

A Gene Regulatory Network for Cellular Reprogramming in Plant Regeneration

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Wounding triggers organ regeneration in many plant species, and application of plant hormones, such as auxin and cytokinin, enhances their regenerative capacities in tissue culture. Recent studies have identified several key players mediating wound- and/or plant hormone-induced cellular reprogramming, but the global architecture of gene regulatory relationships underlying plant cellular reprogramming is still far from clear. In this study, we uncovered a gene regulatory network (GRN) associated with plant cellular reprogramming by using an enhanced yeast one-hybrid (eY1H) screen systematically to identify regulatory relationships between 252 transcription factors (TFs) and 48 promoters. Our network analyses suggest that wound- and/or hormone-invoked signals exhibit extensive cross-talk and regulate many common reprogramming-associated genes via multilayered regulatory cascades. Our data suggest that PLETHORA 3 (PLT3), ENHANCER OF SHOOT REGENERATION 1 (ESR1) and HEAT SHOCK FACTOR B 1 (HSFB1) act as critical nodes that have many overlapping targets and potentially connect upstream stimuli to downstream developmental decisions. Interestingly, a set of wound-inducible APETALA 2/ETHYLENE RESPONSE FACTORS (AP2/ERFs) appear to regulate these key genes, which, in turn, form feed-forward cascades that control downstream targets associated with callus formation and organ regeneration. In addition, we found another regulatory pathway, mediated by LATERAL ORGAN BOUNDARY/ASYMMETRIC LEAVES 2 (LOB/AS2) TFs, which probably plays a distinct but partially overlapping role alongside the AP2/ERFs in the putative gene regulatory cascades. Taken together, our findings provide the first global picture of the GRN governing plant cell reprogramming, which will serve as a valuable resource for future studies.

Keywords: Auxin • Callus formation • Cellular reprogramming • Cytokinin • Regeneration • Wound stress.

Abbreviations: AP2/ERF, APETALA2/ETHYLENE RESPONSE FACTOR; ARR, ARABIDOPSIS RESPONSE REGULATOR; ARF, AUXIN RESPONSE FACTOR; 3-AT, 3-amino-1, 2, 4-triazole; CDK, CYCLIN DEPENDENT KINASE; CIM, callus-inducing medium; ChIP, chromatin immunoprecipitation; CUC, CUP SHAPED COTYLEDON; CYC, cyclin; DAP, DNA affinity purification; DREB, DEHYDRATION RESPONSIVE ELEMENT BINDING PROTEIN; ESR, ENHANCER OF SHOOT REGENERATION; eY1H, enhanced yeast one-hybrid; GR, glucocorticoid receptor; GRN, gene regulatory network; HSE, heat shock element; HSF, HEAT SHOCK FACTOR; HSI, HIGH-LEVEL EXPRESSION OF SUGAR-INDUCIBLE GENE; KRP, KIP-RELATED PROTEIN; LBD, LATERAL ORGAN BOUNDARY DOMAIN; LEC, LEAFY COTYLEDON; LOB/AS2, LATERAL ORGAN BOUNDARY/ASYMMETRIC LEAVES2; LOG, LONELY GUY; MCA, multiple correspondence analysis; NAC, NAM, ATAF1, 2 and CUC2; PSK, PHYTOSULFOKINE; PLT, PLETHORA; SCL, SCARECROW-LIKE; SCZ, SCHIZORIZA; SIM, shoot-inducing medium; TF, transcription factor; WIND, WOUND INDUCED DEDIFFERENTIATION; WOX, WUSCHEL-RELATED HOMEBOX; WUS, WUSCHEL; YUC, YUCCA.

Introduction

Multicellular organisms are often exposed to biotic and abiotic types of stress that cause severe wounding, leading to the partial or complete organ loss. Upon injury, many organisms activate internal mechanisms responsible for initiating wound repair and regeneration. Plant and animal species, however, possess highly varying regeneration capabilities. Arabidopsis roots, for instance, regenerate full apical meristems upon partial loss (Sena et al. 2009, Efroni et al. 2016). Similarly, zebrafish replace amputated fins by regenerating the organ; the new fin grows to be the same size as the original, lost one (Pfefferli and Jaźwińska 2015). Even more strikingly, in many plants,

whole organisms can grow from small pieces of injured tissues, such as detached leaves and cuttings from shoot and roots (Ikeuchi et al. 2016). An important aspect of organ regeneration is the reactivation of cell proliferation at wound sites, which leads to the formation of a cell mass called callus, and subsequent establishment of shoot or root apical meristems (Ikeuchi et al. 2013). It is also possible for de novo organogenesis to occur without the formation of a macroscopic callus but, even in such cases, reactivation of the cell cycle is important for meristem formation and subsequent organogenesis. Another key cellular event in the regeneration of new organs and organisms is the acquisition of pluripotency, i.e. the competence to differentiate into multiple cell types (Kareem et al. 2015). Groups of cells in root explants, for example, can be reprogrammed so that they gain shoot identity during shoot regeneration (Atta et al. 2009).

How exogenous stimuli perturb intrinsic developmental signals, in turn leading to cell reprogramming and initiation of new tissue formation, is one of the central unanswered questions in regenerative biology. Recent studies have started to uncover important molecular crossroads between stress responses and organogenesis. A subclade of APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) transcription factors (TFs) named WOUND INDUCED DEDIFFERENTIATION 1–4 (WIND1–WIND4) are rapidly induced upon wounding and play key roles in callus formation at wound sites (Iwase et al. 2011). Plants overexpressing any one of the WIND genes develop callus that is competent for shoot and root regeneration as well as somatic embryogenesis (Iwase et al. 2011, Ikeuchi et al. 2013). Key to WIND1-triggered shoot regeneration is the ability of this TF to up-regulate directly ENHANCER OF SHOOT REGENERATION 1 (ESR1) (Iwase et al. 2017), which, together with its close homolog, ESR2, plays an important role in shoot development and regeneration (Banno et al. 2001, Ikeda et al. 2006, Chandler et al. 2007). Another wound-inducible AP2/ERF TF, known as ERF115, is required for the replenishment of root stem cells and it activates genes which encode peptide hormones, such as PHYTOSULFOKINE 5 (PSK5) (Heyman et al. 2013, Heyman et al. 2016). PSK peptides were originally identified as diffusible signals that promote cell proliferation in cell culture (Matsubayashi and Sakagami 1996), and later studies demonstrated that they facilitate tissue repair at wound sites (Amano et al. 2007). A DNA-binding with one zinc finger (Dof) TF, OBP1, may have similar physiological functions, as it is also wound inducible and activates cell proliferation through direct transcriptional up-regulation of cell cycle regulators, including CYCLIN D3; 3 (CYCD3; 3) (Skirycz et al. 2008).

Accumulating evidence suggests that wounding also modulates endogenous hormonal homeostasis to promote regeneration. Arabidopsis hypocotyls develop callus at wound sites, and this cellular response involves the transcriptional activation of genes, such as LONELY GUY (LOG); these genes encode enzymes involved in cytokinin biosynthesis (Ikeuchi et al. 2017). This causes cytokinin accumulation, and thus an increase in cytokinin response, leading to cell cycle re-entry and callus formation through the induction of CYCD3 expression. The de novo regeneration of roots from Arabidopsis leaf explants

offers another model system to study wound-induced organ regeneration and, in this case, endogenous auxin plays a central role in promoting regeneration. Recent studies have shown that both basipetal transport of auxin to wound sites and YUCCA (YUC)-dependent de novo biosynthesis contribute to this type of root regeneration (Liu et al. 2014, Chen et al. 2016).

While it is clear that wound stress serves as a primary trigger for organ regeneration, it alone is often insufficient to provoke the entire suite of regenerative responses. Early studies in the 1950s already demonstrated that an exogenous supply of both auxin and cytokinin enhances regenerative capacities (Skoog and Miller 1957), and tissue culture techniques utilizing these plant hormones are now widely employed to propagate a wide range of plant species asexually. A two-step tissue culture method is usually used to induce shoot regeneration in Arabidopsis, in which explants are first incubated on auxin-rich callus-inducing medium (CIM) and subsequently on cytokinin-rich shoot-inducing medium (SIM) (Valvekens et al. 1988). Recent studies have shown that callus formation on CIM follows a developmental program also essential for that of both lateral root formation and adventitious root formation (Atta et al. 2009, Sugimoto et al. 2010, Liu et al. 2014). As reported for lateral root development, auxin in CIM probably activates AUXIN RESPONSE FACTORS (ARFs), such as ARF7 and ARF19, which in turn up-regulate LATERAL ORGAN BOUNDARY DOMAIN 16 (LBD16) and LBD29 (Okushima et al. 2007). In line with this idea, overexpression of LBD16, LBD17, LBD18 or LBD29 promotes callus formation in the wild type, as does LBD16 overexpression in *arf7 arf19*, a mutant which exhibits reduced callus formation on CIM (Fan et al. 2012). LBD18 and a close homolog, LBD33, are also known to activate the expression of *E2Fa*, a key cell cycle regulator (Berckmans et al. 2011), implying that one transcriptional module underlying auxin-induced callus formation is an ARF–LBD–E2Fa pathway. Recent studies identified another auxin-dependent mechanism for shoot regeneration in vitro, which is mediated by AP2/ERF TFs, PLETHORA 3 (PLT3), PLT5 and PLT7 (Kareem et al. 2015). Importantly, these PLTs are prerequisite for both the acquisition of pluripotency and the initiation of shoot meristem fate through the transcriptional up-regulation of *PLT1* and *PLT2* as well as the *NAM*, *ATAF1*, 2 and *CUC2* (NAC) family TF-encoding genes *CUP SHAPED COTYLEDON 1* (*CUC1*) and *CUC2* (Kareem et al. 2015). Cytokinin promotes shoot regeneration through the up-regulation of *WUSCHEL* (*WUS*), and recent studies demonstrated that key cytokinin signaling components, namely type-B ARABIDOPSIS RESPONSE REGULATORS (ARRs), directly bind the *WUS* promoter and regulate transcription of this gene (Meng et al. 2017, Zhang et al. 2017).

Systematic elucidation of a gene regulatory network (GRN) is a powerful approach used to gain a holistic picture of how transcriptional regulation controls specific biological events (Gaudinier and Brady 2016). Chromatin immunoprecipitation-sequencing (ChIP-seq) is one TF-based strategy to identify comprehensively DNA-binding sites in vivo. Performing ChIP-seq with the intent of constructing a GRN is, however, laborious, since a large number of transgenic plants expressing

individual TFs must be generated. Another technique, termed DNA affinity purification sequencing (DAP-seq), can systematically identify TF–DNA interactions in vitro, and publicly available data (http://neomorph.salk.edu/dev/pages/shhuang/dap_web/pages/index.php) provide an excellent platform to search for TF–DNA interactions in silico (O'Malley et al. 2016). In contrast, yeast one-hybrid (Y1H) screening offers an alternative, promoter-based approach to identify potential TFs that bind to a collection of promoters of interest (Gaudinier and Brady 2016). Y1H has been applied to infer a GRN important for cellular differentiation (Taylor-Teeple et al. 2015) and defense response (Li et al. 2014), but similar systematic approaches have not been applied to investigate the GRN underlying plant cell reprogramming. In this study, we performed an enhanced Y1H (eY1H) screen (Gaudinier et al. 2011), using 559 TFs and 48 promoters associated with stress response, hormonal signaling and/or cellular reprogramming. We identified 1,162 TF–promoter interactions, many of which have not been reported in previous studies. Our data have thus revealed the most comprehensive GRN to date which governs cellular reprogramming in plants.

Results

eY1H assays identified 1,162 interactions between TFs and promoters

In order to uncover a GRN important for plant cell reprogramming during plant regeneration, we performed an eY1H screen with 48 bait promoters that are likely to be relevant for regeneration. Considering the rationale that regeneration occurs at the intersection of stress response, hormone signaling and organogenesis, we selected bait promoters based on the following functional annotations: 'stress response' [*WIND1*, *WIND2*, *WIND3*, *WIND4*, *HEAT SHOCK FACTOR A1A* (*HSFA1A*), *HSFA2*, *HSFB1*, *HSFB2A*, *HSFB2B*, *WRKY48* and *DUF313*], 'auxin' (*ARF5* and *ARF19*), 'cytokinin' (*ARR1*, *ARR12*, *LOG1*, *LOG5* and *LOG7*), 'cell proliferation' [*CYCD1*; 1, *CYCD3*; 1, *CYCD3*; 3, *E2Fa*, *KIP RELATED PROTEIN 2* (*KRP2*), *KRP3*, *OBP1*, *PSK1*, *PSK2*, *PSK3*, *PSK4* and *PSK5*] and 'development' [*CUC1*, *CUC2*, *ERF115*, *SCARECROW LIKE 21* (*SCL21*), *WUS*, *WOX3*, *WOX13*, *ESR1*, *ESR2*, *LBD18*, *LBD29*, *PLT3*, *PLT5*, *PLT7*, *LEAFY COTYLEDON 1* (*LEC1*), *LEC2*, *HIGH-LEVEL EXPRESSION OF SUGAR-INDUCIBLE GENE 2* (*HSI2*) and *HSFB4/SCHIZORIZA* (*SCZ*)]. To obtain promoters for each of the aforementioned set of genes, we then cloned 2 kb of upstream sequence (beginning from the start codon) into vectors harboring either *LacZ* or *HIS3* for selection in yeast. We then chose a subset (558) of TFs from the collection described in Pruneda-Paz et al. (2014) as prey, against which to screen our 48 bait promoters. In addition, we cloned *WIND4* and supplemented the prey TF collection with this gene, since it was not present in the library (Supplementary Table S1). We based our TF prey selection on the transcriptional profiles of genes present within the cDNA collection. In particular, we chose genes that are highly expressed in calli (Iwase et al. 2011), during shoot regeneration on SIM (Che et al. 2006) and during protoplast regeneration (Chupeau et al. 2013), as well as several that are responsive to

wounding, auxin or cytokinin (TAIR; <http://www.arabidopsis.org/>). Given that these prey TFs include 15 genes which we also selected as bait promoters, we aimed to uncover multilayered putative regulatory relationships in our GRN.

Using a semi-automated eY1H screening system previously described by Gaudinier et al. (2011), we identified 1,162 interactions between 252 TFs and 48 promoters (Fig. 1; Supplementary Tables S2, S4). In order to test the reliability of these results, we first examined TF–promoter binding for HSFs, a group of TFs that are known to bind their downstream promoters via the heat shock elements (HSEs), 5'-nGAAAnTTCn-3' and 5'-nTTCnnGAAAn-3' (Yoshida et al. 2011). We found that 13 out of 14 HSF targets identified in our eY1H screen harbor HSEs in their promoters (Supplementary Table S5). Further, included in this list are previously reported TF–promoter interactions, such as binding of HSF1 TF to the promoter of *HSFA2* (Yoshida et al. 2011), binding of the HSF1 TF to the *HSFA2* promoter (Ikeda et al. 2011) and binding of an HSF2 TF to its own promoter (Liu et al. 2013) (Supplementary Table S4). These data thus suggest that our eY1H screening successfully reproduced previously described transcriptional relationships and our data can be used to identify novel TF–promoter interactions.

PLT3, ESR1 and HSF1 may act as critical nodes in the reprogramming GRN

In order to reveal network motifs and features that are statistically overrepresented within our GRN, we first ran the NetworkAnalyzer Cytoscape plugin and examined TF

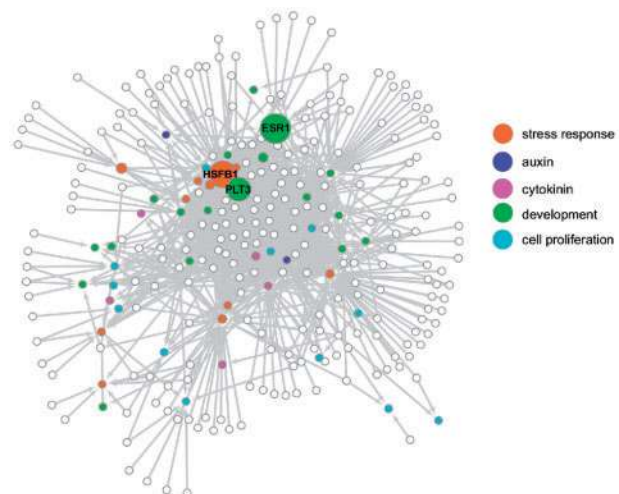


Fig. 1 A graphic summary of the GRN for plant cellular reprogramming identified by eY1H assays. An eY1H screening identified 1,162 interactions (arrows) connecting 286 nodes (circles). Open circles represent prey TFs and filled circles represent bait promoters, with each color indicating a specific functional annotation. The size of circles indicates the betweenness centrality for each node, and three nodes, PLT3, ESR1 and HSF1, that display the highest betweenness centrality are labeled. Prey TFs with a small number of targets are located on the periphery of the network, while those harboring a larger number of targets are located in the center. Bait promoters are often located between the periphery and the center of the network.

interactions for each bait promoter (Assenov et al. 2008). This analysis revealed that each bait promoter has between 1 and 83 incoming TF interactions, with the median number of interactions being 13.5 (Supplementary Table S3). Interestingly, we found that promoters of closely related homologs generally have a comparable number of interactions with TFs (Supplementary Table S3), implying that our interaction data probably reflect genuine biological features. Among the *PSK1*–*PSK5* promoters we tested, for instance, all promoters, with the exception of *PSK5*, have only one or a few TF interactions, and three promoters, *PSK1*, *PSK2* and *PSK3*, are all bound by a NAC family TF encoded by *At3g12910* (Supplementary Table S3). In contrast, among the six *HSF* promoters we tested, five have >40 TF interactions and *HSFB4/SCZ* shares most of its upstream TFs with other HSFs (Supplementary Tables S3, S4). We are aware that generally speaking, Y1H data ought to contain some false-negative or false-positive results and they do not identify, for instance, interactions regulated by TFs acting as heterodimers or in higher order transcriptional complexes. Nevertheless, our data provide the first global TF–promoter relationships that can be further validated and functionally tested.

We next analyzed the number of interactions for each TF and found that the distribution of the number of interactions fits a power-law distribution ($y = 60.742x^{-1.179}$, $R^2 = 0.762$; Supplementary Table S3; Supplementary Fig. S1). This statistical feature suggests that the obtained GRN has a property of being a scale-free network, which has often been reported for other biological networks (Barabási and Oltvai 2004). Given that the impact of nodes can be evaluated by the ‘betweenness centrality’, a measure for the shortest paths within the network passing through specific nodes (Freeman 1978), we calculated this parameter in our GRN (Supplementary Table S6). This analysis revealed that *ESR1*, *HSFB1* and *PLT3* have, by far, the highest betweenness centrality values (0.0120 for *ESR1*, 0.0101 for *HSFB1* and 0.0087 for *PLT3*) among the nodes in our GRN, strongly suggesting that they act as critical nodes (Fig. 1; Supplementary Table S6). Interestingly, we detected that *ESR1* and *PLT3* have >20 incoming and 10 outgoing interactions, while *HSFB1* has 73 incoming but only two outgoing interactions (Supplementary Table S6), implying that the mode of action of these TFs might be different in the GRN.

PLT3- and LOB/AS2-mediated pathways engage in cross-talk and may regulate reprogramming genes

To reveal key regulatory modules within our GRN more comprehensively, we also performed a power graph compression analysis (Ahnert 2013, Ahnert 2014). This compression reduces the GRN into power nodes consisting of groups of highly connected TFs that bind to a common set of targets through a so-called power edge. In our GRN, we identified 34 power edges, together representing 75 TFs interacting with 35 promoters (Supplementary Table S7). Within these power nodes, *AP2/ERF*, *ABI3/VP1*, *HB*, *LOB/AS2* and *NAC* TFs are represented more substantially than other classes of TFs, as they are present in >10 of the 34 power nodes, suggesting that they are the most critical TF families within the cellular reprogramming GRN

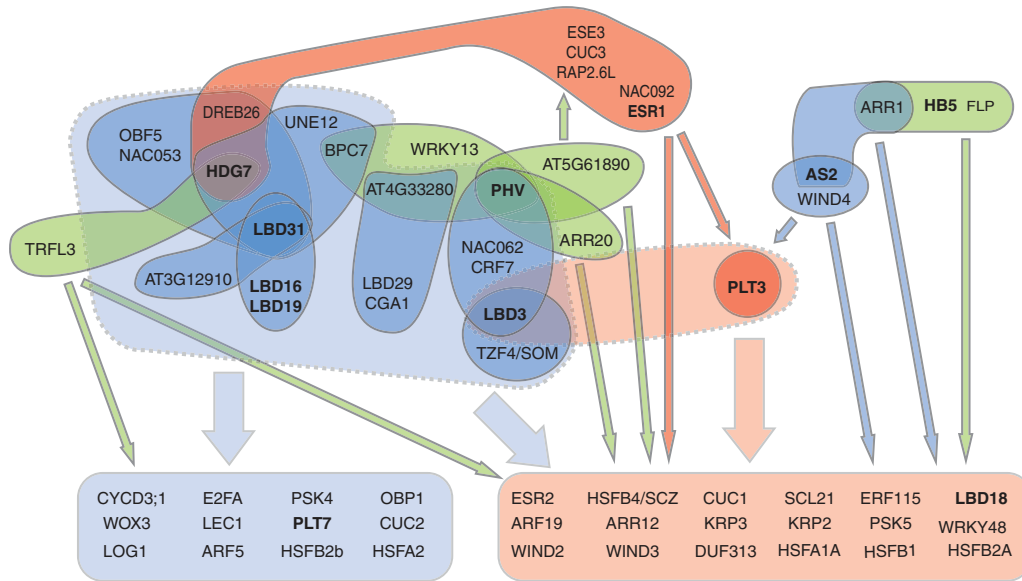
(Supplementary Table S8). To elucidate how these classes of TFs co-occur in our power nodes, we applied a multiple correspondence analysis (MCA) and found two groups of co-occurring TFs, namely, *LOB/AS2*–*HB* and *AP2/ERF*–*ABI3/VP1* (Supplementary Fig. S2, dimension 2). Our MCA also showed that a *NAC* class of TFs exhibit similar co-occurrence with both of these groups (Supplementary Fig. S2, dimension 2).

In order to visualize network characteristics driven by these groups of TF classes, we drew a simplified network depicting the power nodes containing these TFs (Fig. 2). Given our prediction that *PLT3* and *ESR1* are likely to be critical nodes (Fig. 1), we selected them as representative members of the *AP2/ERF*–*ABI3/VP1* group, while we kept all nine *LOB/AS2*–*HB* TFs for further analysis. This analysis confirmed that *PLT3*-containing power nodes and *LOB/AS2*-containing power nodes may constitute two regulatory modules within the network, which appear to target distinct sets of downstream promoters (Fig. 2A). Interestingly, *ESR1* is represented in a single power node that acts upstream of *PLT3*, but the *ESR1* power node also binds many promoters that are targeted by the *PLT3* power nodes (Fig. 2A). Similarly, *WIND4* and *AS2* appear to bind both *PLT3* and many of its targets (Fig. 2A), suggesting that *PLT3*-mediated feed-forward transcriptional regulation is a key feature of the *PLT3* power nodes. The *LOB/AS2* power nodes bind a set of 12 promoters that are not regulated by the *PLT3* power nodes (Fig. 2A). On the other hand, 11 out of 18 targets of the *PLT3* power nodes are also targeted by at least one of the *LOB/AS2* power nodes (Fig. 2B, C), suggesting possible cross-talk between these two regulatory modules. We found that *LBD3* is located at the boundary of *LOB/AS2* and *PLT3* power nodes, thus it might function as a regulator of interactions between both regulatory modules (Fig. 2A). Other candidate regulators that might also act at the boundary between these two regulatory modules include *HDG7*, *NAC053* and *NAC058* TFs, which are represented in both regulatory modules (Fig. 2B, C).

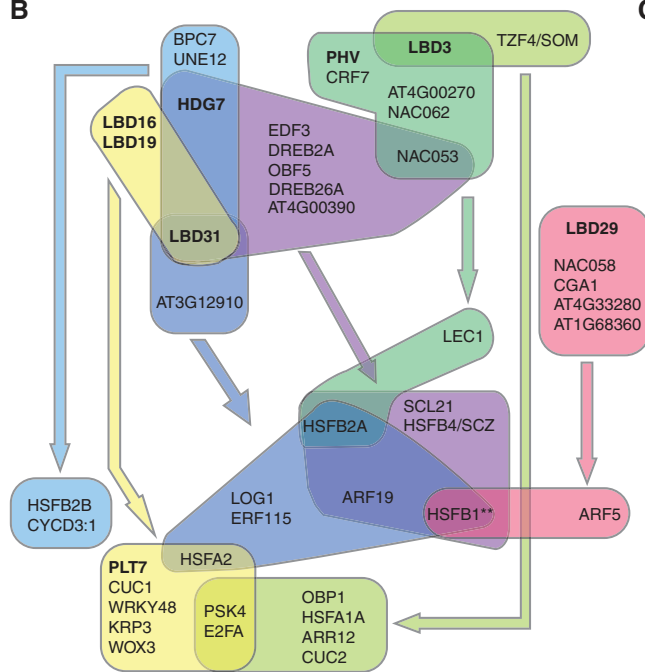
Wound-induced and/or CIM-induced TFs form a heavily overlapping GRN which regulates reprogramming genes

Recent studies have started to uncover some important links among key regulators of regeneration (Ikeuchi et al. 2016), but how wounding stimuli and hormonal pathways converge to control cellular reprogramming is still not known. Having generated the first comprehensive GRN for plant cell reprogramming, we sought to address this question by extracting a subnetwork that includes prey TFs which are highly expressed either after wounding or on CIM. Based on our gene expression profiling during wound-induced callus formation (Ikeuchi et al. 2017), we selected 127 TFs for which expression is induced within 24 h after wounding (Supplementary Table S2). We also selected TFs that are highly expressed upon CIM incubation (Che et al. 2006). In total, we found that 63 of the TFs in our GRN are CIM inducible, of which 37 are also included in our set of wound-induced TFs (Supplementary Table S2). Using the 153 TFs and 45 promoters bound by these TFs, we generated a subnetwork with 188 nodes connected by 707 TF–promoter interactions (Fig. 3). To infer a

A



B



C

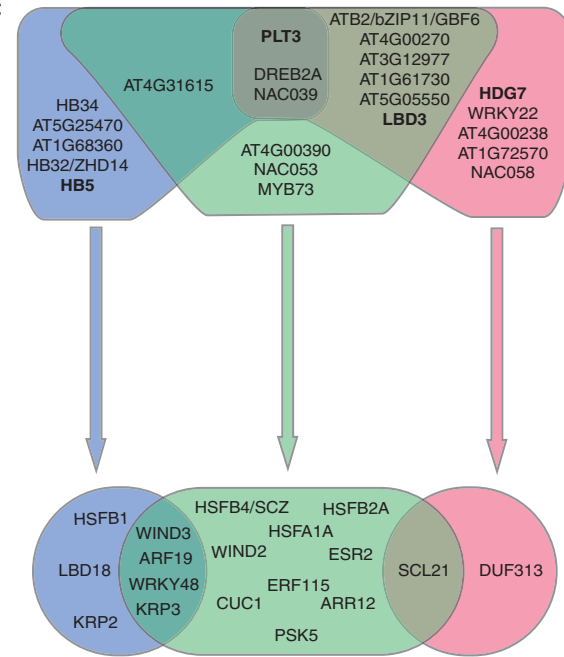


Fig. 2 A power graph of the cellular reprogramming GRN. (A) A simplified representation of a subset of 34 power edges identified in the GRN for plant cell reprogramming using the power graph compression. All power nodes containing members of the LOB/AS2 TFs (colored in blue), HB TFs (colored in green), PLT3 and ESR1 (colored in red), representative of the AP2/ERF TFs, are shown, since they best explain the group structure of the power nodes based on the MCA. For simplicity, three PLT3 power nodes are represented as a single node, and all accompanying TFs, except for HB TFs, in the PLT3 power nodes that overlap with the LOB/AS2 power nodes are not shown. The target promoters are divided into two groups, depending on whether they interact with LOB/AS2 power nodes (colored in light blue) or primarily with PLT3 power nodes (colored in light red). Power edges between the nodes and these two promoter classes are represented by thin arrows, with colors corresponding to their regulatory nodes. Dotted, shaded collections of power nodes indicate the two main regulatory modules, the LOB/AS2-regulatory module (marked in light blue) and the PLT3-regulatory module (marked in light red), and thick arrows indicate their interaction with the promoters. See also Supplementary Table S7 for the full list of represented and non-represented genes. (B) (C) Detailed power edges within the LOB/AS2 (B) and PLT3 (C) regulatory modules, with the different power nodes and their power edges marked by the same colors. TFs belonging to the LOB/AS2 or HB TF classes and PLT3 and ESR1 are shown in bold.

global trend of how wound stress and hormonal cues trigger transcriptional responses, we compared the number of TF–promoter interactions for the three classes of TFs, i.e. those induced only by wounding, only by CIM or by both wounding and CIM,

within this subnetwork. To our surprise, we found that these three classes of TFs all bind, without obvious functional preferences, to promoters associated with stress response, cytokinin, auxin, cell proliferation and development (Fig. 3; Supplementary Table S9).

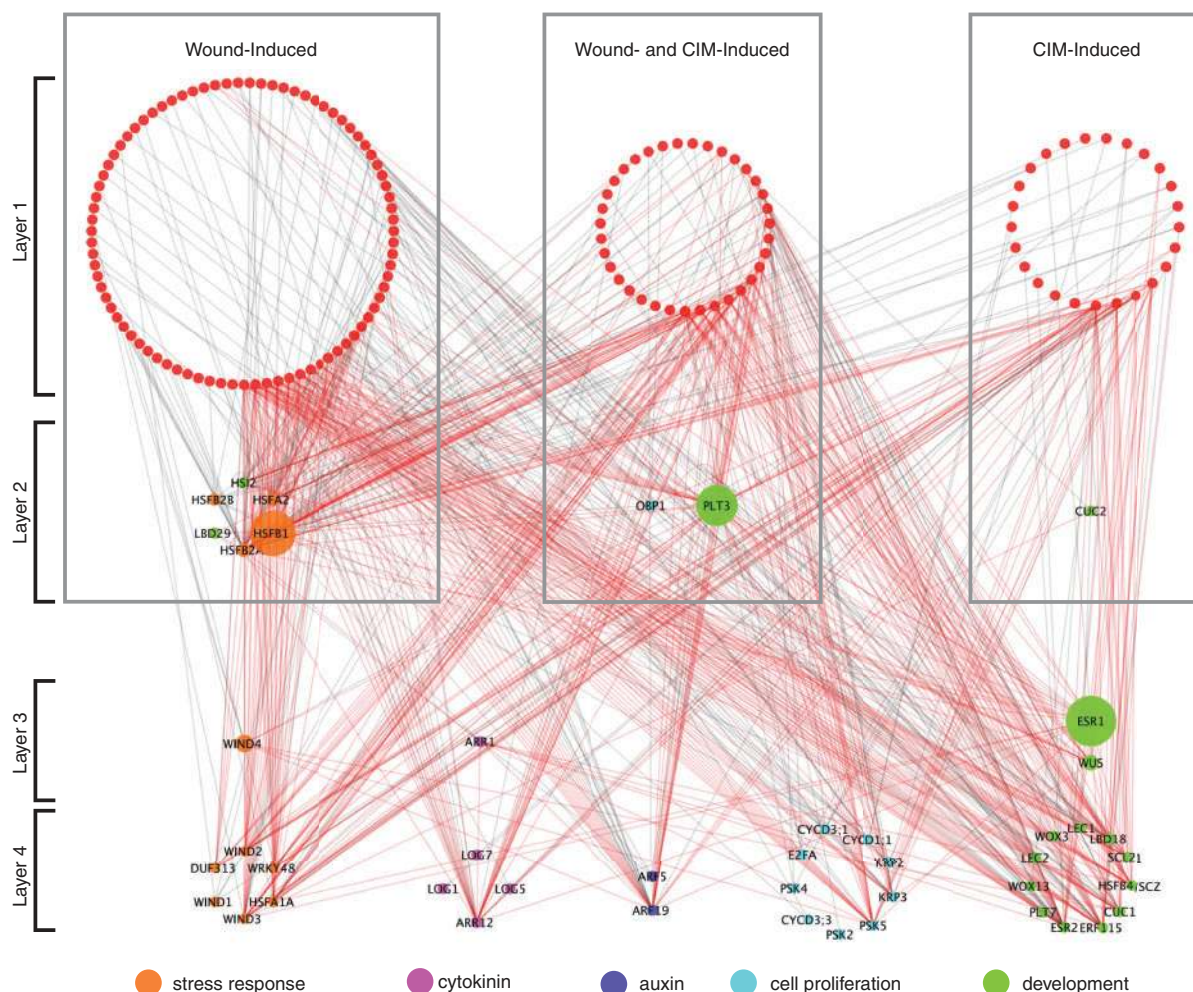


Fig. 3 A gene regulatory subnetwork highlights how wound-induced and/or CIM-induced TFs bind the promoters of downstream target genes. Wound-induced TFs were selected based on their up-regulation within 24 h after wounding as reported in Ikeuchi et al. (2017). CIM-induced TFs were defined as those significantly up-regulated in explants incubated on CIM for 7 d (FDR <0.01 and FC >0) (Che et al. 2006). These TFs were further layered based on their degrees of interactions within the subnetwork. TFs in layer 1 are induced by wounding and/or CIM and they have only outgoing interactions, i.e. binding to target promoters. TFs in layer 2 are also induced by wounding and/or CIM, and they engage in both incoming, i.e. promoter binding by upstream TFs, and outgoing interactions. TFs in layer 3 are not significantly induced by wounding and/or CIM, but they have both incoming and outgoing interactions. Genes in layer 4 represents bait promoters, thus they have only incoming interactions. Genes in layer 1 are colored in red, and those in layers 2, 3 and 4 are colored based on their functional annotations. Node size represents the betweenness centrality of the TFs in the network. Red edges represent TF–promoter interactions that are components of feed-forward loops.

We also detected extensive feed-forward regulatory loops (Fig. 3), and our statistical analysis showed that the number of feed-forward modules is significantly more than expected for a random scale-free network generated by the Watts and Strogatz model (483 vs. 92; P -value <0.02). These data suggest that inducible signals underlying plant cell reprogramming may be transmitted by a robust, multilayered transcriptional network. Taken together, these analyses revealed that the transcriptional cascades underlying wound- or CIM-induced cellular reprogramming are heavily interwoven and target a wide range of promoters associated with both stress response and developmental regulation.

A GRN for wound-induced cellular reprogramming

To investigate how early wound-responsive genes trigger cellular reprogramming more specifically, we next selected prey TFs

for which gene expression is induced within 1 h after wounding (Ikeuchi et al. 2017) and examined their downstream transcriptional subnetwork. For the selected 42 fast wound-induced TFs, our eY1H identified 208 TF–promoter interactions with 30 promoters. Interestingly, we found that nine out of 15 TFs with more than two targets belong to the AP2/ERF family that include previously described key regulators of wound-induced cellular reprogramming, such as WINDs, ERF115 and PLT3/5/7 (Fig. 4). Importantly, many of these AP2/ERF TFs have not been characterized previously or are only known to function in other stress responses. DEHYDRATION RESPONSIVE ELEMENT BINDING PROTEIN 2A (DREB2A), for example, is so far best described for its role in the regulation of drought and heat stress (Sakuma et al. 2006), and we confirmed previously reported binding of DREB2A to *HSFB1* and *HSFB2A* promoters (Fig. 4). Our eY1H data further revealed DREB2A binding to the

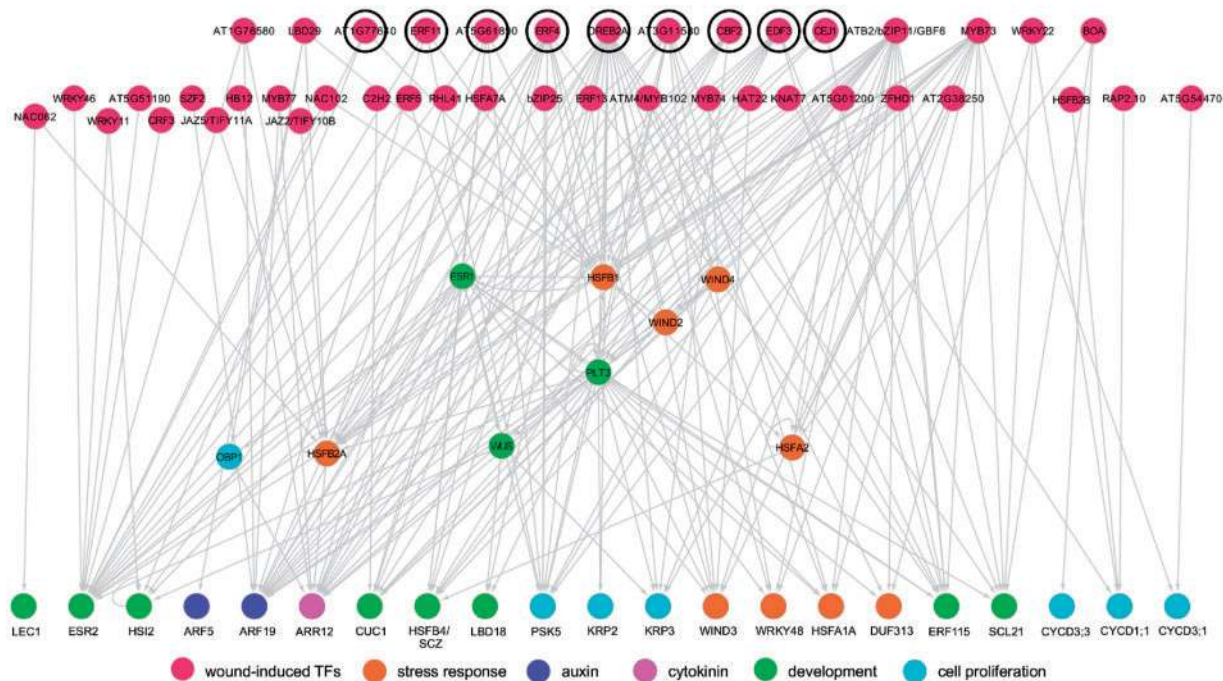


Fig. 4 A subnetwork highlighting how wound-induced TFs bind the promoters of downstream target genes. Wound-induced TFs, marked by red circles, directly or indirectly regulate downstream targets associated with cellular reprogramming. TFs interacting with more than two targets are positioned in the top tier and, among them, AP2/ERF family members are denoted by black circles. Downstream bait promoters are colored according to their functional annotations.

promoters of *PLT3*, *PSK5*, *KRP2*, *KRP3*, *LBD18*, *WIND2* and *WIND3*, suggesting that *DREB2A* is also involved in wound-induced cellular reprogramming. We further noticed that some of these nine AP2/ERFs bind promoters of other AP2/ERF TFs and form multilayered regulatory cascades (Fig. 4). *AT5G61890*, for instance, binds the *ESR1* promoter, and *ESR1*, in addition, binds the promoter of its closely related homolog *ESR2* (Fig. 4). Similarly, *EDF3*, *ERF4* and *DREB2A* all bind the *PLT3* promoter, and *PLT3*, in turn, binds the promoter of *ESR2* and *ERF115* (Fig. 4). We should also mention that some of the wound-induced TFs, e.g. *BOA* and *RAP2.10*, directly bind promoters of core cell cycle regulators, such as *CYCD1;1*, *CYCD3;1* and *CYCD3;3*, implying that early wound signaling also regulates cell cycle progression in a direct manner.

In order to examine whether the detected TF–promoter interactions reflect the actual transcriptional regulation under physiological conditions, we compared our eY1H results with the gene expression profiling data after wounding (Ikeuchi et al. 2017, Iwase et al. 2017). In support of the regulatory relationships inferred from our Y1H data, *AT5G61890* is first induced within 1 h after wounding and its target, *ESR1*, is in turn induced after 3 h (Fig. 4; Supplementary Fig. S3; Iwase et al. 2017). These early transcriptional activations also precede the induction of further downstream target genes such as *PLT3* and *WIND3* (Fig. 4; Supplementary Fig. S3). Our transcriptome data, in addition, show that the expression of *HSFB1* and *HSFA2* is rapidly induced within 1 h upon wounding, followed by the induction of their common target *HSFB4/SCZ* after 6 h (Fig. 4; Supplementary Fig. S3). These data thus suggest that the

TF–promoter interactions identified in this study are relevant for wound-induced transcriptional activation and should help identify novel regulators in wound-induced cellular reprogramming. *HSFB4/SCZ*, for instance, is so far only known to play roles in cell fate determination (Pernas et al. 2010, ten Hove et al. 2010), but our Y1H data suggest that it could also be involved in stress-induced cellular reprogramming downstream of HSFs.

A GRN for auxin-mediated cellular reprogramming

In order to gain further molecular insights into how auxin promotes cellular reprogramming, we selected three key regulators of this process that are highly represented in our GRN; *LBD16*, *LBD29* and *PLT3* (Fig. 2; Fan et al. 2012, Kareem et al. 2015), and examined their downstream subnetworks. As shown in Fig. 5, our eY1H data demonstrated that *PLT3* forms a regulatory feedback loop together with *ESR1* and *HSFB1*, each of which has some unique, but partially overlapping, incoming and outgoing interactions. Our data further support an autoactivation of *PLT3*, which was previously suggested by transcriptional induction of *PLT3*–glucocorticoid receptor (GR) plants (Santuari et al. 2016). Similarly, our data show that *PLT3* directly binds the promoters of *HSFB2A*, *WIND3*, *PSK5* and *HSFB4/SCZ*, which are all misexpressed shortly after *PLT3*–GR activation (Santuari et al. 2016). Interestingly, *PLT3* and *ESR1* share 10 direct targets and they include important regulators of stress response (*WIND3* and *HSFA1A*), auxin (*ARF19*), cytokinin (*ARR12*), development (*ERF115*, *ESR2*, *LBD18*, *WUS* and *CUC1*) and cell proliferation (*PSK5*) (Fig. 5), strongly suggesting that

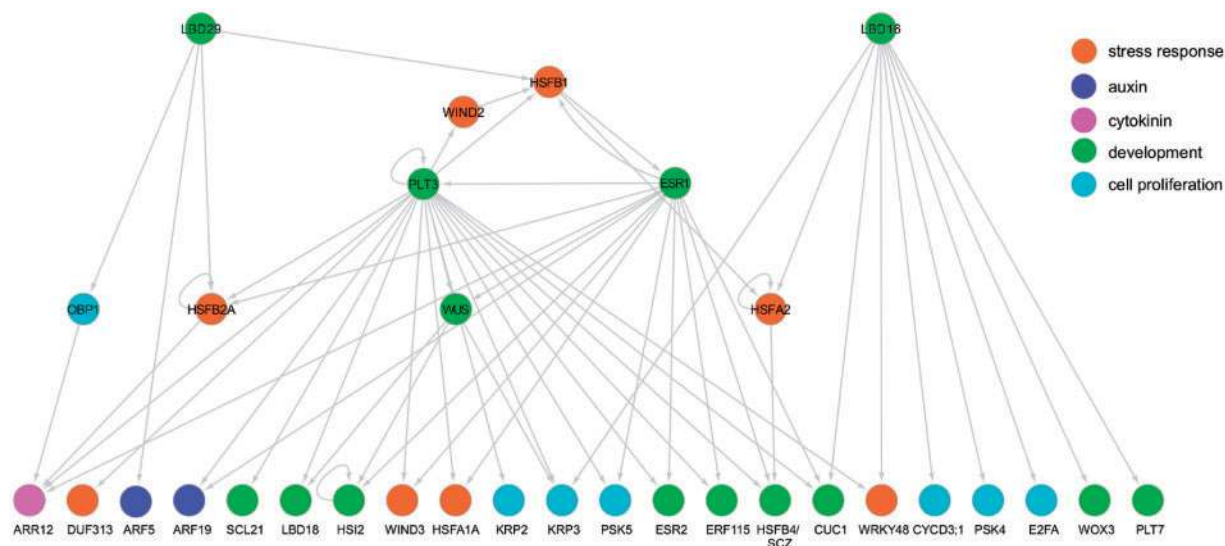


Fig. 5 A subnetwork highlighting how auxin-mediated pathways may regulate plant cell reprogramming. PLT3, ESR1 and HSF1 appear to form a feedback loop which regulates a partially overlapping set of genes involved in development (*ESR2*, *CUC1* and *WUS*) and cell proliferation (*KRP2*, *KRP3* and *PSK5*). LBD29 and LBD16 appear to regulate a largely distinct set of downstream targets involved in cell proliferation. Colored circles indicate functional annotations of prey TFs and bait promoters.

PLT3 and ESR1 together form a robust, overlapping transcriptional network. We have previously shown that ESR1 is required for the expression of *WUS* and *ESR2* on SIM (Iwase et al. 2017). Our eY1H data confirm these results and further suggest that ESR1 regulates *WUS* and *ESR2* expression both directly and indirectly through PLT3 (Fig. 5). It is also interesting to note that PLT3 and ESR1 form several feed-forward loops through *WUS* which regulate downstream genes. PLT3, for instance, appears to regulate *LBD18* and *KRP3* directly and also indirectly through *WUS* (Fig. 5).

As suggested by our power graph analysis (Fig. 2), direct targets of the PLT3–ESR1–HSF1 pathway only overlap slightly with those targeted by LBD16 and LBD29 (Fig. 5). Of note, our data suggest that LBD16 directly targets several genes associated with cell proliferation, including *E2FA*, *CYCD3;1*, *KRP3* and *PSK4* (Fig. 5). A previous study by Berckmans et al. (2011) has shown that a close homolog of LBD16, *LBD18*, directly regulates *E2Fa* expression. Our data thus further substantiate the role of LBDs in the control of cell cycle progression. We also noticed that *CUC1* is among the few common targets of the LBD16/29- and PLT3–ESR1–HSF1-mediated pathways identified in our GRN (Fig. 5). These data confirm previous reports demonstrating direct regulation of *CUC1* expression by ESR1 and PLT3 (Matsuo et al. 2009, Kareem et al. 2015), strongly suggesting that *CUC1* integrates upstream signals from both PLT3–ESR1–HSF1 and LBD16/29 pathways and, in turn, controls cellular reprogramming.

A GRN for cytokinin-mediated cellular reprogramming

It is becoming increasingly clear that activation of both cytokinin biosynthesis and signaling plays key roles in plant cell reprogramming (Ikeuchi et al. 2013, Ikeuchi et al. 2017). We have recently shown that the transcriptional activation of *LOG1*, *LOG5* and *LOG7*, which encode enzymes involved in cytokinin

biosynthesis, is a core process which drives callus formation at wound sites (Ikeuchi et al. 2017). We thus searched for potential upstream regulators of the cytokinin pathway and identified six TFs that bind these types of promoters (Fig. 6A). Interestingly, LBD3 and LBD31 bind to the promoters of *LOG1*, *LOG5* and *LOG7* (Fig. 6A), raising the possibility that they influence cytokinin biosynthesis by up-regulating these genes.

To explore how cytokinin signaling promotes cellular reprogramming, we next screened for downstream targets of the type-B ARRs that were included in our set of prey TFs, ARR1, ARR12, ARR14, ARR18 and ARR20, and found 15 promoters that are bound by ARR1, ARR18 and/or ARR20 (Fig. 6B). Importantly, 12 out of these 15 promoters possess at least one of the previously described cytokinin response motifs 5'-(A/G)GAT(T/C)TT-3' or 5'-AAAGAT(T/C)TT-3' (Ramireddy et al. 2013; Supplementary Table S10), implying that these ARRs bind target promoters via these motifs. As might be expected, targets of these ARRs include those involved in stress response (*HSF1*, *HSFB2A*, *HSFA2*, *WIND3* and *WRKY48*), auxin (*ARF19*), cytokinin (*ARR12*), development (*WUS*, *HSFB4/SCZ*, *CUC1*, *ESR2*, *ERF115*, *HSI2* and *LBD18*) and cell proliferation (*PSK5* and *KRP3*) (Fig. 6B), suggesting that type-B ARR-mediated cytokinin signaling directly regulates genes associated with various aspects of cellular reprogramming. Previous studies have shown that type-B ARRs, including ARR1, ARR10 and ARR12, directly regulate *WUS* expression (Meng et al. 2017, Wang et al. 2017, Zhang et al. 2017, Zubo et al. 2017). Our data did not detect binding of ARR1 and ARR12 to the *WUS* promoter, but we did find that another type-B ARR, ARR20, binds the *WUS* promoter (Fig. 6B). Our data, in addition, showed that ARR1 binds the promoters of *CUC1* and *ESR2*, other key regulators of shoot regeneration (Daimon et al. 2003, Ikeda et al. 2006) (Fig. 6B), suggesting that ARR-mediated cytokinin signaling directs shoot regeneration through multiple

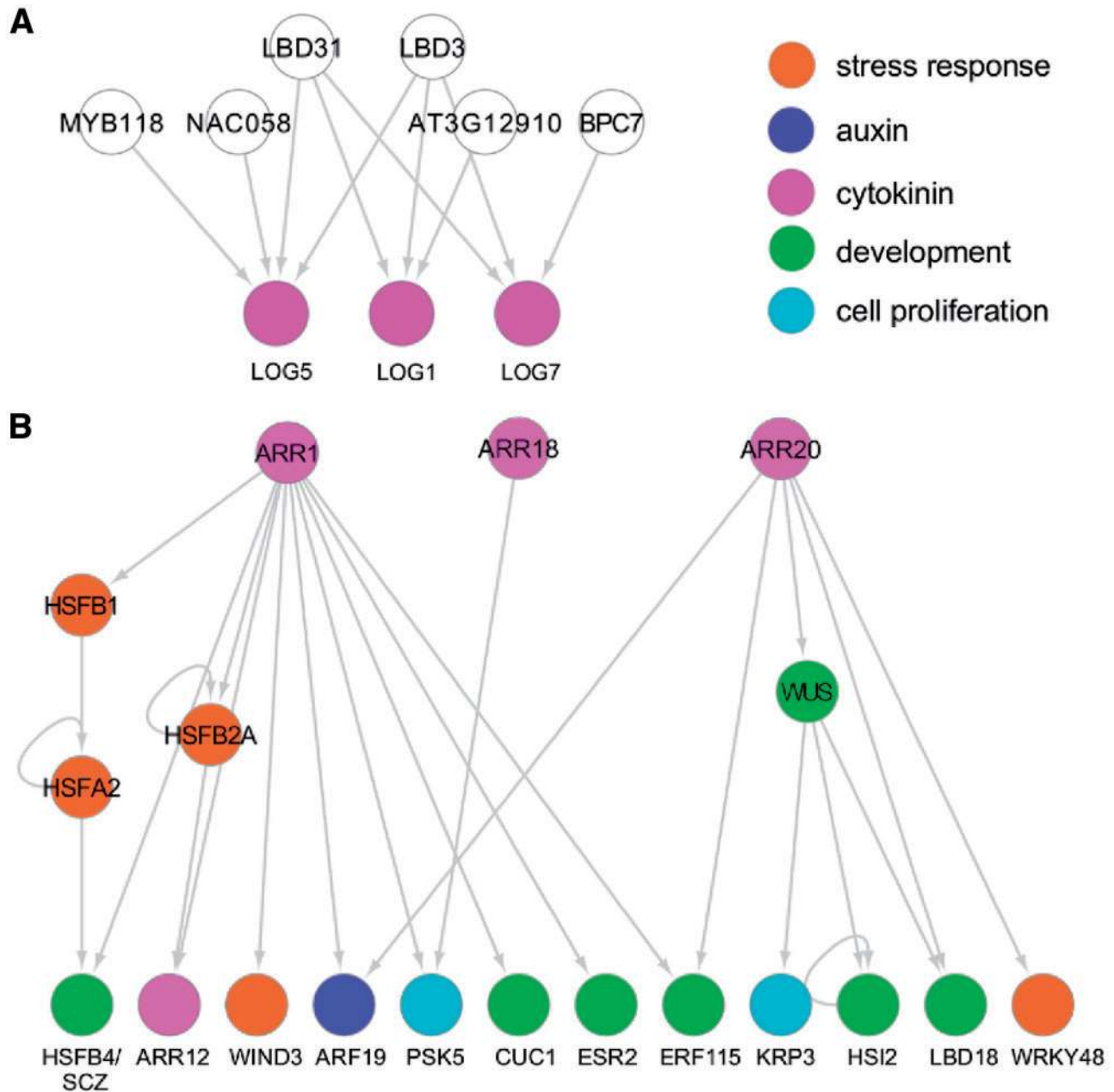


Fig. 6 Subnetworks highlighting how cytokinin-mediated pathways may regulate plant cell reprogramming. (A) Six TFs in our GRN directly bind the promoters of three *LOG* genes involved in cytokinin biosynthesis, potentially regulating their expression directly. Among them, both *LBD3* and *LBD31* bind the promoters of *LOG1*, *LOG5* and *LOG7*. (B) Key regulators of cytokinin signaling, *ARR1*, *ARR18* and *ARR20*, potentially regulate genes associated with stress response (*HSF* genes), development (*WUS*, *CUC1* and *ESR2*), cell proliferation (*PSK5* and *KRP3*) and auxin (*ARF19*). Colored circles indicate functional annotations of prey TFs and bait promoters.

pathways. Our data also highlight the cross-talk between cytokinin and auxin signaling, as we found that both *ARR1* and *ARR20* directly bind the promoter of *ARF19* (Fig. 6B). One unexpected link uncovered in our eY1H data set is the cross-talk between type-B ARRs and HSFs since we detected *ARR1* binding to the promoters of *HSFB1*, *HSFB2A* and *HSFB4/SCZ*, as well as *HSFB2A* binding to the *ARR12* promoter (Fig. 6B). These interactions together form several intersecting feed-forward loops (Fig. 6B), which potentially serve as integrators of stress response and cytokinin signaling.

Discussion

Identification of core regulatory pathways in a cellular reprogramming GRN

In this study we mapped the first GRN for cellular reprogramming in plants, based on large-scale eY1H analysis, and provided comprehensive molecular insights into how wound stress and hormonal cues may integrate and collectively regulate a large collection of genes implicated in stress response and developmental decisions. Further functional validations of new

TF–promoter interactions found in this study should help identify novel regulators of cellular reprogramming and unveil how they shape the heavily interwoven transcriptional cascades. Our network analysis suggests that *PLT3*, *ESR1* and *HSFB1* form a feedback loop and act as the most critical nodes in our GRN, probably integrating upstream signaling to promote coherent downstream developmental transitions (Figs. 3, 5). Our data show that the *PLT3* promoter is bound by many wound- and/or CIM-induced TFs (Fig. 3; Supplementary Table S4). This is in line with previously reported observations that the expression of *PLT3* is strongly induced both by wounding and by incubation on CIM (Kareem et al. 2015, Ikeuchi et al. 2017). In addition, previous genetic evidence suggest that *PLT3* is involved in both wound-induced callus formation and CIM/SIM-induced shoot regeneration (Kareem et al. 2015, Ikeuchi et al. 2017). As expected, our data indicate that *PLT3* has many downstream targets associated with stress response, cytokinin, auxin, cell proliferation and development (Fig. 2; Supplementary Table S4). Similarly, our network analysis revealed that *ESR1* is under the control of multiple inputs from both wound- and CIM-induced TFs (Fig. 3; Supplementary Table S4). This is also in agreement with our previous observation that *ESR1* is up-regulated by wounding and its expression is further enhanced by exogenous application of both auxin and cytokinin (Iwase et al. 2017). Furthermore, our Y1H data include 14 downstream targets of *ESR1* that also range from genes involved in stress response to those associated with developmental regulation (Fig. 2; Supplementary Tables S2, S4). Functional roles of *HSFB1* in stress-induced cellular reprogramming have not been reported before, but it probably participates in this process, since many wound- and/or CIM-induced TFs bind the *HSFB1* promoter, and *HSFB1*, in turn, binds the *ESR1* promoter as part of the *PLT3*–*ESR1*–*HSFB1* regulatory loop (Figs. 3, 5).

Our power graph analysis, in addition, highlighted the *LOB/AS2*-mediated pathway, which appears to play distinct but partially overlapping roles with the *PLT3*–*ESR1*–*HSFB1* pathway in our GRN (Fig. 2). TFs that appear to act in the *LOB/AS2*-mediated pathway include *LBD16* and *LBD29*, which have been implicated in the regulation of auxin-dependent establishment of root meristems (Okushima et al. 2007), as well as many TFs with unknown functions (Fig. 2). Our Y1H data identified a large set of downstream genes that are bound by these TFs and, importantly, uncovered several putative molecular links, such as *LBD3*, *HG7*, *NAC053* and *NAC058*, which might function in both *PLT3*–*ESR1*–*HSFB1*- and *LOB/AS2*-mediated pathways (Fig. 2). Further functional characterization of these regulators should advance our understanding of how the reprogramming GRN responds to inductive cues and collectively promotes organ regeneration through these core regulatory pathways. We should note that many of these regulators such as *PLT3*, *ESR1*, *LBD16* and *LBD29* also play important roles in normal organogenesis (Kirch et al. 2003, Okushima et al. 2007, Prasad et al. 2011) and they might bind their downstream targets identified in our Y1H in either context. Further studies are therefore needed to examine these regulatory relationships under different physiological conditions and reveal context-dependent specificity of TF–promoter binding.

Roles of AP2/ERFs in wound-induced cellular reprogramming

AP2/ERFs form a large family of TFs consisting of 147 members in Arabidopsis (Nakano et al. 2006). Some subfamily members, such as DREBs, are known to mediate various stress responses, while others, such as AP2 and *AINTEGUMENTA*, are more associated with developmental roles in the context of organ growth and differentiation (Jofuku et al. 1994, Mizukami and Fischer 2000). Importantly, previous studies have also identified several AP2/ERF TFs, including *WINDs* and *ERF115*, as important regulators of cellular reprogramming (Iwase et al. 2011, Heyman et al. 2013). A previous study has shown that *ERF115* transcriptionally regulates *WIND1* and *PLT3* (Heyman et al. 2016) but interactions between *ERF115* and these or other tested promoters were not detected in our eY1H experiments. Given that organ regeneration is the process of external stimuli perturbing endogenous developmental programs, it is plausible that there are many more AP2/ERF TFs which will be identified as key players in this process. Our eY1H analysis identified nine AP2/ERF TFs that are rapidly induced by wounding and bind promoters of many genes relevant to cellular reprogramming (Fig. 4; Supplementary Tables S2, S4). A previous study by Yang et al. (2005) has shown that *ERF4* is induced by ethylene, jasmonate and ABA, and, in turn, it modulates cellular sensitivity to ABA. ABA is inhibitory for callus formation at wound sites (Ikeuchi et al. 2017), thus it would be interesting to test whether *ERF4* is involved in ABA-dependent regulation of callus formation. Our eY1H data, in addition, suggest that some AP2/ERFs regulate the expression of other AP2/ERFs in the reprogramming GRN, thus forming multilayered cascades within AP2/ERF TFs (Fig. 4). There is precedent for this in other biological contexts, implying that this is one of the key features of GRNs. Three basic helix–loop–helix TFs, for instance, sequentially regulate stomatal differentiation (Pillitteri et al. 2007), and an interwoven cascade of HSF TFs mediates the heat shock response (Ohama et al. 2017). Further functional annotation of AP2/ERFs identified in this study should reveal the physiological relevance of AP2/ERF-mediated transcriptional cascades in stress-induced cellular reprogramming.

Roles of LBDs in the regulation of cytokinin biosynthesis

De novo biosynthesis of cytokinin promotes wound-induced callus formation, but we have recently shown that *WIND1* is not required for this physiological response, since accumulation of endogenous cytokinin after wounding is not defective in *WIND1*–*SRDX* plants (Ikeuchi et al. 2017). In this study, we have identified *LBD3* and *LBD31* as putative upstream regulators of three wound-induced cytokinin biosynthesis genes, *LOG1*, *LOG5* and *LOG7* (Fig. 6). Intriguingly, however, the expression of *LBD3* and *LBD31* themselves is not wound inducible (Ikeuchi et al. 2017); thus, they might be activated, for example through post-translational mechanisms, in order to induce *LOG* expression upon wounding. It is also possible that other LBD homologs that are induced by wounding, such as *LBD16* and *LBD29* (Ikeuchi et al. 2017), also bind these promoters in planta and induce *LOG* genes, although our eY1H

assay did not detect such TF–promoter interactions. It is worth noting that the expression of *LBD3* is induced by the elevation of endogenous cytokinin levels (Wang et al. 2015). Therefore, *LBD3* might act as a part of a feedback loop which enhances cytokinin-mediated signaling pathways.

Materials and Methods

Promoter cloning

The 2 kb promoter sequence upstream of the start codon was amplified by PCR using Prime star max (TAKARA) and subjected to A-tailing by *Ex Taq* polymerase (TAKARA). Amplicons were then introduced into either the pENTR 5'-TOPO (Thermo Fisher Scientific) or pDONR P4-P1r (Oshima et al. 2011) vector. Promoter sequences were then subcloned into pMW2 and pMW3 (Addgene) by LR reactions (Thermo Fisher Scientific) and transformed into *Escherichia coli* (DH5 α). After verifying the border sequence, purified plasmids were transformed into yeast (YM4271) as described in Gaudinier et al. (2017). A set of primers used for cloning and sequencing in this study are listed in Supplementary Table S11.

eY1H screening

The 48 bait promoters were screened against the 559 TFs as described previously (Gaudinier et al. 2011, Gaudinier et al. 2017). The 558 transcription factors were obtained from the Arabidopsis TF collection at the proteomics core facility of UC Davis (<http://proteomics.ucdavis.edu/yeast-one-hybrid-services-brady-lab/>). To supplement this library, *WIND4* cDNA was cloned into the pDONR221 vector and recombined into the pDESTAD-2 μ vector by an LR reaction (Thermo Fisher Scientific). The recombined plasmid was transformed into yeast cells (Y α 1867) as described in Gaudinier et al. (2017). Yeast cells containing a bait vector were mated with yeast cells containing a prey vector. Mated yeast cells were spotted at two independent foci for experimental replication. Mating and transferring of yeast cells were assisted by an automated system using ROTOR HDA (Singer Instruments). Diploid yeast cells were then subjected to the X-gal assay and 3-amino-1, 2, 4-triazole (3-AT) resistance assay. For the X-gal assay, plates were photographed at the earliest time point when any colonies turned blue, with the plates checked at 30 min and at 1, 2, 3, 4, 7 and 24 h. If no colony turned blue, plates were photographed at 7 and 24 h. For the 3-AT resistance assay, plates were kept in the dark at room temperature and observed every 2 d up to 10 d. Plates were photographed at 10 d and the 3-AT resistance was scored as 'positive' when both duplicate yeast colonies showed normal growth. The TF–promoter binding was regarded as 'positive' if either the X-gal assay or 3-AT resistance assay was positive compared with the pDESTAD-2 μ plasmid control with no TF.

Visualization of network graphs and power graph compression analysis

The network graphs were visualized by Cytoscape version 3.5.1 (<http://www.cytoscape.org/>) as previously described in

Shannon et al. (2003). The power graph compression analysis was performed as previously described in Ahnert (2013) and Taylor-Teeples et al. (2015).

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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