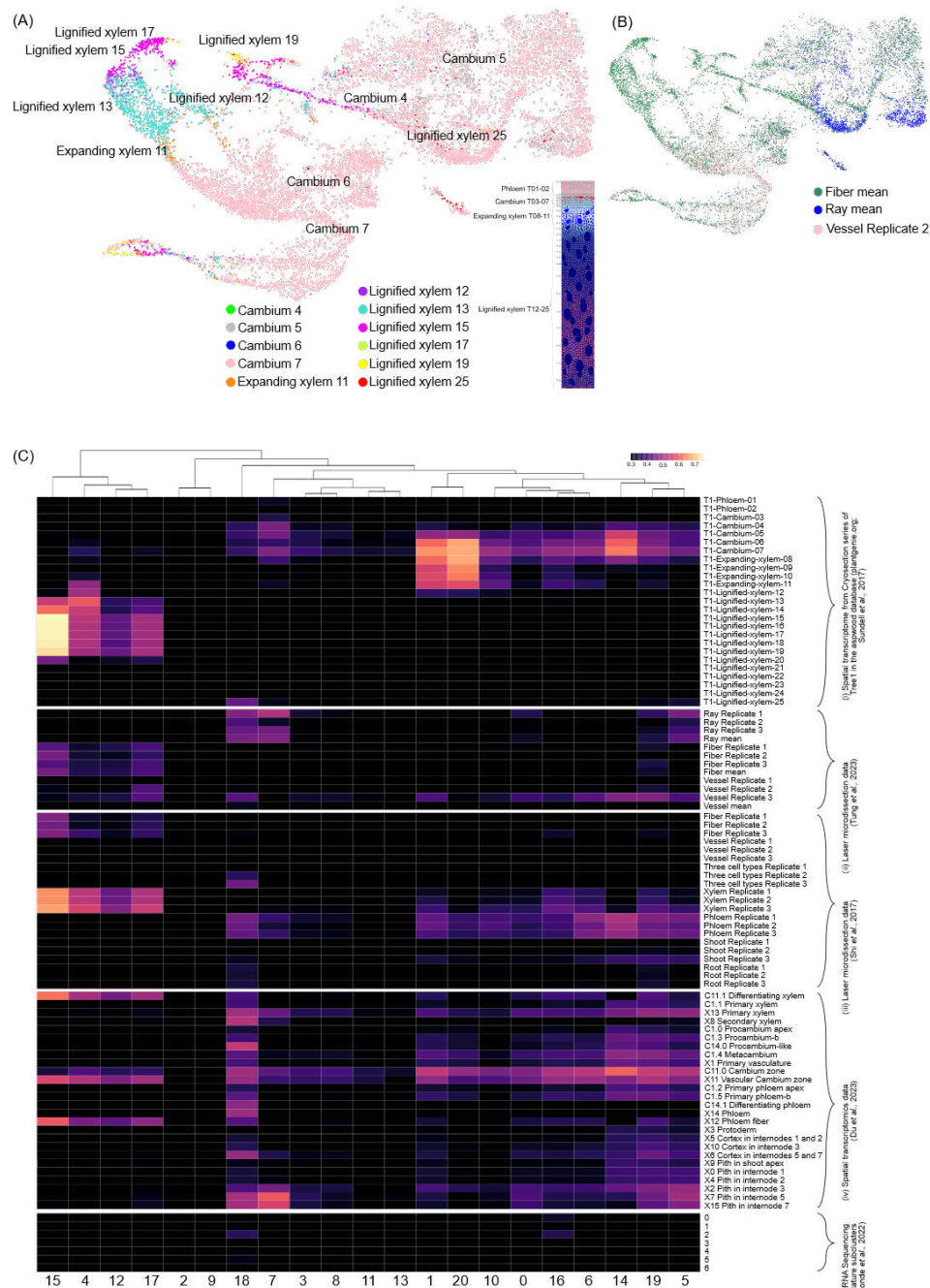


Figure S2 Correlation of clusters identified from our scRNA-seq experiment with reported spatial and cellular transcriptomes in *Populus* species. (A) Correlation dotplot of cells from our cell clusters with Tree1 in the Aspwood database (Sundell et al., 2017). (B) Correlation dotplot of cells from our cell clusters with transcriptome profiles of laser capture microdissected (LCM) xylem rays, fibers, and vessels from *P. trichocarpa*, reported in Tung et al. (2023). Note that for the vessels, we show only replicate 2 that most likely is free from other cell types. (C) Correlation heatmap of our scRNA-seq clusters (below) to various cellular and spatial profiles available in the public domain. (i) Correlation to RNA-seq data from Aspwood sections from Tree 1 where sections 1 and 2 consist of phloem, 3-7 vascular cambium, 8-11 expanding xylem and the rest lignified xylem. Vessel cell death is estimated to occur in section 18 and fiber cell death in section 24. Section 25 comprises exclusively of the living rays. (ii) Correlation to RNA-seq data of three biological replicates of each of the xylem cell type (fiber, vessel and ray) captured by LCM and their respective means (Tung et al., 2023). (iii) Correlation to RNA-seq data of various xylem cells and tissues captured by LCM (Shi et al., 2017). (iv) Correlation to clusters obtained from spatial transcriptome profiles from shoot apex till the 7th internode of wood by Du et al. (2023). (v) Correlation to clusters obtained from single nuclei RNA-seq from vasculature of the shoot apex (Conde et al., 2022). Gene expression data utilized in the analysis are $\log_2(\text{TPM}+1)$ -transformed expression values.



Figure S3 Gene expression in response to nitrate in xylem cells. Heatmap for cluster-wise expression profile showing log₂ fold change (LFC) of differentially expressed genes (DEGs) for the comparison between nitrate treatment versus control. DEGs fitting the significance criteria (LFC >1 and false discovery rate <0.01) in a specific cluster are shown with up and downregulated genes in red and blue, respectively. Gene annotations are based on the closest Arabidopsis homologs. See Table S2E for more details.

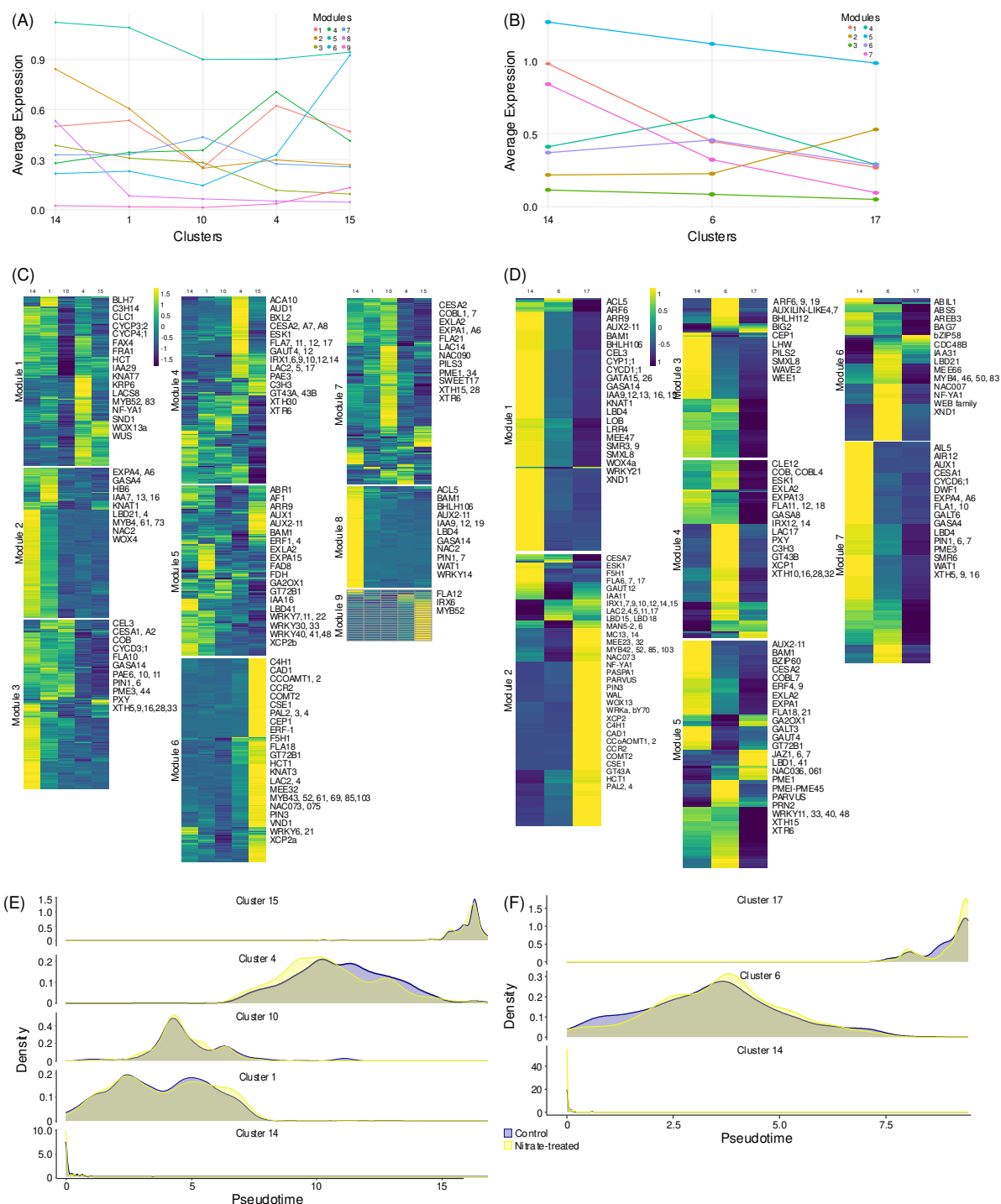


Figure S4 Pseudotime developmental trajectory for xylem fiber and vessel. (A-B) Monocle was used to identify modules of genes that co-vary across pseudotime during fiber (A) and vessel (B) cell differentiation. (C-D) Heatmap of pseudotime-dependent genes in each module during fiber (C) and vessel (D) differentiation. Example of genes in each module are shown. See Table S2G for full set of genes in each module. (E-F) Effect of nitrate treatment on the developmental trajectory. Density plot showing fiber (E) and vessel (F) developmental trajectories for control and nitrate-treated samples over pseudotime.

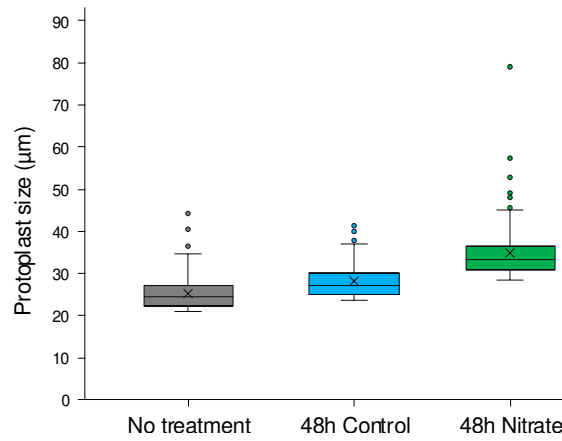


Figure S5 Xylem cells expand after nitrate application. Protoplasts were isolated from developing xylem 48h after nitrate treatment and their sizes were determined. The diameters of the top 10% largest cells, likely to represent the xylem vessels, are shown.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS1.xlsx](#)
- [TableS2.xlsx](#)
- [TableS3.xlsx](#)