Tree Physiology 38, 877–894
doi:10.1093/treephys/tpx175

Research paper

Localized gene expression changes during adventitious root formation in black walnut (*Juglans nigra* L.)

Micah E. Stevens[1], Keith E. Woeste[2] and Paula M. Pijut[2,3]

[1]Department of Forestry and Natural Resources, Purdue University, Hardwood Tree Improvement and Regeneration Center (HTIRC), 715 West State Street, West Lafayette, IN 47907, USA; [2]USDA Forest Service, Northern Research Station, HTIRC, 715 West State Street, West Lafayette, IN 47907, USA; [3]Corresponding author (ppijut@purdue.edu)

Received May 31, 2017; accepted December 20, 2017; published online January 25, 2018; handling Editor Danielle Way

Cutting propagation plays a large role in the forestry and horticulture industries where superior genotypes need to be clonally multiplied. Integral to this process is the ability of cuttings to form adventitious roots. Recalcitrance to adventitious root development is a serious hurdle for many woody plant propagation systems including black walnut (*Juglans nigra* L.), an economically valuable species. The inability of black walnut to reliably form adventitious roots limits propagation of superior genotypes. Adventitious roots originate from different locations, and root induction is controlled by many environmental and endogenous factors. At the molecular level, however, the regulation of adventitious root formation is still poorly understood. In order to elucidate the transcriptional changes during adventitious root development in black walnut, we used quantitative real-time polymerase chain reaction to measure the expression of nine key genes regulating root formation in other species. Using our previously developed spatially explicit timeline of adventitious root development in black walnut softwood cuttings, we optimized a laser capture microdissection protocol to isolate RNA from cortical, phloem fiber and phloem parenchyma cells throughout adventitious root formation. Laser capture microdissection permitted high-resolution, site-specific analysis of gene expression that differentiated between participatory and non-participatory root progenitor cells. Results indicated mRNA abundance was altered in all nine rooting-related genes in response to auxin treatment in both juvenile and mature cuttings. *SCARECROW LIKE-1* (*SCL*) had the greatest change in expression in juvenile rooting-competent cells at days 16 and 18, with a 24- and 23-fold increase relative to day 0, respectively. Tissues not linked to root organogenesis had little change in *SCL* expression at similar time points. *AUXIN RESPONSE FACTOR* (*ARF*)*6* and *ARF8* as well as *SHORTROOT* expression also increased 2- to 4-fold in rooting-competent tissue. The greatest transcript abundance in rooting-competent cuttings was restricted to root progenitor cells, while recalcitrant cuttings had a diffuse mRNA signal among tissue types.

Keywords: adventitious roots, gene expression, *Juglans nigra*, laser capture microdissection, qRT-PCR.

Introduction

Vegetative propagation is widely used to clonally reproduce plants, and is ideal for quickly producing large numbers of unique genotypes. It circumvents long generation times and variable seed production while maintaining genetic diversity, accelerating breeding programs, and conserving endangered or threatened species (Macdonald 1986, Timmis et al. 1987, Merkle et al. 2007, Hartmann et al. 2011). Cutting propagation relies on the ability of cuttings to form adventitious roots, which can be defined as roots that develop on any aerial plant tissue. Adventitious root development proceeds in stages that require cells, typically in proximity to the vascular cambium, to change their cell fate and to reorganize into new meristems (Jackson 1986, Hartmann et al. 2011). Adventitious roots may form naturally, such as brace- or crown-roots in corn (*Zea mays* L.), or through wounding, nutrient deficiency or flooding (Steffens and Rasmussen 2016), with each type presenting a unique developmental physiology. The ability to form adventitious roots is not

universal, but instead is highly species- and genotype-specific, and adventitious root development has been directly linked to the ontogenetic age of the cutting (Diaz-Sala et al. 1996, Greenwood et al. 2001, Vidal et al. 2003, Pijut et al. 2011). Adventitious root development recalcitrance is a significant impediment to vegetative propagation.

Black walnut (*Juglans nigra* L.) is a fine hardwood tree that grows throughout central and eastern forest regions of the United States. Black walnut is highly prized for its valuable timber. Its unique wood properties make it ideal for cabinetry, veneer, furniture and other high-end wood products that are traded in both regional and global markets (Cassens 2004, Michler et al. 2007). As such, black walnut has long been grown commercially for timber production. Genetic improvement programs have also successfully selected individuals with improved marketable traits (Beineke 1983, Victory et al. 2004). It became quickly recognized, however, that routine propagation of superior black walnut genotypes was extremely difficult, and success was often variable (Coggeshall and Beineke 1997). Despite their economic value, vegetative propagation methods such as rooted cuttings, grafting and tissue culture have been suboptimal for black walnut. Rooted cuttings and tissue culture propagation can be superior to grafting, but their use is seriously hindered by recalcitrance to adventitious root development (Beineke 1984, Coggeshall and Beineke 1997, Hasey et al. 2001, Lopez 2001). Using a fog chamber and a 93.2 mM indole-3-butyric acid-potassium salt (K-IBA) induction treatment, softwood cuttings were rooted at 72%, which allowed for the development of a spatially explicit timeline of adventitious root formation in black walnut (Stevens and Pijut 2017). This timeline provides valuable context for a more precise evaluation of the changes in gene expression of adventitious root progenitor cells.

Initiating and regulating adventitious root development involves many endogenous and environmental factors; unfortunately, how these mechanisms interact, promote or inhibit adventitious roots is not well understood (da Costa et al. 2013, Guan et al. 2015, Christiaens et al. 2016). Central to the stimulation of adventitious root development in most species has been the application of exogenous auxin, which acts in concert with many other phytohormones in complex regulatory networks to control root initiation (Hartmann et al. 2011, Păcurar et al. 2014). In *Arabidopsis thaliana* (L.) Heynh, Gutierrez et al. (2009) identified *AUXIN RESPONSE FACTOR* (*ARF*)*6* and *ARF8* as promoters of adventitious root development, whereas *ARF17* inhibited root formation. In *Arabidopsis*, auxin regulates adventitious root development through cross-talk with jasmonic acid, light and micro-RNA (miRNA)160 and miRNA167 abundance. In *argonaute1* (*ago1*) knock-out mutants, which rarely form adventitious roots, *ARF17* overexpression led to a perturbation of auxin homeostasis as a result of the down-regulation of *Gretchen Hagan3* (*GH3*) type genes, which regulate free indole-3-acetic acid (IAA) (Sorin et al. 2005, Zhang et al. 2011). *GH3* genes have also been shown to regulate adventitious root development through jasmonic acid homeostasis. *GH3* genes were positively regulated by *ARF6* and *ARF8*, and negatively regulated by *ARF17* (Gutierrez et al. 2012). Decreased expression of *PtaERF003* in a hybrid poplar clone (*Populus tremula* × *P. alba*) and ATP-*binding cassette B19* in *Arabidopsis* led to a reduction in the auxin signal cascade and auxin accumulation in competent tissue, respectively, and these decreases resulted in less adventitious root development (Sukumar et al. 2013, Trupiano et al. 2013). *PINFORMED1* (*PIN1*) and its analogs act as auxin efflux carriers, and coordination among PIN proteins can generate local auxin maxima that are thought to play a central role in adventitious root development (Xu et al. 2005, Kitomi et al. 2008, Guan et al. 2015).

SCARECROW (*SCR*) and *SHORTROOT* (*SHR*), members of the plant-specific GRAS family of transcription factors, are important for early root meristem patterning and maintenance. Knock-out *SCR*/*SHR* mutants display phenotypes characterized by abnormal stele patterning and cytokinin levels. In *Castanea sativa* Mill. (European chestnut) shoots capable of root initiation, *SCL1* expression was localized to the cambial region, while shoots incapable of root formation had a more diffuse pattern of *SCL1* expression (Vielba et al. 2011). *SCL1* expression was shown to be highly auxin inducible with similar expression patterns across diverse tree lineages (Sánchez et al. 2007), demonstrating a conserved genetic function. Also key to adventitious root development, the SCR/SHR complex was shown to activate D-type *CYCLIN* genes in the *Populus* clone 'Nanlin 895' (*Populus deltoides* × *P. euramericana*), an integral step in cellular dedifferentiation (Xu et al. 2016). It has also been demonstrated that adventitious root development can be controlled indirectly through regulation of secondary metabolites. Adventitious root formation in *Juglans regia* (English walnut) microshoots was increased with down-regulation of chalcone synthase (CHS), a key enzyme in flavonoid biosynthesis (El Euch et al. 1998, Cheniany et al. 2012). Increase in expression of genes controlling alternative oxidases and nitrate reductases led to increased ability to form adventitious roots (Abu-Abied et al. 2012, Santos Macedo et al. 2012).

While these studies, usually in model species, have shown the influence that one or two genes can have on adventitious root development individually; it is clear that root induction, primordia development and meristem organization relies on a functionally complex network of molecular regulation. The aim of this study was to determine how spatial and temporal gene expression patterns influence adventitious root development in black walnut cuttings. To better understand adventitious root regulation, we compared expression profiles for nine genes during the early stages of adventitious root development. Days for sampling were selected based upon our previous research that showed root initials were present by day 16 after induction treatment, and root primordia developed by day 18 (Stevens and Pijut 2017). By using laser capture microdissection

we were able to refine our understanding of the importance of spatial regulation of gene expression in producing and maintaining competency to form roots. Although not widely applied to woody plant tissues, laser capture microdissection allows for precise targeting and excision of individual cells or groups for gene expression assays (Emmert-Buck et al. 1996). Laser capture microdissection allows RNA to be isolated only from cells anatomically shown to give rise to adventitious roots, while eliminating background noise from non-progenitor cells. Stevens and Pijut (2017) found that adventitious roots in black walnut cuttings originated solely from phloem parenchyma cells within gaps of phloem fibers. When gaps were not present, as in the case of mature cuttings, roots failed to form. While gene expression is influenced by factors such as RNA stability, degradation and translation rate, here we use it to mean complementary DNA (cDNA) abundance at the time of sampling. Our objective was to measure spatial and temporal expression changes with a diverse candidate gene panel in easy-to-root juvenile cuttings and recalcitrant hard-to-root mature cuttings during adventitious root development. Water treatment typically resulted in differences in timing of onset, range and amount of expression of genes, but no differences in tissue specificity or duration of expression in juvenile (competent) shoots compared with auxin-treated shoots. We propose to test a model where adventitious root development protagonist genes are preferentially expressed in adventitious root progenitor cells only in competent juvenile cuttings 16–18 days after auxin treatment, but not following control treatments (Figure 1).

Materials and methods

Plant material

Softwood cuttings (mature) were collected from 1- to 3-year-old grafted black walnut stock plants grown in the greenhouse. Scion wood was originally collected from the canopy of mature, fruit bearing, elite black walnut genotypes at the Martell Forest (40.4322°N, 87.0389°W, West Lafayette, IN, USA) in March 2012 and 2014, and grafted onto wild-type seedling rootstocks. Successfully grafted plants were overwintered 1 year in cold storage (3–4 °C) prior to use. Softwood cuttings (juvenile) were taken from 5- to 8-week-old seedlings (half-sibs) of the same elite genotypes. Seeds were collected at the Martell Forest from mature black walnut trees, stratified, germinated and grown in seedling trays (Polyflat 40 cm × 40 cm × 12.7 cm deep; Anderson Die and Mfg. Co., Portland, OR, USA) in a potting mix (1:1:1 (v/v/v), peat moss:perlite:vermiculite) under ambient greenhouse conditions (22 ± 2 °C).

Tissue collection

Softwood cuttings (15–20 cm in length) were collected from new growth of mature and juvenile walnut genotypes. Leaflets were trimmed to reduce total leaf area by 25–50%. The basal 5 cm of cuttings were dipped for 60 s in 93.2 mM indole-3-butyric acid-potassium salt (K-IBA) dissolved in deionized water or deionized water (control), and then inserted into a moist medium of 3 perlite:1 coarse vermiculite (v/v). Cuttings were placed in a bench-top fog chamber for the duration of root formation under ambient light conditions (1000–1200 μmol m^{-2} s^{-1}). This root induction was found to be optimal for black walnut cuttings, when after 5 weeks after root induction treatment 72% of juvenile cuttings had rooted vs 0% of mature cuttings (Stevens and Pijut 2017). To understand spatial and temporal gene expression changes during adventitious root development, stem segments (3 cm) were collected on day 0, 8, 16 and 18, and day 26 (for mature cuttings only in case adventitious root development was delayed), partitioned into 0.5 cm segments, and immediately frozen in liquid nitrogen and stored at −80 °C. Through previous histological analysis, key stages of root development were observed on these days. Three biological replicates were taken for each day.

RNA isolation and cDNA synthesis from whole tissue samples

RNA was extracted from the basal 5 cm of actively growing roots and from fully expanded leaves, to act as positive and negative controls, respectively. Tissue was collected, immediately frozen in liquid nitrogen, and homogenized using an RNase-free mortar and pestle. Total RNA was isolated following the recommended protocol for the Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA; No. 74903), quantified and purity checked using a NanoDrop™ 2000c (Thermo Fisher Scientific, Waltham, MA,

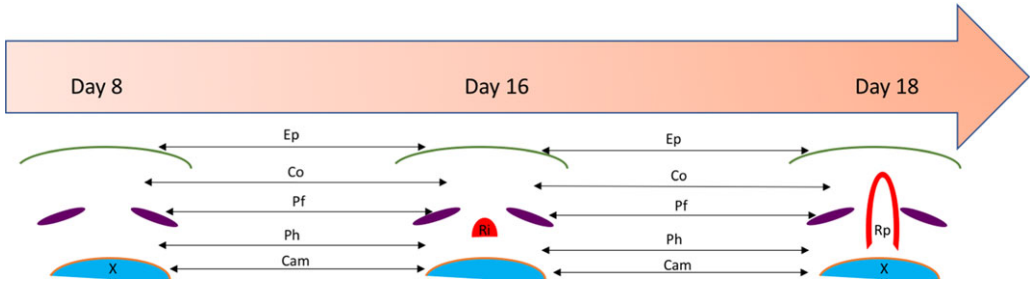

Figure 1. Spatial representation of adventitious root formation in juvenile black walnut (*Juglans nigra*) cuttings. Sixteen days after 93.2 mM indole-3-butyric acid-potassium salt treatment, adventitious roots began to form from phloem parenchyma cells between phloem fiber bundles only. Cam, cambium; Co, cortex; Ep, epidermis; Ph, phloem; Pf, phloem fibers; Ri, root initial; Rp, root primordia; X, xylem.

USA), and quality checked by agarose gel electrophoresis. RNA was then treated with DNase (Qiagen; No. 79254), and 2–2.5 μg RNA was used as a template for reverse transcription to generate first-strand cDNA using the SuperScript® VILO™ Master Mix (Thermo Fisher Scientific; No. 11755050). cDNA from roots and leaves was then used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

Laser capture microdissection of black walnut stems

Flash-frozen, fresh black walnut stem segments were sectioned (10 μm) using a cryostat rotary microtome (Leica, Buffalo Grove, IL, USA; Model 1950), and then sections were fixed to RNase-free PEN membrane microscope slides (Leica; No. 11505189) with a graduated ethanol series up to 100%, at which point they came to ambient temperature. Microdissection was performed using an LMD6000 machine (Leica) using the ×10 objective. The operating laser parameters were 45 mW power, aperture 1 and speed 7 ms to isolate solely cortex, phloem fiber or phloem parenchyma cells from auxin- or control-treated stems for all collection days for mature and juvenile cuttings. Microdissected cells fell directly into the cap of a 0.5 ml RNase-free PCR tube cap filled with 50 μl extraction buffer (Thermo Fisher Scientific; PicoPure® RNA Isolation Kit, No. KIT0204) beneath the microscope stage. Approximately 250 cells were collected per replicate, and each replicate was collected in an individual tube. Tubes were then centrifuged at 5000*g* for 30 s, and immediately frozen and stored at −80 °C until RNA isolation.

RNA isolation and cDNA synthesis from microdissected samples

RNA was isolated following the recommended instructions for the PicoPure® RNA Isolation Kit and eluted in 15 μl RNase-free water. The cells in the three tubes obtained from laser capture microdissection were pooled after the lysis step, and genomic DNA was removed using an RNase-free DNase Kit (Qiagen; No. 79254). The RNA was then quantified and purity checked using a NanoDrop™ 2000c.

For each sample, 10 μl (1.5–18.6 ng μl^{-1}) total RNA extracted from microdissected cells was processed through one round of RNA amplification using the MessageAmp™ II asRNA Amplification Kit (Thermo Fisher Scientific; No. AM1751). The antisense RNA (asRNA) synthesis reaction was conducted at 37 °C for 14 h. The asRNA was then purified according to the manufacturer's instructions, eluted in 200 μl RNase-free water, and quantified using a NanoDrop™ 2000c. Microdissected asRNA was then concentrated using Ultra Pure™ Glycogen (Thermo Fisher Scientific; No. 10814010) to a final volume of 50 μl. First-strand cDNA was synthesized using the SuperScript® VILO™ Master Mix using 14 μl asRNA (1–1.5 μg) in a final volume of 20 μl. cDNA was then stored at −20 °C.

Primer design

Primer pairs (Table 1) for the qRT-PCR analysis of adventitious rooting genes in black walnut and β-tubulin for expression normalization, were derived from RNA-seq data obtained from The Hardwood Genomics Project (https://hardwoodgenomics.org/).

Table 1. Primer sequences used for quantitative real-time polymerase chain reaction expression analysis.

Gene abbreviation	Gene name	Black walnut gene[1]	Primer sequence[2]
ARF6	*Auxin response factor6*	*Juglans_nigra*_120313_comp34183_c0_seq1	For: ATGCACATCGGACTCCTAGC Rev: CGCATCCCAACTGATACACG
ARF8	*Auxin response factor8*	*Juglans_nigra*_120313_comp35071_c0_seq2	For: ATCCGGGCTTCTACTCCCTA Rev: CACTCCGCAACAACTCAGAA
ARF17	*Auxin response factor17*	*Juglans_nigra*_120313_comp209493_c0_seq1	For: TACATTTCCTCCCGCAAAGA Rev: CATGCCAGCAGGAAAAGAGT
CHS	*Chalcone synthase*	*Juglans_nigra*_120313_comp28055_c0_seq1	For: TTCCTGGGCTCATTTCAAAG Rev: CTTCAGCTCCAGCTTCGACT
CYCD	*Cyclin-D1*	*Juglans_nigra*_120313_comp34401_c1_seq1	For: CCGCATAGTAGGTCGGAGAA Rev: CACACACACGCACAGTTACA
PIN3	*PIN-FORMED3*	*Juglans_nigra*_120313_comp32277_c0_seq4	For: ACGTCACGGTGAGGAAGTCT Rev: CGGCGTTGTTAAAATTCGAT
PIN7	*PIN-FORMED7*	*Juglans_nigra*_120313_comp32276_c0_seq9	For: GCTGAGCTCCACCCAAAATA Rev: AGGGACCAAACGAGACCAAT
SCL	*SCARECROW-like*	*Juglans_nigra*_120313_comp66749_c0_seq1	For: TTCACGAGCTCTTGGTTCAA Rev: ATGGGAGGGAGGCTATGAAT
SHR	*SHORTROOT*	*Juglans_nigra*_120313_comp96649_c0_seq1	For: GAATGGAAAAGTTCGCTAGGC Rev: CAATGGGTGTGATTGAATGC
TUB	*β-tubulin*	*Juglans_nigra*_120313_comp32560_c0_seq2	For: CCGTGGGAAAGGAATTAGGT Rev: GGCGATTTGAACCATCTCAT

[1]Black walnut sequence ID in the RNA-seq database of the Hardwood Genomics project (https://hardwoodgenomics.org/).
[2]Forward (For) and Reverse (Rev) primer pair.

β-tubulin was chosen from a group of references genes based on its detection levels in untreated day 0 black walnut leaf tissue. Candidate genes (Table 2) were run against a custom BLAST database of the RNA-seq data. Black walnut sequences that displayed a high level of similarity were selected; a nucleotide BLAST search was run against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), translated to an amino acid sequence using the ExPASy software (http://web.expasy.org/translate/), and then a protein BLAST search was run against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the black walnut sequence represented our candidate gene of interest. Confirmed sequences were then used to design primers using the Primer3 software v. 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/).

Quantitative real-time PCR expression analysis

iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA; No. 172–5121) was used to perform qRT-PCRs with specific primer pairs designed for the nine genes of interest and one internal β-tubulin control gene (Table 1). The reaction conditions were as follows: 95 °C for 3 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and were performed using a CFD-3200 Opticon™ II Detector (Bio-Rad). Each sample was examined in two technical replicates, and cycle threshold (Ct) values were calculated using Opticon™ Monitor Analysis Software v.1.4. Black walnut β-tubulin was used for expression normalization. The fold difference value was found using the formula: fold difference = $2^{-\Delta\Delta Ct}$. The delta Ct value was derived by subtracting the average Ct value for tubulin from the average Ct of the gene of interest. Three separate calculations were run to analyze fold differences: day 0, matching day control treatment or juvenile matching day as the baseline with a value of one. Two technical replicates of qRT-PCR analysis were performed by ProNovus Bioscience LLC, Mountain View, CA, USA. Statistical analysis was not performed.

Results

Adventitious root formation in juvenile and mature black walnut stem cuttings was investigated using qRT-PCR coupled with laser capture microdissection to measure changes in expression of nine genes believed to contribute to adventitious root development (Table 1). RNA was collected from cortex, phloem fiber, or phloem parenchyma cells within phloem fiber gaps on day 0, 8, 16 and 18, and day 26 for mature cuttings only, after stems were treated with 93.2 mM K-IBA or water (Figure 2). As expected, expression changed with tissue type, physiological age and treatment (Figures 3–7). A spatially explicit timeline in easy-to-root juvenile cuttings and hard-to-root mature cuttings was previously established as to key anatomical changes during root development (Stevens and Pijut 2017). Briefly, root organogenesis only originated in juvenile phloem parenchyma cells within phloem fiber gaps. Root organogenesis began with organized cell division on day 8 after auxin treatment. Adventitious roots were first detected on day 16, and were fully formed on day 18. Cortex and phloem fiber cells did not participate in root development, so expression changes in those tissues were not anticipated in auxin-treated, rooting-competent juvenile cuttings. Expression changes were expected in root progenitor phloem parenchyma tissue. Root-tip and leaf RNA were used as controls to support the role of the nine candidate genes

Table 2. Genes evaluated for their role in adventitious roots (AR) and AR development (ARD) in *Juglans nigra*.

Gene	Trait/function	Species	Role in ARD	Reference
AUXIN RESPONSE FACTOR (*ARF6*, *ARF8* and *ARF17*)	Transcription factors	*Arabidopsis thaliana*	*ARF6* and *ARF8* promoted AR regulated by *ARF17* (*ARF6* only), miRNA167; *ARF6* promoted miRNA167 and miRNA160, *ARF8* and *ARF17*; *ARF17* inhibited AR, regulated by miRNA160, *ARF8* and light	Gutierrez et al. (2009)
Castanea sativa SCARECROW LIKE1 (*CsSCL1*) *Pinus radiata SCRL1* (*PrSCL1*)	Root meristem organization and maintenance (GRAS family of transcription factor)	*Castanea sativa* and *Pinus radiata*	*CsSCL1* transcripts localized to cambial region in rootable shoots, diffuse signal in non-rootable shoots; both *SCL1* genes are auxin inducible in rooting-competent cuttings; early formation of root initials were determined the first 24 h prior to root primordia organization	Sánchez et al. (2007), Vielba et al. (2011)
SHORTROOT (*SHR*)	Auxin induced, radial patterning in root meristem organization	*Arabidopsis thaliana*	Mutant *SHR* plants had abnormal stele patterning; proper *SHR* function was needed to diminish cytokinin levels in xylem, this promoted vascular differentiation independent of *SCR*	Cui et al. (2011)
CHALCONE SYNTHASE (*CHS*)	Key enzyme in flavonoid biosynthesis	*Juglans regia*	Decrease in flavonoids led to an increase in ARD	El Euch et al. (1998), Cheniany et al. (2012)
PIN-FORMED (*PIN3* and *PIN7*)	Auxin efflux	*Oryza sativa*	Generation of local auxin maxima	Xu et al. (2005)
CYCLIN-D	Cell cycle initiation	*Oryza sativa*	Expression preceded ARD	Lorbiecke and Sauter (1999)

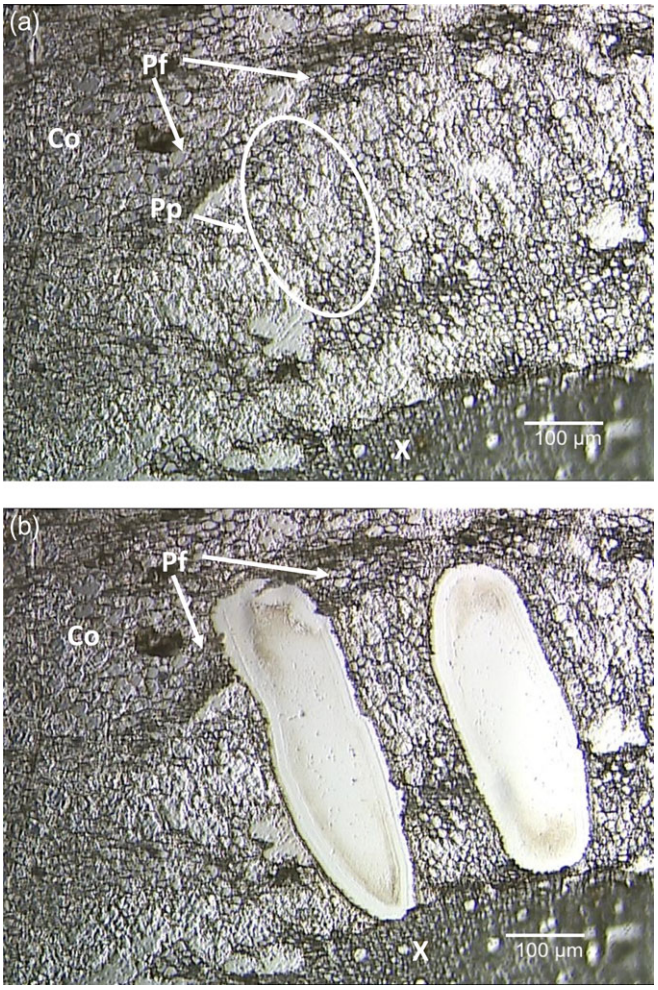

Figure 2. Cross-section (10 µm) of a juvenile black walnut stem (*Juglans nigra*) 16 days after treatment with 93.2 mM indole-3-butyric acid-potassium salt. (a) Cross-section prior to microdissection displaying normal stem anatomy, (b) cross-section after microdissection in which phloem parenchyma cells were removed by laser capture microdissection. Co, cortex; Pf, phloem fibers; Pp, phloem parenchyma; X, xylem. Scale bar 100 µm.

in root development and growth. The candidate genes generally displayed greater differences in expression beginning on day 0 across tissue types and treatments in root-derived RNA than leaf-derived RNA (Figures 8–10).

Gene expression changes of SHR and SCL

SCL and *SHR*, root meristem organization genes, exhibited similar expression patterns during root development. In juvenile cortex and phloem fiber auxin-treated tissue, expression of both genes increased slightly from day 0 levels by day 8, and remained steady through day 18, but expression increased continuously in phloem parenchyma auxin-treated tissue through day 18 (Figure 3a and d). *SHR* and *SCL* expression increased in control (water-treated) shoots on days 8, 16, and 18 compared with day 0, but expression changes were not confined to the parenchyma cells (Figure 3a and d). Mature shoots expressed *SCL* and *SHR* at levels greater than juvenile stems after auxin treatment, but expression changes were not tissue specific (Figure 3a and d). To further elucidate the cell-specificity of expression changes caused by auxin treatment and the molecular controls initiating adventitious root development in black walnut stems, relative expression changes between treatments (auxin or water) on each collection day were analyzed. In juvenile auxin-treated phloem parenchyma cells, by days 16 and 18 there was a significant increase of *SCL*, and a slight increase in *SHR* compared with water-treated cells (Figure 3b and e). Expression of *SCL* and *SHR* changed little in non-rooting-competent cortex and phloem fiber cells of auxin-treated juvenile cuttings vs water-treated cuttings (Figure 3b and e). Tissue-specific expression of *SHR* and *SCL* was not observed in mature cuttings, however. Phloem parenchyma, phloem fiber and cortex cells all showed an increase of *SHR* and *SCL* by day 8 that returned to standard levels by day 26 (Figure 3b and e). Auxin-treated mature stems surprisingly had slightly greater *SHR* and *SCL* expression in phloem parenchyma, phloem fiber and cortex cells compared with juvenile stems on the same days but, unlike juvenile stems expression was not cell-type specific (Figure 3c and f). Mature water-treated stems, however, showed lower expression of *SCL* and *SHR* across all days and sampled tissues compared with juvenile tissue.

Gene expression changes of ARF6, ARF8 and ARF17

We expected expression of the adventitious root promoting genes *ARF6* and *ARF8* to be induced by auxin and to be elevated only in competent phloem parenchyma tissue. We also expected the expression of the adventitious root inhibiting gene *ARF17* to decrease in phloem parenchyma cells after auxin treatment. In auxin-treated stems, *ARF6* and *ARF8* expression increased 1.5- to 3-fold over day 0, beginning day 8 in the phloem parenchyma cells. We observed little to no change in expression in comparable cortex or phloem fiber cells at the same time periods (Figure 4a and d). Expression of *ARF6*, *ARF8* and *ARF17* changed little compared with day 0 (control) levels in cells sampled from mature auxin-treated stems irrespective of tissues or collection day (Figures 4a and d, 5d). In contrast, *ARF17* expression in auxin-treated juvenile stems decreased 50% and 40% in parenchyma cells on days 16 and 18, respectively, but there was little change in the cortex or fiber cells compared with day 0 (Figure 5d).

Tissue type and time after auxin exposure had a clear effect on gene expression of the black walnut rooting-related genes *ARF6*, *ARF8* and *ARF17*. Exogenous auxin application influenced expression changes for both mature and juvenile stems and expression patterns differed among cell types. *ARF6* expression in auxin-treated juvenile stems differed little from the water-treated controls on days 8 and 16, but *ARF6* decreased 2-fold on day 18 in phloem fiber and cortex tissue (Figure 4b). *ARF8* expression after auxin treatment, however, changed little for all collection days except day 16, when it increased 3- to 5-fold

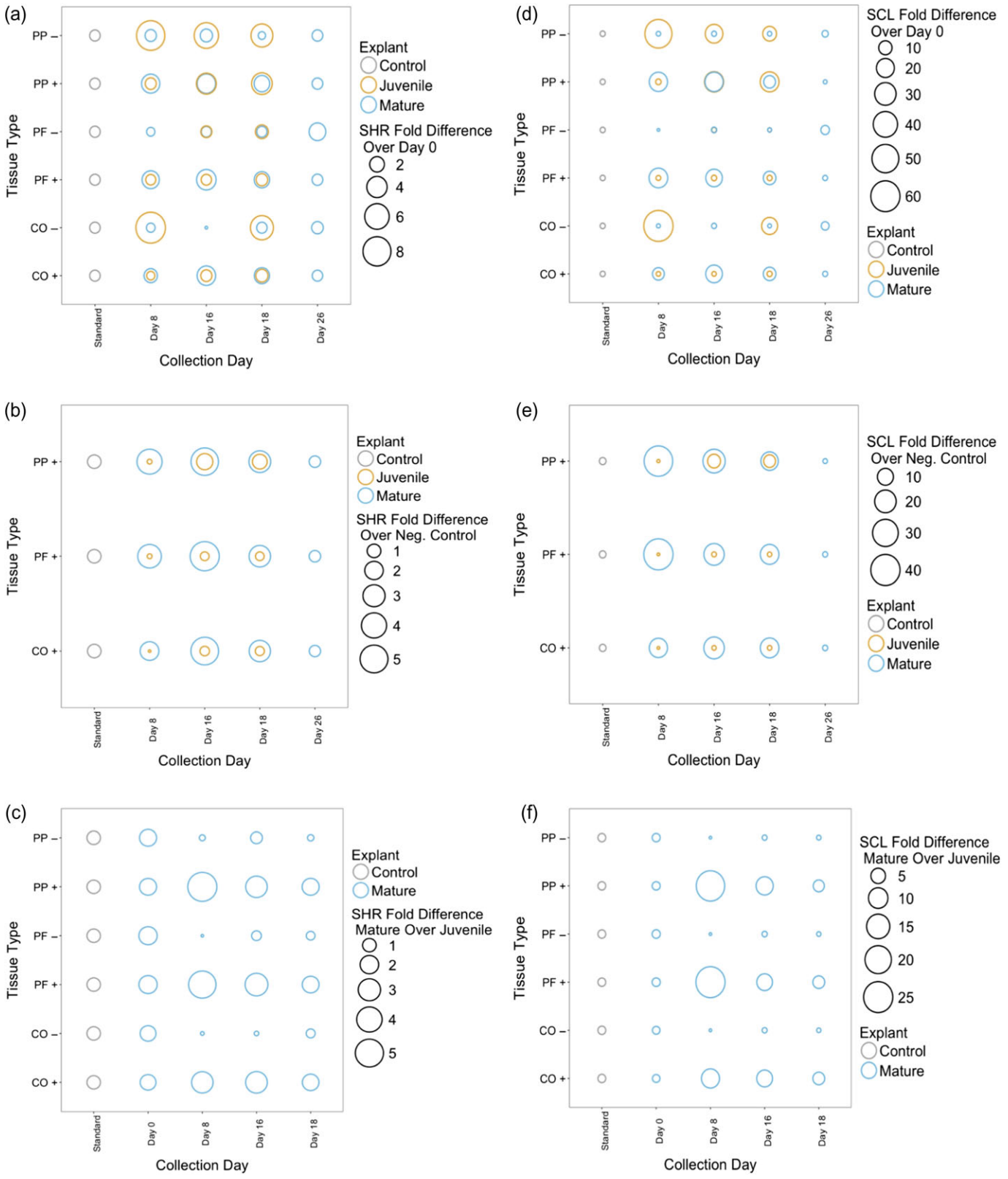

Figure 3. qRT-PCR relative fold differences among sample collection days over day 0 (a, d), among samples treated with 93.2 mM indole-3-butyric acid-potassium salt and water (negative control) (b, c), and between non-rooting mature samples over rooting-competent juvenile samples (c, f) for nine known adventitious rooting-related genes. Change in circle size for both juvenile and mature stems from an internal β-tubulin standard value of one represents a relative decrease or increase in expression for each gene studied. (a–c) *SHORTROOT* (SHR), (d–f) *SCARECROW*-like (SCL), (+) stems treated with 93.2 mM indole-3-butyric acid-potassium salt, (–) stems treated with water (control). PP, phloem parenchyma; PF, phloem fibers; CO, cortex.

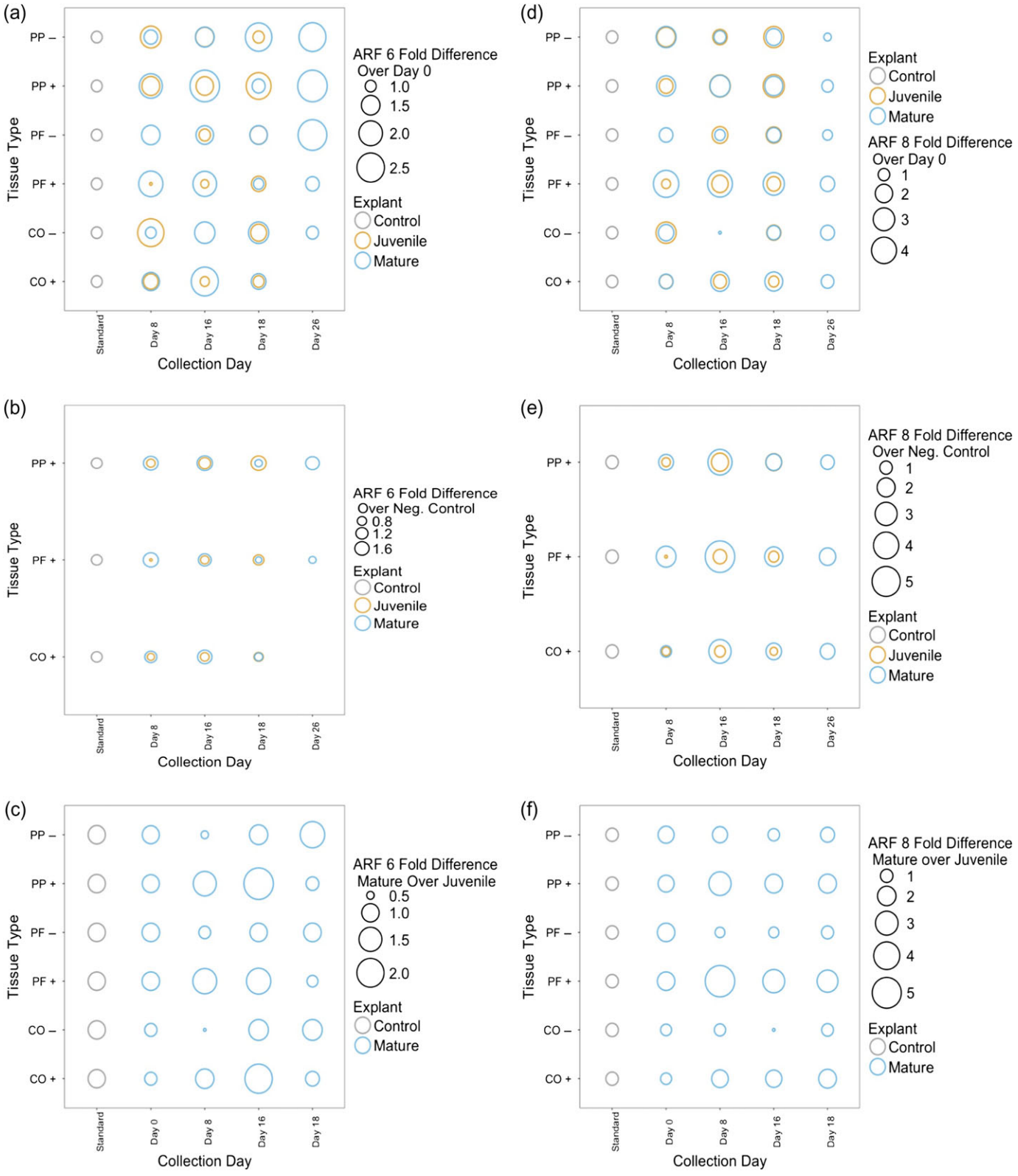

Figure 4. qRT-PCR relative fold differences among sample collection days over day 0 (a, d), among samples treated with 93.2 mM indole-3-butyric acid-potassium salt and water (negative control) (b, c), and between non-rooting mature samples over rooting-competent juvenile samples (c, f) for nine known adventitious rooting-related genes. Change in circle size for both juvenile and mature stems from an internal β-tubulin standard value of one represents a relative decrease or increase in expression for each gene studied. (a–c) *Auxin response factor6* (ARF6), (d–f) *Auxin response factor8* (ARF8), (+) stems treated with 93.2 mM indole-3-butyric acid-potassium salt, (–) stems treated with water (control). PP, phloem parenchyma; PF, phloem fibers; CO, cortex.

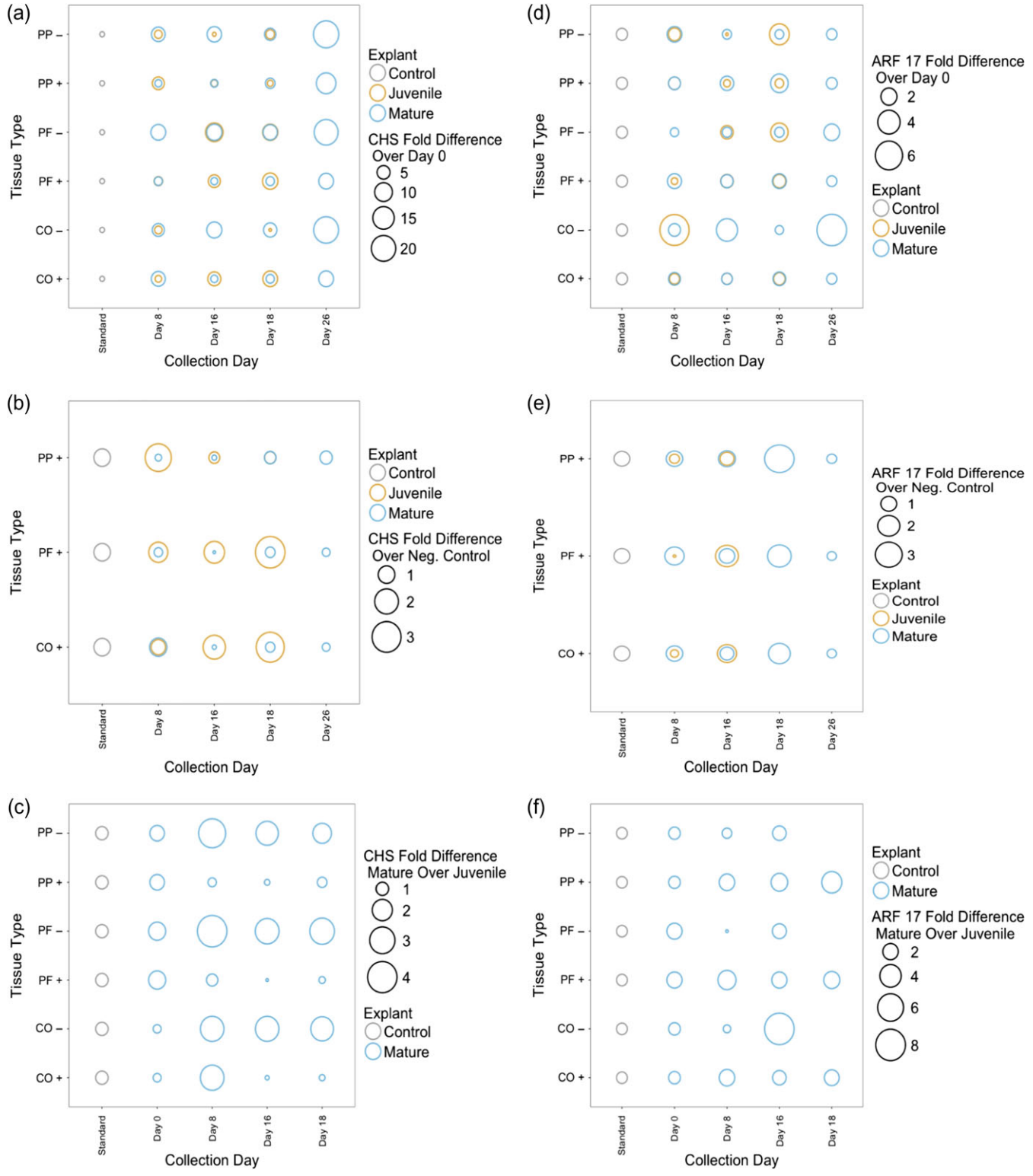

Figure 5. qRT-PCR relative fold differences among sample collection days over day 0 (a, d), among samples treated with 93.2 mM indole-3-butyric acid-potassium salt and water (negative control) (b, c), and between non-rooting mature samples over rooting-competent juvenile samples (c, f) for nine known adventitious rooting-related genes. Change in circle size for both juvenile and mature stems from an internal β-tubulin standard value of one represents a relative decrease or increase in expression for each gene studied. (a–c) *Chalcone synthase* (CHS), (d–f) *Auxin response factor17* (ARF17), (+) stems treated with 93.2 mM indole-3-butyric acid-potassium salt, (–) stems treated with water (control). PP, phloem parenchyma; PF, phloem fibers; CO, cortex.

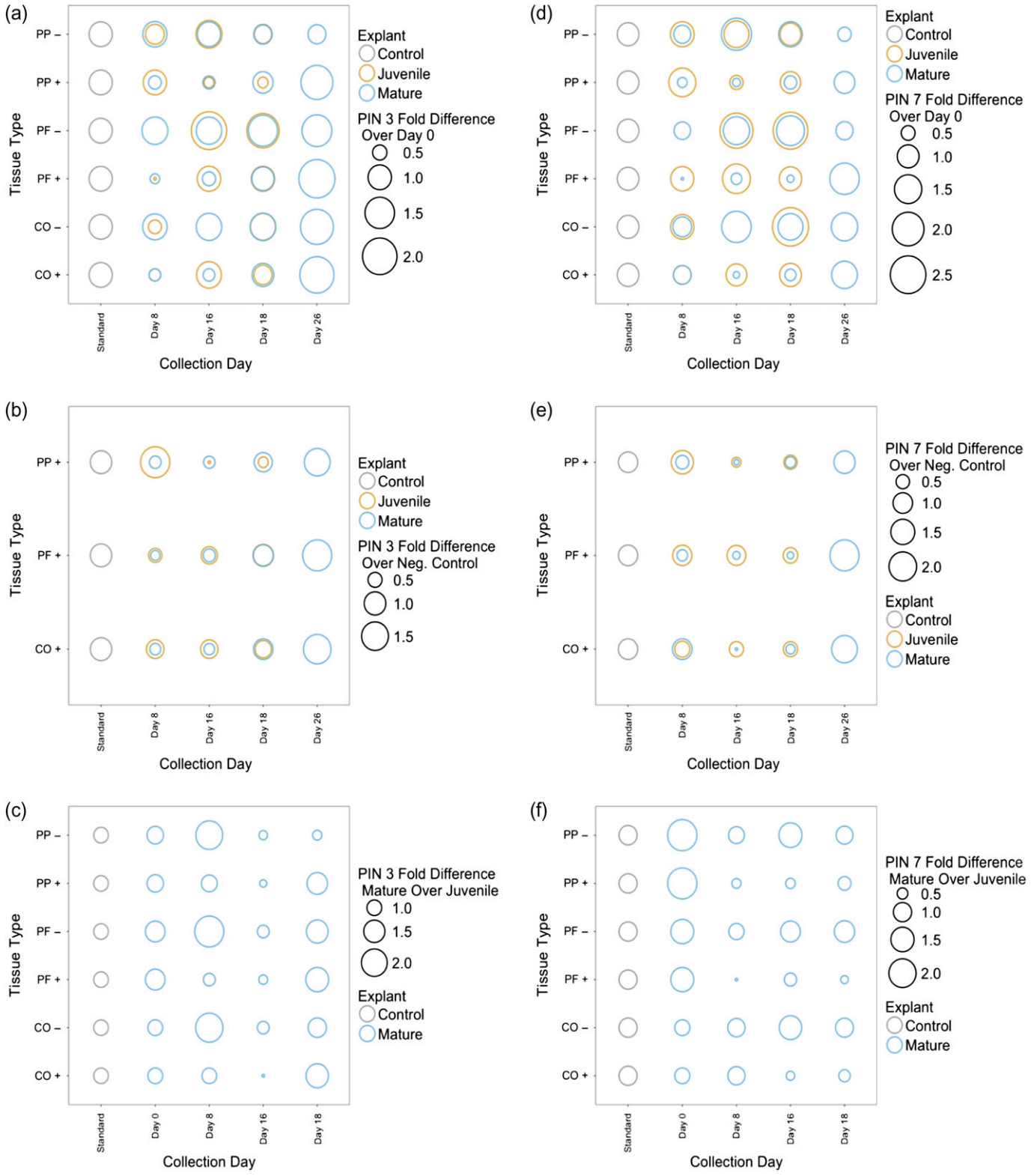

Figure 6. qRT-PCR relative fold differences among sample collection days over day 0 (a, d), among samples treated with 93.2 mM indole-3-butyric acid-potassium salt and water (negative control) (b, c), and between non-rooting mature samples over rooting-competent juvenile samples (c, f) for nine known adventitious rooting-related genes. Change in circle size for both juvenile and mature stems from an internal β-tubulin standard value of one represents a relative decrease or increase in expression for each gene studied. (a–c) *PIN-FORMED3* (PIN3), (d–f) *PIN-FORMED7* (PIN7), (+) stems treated with 93.2 mM indole-3-butyric acid-potassium salt, (–) stems treated with water (control). PP, phloem parenchyma; PF, phloem fibers; CO, cortex.

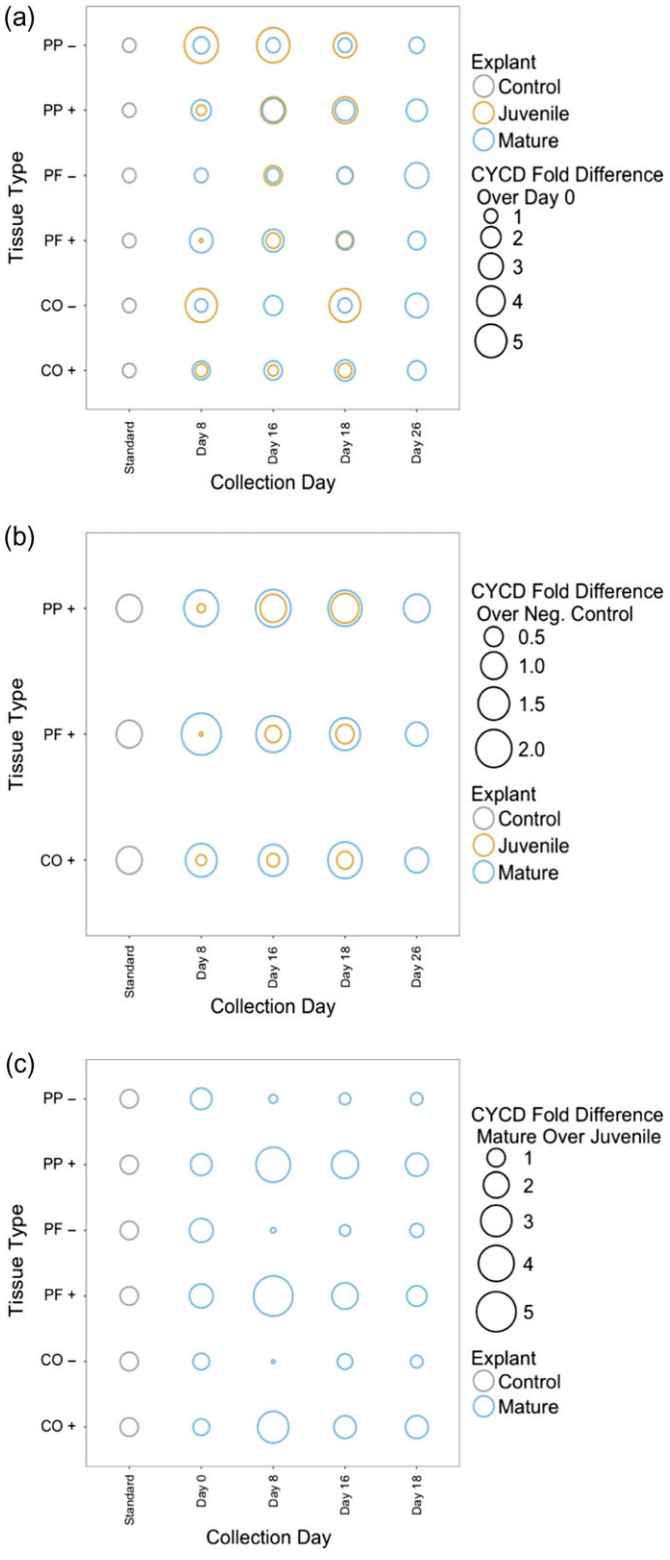

Figure 7. qRT-PCR relative fold differences among sample collection days over day 0 (a), among samples treated with 93.2 mM indole-3-butyric acid-potassium salt and water (negative control) (b), and between non-rooting mature samples over rooting-competent juvenile samples (c) for nine known adventitious rooting-related genes. Change in circle size for both juvenile and mature stems from an internal β-tubulin standard value of one represents a relative decrease or increase in expression for each gene studied. (a–c) *Cyclin-D1* (CYCD), (+) stems treated with 93.2 mM indole-3-butyric acid-potassium salt, (–) stems treated with water (control). PP, phloem parenchyma; PF, phloem fibers; CO, cortex.

compared with water-treated stems (Figure 4e). Auxin treatment had little effect on expression of *ARF17* vs water treatment except for day 18, when its expression increased 4-fold in phloem parenchyma cells (Figure 5f). *ARF6* and *ARF8* in auxin-treated stems increased 2-fold on days 16 and 18 compared with water controls on matching days in phloem parenchyma cells, while expression in the cortex and phloem fiber cells decreased (Figure 4b and e). *ARF17* expression was greatly reduced in juvenile auxin-treated stems compared with water-treated controls on day 8, but *ARF17* expression changed only minimally in juvenile auxin-treated stems compared with water-treated controls on day 16 in all tissues (Figure 5e).

To understand how physiological age affected the expression of adventitious root development-related genes, we compared hard-to-root mature cuttings to easy-to-root juvenile cuttings. In auxin-treated mature stems, a 2-fold *ARF6* increase over juvenile stems was recorded on day 16 in all sampled tissues, but there was little difference in expression for the other days and treatments (Figure 4c). *ARF8* expression varied little, except after auxin treatment on days 8, 16 and 18 in the mature phloem fiber cells, where it increased 5-, 3- and 3-fold, respectively, over juvenile tissues (Figure 4f). In contrast, *ARF17* expression was 2- to 3-fold higher in auxin-treated mature shoots on days 8, 16 and 18 in cortex, fiber and parenchyma cells than in comparable tissues of juvenile stems on matching days (Figure 5f). Physiological age of the cuttings was directly related to the expression levels of rooting-related genes, and in which tissues they were expressed.

Gene expression changes of CHS

Expression of *CHS*, a key flavanoid biosynthesis gene, was consistent with an antagonistic role in adventitious root formation. Juvenile cuttings had distinct, tissue-specific patterns of *CHS* expression. Juvenile root progenitor phloem parenchyma cells had a 72–75% reduction in *CHS* expression on days 16 and 18, respectively, compared with day 0. *CHS* expression in non-competent juvenile cortex and phloem fiber cells did not change or increased only slightly after auxin treatment compared with day 0 (Figure 5a). Expression levels through time were also influenced by treatment. Water-treated juvenile stems had a marginal change or a slight increase in all three sample tissues compared with day 0 (Figure 5a). Beginning on day 8 through to day 26, *CHS* expression increased markedly in cells from mature samples independent of tissue type (Figure 5a). Auxin, however, mitigated the amount of *CHS* expression increase compared with day 0 in mature stems. Auxin-induced gene expression changes in juvenile stems were not uniform among the tissues sampled. The influence of auxin was most pronounced in juvenile phloem fiber and cortex cells.

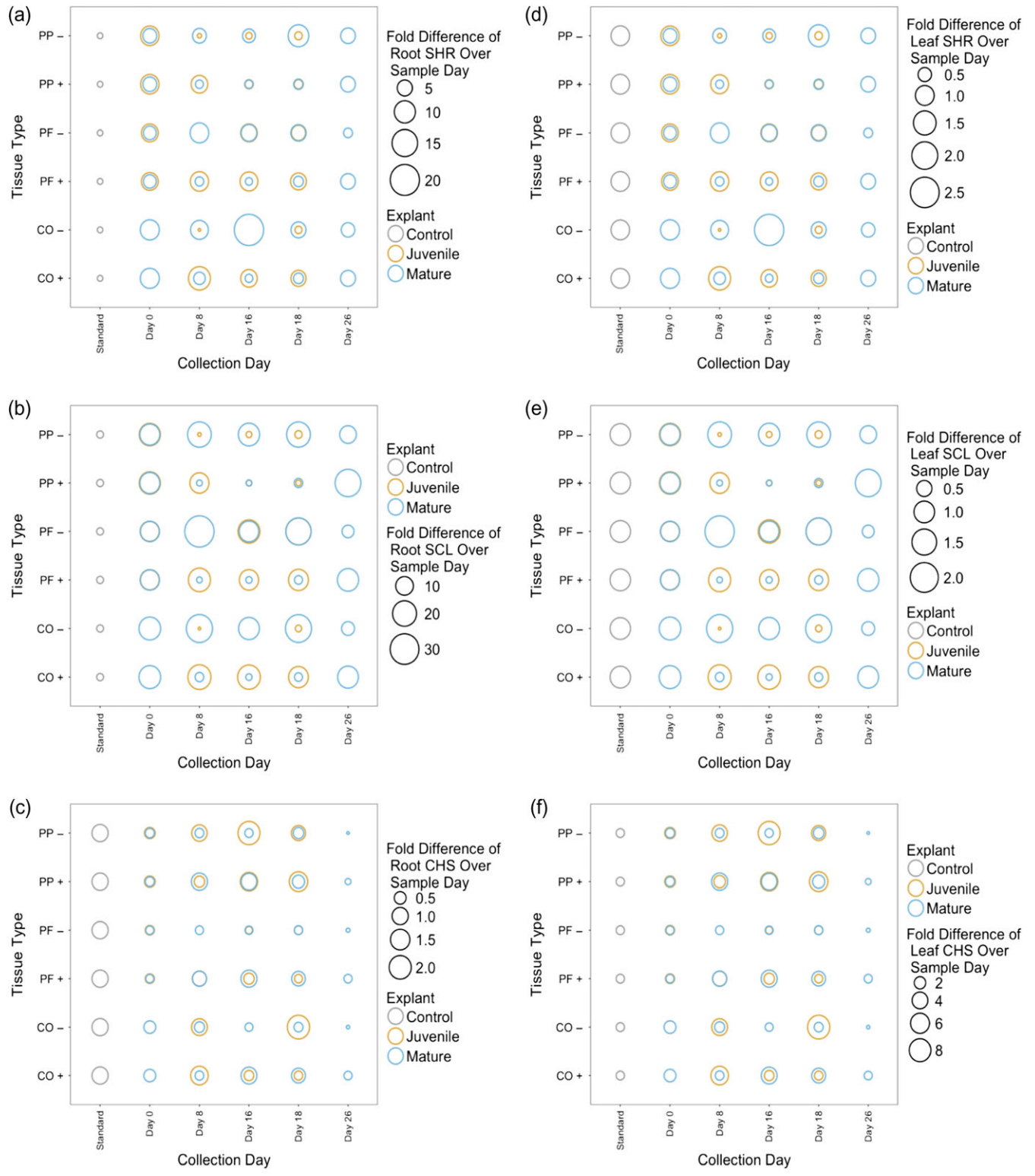

Figure 8. qRT-PCR relative fold differences for RNA extracted from actively growing root tips (a–c) and fully expanded leaves (d–f) and juvenile and mature stem tissue among sample collection days over day 0 for nine known adventitious rooting-related genes. Change in circle size for both juvenile and mature stems from an internal β-tubulin standard value of one represents a relative decrease or increase in expression for each gene studied. (a, d) *SHORTROOT* (SHR), (b, e) *SCARECROW*-like (SCL), (c, f) *Chalcone synthase* (CHS), (+) stems treated with 93.2 mM indole-3-butyric acid-potassium salt, (–) stems treated with water (control). PP, phloem parenchyma; PF, phloem fibers; CO, cortex.

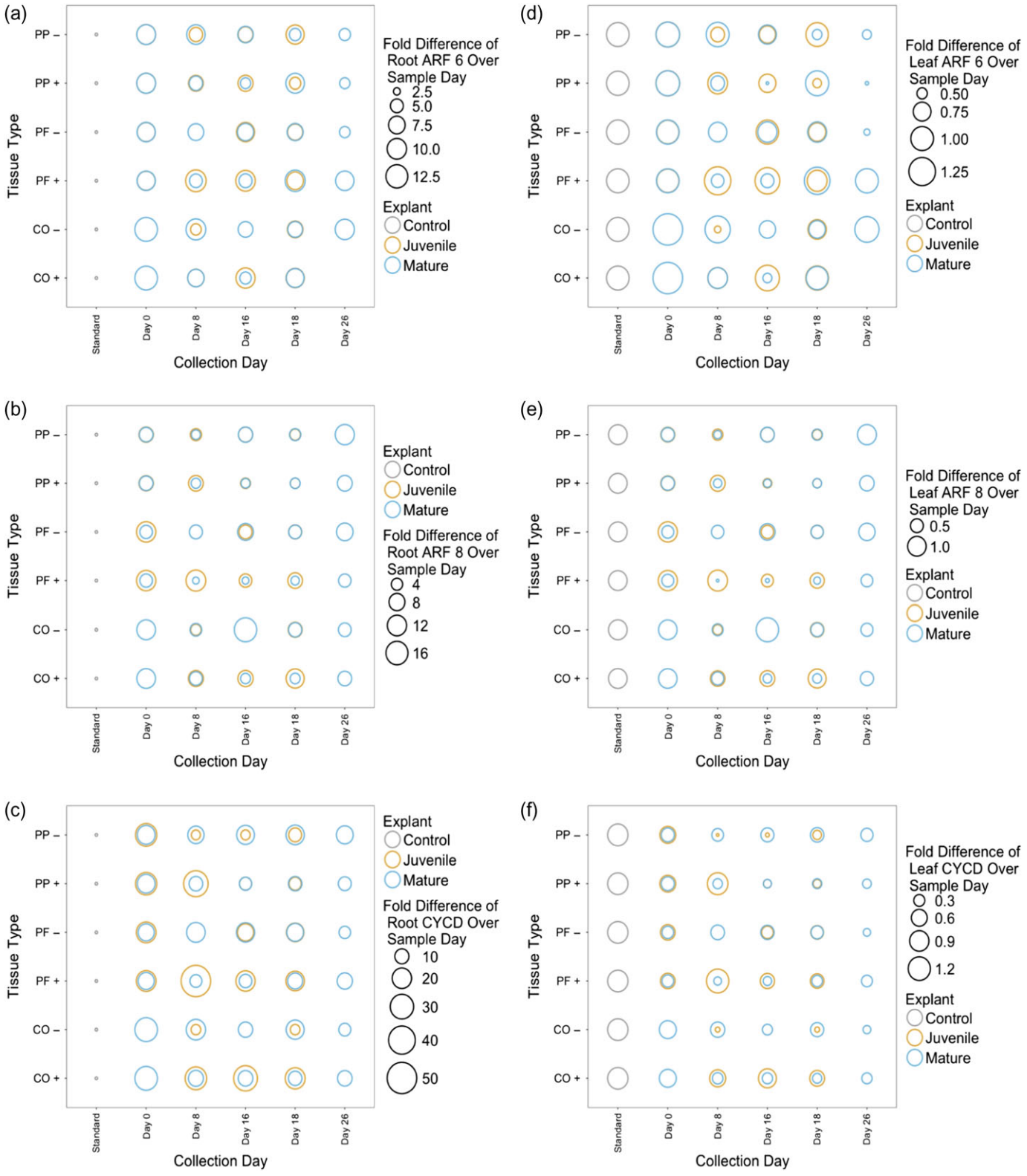

Figure 9. qRT-PCR relative fold differences for RNA extracted from actively growing root tips (a–c) and fully expanded leaves (d–f) and juvenile and mature stem tissue among sample collection days over day 0 for nine known adventitious rooting-related genes. Change in circle size for both juvenile and mature stems from an internal β-tubulin standard value of one represents a relative decrease or increase in expression for each gene studied. (a, d) *Auxin response factor6* (ARF6), (b, e) *Auxin response factor8* (ARF8), (c, f) *Cyclin-D1* (CYCD), (+) stems treated with 93.2 mM indole-3-butyric acid-potassium salt, (–) stems treated with water (control). PP, phloem parenchyma; PF, phloem fibers; CO, cortex.

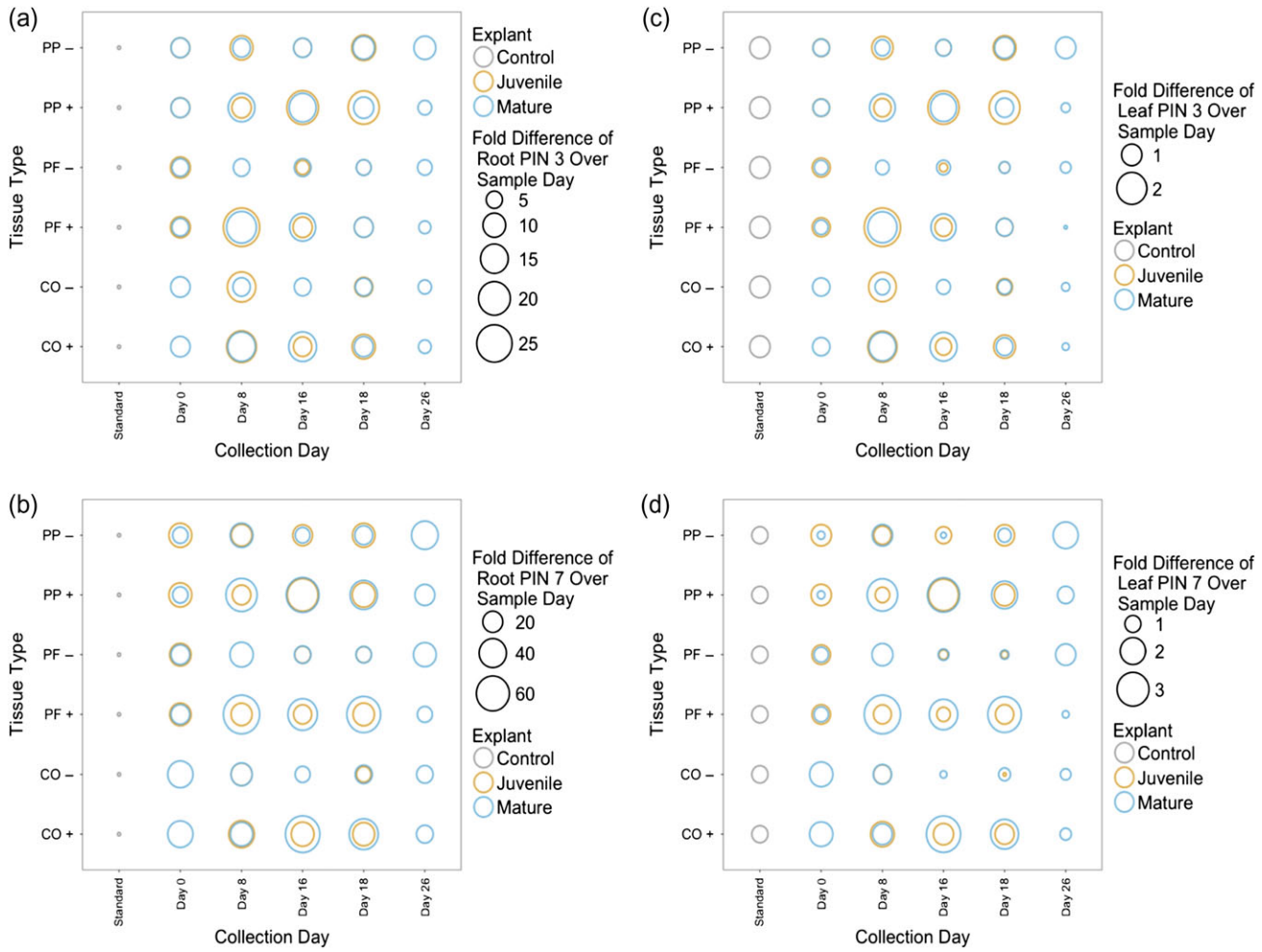

Figure 10. qRT-PCR relative fold differences for RNA extracted from actively growing root tips (a, b) and fully expanded leaves (c, d) and juvenile and mature stem tissue among sample collection days over day 0 for nine known adventitious rooting-related genes. Change in circle size for both juvenile and mature stems from an internal β-tubulin standard value of one represents a relative decrease or increase in expression for each gene studied. (a, c) *PIN-FORMED3* (PIN3), (b, d) *PIN-FORMED7* (PIN7), (+) stems treated with 93.2 mM indole-3-butyric acid-potassium salt, (–) stems treated with water (control). PP, phloem parenchyma; PF, phloem fibers; CO, cortex.

Compared with water-treated stems on the same day, auxin-treated juvenile stems had an increase in *CHS* expression on day 8 that peaked on day 18 (Figure 5b). Phloem parenchyma cells from juvenile auxin-treated stems showed an increase in *CHS* expression over water-treated stems on day 8, but *CHS* expression on days 16 and 18 when primordia formed was negligible. *CHS* expression in mature auxin-treated stems was no different than in water-treated stems on the same day (Figure 5b). We expected that in recalcitrant mature stems, *CHS* would be more highly expressed than in juvenile stems. This outcome was evident only for water-treated stems. Beginning on day 8, mature water-treated stems had a 2- to 4-fold increase in *CHS* expression compared with juvenile water-treated stems on corresponding days (Figure 5c).

Gene expression changes of PIN3 and PIN7

PIN3 and *PIN7*, auxin efflux carrier genes, exhibited limited changes in expression during root development. In juvenile auxin-treated cuttings, *PIN3* and *PIN7* both decreased slightly in rooting-competent phloem parenchyma cells beginning on day 8 and continuing through day 18. Eight days following auxin treatment, expression of *PIN3* and *PIN7* in phloem parenchyma cells was about 5-fold greater than in cortex cells in juvenile cuttings (Figure 6a and d). Eighteen days following treatment with water (control), expression of *PIN3* and *PIN7* in phloem parenchyma cells was only about 2-fold greater in phloem parenchyma cells vs cortex cells, irrespective of whether the cuttings were juvenile or mature. *PIN3* had the greatest change in expression on day 8 after auxin treatment compared with water-treated stems, but *PIN3* expression decreased below baseline conditions by day 16 (Figure 6b). Relative expression of *PIN7* in juvenile auxin-treated stems was reduced by as much as 2-fold over water controls (Figure 6b). Expression of *PIN3* and *PIN7* was about the same in mature and juvenile stems in all three tissue types for both treatments. The greatest difference between mature and juvenile stems

in *PIN3* expression was on day 8 after water treatment in phloem parenchyma, phloem fiber and cortex cells (Figure 5c). *PIN3* and *PIN7* expression in black walnut during adventitious root development was only marginally influenced by time after induction, physiological age or auxin treatment.

Gene expression changes of CYCD

We saw evidence that *CYCD*, a cell cycle initiation gene, has a role in adventitious root development. There was little change in *CYCD* expression in mature samples irrespective of treatment or collection day (Figure 7a). On days 16 and 18, *CYCD* expression in phloem parenchyma cells of auxin-treated juvenile shoots increased 3-fold over day 0, but little change was detected in cortex or phloem fiber cells on days 16 and 18 (Figure 7a). Fold increases of *CYCD* were uniform across tissue and day for water-treated shoots except day 18, when a 5-fold increase over day 0 in expression was observed in the cortex (Figure 7a). This was consistent with the observed origin of callus formation in juvenile cuttings (Figure 1). *CYCD* expression decreased on all collection days and in juvenile auxin-treated tissues compared with the water-treated stems, except for phloem parenchyma cells on days 16 and 18 when root primordia formed and it increased (Figure 7b). We found auxin treatment had a limited effect on *CYCD* expression in mature recalcitrant stems. Indeed, on days 16 and 18 when juvenile auxin-treated stems were undergoing directed cell divisions to form adventitious root initials, mature stems only experienced a slight increase in *CYCD* expression compared with water-treated (Figure 7b). Physiological age influenced expression of *CYCD* after auxin or water treatment during root development. There was more *CYCD* expression in mature auxin-treated stems than juvenile stems, but mature water-treated stems expressed less *CYCD* than juvenile stems in all tissues beginning on day 8 (Figure 7c).

Discussion

The changes in cell fate that are required for adventitious root development necessitate extensive transcriptional reprograming. Although adventitious roots typically arise from a small number of founder cells, past studies examining molecular controls of adventitious root development have analyzed gene expression changes in whole tissue samples (Sorin et al. 2005, Sánchez et al. 2007, Gutierrez et al. 2009, 2012, Cui et al. 2011, Vielba et al. 2011, Rigal et al. 2012, You et al. 2013). Such approaches risk masking or diluting important molecular checkpoints by the inclusion of cells not directly involved in adventitious root development. Previous research found adventitious roots predictably formed from specific founder cells in black walnut cuttings, but was highly dependent upon induction treatment and age of the cutting (Stevens and Pijut 2017). These findings then allowed us to use laser capture microdissection to describe the changes in transcript levels with high spatial resolution. This unique approach confirmed visual evidence of the necessity for rooting-competent cells to be present in order for root organogenesis to occur.

Past research showed that laser capture microdissection was effective for understanding transcriptional regulation of complex developmental processes such as disease response (Lenzi et al. 2016) and terpenoid synthesis (Abbott et al. 2010). Despite its utility, laser capture microdissection can be technically demanding because it often requires species-specific fixation protocols (Nelson et al. 2006). Although samples may be fixed and embedded in paraffin, we found this step unnecessary. Fresh samples that were flash-frozen and sectioned with a cryostat yielded adequate quantities of high-quality RNA. This approach risks cellular damage from ice crystals, limiting visualization of anatomical structures (Kerk et al. 2003, Balestrini and Bonfante 2008), but this did not prove to be an issue with black walnut stem sections.

Transcriptional modulation in microdissected cortex, phloem fiber or phloem parenchyma cells was examined for nine black walnut genes known to participate in adventitious root development (Table 2). We determined the expression patterns of two transcription factors that regulate meristem organization (*SCL* and *SHR*), one gene related to secondary metabolite synthesis (*CHS*), one gene associated with cell cycle initiation (*CYCD*), two auxin efflux carriers (*PIN3* and *PIN7*) and three auxin responsive transcription factors (*ARF6*, *ARF8* and *ARF17*). Reduction in *CHS* expression has been shown to increase adventitious rooting in *J. regia* (El Euch et al. 1998, Cheniany et al. 2012). A similar effect was noted in black walnut. In juvenile cuttings, root founder cells treated with auxin showed a 4-fold decrease in *CHS* compared with day 0, while non-participatory cells had no change in expression. This decrease was concurrent with the first known observations of root initials and primordia (days 16 and 18) (Stevens and Pijut 2017). Non-rooting mature cuttings that were treated with auxin also showed a decrease in *CHS* expression for days 16 and 18, but it was not tissue specific. These results indicated the importance of a localized reduction in flavanoid biosynthesis, induced by exogenous auxin application, to adventitious root development in black walnut cuttings.

Some, but not all, observed patterns of expression during adventitious root development corresponded with previous research. In contrast to past research, we saw little interaction between auxin and flavanoids through *CHS* and *PIN* expression. Specifically, flavonoids can interact with and modify auxin efflux, influencing adventitious root development (Peer and Murphy 2007, Guan et al. 2015). Interestingly, however, we found little evidence of a relationship between the transcript abundance of *CHS* and *PIN3* or *PIN7*. *PIN* transcript levels were reduced to the same degree as *CHS* in juvenile auxin-treated parenchyma cells on days 16 and 18. It was possible that the decrease in *PIN* expression was influenced by collection day; for example, *PIN3* and *PIN7* may not be necessary until later in root formation. Also, the PIN protein family is large (Křeček et al. 2009), and these

two *PIN* genes may not be directly involved in adventitious root development in black walnut. *CYCD* expression patterns supported conclusions from previous studies of rooting in black walnut that showed water-treated cuttings were capable of undergoing cellular dedifferentiation, but the subsequent proliferation was undirected, leading to callus formation (Stevens and Pijut 2017). In cortex cells of juvenile water-treated cuttings, *CYCD* expression increased 5-fold on all collection days, while auxin-treated cuttings showed an expression increase only in parenchyma cells.

Black walnut cuttings do not root without exogenous auxin (Stevens and Pijut 2017), and as such, *SHR* and *SCL* expression was influenced by auxin treatment in mature and juvenile stems. Concomitant to *CYCD* expression increases in auxin-treated juvenile parenchyma cells, we also observed an increase in expression of *SCL* and *SHR* compared with day 0. *SCL* and *SHR* interact to direct root meristem maintenance and cell identity (Cui et al. 2011), while also promoting expression of *CYCD* (Sozzani et al. 2010, Xu et al. 2016). The role of *SCL*/*SHR* in adventitious root formation has been demonstrated in diverse tree lineages including *Populus* (Xuan et al. 2014), *Pinus* (Sánchez et al. 2007) and *Castanea* (Sánchez et al. 2007, Vielba et al. 2011). Water-treated juvenile shoots showed an increase in *SHR* and *SCL* by day 16, but the increase was not confined to the parenchyma. This indicated auxin was necessary to confine *SCL*/*SHR* and *CYCD* upregulation to rooting-competent cells. Auxin-treated mature shoots showed an increase in *SHR* and *SCL* expression, but again transcript abundance was uniform across all sampled tissues, similar to results in *Castanea* (Vielba et al. 2011). Auxin application and an increase in *SHR*/*SCL* and *CYCD* expression were not sufficient to promote adventitious root development in black walnut.

The transcription factors *ARF6*, *ARF8* and *ARF17* influence adventitious root development through modulation of jasmonic acid homeostasis (Gutierrez et al. 2012). These three *ARF*s act as a complex network with overlapping expression profiles. In *Arabidopsis, ARF6* and *ARF8* promote adventitious root development, while *ARF17* inhibited adventitious root development (Gutierrez et al. 2009). Our results indicated these genes have a similar function in controlling adventitious root development in black walnut. In rooting-competent parenchyma cells only, *ARF6* and *ARF8* expression increased beginning at day 8 and continued until day 18, when root primordia were first visible. In addition, *ARF17* expression decreased concomitant with the increase in *ARF6* and *ARF8* expression. Auxin was necessary for localizing expression changes, as all cell types responded equally and similarly in water-treated cuttings. Again, transcript abundance across all tissues in physiologically mature stems was uniform with regard to sample day and treatment.

Using laser capture microdissection, we showed that site-specific transcriptional activation accompanied adventitious root formation. These findings support our conclusion that the recalcitrance of black walnut to adventitious root development was a result of the failure of rooting-competent cells to perceive molecular signals initiated by auxin. This conclusion was supported by Rasmussen et al. (2015), where in the model species *Pisum sativum* the authors found reduction in root formation in recalcitrant mature tissue was linked to changes in auxin metabolism, specifically endogenous auxin becoming inactivated at rooting sites. Auxin alone was not sufficient to induce rooting; rooting-competent cells must also be present. However, when cells lose competency to root was unclear and can be species dependent. Rooting was not achieved in softwood cuttings from $\geq$30-year-old physiologically mature black walnut trees (Stevens and Pijut 2017), and as such had unique patterns of expression in cells known to produce roots compared with rooting-competent juvenile cuttings. Loblolly pine (*Pinus taeda*) epicotyls, however, lost the ability to root after only 50 days (Diaz-Sala et al. 1996). Epicotyls demonstrated no difference in auxin availability or polar auxin transport. Adventitious root development remains a complex biological process that is often species- and life-stage-dependent, but nonetheless requires rooting-competent sites to be present and perceptive to inductive signals.

Acknowledgments

The authors would like to acknowledge the contributions of Dr Steven Boehm and Ping Zhang at Indiana University Purdue University Indianapolis for their help optimizing the laser capture microdissection protocol. The mention of a trademark, proprietary product or vendor does not constitute a guarantee or warranty of the product by the US Dept of Agriculture and does not imply its approval to the exclusion of other products or vendors that also may be suitable.

Conflict of interest

None declared.

Funding

Financial support for this work came from a Fred M. van Eck scholarship for Purdue University to M.E.S.

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