DOF2.1 Controls Cytokinin-Dependent Vascular Cell Proliferation Downstream of TMO5/LHW

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Highlights
- DOF2.1 acts as a major transcriptional hub downstream of TMO5/LHW
- The CK-inducible DOF2.1 is sufficient to trigger periclinal and radial cell divisions
- DOF transcription factors redundantly regulate specific procambium divisions

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In Brief
Smet et al. capture the transcriptional responses upon simultaneous TMO5/LHW induction and identify DOF2.1 as part of the cytokinin-dependent downstream responses. Furthermore, they show that DOF2.1 and its closest homologs control periclinal and radial procambium divisions in distinct zones of this tissue.
DOF2.1 Controls Cytokinin-Dependent Vascular Cell Proliferation Downstream of TMO5/LHW

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SUMMARY

To create a three-dimensional structure, plants rely on oriented cell divisions and cell elongation. Oriented cell divisions are specifically important in procambium cells of the root to establish the different vascular cell types [1, 2]. These divisions are in part controlled by the auxin-controlled TARGET OF MONOPTEROS5 (TMO5) and LONESOME HIGHWAY (LHW) transcription factor complex [3–7]. Loss-of-function of tmo5 or lhw clade members results in strongly reduced vascular cell file numbers, whereas ectopic expression of both TMO5 and LHW can ubiquitously induce periclinal and radial cell divisions in all cell types of the root meristem. TMO5 and LHW interact only in young xylem cells, where they promote expression of two direct target genes involved in the final step of cytokinin (CK) biosynthesis, LONELY GUY3 (LOG3) and LOG4 [8, 9]. Therefore, CK was hypothesized to act as a mobile signal from the xylem to trigger divisions in the neighboring procambium cells [3, 6]. To unravel how TMO5/LHW-dependent cytokinin regulates cell proliferation, we analyzed the transcriptional responses upon simultaneous induction of both transcription factors. Using inferred network analysis, we identified AT2G28510/DOF2.1 as a cytokinin-dependent downstream target gene. We further showed that DOF2.1 controls specific procambium cell divisions without inducing other cytokinin-dependent effects such as the inhibition of vascular differentiation. In summary, our results suggest that DOF2.1 and its closest homologs control vascular cell proliferation, thus leading to radial expansion of the root.

RESULTS

Simultaneous Induction of TMO5/LHW Results in Cell Proliferation

To dissect the transcriptional responses downstream of TMO5/LHW, we generated a double dexamethasone (DEX)-inducible line by fusing TMO5 and LHW to the glucocorticoid receptor (GR) tag and driving them from the strong meristematic RPS5A promoter [10]. As expected, the number of cell files (quantified as total number of cell files in radial sections halfway between the quiescent center and the elongation zone) in the root apical meristem was strongly increased in the pRPS5A::TMO5:GR x pRPS5A::LHW:GR (henceforth named double-GR or dGR) line compared to the control line upon a 24 h induction (Figures 1A–1D). In our experimental conditions, the dGR line resulted in much stronger induction of periclinal and radial divisions (PRD, Figure S1A) compared to the single TMO5-GR or LHW-GR lines (Figures S1B and S1C). Although the constitutive TMO5/LHW misexpression line [4] resulted in significantly more divisions, the dGR line showed a very predictable increase in the number of cell divisions (Figures S1A and S1C). Therefore, CK was hypothesized to act as a mobile signal from the xylem to trigger divisions in the neighboring procambium cells [3, 6]. To unravel how TMO5/LHW-dependent cytokinin regulates cell proliferation, we analyzed the transcriptional responses upon simultaneous induction of both transcription factors. Using inferred network analysis, we identified AT2G28510/DOF2.1 as a cytokinin-dependent downstream target gene. We further showed that DOF2.1 controls specific procambium cell divisions without inducing other cytokinin-dependent effects such as the inhibition of vascular differentiation. In summary, our results suggest that DOF2.1 and its closest homologs control vascular cell proliferation, thus leading to radial expansion of the root.
Identification of DOF2.1 as Transcriptional Hub Downstream of TM05/LHW

We next interrogated the genome-wide transcriptional responses upon TM05/LHW induction in a high-density time course experiment. After statistical analysis (fold change > 2, q value < 0.05, see STAR Methods for details), 237 genes were identified as significantly upregulated at one or more of the time points (Table S1). We confirmed the regulation of a selection of 25 genes by qRT-PCR analysis, which included known TM05/LHW target genes (Table S2). In line with previous data [3, 6, 11, 12], the cytokinin (CK) biosynthesis genes, LOG3 and LOG4, and other known targets, SACL3 and AT4G38650, were quickly and strongly upregulated (Figure 1J and Table S2). Intriguingly, following this first wave of direct target responses at 0.5–1 h of induction, a second wave of gene expression including A-type ARABIDOPSIS RESPONSE REGULATORS (ARRs) [13, 14] was observed between 1–3 h of DEX treatment (Figure 1J and Table S2). Given that CK biosynthesis through LOG3 and LOG4 genes is activated at the 0.5–1 h time point, induction of downstream CK signaling reported by A-type ARR genes was expected, but not reported so far. Next, to understand the spatial aspects of dGR induction, reporters for CK biosynthesis (pLOG4:::tdTomato) and CK signaling (pPTCSn:::tdTomato) were analyzed. LOG4 is expressed along the xylem axis and in protoxylem associated pericycle and endodermis, whereas TCSn is expressed in procambium, columella, epidermis, and root cap cells (Figures 1K and 1M). Upon induction, LOG4 and TCSn were ectopically expressed outside of their normal domain in the root meristem (Figures 1L and 1N), confirming the activation of CK biosynthesis and CK signaling in all cell types in the root meristem upon induction of dGR.

As our high-resolution time-course dataset allowed the identification of consecutive waves of gene expression upon TM05/LHW induction, we wanted to identify downstream transcriptional hubs using network inference analysis [15]. To infer relationships and relative importance in the differentially expressed genes, we utilized the GENIST regulatory network inference algorithm [15]. The application of GENIST resulted in 6 individual networks, corresponding to pairwise comparisons between the 0 h and all consecutive time points of the TM05/LHW induction time course in which 0.5 h and 1 h were combined into one set (0–0.5+1 h, 0–2 h, 0–3 h, 0–4 h, 0–5 h, and 0–6 h) (see STAR Methods for details). Both TM05 and LHW were included in the network to provide a starting point for the transcriptional cascade. To illustrate the cascade of regulations through time, the networks were color coded for each time point (Figure S2, see Data S2 for more information). A first wave of gene expression (red), starting from TM05, includes its direct target genes. This is followed by a second wave (green), including CK response marked by ARRs. A third wave of gene expression (blue) includes several major nodes each controlling a high number of genes and thus marking the point where a large transcriptional change occurs. Notably, one of the most prominent nodes corresponded to AT2G28510/DOF2.1; a DOF-type TF previously suggested to be expressed in vascular tissues [16, 17]. To investigate this regulatory network in more detail and highlight significant regulations, we generated a subnetwork focusing only on TM05, ARR12, DOF2.1, and their predicted direct target genes (see Data S2 for more information). First, this network correctly predicts TM05 to regulate all known target genes, including SACL3 (represented as uORF34 and uORF35), BUD2, LOG3, and LOG4, thus confirming earlier results [3, 6, 11, 12]. Second, DOF2.1 is predicted to act downstream of ARR12, not TM05, suggesting that this gene might not be a direct target gene but is likely regulated by CK signaling pathway, which is reported to be activated by TM05/LHW (see Data S2 for more information).

DOF2.1 Expression Is Controlled by TM05/LHW-Dependent CK

To investigate the role of DOF2.1 as a downstream target of TM05/LHW, we first generated a transcriptional pDOF2.1::GUS-GFP reporter line. During embryogenesis, DOF2.1 is first expressed in the upper tier in heart stage (Figures 2A–2C) and shows expression in the embryonic root at torpedo stage (Figure 2C). Post-embryonically, DOF2.1 shows weak expression in the aerial tissues and high expression throughout the root (Figures S3A and S3B). Specifically, in the root apical meristem, DOF2.1 is strongly expressed in xylem pole pericycle cells, in specific neighboring procambium cells, and in the flanking endodermal cells (Figures 2D and 2E), suggesting that the bilateral symmetry of the root might extend beyond the central vascular cylinder [18]. Given that some DOF-type TFs have been reported to be mobile [19], we next investigated if the 31.8 kDa DOF2.1 protein might be a mobile factor. Protein accumulation of a translational pDOF2.1::DOF2.1::YFP fusion recapitulated the expression pattern of the transcriptional reporter line (Figures 2F and 2G), suggesting that DOF2.1 is not moving outside of its domain of expression. We next examined DOF2.1 expression upon TM05/LHW induction and observed that DOF2.1 expression extended outside its normal domain and into the ground tissue cells neighboring the phloem poles (Figures 2H and 2I). Notably, no expression of DOF2.1, both prior and upon induction of TM05/LHW, was observed in xylem, centrally located procambium or the phloem lineage cells. However, the RPS5A promoter is expressed in this zone (Figure 2J), and CK signaling...
Figure 2. DOF2.1 Expression in Xylem-Pole-Associated Cells

(A–C) Expression of pDOF2.1::GFP-GUS during embryogenesis. Arrows indicate expression in the embryonic root. (D–I) Optical sections of the primary root meristems of pDOF2.1::GUS-GFP (D and E), pDOF2.1::DOF2.1::YFP (F and G) and pDOF2.1::GUS-GUS in dGR upon 24h DEX treatment (H and I). (J) Optical cross section of pRPS5A::nGFP-GUS in the root meristem. (K and L) TCSn::ntdTomato-DR5revV2::n3GFP expression in dGR after 24h DEX treatment. The line in (K) represents the optical cross section shown in (L). (M and N) pDOF2.1::GUS-GFP expression in Col-0 and tmo5 tmo5-like1 double mutant background. (O) Relative expression levels of DOF2.1 upon treatment with 10 μM Benzyl Adenine (BA) for 0, 1, 2, 6, and 24 h as determined by qRT-PCR analysis. (P) Relative expression levels of DOF2.1 upon treatment with 10 μM BA in Col-0 and wol mutant backgrounds.

In all qRT-PCR experiments, data are represented as mean ± SEM and asterisks indicate significance (***p < 0.001; n.s.: not significant) as determined by two-sided t-tests. In all confocal images, asterisks indicate endodermis and crosses indicate xylem cells; scale bars, 25 μm. See also Figures S1, S2, S3 and Data S1.
Figure 3. DOF2.1 Controls Specific Procambium Divisions

(A, B, and E) Optical sections of root meristems of Col-0, *dof2.1-1* and *dof2.1-2 tmo6-1 dof6-2*, respectively.

(C) Quantification of the cell file number of *dof2.1* (left panel) and *dof2.1-2 tmo6-1 dof6-2* (triple mutant) (right panel) with respective controls.

(D) Relative expression levels of *DOF2.1*, *DOF6* and *TMO6* in Col-0 and *dof2.1-1* as determined by qRT-PCR analysis.

(F–I) *pDOF6::erVENUS* and *pTMO6::erRFP* expression in root apical meristems. Location of cross sections in G and I are indicated with lines in F and H respectively.

(J) Relative expression levels of *DOF6* and *TMO6* upon treatment with 10 μM BA for 0, 1, 2, 6 and 24h as determined by qRT-PCR analysis.

(K) Relative expression levels of *DOF6* and *TMO6* upon 2 h treatment with 10 μM BA in Col-0 and w/ot mutant backgrounds.

(L) Schematic overview of different cell types in the vascular bundle, indicating OPC and IPC cells.

(M) Quantification of OPC cell numbers in the mutant backgrounds indicated with the respective controls.

(legend continued on next page)
(but not auxin signaling) is activated in these cells by TMO5/LHW [3, 6] as visualized by a newly generated dual color, single locus auxin/CK-signaling reporter line (pTCSn:ntdTomato – pDR5revV2::n3GFP) (Figures 2K and 2L). These results suggest an active suppression of DOF2.1 expression in the center of the root vasculature. To provide additional evidence that DOF2.1 acts downstream of TMO5/LHW, we first analyzed its relative expression levels in *lhw* single and *tmo5 tmo5-like1* double mutant backgrounds by qRT-PCR and found that these were reduced (Figure S3F). Because these mutants have a reduced vascular bundle with only one xylem pole and DOF2.1 is mostly expressed in this area, it could well be that the observed result is due to the altered anatomy of these mutants. Hence, we introduced the pDOF2.1::GUS-GFP reporter line in the *tmo5 tmo5-like1* double mutant background to observe changes in the tissue specific expression levels. Expression level was reduced in this mutant backgrounds supporting that DOF2.1 expression depends on functional TMO5/LHW (Figures 2M and 2N).

Next, given that DOF2.1 is induced later than LOG4 upon TMO5/LHW induction (3–4h for DOF2.1 compared to 0.5–1h for LOG4, see Table S1-2); that the DOF2.1 expression pattern is very similar to that of LOG3 and LOG4 [3] and that our network analysis predicts DOF2.1 to act downstream of ARR12 (Table S2), we questioned if DOF2.1 could act downstream of the TMO5/LHW-dependent CK biosynthesis. Indeed, DOF2.1 transcript levels were quickly induced in root meristems by exogenous CK treatments in a qRT-PCR experiment (Figure 2O) and in seedlings [20, 21]; but this induction was abolished in a double mutant background (Figure 3K) and found that these were mildly regulated in the transcriptome data-set (Figure S3J). To investigate the potential that these factors regulate specific divisions in the procambium based on their expression domains, we quantified the number of procambium cells in single-, double-, and triple-mutant combinations using optical cross sections. Additionally, we quantified both procambium cells associated with the protoxylem pole next to the pericycle (where DOF2.1 is mostly expressed: outer procambium cells - OPC) as well as those associated with the phloem pole toward the inside of the vascular bundle (showing no DOF2.1 expression: inner procambium cells - IPC) (Figure 3L). The number of OPC cell files was significantly reduced in *dof2.1-1* (Figure 3M). Although a second *dof2.1-2* allele did not show a reduction by itself, it did significantly enhance the effect of the *tmo6-1 dof6-2* double mutant, suggesting that both alleles are functional. On the contrary, neither of the *dof2.1* alleles had a significant effect on the number of IPC cells (Figure S3J), while multiple *dof6* *tmo6* double mutant combinations significantly reduced IPC numbers (Figure S3J). Thus, these results suggest that DOF2.1 specifically controls the number of OPC divisions, while TMO6 and DOF6 show the strongest effect toward the IPC cell numbers. Intriguingly, the *dof2.1-2 tmo6-1 dof6-2* triple mutant also showed a mild reduction in the number of vascular cell files in mature embryos (Figure S3H), suggesting that these DOF-type TF might act from embryogenesis onward. Taken together, multiple DOF-type TFs control cell divisions in specific sets of procambium cells during early vascular development.

**DOF2.1 and its Close Homologs Control Vascular Cell Proliferation**

As the inferred network analysis predicts that DOF2.1 would act as a major downstream transcriptional hub, we next questioned whether this CK-inducible DOF-type TF is indeed involved in controlling vascular cell proliferation. Given that a single *dof2.1* loss-of-function did not result in obvious phenotypes (Figures 3A–3C) and the possible redundancy in the large DOF-type TF family, we quantified the relative expression levels of the two closest homologs, namely TMO6 and DOF6 (Figure S3E), in the *dof2.1* line. The relative expression levels of the latter were upregulated (Figure 3D), hinting toward compensatory regulation. Hence, we generated a triple mutant of *DOF2.1, DOF6*, and *TMO6* to overcome the redundancy within this subclade of the DOF transcription factor family using the CRISPR/Cas9 system (Figure S4). Using confocal cross sections to quantify the number of cell files in the root meristem, the *dof2.1-2 tmo6-1 dof6-2* triple mutant showed a significant reduction in the total number of cell files and in the number of vascular cell files when compared to Col-0 (Figures 3C and 3E), suggesting that these closely related DOF-type TFs act in a redundant manner to control vascular proliferation. To further examine the redundancy of these factors, we analyzed the expression patterns of *pTMO6* and *pDOF6* and found that they are only partly overlapping but mostly distinct from the *DOF2.1* expression domain. *DOF6* shows sieve element specific expression, and the TMO6 reporter line is more broadly expressed in phloem-associated procambium cells (Figures 3F–3I). Intriguingly, TMO6 and DOF6 are also CK inducible (Figure 3J) in a wild-type, but not a *wol*, background (Figure 3K) and are mildly regulated in the transcriptome data-set (Figure S3J).

In all qRT-PCR experiments and cell number quantifications, data are represented as mean ± SEM and asterisks indicate significance (**p < 0.001; *p < 0.05; n.s.: not significant) as determined by two-sided t-tests; small case letters in M indicate significantly different groups as determined using a one-way ANOVA with post hoc Tukey HSD testing and sample numbers are indicated above the x axis. In all confocal images, asterisks indicate endodermis and crosses indicate xylem cells; scale bars, 25 μm. See also Figure S3 and S4 and Data S1.
Although a similar effect was observed upon inducing TMO5/LHW or DOF2.1, the former involves the activation of CK biosynthesis. As such, the effects of constitutive TMO5/LHW overexpression are not limited to induction of PRD but also include other CK-related phenotypes such as inhibition of protoxylem cell differentiation [3, 6]. Following the logic that DOF2.1 acts downstream of CK signaling, plants with elevated DOF2.1 levels exhibit normally differentiated protoxylem cells (Figures 4H–4J), suggesting that DOF2.1 specifically controls vascular proliferation without causing other pleiotropic CK-related effects. Nevertheless, likely due to divisions of the young xylem cells, constitutive misexpression of DOF2.1 occasionally showed the formation of additional protoxylem cell files with normal differentiation (Figures S3C and S3D). Next, we introduced the pRPS5A::DOF2.1::GR line into the tmo5 tmo5-like1 mutant backgrounds harboring the pRPS5A::DOF2.1::GR rescue construct with (DEX) and without (MS) treatment with 10 μM DEX. In all cell number quantifications, asterisks indicate significance (**p < 0.001) as determined by two-sided t-tests and sample numbers are indicated above the x axis. In all confocal images, asterisks indicate endodermis; scale bars, (A)–(D) 25 μm, (E)–(G) 10 μm. See also Figure S3 and Data S1.

(Figures 4E–4G and S3I), Although a similar effect was observed upon inducing TMO5/LHW or DOF2.1, the former involves the activation of CK biosynthesis. As such, the effects of constitutive TMO5/LHW overexpression are not limited to induction of PRD but also include other CK-related phenotypes such as inhibition of protoxylem cell differentiation [3, 6]. Following the logic that DOF2.1 acts downstream of CK signaling, plants with elevated DOF2.1 levels exhibit normally differentiated protoxylem cells (Figures 4H–4J), suggesting that DOF2.1 specifically controls vascular proliferation without causing other pleiotropic CK-related effects. Nevertheless, likely due to divisions of the young xylem cells, constitutive misexpression of DOF2.1 occasionally showed the formation of additional protoxylem cell files with normal differentiation (Figures S3C and S3D). Next, we introduced the pRPS5A::DOF2.1::GR line into the tmo5 tmo5-like1 double and lhw single mutants [4, 23]. Induction of DOF2.1 led to an increase in the total number of cell files of the root meristem in both tmo5 tmo5-like1 double and lhw single mutant backgrounds (Figure 4K). Thus, these results suggest that DOF2.1 acts as a transcriptional hub downstream of TMO5/LHW and is both required and sufficient to controlling vascular cell proliferation.

DISCUSSION

Although over 200 genes were identified as being transcriptionally upregulated upon simultaneous induction of TMO5 and LHW, only a few were suggested to act as major transcriptional hubs in the inferred network analysis. We focused our attention to one of these, DOF2.1, previously reported to be expressed in vascular tissues [17]. We showed that DOF2.1 acts down- stream of the TMO5/LHW-dependent cytokinin response and, together with its closest homologs, controls vascular proliferation. Several other DOF-type transcription factors have been reported to be expressed in vascular tissues [17, 19], suggesting a more prominent role for this family of transcription factors in regulating vascular development. For example, DOF5.6/HCA2 was reported to be involved in controlling divisions in the interfascicular cambium although no phenotypes were observed in root tissues [24]. Given that the observed reduction in cell file
number is weaker in the triple *dof2.1-2 tmo6-1 dof6-2* mutant compared to the reduction observed in higher order mutants of the *tmop5* or *lhw* subclades [4]. It is likely that more DOF family members are involved in controlling this process. However, DOF2.1 is the only DOF-type transcription factor we clearly identified as TMOS/LHW target, suggesting that other members of this large transcription factor family are likely to be under control of different signals or might act during different stages of development.

Here, we showed that at least three DOF-type transcription factors DOF2.1, TMO6, and DOF6 control specific subsets of procambium cell divisions leading to vascular proliferation in the root meristem, with DOF2.1 specifically controlling OPC divisions, while TMO6 and DOF6 have the strongest effect on IPC cell numbers. This differential response suggests that the procambium is not a homogeneous pool of cells. Indeed, distinct zones might exist along the longitudinal and radial axis of procambial tissue, showing differential properties in cell division potential and thus also regulatory mechanisms. In this aspect, it would be interesting to investigate the contribution of these cell populations to secondary growth when procambium cells are reactivated.

**DOF2.1** is specifically expressed in cells surrounding the xylem poles but remains absent from the central region of the vascular bundle even when ectopically expressed in this domain. This observation suggests that **DOF2.1** expression levels might be actively repressed in this zone. Intriguingly, CLASS III HOMEODOMAIN LEUCINE ZIPPER (HD-ZIPIII) transcription factors are expressed in this central region and have been shown to act as negative regulators of cell proliferation [25]. Besides the fact that several DOF factors have been identified as binding to the promoter regions of HD-ZIPIII genes [26], it would be interesting to investigate if these factors might at the same time act as negative regulators of DOF-type TF expression.

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**AUTHOR CONTRIBUTIONS**

B.D.R. and Y.H. conceived the project; W.S. and B.D.R. designed experiments; W.S., I.S., B.W., E.M, P.W. and S.M. performed most of the experiments; T.B.J. assisted setting up the CRISPR/Cas9 system; B.B. assisted in generated mutant alleles; M.A.d.L.B. and R.S. performed the network inferences; M.B. and G.H. processed and analyzed the transcriptome data; B.D.R. supervised the project; W.S. and B.D.R. wrote the paper with input from all authors.

**DECLARATION OF INTERESTS**

Part of this work is included in a patent filing: GB 1817464.9; MEANS AND METHODS TO INCREASE ORGAN SIZE IN PLANTS.

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STAR METHODS

KEY RESOURCES TABLE

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by Bert De Rybel (beryb@psb.vib-ugent.be).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

*Arabidopsis thaliana* (L.) Heynh. background lines Columbia-0 (Col-0), were used for experimentation, with mutants and transgenic lines in these backgrounds as detailed in the Key Resources Table. *Arabidopsis* seedlings were cultivated at 22°C under continuous light conditions.

METHOD DETAILS

**Plant material and growth conditions**

All seeds were surface sterilized, sown on solid ½ MS plates without sucrose, and stratified for 24h at 4°C two days before they were grown at 22°C in continuous light conditions. Ten day old seedlings were transferred to soil and grown in greenhouse conditions. Dexamethasone (DEX) treatment was performed by either germinating seeds on 10µM DEX-supplemented medium or by transferring plants from ½ MS to 10µM DEX supplemented medium and continuing growth for the indicated time. Benzyl Adenine (BA) treatment...
Cloning and plant transformation

The pRPPSA::TM05:GR x pRPPSA::LHW:GR or dGR line was generated by first fusing the LHW coding sequence to the mammalian glucocorticoid receptor (GR) and driving this from the strong meristematic RPPSA promoter [10] using LIC cloning [33, 34]. This pRPPSA::LHW:GR line was next crossed into the existing pRPPSA::TM05:GR line [4] to obtain the dGR line. The pTCSn::ntdTomato - pDR5revV2::n3GFP [30, 31] construct was constructed by using PCR to generate the TCSn promoter fragment with appropriate LIC adapters. This was inserted into the pGIIIL/IC_Swal-ntdTomato - pDR5revV2-n3GFP destination vector [30] using the LIC cloning system. Other vectors were generated using Gateway Technology (Thermo-Fisher). pRPPSA::DOF2.1 was generated by cloning the DOF2.1 genomic sequence in the pDONR221 entry vector and subsequently recombining it with the pRPPSA pDONRP4P1R in the pHm42GW destination vector. pRPPSA::DOF2.1-GR was generated by cloning DOF2.1 genomic sequence without stop in the pDONRP4P1R entry vector and subsequently recombining it with the pRPPSA pDONRP4P1R and GR pDONRP2RP3 in a pHm43GW destination vector. The DOF2.1 promoter was obtained by amplifying the 3711bp upstream region of the transcriptional start and cloning this into the pDONRP4R1 entry vector. pDOF2.1::GFP/GUS was generated by cloning the DOF2.1 promoter region in pHGWF57 destination vector using Gateway cloning. The TMO6 and DOF transcriptional fusions were generated by cloning the promoter regions into the pDONRP4R1 entry vector and combining these with erRFP and eRVENUS respectively into the pHm43GW destination vector. pDOF2.1::DOF2.1:sYFP was obtained by cloning the pDOF2.1, DOF2.1 genomic sequence minus stop, and sYFP entry clones in the pHm43GW destination vector using Gateway cloning. pRPPSA::nGFP-GUS was generated by amplifying the pRPPSA promoter sequence [10] and cloning this into the pDONRP4P1R and subsequently cloning this into the pMK7S-NFm14GW.0 destination vector. All constructs were verified by Sanger sequencing and were transformed into Col-0 using simplified floral dipping. All primer sequences used for cloning and sequencing can be found in Data S1.

Crispr/Cas9 Mutant Generation

Two guide RNAs (gRNAs) were designed per gene using the CRISPR-P tool [32]. Cloning of gRNA vectors was performed essentially as previously described [29]. Briefly, gRNA oligos were appended with the corresponding overlaps (FW: 5’-ATTG REV: 5’-AAAC) to enable annealed-oligo cloning. Primers used for cloning and sequencing can be found in Data S1. Oligos were annealed and ligated into Golden Gate gRNA entry modules using standard DNA ligation and sequenced verified. The gRNA entry plasmids were cloned into the pFASTRK_AtCas9_AG destination vector (https://gateway.psb.ugent.be) using Golden Gate array expression. Oligos were sequenced to verify successful insertion of the gRNAs. Positive expression vectors were transformed into Agrobacterium tumefaciens C58 PMP90. Plants were transformed with Agrobacterium cultures using floral dip. Transformed events were selected based on red seed fluorescence and sown on soil. Knockout mutations in the target genes were confirmed in the T1 generation by PCR amplification and Sanger sequencing followed by TIDE analysis [35]. T2 seeds lacking red seed fluorescence (Cas9 null segregants) were sown on soil and plants again screened for the desired editing events. Cas9-free, homozygous knockout plants were selected and seeds harvested. Desired editing events were confirmed in T3 generation by Sanger sequencing. T3 seeds were used for all experiments.

Plant Imaging and Image Processing

For differential interference contrast (DIC) microscopy, samples were mounted in a solution of 20% glycerol 60% lactic acid and imaged using an Olympus BX53 microscope equipped with DIC optics. Expression of pDOF2.1::GFP-GUS for DIC analysis was visualized using GUS staining as described in [36]. Cell wall staining for optical cross sections was done using modified Pseudo Schiff – Propidium Iodide (mPS-PI) [37]. Marker lines were cleared using the ClearSee protocol [38] including a cell wall staining with 0.1% Calcofluor White [39]. Confocal microscopy was performed on Leica SP8 (40X) and Leica SP2 (63X) (all water corrected objective lenses with NA 1.2) confocal microscopes. Calcofluor White, GFP, sYFP, tandemTomato (tdT) and propidium iodide (PI) samples were imaged at an excitation of 405nm, 488nm, 514nm, 516nm and 514nm respectively. Calcofluor White, GFP, tdT and PI were visualized at an emission of 425-475 nm, 500-535nm, 580-630nm and 600-700nm respectively. Embryos were fixed and stained using Renaissance [40]. Embryos were imaged at excitation of 405 and 488 nm and detection between 430-470 nm and 500-535 nm, respectively.

qRT-PCR

RNA was extracted with the RNeasy kit (QIAGEN). Poly(dT) CDNA was prepared from 1 mg of total RNA with an iScript cDNA Synthesis Kit (Bio-Rad) and analyzed on a LightCycler480 apparatus (Roche) with SYBR GREEN I Master kit (Roche) according to
the manufacturer’s instructions. Primer pairs were designed with the Universal Probe Library Assay Design Center (Roche). Experiments were repeated in triplicate, each with three technical replicates. Data was analyzed using qBase+ software package (Bio-gazelle). Expression levels were normalized to those of EEF1α4 and CDKA1;1. All primers used for qRT-PCR analysis can be found in Data S1

Whole genome transcriptome analysis
pRPS5A::TMO5:GR × pRPS5A::LHW:GR (dGR) and Col-0 seeds were bleach sterilized and stratified for 24 h at 4°C. Seeds were sown on ½ MS plates and grown for 5 days in a growth room at 22°C. 5-day old plants of both Col-0 and dGR were transferred to ½ MS plates containing 10 μM DEX and mock-plates and were sampled at the following time points: 0 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h and 6 h. 300 individual root tips were sampled per sample and three biological repeats per time point were used. Root tips were harvested directly into liquid nitrogen, RNA was extracted using the RNeasy kit (QIAGEN). Total RNA (100 ng) was labeled using an Ambion WT expression kit (Life Technologies) and hybridized to Arabidopsis Gene 1.0 ST arrays (Affymetrix), that probes the expression of 27,827 unique genes. Sample labeling; hybridization to chips and image scanning was performed according manufacturer’s instructions. Microarray analysis was performed using MADMAX pipeline for statistical analysis of microarray data [41]. Expression values were calculated using robust multichip average (RMA) method, which includes quantile normalization [42, 43]. Probe sets on the array were redefined using current genome information [44]. In this study, probes were reorganized on the basis of the gene definitions as available in the TAIR10 database.

Network inference
To infer a gene regulatory network (GRN) and predict the causal relationships of genes regulated by TMOS and LHW, differentially expressed genes (DEGs) were identified using q < 0.05 & fold change > 2 as our selection criteria, when performing pairwise comparisons between hours 0-0.5, 0-1, 0-2, 0-3, 0-4, 0-5, and 0-6 of the TMOS/LHW induction time course. This resulted in the identification of 237 genes differentially expressed at 0.5, 1, 2, 3, 4, 5, and 6 hours after TMOS/LHW induction, which contained 22 transcription factors (Table S2). To preserve the temporal cascade of regulations, the network was inferred as individual GRNs containing the DEGs at each time point, as opposed to predicting a GRN containing the 237 DEGs together. Specifically, because we assume that regulation between genes can occur, not only during concurrent time points, but also between consecutive time points, the DEGs from consecutive time points were grouped (0.5-1, 1-2, 2-3, 3-4, 4-5, 5-6 hours), and GRNs from each of the 6 resulting lists of genes were inferred. The GRN inference on each of the 6 sets of DEGs was performed by applying a dynamic Bayesian network (DBN)-based inference algorithm, GENIST [15]. Since GENIST offers the possibility of clustering genes based on their co-expression prior the inference step to improve the performance of the algorithm, GENIST was ran using a previously published TMOS-GR dataset (TMOS induced for short time points and a cell sorted set) [3] for the clustering step. Details about the application of GENIST to each of the 6 sets of genes are provided below.

1. Gene selection
The genes differentially expressed at each time point after induction of TMOS/LHW, gt, for t ∈ {0.5, 1, 2, 3, 4, 5, 6} hours were selected. Then, the DEG from every two consecutive time points, gs and gt+1, were combined in sets Sτ for τ ∈ {0.5 & 1, 1 & 2, 2 & 3, 3 & 4, 4 & 5, 5 & 6}. Steps 2 and 3 were applied to the genes in each set Sτ individually.

2. Clustering
The expression values from the TMOS induction from De Rybel et al., 2014 were used as the input data. Clustering of the genes in Sτ was implemented by using the Silhouette index followed by linkage clustering. This resulted in a division of the Sτ genes into c clusters.

3. GRN inference
3.1. Inferring intra-cluster connections for each cluster Cτ, for n∈[1, c]: The expression values in the TMOS/LHW induction time course for all genes in cluster Cτ were used as the input data.

3.1.1. Selecting potential regulators: A gene gs was selected as a potential regulator of a target gene gt (denoted gt → gs) if it exhibited a ± p×gs change of expression immediately prior a change of expression of gt of ± p × gs:

\[
g_t \rightarrow g_s \iff (g_s(t) > (1 + p) \times g_t(t - 1) || g_s(t) < (1 - p) \times g_t(t - 1)) \& (g_t(t + 1) > (1 + p) \times g_s(t) || g_t(t + 1) < (1 - p) \times g_s(t))
\]

(1)

where we set a low threshold (p = 0.1) to ensure that no regulators were missed.

3.1.2. DBN modeling: The GRN inference step was implemented as a Dynamic Bayesian Network (DBN) learning problem, where the dependencies among the variables (genes) can be derived over adjacent time steps. Assuming stationarity and the genes to be modeled obeyed the first order Markov assumption, the joint probability distribution could be expressed as:

\[
P(X_1, ..., X_m) = \prod_i P(X_i | X_1, ..., X_{i-1}) = \prod_i P(X_i | Pa(X_i))
\]

(2)

where Xi is the expression of gene i, m = n(T − 1) is the number of genes (nodes), and Pa(Xi) is the set of regulators of gene i (parents of node i).
Given some observations of the variables over time, the DBN estimation was implemented by finding the structure of (2) that maximized the Bayesian Dirichlet equivalence uniform (BDeu) score [3]. Since the BDeu score of a DBN can be decomposed as the sum of the scores of the log conditional probabilities of each node, the log of the BDeu, BDeul, was used:

$$
\text{BDeul}(D, G) = \log(P(G)) + \sum_{i=1}^{n} \sum_{j=1}^{q_i} \left( \log \left( \frac{\Gamma \left( \frac{\delta}{q_j} \right)}{\Gamma \left( \sum_{k=1}^{n} \frac{N_{ik}}{q_k} + \frac{\delta}{q_j} \right)} \right) \right) + \sum_{k=1}^{r} \log \left( \frac{\Gamma \left( \frac{N_{jk}}{q_k} + \frac{\delta}{q_j} \right)}{\Gamma \left( \frac{\delta}{q_j} \right)} \right)
$$

(3)

where G refers to the Bayesian graph, D refers to the dataset containing the observations of the system, N_{ijk} indicates the number of data vectors in which gene i, X_i, has the value k while its parents are in the jth configuration, and \( \delta \) refers to the hyperparameters of the Dirichlet distribution.

From (2) and (3), the problem of deriving the DBN can be decomposed into finding the parents for each node. For this, the expression values of each gene were discretized in 2 levels (high and low). Then, for each gene, a list of all possible subsets of potential regulators was generated. To lower the complexity of the algorithm, which increases exponentially with the number of genes, the maximum size allowed for any subset (maximum number of regulators of a gene) was set to 3. The BDeul was used to evaluate the likelihood that each gene was due to each subset of potential regulators. The regulators of gene i were selected as the ones contained in the subset that led to the highest value of the BDeul.

3.2. Inferring inter-cluster connections: Steps 3.1.1-3.1.2 were repeated for all hubs (cluster node with the largest degree of edges leaving the node (out-degree)) in all clusters C_n, for \( n \in [1,c] \). This resulted in inter-cluster interactions among the cluster hubs.

3.3. Determining the sign of the interactions: A score was implemented to estimate whether the inferred interactions (edges) were activations or repressions. The score was calculated for each edge as the conditional probability that a gene is expressed (or not expressed) given that a parent was expressed (not expressed) in the prior time point, relative to the probability that a gene is expressed (or not expressed) given that a parent was not expressed (or vice versa expressed) in the prior time point. If the first conditional probability is larger (or smaller) than the second one, then the parent was found to be an activator (or repressor). In the case of a tie, the edge was found to have an undetermined sign.

The application of GENIST with this data resulted in 6 networks, corresponding to the 6 sets of DEGs, S_{ib}. To illustrate the cascade of regulations through time, the networks for each set were jointly visualized in Cytoscape [45]. The final network has 237 nodes, corresponding to the 237 DEGs, and 532 edges (regulations). The thickness each edge correlates with the probability with which the corresponding regulation was calculated (as in (3)), and the size of each node correlates with the number of genes that it directly regulates. The predicted most important regulators can be identified as the largest nodes in the network. The network depicts the time cascade by color-coding the regulations inferred in the different time points: red (0.5-1), green (1-2), blue (2-3), yellow (3-4), pink (4-5), orange (5-6) hours. Overall, the network places the initial time points at the center, and shows how the cascade of regulations expands outward over time.

QUANTIFICATION AND STATISTICAL ANALYSIS

All violin plots were generated using the BoxPlotR webtool using standard settings (http://shiny.chemgrid.org/boxplotr). In all plots, center lines represent the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots. The number of samples analyzed is indicated on the top of the x axis for each sample when relevant. In the quantification of IPC cell number, one single outlier was removed before statistical analysis. Pairwise comparisons were performed using standard two-sided Student t testing. In all cases, * indicates a p value < 0.05 and ** indicates a p value < 0.001. In case of multiple samples, a one-way ANOVA analysis with post hoc Tukey HSD testing was performed. Significantly different groups (p value < 0.001) of samples are indicated using lower case letters.

DATA AND SOFTWARE AVAILABILITY

The transcriptomics data files are deposited on the Gene Expression Omnibus (accession number GEO: GSE116858).