RESEARCH PAPER

Molecular basis of differential adventitious rooting competence in poplar genotypes

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Abstract

Recalcitrant adventitious root (AR) development is a major hurdle in propagating commercially important woody plants. Although significant progress has been made to identify genes involved in subsequent steps of AR development, the molecular basis of differences in apparent recalcitrance to form AR between easy-to-root and difficult-to-root genotypes remains unknown. To address this, we generated cambium tissue-specific transcriptomic data from stem cuttings of hybrid aspen, T89 (difficult-to-root) and hybrid poplar OP42 (easy-to-root), and used transgenic approaches to verify the role of several transcription factors in the control of adventitious rooting. Increased peroxidase activity was positively correlated with better rooting. We found differentially expressed genes encoding reactive oxygen species scavenging proteins to be enriched in OP42 compared with T89. A greater number of differentially expressed transcription factors in cambium cells of OP42 compared with T89 was revealed by a more intense transcriptional reprogramming in the former. PtMYC2, a potential negative regulator, was less expressed in OP42 compared with T89. A greater number of differentially expressed transcription factors in cambium cells of OP42 compared with T89 was revealed by a more intense transcriptional reprogramming in the former. PtMYC2, a potential negative regulator, was less expressed in OP42 compared with T89. Using transgenic approaches, we demonstrated that PttARF17.1 and PttMYC2.1 negatively regulate adventitious rooting. Our results provide insights into the molecular basis of genotypic differences in AR and implicate differential expression of the master regulator MYC2 as a critical player in this process.

Keywords: Adventitious roots, cambium, hybrid aspen, hybrid poplar, Populus spp., stem cuttings

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Introduction

In the 1990s, only 3% of the world’s forested land were plantations for wood production. However, despite this small percentage, it still provided more than one third of total industrial wood production (Kirilenko and Sedjo, 2007). The shift of production from natural forests to plantations is projected to accelerate and is expected to rise to 75% in the 2050s (Kirilenko and Sedjo, 2007). Operating plantations is expensive and requires high productivity per hectare, which in turn requires good quality, i.e. genetically improved planting stock. Many forest companies are therefore currently considering clonal propagation in addition to, or in conjunction with, their breeding programmes. This aims to propagate elite genotypes from available genetic diversity and maximise the productivity of selected high-value hybrid clones (Bozzano et al., 2014). The genus Populus comprises about 30 species; its wood forms an abundant and renewable source of biomaterials and bioenergy (Ragauskas et al., 2006). The propagation of poplar species depends primarily on adventitious root (AR) formation from detached stem cuttings (Dickmann, 2006), but one major constraint for vegetative propagation of some economically important elite genotypes is incompetence or rapid loss of capacity in forming AR (Bellini et al., 2014; Brunoni et al., 2019; Bannoud and Bellini, 2021). AR development is a complex, heritable trait controlled by many endogenous regulatory factors, and quite influenced by environmental factors (Bellini et al., 2014; Bannoud and Bellini, 2021). The rooting capacity of cuttings varies among individuals within species, populations, or even clones (Abarca and Díaz-Sala, 2009a, 2009b). Few studies have reported the genetic variability of AR development of Populus hardwood cuttings. Zhang et al. (2009) reported quantitative trait loci (QTL) that control two AR growth parameters in a full-sib family of 93 hybrids, derived from an interspecific cross between two Populus species, P deltoides and P euramericana, which are defined as difficult-to-root and easy-to-root, respectively. They showed that the maximum root length and the total AR number correlated and were under strong genetic control, which supports earlier QTL analysis performed on forest trees (reviewed in Geiss et al., 2009). Several studies focusing on AR development in poplar have identified a number of genes involved in its regulation (Ramírez-Carvajal et al., 2009; Rigal et al., 2012; Trupiano et al., 2013; Wuddineh et al., 2015; Xu et al., 2015; Yordanov et al., 2017; Li et al., 2018; Liu et al., 2020; Wang et al., 2020; Wei et al., 2020; Yue et al., 2020; Zhang et al., 2020; Xu et al., 2021) including large-scale data analyses identifying regulators (Ribeiro et al., 2016; Zhang et al., 2019) and pharmacological assays of physiological regulators (Gou et al., 2010; Mauriat et al., 2014; Zhang et al., 2019). All these studies resulted in a substantial increase in our understanding of the molecular mechanisms that control successive steps of AR development, but the molecular differences in recalcitrance to form AR between easy-to-root and difficult-to-root genotypes remains unknown. To address this question, we compared the transcriptome of cambium cells obtained immediately after cutting and 24 h later, by laser capture microdissection (LCM) from P trichocarpa × P maximowiczii (clone OP42) which we defined as ‘easy-to-root from woody stem cuttings’, and the hybrid aspen P tremula × P tremuloides (clone T89) which we defined as ‘difficult-to-root from woody stem cuttings’. OP42 is one of the poplar clones used most widely, both in Northern Europe and worldwide (Taereoe et al., 2015). It can easily be propagated from dormant stem cuttings. In contrast, the hybrid aspen T89 cannot be propagated via dormant stem cuttings but can be easily propagated in vitro, and is very amenable to genetic transformation (Nilsson et al., 1992). The analysis of the transcriptomic dataset showed more differentially expressed genes encoding transcription factors (TFs) in OP42 than in T89. We identified several TFs that could explain differences in ability to produce adventitious roots. We showed that up-regulation of the jasmonate (JA) signalling pathway in the cambium of T89 could be one cause of the failure to produce adventitious roots.

Materials and methods

Plant growth conditions and rooting assays

The hybrid aspen (P tremula L. × P tremuloides Michx.), clone T89, and the hybrid poplar (P trichocarpa × P maximowiczii) clone OP42, were propagated in vitro for 4 weeks as described in Karlberg et al. (2011) and shown in Supplementary Fig. S1A. More precisely, plants were grown in plastic jars containing sterile half-strength Murashige and Skoog medium (Duchefa, The Netherlands), pH 5.6, at an average temperature of 25 ± 1 °C, under an 18 h/6 h light/dark cycle. Light at 150 μmol m-2 s-1 was provided by warm white fluorescent tubes. For in vitro rooting assays, 3 cm cuttings with four to five leaves in the case of T89, and two to three leaves in the case of P trichocarpa × P maximowiczii clone OP42 plantlets, were collected and transferred into smaller rectangular jars containing fresh sterile medium, as above (Supplementary Fig. S1B, D). The number of ARs was scored from day five after cutting, until day 14. Three replicates of 15 stem cuttings each were analysed. For the jasmonic acid and auxin treatments, cuttings from 4-week-old in vitro grown T89 and OP42 plantlets were transferred to fresh sterile medium with or without methyl jasmonate (MeJA) at 5 μM, 10 μM, or 20 μM, or with or without indole acetic acid (IAA) at 0.1 nM or 10 nM.

For the rooting assay in hydroponic conditions, in vitro plants of hybrid aspen (P tremula L. × P tremuloides Michx.), clone T89, and hybrid poplar (P trichocarpa × P maximowiczii) clone OP42, that had been propagated in vitro for 4 weeks were transferred to soil and kept in the greenhouse for three months (16 h light, 21°C; 8 h dark 18°C). After 3 months, 20 cm long stem cuttings were taken from the third internode below the shoot apex from T89 and OP42 plants. After removal of all leaves and buds except for the higher axillary bud (Supplementary Fig. S1C, E), the cuttings were transferred to hydroponic conditions in the greenhouse. The nutrient solution was composed of a modified Hoagland solution, as described in Plitt et al. (2011). Photos of the ARs were taken using a Canon EOS 350 digital camera and Discovery V8.8 stereomicroscope fitted with a Zeiss camera (Zeiss, Sweden; Supplementary Fig. S1C, E).

Histological analysis of stem cuttings in vitro

For histological analysis of stems, 5 mm stem fragments were taken at the base of cuttings 4 or 5 d after cutting. Samples were vacuum infiltrated with a fixation medium (10 ml of 37% formaldehyde, 5 ml of 5% acetic acid, 50 ml of 100% ethanol and 35 ml of water) for 20 s.
and left for 24 h at 20 °C. The samples were then washed in 70% ethanol for 10 min and transferred into fresh 70% ethanol until required for use. Samples were then gradually dehydrated in an ethanol series (80%, 90%, 96% for 2 h each, and 100% overnight at 20 °C). The 100% ethanol was gradually replaced by HistoChoice tissue fixative (VWR Life, Sweden) in three steps of 1:3, 1:1, 3:1 (EtOH: HistoChoice), then with pure HistoChoice twice in 1 h. The HistoChoice fixative was gradually replaced with Parafilm Plus for tissue embedding (Sigma-Aldrich, USA) over 6 d. Ten μm cross or longitudinal sections were made with a rotary microtome (Zeiss, Germany) and stained with safranin and alcian blue (Sigma-Aldrich, USA) in a ratio of 1:2; using methods from Hamann et al. (2011). Stem sections taken from cuttings in hydroponic conditions were obtained using a vibratome (Leica Biosystems, UK). Following this, 20 μm sections were stained as described above.

Tissue preparation before laser capture microdissection

Sampling, fixation, and cryoprotection steps

The basal 5 mm stem pieces of T89 and OP42 cuttings were harvested immediately after excision from greenhouse-grown plants (time T0) and after 24 h of hydroponic culture (time T1; Supplementary Fig. S2A–C). Three biological replicates of tissue samples were collected at each time point (T0 and T1) from both OP42 and T89 (12 samples in total = three biological replicates × two genotypes × two time points). Immediately after the sampling, stem pieces were split in half longitudinally and subjected to fixation and cryoprotection steps before the laser microdissection. We used the protocol described at https://schnablelab.plantgenomics.iastate.edu/resources/protocols/, slightly modified as follows: samples were soaked in cold ethanol-acetic acid (EAA) Farmer’s fixative solution, containing 75% (v/v) ethanol and 25% (v/v) acetic acid, and vacuum infiltrated on ice at 400 mm Hg for 20 min. After 1 h incubation at 4 °C, another step of vacuum infiltration with fresh Farmer’s solution was performed (400 mm Hg for 20 min). Samples were then kept at 4 °C overnight. The following day, the fixative solution was removed and the samples transferred into a 10% sucrose solution (400 mm Hg for 20 min). After 1 h incubation at 4 °C, another step of vacuum infiltration with fresh Farmer’s solution was performed (400 mm Hg for 20 min). Samples were then kept at 4 °C overnight. The following day, the fixative solution was removed and the samples transferred into a 10% sucrose solution prepared with 1× phosphate buffered saline (PBS, 137 mM NaCl, 8 mM Na2PO4, 2.68 mM KCl, 1.47 mM KH2PO4), vacuum infiltrated on ice at 400 mm Hg for 20 min. After 1 h incubation at 4 °C, another step of vacuum infiltration with fresh Farmer’s solution was performed (400 mm Hg for 20 min). Samples were then kept at 4 °C overnight. The following day, the fixative solution was removed and the samples transferred into a 10% sucrose solution prepared with 1× phosphate buffered saline (PBS, 137 mM NaCl, 8 mM Na2PO4, 2.68 mM KCl, 1.47 mM KH2PO4), vacuum infiltrated on ice at 400 mm Hg for 15 min. Samples were then left incubating for 1 h at 4 °C, then vacuum infiltrated with a 15% sucrose solution (400 mm Hg for 15 min). Samples were then incubated overnight at 4 °C; then frozen in liquid nitrogen and stored at −80 °C until cryosectioning.

Cryosectioning

The day before cryosectioning, membrane slides for laser microdissection (FrameSlide PET, Zeiss; Fisher Scientific, UK) were treated with RNaseZap (Sigma-Aldrich, USA), rinsed twice with diethylpyrocarbonate (DEPC) water and dried for 2 h at 37 °C. Immediately before sectioning, slides were further treated with UV light for 30 min to improve adhesion of sections. Tweezers and a cryostat knife were sterilised at 180 °C for 4 h. The chamber temperature of the cryostat (Leica CM1850, Germany) was set at −25 °C. The instruments including tweezers, knives, and polystyrene teraphthalate (PET)-membrane coated slides were transferred into the chamber 20 min before sectioning. Samples were transferred from a −80 °C freezer to the cryostat in liquid nitrogen. They were fixed with Tissue-Tek® optimal cutting temperature (OCT) compound onto a specimen stage directly in the cryochamber. To avoid embedding and the presence of OCT compound on membrane slides, stem sections were mounted to allow cambium collection from tangential cryosections (Supplementary Fig. S2D). Sections of 25 μm were transferred with tweezers onto membrane slides, then moved to a Petri dish at 20 °C. Sections were then treated with 70% ethanol for 5 min at room temperature, followed by 95% ethanol for 2 min on ice, and 100% ethanol for 2 min on ice. In these dehydration steps, ethanol was applied and removed directly onto the membrane slide chamber with a sterile plastic Pasteur pipette, being careful not to damage the membrane. After ethanol removal, sections were air-dried for 5 min before being cut at the microdissector (Zeiss MicroImaging, Germany).

Laser capture microdissection (LCM), RNA extraction, and RNA sequencing

LCM was performed with a PALM Robot-Microbeam system (Zeiss MicroImaging, Munich, Germany). Cambium microdissected cells were catapulted into the adhesive caps of 500 μl tubes (Supplementary Fig. S2E–K). Total RNA was isolated using the PicoPure RNA Isolation Kit (Thermo Fisher Scientific, Sweden). Quality and quantity of RNA samples were assessed using the Bio-Rad Experion assayer and Experion RNA high-sense analysis kit (Bio-Rad, USA). Total RNA from each biological replicate was amplified using the MessageAmp II aRNA amplification kit (Ambion, Austin, TX, USA). Amplified RNA profiles were verified using the Experion assayer and Experion RNA standard-sense analysis kit (Bio-Rad, USA). In total, 12 cDNA paired-end libraries were generated using the mRNA-Seq assay for transcriptome sequencing on an Illumina HiSeq™ 2000 platform at Beijing Genome Institute (BGI, China), but only 11 were sequenced as one T89 (T1) sample failed the quality check.

Pre-processing of RNA-seq data

The data pre-processing was performed as described in Delhomme et al. (2014). Briefly, the quality of the raw sequence data was assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Residual ribosomal RNA (rRNA) contamination was assessed and filtered using SortMeRNA (v2.1; Kopylova et al., 2012; settings --log --paired in --fastx --sam --num_alignments 1) using the rRNA sequences provided with SortMeRNA (rfam-5s-database-id98.fasta, rfam-5.8s-database-id98.fasta, rfam-16s-database-id98.fasta, rfam-18s-database-id98.fasta, rfam-23s-database-id98.fasta, rfam-23s-database-id98.fasta, rfam-23s-database-id98.fasta). Data were then filtered to remove adapters and trimmed for quality using Trimmomatic (v0.32; Bolger et al., 2014; settings TruSeq3-PE-2, 2:3:10 LEADING:3 SLIDINGWINDOW:5:20 MINLEN:50). After both filtering steps, FastQC was run again to ensure that no technical artefacts were introduced. Filtered reads were aligned to v3.0 of the P. trichocarpa genome (Phytozome) using STAR (v2.5.2b; Dobin et al., 2013; non default settings: --outSAMstrandField intronMotif --readFilesCommand zcat --outSAMpq Unique 254 --quantMode TranscriptomeSAM --outFilterMultimapNmax 100 --outReadsUnmapped Fastx --chimSegmentMin1 --outSAMtype BAM SortedByCoordinate --outFilterMultimapNmax 100 --quantMode TranscriptomeSAM --outFilterMultimapNmax 100 --outReadsUnmapped Fastx --chimSegmentMin1 --outSAMtype BAM SortedByCoordinate --outWigType bedGraph --alignIntronMax 11000). The annotations obtained from the P. trichocarpa v3.0 GFF file were flattened to generate ‘synthetic’ gene models. This synthetic transcript GFF file and the STAR read alignments were used as input to the HTSeq (Anders et al., 2015) htsseq-count python utility to calculate exon-based read count values. The htsseq-count utility takes only uniquely mapping reads into account.

Differential gene expression analysis

Statistical analysis of single-gene differential expression between conditions was performed in R (v3.4.0; Team, 2018) using the Bioconductor (v3.5; Gentleman et al., 2004) DESeq2 package (v1.16.1; Love et al., 2014). FDR-adjusted P values were used to assess significance; a common threshold of 1% was used throughout. For the data quality assessment and visualization, the read counts were normalized using a variance stabilising transformation (vst) as implemented in DESeq2. The biological relevance of the data, such as similarity of biological replicates (Supplementary Fig.
The gene list encoding *P. trichocarpa* transcription factors was downloaded from the plant transcription factor database v4.0 (http://planttfdb.gaelab.org/).

Dendrograms and heat maps were generated using the function heatmap.2 from the gplots R library. Heat maps of differentially expressed genes (DEGs, DE cut-offs of FDR ≤0.01 and [LFC] ≥ 0.5), were generated using the function heatmap.2 from the gplots R library. The 17 997 genes, which were detected in all biological replicates, were used for further analysis. Genes which were expressed only in one or two biological replicates for each genotype, but which were significant for differential expression between T89 and OP42, were analysed separately. The gene expression mean values are listed in Supplementary Dataset S3 (sheet 6).

**Gene Ontology analysis**

The REVIGO web server (http://revigo.irb.hr/) was used to summarize Gene Ontology (GO) terms from differentially expressed genes (Supek et al., 2011). The GO terms with a false discovery rate (FDR; e-value corrected for list size) of ≤0.05 were submitted to the REVIGO tool, and the ‘small allowed similarity’ setting was selected to obtain a compact output of enriched GO terms. The overall significance of enriched processes was expressed as the sum of 100 × –log10(FDR) for each enriched GO term counted within that process. This technique was adapted from the method used to visualise enriched GO terms as a percentage of the total enriched terms in the TreeMap function of the REVIGO web server.

**Identification of poplar homologues of Arabidopsis ARFs and MYC transcription factors**

To identify poplar homologues of Arabidopsis ARFs, the complete amino acid sequences from Arabidopsis *AtARF6* (AT1G30330), *AtARF8* (AT5G37020) and *AtARF17* (AT1G77850), were used in BLAST searches of the *Populus trichocarpa* proteome (https://phytozone.jgi.doe.gov/pz/portal.html) and popgenie (https://popgenie.org/). Full-length amino acid sequences of the selected poplar and Arabidopsis ARFs were subjected to phylogenetic analysis using MEGA8.0 software. The phylogenetic analysis was performed with the MEGA8.0 software using the Neighbor-Joining method on the p-distance model with 1000 iterations. The most closely related orthologues were chosen for the study (Supplementary Fig. S4A). We used poplar *ARF* gene names according to the nomenclature in PopGenIE. Corresponding Arabidopsis names are as follows: *PnARF6.1*, *PnARF6.2*, *PnARF6.3*, *PnARF6.4*; *PnARF8.1*, *PnARF8.2*, *PnARF8.3*, *PnARF8.4*; *PnARF17.1*, *PnARF17.2*, *PnARF17.3*. Similarly, the poplar homologues of Arabidopsis *AtMYC2* were analysed; their corresponding gene names are as follows: *PnMYC2.1*, *PnMYC2.2*, *PnMYC2.3*, *PnMYC2.4*, *PnMYC2.5*, *PnMYC2.6*. The *ARF* and *MYC*€™s paralogues together (*PnARF6.1/2*, *PnARF6.3/4*, *PnARF8.1/2*, and *PnARF17.1/2*) were further quantified by qPCR. Two independent RNAi lines for each construct were selected and analysed for their adventitious rooting ability.

**Quantitative real-time PCR analysis**

To determine overexpression or down-regulation of the selected genes in the transgenic lines, five 5 mm stem pieces were taken at the base of cuttings from T89 (three biological replicates) and transgenic lines (three biological replicates for each line) at the time of adventitious rooting assay, and pooled. Each biological replicate was formed by a pool of stem pieces collected from three different plants. Total RNA was extracted using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich). A total 10 μg of RNA samples was treated with TURBO DNA-free Kit (Ambion) to remove contaminating DNA from RNA preparations, and to remove the DNase from the samples. cDNA was synthesized using SuperScript® III Reverse Transcriptase Kit (Invitrogen) following the DNase and reverse transcription analysis. Genes which were detected in all biological replicates were used for further analysis. Genes which were expressed only in one or two biological replicates for each genotype, but which were significant for differential expression between T89 and OP42, were analysed separately. The gene expression mean values are listed in Supplementary Dataset S3 (sheet 6).

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To amplify the candidate genes, cDNA was synthesized (SuperScript II Reverse Transcriptase, Thermo Fisher Scientific, USA) starting from total RNA extracted from hybrid aspen T89 (*P. tremula × P. tremuloides*) leaves using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA) followed by DNase treatment (TURBO DNA-free Kit, Ambion). As it is not possible to distinguish the *P. tremula* sequence from that of *P. tremuloides*, the genes are referred to as *PnARF6.4*, *PnARF8.2*, *PnARF17.2*, and *PnMYC2.1*, and the corresponding primers used for amplification of the coding sequences are listed in Supplementary Table S1.

The amplified cDNAs of *PnARF6.4*, *PnARF8.2*, and *PnMYC2.1* were cloned independently into the pENTR/D-TOPO donor vector (Thermo Fisher Scientific, USA) and transferred into the pK2GW7 plant transformation vector (Gateway Vectors, VIB-Ugent Center for Plant Systems Biology, Belgium). *PnARF6.4* and *PnARF8.2* coding sequences were also cloned into the pK2GFW57 vector (Gateway Vectors, VIB-Ugent Center for Plant Systems Biology, Belgium) in which the CaMV35S promoter had been replaced by a 2 kb promoter fragment from the *PtHb3a* gene for specific expression in the cambium (Schnader et al., 2004). To down-regulate the *ARF* genes, we generated RNAi constructs with 578 bp, 624 bp, and 480 bp fragments from *PnARF6.4*, *PnARF8.2*, and *PnARF17.2*, respectively. These fragments were amplified using primers listed in Supplementary Table S1 and T89 cDNA as a template. Due to high coding nucleotide sequence similarity, RNAi constructs targeting both *PnARF6.3* and *PnARF6.4* paralogues, *PnARF8.1* and *PnARF8.2* paralogues, or *PnARF17.1* and *PnARF17.2* paralogues were generated. The amplified fragments were cloned into pENTR/D-TOPO (Thermo Fisher Scientific, USA) and then transferred into the plant transformation vector pK7GWIWG2.

All the different constructs were transformed independently into *Agrobacterium tumefaciens* GV3101 pmp90RK, which was used to transform the hybrid aspen T89. In total, 14 independently transformed lines for each construct were generated. The relative expression of *PnARF6.1/2*, *PnARF6.3/4*, *PnARF17.1/2*, and *PnARF17.1/2* in the respective transgenic lines were further quantified by qPCR. Two independent RNAi lines for each construct were selected and analysed for their adventitious rooting ability.

**Statistical analysis**

Statistical analysis was performed using the GraphPad Prism version 9.0 for Mac (www.graphpad.com). Unless specified one-way ANOVA followed by Tukey’s multiple comparison post-hoc were used to compare means.
Results

Hybrid aspen and hybrid poplar show different patterns of adventitious root formation

To understand why some genotypes readily develop AR and others do not, we compared the rooting efficiency of cuttings from the poplar clone OP42 (P. trichocarpa × P. maximowiczii) and the hybrid aspen clone T89 (P. tremula × P. tremuloides) from juvenile plants kept in vitro (Fig. 1; Supplementary Fig. S1A, B, D) and from stem cuttings of 3-month-old plants grown in the greenhouse (Fig. 2; Supplementary Fig. S1C, E). When cuttings were taken from juvenile in vitro plants, no significant difference was observed between the two clones (Fig. 1A; P<0.05). Nevertheless, in T89 in vitro cuttings, AR developed at the base of the cuttings in a crown-like arrangement (Fig. 1B–E), while in OP42, AR developed a few mm above the base of the cuttings and along the stem (Fig. 1F–I, O, Q). Cross-sections of juvenile in vitro cuttings revealed the presence of bulges on the stems that were visible as early as 3 d after cutting (Fig. 1; Supplementary Fig. S1A, B, D). In contrast, when cuttings were taken from greenhouse-grown 3-month-old plants (Supplementary Fig. S1C) and kept in a hydroponic culture system as described elsewhere (Merret et al., 2010; Rigal et al., 2012; Supplementary Fig. S1E), T89 cuttings were unable to develop ARs (Fig. 2A, B), while 100% of OP42 cuttings did root (Fig. 2A, C). For OP42 cuttings, the first indication that AR primordia were emerging was the presence of bulges on the stems that were visible as early as 3 d after cutting, and AR emerged after around 5 or 6 d (Fig. 2C), and fully developed and formed secondary roots at 13 d after cutting (Fig. 2C). In the case of T89, we never observed any bulge on the surface of the cuttings, and to check if there were any arrested primordia, cross sections were made at different levels in the stem cuttings 6 and 26 d after being cut. No arrested primordia were observed, suggesting that the repression of AR development occurred at very early stages of AR initiation.

In both T89 and OP42, we observed the formation of lenticles; these correspond to cell proliferation regions in the cortex due to the high humidity in hydroponic conditions (Fig. 2D, E).

Transcriptomic profile and functional classification of differentially expressed genes from cambium tissue between OP42 and T89 poplar genotypes

To explain the extreme difference in rooting performance, we performed a transcriptomic analysis of the cambium of OP42 and T89 cuttings 3-month-old plants grown in the greenhouse (Supplementary Fig. S2A). According to Ramirez-Carvajal et al. (2009) the highest number of DEGs in stem cuttings of Populus tremula × Populus alba was observed between 6 and 24 h after cutting. Therefore, to target the early events of AR initiation, before the occurrence of primordia, we decided to analyse the cambium transcriptome of OP42 and T89 cuttings 24 h after cutting. We performed LCM (Supplementary Fig. S2D-I) to dissect and collect homogenous and specific cambium tissues from the basal 5 mm of stem cuttings at time T0 (immediately after cutting; Supplementary Fig. S2B) and T1 (24 h after transfer in hydroponic conditions; Supplementary Fig. S2C).

We mapped the RNA-seq reads to the P. trichocarpa reference genome (Supplementary Dataset S1, sheet1) and classified 17 997 genes in the current annotation as being expressed significantly in all biological replicates in both genotypes at times T0 and T1 (Supplementary Dataset S1, sheet 2). These 17 997 genes represent approximately 43% of the annotated genes in the Populus genome (poplar v3 assembly version; Tuskan et al., 2006). Interestingly, there were more DEGs in OP42 after 24 h in hydroponic conditions than in T89 (Fig. 3). In the case of T89, a total of 1198 (6.6% of the 17 997) genes were differentially expressed; 824 were up-regulated in OP42 compared with T89 at T1 (Fig. 3A; Supplementary Dataset S2, sheets 11–13). GO enrichment analysis of DEGs showed a significant enrichment of GO terms related to biological processes, and molecular functions related to carbohydrate catabolism or redox mechanisms, regulation of transcription, response to abiotic stresses, cation binding, nucleic acid binding activity, or electron carrier activity (Supplementary Dataset S3, sheets 4, 5). In contrast, in OP42, a total of 5464 genes (30% of the 17 997 genes) were found to be differentially expressed, among which 3242 were up-regulated, and 2222 down-regulated at time T1 compared with T0 (Fig. 3A; Supplementary Dataset S2, sheets 8–10). Interestingly, among the 3242 DEGs, 2420 (74.6%) were exclusively up-regulated in OP42 at T1 (Fig. 3B), suggesting a specific remodulation of the transcriptome in OP42 during the 24 h timeframe that did not occur in T89. The GO enrichment analysis of these up-regulated DEGs showed a significant enrichment of GO in cellular components, biological processes or molecular functions related to cell metabolism or cell biology, such as transcription regulation, translation and post translation regulation (Supplementary Dataset S3, sheet 4). Similarly, 66% of the 2222 DEGs that were down-regulated in OP42 at T1 compared with T0 were specifically differentially expressed in OP42 (Fig. 3C). In contrast to the up-regulated genes, the GO enrichment analysis showed a significant enrichment of GO in cellular components, biological processes or molecular functions related to abiotic stress responses (Supplementary Dataset S3, sheet 5). When the two genotypes were compared with each other, 25% of the 17 997 genes were differentially expressed between OP42 and T89 at T0 (Fig. 3A; Supplementary Dataset S2) among which, 2007 were up-regulated in T89 compared with OP42 (Fig. 3A) while 2533 were down-regulated (Fig. 3A; Supplementary Dataset S2, sheets 2 to 4). This difference between the two genotypes was reduced to 14% 24 h after
Fig. 1. Pattern of adventitious rooting in hybrid aspen and hybrid poplar in vitro. (A) Average number of adventitious roots (ARs) and percentage of rooted cuttings in T89 and OP42. Fifteen 3 cm long cuttings, starting from the shoot apex, were taken from 4-week-old plantlets, propagated in vitro, and transferred onto half-strength MS medium as shown in Supplementary Fig. S1A, B, D). The emerged ARs were scored starting on day 5 after transfer to fresh medium, until day 15. Data from three independent biological replicates, each of 15 stem cuttings, were pooled and averaged. Error bars indicate standard error. (B to E) Pictures of the base of T89 cuttings taken at day 5, 6, 7 and 8 showing AR emerging primordia (arrows in B, C) and elongating AR (D, E). (F to I) Pictures of the base of OP42 cuttings taken at day 5, 6, 7 and 8 showing AR emerging primordia (arrows in F, G, H) and elongating AR (I). Scale bars in B to I = 2 mm. (J to Q) Cross- (J, L, N, P) and longitudinal (K, M, O, Q) sections show that in both cases the AR primordia develop from cells situated in the cambium/phloem region. Scale bars in J to P = 100 μm CZ = cambial zone; P = Phloem; X = Xylem; ARP = Adventitious root primordium; AR = Adventitious root.
Fig. 2. Adventitious root development in woody stem cuttings under hydroponic conditions. (A) Average number of adventitious roots (AR) and rooting percentage in T89 and OP42. About 20 cm lengths of stem from 3-month-old greenhouse-grown hybrid aspen T89 and OP42 plants were used. The stem cuttings were kept in hydroponic conditions for 5 weeks and the number of ARs was scored every day after cutting (DAC). Data from three biological replicates, each of at least 15 stem cuttings, were pooled and averaged. Error bars indicate standard error. (B) In T89 only lenticels were observed (white arrows). (C) In OP42, bulges of AR primordia were observed 3 DAC, and fully developed into ARs at 13 DAC (black arrows). Lenticels were also observed in OP42 cuttings (white arrows). (D, E) Cross-sections at the level of a lenticel (white arrows) in T89 (D) and OP42 (E). X = xylem; C = cambium; P = phloem. Scale bars are 1.5 cm in B and C panels and 200 µm in D and E.
Molecular basis of differential adventitious rooting competence in poplar genotypes

transfer into hydroponic conditions, with 1156 up-regulated and 1330 down-regulated in T89 compared with OP42 (Fig. 3A; Supplementary Dataset S2, sheets 5 to 7). The genes that were differentially expressed between T89 and OP42 are mostly involved in cellular and chemical homeostasis, photosynthesis, dioxygenase activity and protein synthesis (Supplementary Dataset S3, sheets 4 and 5).

Genes related to cambium or vascular tissues behave similarly in both genotypes

After checking the similarity of the biological replicates of RNA-seq data (Supplementary Fig. S3A, B), we also confirmed the quality and the specificity of the datasets. For this, we selected a list of 40 Arabidopsis genes described as being expressed in the cambium or vascular tissues, and checked the expression of their putative *Populus* orthologues in our data (Supplementary Fig. S3C; Supplementary Dataset S3, sheet 1). All were found to be expressed (and most behaved similarly) in the two genotypes, showing a slight up-regulation or down-regulation in both T89 and OP42 (Supplementary Dataset S3, sheet 1). A few exceptions to this general pattern included Potri.003G111500 (PtrPPNRT1.2), Potri.004G223900 (similar to *AtCLAVATA1*-related gene) and Potri.014G025300 (similar to *AtWOX4b*), which were slightly down-regulated in T89 but up-regulated in OP42 24 h after cutting; additionally, a few genes were up-regulated in T89 compared with OP42 at T0 and T1. They comprise Potri.003G111500 (PtrPPNRT1.2), Potri.001G131800 (similar to Arabidopsis *BREVIS RADIX* gene) and Potri.002G024700 (ARF5), Potri.009G017700, which is similar to *AtLONESOME HIGHWAY*, a bHLH master transcriptional regulator of the initial process of vascular development.

**Genes encoding reactive oxygen species scavenging proteins are mostly up-regulated in OP42 compared with T89**

Reactive oxygen species (ROS) are signalling molecules involved in the response to biotic and abiotic stresses as well as many aspects of plant development, including AR formation, as shown by recent studies (reviewed in Nag *et al.*, 2013; Li *et al.*, 2017; Velada *et al.*, 2018). We therefore searched for genes encoding ROS scavenging proteins among all DEGs in T89 and OP42. We identified 43 DEGs encoding ROS scavenging proteins, 33 of which belong to the GLUTATHIONE S-TRANSFERASE superfamily (GSTs) and 10 to the PEROXIDASE superfamily (Supplementary Dataset S3 sheet 3). Twenty of these genes were up-regulated at T1 compared with T0 in both genotypes, but on average the fold change was higher in OP42 than in T89 (Supplementary Fig. S5; Dataset S3, sheet 3); nine genes were repressed 24 h after cutting in both genotypes. The most striking observation was that 32 out of 43 genes were significantly up-regulated in OP42 compared with T89 at T1, and 21 of those were also up-regulated in OP42 at T0 (Supplementary Dataset S3, sheet 3); only six were up-regulated in T89 compared with OP42 at T0 and T1; four were up-regulated in T89 compared with OP42 at T0, but down-regulated in T89 compared with OP42 at T1; and five were up-regulated in OP42 compared with T89 at T0 - but by contrast, up-regulated in T89 at T1.

**The easy-to-root OP42 shows increased transcriptional activity in the cambium compared with the difficult-to-root T89**

The different stages of AR initiation (ARI) in *Populus* are associated with substantial remodelling of the transcriptome (Ramirez-Carvajal *et al.*, 2009; Rigal *et al.*, 2012). We therefore
focused our analysis on the expression of TFs. From the 58 families of TFs identified in *Populus*, 49 families were represented in the DEG list (Table 1; Supplementary Dataset S2, Dataset S3, sheet 2) and most of the DEGs were observed in OP42 (Table 1). Furthermore, 24 h after cutting, 210 and 209 TFs were up- or down-regulated respectively in OP42, while in T89 there were only 89 up-regulated and 43 down-regulated DEGs (Table 1). The most represented DEGs belong to the *ARF*, *bHLH*, *bZIP*, *C2H2*-, and *C3H*—type zinc-finger family, *ERF*, *LBD*, *MYB*, *MYB-related*, *NAC* and *WRKY* families. Several genes belonging to these TF families have been shown to be involved in the control of adventitious rooting in *Populus* species (reviewed in Legue et al., 2014).

Genes from the *LATERNAL BOUNDARY* (*LBD*) gene family have been shown to be involved in the development of lateral organs in Arabidopsis (reviewed in Matsumura et al., 2009). In particular *AtLBD16*, *AtLBD17*, *AtLBD18* and *AtLBD29* were shown to be involved in lateral root, adventitious root or regeneration processes in Arabidopsis (Okushima et al., 2007; Liu et al., 2018). Interestingly we observed that 10 *PtrLBD* genes were specifically up-regulated at T1 in OP42, among which the putative orthologue of *AtLBD16*, *PtrLBD16* (Potri.002G041200), was up-regulated in OP42 at T1 with a log2 FC of 4.3 (Supplementary Dataset S2, sheet 6). In addition, *PtrLBD11* (Potri.010G217700) was also up-regulated in OP42 at T1 with a log2 FC of 8.5 (Supplementary Dataset S2, sheet 6). *PtrLBD11* is the putative orthologue of *AtLBD11* which was shown to be involved in secondary growth and stem cell maintenance in the cambium during root development (Ye et al., 2021). The expression of other genes involved in the control of vascular differentiation that could contribute to the rooting difference between T89 and OP42 were specifically up- or down-regulated in OP42.

The NAC family of transcription factors is one of the largest plant-specific families of transcriptional regulators involved in various aspects of plant development and responses to biotic and abiotic stresses (reviewed in Olsen et al., 2005). Twenty-four genes from the NAC family were differentially expressed in OP42 at T1 compared with T0 (Table 1; Supplementary Dataset S2, sheet 6). Among the up-regulated genes encoding NAC transcription factors in OP42, Potri.001G080900 (log2 FC of 7.5) and Potri.002G057200 (log2 FC of 9) encode putative orthologues of *AtJUNGBRUNNEN1* (*AtJUB1*/AtNAC042), a transcription factor induced by ROS, and that represses senescence in Arabidopsis (Wu et al., 2012). This up-regulation could be related to the up-regulation of genes encoding ROS scavenging proteins, as described above. Fifteen NAC genes were specifically down-regulated in OP42 (Supplementary Dataset S2, sheet 6). Potri.001G404400 and Potri.017G063300 were down-regulated with a log2 FC of ~3 and ~2, respectively. These two genes encode putative orthologues of the Arabidopsis *VND-INTERACTING2* (*AtVNI2*/AtNAC83) protein which was shown to interact with the AtVASCULAR-RELATED NAC-DOMAIN7 (AtVND7) transcription factor regulating the differentiation of xylem vessels (Yamaguchi et al., 2008) and to repress its activity (Yamaguchi et al., 2010). A third putative orthologue of *AtVNI2* (Potri.003G166500) was in contrast up-regulated with a log2 FC of 2.6 (Supplementary Dataset S2, sheet 6). Potri.001G404400 and Potri.011G121300 encode two other NAC transcription factors involved in vascular development, both of which were also down-regulated in OP42 at T1 (Supplementary Dataset S2, sheet 6). Potri.001G404400 is a putative ortholog of AtNAC-RELATED SEED MORPHOLOGY 1 (*AtNARS1*/AtNAC2) which was shown to be involved in the regulation of asymmetric cell divisions of sieve element precursors in the phloem downstream of AtSHORTROOT (*AtSHR*), a GRAS family TF (Kim et al., 2020). Potri.011G121300 encodes a putative orthologue of AtNAC86 involved in the differentiation of sieve elements (Furuta et al., 2014). Interestingly Potri.007G132000, the orthologue of *AtSHR*, was up-regulated in OP42 at T1 with log2 FC of 3 (Supplementary Dataset S2, sheet 6). In addition, seven *SCARECROW-Like* (*SCL*) genes of unknown function were down-regulated in OP42 at T1 (Supplementary Dataset S2, sheet 6). In Arabidopsis, *AtSHR* together with its closely related member *AtSCARECROW* (*AtSCR*) controls radial patterning during root development (Nakajima et al., 2001). They are also important for the maintenance of the root apical meristem and the quiescent centre (reviewed in Vernoux and Benfey, 2005) as well as the positioning of the stem cell niche (Lucas et al., 2011). In *Pinus radiata* the expression of several *PrSCL* genes was associated with the maturation-related decline of competence to develop adventitious roots (Abarca et al., 2014). In addition, several *SCARECROW-LIKE* (*SCL*) family genes, such as *PrSCL1* from *Pinus radiata* and *CsSCL1* from *Castanea sativa* (Sanchez et al., 2007; Solé et al., 2008; Vielba et al., 2011), *PrSHR* from *Populus radiata* (Solé et al., 2008), and *PtrSCLR* from *Populus trichocarpa* (Rigal et al., 2012), were shown to be induced during the earliest stages of AR formation in cuttings generated in vitro. In OP42, Potri.001G2422000, which is similar to *AtSCL30/SCL14*, an essential gene for the activation of stress induced response (Fode et al., 2008), was up-regulated with log2 FC of 9 (Supplementary Dataset S2, sheet 6). In T89, three *SCL* genes encoding DELLA proteins involved in the gibberellin acid signalling pathway were up-regulated compared with OP42 at T1 (Supplementary Dataset S2, sheet 6). Gibberellic acid has been shown to be a negative regulator of adventitious root development in *Populus* (Mauriat et al., 2014). Whether a difference in the regulation of gibberellic acid signalling pathway explains the rooting difference between OP42 and T89, requires further investigation.

The *APETALAL2/ETHYLENE RESPONSE FACTOR* (*AP2/ERF*) family was the most represented, with 21 and 42 *ERF* genes up-regulated at T1 in T89 and OP42, respectively (Table 1; Supplementary Dataset S3, sheet 2). Twenty of the *ERFs* up-regulated in T89 were also up-regulated in OP42 at 24 h after cutting. Among the 22 genes specifically up-regulated in OP42, we found *PtrERF003* (Potri.018G085700; log2...
### Table 1. Numbers of differentially expressed transcription factors which were either up- or down-regulated in T89 and OP42.

| Families       | T89 T1-vs-T0 | OP42 T1-vs-T0 | T0 T89-vs-OP42 | T0 T89-vs-OP42 |
|----------------|--------------|---------------|----------------|---------------|
|                | Up-regulated | Down-regulated | Up-regulated | Down-regulated |
| AP2            | 0            | 1             | 2             | 2             |
| ARF            | 2            | 3             | 0             | 12            |
| ARR3           | 0            | 0             | 1             | 1             |
| B3             | 0            | 1             | 1             | 4             |
| BBR-BPC        | 0            | 0             | 1             | 0             |
| BES1           | 0            | 0             | 3             | 0             |
| bHLH           | 15           | 2             | 32            | 14            |
| bZIP           | 3            | 1             | 6             | 11            |
| C2H2           | 4            | 2             | 10            | 6             |
| C3H            | 0            | 2             | 3             | 9             |
| CAMTA          | 0            | 0             | 0             | 1             |
| CO-like        | 0            | 1             | 0             | 3             |
| CPP            | 0            | 0             | 0             | 0             |
| DBB            | 1            | 1             | 4             | 0             |
| Dof             | 1            | 3             | 3             | 7             |
| EIL            | 1            | 0             | 0             | 0             |
| ERF            | 21           | 5             | 42            | 12            |
| FAR1           | 0            | 0             | 0             | 0             |
| G2-like        | 1            | 0             | 2             | 7             |
| GATA           | 1            | 1             | 4             | 2             |
| GeBP           | 0            | 0             | 1             | 0             |
| GRAS           | 1            | 1             | 3             | 8             |
| GRF            | 0            | 1             | 1             | 5             |
| HB-other       | 0            | 0             | 0             | 2             |
| HD-ZIP         | 2            | 0             | 6             | 6             |
| HRT-like       | 0            | 0             | 0             | 1             |
| HSF            | 1            | 1             | 5             | 2             |
| LBD            | 3            | 0             | 11            | 2             |
| LSD            | 0            | 0             | 1             | 0             |
| M-type_MADS    | 0            | 0             | 1             | 1             |
| MIKC_MADS      | 2            | 3             | 0             | 5             |
| MYB            | 7            | 2             | 19            | 13            |
| MYB-related    | 1            | 4             | 5             | 16            |
| NAC            | 3            | 1             | 8             | 16            |
| NF-YA          | 0            | 3             | 0             | 9             |
| NF-YB          | 0            | 1             | 0             | 2             |
| NF-YC          | 0            | 0             | 1             | 0             |
| Nin-like       | 0            | 0             | 0             | 1             |
| SBP            | 0            | 3             | 1             | 7             |
| TALE           | 0            | 0             | 1             | 7             |
| RAV            | 1            | 0             | 2             | 0             |
| S1Fa-like      | 0            | 0             | 1             | 0             |
| TCP            | 1            | 0             | 0             | 3             |
| Trihelix       | 5            | 0             | 7             | 2             |
| WOX            | 0            | 0             | 2             | 0             |
| VOZ            | 0            | 0             | 0             | 0             |
| WRKY           | 13           | 0             | 23            | 3             |
| ZF-HD          | 0            | 0             | 2             | 0             |
| YABBY          | 0            | 0             | 0             | 0             |

| Total          | 89           | 43            | 210           | 209           | 115           | 137           | 71            | 49            |
Several *WUSHEL*-Like Homeobox genes, have been shown to positively control AR development in *Populus* species (Li et al., 2018; J. Liu et al., 2014; B. Liu et al., 2014; Xu et al., 2015). More specifically, the *P. tomentosa* *PtrWOX5a* (*Potri.008G065400*) gene (Li et al., 2018), and the *Populus × euramericana* *PeWOX11/12b* (*Potri.013G066900*) and *PeWOX11/12b* (*Potri.019G040800*) genes (Xu et al., 2015) are involved in AR development in poplar; nevertheless, they were not expressed in the cambium cells of OP42 or T89 (Supplementary Dataset S1). In contrast, we found that two paralogues of *PtrWOX13, PtrWOX13a* (*Potri.005G101800*) and *PtrWOX13b* (*Potri.005G252800*) were up-regulated in OP42 at 24 h after cutting and transfer into hydroponic conditions (Supplementary Dataset S3, sheet 2). *PtrWOX13* belongs to an ancient clade of *PtrWOX* genes (B. Liu et al., 2014) and the Arabidopsis *ArRWOX13* and *ArRWOX14* are involved in the regulation of primary and lateral root development in Arabidopsis (Deveaux et al., 2008).

Recently Wei et al. (2020) showed that the *P. ussuriensis* *PtrHox52* gene, which belongs to the HD–Zip sub-family of TFs, positively controls adventitious rooting in *P. ussuriensis*. It acts by inducing nine regulatory hubs, including the jasmonic acid (JA) signalling pathway *PtrMYC2* (MH644082; *Potri.002G176900*), a TF from the *bHLH* family, which has been demonstrated to be a positive regulator of AR development in *P. ussuriensis*. In contrast, in our dataset, we found that *PtrMYC2* (*Potri.014G103000*) was down-regulated in the cambium of the easy-to-root genotype OP42 at T1, i.e. 24 h after cutting and transfer to hydroponic conditions (Supplementary Dataset S3, sheet 2). *PtrHox52* was also up-regulated in the difficult-to-root genotype T89 compared with OP42 at T1 (Supplementary Dataset S3, sheet 2). Accordingly, we observed that *PtrMYC2.5* (*Potri.003G147300*) was up-regulated in the cambium of T89 compared with OP42 at T1. There are six paralogues of *MYC2* in *Populus*. Three of these paralogues - *PtrMYC2.1* (*Potri.003G092200*), *PtrMYC2.2* (*Potri.001G142200*), and *PtrMYC2.4* (*Potri.001G083500*), were up-regulated in both T89 and OP42 at T1, but with a higher fold change in T89, while *PtrMYC2.5* (*Potri.003G147300*) was exclusively up-regulated in T89 at T1, which led to a significant increase in *PtrMYC2* expression in T89 compared with OP42 (Supplementary Dataset S3, sheet 2). The potential up-regulation of JA signalling in T89 was corroborated by a higher fold change in the expression of several JA-inducible *JA ZIM DOMAIN* (*JAZ*) genes 24 h after cutting in T89, compared with OP42. *PtrJAZ6* (*Potri.003G068900*), *PtrJAZ8* (*Potri.011G083900*) and *PtrJAZ10* (*Potri.001G062500*) were up-regulated in T89 compared with OP42 at T1, with a respective log2 FC of 4.25, 5.5, and 4.7 (Supplementary Dataset S2, sheet 6). These results suggest a negative role of JA signalling on AR development, as described in Arabidopsis (Gutierrez et al., 2012; Lakehal et al., 2020a) and contradict the positive role of JA on AR development, as described for *P. ussuriensis* (Wei et al., 2020).

Several genes from the *AUXIN RESPONSE FACTOR* (*ARF*) family have been shown to be involved in AR development in Arabidopsis and *Populus* (Gutierrez et al., 2009, 2012; Cai et al., 2019; Lakehal et al., 2019; Liu et al., 2020). *AtARF6* and *AtARF8* are positive regulators of adventitious root initiation (*AR1*), while *AtARF17* negatively regulates adventitious rooting (Gutierrez et al., 2009). In *Populus*, *PeARF8* also positively regulates AR formation (Cai et al., 2019) but *PeARF17*, in contrast to the Arabidopsis gene, acts as a positive regulator of AR development in the hybrid poplar *P. davidiana × P. bolleana* (Liu et al., 2020). We identified 36 *PtrARF* genes encoding paralogues of 15 out of the 27 Arabidopsis *ARF* orthologues. Although some of them were more significantly down-regulated in OP42 than in T89 24 h after cutting, they mostly behaved in a similar manner in both genotypes (Supplementary Fig. S6; Supplementary Dataset S3, sheet 6). In particular, expression of *PtrARF6.2* (*Potri.002G055000*) and *PtrARF6.3* (*Potri.001G358500*) was up-regulated, while *PtrARF6.1* (*Potri.005G207700*) and *PtrARF6.4* (*Potri.011G091900*) were down-regulated in both T89 and OP42 at T1 compared with T0 (Supplementary Fig. S6; Supplementary Dataset S3, sheet 6). Similarly, both *PtrARF8.1* (*Potri.004G078200*) and *PtrARF8.2* (*Potri.017G141000*) were down-regulated at time T1 compared with T0 in both T89 and OP42. Interestingly, *PtrARF17.1* (*Potri.002G089900*) was significantly less expressed in the cambium of the difficult-to-root T89 than in OP42, at both T0 and T1, which agrees with a potential positive role of *PtrARF17.1* in AR development.

*PtrARF6* and *PttARF8* positively control, while *PttARF17* negatively controls, adventitious rooting in hybrid aspen

To assess the role of *PtrARF6, PttARF8*, and *PtrARF17* in adventitious rooting in *Populus*, we produced transgenic plants that either overexpressed these genes or down-regulated their expression. Using the PopGenIE data base (http://popgenie.org) we identified the *Populus* genes most closely related to the corresponding Arabidopsis genes (Supplementary Fig. S4A), and checked their expression pattern in the cambium and wood-forming region in the PopGenie database (http://aspwood.popgenie.org/aspwood-v3.0/; Sundell et al., 2017). AspWood provides high resolution *in silico* transcript expression profiling of the genes expressed over the phloem, cambium, and other xylem development zones in aspen trees. We observed, *PtrARF6.1/2* 3/4 and *PtrARF8.1/2* to be highly expressed in the phloem/cambium region, while *PtrRF17.1/2*...
exhibited very low expression in the same region (Supplementary Fig. S4B-D).

For the lines overexpressing *PttARF6.4* and *PttARF8.1*, coding sequences were cloned under the control of the 35S promoter of the Cauliflower Mosaic Virus (CaMV) or the promoter of the cambium specific gene *PtrHB3a* (Schrader et al., 2004). For down-regulated lines, RNAi constructs were made to target *PttARF6_3* and 4, *PttARF8.1* and 2, and *PttARF17.1* and 2 paralogues. We had previously shown that in Arabidopsis hypocotyl, *AtARF6, AtARF8* and *AtARF17* regulate the expression of each other at the transcriptional and post-transcriptional level, and that the balance between positive and negative regulators determined the average number of ARs (Gutierrez et al., 2009). As in Arabidopsis, the *Populus* ARFs are regulated by microRNAs (Cai et al., 2019; Liu et al., 2020). We therefore checked the relative transcript amount of the un-cleaved transcript of the three *ARF* types in each transgenic line. A multiple sequence alignment analysis revealed that the coding sequences (CDS) of *PttARF6.1* and *PttARF6.2* paralogues were highly similar, and we were unable to differentiate their expression by qPCR. A similar situation occurred with *PttARF6.3* and *PttARF6.4, PttARF8.1* and *PttARF8.2, PttARF17.1* and *PttARF17.2*. We therefore designed primers to span the microRNA cleaving site and measured the cumulative expression of the two paralogues (designated *PttARF6_1 + 2, PttARF2_3 + 4; and PttARF17_1 + 2*) (Fig. 4; Supplementary Fig. S7A, B).

We confirmed the overexpression of *PttARF6_3 + 4* and *PttARF8_1 + 2* in the overexpression lines (Fig. 4A, B; Supplementary Fig. S7A, B), and the down-regulation of *PttARF6_3 + 4, PttARF8_1 + 2* and *PttARF17_1 + 2* in the RNAi lines (Fig. 4C-E). Interestingly, we observed that, as in Arabidopsis, when the expression of one of the three ARFs was modified, the expression of the others was also affected, establishing a different ratio between potential positive and negative regulators (Fig. 4; Supplementary Fig. S7).

We performed rooting assays to assess the ability of the different transgenic lines to produce AR. When either *PttARF6.4* or *PttARF8.2* was overexpressed in the cambium under the control of the *PtrHB3* promoter, the transgenic lines produced more AR than the control T89 (Fig. 5A, B). Similar results were obtained with *PttARF6.4* overexpressed under the control of the 35S promoter (Supplementary Fig. S7C), but not with p35S*p ttARF8.2* (Supplementary Fig. S7D). The positive effect of *PttARF6* and *PttARF8* was confirmed in the RNAi lines, which produced fewer ARs than the control line T89 (Fig. 5C, D). The role of *PttARF17* was unclear, although it has been described as a positive regulator in the hybrid poplar *P. davidiana × P. bolleana* (Liu et al., 2020). However, our results show that when *PttARF17_1 + 2* are down-regulated, the transgenic lines produce more ARs (Fig. 5E), suggesting that *PttARF17.1* or *PttARF17.2* could be negative regulators. Nevertheless, because *PttARF6_3 + 4* were up-regulated in the *PttARF17* RNAi lines (Fig. 4E), it is difficult to conclude whether the increased AR average number was solely due to the down-regulation of *PttARF17*, the overexpression of *PttARF6_3 + 4*, or to a combination of both.

*PtMYC2.1* is a negative regulator of adventitious root development in hybrid aspen

In Arabidopsis, the *AtARF6, AtARF8*, and *AtARF17* genes have been shown to act upstream of *AtMYC2*, which is a negative regulator of AR development (Gutierrez et al., 2012; Lakehal et al., 2020a). In our present study, five out of the six *PtMYC2* paralogues are shown to be among the DEGs
Ranjan et al. (Fig. 6A; Supplementary Dataset S3, sheet 2). They mostly behaved the same way in both T89 and OP42, but the fold change induction was higher for four of them at T1 in the difficult-to-root genotype T89, and PtMYC2.5 was significantly up-regulated in T89 compared with OP42 at 24 h after cutting (Fig. 6A; Supplementary Dataset S3, sheet 2). These results suggest that PtrMYC2 could be a negative regulator of adventitious rooting in hybrid aspen. To confirm this hypothesis, we produced transgenic hybrid aspen trees overexpressing PttMYC2.1 under the control of the 35S promoter. The overexpression was confirmed in two independent transgenic lines by qPCR (Fig. 6B), and the rooting assays confirmed that overexpressing PttMYC2.1 repressed AR development (Fig. 6C). The up-regulation of the JA signalling pathway in T89 (Fig. 5). PtARF6 and PtARF8 positively control adventitious root (AR) development while PtARF17 is a negative regulator. (A, B) Average number of ARs on cuttings of transgenic plants expressing PtARF6.4 (A) and PtARF8.2 (B) under the cambium specific promoter pPtHB3. Rooting assay was performed as described in Material and Methods. Two independent transgenic lines were compared with the control T89. AR number was scored every day starting at day 5 after cutting (DAC) until 14 DAC. For each line, 15 cuttings were analysed. (C-E) Average number of ARs on cuttings of transgenic plants expressing the p35S:PtARF6.2-RNAi (C), p35S:PtARF8.4-RNAi (D) or p35S:PtARF17.2-RNAi (E) constructs. Two independent transgenic lines were compared with the T89 control. AR number was scored every day starting at day 5 until 14 DAC. For each line 15 cuttings were analysed. Data are means ±SE, n=15, corresponding to two independent lines per construct. A two-way ANOVA followed by Tukey’s multiple comparisons test indicated that the difference between the transgenic lines and the control were significant, except for PttHB3a:ARF6.4 line 779-L-9 for which the difference was significant only from day 8 to 12, and PttARF8-RNAi L-1 for which no significant difference was observed.
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cambium compared with OP42 could contribute to the recalcitrance of stem cuttings from greenhouse-grown plants to produce AR. This led us to compare the behaviour of OP42 and T89 in response to exogenous JA. Rooting assays were performed with in vitro propagated T89 and OP42 plants in the absence or presence of increasing concentrations of JA (Fig. 6C, D). We observed that even though the two genotypes rooted similarly and responded similarly to exogenous auxin (Supplementary Fig. S8) under in vitro conditions, they showed a different response to exogenous JA. The difficult-to-root T89 was more sensitive to exogenously applied JA compared with OP42 (Fig. 6D, E).

Discussion

*Populus* species are among the most economically utilized trees. Their ability to be propagated vegetatively means that novel genotypes can be rapidly multiplied. Nevertheless, tree cloning is often limited by the difficulty of developing ARs from stem cuttings. Adventitious rooting is a complex multifactorial process. Many QTLs have been detected for adventitious rooting–related traits (Ribeiro et al., 2016; Sun et al., 2019; Zhang et al., 2009), highlighting the genetic complexity of this trait. With the emergence of Arabidopsis as a genetic model, many genes and signalling pathways involved in the control of AR development have been identified (Sorin et al., 2005; Gutierrez et al., 2009, 2012; B. Liu et al., 2014; Hu and Xu, 2016; Lakehal et al., 2019, 2020a, b), and lately, several groups have focused on AR development in *Populus* and identified genes and gene networks involved in this process (Ramirez-Carvajal et al., 2009; Trupiano et al., 2013; Legue et al., 2014; Xu et al., 2015; Yordanov et al., 2017; Li et al., 2018; Cai et al., 2019; Wei et al., 2020; Yue et al., 2020; Zhang et al., 2020; Xu et al., 2021). Nevertheless, most research has so far focused on successive AR development stages in a given genotype; there have been no comparisons between easy-to-root and difficult-to-root genotypes.

To understand the underlying causes of poor-rooting and good-rooting in different genotypes, we compared the hybrid poplar clone OP42, which is easily propagated from dormant stem cuttings, and the hybrid aspen clone T89, which is unable to develop ARs under the same conditions.

Previous research has revealed that, predictably, ARs form from specific founder cells in poplar stem cuttings, but that the process is highly dependent upon induction treatment and age of the cutting (Rigal et al., 2012). Cambium cells have also been shown to be competent initiators of ARs in *Eucalyptus* or *Populus* (Chiatante et al., 2010; Chao et al., 2019). Transcriptomic profiling of vascular tissues including the cambium region in *Populus* have been reported in several studies (Schrader et al., 2004; de Almeida et al., 2015; Kim et al., 2019), but little attention has been given to gene expression in *Populus* cambial cells during AR development. Rigal et al. (2012) showed that changes in the transcriptome occur in the cambium during the early stages of AR development in *Populus*. In our present study we performed a global comparative transcriptomic analysis of the cambium of cuttings taken from OP42 and T89 clones.

Interestingly, the juvenile plants from the two clones rooted similarly when grown in vitro (Fig. 1). In both cases the ARs originate from the cambium region (Fig. 1). But the hybrid aspen T89, unlike the hybrid poplar OP42, was unable to develop roots from 3-month-old plants grown in the greenhouse (Fig. 2). Aging is a well-known limiting factor for AR development (reviewed in Diaz-Sala et al., 2002; Bellini et al., 2014; Aumond et al., 2017) and this could be one explanation to the different behaviours observed between plants grown in vitro and those grown in the greenhouse for 3 months.

Interestingly, among the differentially expressed TFs, we found that the *P. trichocarpa* PtHox52 gene (Potri.014G103000) was down-regulated in the cambium of the easy-to-root genotype OP42, and up-regulated in the difficult-to-root genotype T89, compared with OP42 at T1. This is surprising, since the *P. ussuriensis* PuHox52 gene product has been described as a positive regulator of adventitious rooting in *P. ussuriensis* (Wei et al., 2020). It was shown to induce nine regulatory hubs, including the JA signalling pathway driven by the *PuMYC2* gene (MH644082; Potri.002G176900), which was confirmed to be a positive regulator of AR development in *P. ussuriensis*. In contrast, JA signalling appears to be up-regulated in the cambium of the difficult-to-root T89 genotype compared with OP42, and we confirmed that *PMYC2* t negatively controls AR development in the hybrid aspen T89 (Fig. 6), as we had previously shown in Arabidopsis (Gutierrez et al., 2012; Lakehal et al., 2020a). These are intriguing results, but the role of JA in the control of AR development is still unclear, and seems to be context- and species-dependent (Lakehal et al., 2020b). It will be interesting in the future to study whether *Populus* MYC2 paralogues have acquired different functions depending on the species, growth and vegetative propagation conditions. Although T89 and OP42 clones rooted similarly in vitro, T89 was more sensitive to exogenously applied JA (Fig. 6). This result suggests that the higher up-regulation of the JA pathway in the cambium of T89 24 h after cutting could contribute to repress adventitious root initiation.

Interestingly, the orthologues of the three Arabidopsis *ARF* genes that were shown to be either positive (*AtARF6, AtARF8*) or negative (*AtARF17*) regulators of ARI in Arabidopsis (Gutierrez et al., 2009, 2012; Lakehal et al., 2019) behaved similarly in both T89 and OP42 (Fig. S6). An exception is *PttARF17.1*, which was significantly less expressed in the cambium of the difficult-to-root T89 compared with OP42 at both time points T0 and T1. This result agrees with a potential positive role of *PttARF17.1* in ARI, as described for *PeARF17* in the hybrid poplar *P. davidiana* × *P. bollcana* (Liu et al., 2020). Nevertheless, down-regulation of *PttARF17.1* and *PttARF17.2* expression in T89 induced ARI (Fig. 5E), suggesting a negative role for
As in Arabidopsis (Gutierrez et al., 2009), when the expression of one of the three PttARFs was perturbed, the expression of the others was modified (Fig. 4). In this study, when down-regulation of PttARF17 occurred, PttARF6 paralogues were up-regulated, which probably contributed to increase ARI (Fig. 4E). As for MYC2 genes, it is possible that different paralogues of ARF17 have different functions, depending on the species or the context. We also observed that, as in Arabidopsis (Gutierrez et al., 2009), PttARF6, PttARF8 and PttARF17 are likely to regulate the expression of one another at the transcriptional and post-transcriptional level through the microRNA pathway, suggesting that at least part of the regulatory mechanisms is conserved.

There were many TFs that were either up- or down-regulated in OP42 at T1 compared with T0, but not in T89, and their further characterization may certainly further advance our understanding of the mechanisms differentiating difficult-to-root from easy-to-root genotypes. In particular, several
genes from the LBD, NAC and GRAS families of TFs, involved in root or vascular development, were found more specifically differentially expressed in OP42. Whether these genes account for the difference between the two genotypes requires additional functional characterization.

Another interesting difference we observed between T89 and OP42 concerns the expression of genes encoding ROS scavenging proteins. We identified 43 of these genes among the DEGs, 33 of which belong to the GST superfamily, and 10 to the PEROXIDASE superfamily. The most striking observation was that 32 were significantly up-regulated in OP42 compared with T89 at T1, and 21 of those were also up-regulated in OP42 at T0 (Supplementary Fig. S5). Recent studies have shown that peroxidase activity positively regulates AR formation in different species (reviewed in Nag et al., 2013; Li et al., 2017; Velada et al., 2018). It is therefore possible that the up-regulation of most of these genes in the cambium of OP42 compared with T89 partially explains the difference in rooting competence.

In conclusion, the comparison of the transcriptomes of the cambium region from two Populus species with opposite adventitious root phenotypes, showed a higher number of DEGs in the easy-to-root genotype compared with the difficult-to-root genotype. In particular, there were three times as many differentially expressed transcription factors in the easy-to-root genotype, several of which are known to be involved in adventitious root development, but many for which the function still needs to be addressed. Further functional characterization will shed light on their role in the differential competence to develop adventitious roots.

Supplementary data
The following supplementary data are available at JXB online.

Fig. S1. Conditions for adventitious rooting assays from in vitro plants and greenhouse-grown plants.

Fig. S2. Workflow for laser capture microdissection (LCMS) of cambium tissues from stem cuttings.

Fig. S3. Quality assessment of the RNAseq data in the different biological replicates.

Fig. S4. Populus Arabidopsis orthologues of ARF6, ARF8 and ARF17 and their expression pattern in wood-forming tissues.

Fig. S5. Heat map showing the average expression of genes encoding ROS scavenging proteins in the cambium of T89 and OP42 genotypes.

Fig. S6. Heat map showing the average expression of PtARF genes in the cambium of T89 and OP42 genotypes.

Fig. S7. Overexpression of PtAF6.4 and PtARF8.2 under the control of the 35S promoter.

Fig. S8. Effect of exogenous auxin on the development of adventitious roots on T89 and OP42 cuttings.

Table S1. Primer list used in the present study.

Dataset S1. RNA-seq raw data:
Dataset S2. Lists of differentially expressed genes.
Dataset S3. Gene Ontology and list of differentially expressed transcription factors.

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Author contributions
AR, IP, SA, VL, and CB conceived and designed the experiments; AR, IP, SA, RS, AK, and FB performed the experiments; RB, VL, and CB supervised the work; SA, FM, AK, RB, and CB acquired funding; AR and CB wrote the manuscript; SA and IP reviewed and edited the manuscript. All authors read, commented and approved the final article for publication.

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Conflict of interest
The authors have no conflicts to declare.

Data availability
The RNA-seq data have been deposited at the European Nucleotide Archive (http://www.ebi.ac.uk/ena/) under the accession number PRJEB21558. RNA-seq data for OP42 and T89 can be accessed with the accession numbers PRJEB21549 and PRJEB21557, respectively.

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