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An Arabidopsis gene regulatory network for secondary cell wall synthesis

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The plant cell wall is an important factor for determining cell shape, function and response to the environment. Secondary cell walls, such as those found in xylem, are composed of cellulose, hemicelluloses and lignin and account for the bulk of plant biomass. The coordination between transcriptional regulation of synthesis for each polymer is complex and vital to cell function. A regulatory hierarchy of developmental switches has been proposed, although the full complement of regulators remains unknown. Here we present a protein–DNA network between Arabidopsis thaliana transcription factors and secondary cell wall metabolic genes with gene expression regulated by a series of feed-forward loops. This model allowed us to develop and validate new hypotheses about secondary wall gene regulation under abiotic stress. Distinct stresses are able to perturb targeted genes to potentially promote functional adaptation. These interactions will serve as a foundation for understanding the regulation of a complex, integral plant component.

Plant cell shape and function are in large part determined by the cell wall. Almost all cells have a primary wall surrounding the plasma membrane. Specialized cell types differentiate by depositing a secondary cell wall upon cessation of cell elongation. In addition to providing mechanical support for water transport and a barrier against invading pathogens, the polymers contained within the wall are an important renewable resource for humans as dietary fibre, as raw material for paper and pulp manufacturing, and as a potential feedstock for biofuel production. Secondary cell walls account for the bulk of renewable plant biomass available globally.

The secondary cell wall consists of three types of polymer—cellulose, hemicelluloses and lignin—and is found in xylem, fibres and anther cells. Cellulose microfibrils form a main load-bearing network. Hemicelluloses include xylans, glucans, and mannans. Lignin is a complex phenylpropanoid polymer that imparts ‘waterproofing’ capacity as well as mechanical strength, rigidity and environmental protection. Despite the importance of the plant secondary cell wall, our knowledge of the precise regulatory mechanisms that give rise to these metabolites is limited. The expression of cell wall-associated genes is tightly spatiotemporally co-regulated1,2. However, the pervasive functional redundancy within transcription factor families, the combinatorial complexity of regulation, and activity in a small number of cell types render functional characterization from single gene experiments difficult. A model of master regulators has been proposed with NAC domain and homeodomain HD-ZIP Class III (HD-ZIPIII) transcription factors initiating cell specification and secondary cell wall synthesis in Arabidopsis thaliana. In this model, VASCULAR-RELATED NAC DOMAIN6 (VND6) and VND7 are sufficient but not necessary to regulate xylem vessel formation; additionally, the HD-ZIPIII transcription factor PHABULOSA (PHB) also regulates vessel formation, and acts in a highly redundant manner with four other HD-ZIPIII factors1. In anthers, two NAC domain transcription factors, NAC SECONDARY WALL THICKENING1 (NST1) and NST2, are sufficient to drive the secondary cell wall biosynthetic program, but act redundantly4. Thus, regulation of this process is highly redundant and combinatorial. However, no comprehensive map of interactions has been developed at cell-type-resolution over time, nor have upstream regulators been identified. We therefore chose to pursue a network-based approach to comprehensively characterize the transcriptional regulation of secondary cell wall biosynthesis.

Mapping the secondary cell wall synthesis regulatory network

To systematically map this regulatory network at cell-type-resolution, we used a combination of high-spatial-resolution gene expression data3 and the literature1,2 to identify fifty genes implicated in xylem cell specification. These included transcription factors and enzymes involved in cellulose, hemicellulose and lignin biosynthesis that are expressed in root xylem cells (Supplementary Table 1; Methods). Selection of both developmental regulators and downstream functional genes allowed us to interrogate upstream regulatory events that determine xylem specification and differentiation associated with secondary cell wall synthesis. Promoter sequences were screened using an enhanced yeast one hybrid (Y1H) assay against 467 (89%) of root-xylem-expressed transcription factors2. Protein interactions were identified for 45 of the promoters (Supplementary Table 2). The final network comprises 242 genes and 617 protein–DNA interactions (Fig. 1a; http://gturco.github.io/trenzalore/stress_network). Thirteen of the transcription factors have been previously identified as having a role in xylem development or...
Regulators of xylem development and secondary cell wall biosynthesis. a, Gene regulatory network for secondary cell wall biosynthesis in Arabidopsis root xylem. Nodes, transcription factors or promoters; edges, protein–DNA interactions. Edges in feed-forward loops are red. b, A sample feed-forward loop in red. c, ‘Power edges’ between node sets. d, The secondary cell wall network from sub-fragments of cell wall promoters.

Testing interactions predicted by the network

Using our network, we hypothesized that E2Fc is a key upstream regulator of VND6, VND7 and secondary cell wall biosynthesis genes. This hypothesis is based on our finding that E2Fc bound to 23 promoters including those of VND6, VND7 and MYB46, and cellulose-, hemicellulose- and lignin-associated genes (Fig. 2a). VND7 and MYB46 are also known to bind to the promoters of many of these genes as well8,13,15, creating a suite of feed-forward loops. E2Fc can act as a transcriptional repressor16–18 as well as a transcriptional activator19–22 and here we report both. E2Fc activated VND7 expression in a dose-dependent manner (Fig. 2b and Extended Data Fig. 2a, b) in transient assays, but not in the presence of RETINOBLASTOMA-RELATED (RBR) protein, as is typical of E2F transcription factors (Extended Data Fig. 2c). In an E2Fc-overexpressor line with the amino terminus deleted to overcome post-translational degradation16,17, regulation of VND7 expression by extremely high or

Figure 1 | Regulators of xylem development and secondary cell wall biosynthesis. a, Gene regulatory network for secondary cell wall biosynthesis in Arabidopsis root xylem. Nodes, transcription factors or promoters; edges, protein–DNA interactions. Edges in feed-forward loops are red. b, A sample feed-forward loop in red. c, ‘Power edges’ between node sets. d, The secondary cell wall network from sub-fragments of cell wall promoters.

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Based on our results, we propose that E2Fc acts in a complex.

Figure 3 | Tissue-specific VND7 regulation and VND7 targets. a, REV and PHB expression relative to β-tubulin control following dexamethasone treatment of 35S::VND7:VP16:GR relative to untreated. n = 3 biological replicates; * significantly different from 1, † significantly different from ‡, and ¶ significantly different from †. P < 0.01. b, PAL4 expression relative to AT5G15710 control in rev-5 relative to wild-type. c, PAL4 expression relative to UBC21 control following one hour dexamethasone (Dex) treatment of 35S::REV:GR relative to untreated. *P < 0.05 for panels b and c, n = 2 biological replicates with 3 technical replicates. All panels show data as means ± s.d., with P calculated from Student’s t-test.

Abiotic stress can co-opt the xylem regulatory network

Having generated a gene regulatory network supported by in vivo and in vitro approaches, we sought to test if the model could allow us to predict responses under abiotic stress perturbation. Co-opting a developmental regulatory network is likely a key mechanism to facilitate adaptation in response to stress. Thus, we hypothesized that stress responses are likely integrated into the gene regulatory network that determines xylem cell specification and differentiation and that we can predict the exact genes that these stresses manipulate within our network.

In concert with VNI2, can repress REV expression, and REV can repress expression of PAL4. This series of interactions predicted by the network model and tested by perturbation analyses ensures that activation of VND7 and coordination of lignin biosynthesis is tightly regulated.

We next sought to identify all transcription factors that potentially regulate secondary cell wall biosynthesis genes, not just in root xylem cells but also in above-ground cell types including xylary fibres, interfascicular fibres and anthers. Many of the biosynthetic genes downstream of the key NAC domain transcription factors act in both the root and the shoot. To expand the network, we used Y1H to screen multiple smaller promoter fragments of a subset of promoters included in the root xylem network, including genes associated with cellulose, hemicellulose and lignin biosynthesis against a library of 1,664 full-length Arabidopsis transcription factors (Supplementary Tables 5, 6). We observed a total of 413 interactions that included proteins from 36 of the 75 protein families tested (Supplementary Table 7; Fig. 1d; http://gturco.github.io/trenzalore/secondary_cell_wall). We found an over-representation of AP2-EREBP, bZIP, ZF-HD, MYB and GeBP families (Supplementary Table 8). Each promoter interacted with an average of 38 different proteins, generating even more possibilities for combinatorial, redundant or condition-specific gene regulation. Like the root xylem network, previously reported protein–DNA interactions were observed in this screen including MYB46 and MYB83 binding the promoters of CESA genes (Supplementary Table 7). Since most of these interactions were novel, we tested three regions of the CESA4 promoter with two NAC family proteins, SNII2 and NST2 (4b, c), using an in vitro electrophoretic mobility shift assay (EMSA). Extracts of Escherichia coli expressing either glutathione-S-transferase-conjugated NST2 (GST:NST2) or GST:SND1 in the presence of a CESA4-2 pr promoter probe produced DNA species with retarded mobility (Fig. 4b, c). We also observed binding of the CESA7, CESA8 and KOR promoter fragments with the NST2 protein and CESA8 with the SND1 protein (Extended Data Fig. 3). These interactions between NST2 and CESA4, CESA8, and KOR promoters were further confirmed in planta by chromatin immunoprecipitation (ChIP). An antibody to green fluorescent protein (GFP) was used to immunoprecipitate NST2 protein from extracts of 35S::NST2::GFP plants. The complex was significantly enriched for fragments from the CESA4, CESA8 and KOR promoters (Fig. 4d). The trachyoe element-regulating cis-element (TERE, CTTNAAGCNA) is a direct target of VND6 and VND7. A perfect TERE is present in the CESA4 promoter and TERE-like sequences are present in CESA8 (CTTCAATGTGA) and KOR (CTTGAAATAGTA). Taken together, these data clearly demonstrate that the expression of CESA4 and other secondary cell wall genes is mediated by the direct binding of the NAC-domain binding transcription factors NST2 and SND1 to the target gene promoters via the TERE.
deprivation and salt stress gene responses and was further characterized (Fig. 5a). We filtered the xylem network to include only genes differentially expressed in salt or iron, creating stress-specific sub-networks (Extended Data Fig. 4). Previously, we determined that key developmental transcription factors have significantly more upstream regulators compared to other genes\(^5\). In response to iron deprivation, REV has the most upstream regulators, while in response to salt stress, VND7 and MYB46 have the most upstream regulators.

On the basis of these data from the iron-deprivation sub-network, we hypothesized that REV plays a key role in regulating secondary cell wall development in response to iron deprivation. To additionally determine directionality and sign (activation or repression) in the network, we constructed a network of 16 key nodes using the consensus network from four unsupervised and one supervised network inference method. REV was also predicted to be an important regulator of lignin biosynthesis gene expression in response to iron deprivation using these methods (Extended Data Fig. 5). First, to test the model-generated prediction that lignin biosynthetic gene expression is altered in response to iron deprivation, we measured phenylpropanoid-related gene expression. An increase in 4CL1, PAL4 and HCT gene expression was observed (Fig. 5b). Additionally, iron deprivation stress altered the timing and spatial distribution of the 4CL1 transcript (Fig. 5c). These expression changes are accompanied by an increase in fuchsin staining, indicative of increased phenylpropanoid deposition (Extended Data Fig. 6b). Expression in a rev-5 loss-of-function mutant in iron-deficient conditions revealed a REV- and stress-dependent influence on CCoAOMT1, PAL4 and HCT expression (Fig. 5d), thus validating our model predictions.

In the high-salinity sub-network VND7 and MYB46 contain the most upstream regulators (Extended Data Fig. 4). VND7 and MYB46 expression is greatly increased in roots in response to salt stress, but lignin biosynthetic gene expression is unaltered (Fig. 5e; Extended Data Fig. 6a). In corroboration with this hypothesis, the network model constructed using the described in silico methods also predicts VND7 and MYB46 as main regulators in response to salt stress but not iron deprivation (Extended Data Fig. 7), and indeed this was observed with an expansion of the domain of VND7 expression after salt treatment but not iron deprivation (Fig. 5e, f; Extended Data Fig. 6c). In conjunction with this ectopic increase, we observed an additional strand of metaxylem in roots exposed to high salinity (Fig. 5g).

**Discussion**

Owing to functional redundancy among regulators of secondary cell wall biosynthesis, transcription factors have largely eluded identification by loss-of-function genetic screens. Our network approach has identified hundreds of novel regulators and provided considerable insight into the developmental regulation of xylem cell differentiation. The network, which includes a cell cycle regulator, is comprised of many feed-forward loops that are likely to ensure robust regulation of this process (Fig. 5h). Accordingly, we revealed that perturbation at distinct

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**Figure 4** | Multiple transcription factors bind the CESA4 promoter. a, Activation of CESA4::LUC by transcription factors in tobacco (n = 5). *P < 0.05 based on Student’s t-test. Data are means ± s.d. b, c, EMSA with NST2 and SND1 (c), with promoters. Arrowheads indicate protein–DNA complexes, arrows indicate free probe. d, ChIP of NST2–GFP with CESA4, CESA7, CESA8 and KOR promoters. 3′D, 3′ downstream; 5′U, 5′ upstream.

**Figure 5** | The xylem-specific gene regulatory network is responsive to high salinity and iron deprivation. a, Network genes responsive to high salinity and/or iron deprivation. b, VND7, HCT, 4CL1, PAL4 expression after iron deprivation. c, 4CL1::GFP expression after iron deprivation (representative images shown, n = 4 per line). d, Lignin gene expression after iron deprivation in rev-5. *P ≤ 0.01; **P ≤ 0.001; ***P ≤ 0.0001; †P ≤ 0.0001; P values from ANOVA. e, VND7, HCT, 4CL1, PAL4 expression after NaCl. f, g, Expression relative to UBC10 and PP2A A3 controls. n = 2 biological replicates with 3 technical replicates. b, e, *P ≤ 0.01 based on Student’s t-test and data are means ± s.d. f, g, Representative images of VND7::YFP (n = 5) (f) and fuchsin-staining (n = 5) (g) after NaCl. Arrows, non-stele cells (f) and extra metaxylem strand (g). h, Proposed regulation of secondary wall biosynthesis.
nodes changes the network subtly, including phenylpropanoid biosynthesis in response to iron deprivation and ectopic xylem cell differentiation in response to salt stress (Fig. 5h). We anticipate that these findings will be instrumental in biotechnology and in our understanding of cell fate acquisition.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to S.M.B. (sbrady@ucdavis.edu) and S.P.H. (hazen@bio.ummass.edu).
**METHODS**

**Yeast one-hybrid (Y1H) protein–DNA interaction assays.** The root vascular-expressed transcription factor collection is described in ref. 7. The 1,663 transcription factor collection was assembled primarily from clones deposited in the Arabidopsis Biological Resource Center by various collaborative projects including the Peking-Yale Consortium34, REGIA35, TIGR36, and the SSP Consortium37. Translational fusions to the GAL4 activation domain were generated as described in ref. 38. A total of 1,663 E. coli strains harbouring different Arabidopsis transcription factors (Supplementary Table 5) were arrayed in 96-well plates and plasmids were prepared using the Promega Wizard SV 96 plasmid purification DNA system according to manufacturer’s recommendations.

Root secondary cell wall gene promoters (2–3 kb of upstream regulatory region from the gene’s transcriptional start site, or the next gene, whichever comes first) were cloned and recombined with reporter genes according to ref. 33. Promoter sequences and primers used are described in Supplementary Table 1. AT1G30490, ATSG06090, AT2G34710, AT1G71930, AT1G62990 promoter sequences and primers are described in ref. 33, while the promoter sequences and primers for AT5G16360 are described in ref. 5. For dissection of cell wall biosynthesis promoters, approximately 1,000 bp of sequence upstream of the translational start site was tested for interactions with the transcription factor library. Three overlapping fragments of approximately equal and average size of 419 bp were independently cloned for each promoter according to ref. 38. The oligonucleotides used to amplify promoter fragments and details of their coordinates for 4CL (At1G51680), CES4/IRX5 (At4g40330), CES7/IRX3 (At5g17420), CES8/IRX1 (At4g18780), COBL4/IIX6 (At5g15630), HCT (At5g89330), IRX9 (At2g37660), IRX14 (At4g36890), KOR/IRX2 (At5g49720), LAC4/IRX12 (At2g38080), and REI8 (At2g40890) are described in Supplementary Table 6.

Root bait promoters were screened against the stele-expressed transcription factor collection using the Y1H protocol as previously described38. The 1,665 transcription factor library was transformed into each yeast strain and the resulting 1,665 yeast transformants were replica-plated onto hygromycin and ampicillin to reconfirm identity. All interacting transcription factors were assembled into a cell wall interaction library and the screen was repeated to confirm the results and each clone was sequenced to reconfirm identity.

**Statistical analysis for protein family enrichment.** Enrichment was determined using the hypergeometric distribution online tool http://stattrek.com/). The population size is the number of transcription factors in the xylem transcription factor collection while the successes within the population is the number of transcription factors within that family in the xylem. The number of successes in the sample was the number of proteins belonging to that family, and the number in the sample is the total number of transcription factors within the network. The A. thaliana transcription factor list is as described in ref. 7.

**Power graph compression approach.** The power graph compression was performed using the algorithm as previously described38.

**Plant material.** The E2Fc RAI line is described in ref. 23 and was verified by quantifying E2Fc transcript abundance relative to the Col-0 control using an E2Fc primer compared to an ACTIN control primer (Supplementary Table 1). VND7::YFP lines are described in ref. 39. The VND7 glucocorticoid induction line is described in ref. 9. The rev-5 loss-of-function mutant was described in ref. 40.

Cloning and insertion of the 4CL1 promoter into a pENTR p4-p1R donor vector was performed according to ref. 33 (for sequence, see Supplementary Table 1). The promoter was then recombined into binary vector pKm234GW3 along with pENTR 221 ER-GFP::NOS. The resulting 4CL1::GFP vector was transformed into Agrobacterium strain GB3101. Col-0 plants were then transformed using the floral dip (Horsch et al., 1985) protocol. Plant growth conditions. All plants were grown vertically on plates containing 1 × Murashige and Skoog salt mixture, 1% sucrose, and 2 mm 3-Morpholino)ethanesulfonic acid (pH 5.8) in 1% agar. NaCl plates were made by adding 140 mM NaCl to this standard media. Iron control and deprivation media were made according to ref. 39. Plants grown on stress media (iron or salt) were first germinated on nylon mesh placed over control media for four days before transferring mesh with seedlings to iron deprivation or NaCl plates. Plants used for RNA isolation were also grown on nylon mesh placed over the agar to facilitate the collection of root material38.

**Determination of crystalline cellulose.** Roots of 7-day-old plants were harvested and lyophilized. Six to ten plates of seedlings grown at the same time on the same media were pooled to make a single biological replicate. Crystalline cellulose was measured according to ref. 41. After hydrolysis of non-cellulosic polysaccharides (acetic acid/nitric acids/water, 8:1:2 v/v), the remaining pellet was hydrolysed in 72% sulfuric acid. The resulting glucose quantity was determined by the anthrone method38.

**Phloroglucinol staining.** Five day after imbibition seedlings to be stained with phloroglucinol were fixed in a 3:1 95% ethanol/glacial acetic acid solution for 5 min. Samples were then transferred to a solution of 1% phloroglucinol in 50% HCl for 1–2 min. Whole seedlings were then mounted in 50% glycerol on slides and viewed using an Olympus Vanox microscope. Images were captured with a PIXERA Pro-6000ES camera.

**Confoocal laser scanning microscopy.** Confocal laser scanning microscopy was carried out on a Zeiss LSM700. Cell walls were stained using propidium iodide as previously described39.

**Transient protein–DNA interaction detection in tobacco.** β-galuronidase. For transient transactivation expression assays, the VND7, GALA, and/or CyclinB1 promoters were cloned into pGWB3 to generate α-GUS (β-galuronidase gene) fusion reporter vectors for E2Fc transcriptional activity. The E2Fc effector vector36 was provided by S. D. Kumar (UC Davis, CA). The effector and reporter constructs were transformed into Agrobacterium tumefaciens strain GV3101 and co-infiltrated with the p19 silencing inhibitor into 3-week-old Nicotiana benthamiana leaves at 0.6:0.1, respectively. Leaves were harvested 3 days after agro-infiltration and homogenized in GUS extraction buffer (50 mM Na2PO4, pH 7.0, 10 mM Na2EDTA, 0.1% SDS, 0.1% Triton X-100 and 10 mM β-mercaptoethanol). Quantitative MUG fluorescent assay for GUS determination was performed using 100 μg of protein/sample in 500 μl of GUS assay buffer (1 mM 4-methylumbelliferone (β-D-glucuronide, Sigma, in Extraction Buffer)). Samples were covered in aluminium foil and incubated at 37 °C. Reaction was stopped at different time points by transferring 50 μl to a tube with 450 μl of Stop Buffer (0.2 M Na2CO3). 4-methylumbelliferyl fluorescence was determined using a Infinite 200 Pro-series reader (excitation at 360 nm, emission at 455 nm).

Luciferase (Fig. 2). Overnight cultures of Agrobacterium (GV3101, D0.00n = 0.6) carrying VND7 promoter fused to luciferase (LUC) and 35S::E2Fc were prepared in fermentation medium (2 mM Na2PO4, 50 mM MES, 0.5% glucose, 100 μM acetyl-syringone) at D0.00n = 0.1. Subsequently, cultures containing VND7::LUC and 35S::E2Fc at respective ratios of 1:0, 1:0.5, 1:1, 1:2, 1:3, and 1:10 were spot-infiltrated into 6-week-old Nicotiana benthamiana leaves. To prevent gene silencing, Agrobacterium strain carrying the pBIN19 suppressor from tomato bushy stunt virus was included in each of the combinations38. The LUC activity was inspected at 72 to 96 h post infiltration using CCD camera (Andor Technology).

Luciferase imaging of VND7::LUC was performed as previously described with modifications39. Briefly, tobacco leaves were cut off after 3 days of transient transformation and sprayed with 1 mM luciferin (Promega) in 0.01% Tween-80, then were imaged using an Andor DU434-BV CCD camera (Andor Technology). Images were acquired every 10 min for 12 pictures. Luciferase activity was quantified for a defined area as mean counts per pixel per exposure time using Andor Solis image analysis software (Andor Technology). Statistical analyses were performed using two-tailed Student’s t-tests. The difference was considered significant if P < 0.05.

Luciferase (Fig. 4). A vector system was created to generate a single vector with the CaMV 35S constitutive promoter (35S) fused to a transcription factor, a promoter fragment fused to the firefly luciferase reporter gene, and 35S fused to the Renilla luciferase reporter gene. The constitutively expressed Renilla gene served as a control to normalize for transformation efficiency. This system includes one destination vector pLAH-LARm and three entry vectors pLAH-TF-pLAH-PROM and pLAH-VP6435T using MultiSite Gateway Pro Technology (Invitrogen) to simultaneously clone three DNA fragments (Extended Data Fig. 8). To develop the expression vector, promoter fragments and transcription factors were cloned, using the BP system (Invitrogen), into pDONR-P3-P2 and pDONR-P1-P4 to create pLAH-TF and pLAH-Prom, respectively. Pacl-digested pMDC32 was ligated with the 2.427 kb pFLASH fragment following HindIII and SacI digestion to yield pLAR-L with the constitutively active luciferase (LUC) reporter gene. The 3 kb pRTL2-Renilla HindIII-digested fragment was inserted into SacI-digested pLAH-L to create pLAR-LR with both firefly LUC and Renilla luciferase (REN) genes. To generate pLAH-LAR, a SpeI-digested PCR fragment containing the AmpR gene amplified from pDEST22 was ligated with SpeI-digested pLAR-LR. To add the minimal CaMV 35S fragment (Mini35S) before the LUC reporter gene, the gateway cassette ccdB/Cmr of pLAR-LAR was replaced by a HindIII-digested PCR fragment Mini35S-ccdB-Cmr amplified from pMDC32 using specific primer pHindIII-Rv and primer Mini35S-attR2. The final destination vector is referred to as pLAH-LARm.

The protein coding regions of select transcription factor genes were amplified. Each transcription fragment was combined with pDONR-P1-P4 by performing BP reactions to produce pLAH-TF. Target promoter fragments were amplified from A. thaliana genomic DNA using appropriate primers with attB3 and attB2 sites (Supplementary Table 1). Each amplified fragment was cloned into pDONR-P3-P2 vector by performing BP reactions to produce pLAH-PROM. A third pDONR
vector (pLAH-VP64Ter) was designed to create a carboxy-terminal fusion of the strong transcription activation domain VP64 to the transcription factor followed by the 3SS transcription terminator (3SS). A PCR fragments containing VP64 region and 3SS terminator was amplified from pBlP-VP64 using specific primers with attB4r and attB43r sites (Supplementary Table 10) into pDONR P4r-P3r to produce pLAH-VP6435T. Finally, the fully functional expression vector was generated by Gateway LR cloning of destination vector and the three entry clones: pLAH-LAHm, pLAH-TF, and pLAH-VP64Ter (Extended Data Fig. 7).

Agrobacterium tumefaciens was grown strain GV3103 (MP90) carrying expression constructs were grown in Luria–Bertani media with rifampicin and ampicillin and suspended in infiltration buffer 10 mM MES, pH 5.7, containing 10 mM MgCl₂ and 150 μM acetoxyrindone. The cultures were adjusted to a OD₆₀₀ of 0.8 and incubated at room temperature for at least 3 h before infiltration. The cultures were hand infiltrated using a 1 ml syringe into 3–4-week-old N. benthamiana leaves. Leaf samples were harvested 36 h after infiltration and assayed for luciferase activity according to manufacturer instructions using the Dual-Luciferase Reporter Assay (Promega). Approximately 100 μg of tissue was frozen in liquid nitrogen and homogenized using a Retsch Mixer Mill MM400 for 1 min at 30 Hz. Ground tissue was then thawed in lysis buffer (0.1 M HEPES, pH 7.8, 1% Triton X-100, and homogenized using a Retsch Mixer Mill MM400 for 1 min at 30 Hz. Ground tissue was then thawed in lysis buffer (0.1 M HEPES, pH 7.8, 1% Triton X-100, 100 mM CaCl₂ and 1 mM MgCl₂) at 25 °C for 15 min. Then 50 μl of Luciferase Assay Reagent II was added to 10 μl aliquots of the lysate to measure firefly luciferase activity, 1,000 ms integration time, using a Spectra Max M5/M5e plate reader to measure total light emission. Firefly luciferase activity was quantified with 50 μl of Stop & Glo Reagent, which contains Renilla luciferase substrate, also measured, 100 ms integration time, as total light emission. An expression vector containing part of the coding sequence (+1/+Y) of the β-galuronidase reporter gene rather than a transcription factor gene was used for baseline measurement of firefly luciferase activity. To estimate relative transcription factor affinity with each promoter fragment, three biological replicates of transcription factor expressing vectors were compared to the average results for the GUS expression vector. First, dividing firefly luciferase activity by Renilla luciferase activity normalized the transformation efficiency of each infiltrated leaf sample. Relative binding of the transcription factors to the promoter bait sequences was determined relative to the GUS control using a Student’s t-test in R v2.11.0.

Electrophoretic mobility shift assays. To express recombinant NST2 or SND1 protein, coding sequence was cloned and fused to glutathione S-transferase tag in the pDONR221 vector and then transformed into pDEST15 (Invitrogen). E. coli strain BL21-AI (Invitrogen) transformed with pDEST15-GST:NST2 were grown in liquid media to an OD₆₀₀ of 0.4, treated with 0.2% L-arabinose to induce expression overnight and harvested by centrifugation the following day. Cells were treated with 1 mg ml⁻¹ lysozyme on ice for 30 min in minimal volume of 1× PBS buffer and lysed by sonication. Cell lysates were clarified by centrifugation and incubated with 100 μl of glutathione Sepharose beads (GE Healthcare) for 30 min at 4 °C with rotation. The beads were transferrred to a column, washed with 10 volumes of 1× PBS. Protein was eluted in 100 mM Tris-HCl pH 8.0, 100 mM NaCl and 3 mg ml⁻¹ glutathione buffer and purified protein was resuspended in 50% glycerol and stored at −80 °C.

Three overlapping probes were generated for CESAs7, CESAs8 and KOR promoters using the same oligonucleotides described in Supplementary Table 1, whereas three probes were generated for CESAs4 using the following primers: CESAs4-pr1fw, CACGGGGCGCTTGTGAAATTTGTGGGC; CESAs4-pr2rev, TGTAGTTCTCTTCACTGTCTTC; CESAs4-pr3fw, CGAGATTGTGAAAGTGATTAC; CESAs4-pr2rev, GTGCTGTCATAAGAACTTTCAAG; CESAs4-pr3fw, TCCTCCTAGACACAAACCTTGAG; CESAs4-pr3rev, ACACAGGGACTCTCCGAAGC AGACAG. Reactions were carried out in binding buffer (10 mM Tris, pH 7.5, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM MgCl₂, 0.1% IGEPA CL CA-630, and 0.05 μg ml⁻¹ calf thymus DNA). Following the addition of 150 ng of protein from the GST purification eluate, reactions were incubated at room temperature for 30 min. Protein–DNA complexes were separated from free DNA on 1% agarose/1× TAE gels at 0.45 V cm⁻¹. The agarose gels were stained with ethidium bromide and bands visualized under ultraviolet light. For the titration of promoter DNA with NST2 protein, CESAs4 promoter fragment-2 DNA and KOR promoter fragment-1 DNA in 30 ng were titrated with increasing amounts of NST2 protein: 25, 50, 150, 300, and 600 ng. Binding reaction and the separation of protein–DNA complexes were carried out as described above.

Chromatin immunoprecipitation of NST2. Chromatin immunoprecipitation was conducted as described in ref. 46 with the following modifications. Roughly 5 g (fresh weight) whole stems from six-week-old Arabidopsis were harvested and crosslinked for 15 min under vacuum in crosslinking buffer (10 mM Tris, pH 8.1, 1 mM EDTA, 250 mM sucrose, 1 mM PMSF and 1% formaldehyde). Technical replicates containing approximately 1.5 mg DNA were resuspended in 800 μl SII buffer, incubated with 2 μg anti-GFP antibody (ab290, Abcam) bound to Protein G Dynabeads (Invitrogen) for 1.5 h at 4 °C and then washed five times with SII buffer. Chromatin was eluted from the beads twice at 65 °C with Stop buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20 mM EDTA and 1% SDS). RNase- and DNase-free glycerogen (2 μg) (Boehringer Mannheim) was added to the input and eluted chromatin before they were incubated with DNase- and RNase-free proteinase K (Invitrogen) at 65 °C overnight and then treated with 2 μg RNase A (Qiagen) for 1 h at 37 °C. DNA was purified by using Qiagen PCR Purification kit and resuspended in 100 μl H₂O. Quantitative PCR reactions of the technical replicates were performed using Quantifast SYBR Green PCR Kit (Qiagen), with the following PCR conditions: 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 55 °C and 20 s at 68 °C. Primers used in this study are listed in Supplementary Table 4. Results were normalized to the input DNA, using the following equation: 100 × 2⁻³⁰ΔCt (input − 3.32 − Ct ChIP).

Quantitative RT–PCR. Primers for qRT–PCR were designed to amplify a 100 bp region (or a 400 bp region for REV, PHB, and PHV transcripts due to sequence similarity) on the 3′ end of each transcript. Primer sets used for qRT–PCR are listed in Supplementary Table 1. Each plate was considered a biological replicate and Columbia and reference genotypes were plated on the same plate. Five days after imbibition, total RNA was extracted from seedling roots using an RNasey Kit (Qiagen). cDNA was synthesized by treatment with reverse transcriptase and oligo(dT) primer (SuperScript III First-Strand Synthesis System; Invitrogen). qRT–PCR was performed in an iCycler iQ Real-Time PCR Detection System (Bio-Rad) using the iQ SYBR Green Supermix. Gene expression was measured between wild-type and mutant pairs across at least two biological replicates with three technical replicates using the Δ–ΔCt method.

VND7 induction experiments. VND7–VP64 GR plants were grown vertically on sterile mesh placed on top of MS media with sucrose. Five days after imbibition, seedlings were transferred, with the mesh, to MS media containing 10 μM demethasone and roots were collected for qRT–PCR (RNasey Kit; Qiagen) after 0, 1, 2, 3, or 4 h on demethasone (n = 3). As a positive control, upregulation of MYB66 expression was confirmed using qRT–PCR.

Nitrogen influx, salt stress, iron deprivation, sulphur stress, pH stress analysis. The data sets used contained mean expression values for each gene in both control and treatment, and a q value for each gene indicating the significance of the hypothesis that the expression values of control and treatment are drawn from distributions with the same means. These data sets were filtered to extract only those genes whose q value was ≤ 0.01 and whose fold change between mean expression values was ≥ 1.5 in either direction. Fisher’s exact test was used to test whether the number of such genes is overrepresented in the xylem cell specification and differentiation gene regulatory network.

Gene regulatory network inference. Expression data were used, after normalization with the mmgMOS method from the PUMA R package. The supervised regulatory interactions network was constructed using SIRENE. The directionality of the interactions is defined by the protein–DNA interactions from YH data. The interaction sign (activation or repression) is derived by Pearson’s correlation coefficient for each protein–DNA interaction. The expression was categorized as (1) supervised tier Ia, network inferred with SIRENE with the provided YH1 gene regulatory connections and the corresponding gene expression profiles (16 genes, 4 transcription factors); (2) supervised tier Ib, an additional three verified connections from the supervised tier Ia and unsupervised tier I were considered in the inference. The unsupervised regulatory interaction network was constructed using the consensus from four different gene regulatory network inference methods, GENIE³⁵, Inferelator⁵⁴, TIGRESS⁵⁵ and ANOVerence⁵⁶. The data used were the same as the supervised TIERIa network. The default parameters were used in all methods and a rank-based method was used to build the consensus network as in ref. 53.
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Extended Data Figure 1 | Number of novel and previously described protein–DNA interactions and transcription factors involved in secondary cell wall biosynthesis and xylem development. a, b, Venn diagrams of overlap between previously reported interactions (a) or transcription factors (b) and those of the xylem-specific gene regulatory network. *Includes genes that were not included in the yeast one hybrid screen.
Extended Data Figure 2 | Activation or repression of VND7 by E2Fc is dynamic and dose-dependent. a, Intensity of LUC bioluminescence quantified using Andor Solis image analysis software. Data are means ± s.d. (n = 20). Asterisks denote significance at P < 0.05 determined by Student’s t-test. b, Quantitative PCR with reverse transcription of E2Fc and VND7 transcripts in ΔN-E2Fc (E2Fc overexpressor line lacking the N-terminal domain) expressing plants versus Col-0 control. Each data point is an individual biological replicate with 3 technical replicates. c, 3-week-old tobacco leaves were infiltrated with the p19 silencing inhibitor and either the reporter VND7p::GUS or VND7p::GUS and either 35S::E2Fc::MYC or 35S::RBR::GFP, or both. Extracted protein was then used in a quantitative MUG fluorescent assay, where relative fluorescence was measured 60 min after incubation with substrate. Data are means ± s.d., n = 3.
Extended Data Figure 3 | Binding of NST2 and SND1 to fragments of CESA7, CESA8, and KOR promoters. a–f, Electrophoretic mobility shift assays showing NST2 (a–d) and SND1 (e–f) protein specifically binds the promoters of cellulose-associated genes. Probe was incubated in the absence or presence of GST or GST:SND1 protein extracts. The arrowheads indicate the specific protein–DNA complexes, while arrows indicate free probe.
Extended Data Figure 4 | Sub-networks of network genes differentially expressed in response to iron deprivation of high salinity. a, b, Sub-network of genes with q values of \( \leq 0.01 \) and whose fold change between mean expression values was \( \geq 1.5 \) in either direction in iron deprivation (a) or high NaCl (b) stress microarray data set. Nodes are coloured according to in-degree as shown on scale bars below sub-networks. Transcription factors with the highest in-degree are labelled and indicated with a black circle.
Extended Data Figure 5 | The reconstructed gene regulatory consensus network based on analysis of the iron-deprivation expression data set by different network inference methods. a, Unsupervised; b, supervised in the first pass; c, supervised after the validated two connections have been added in the training set. Edge transparency denotes $P \approx 0.06$ for the Pearson correlation coefficient (PCC); edge width is proportional to PCC; edge value corresponds to the total edge score; a greater value corresponds to a more significant score. Yellow and red nodes correspond to transcription factor and target gene nodes, respectively; black and blue edges denote Y1H-derived and inferred interactions, respectively.
Extended Data Figure 6 | Iron deprivation and NaCl stress influences lignin and phenylpropanoid biosynthesis associated gene expression.  

**a**, No change was observed in the expression of 4CL1::GFP in 4 days after imbibition (DAI) roots transferred to a control media (left, \(n = 4\)) or media with 140 mM NaCl for 48 h (right, \(n = 4\)). 

**b**, Increased fuchsin staining of xylem cells as well as of cell walls of non-vascular cells in 4 DAI roots transferred to a control media (left) or media with an iron chelator for 72 h (right).

**c**, No change was observed in the expression of VND7::YFP in 4 DAI roots transferred to a control media (left, \(n = 4\)) or media with an iron chelator for 72 h (right, \(n = 5\)).
Extended Data Figure 7 | The reconstructed gene regulatory consensus network based on analysis of the salt-stress expression data set by different network inference methods. a, Unsupervised; b, supervised in the first pass; c, supervised after the validated two connections have been added in the training set. Edge transparency denotes $P \leq 0.06$ for the Pearson correlation coefficient (PCC); edge width is proportional to PCC; edge value corresponds to the total edge score; a greater value corresponds to a more significant score. Yellow and red nodes correspond to transcription factor and target gene nodes, respectively; black and blue edges denote Y1H-derived and inferred interactions, respectively.
Extended Data Figure 8 | Schematic diagram of dual-luciferase reporter vector development. a, Three distinct donor vectors harbouring either the transcription factor, VP64 activation domain fused to the 35S minimal promoter, or a promoter fragment. b, The dual reporter vector, pLAH-LARm, is then recombined with the three donor vectors to generate the single reporter vector (c).