Running title: PtRR13 and adventitious root development

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The cytokinin type-B response regulator PtRR13 is a negative regulator of adventitious root development in *Populus*.

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1ABSTRACT

Adventitious root formation at the base of plant cuttings is an innate de novo organogenesis process that allows massive vegetative propagation of many economically and ecologically important species. The early molecular events following shoot excision are not well understood. Using whole-genome microarrays, we detected significant transcriptome remodeling during 48 hours following shoot removal in Populus tremula x Populus alba softwood cuttings in the absence of exogenous auxin, with 27% and 36% of the gene models showing differential abundance between 0 and 6 hours, and 6 and 24 hours, respectively. During these two time intervals, gene networks involved in protein turnover, protein phosphorylation, molecular transport and translation were among the most significantly regulated. Transgenic lines expressing a constitutively active form of the Populus type-B cytokinin response regulator PtRR13 (ΔDDKPtRR13) have a delayed rooting phenotype and cause misregulation of COV1, a negative regulator of vascularization; PDR9, an auxin efflux transporter; and two AP2/ERF genes with sequence similarity to TINY. Inappropriate cytokinin action via ΔDDKPtRR13 expression appeared to disrupt adventitious root development 24 hours after shoot excision, when root founder cells are hypothesized to be sensitive to the negative effects of cytokinin. Our results are consistent with PtRR13 acting downstream of cytokinin to repress adventitious root formation in intact plants, and that reduced cytokinin signaling after shoot excision enables coordinated expression of ethylene, auxin and vascularization pathways leading to adventitious root development.
Adventitious rooting is an ecologically and economically important developmental process. *Populus* and other perennial species adapted to riparian ecosystems naturally propagate clonally when stems or branches detached by a natural disturbance are carried downstream and lodge in a moist environment conducive to rooting. For many species, therefore, clonal propagation via adventitious root formation is a natural complement to sexual propagation by seeds. The stimulation of adventitious rooting to facilitate clonal propagation is a cornerstone of the ornamental horticulture and forest products industries. Treatment of cuttings with synthetic auxins has been used for more than 60 years to induce and accelerate rooting in hard-to-root species (Kevers et al., 1997). Genetic improvement programs for forest trees including *Eucalyptus* spp., *Populus* spp. and *Pinus radiata* are almost exclusively based on the production of rooted cuttings for breeding and deployment in operational plantations (Davis and Becwar, 2007).

In woody plants, adventitious root primordia primarily arise from ray cells adjacent to the vascular cambium, from buds or leaf gaps, or from callus formed at the base of cuttings (Lovel and White, 1986). Ectopic formation of roots is regulated by both endogenous (e.g. hormones, sugars, phenolic compounds) and exogenous (e.g. temperature, light) factors (Eliasson, 1978; Davies and Hartmann, 1988; Kevers et al., 1997; De Klerk et al., 1999). Among the plant hormones, it is well established that basipetal transport and accumulation of auxin at the base of cuttings precedes adventitious root formation (Liu and Reid, 1992; Hausman et al., 1995; Guerrero et al., 1999). Auxin synthesized in shoot tips is important during adventitious rooting since removal of the shoot apex decreases both the level of endogenous auxin in the basal portion of a cutting and the number of adventitious roots produced (Nordstrom and Eliasson, 2001). Elevated auxin concentrations give rise to new root primordia by activating the differentiation and elongation of phloem parenchyma cells adjacent to vascular bundles in the stem (Lund et al., 1996; De Klerk et al., 1999).

Adventitious root formation can be seen as a three-stage process: (1) activation, where the cells originating the root primordia become competent to respond to the rhizogenic action of auxin, (2) induction, comprising the determination of root primordia and initial cell divisions,
and (3) outgrowth, where root primordia elongate and vascular connections are established to preexisting vasculature within the stem. Auxin and cytokinin appear to play antagonistic roles in more than one stage of the adventitious rooting process (De Klerk et al., 1997). Quantification of the endogenous levels of auxin and cytokinins in the basal region of cuttings from diverse woody and herbaceous plants including *Populus*, *Malus*, *Solanum* and *Phaseolus*, reveal that the concentrations of these two hormones follow opposite patterns during the initial 48 hours of rooting. Auxin concentrations are high during activation and induction stages, and low during outgrowth, while cytokinin levels are low during activation and induction stages, and high during outgrowth (Blakesley et al., 1985; Bollmark and Eliasson, 1986; Maldiney et al., 1986; Hausman et al., 1997; Kevers et al., 1997). In addition, exogenous application of cytokinin to cuttings during the induction phase strongly inhibited root formation (De Klerk et al., 1999). A naturally occurring cytokinin in cucumber root xylem sap (*trans*-zeatin riboside) was identified as a major suppressor of adventitious root formation in hypocotyls (Kuroha et al., 2002). Histological studies indicate that cytokinin inhibits the differentiation of primordia at an early stage in development (Bollmark and Eliasson, 1986).

Cytokinin signaling resembles two-component systems first identified in bacteria and yeast. In two-component systems, an extracellular cue is sensed by a plasma membrane localized histidine kinase (HK) which transfers the signal to a response regulator (RR) in the form of a phosphoryl group (Mizuno, 1998; West and Stock, 2001). In plants, these signaling pathways include a third component, a histidine phospho-transfer protein, which functions as a carrier of the phosphoryl group from the HK to the RR (Mok and Mok, 2001; Kakimoto, 2003; Ferreira and Kieber, 2005). The RRs are the final step of this phosphorelay and have been traditionally divided into two groups; the type-As and the type-Bs. The type-As are cytokinin primary response genes whose proteins consist mostly of a receiver domain with conserved D-D-K residues (Brandstatter and Kieber, 1998; Taniguchi et al., 1998; D'Agostino et al., 2000). The type-Bs are more complex proteins that contain, in addition to the receiver domain, a DNA-binding motif (GARP domain) that resembles a domain originally found in the mammalian oncoprotein c-Myb (Imamura et al., 1998; Sakai et al., 1998). While phosphorylated type-Bs act as transcriptional activators of cytokinin-regulated genes, the type-As down regulate cytokinin
signaling by competing with the type-Bs for phosphoryl groups (reviewed in To and Kieber, 2008). Genetic analyses to define the function of RRs in Arabidopsis suggest functional redundancy among family members since phenotypes are not obvious unless mutant lines contain null alleles from several loci (To et al., 2004; Argyros et al., 2008; Ishida et al., 2008). Because of their potential functions as transcriptional activators, type-Bs are thought to represent key elements orchestrating transcriptome changes triggered by cytokinin. We previously identified 22 genes in *Populus* that exhibit the typical features of plant RRs (Ramirez-Carvajal et al., 2008).

Although the physiological and anatomical processes associated with adventitious rooting have been described in a variety of genera including *Eucalyptus, Pinus* and *Malus* (De Klerk et al., 1999; Fett-Neto et al., 2001), the molecular mechanisms involved in determining the competence of cells to generate adventitious roots as well as the development of adventitious roots per se, are not well defined. Studies in Arabidopsis and tobacco have provided compelling evidence linking cytokinin signaling to adventitious root formation. Arabidopsis mutants lacking the cytokinin HK receptors AHK2, AHK3 and AH4 exhibit enhanced adventitious root growth, whereas the elongation of primary and lateral roots is virtually eliminated (Higuchi et al., 2004; Nishimura et al., 2004). Similar phenotypes have been observed in cytokinin deficient tobacco plants where overexpression of the cytokinin degrading enzyme cytokinin oxidase (AtCKX) results in abundant adventitious roots (Werner et al., 2001; Werner et al., 2003). The Arabidopsis triple mutant *arr1 arr10 arr12* lacks the function of three type-B RRs and shows almost complete insensitivity to exogenously applied cytokinin and spontaneously produces adventitious roots in hypocotyls (Argyros et al., 2008). While these genetic studies imply a potential role of cytokinin in adventitious rooting, they do not illuminate when or where these effects occur. Analysis of shifts in gene expression in conjunction with adventitious rooting (Kohler et al., 2003; Brinker et al., 2004) examined relatively small sets of genes or targeted relatively late stages of root formation. Thus the potential role of cytokinin in regulating adventitious root development remains obscure, particularly the activation and induction phases comprising the very early stages of adventitious root development.
As part of a comprehensive analysis of the primary molecular mechanisms orchestrating de novo adventitious root formation in *Populus* cuttings, we report transcriptome remodeling involving about half of the transcriptional units annotated in the poplar genome within 24 hr of shoot excision. We investigated the role of cytokinin signaling by contrasting whole-genome expression data acquired from nontransgenic (NT) poplar, with transgenic lines delayed in rooting due to ectopic expression of a constitutively active form of PtRR13, referred here as ΔDDKPtRR13. Perturbation of rooting due to alteration of cytokinin signaling through the expression of ΔDDKPtRR13 appears to be physiologically relevant at 24 hours where a large set of signaling, developmental and metabolic networks are differentially regulated in the transgenic and NT lines. Promoter sequence examination of differentially regulated genes across all time points between NT and ΔDDKPtRR13 lines reveal putative direct and indirect targets of PtRR13. These putative targets include a negative regulator of vascularization COV1, an auxin efflux transporter PDR9 and two AP2/ERF proteins similar to TINY.

**RESULTS**

**The poplar transcriptome is remodeled in the base of excised shoots**

*Populus* softwood cuttings of the genotype used in this study form an adventitious root system that is sufficiently developed within 10 to 14 days that plants can be removed from periodic mist treatments. No exogenous auxin treatment is required; the lack of a requirement for auxin treatment may be due to high rates of auxin synthesis and/or transport in the actively growing shoots used to generate cuttings. Observations in *Malus* and *Populus* microcuttings reveal cell divisions as early as 48 hours after auxin exposure and the presence of organized meristemoids by 96 hours (De Klerk et al., 1995; Wu, 2004). Because early physiological and biochemical evidence indicates there are significant changes in endogenous hormone pools, including ethylene, auxin and cytokinin, during the first 48 hours after excision (Maldiney et al., 1986; Selby et al., 1992; Hausman, 1993; De Klerk et al., 1997), we predicted that changes in gene expression during this time frame would provide information about the early and poorly defined molecular networks involved in adventitious root formation.
To analyze transcriptome changes during adventitious root formation in *Populus*, the basal 5 mm of softwood cuttings, where adventitious roots originate, were harvested at 0, 6, 24 and 48 hours after excision. RNA extracted from these samples was used for microarray analyses with a custom designed NimbleGen array containing features representing 55,793 annotated gene models from the sequenced genome of *P. trichocarpa*, including 45,555 predicted gene models reported previously (Tuskan et al., 2006) plus an additional 10,238 gene models showing evidence of transcription (Quesada et al., 2008). Transcripts showing significant differences in abundance at a false discovery rate (FDR) (Storey and Tibshirani, 2003) of 5% between adjacent time points (0h-6h, 6h-24h and 24h-48h) were identified. A total of 1015,134 transcripts (representing 27% of the predicted gene models used in this study) were differentially regulated between 0 and 6 hours; 20,111 (36%) between 6 and 24 hours, and 122,474 (4%) between 24 and 48 hours (Figure 1). This high proportion of differentially regulated genes is consistent with massive transcriptome remodeling after shoot excision. Gene-set enrichment analysis of these genes suggests differential regulation of functional networks with potential roles in protein degradation, protein phosphorylation and RNA metabolism (Supplemental Table 1, Supplemental Figure 1A-B).

Gene clustering reveals diverse patterns of gene expression and reflects contrasting roles of ethylene, auxin and cytokinin

To discern global patterns of differential transcript abundance over the time course, transcripts with contrasts significant at FDR <5% were further filtered to include only those with greater than 2-fold changes in transcript abundance at adjacent time points (0h-6h, 6h-24h and 24h-2348h). Transcripts meeting these criteria were clustered using the Gaussian Clustering application of ArrayMiner5 (Optimal Designs, Belgium; Figure 2). We chose to present N=7 clusters since the number of transcripts not included in any cluster (Figure 2, “Unc.”) was similar with clusters N≥7. Cluster size ranged from 3796 from 1330 transcripts. Current research indicates that the early (activation and induction) stages of adventitious root development probably involve complex interactions among ethylene, auxin and cytokinin. Therefore, we evaluated the clusters to determine if and how these hypothesized hormone interactions might
be reflected in the transcript data. For clarity, we distinguish clusters reflecting general trends toward increasing transcript abundance (red; clusters 1, 4 and 7) and decreasing transcript abundance (green; clusters 2, 3, 5 and 6) over time.

Ethylene biosynthesis is predicted to increase after shoot excision (Bollmark et al., 1988; O’Donnell et al., 1996; Van Loon et al., 2006). We observed an increase in transcript abundance for 9 of the 12 members of the aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase gene families that were placed in clusters (Figure 2). Seven of these 9 transcripts were located in either cluster 4 or 7 (both clusters with the lowest transcript abundance at 0 hr). These data are consistent with an increase in ethylene biosynthesis following shoot excision.

Two families of transcriptional regulators (IAAs and ARFs) are involved in auxin signaling. IAAs negatively modulate auxin signaling by repressing the transcriptional activity of ARFs through heterodimerization (Ulmasov et al., 1999). Auxin can induce the transcription of specific IAAs which is thought to establish a negative feedback loop and ensure a transient response (Abel and Theologis, 1996; Liscum and Reed, 2002). Poplar IAAs and ARFs have been annotated and evaluated for auxin inducibility (Kalluri et al., 2007). We found that transcripts for IAA gene family members increased (4 of 11 clustered genes) in concert with transcripts for ethylene biosynthesis or decreased (7 of 11; Figure 2). In contrast, all ARF gene family members that were placed in clusters (all 8 of the clustered genes) decreased in transcript abundance.

Physiologically relevant quantities of cytokinin are exported from roots to aboveground organs (Hirose et al., 2008). Since type-A RRs are postulated to be transcriptionally regulated by cytokinin, changes in the transcript abundance of these genes may operate as reporters for cytokinin availability. Two subfamilies of RRs (type-A and type-B) have been annotated in the poplar genome and evaluated for cytokinin inducibility (Ramirez-Carvajal et al., 2008). Of the 8 RR family members that occurred in clusters, all of them decreased in transcript abundance over time (Figure 2, clusters 2 and 3). While this general pattern coincides with the ARFs in that the greatest transcript abundance was at time 0, the transcript abundance for most of the RRs decreased within 6 hr (7 of the 8 RR transcripts grouped in clusters 3 and 5). In contrast, most of the ARFs decreased at 24 hr (5 of the 8 ARF transcripts grouped in cluster 2).
PtRR13 lacking a receiver domain generates a phenotype, but not full-length or RNAi constructs.

PtRR13 displays the typical domain organization of type-Bs: an N-terminal receiver domain with invariable D-D-K residues, a GARP domain, and a long C-terminal transactivation domain (Figure 3A). Sequence similarity and neighboring gene colinearity suggest PtRR13 is an ortholog of Arabidopsis ARR1 (Supplemental Figure 2). To develop knowledge about the in vivo roles of PtRR13, three different constructs were designed to direct gain or loss of PtRR13 function. Two different overexpression constructs, one for production of full-length PtRR13 (FLPtRR13) and a second for production of a truncated version in which the receiver domain was deleted (ΔDDKPtRR13), were engineered. Since the type-B receiver domain is proposed to inhibit the DNA binding activity of the GARP domain in the absence of the phosphorylated aspartate residue associated with cytokinin signaling (Sakai et al., 2000; Sakai et al., 2001), engineering a construct in which this domain is missing is predicted to create a constitutively active version of PtRR13. The third construct, consisting of a transitive RNAi vector containing ~200 bp of the last exon of PtRR13, was generated for a loss-of-function approach. Transgenic P. tremula x alba plants were generated for all three constructs via Agrobacterium-mediated transformation and the two strongest lines from each construct were chosen for detailed phenotyping. To identify these, sixty lines (twenty independent transgenic events per construct) were regenerated, hardened off to greenhouse conditions, propagated as rooted cuttings and then screened for transgene expression levels. Transgene transcript abundance for the FLPtRR13 and ΔDDKPtRR13 lines was assayed by real-time quantitative PCR and the two lines with highest expression for each construct were chosen for further phenotyping (FLPtRR13-1 and FLPtRR13-14 for the full length and ΔDDKPtRR13-16 and ΔDDKPtRR13-20 for the truncated; Figure 3B). The two RNAi lines with the greatest reductions in endogenous PtRR13 expression compared to the nontransgenic controls were also selected for further phenotyping (lines RNAi45 and RNAi57 with 73% and 62% reduction, respectively) (Figure 3C).

A perturbation in adventitious rooting during propagation was also observed. Measuring total root length of apical cuttings 10 days after shoot excision revealed that the two ΔDDKPtRR13 lines had significantly lower total root lengths than the nontransgenic line (36.3±
5.2 and 27.3±6.5 for ΔDDKPtRR13-16 and ΔDDKPtRR13-20 respectively, vs. 84.2±10.5 for the NT; Figure 4A). No significant differences were found between the FLPtRR13 and RNAi and the NT lines. The absence of phenotypes for the FLPtRR13 lines may be because ectopically expressed PtRR13 does not alter cytokinin signaling, which may be driven by shifts in RR phosphorylation status. The lack of phenotypes in the PtRR13 RNAi lines could be a consequence of incomplete gene silencing since the line with the most severe reduction may retain normal cytokinin signaling. Alternatively, PtRR13 may be functionally redundant with another RR. Based on the observed delay in adventitious rooting, subsequent analyses focused on the ΔDDKPtRR13 lines.

PtRR13 is a negative regulator of adventitious root formation

The differences in total root length observed above were a consequence of decreased numbers of roots in the ΔDDKPtRR13 lines. While no differences were observed in plant growth, cuttings of equivalent biomass (Figure 4B) revealed consistent differences in root number between the control NT line, which formed, on average, 11.66 (± 0.84 SE) roots per cutting, whereas the ΔDDKPtRR13-16 and ΔDDKPtRR13-20 lines formed 4.92 (± 0.65 SE) and 6.07 (± 0.99 SE) roots per cutting, respectively. This indicates that the differences observed in rooting were not driven by disparities in plant size or shoot biomass. The reduction in root number in the ΔDDKPtRR13 lines (Figure 4D-E) was typically accompanied by callus formation at the base of the cutting adjacent to the wound site (Figure 4F), implying a discrete disruption in adventitious root development.

To evaluate whether the proliferation of unorganized cells at the base of the cuttings reflected an altered response to hormones, stem explants from the ΔDDKPtRR13 lines were cultured under six different auxin:cytokinin ratios (0:0, 10:0, 100:0, 0:10, 10:10 and 10:100 μM IBA: μM zeatin, respectively; Supplemental Figure 3). The ΔDDKPtRR13 lines showed greater tissue growth in the absence of exogenous cytokinins, implying an upregulation of cytokinin signaling in the transgenic lines. Collectively, the results obtained in these experiments imply a perturbation of cytokinin signaling in transgenic poplars expressing ΔDDKPtRR13, with an apparent negative role in adventitious root formation.
ΔDDKPtRR13 expression has minor pleiotropic effects on the transcriptome

To gain insights into the molecular mechanisms altered by a constitutively active version of PtRR13, whole-transcriptome monitoring data were generated to detect shifts in transcript abundance. Line ΔDDKPtRR13-16 was arbitrarily selected since both ΔDDKPtRR13-16 and ΔDDKPtRR13-20 lines displayed identical phenotypes with respect to the adventitious rooting and the tissue culture response to cytokinin. Expression data at 0, 6, 24 and 48 hours after shoot excision for line ΔDDKPtRR13-16 was contrasted with NT and transcripts significantly altered across all four time points (transgene effect in the ANOVA model) were identified.

Consistent with our whole-plant studies that collectively implied a lack of pleiotropic effects of the transgene, we identified only 11 transcripts that showed differential abundance across all time points at a 10% FDR and a 2-fold regulation threshold. Of the 11 genes differentially regulated across all time points, 5 were up-regulated (including transgene ΔDDKPtRR13) and 6 were down-regulated (Table I). The two transcripts most increased were COV1 (CONTINUOUS VASCULAR RING 1), a negative regulator of vascular formation in stems (Parker et al., 2003) and PDR9 (PLEIOTROPIC DRUG RESISTANCE TRANSPORTER 9) (Ito and Gray, 2006) encoding a protein involved in auxin efflux. The other genes up-regulated by ΔDDKPtRR13 were FIM2 (FIMBRIN-LIKE 2) encoding an actin-binding protein, and MSL10 (MECHANOSENSITIVE CHANNEL OF SMALL CONDUCTANCE-LIKE 10) encoding an ion channel protein. The transcripts with lower abundance in the ΔDDKPtRR13 line (or increased in NT) included two TINY-like transcription factors, the cytochrome p450 CYP94B3, a caffeic acid O-methyltransferase family 2 protein, a metal-nicotinamide transporter YSL7 (YELLOW STRIPE-LIKE 7), and a homeodomain 2 protein, BELL1 (BEL1).

Arabidopsis ARR1 binds DNA in a sequence specific manner to the semi-palindromic motif AGATC (Sakai et al., 2001). This motif has been detected in the promoters of several Arabidopsis type-As and in several cytokinin-regulated genes (Rashotte et al., 2003). A manual search for this ARR1 motif in the promoters of the 10 genes (less the ΔDDKPtRR13 transgene) regulated across all time points revealed that the COV1 promoter had the highest number of AGATC motifs (5 motifs) that all occurred in a 374 bp segment approximately 680 bp upstream of the presumed translation start (Supplemental Figure 4). The number of ARR motifs in the
other gene promoters ranged from 1 to 4. Because Arabidopsis TINY has been demonstrated to bind the dehydration responsive element (DRE) with a core sequence of A/GCCGAC as well as the ethylene responsive element (ERE) with a core sequence of AGCCCGCC (Sun et al., 2008) we manually searched for these motifs but found no indication of enrichment.

Cytokinin action may interfere with adventitious rooting at 24h

To gain insights into the molecular mechanisms altered during early adventitious root formation in transgenic plants, we identified genes significantly regulated at each time point (transgene by time interaction terms in the ANOVA model). These analyses revealed no significant gene regulation differences at 6 and 48 hours (above and beyond that incited by the transgene alone), however a significant transcriptional response at 24 hours was identified (273 genes at 1% FDR and 2-fold abundance threshold, or 5111 genes at 5% FDR and 2-fold abundance threshold) (Figure 5). Of the 273 genes differentially regulated at 24 hours at 1% FDR (Supplemental Table II), 160 (59%) had significantly greater expression in ΔDDKPtRR13-16 than in NT, while the remaining 113 (41%) were more abundantly expressed in NT. The top 5 most significantly up-regulated transcripts, and the top 5 most significantly down-regulated transcripts were identified (Table II). These transcripts encode genes involved in diverse biological processes such as auxin homeostasis (IAA conjugating enzyme GH3-8), brassinosteroid signaling (BEE transcription factor), transcriptional regulation (Myb102), calcium signaling (IQD33), defense responses (SOBER1), and transport (CAT7 and MATE efflux protein).

To determine if there was significant enrichment of any particular cis-element(s) in these genes differentially regulated at 24 hours, we adapted the overrepresentation computational analysis described by Nemhauser et al. (2004) to Populus. In genes up-regulated by ΔDDKPtRR13, a significant enrichment of the element OSE2ROOTNODULE (CTCTT) (Fehlberg et al., 2005) originally found in promoters of genes activated during root nodule formation was detected (Z-score = 3.44 and p-value < 0.001, data not shown). For genes down-regulated by ΔDDKPtRR13, we detected enrichment of the root-hair specific cis-element RHERPATEXPA7 (KCACGW; Z-score = 2.37 and p-value = 0.009) (Kim et al., 2006). Interestingly, cytokinin
receptors are required to activate cell divisions during nodule organogenesis (Murray et al., 2007), implying potential biological significance of these promoter motifs. We developed a model (Figure 6) to organize interpretation of these results, and to serve as a set of working hypotheses to guide further dissection of adventitious root development.

**DISCUSSION**

Adventitious rooting is a complex process that encompasses signaling networks involved in physiological responses to mechanical injury, wound repair and organ development. In this study, we monitored the *Populus* transcriptome during early stages of adventitious rooting and gained new insights into the regulation of endogenous hormone signaling cascades during this developmental process. We investigated the poorly understood effect of cytokinin on adventitious root formation by altering the expression of the type-B response regulator PtRR13. Significant transcriptional alterations at discrete times may imply when cytokinin antagonizes adventitious root development, and identifies potential points at which cytokinin may interact with ethylene and auxin pathways.

**A molecular genetic roadmap: from intact plants to adventitious roots**

Our results are consistent with a model in which cytokinin, synthesized in the roots and transported throughout the plant body, acts through PtRR13 to repress adventitious root development in intact *Populus* plants. Although cytokinins can be synthesized in various plant organs, significant amounts of root-borne cytokinins are continuously exported to above-ground organs in the xylem sap (Kuroha et al., 2002; Matsumoto-Kitano et al., 2008). Such root-to-shoot transport is thought to signal the nitrogen and nutrient status of the soil and thus coordinate soil nutrient availability with metabolism and development (Samuelson et al., 1992; Hirose et al., 2008). Our finding that constitutive cytokinin activity, implemented via ΔDDKPtRR13 expression, disrupts adventitious root development is consistent with observations that Arabidopsis mutant lines lacking cytokinin receptors develop numerous adventitious roots, and that the cytokinin-insensitive triple mutant *arr1 arr10 arr12* spontaneously develops adventitious roots from the hypocotyl (Werner et al., 2003; Higuchi et
al., 2004; Nishimura et al., 2004; Argyros et al., 2008). While an impact on adventitious root development was not reported, transgene-directed cytokinin biosynthesis in lateral root founder cells disrupted their development from primary roots (Laplaze et al., 2007). We speculate that in intact plants the supply of cytokinin might maintain PtRR13 in an active, phosphorylated state. We note that ΔDDKPtRR13 plants lack a visible phenotype, perhaps because the transgene-encoded protein mimics the predominant form of endogenous PtRR13 in intact plants. In the phosphorylated state, PtRR13 would be expected to transcriptionally regulate downstream targets that collectively repress adventitious root development.

Shoot excision caused a significant reduction in type-A RR transcripts in the stem base, a possible explanation is a disruption in cytokinin supply from the roots. Cytokinin progressively declines within basal portions of cuttings during the first 24 hr after shoot excision, a time period known to be critical for adventitious root development (Blakesley et al., 1985; Bollmark and Eliasson, 1986; Maldiney et al., 1986; Hausman et al., 1997). The results of gene set analysis suggested potential roles for protein phosphorylation (known to alter global protein activity or degradation processes associated with hormone action; Jonak et al., 2002; Ouaked et al., 2003; Nakagami et al., 2005; Yoo et al., 2008) and protein turnover (a mechanism for rapid removal of protein inhibitors and effectors of diverse hormone signaling and stress responses; Dreher and Callis, 2007) which, if they were to involve PtRR13 itself, may target phosphorylated PtRR13 for proteasomal degradation. Accompanied by de novo translation of PtRR13 transcripts, the PtRR13 pool might be quickly shifted to a dephosphorylated (i.e. inactive as a transcription factor) form. Accompanying the removal of cytokinin-mediated repression, adventitious root development could then proceed. The phosphorylation status of RRs affects the extent to which they are proteasomally degraded in plants (Murray et al., 2007), as well as in yeast (Sato et al., 2003) where it is thought to be a mechanism for rapid response to acute changes in osmolarity of the growth medium. Rapid acquisition of tissue capacity for adventitious root development could be similarly adaptive in woody plants, since the acquisition of competence for rooting would occur shortly after shoot detachment in coordination with cellular adaptation to desiccation and tissue repair.
Interactions between cytokinin and other factors

The two most significantly up-regulated genes in the ΔDDKPtRR13 transgenic lines, COV1 and PDR9, may imply effects of cytokinin on vascular tissue formation and cross-talk with auxin signaling, respectively. COV1 is an integral membrane protein that negatively regulates vascular tissue differentiation in stems (Parker et al., 2003). In Arabidopsis, COV1 mutants lack appropriate definition of vascular bundles in the stem, resulting in a continuous ring-like pattern of xylem and phloem with little interfascicular tissue (Parker et al., 2003). The phenotypes of these mutants are independent of auxin and were attributed to the action of an unknown inhibitory factor. If our hypothesis is correct, then the unknown inhibitory factor proposed by Parker et al. (2003) may be cytokinin or cytokinin-dependent. We hypothesize that ΔDDKPtRR13 transcriptionally activates the Populus COV1 homolog, which interferes with establishment of vascular continuity between root primordia and stem vascular tissue.

ΔDDKPtRR13 upregulation of PDR9 could suggest direct effects on auxin distribution. Arabidopsis PDR9 is exclusively expressed in roots and the protein is predicted to act as a 2,4-dichlorophenoxyacetic acid efflux pump (Ito and Gray, 2006). Recent studies by Delker et al. (2008) indicate PDR9 is a genetic suppressor of the SCF complex mutant tir1-1 that is able to restore wild type root growth when supplied with auxin. Similarly in Arabidopsis, transgene-directed cytokinin accumulation in root founder cells caused disorganized cell divisions and disrupted PIN-mediated auxin gradient establishment (Benkova et al., 2003; Geldner et al., 2004). Our results suggest the Populus PDR9 homolog is expressed in stems of ΔDDKPtRR13 lines and may perturb an auxin gradient required for adventitious root initiation or organization.

Ethylene synthesis and action coordinate early responses to tissue injury (Adie et al., 2007; Jackson, 2008), which are likely reflected in the up-regulation of the ethylene biosynthetic enzymes ACC synthase and ACC oxidase. A clue as to how cytokinin interacts withethylene may be found in the observation that ΔDDKPtRR13 expression reduced the transcript abundance of two TINY-like transcription factors. TINYs belong to the DREB (dehydration-responsive element binding protein) subfamily of AP2/ERF transcription factors. Among ERF/AP2 family members, the DREB and ERF (ethylene-responsive element binding factor)
Subfamilies participate in responses to abiotic stress such as mechanical injury and reduced water availability (Shinozaki and Yamaguchi-Shinozaki, 2000; Lorenzo et al., 2003). \textit{TINY} appears to be a positive effector of ethylene action based on its transcript induction in response to ethylene treatment and the triple response phenotype observed when \textit{TINY} is overexpressed in seedlings (Wilson et al., 1996; Sun et al., 2008). Genes encoding other members of the AP2/ERF family, \textit{PLETHORA1} and -2, and \textit{BABY BOOM (BBM)} are up-regulated during actual formation of adventitious roots in culture (Imin et al., 2007). The mechanism by which ΔDDKPtRR13, or other type-B RRs, reduce transcript abundance in the presence of cytokinin are not known, however Arabidopsis lines that lack type-B RRs are impaired in their normal responses to cytokinin treatments, including cytokinin down-regulated genes (Argyros et al., 2008). Our model proposes that shoot excision induces expression of \textit{TINY-like} genes (due to rapid loss of active PtRR13), reflecting a possible mechanism for integrating ethylene and cytokinin signaling.

\textbf{Stages of adventitious root development}

Early responses to shoot excision are complex, as revealed by the differential regulation of about half of the nuclear genes within 24 hr. Adventitious rooting is generally thought to progress in a stepwise manner with discrete stages identified using a combination of hormone measurements and treatments. \textit{In vitro} rooting experiments of apple microcuttings revealed that following a lag period during the initial 24 hours after excision, founder cells acquired a transient state of higher sensitivity to auxin and cytokinin, since exogenous applications of auxin or cytokinin at this time strongly stimulate or inhibit root formation, respectively (De Klerk et al., 1999). Likewise, cytokinin effects on lateral root formation have been shown to be stage-specific with lateral root founder cells sensitive to cytokinin, and lateral root primordia not sensitive (Laplaze et al., 2007). Consistent with these observations, we found a significant (time point X transgene) interaction for gene expression was specific to the 24h time point. We hypothesize that these significant effects reveal the ability of adventitious root founder cells to respond to the rhizogenic stimulus of auxin, and that a low cytokinin:auxin ratio might be required for normal induction of rooting. High cytokinin availability mimicked by the constitutively active PtRR13 at 24h post excision might disrupt this ratio. The transient effects
of ΔDDKPtRR13 on downstream gene expression reinforce the stage-specific nature of cytokinin inhibition of adventitious root development.

The discrete effect of the ΔDDKPtRR13 construct on the timing of adventitious root development merits attention, because it stands in contrast to the pleiotropic effects of ARR1ΔDDK expression in Arabidopsis that includes reduced plant growth, disordered cellular proliferation around the shoot apex and ectopic shoots on cotyledons (Sakai et al., 2001). Distinct phenotypes may reflect functional divergence of *Populus* PtRR13 and Arabidopsis ARR1, or reveal differences in the transformation/regeneration procedures that facilitate the recovery of lines with stronger “alleles” with the Arabidopsis floral dip method compared to the *Populus* tissue culture-based regeneration method. The effect of ΔDDKPtRR13 expression in delaying, but not abolishing, adventitious root development may also reflect selection of comparatively weak transgenic events that could be propagated. Alternatively, ΔDDKPtRR13 may completely disrupt one developmental pathway that is independent of alternative pathways, potentially involving diverse cell type precursors of adventitious roots (Lovel and White, 1986). If this hypothesis is correct, then this study has illuminated only one of several distinct pathways for adventitious root development.

**MATERIALS AND METHODS**

**Plant material and growth conditions**

Root induction experiments were conducted in a greenhouse at ambient temperature between the months of September and December in Gainesville, FL. *Populus tremula* × *Populus alba* INRA-clone No. 717-1-B4 plants were given 12-14 hours of natural light, supplemented in the winter with artificial illumination to maintain indeterminate growth. Plants were grown in 11.4 liter pots on flood benches subirrigated once daily with a nutrient solution containing Peters Professional Blend 20-10-20 fertilizer solution (adjusted to 4 mM nitrogen). When plants reached 60-80 cm tall, single cuttings per plant were collected, planted in 25 cm² pots containing Fafard’s-4 mix potting media and placed under mist until harvested.
1 Real-time PCR

Total RNA was isolated using a cetyltrimethylammonium bromide (CTAB) method (Chang et al., 1993). RNA samples were subjected to DNase treatment with RQ1 RNase-free DNase (Promega, Madison, WI, USA) and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). One μg of DNA-free RNA was used to synthesize first strand cDNAs using oligo-dT primers and M-MLV reverse transcriptase (Promega, Madison, WI, USA). Gene specific primers were designed using the Joint Genome Institute (JGI) assembly for *P. trichocarpa* v1.1 and are given in Supplemental Table III. To avoid non-specific PCR amplification, primers were designed against the most variable regions in the coding sequences using NetPrimer (Premier Biosoft International, Palo Alto, CA, USA), followed by melt curve analysis to verify single products. For each biological sample (i.e., RNA extracted from an individual plant), 3 technical replications were performed, each using 1 μl of a 20 μl reverse transcription reaction on that template RNA. The number of biological replications (samples derived from individual plants) varied from n=3 to n=5 depending on the experiment, and these values are indicated in the figure legend showing the results. Gene expression was quantified using the SYBR Green kit (Stratagene, La Jolla, CA) and Mx3000P thermo-cycler (Stratagene) as per manufacturer’s instructions. The obtained expression values were scaled to the mean expression of the actin2 and ubiquitin control genes.

19 Tissue culture experiments

Stems were harvested from plants grown as described to a height of 60 cm. Leaves were removed and stems were surface-sterilized by four consecutive washes of 70% (v/v) of 95% ethanol; 30% (v/v) of bleach (=5.25% sodium hypochlorite); and two washes with autoclaved deionized water. Stems were cut into 0.5 cm sections and placed on MS-agar plates (0.443 % w/v Murashige and Skoog medium, 0.01 % w/v Myo-inositol, 3% w/v sucrose, 0.65% w/v phytoagar) (w/v) containing the indicated cytokinin:auxin concentration. Plates were placed in a Percival growth chamber under 12 hr light-dark cycles at a constant temperature of 25°C and 775% relative humidity.

29 PtRR13 plasmid construction and transgenic line generation
Complementary DNAs encoding the full-length (FLPtRR13), DDK truncation (ΔDDKPtRR13) and RNAi fragment were amplified by PCR and cloned into the TOPO entry vector (Invitrogen USA, Carlsbad, CA). Cloned sequences were transferred to destination vectors using Gateway technology (Invitrogen USA, Carlsbad, CA) with the FLPtRR13 and ΔDDKPtRR13 cDNA sequences cloned into pZKY1 Overexpress and the RNAi sequence into pZKY2 Direct. Vectors were kindly provided by Oak Ridge National Laboratories and sequence information can be found at http://www.esd.ornl.gov/PGG/foo_vectors.htm#35s%20Overexpression. *Populus tremula* x *Populus alba* INRA-clone No. 717-1-B4 was transformed via an *Agrobacterium*-mediated protocol developed by Han et al. (2000). Twenty independent transformations (lines) per construct were obtained and explants grown on kanamycin selection media. Transgenic lines were screened using PtRR13 specific primers and primers directed against the 35S promoter and octopine synthase inverted repeats (for full-length and DDK truncated lines) or the PIV2 intron (for RNAi lines).

### Microarray analysis

For these analyses, apical cuttings 14 cm in length were collected from NT and ΔDDKPtRR13 plants 60 cm in height. Cuttings were placed in 25 cm$^2$ pots containing Fafard’s-4 mix potting media and placed on a bench with intermittent mist to prevent shoot desiccation. Samples consisting of a 5 mm section measured up from the base of the cutting (one sample per cutting) were collected at the indicated time points. Total RNA was extracted with the RNeasy mini kit (Qiagen USA) and DNase treated in-column with the RNase-Free DNase set (Qiagen USA). Double-stranded cDNA was synthesized using SuperScript Double Strand cDNA Synthesis Kit (Invitrogen USA, Carlsbad, CA) with oligo-dT primers following the manufacturer’s protocol except that the synthesis step was extended to 16 hours. Cy-3 labeling and hybridization steps were performed by NimbleGen using their standard procedures. A custom-designed microarray platform was used comprising single 60-mer probes designed against 55,793 annotated gene models from the sequenced genome of *P. trichocarpa*. Each 60-mer probe was chosen from a group of 6-7 non-overlapping probes designed against different parts of the gene model. The probe whose value was the most similar to the average of the 6-7 non-overlapping probes was used for analysis.
experimental probes was assumed to be the most reliable for transcript level estimation. A total of 40 microarray chips were used in these experiments: 40 chips = 2 genotypes (NT and ΔDDK) x 4 time points (0, 6, 24 and 48 hours) x 5 biological replications. Signal intensities were \( \log_2 \) transformed and quantile normalized (Bolstad et al., 2003). Normalized signals were analyzed in SAS 9.1 (SAS Institute, Cary, NC) using a mixed model analysis of variance (ANOVA) with genotype and genotype by time interactions as fixed effects and biological replication as a random effect. Data are available through the Gene Expression Omnibus (accession GSE15049). Microarray expression data were verified for a subset of significantly regulated genes by real-time PCR obtaining similar gene expression patterns with both analysis methods (Supplemental Figure 5).

**Gene set analysis**

Gene set analysis was performed using the over-representation analysis (ORA) application of the ErmineJ software (Version 2.1.16) which uses the binomial approximation to the hypergeometric distribution to find gene-set enrichment. The corresponding GO annotations of the *Populus* genes were obtained by querying the GO annotation tool of TAIR [http://www.arabidopsis.org/tools/bulk/go/index.jsp](http://www.arabidopsis.org/tools/bulk/go/index.jsp) with the closest Arabidopsis hit for each *Populus* gene. GO annotations were downloaded from the Gene Ontology website [http://www.geneontology.org/GO.downloads.ontology.shtml](http://www.geneontology.org/GO.downloads.ontology.shtml). Only biological processes-related GO categories were considered in the analysis. Genes with contrasts significant at a FDR < 0.5% were used for gene-set enrichment analysis. Gene-sets were determined significant at a FDR < 10%.
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FIGURE LEGENDS

Figure 1. Differentially expressed genes during early stages of adventitious root formation. Microarray expression data was contrasted between 0 and 6h, 6 and 24h and 24 and 48h after shoot excision. Genes whose contrasts were significant at a FDR lower than 5% are displayed in the Venn diagram.

Figure 2. Clusters of differentially regulated genes during early stages of adventitious root formation. Microarray expression data were contrasted between 0 and 6h, 6 and 24h and 24 and 48h after shoot excision. Genes whose contrasts were significant at a 5% FDR and 2-fold regulation at any of the three contrasts were clustered using the Gaussian clustering application from Arrayminer5. Clusters of genes showing overall down-regulation are in green while up-regulated clusters are shown in red. The number of genes in each cluster (n) is displayed. Selected gene families involved in cytokinin, auxin and ethylene signaling/biosynthesis are displayed. Unclustered genes are labeled ‘Unc’.

Figure 3. PtRR13 domain organization and screening of transgenic lines. A, Domain organization of PtRR13 was deduced by amino acid sequence similarity with Arabidopsis RRs (Ramirez-Carvajal et al., 2008). The three coding regions used for construct synthesis included the full-length protein (FL), a truncated version missing the receiver domain (ΔDDK), and a short region (60 a.a.) in the last exon (RNAi). B-C, Transgenic line screening. Transgenic lines carrying the FL or ΔDDK (B) and RNAi (C) constructs were screened for transgene/endogenous PtRR13 expression by real-time PCR and compared to the non transgenic control NT. For all lines, RNA extracted from shoots was reversed-transcribed and used as template in real-time PCR.
1reactions. Fluorescence intensities were normalized to the intensity of the actin control and are
2presented relative to the NT. Error bars represent SE where n = 3.

3Figure 4. PtRR13 is a negative regulator of adventitious root formation. Total root length was
4measured in 14 cm/10 day old cuttings for two independent transgenic lines per construct (FL,
5ΔDDK and RNAi) and in NT (A). The ΔDDKPtRR13 lines exhibit fewer roots than the NT (B), and
6the phenotype is specific to roots (C). Overview of the rooting phenotypes (D). Phloroglucinol-
7HCl stained stem sections at rooting sites (E). Abnormal callus formation at the base in ΔDDK
8lines (F). The rooting experiments described here were repeated a minimum of five times to
9confirm the phenotypes. A typical experiment is shown. Error bars represent SE where n = 15.

10Figure 5. Overall effects of ΔDDKPtRR13 overexpression on gene expression. 11 genes were
11differentially regulated between NT and ΔDDKPtRR13 across all time points (blue bars) at a 10%
12FDR and 2-fold regulation threshold; 4 were up-regulated while 6 were down-regulated in
13ΔDDKPtRR13. An additional 273 genes were differentially regulated between NT and
14ΔDDKPtRR13 at 24 hours at a 1% FDR and 2-fold regulation threshold (orange bars); 160 were
15up-regulated while 113 were down-regulated in ΔDDKPtRR13.

16Figure 6. Hypothesized role of PtRR13 in adventitious root development. In intact plants,
17cytokinin:auxin ratios regulate growth and development. Under these circumstances, a
18continuous supply of root-borne cytokinins maintains RRs, such as PtRR13, in a phosphorylated
19(active) state. Shoot excision alters the normal hormone balance by stimulating ethylene- and
20auxin-dependent pathways (red triangles) and decreasing cytokinin to auxin ratios (blue
21triangles). Abolition of cytokinin supply from the roots down-regulates cytokinin signaling
22leading to inactivation of PtRR13. Adventitious root formation requires that a small group of
1. Root founder cells (FC) become competent to form a root meristematic cell cluster (MCC).

2. Active PtRR13 (phosphorylated or constitutively active ΔDDKPtRR13) plays a negative role by activating transcription of COV1, a negative regulator of vascularization. At the same time, PtRR13 also potentially perturbs root primordia formation by interfering with auxin gradient establishment by stimulating transcription of the auxin efflux pump PDR9, and also by inhibiting stress-ethylene inducible expression of TINY-like transcription factors.

3. Table I. Differentially regulated genes between NT and ΔDDKPtRR13 from 0 to 48 hours.

4. Transgene effects on gene expression during 48 hours following shoot excision were determined by contrasting expression estimates of the ΔDDKPtRR13 line relative to NT across all time points. Genes with contrasts significant at a FDR < 10% and 2-fold regulation are displayed. Significantly regulated Populus gene models were annotated by identifying putative orthologs from GenBank databases (closest hit). Positive fold changes indicate higher transcript abundance in ΔDDKPtRR13, whereas negative fold changes indicate higher transcript abundance in NT.

5. Table II. Top 10 differentially regulated genes between NT and ΔDDKPtRR13 at 24 hr. The genotype x time point interaction was significant only at 24 hr after shoot excision. The top 5 up- and down-regulated genes at a 1% FDR and 2-fold abundance threshold are displayed. Positive fold changes indicate higher transcript abundance in ΔDDKPtRR13 whereas negative fold changes indicate higher transcript abundance in NT.
| Gene name | Gene model | Cluster |
|-----------|------------|---------|
| **Type-A RRs** | | |
| PtRR1 | gw1.VIII.35.1 | 3 |
| PtRR2 | gw1.VIII.329.1 | 3 |
| PtRR3 | gw1.II.42.1 | 3 |
| PtRR10 | grail3.0005005001 | 3 |
| PtRR7 | gw1.XVI.1327.1 | 5 |
| PtRR5 | estExt_Genewise1_v1.C_LG_I7064 | Unc. |
| PtRR9 | gw1.XIII.278.1 | Unc. |
| **Type-B RRs** | | |
| PtRR22 | estExt_fgenesh4_pg.C_LG_XVIII0466 | 2 |
| PtRR14 | gw1.VIII.612.1 | 3 |
| PtRR21 | gw1.X.5015.1 | 3 |
| PtRR13 | estExt_Genewise1_v1.C_LG_X3573 | Unc. |
| **IAAs** | | |
| PoptrIAA3.2 | estExt_fgenesh4_pg.C_LG_XIII0196 | 2 |
| PoptrIAA3.3 | fgenesh4_pm.C_LG_I1000215 | 2 |
| PoptrIAA27.2 | estExt_Genewise1_v1.C_LG_I100268 | 3 |
| PoptrIAA16.4 | grail3.0003037201 | 3 |
| PoptrIAA20.1 | grail3.0050017401 | 4 |
| PoptrIAA3.5 | eugene3.00081508 | 4 |
| PoptrIAA34 | gw1.X.53.1 | 5 |
| PoptrIAA27.1 | eugene3.01570047 | 6 |
| PoptrIAA12.2 | fgenesh4_pm.C_LG_VIII000731 | 6 |
| PoptrIAA20.2 | grail3.0061005101 | 7 |
| PoptrIAA29.2 | eugene3.00181144 | 7 |
| PoptrIAA29.3 | fgenesh4_pg.C_LG_VI000485 | Unc. |
| PoptrIAA33.1 | eugene3.00180818 | Unc. |
| PoptrIAA7.1 | eugene3.00100709 | Unc. |
| PoptrIAA7.2 | estExt_Genewise1_v1.C_LG_VIII2464 | Unc. |
| PoptrIAA15 | estExt_Genewise1_v1.C_LG_I9550 | Unc. |
| PoptrIAA19.3 | estExt_fgenesh4_pm.C_LG_I100099 | Unc. |
| **ARFs** | | |
| PoptrARF1.2 | estExt_fgenesh4_pm.C_860029 | 2 |
| PoptrARF2.2 | eugene3.00150845 | 2 |
| PoptrARF6.2 | estExt_Genewise1_v1.C_LG_XI2869 | 2 |
| PoptrARF6.3 | fgenesh4_pg.C_scaffold_1006000001 | 2 |
| PoptrARF8.1 | gw1.IV.3880.1 | 2 |
| PoptrARF3.1 | estExt_Genewise1_v1.C_LG_IV2935 | 3 |
| PoptrARF6.1 | fgenesh4_pg.C_LG_I002802 | 3 |
| PoptrARF3.2 | fgenesh4_pg.C_scaffold_18700006 | Unc. |
| **ACO oxidases** | | |
| ACO6 | estExt_Genewise1_v1.C_1660131 | 1 |
| ACO2 | eugene3.00002047 | 4 |
| ACO7 | eugene3.00110176 | 4 |
| ACO3 | eugene3.00141061 | 4 |
| ACO4 | grail3.0021002201 | 5 |
| ACO5 | fgenesh4_pg.C_LG_VI000988 | 7 |
| ACO4 | eugene3.00050903 | Unc. |
| ACO7 | eugene3.00051288 | Unc. |
| ACO2 | eugene3.00130446 | Unc. |
| ACO3 | gw1.X.5507.1 | Unc. |
| **ACC synthases** | | |
| fgenesh4_pg.C_scaffold_2423000001 | 1 |
| gw1.XV.3020.1 | 3 |
| ACS8 | fgenesh4_pg.C_LG_I1000152 | 4 |
| gw1.XII.1085.1 | 5 |
| ACS2 | estExt_fgenesh4_pm.C_LG_I10741 | 7 |
| ACS1 | estExt_fgenesh4_pg.C_LG_I0872 | Unc. |
| ACS3 | fgenesh4_pg.C_LG_VI000500 | Unc. |
**A**

- **Total root length (mm)**
  - RNAi
  - FL
  - ΔDDK

**B**

- Above-ground dry weight (g)
  - NT
  - ΔDDK20
  - ΔDDK16

**D**

- Average root number per cutting
  - NT
  - ΔDDK20
  - ΔDDK16

**E**

- Images of plant tissues labeled NT and ΔDDK16.
Auxin

CK

PtRR13

(aactive)

Cytokinin

PDR9

COV1

FC

MCC

TINY-like

PtRR13

(inactive)

Ethylene

Cytokinin

Auxin

Root primordium formation

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