The auxin receptor TIR1 homolog (PagFBL 1) regulates adventitious rooting through interactions with Aux/IAA28 in Populus

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Introduction

Roots play a crucial role in water and nutrient acquisition to support growth of the aerial parts of the plant, and healthy root systems contribute in maximizing plant biomass (Jansen et al., 2013). In contrast to lateral roots (LRs) that occur on primary roots and originate from pericycle cells of primary roots, adventitious roots (ARs) can be formed from above-ground organs such as leaves, hypocotyls and stems, and are initiated from cambial or adjacent vascular cells (Legué et al., 2014; Verstraeten et al., 2014). AR founder cells are believed to dedifferentiate from nonroot differentiated tissues (Srivastava, 2002). AR formation occurs naturally in many monocotyledonous species and many species of tropical and temperate wet forest trees as a part of the normal development. Commercially, ARs are produced during vegetative propagation by artificial induction using wounding or hormone application treatments in many dicotyledonous species (Nadkarni, 1994; Pacurar et al., 2014). The biological processes involved in AR formation are complex, and the temporal phases can be described as induction, initiation, activation of root primordium and out-growth (Legué et al., 2014). These processes are influenced by multiple factors, such as the genetic background and the physiological status of the mother plants, the application of hormones and environmental conditions (Geiss et al., 2010; Pacurar et al., 2014).

Phytohormones are the most important modulators of AR development (Bellini et al., 2014). Plant hormones, such as abscisic acid (Da Costa et al., 2013; Mehrotra et al., 2014), cytokinin (Della Rovere et al., 2013), ethylene (Muday et al., 2012; Negi et al., 2010), gibberellins (Mauriat et al., 2014; Niu et al., 2013), jasmonic acid (Da Costa et al., 2013) and strigolactones (Rasmussen et al., 2012; Sun et al., 2015), form a signalling network influencing cell fate determination and specification in which auxin plays the crucial role (Da Costa et al., 2013; Pacurar et al., 2014; Pop et al., 2011). Early in the 1930s, indole-3-acetic acid (IAA) was shown to be effective in promoting the formation of AR primordia (Thimann and Koenig, 1935), and since then IAA has been widely used to induce AR formation in the clonal propagation of various tree species, including poplar (Preece, 2003; Rademacher et al., 2011). On the other hand, anti-auxin agents applied at AR early phases cause significant inhibition of AR in poplar cuttings (Bellamine et al., 1998). In addition, the IAA content of an easily rooted genotype was higher than that of a difficult to root genotype of Eucalyptus globulus (De Almeida et al., 2015; Negishi et al., 2011). These observations have demonstrated the important role of auxin in AR induction.

It is well-established that auxin is perceived by a receptor (SCFTIR1/AFB), which upon binding auxin targets AUXIN/INDOLE-3-ACETIC ACID INDUCIBLE (Aux/IAA) proteins for degradation. Aux/IAA proteins repress auxin response factors (ARFs), the latter activate or repress downstream auxin signalling genes upon released from repression Aux/IAAs (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Chapman and Estelle, 2009, 2014; Wang and Estelle, 2014; Korasick et al., 2015; Salehin et al., 2015). The three key signalling elements TIR1/AFBs, Aux/IAAs and ARFs are encoded by gene families of 6, 29 and 23 members in Arabidopsis.
The regulation of root development by auxin in *Arabidopsis* has been described in a recent review by Quint et al. (2007). The induction of auxin-inducible acyl amido gene expression can lead to a differential auxin signalling (Quint et al., 2007), respectively. A different context of signalling pathway is conserved in cereals and *Arabidopsis*. The induction of auxin signalling is also observed in rice and maize mutants that are altered in AR and LR initiation (Ramírez-Carvajal and Davis, 2010; Verstraeten et al., 2013). It is anticipated that the underlying mechanisms of some genes, such as auxin receptors and foundry genes, are conserved in *Arabidopsis* and *Populus* (Verstraeten et al., 2014). AR formation, in contrast, has proved difficult to study, and the mechanisms controlling AR initiation and development are poorly understood. Recent studies in *Arabidopsis* showed that auxin is likely to induce AR initiation through the activation of an auxin signalling network similar to that in LR (see the recent review by Bellini et al., 2014). In LR formation, there are two signalling pathways, namely TIR1/AFB2-IAA12,28-ARF5 and TIR1-IAA14-ARF7,19 (Bellini et al., 2014). ARF6, 8 and 17 have been identified in auxin signalling pathways during AR formation in *Arabidopsis*, but which SCF$\text{TRI1/AFB}^\text{AR}$ or Aux/IAA members are involved in this signalling process remains elusive (Bellini et al., 2014; Gutierrez et al., 2012). Characterization of rice and maize mutants that are altered in AR and LR development showed that the transcriptional regulatory pathway is conserved in cereals and *Arabidopsis*, involving TIR1/AFB2 auxin receptors and the Aux/IAA, ARF and LBD (LATERAL ORGAN BOUNDARIES DOMAIN) transcription factors (Ormanliegeza et al., 2013). Analysis of gene expression in poplar cuttings indicated that the context of genes encoding Aux/IAA and ARF proteins were remodelled during the first 2 days after excision of stems (Ramírez-Carvajal et al., 2009), and some ARF family members were specifically expressed during adventitious rooting in *P. trichocarpa*, based on transcriptomic data (Rigal et al., 2012).

Understanding AR formation in trees is important (Leguedé et al., 2014), because this capability underlies the ability to vegetatively propagate millions of cuttings from elite clones for commercial production (Li et al., 2009). Recent advances in *Populus* suggest that it is feasible to identify genes and their pathway regulating adventitious rooting, which are remodelled in cells prior to AR initiation (Ramírez-Carvajal and Davis, 2010; Verstraeten et al., 2013). It is anticipated that the underlying mechanisms of some developmental aspects of the induction and formation of ARs may be common between *Arabidopsis* and *Populus* (Verstraeten et al., 2013). However, whether it will be possible to translate what is known about AR development in herbaceous species to woody species still needs to be investigated (Bellini et al., 2014).

We previously performed a comprehensive analysis of the poplar auxin receptors and found the *TIR1* homolog *PtrFBL1* from *P. trichocarpa* plays an important role in growth rate and development (Shu et al., 2015). Here, we report that FBL1 (PagFBL1) from hybrid poplar (*P. alba* × *P. glandulosa*) clone 84K regulates AR formation from stem cuttings. We demonstrate that PagFBL1 is a key regulator in auxin signalling pathway to induce adventitious rooting, and the potential downstream regulators, including candidate Aux/IAAs in the auxin signalling pathway, are also identified in poplar.

### Results

**PagFBL1** exhibits spatially distinct expression patterns during adventitious rooting

The expression patterns of genes can provide useful clues to their functions. Therefore, we generated *Ppag*:GUS transgenic *Populus* B4K plants to investigate PagFBL1 expression pattern (Materials and methods). GUS signal was mainly observed in the cambial zone and immature xylem in stems at time zero after cutting (Figure 1a, b), and then became more broadly expressed in the cambial zone and secondary phloem 2 days after AR induction (Figure 1c, d). Three to four days after AR induction, GUS signal was observed in the AR primordium which included cells within the cambial zone, secondary phloem and cortex (Figure 1e–h). GUS signal decreased within the enlarged root primordium by 5 days after AR induction (Figure 1i, j) and was undetectable within the AR 6 days after AR induction (Figure 1k, l). This indicates that PagFBL1 may be involved in the formation of ARs at early stages, that is induction and initiation phases. In addition, in comparison with our earlier study on AR formation using DRS::GUS auxin response reporter lines, the expression of PagFBL1 showed similar dynamic changes with the auxin distribution during AR formation (Liu et al., 2014). These results suggest that the PagFBL1, the auxin receptor, could participate in the auxin signalling pathway to regulate AR initiation and initiation.

**Overexpression and knock-down of PagFBL1 affect AR formation in transgenic poplar**

The expression of PagFBL1 during early AR development prompted us to study its role in this process. In total, 19 independently transformed overexpression (OE) lines and 20 lines with knock-down transcript levels (KD) lines were generated, and the relative up- and downexpression of PagFBL1 in these lines was quantified using real-time qRT-PCR (Materials and methods). We selected eight OE lines and eight KD lines with moderate change in expression levels, respectively (Figure S1a), and investigated their rooting ability. The OE lines exhibited earlier AR emergence (Figure 2a, Figure S1b), higher percentage of leafy stem explants with ARs at different times after induction and 6 h earlier in reaching to 100% than wild-type controls (WTs) (Figure 2b, Figure S1c). In addition, the number of ARs generated from leafy stem explants of OE lines was significantly increased (Figure 2c, e) and supported larger root systems (Figure 2d) as measured by total root length, root area, fresh and dry weight (Figure 2e) 5 months after planting in soil. OE lines produced earlier and more ARs than WTs, and this phenomenon was even pronounced under IAA treatment using leaf explants (Figure S2a–e). In PagFBL1 KD lines, the emergence time was delayed (Figure 2f, g, Figure S1d, e), even under IAA treatment using leaf explants (Figure S2f-h). In addition, the number (Figure 2h, j) and biomass of ARs (Figure 2i, j) were significantly decreased compared with WTs as well as OE lines. These findings suggest that PagFBL1 plays a significant role in AR formation in poplar.

**Overexpressing PagFBL1 stimulates the remodelling of gene expression in transgenic poplar**

To gain molecular insights into the roles of PagFBL1 in adventitious rooting, a transcriptome analysis was performed using RNA sequencing to identify differentially expressed genes (DEGs) in AR formation (Materials and methods). For WT, a total of 8855 genes were significantly differently expressed between 0 and 12 h after AR induction, with 4488 up-regulated and 4367 down-regulated in nontransgenic controls (Figure 3a, b). However, only 1,607 DEGs including 881 up- and 726 down-regulated genes were detected from 12 to 24 h after induction (Figure 3a, b), and 1121 DEGs with 814 up- and 350 down-regulated from 24 to 48 h were obtained (Figure 3c, d). Similarly, for OE line #18, a total of 10 373 DEGs with 5357 (2546 shared with WT) up- and 5,016
down-regulated were detected from 0 to 12 h (Figure 3a, b). Only 2441 DEGs including 1099 up- and 1342 down-regulated (Figure 3a, b) from 12 to 24 h, and 949 DEGs with 632 up- and 317 down-regulated from 24 to 48 h were found after AR induction (Figure 3c, d). The numbers of DEGs between 0 and 12 h in both non- and transgenic plants were much larger than that between 12 and 24 h or 24 and 48 h. Therefore, the remodelling of expression of a larger numbers of genes occurred in the first 12 hours of AR induction and initiation. Notably, 1518 more DEGs appeared in first 12 h in the OE #18 line, 119 of which appeared later (from 12 to 24 h) in WT (Figure 3a, Table S1). These results suggest that the high level of PagFBL1 could potentiate the shift of gene expression in favour of AR formation.

To understand the significance of DEGs, clusters of orthologous groups (COG) classification was determined (Figure S3a-f). A large number of genes involved in signal transduction mechanisms were induced in the first 12 hours of AR induction and initiation. Notably, 1518 more DEGs appeared in first 12 h in the OE #18 line, 119 of which appeared later (from 12 to 24 h) in WT (Figure 3a, Table S1). These results suggest that the high level of PagFBL1 could potentiate the shift of gene expression in favour of AR formation.

Figure 1 Expression patterns of PagFBL1 during AR formation. GUS staining of ProPagFBL1::GUS leafy stems (a, c, e, g, i, k) and their transverse sections (b, d, f, h, j, l); the samples were collected at 0 day (a, b), 2 days (c, d), 3 days (e, f), 4 days (g, h) 5 days (i, j) and 6 days (h, l). Experiments were repeated three times for each, and the representative phenotypes are shown. Scale bars: (a, c, e, g, i, k) 1 mm; (b, d, f, h, j, l) 200 µm.

PagFBL1 executes auxin signalling by interacting with PagIAA28.1 and PagIAA28.2

Auxin signalling starts from the FBL1-mediated Aux/IAA degradation, prompting us to identify Aux/IAA proteins that might be targeted by PagFBL1. Aux/IAA genes exhibit differential expression under auxin treatment and are generally more responsive than ARF or TIR1/AFB genes (De Almeida et al., 2015; Ivan et al., 2008; Trenner et al., 2016; Villacorta-Martín et al., 2015; Wen et al., 2016; Xu et al., 2017a). We screened the candidate Aux/
Poplar auxin receptor promotes adventitious rooting

IAA genes that showed changes in expression during the early stages of AR formation, or else show differential expression in WT control vs OE plants undergoing AR formation. Based on the transcriptome in this study (Figure S5) and that in P. trichocarpa (Ramirez-Carvajal et al., 2009), we selected 15 genes (and their alternative transcripts) with such expression patterns during AR formation, including PagIAA7.1, PagIAA7.2, PagIAA9, PagIAA12.1, PagIAA16.1, PagIAA16.2, PagIAA16.3, PagIAA16.4, PagIAA19.1, PagIAA20.1, PagIAA27.1, PagIAA28.1, PagIAA28.2, PagIAA29.2 and PagIAA29.3. Their expression was further checked during AR formation in both WT and #18 using qRT-PCR (Figure S6). The results showed all these genes’ transcript levels were significantly changed in during AR induction in WT and #18 (Figure S6); thus, their proteins were then tested as candidate targets of PagFBL1.

To determine which PagIAA members are targeted by PagFBL1, we used a bimolecular fluorescence system, in which PagFBL1 and one of the 15 PagIAA members were fused to each
Figure 3  Venn diagrams showing the number of DEGs classified into groups of 0, 12, 24, 48 h after AR induction. (a) Up-regulated genes from 12 h vs 0 h and 24 h vs 12 h. (b) Down-regulated genes from 12 h vs 0 h and 24 h vs 12 h. (c) Up-regulated genes from 24 h vs 12 h and 48 h vs 24 h. (d) Down-regulated genes from 24 h vs 12 h and 48 h vs 24 h. (e) COG classification of DEGs in signal transduction mechanisms. (f) DEG percentages for major hormones in plant hormone signal transduction based on KEGG pathway.

half of the yellow fluorescence protein (YFP) and co-expressed transiently in tobacco leaves (Materials and methods). The complemented YFP fluorescence signals were checked for the 15 combinations tested (Figure 5a, Figure S7). The YFP signal was only observed in the nucleus (merged with DAPI signal) when nYFP-PagFBL1 was cotransformed with cCFP-PagIAA28.1 or cCFP-PagIAA28.2 (Figure 5a), and intensified in higher auxin concentration, but only DAPI signals were observed in other combinations (Figure S7).

Previous studies showed that the LexA yeast two-hybrid system can be used to study the interaction between auxin receptors TIR1/AFB and their substrates Aux/IAAs (Calderón Villalobos et al., 2012; Yu et al., 2015). To further verify the above interactions identified by the bimolecular fluorescence system, PagFBL1 was fused to the LexA DNA-binding domain and introduced into a strain expressing the PagIAA28.1 or PagIAA28.2 protein fused with LexA activating domain. The interaction between PagFBL1 and PagIAA28.1 or PagIAA28.2 was confirmed and its strength increased as measured by galactosidase activity following the elevated auxin concentrations (Figure 5b). This result demonstrates that PagFBL1 can interact strongly with both PagIAA28.1 and PagIAA28.2 in the presence of NAA, thus are candidates for participating in AR induction in poplar stem segments.

Discussion

The mechanisms underlying AR formation and the cause for variation among plant species and genotypes in AR production are poorly understood (Hu and Xu, 2016; Liu et al., 2014; Sena et al., 2008). The details of the mechanisms underlying AR formation are of interest by virtue of their relevance to basic plant biology, but are also crucial for applied aspects for commercial woody plants, like poplar, for propagation of
superior cultivars and capture of both additive and nonadditive genetic variance in tree improvement programmes (Dickmann et al., 2006). In this study, we provided details about the roles of auxin signalling pathways involved in regulation of AR formation in poplar.

In this study, we found the presumed poplar auxin receptor-encoding gene, PagFBL1, was expressed in the cambium zone and secondary phloem during the AR induction phase to the early AR primordium formation, but down-regulated in the enlarged AR primordium. This is a similar pattern to auxin distribution during AR induction (Liu et al., 2014). Previous studies suggest that strong auxin signalling is required during the induction phase of both LR and AR in Arabidopsis (Bustilloavendano et al., 2017; De Klerk and De Jong, 1999; Du and Scheres, 2017; López-Bucio et al., 2015; Sánchez et al., 2007). In woody plants, IAA was mostly located in the cambial region of rooting-competent pine hypocotyls, and IAA content was higher in the cambium zone in E. grandis during the initial 24 h of AR induction (Abarca et al., 2014; De Almeida et al., 2015). In addition, compared to the hard-to-root species E. globulus, the easy-to-root species E. grandis had a peak of TIR1 expression after 6 h of exposure to exogenous auxin (De Almeida et al., 2015), and FB1 (homolog of TIR1) was also involved in the early induction of AR primordium by auxin and regulated the elongation of ARs by auxin in Liriodendron hybrids (Zhong et al., 2016). Together these results show that the auxin receptor FBL1 follows auxin distribution and acts at the very beginning of adventitious rooting. Indeed, overexpression of PagFBL1 dramatically stimulated early AR formation and led to high number of ARs in OE poplars (Figure 2). Therefore, we conclude that PagFBL1 may serve as a key regulator promoting the formation of root primordia in poplar.

The initiation stage of AR is characterized by cell division and organization of the root primordia (Li et al., 2009). Studies in apple (De Klerk and De Jong, 1999; De Klerk et al., 1995), chestnut (Sánchez et al., 2007), Populus (Ribeiro et al., 2016; Rigal et al., 2012), Petunia (Ahkami et al., 2014; Druge et al., 2016), and other species indicate that auxin plays a key role in the initial stages of AR formation.

Figure 4 Expression profiles of the genes (Table S2) related to auxin signalling pathways at different time points during AR formation by both qRT-PCR and RNA-Seq (fold change for FPKM).
Interactions of PagFBL1 and PagIAAs revealed by BiFC assay and LexA yeast two-hybrid assay. (a) PagFBL1 and PagIAA 28.1 or 28.2 by BiFC assay. Bars = 12 μm. (b) PagFBL1 and PagIAA28.1 or 28.2 by LexA yeast two-hybrid assay.

**Figure 5** Interactions of PagFBL1 and PagIAAs revealed by BiFC assay and LexA yeast two-hybrid assay. (a) PagFBL1 and PagIAA 28.1 or 28.2 by BiFC assay. Bars = 12 μm. (b) PagFBL1 and PagIAA28.1 or 28.2 by LexA yeast two-hybrid assay.
carnation (Dianthus caryophyllus) (Villacorta-Martín et al., 2015), Catalpa bungei (Wang et al., 2016) and mung bean (Steffens and Rasmussen, 2016) reveal that the critical events that culminate in the formation of ARs in cuttings occur in the first 3–24 h, and the induction stage comprises molecular and biochemical events without visible changes. Auxin signal transduction was revealed in the transcriptome during the formation of ARs in cuttings in the first 24 h after induction of Populus (Ramírez-Canvajal et al., 2009), Petunia (Afkami et al., 2013; Druege et al., 2014), mung bean (Vigna radiata) (Li et al., 2015), carnation (Villacorta-Martín et al., 2015) and Malus xiaojingshini (Xu et al., 2017b). These transcriptome analyses in AR induction stage provide a meaningful tool for investigating the auxin signalling pathway to regulate AR formation. Higher concentration of IAA is required in the AR induction stage to stimulate auxin-induced cell division but may not be required during root meristem organization (Goldfarb et al., 2010); thus, auxin signal transduction was strengthened in the initiation of ARs of cuttings. In this study, consistent with the above observations, significant changes in gene expression patterns were found, particularly in the induction phase. DEGs enriched in plant hormone signal transduction were predominant at this stage. This also illustrates that a fast reprogramming of gene expression is required to support AR formation, with plant hormone signalling playing a critical role. In addition, OE lines exhibited more DEGs in auxin signalling pathways in the early induction stage, which appeared later in WT plants (Figure 3f, Figure S4). This result is consistent with the earlier formation and higher number of ARs in OE lines. These results further emphasize that PagFBL1 can stimulate this transition by strengthening the auxin signalling pathway.

It is well documented that auxin is perceived by SCF\textsuperscript{TIR1/AFB}-Aux/IAA complexes, and releases bound ARFs to activate auxin-inducible gene transcription through the degradation of Aux/IAA repressors (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Salehi et al., 2015). Although Aux/IAA family proteins are highly redundant, they have different affinity to TIR1 and drive degradation at varied rates, leading to different responses to auxin in diverse biological processes (Chen et al., 2017). Therefore, we further investigated which IAAAs are targeted by PagFBL1 to activate auxin signalling. We found that only PagIAA28.1 and PagIAA28.2 interacted with PagFBL1 using BIFC assay and LexA yeast two-hybrid assay in the presence of IAA in a dose-dependent manner. In Arabidopsis, iaa28-1 mutant showed reduced AR formation (Bustilloavendano et al., 2017; López-Bucio et al., 2015), suggesting IAA28 is required to be degraded in order to release ARFs to initiate both LR and AR formation. Supporting to this suggestion, we found that it is necessary to degrade IAA28 to initiate auxin signalling in AR formation in poplar. Previous studies have also shown that unlike most known auxin-inducible Aux/IAAs, both IAA28 expression levels and protein abundance have been reported to be reduced by auxin treatment in the LR formation (De Rybel et al., 2010; Parizot et al., 2010; Rogg et al., 2001). In addition, IAA28 was suggested to release ARF5, ARF6, ARF7, ARF8 and ARF19 in LR initiation in Arabidopsis (De Rybel et al., 2010), and three of them (PagARF5.1, PagARF5.2 and PagARF7.3) were found specifically expressed in AR initiation in poplar in this study, indicating high level of these ARFs is required in AR initiation. Except the release of IAA-bond ARFs mediated by FBL1, high expression of these ARFs may also be needed in the initiation of ARs. Indeed, PagARF5.1 and PagARF5.2 were highly expressed in OE lines comparing to WT lines, which may contribute to their early AR initiation and higher number of ARs, although the positive regulation of these ARFs in FBL1 OE lines needs to be elucidated. The induction of auxin-inducible GH3 by the ARF family (Zhang et al., 2016) is an early event in the auxin signalling cascade. Previous studies have found that auxin-inducible Gretchen Hagen3 (GH3) genes, GH3.3, GH3.5 and GH3.6, are required for fine-tuning the AR initiation by modulating JA homeostasis and regulated by ARF6, ARF8 and ARF17 in Arabidopsis (Gutierrez et al., 2012; Sorin et al., 2006). In this study, PagGH3.1, PagGH3.5 and PagGH3.6 were found up-regulated during AR initiation in poplar and even pronounced in OE lines based on the RNA sequencing (Table S2, Figure 4), suggesting GH3 plays a key role in auxin signalling in both Arabidopsis and poplar. Due to the biological similarity between the initiation process of LR and ARs (Legué et al., 2014; Verstraeten et al., 2014), the same signalling module may be shared in both processes. Our results thus provide an evidence that the FBL1-IAA28.1,2-dependent auxin signalling module involves in regulation of AR induction in poplar, which shares at least partly with mechanism in LR formation in Arabidopsis.

This study addresses the role of auxin in AR formation in poplar and suggests that FBL1 participates in an FBL1-IAA28.1,2 module regulating AR formation in poplar, which shares similarity with the regulatory mechanisms of LR induction in Arabidopsis. PagFBL1 acts in auxin signalling required early in AR development, representing a potential biotechnological target for the improvement of poplar propagation by cuttings.

Materials and methods

Plant materials and growth conditions

P. alba × P. glandulosa clone 84K was used as the plant material for the cloning of PagFBL1 and its transformation. Plants were propagated by microcuttings in bottles and cultured on 1/2 × MS (Murashige and Skoog) medium at 24 ± 1 °C under cool-white light (60 ± 5 μmol photons m\textsuperscript{-2}s\textsuperscript{-1}) for 16-h light/8-h dark (Shu et al., 2015), and leafy stems from 3-week-old plants were used in induction experiments for ARs.

To explore the role of PagFBL1 in AR formation, we used a PagFBL1 promoter::GUS assay to monitor the expression of PagFBL1 during AR formation in a time course after AR induction from leafy stem segments. A 2.0 kb 5′-UTR fragment of PagFBL1 (KY020444) was amplified from the genomic DNA of 84K and used to investigate the tissue-specific expression using the sequence-specific primers listed in Table S3. The promoter fragment was then cloned into pMD18T and inserted into pMD18T to produce Pro\textsubscript{PagFBL1}:::GUS constructs using the Gateway cloning system (Invitrogen) for transformation into poplar 84K via Agrobacteria (Shu et al., 2015). Derooted leafy stems of Pro\textsubscript{PagFBL1}:::GUS lines were cultured on 1/2 MS for 0, 2, 3, 4, 5, 6 days, and the GUS staining was performed on the lower parts of stems. GUS staining during AR formation was performed as described by Shu (Shu et al., 2015). In brief, the samples were incubated in staining solution (20 mM X-Gluc in phosphate buffer) for 12 h at 37 °C with gentle agitation at 70 r/min and then rinsed in 70% ethanol for visual observation and microscopy. Three replicates were included for each time point.

To explore the role of PagFBL1 in AR formation in poplar 84K, PagFBL1 cDNA from the cDNA of 84K was amplified using the sequence-specific primers (Table S3), cloned into a plant overexpression vector pCAMBIA2301 (OE construct) and a binary pBlues vector with antisense orientation (KD construct).
respectively, as described in previous studies (Tang et al., 2010; Zhao et al., 2013), and transformed into poplar clone 84K. After obtaining the regenerated buds, we first induced their rooting using the screening medium (1/2 × MS) with the vector-specific antibiotic (Hygromycin) and Timentin (for inhibiting Agrobacteria). The rooted transgenic plants were verified by genomic PCR and propagated by cutting. Then the expression level of FBL1 in these transgenic lines was determined by qRT-PCR, and the lines with intermediate change in expression level were selected in the following experiments. More than 19 lines have been generated for both OE and KD construct and the eight OE lines (#4, #5, #7, #11, #15, #16, #17 and #18) and eight KD lines (#2, #5, #8, #10, #11, #12, #16 and #19) with intermediate FBL1 expression levels in OE or KD lines were used for the experiments in this study. The root induction from leafy stems was performed on the OE, KD transgenic plants and 84K controls (WT) in 1/2 × MS at 24 ± 1 °C under cool-white light (60 ± 5 μmol photons m⁻²s⁻¹ at the top leaves surface, 16-h light/8-h dark) (Shu et al., 2015), and the plants were checked and photographed after 96, 102, 108, 114, 120, 126, 138 h and 10 days. In addition, leaf explants for the OE transgenic plants (#4 and #18) and WT from 3-week-old seedlings were cultured on 1/2 × MS medium with sucrose in 0 μM and 5 μM IAA for de novo regeneration of ARs; leaf explants for the KD transgenic plants (#2 and #12) and WT from 3-week-old seedlings were cultured on 1/2 × MS medium with sucrose in 0 μM and 10 μM IAA for de novo regeneration of ARs. The cultured leaves were photographed after 11, 12, 14, 16 days. The OE transgenic plants (#4 and #18) and KD transgenic plants (#2 and #12) were propagated by cuttings in soil as previously described (Shu et al., 2015) and grown for 5 months (OE #4, #18 and WT) and 2 months (KD #2, #12 and WT) in a glasshouse at Chinese Academy of Forestry. The ARs from the transgenic plants and WT were collected and measured. The experiments were performed with at least 30 clonal plants for each line.

Plant phenotypic determination

The numbers of ARs were directly counted as described by Song and Xu (2011) to survey the difference in emerged and outgrown roots among the plant materials. Roots from cuttings grown on TS1 (Klasmann Deilmann, Germany) at 24 ± 1 °C with well-watered and natural light from April to August in the glasshouse (Chinese Academy of Forestry, Beijing) were scanned using a root analysis machine (WinRhizoV4.0b; Regent instrument Inc., Quebec, Canada), and then, the roots were dried in an oven at 105 °C to a constant weight for measuring the root biomass. The measurements were performed on six individual plants for each line, and their mean and standard error were calculated (Data analysis).

Sequence annotation and differential expression analysis

Leafy stems from 1-month-old seedlings (WT and #18) were subcultured into 1/2 × MS media. The bases of the stems (lowest 0.5 cm portion of the stem) were sampled at 0, 12, 24, 48 h during AR induction, frozen immediately in liquid nitrogen and stored at −80 °C before use. Three replicates were analysed consisting of about 100 stem segments for each. The RNAs were extracted using the RNeasy Plant Mini Kit and treated with RNase-free DNase I (Qiagen, Hilden, Germany). RNA quality and quantity were determined using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v4-cBot-HS (Illumia) according to the manufacturer’s instructions, generating 2 × 150 bp and 1 × 60 bp reads. After cluster generation, the libraries were prepared and sequenced on an Illumina Hiseq 2500 platform. Image analysis and base calling were performed using the HiSeq Control Software version 1.4, and the Off-Line Base Caller v1.9 ~ 120 million high quality RNA-Seq reads (with quality score > 30 for each base) were pooled from Illumina sequencing of each of the 24 samples (three biological replicates of four stages) and were then assembled into contigs using Trinity. The paired-end reads were generated by Biomarker Technologies (Fan et al., 2015). Gene function was annotated based on the following databases: Nr (ftp://ftp.ncbi.nih.gov/blast/db/); COG (http://www.ncbi.nlm.nih.gov/COG/); Swiss-Prot (http://www.uniprot.org/); GO (http://www.geneontology.org/); KEGG (http://www.genome.jp/kegg/); and KOG (http://www.ncbi.nlm.nih.gov/KOG/). Differential expression analysis of two conditions was performed using the DESeq software package in which a MA-plot-based method coupled to a random sampling model (MARS) method was mainly used. This approach was supplemented by the likelihood ratio test (LRT), Fisher’s exact test (FET) and the fold-change threshold on MA-plot (FC) method (Li et al., 2015). The resulting P values were adjusted using the Benjamini and Hochberg’s approach for controlling the false discovery rate (Storey and Tibshirani, 2003). Genes with an adjusted P value <0.05 found by DESeq were assigned as differentially expressed with three biological replicates (Anders and Huber, 2010). The RNA-Seq data were deposited in SRA database of NCBI with accession number SRP101893.

RNA isolation and qRT-PCR

Total RNAs from above stem samples at 0, 12, 24, 48 h were extracted, and their quality and quantity were checked as previously described. First-strand cDNA synthesis was carried out with approximately 3 μg RNA using Superscript III reverse transcription kit (Life Technologies, Carlsbad, CA) according to the manufacturer’s instruction. The amplified fragments were confirmed using agarose gel electrophoresis. Real-time qRT-PCR was performed as described by Shu (Shu et al., 2015) using PagUBQ gene as an internal reference (Table S4). All primer sequences used in the qRT-PCR were described in Table S5. To confirm their expression patterns, nine auxin signalling-related genes were selected for qRT-PCR.

Bimolecular fluorescence complementation (BiFC) assay

BiFC assay was performed as previously described (Sparkes et al., 2006). Complementary DNA of PagFB1 and PagIAAs, including PagIAA7.1, PagIAA7.2, PagIAA9, PagIAA12.1, PagIAA16.1, PagIAA16.2, PagIAA16.3, PagIAA16.4, PagIAA19.1, PagIAA20.1, PagIAA27.1, PagIAA28.1, PagIAA28.2, PagIAA29.2, and PagIAA29.3, were amplified using the primers listed in Table S3 and cloned into BiFC vectors pYFP-X for PagFB1 and pCPC-P for PagIAAs using the GATEWAY recombination system (Invitrogen). The pairs of constructs were cotransformed into leaves of 2-month-old tobacco (Nicotiana benthamiana) by infiltration as described previously (Shu et al., 2015). After 3 days, the leaves were treated with 0, 10, 100 μM, 1 μM NAA (Calderon Villalobos et al., 2012) (Sigma-Aldrich, St. Louis, MO) and further incubated in a glasshouse for 3 days. The leaves were immersed in 50 μM DAPI (4′, 6-diamidino-2-phenylinodole), a nuclear marker, for 60 min. Fluorescence was observed using an UltraVIEW VoX 3D Live Cell Imaging System (PerkinElmer). For confocal imaging YFP
and DAPI fluorescence, 488 and 405-nm laser and a 488 and 405-nm band-pass emission filter were used, respectively (Shu et al., 2015). The experiments were performed on three tobacco leaves for each pair of constructs and repeated three times.

LexA yeast two-hybrid assays

FBL1 and Aux/IAA coding regions were cloned into the Y2H bait vector pGILDA and the prey vector pB42AD (Clontech), respectively, after amplifying using the primer pairs shown in Table S3. Bait and prey constructs were cotransformed into S. cerevisiae strain EGY48[p8opLaCZ] (Clontech), and transformants were selected on SD supplemented with -Ura/-His/-Trp dropout solution (BD Biosciences) and glucose medium. To test the interaction between FBL1 and Aux/IAA proteins, transformed yeast colonies were plated on SD-galactose/raffinose-inducing medium containing -Ura/-His/-Trp dropout solution, 80 µg/ml X-Gal and NAA in the different concentrations of 0 µM, 1 µM, 10 µM, 100 µM and incubated for 3-4 days at 30 °C (Calderón Villalobos et al., 2012; Yu et al., 2015).

Data analysis

Data were analysed by ANOVA using the SPSS 10 program (SPSS Inc., Chicago, IL). All data in the figures are given as means ± SE. Significance of differences between means was analysed by the two-sample t-test at P < 0.05 or P < 0.01. Asterisks on the histograms or after the mean value between the transgenic and WT, or among different treatments indicate they are statistically different.

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**Supporting information**

Additional supporting information may be found online in the Supporting information section at the end of the article.

**Figure S1** AR formation in 8 PagFBL1 OE and KD lines. (a) Expression of FBL1 by qRT-PCR analysis respectively. (b, c) AR rooting rates from leafy stems of 8 OE lines. (d, e) AR rooting rates of 8 KD lines. Bars = 1 cm. The values are means ± SE of 3 replicates. Significant differences between WT and transgenic lines are indicated with asterisks (*P < 0.05 and **P < 0.01).

**Figure S2** ARs from leaves of WT, PagFBL1 OE lines #4 and #18 and KD lines #2 and #12 treated with (d, e, h) or without (b, c, g) IAA. (b, d) The induction rates of AR of WT and OE lines accessed during 11 and 16 days. (a, c, e) The number of ARs counted after 1 month for OE and WT lines. (f, g, h) ARs induction rate of WT and KD lines treated with or without IAA (10 μM). Bars = 5 mm. The values are means ± SE of 3 replicates. Significant differences between WT and transgenic lines are indicated with asterisks (*P < 0.05 and **P < 0.01).

**Figure S3** COG classification of DEGs. (a) 12 h vs 0 h in WT. (b) 24 h vs 12 h in WT. (c) 48 h vs 24 h in WT. (d) 12 h vs 0 h in #18. (e) 24 h vs 12 h in #18. (f) 48 h vs 24 h in #18.

**Figure S4** KEGG pathway of DEGs. (a) 12 h vs 0 h in WT. (b) 24 h vs 12 h in WT. (c) 48 h vs 24 h in WT. (d) 12 h vs 0 h in #18. (e) 24 h vs 12 h in #18. (f) 48 h vs 24 h in #18.

**Figure S5** Heat map showing the expression patterns of PagIAAs during AR formation from OE transgenic line (#18) and WT at four time points.

**Figure S6** The expressions of 15 PagIAAs during AR induction by qRT-PCR.

**Figure S7** Interaction between PagFBL1 and PagIAA7.1, 7.2, 9, 12.1, 16.1, 16.2, 16.3, 16.4, 19.1, 20.1, 27.1, 29.2 or 29.3 with 100 μM NAA, respectively. Bars = 12 μm.

**Table S1** The same up-regulated DEGs appeared from 0 h to 12 h after AR induction in #18 and from 12 h to 24 h after AR induction in WT.

**Table S2** Up-regulated and down-regulated genes of auxin signaling pathways related to AR induction at different time points.

**Table S3** The primer sequences used in real-time quantitative PCR.