Poplar MYB transcription factor PtrMYB012 and its Arabidopsis AtGAMYB orthologs are differentially repressed by the Arabidopsis miR159 family

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A phenotype-based screening of the T1 transgenic Arabidopsis population transformed by overexpression constructs of the entire poplar MYB transcription factor family found that overexpression of a poplar MYB transcription factor, PtrMYB012, in Arabidopsis resulted in upwardly curled rosette leaves, dwarfishism and male sterility. Sequence analysis identified that PtrMYB012 is homologous to the Arabidopsis GAMYB genes (e.g., AtMYB65 and AtMYB33). Gene expression analysis revealed that PtrMYB012 is specifically expressed in floral tissues, especially in male catkins, similar to AtMYB65. It was well known that Arabidopsis GAMYBs are negatively regulated by microRNA159 (miR159) during vegetative growth; thus, the typical phenotypes of upwardly curled leaves, dwarfishism and male sterility were only shown in overexpression of GAMYBs with mutations in the miR159 target sequence. To confirm our phenotypic consequences, we independently re-produced transgenic Arabidopsis plants overexpressing PtrMYB012 without mutations in the miR159 target sequence. The resulting 35 S::PtrMYB012 Arabidopsis plants phenocopied the previous transgenic Arabidopsis plants, suggesting that PtrMYB012 is probably not a target of Arabidopsis miR159 despite containing the conserved miR159 target sequence. To gain further insight, we produced transgenic poplars overexpressing the intact PtrMYB012. As a result, no conspicuous phenotype was found in 35 S::PtrMYB012 poplar plants. These results suggest that PtrMYB012 transcripts are down-regulated by miR159 in poplar but not in Arabidopsis. Indeed, subsequent 5′-RACE analysis confirmed that PtrMYB012 transcripts are completely degraded in poplar, probably by miR159, but not in Arabidopsis. These results suggest that species-specific family members of miR159 are important for the regulation of normal growth and development in plants.

Keywords: Arabidopsis, AtMYB33/65, GAMYB, microRNA159, Poplar, PtrMYB012.

Introduction

The R2R3 MYB transcription factor (TF) family has been identified as one of the most abundant gene families in plants (Stracke et al. 2001) and is involved in various aspects of plant biology, such as cell fate determination, biotic/abiotic stress resistance, hormone signal transduction and regulation of secondary metabolism (Martin and Paz-Ares 1997, Jin and Martin 1999, Du et al. 2009, Wilkins et al. 2009, Dubos et al. 2010). Poplars are widespread in the northern hemisphere and are used as raw materials in the construction, pulp and bioenergy industries. Poplars have several advantages as a model genetic system of perennial woody plants, including the availability of a wide range of genetic resources, fast growth, prolific sexual reproduction, small genome size and relative ease of genetic modification (Bradshaw et al. 2000, Taylor 2002, Cronk 2005, Jansson and Douglas 2007). Wilkins et al. (2009) reported that Populus
trichocarpa has a total of 192 R2R3 MYB genes; however, many of them have not been investigated yet.

Gibberellins (GA), a group of tetracyclic diterpenoid compounds, play critical roles as plant hormones in plant growth and development, including seed germination, stem elongation, wood formation, and floral and anther development (Jacobsen and Olszewski 1991, Eriksson et al. 2000, MacMillan 2001, Olszewski et al. 2002, Israelsson et al. 2004, Yamaguchi 2008, Plackett et al. 2011), mostly through GAMYBs. GAMYB, a MYB family TF up-regulated by GA, is a transcriptional activator that binds to the GA-response element (e.g., TAACAAA box) in the alpha-amylase promoter in barley (Gubler et al. 1995). HvGAMYB was reported to be involved in GA signaling in the cereal aleurone (Gubler et al. 1995). In addition, HvGAMYB is strongly expressed in anthers, and overexpression of HvGAMYB decreases the size and length of the anther and causes a failure of anthers to dehisce, resulting in a male infertile phenotype (Murray et al. 2003). Later, it was found that GAMYB promotes programmed cell death (PCD) in seeds (aleurone) and anthers (tapetum) (Beers et al. 2000, Fath et al. 2000, Kaneko et al. 2004, Aya et al. 2009, Alonso-Peral et al. 2010). In rice, a loss-of-function gamyb mutant has shorter internodes and is male sterile as the tapetum fails to undergo PCD and degenerates (Kaneko et al. 2004). Similarly, in Arabidopsis, AtMYB33 (AT5G06100.1), AtMYB65 (AT3G11440.1) and AtMYB61 (AT2G32460.1) were identified as GAMYB-like genes that have been implicated in GA signaling in anthers and germinating seeds (Gocal et al. 2001, Woodger et al. 2003, Millar and Gubler 2005). AtMYB33 and AtMYB65 are expressed in seeds and anthers and are specifically regulated by two major miR159 family members, miR159a and miR159b. In other words, miR159 completely represses AtMYB33 and AtMYB65 in vegetative tissues (Alonso-Peral et al. 2010, Li et al. 2016), with weak repression in seeds and anthers (Tsui et al. 2006, Alonso-Peral et al. 2012). In seeds, AtMYB33 and AtMYB65 participate in aleurone vacuolation, a GA-mediated PCD process required for germination (Alonso-Peral et al. 2010). AtMYB33/65 transgenic plants with a mutated target sequence of miR159 exhibited increased expression of AtMYB33/65, upwardly curled leaves, shortened internode length, reduced apical dominance and failure to produce seeds (Millar and Gubler 2005).

To understand the molecular function of the entire poplar MYB TF family, we employed a small-scale forward genetics approach using Arabidopsis as a surrogate. That is, we produced a transgenic Arabidopsis population by overexpressing all of the poplar MYB TFs (a total of 148 genes, selected by removing redundant genes with over 90% identity of amino acid sequence as a criteria) and performed a phenotype-based screening. Among many interesting phenotypic changes (data not shown, unpublished results), in this study, we focused on transgenic plants with upwardly curled rosette leaves and dwarfism. Subsequent analysis identified that the responsible poplar MYB TF gene was PtrMYB012 (Potr001G224500), which is homologous to AtMYB33 and AtMYB65, well known as a GAMYB-like gene in Arabidopsis. Until now, no GAMYB-like genes have been investigated in poplars. In addition, to our knowledge, overexpression of an intact GAMYB does not show any phenotypic changes because of robust negative post-transcriptional regulation by miR159 in vegetative tissues (Alonso-Peral et al. 2010, Li et al. 2016). To understand the molecular basis of PtrMYB012 function, we produced transgenic Arabidopsis and poplar plants overexpressing PtrMYB012. Subsequent experimental results suggest that PtrMYB012 transcripts are completely degraded in poplar, probably by miR159, but not in Arabidopsis. The underlying molecular mechanism will be discussed.

Materials and methods

Plant materials and growth conditions

Hybrid poplars (Populus alba × P. tremula var. glandulosa, clone BH) and Arabidopsis thaliana, ecotype Columbia (Col-0), were used for both non-transformed control plants (WT) and transgenic plants in this study. Plants were grown in either soil or a half-strength MS medium (Murashige and Skoog, Sigma-Aldrich, St. Louis, MO, USA) in controlled conditions in a growth room (14 h light; light intensity, 150 μmol m⁻² s⁻¹; 24 °C). For gibberellin treatment, young leaves of hybrid poplar (30-day-grown in a 15 cm-long test tube) were detached and incubated in a half-strength MS liquid media for 6 h, and then treated GA3 (G7645, Sigma-Aldrich, St. Louis, MO, USA) as indicated (see Figure 3). Samples were immediately frozen in liquid nitrogen until use.

Genomic DNA extraction and DNA sequencing

Genomic DNA was extracted from 3-week-old Arabidopsis leaves using Edward’s buffer (200 mM Tris (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5% SDS) as previously described (Edwards et al. 1991). One microliter of extracted genomic DNA was used as a template for PCR (50 μl reaction) with a pK2GW7 vector-specific primer. The PCR products were electrophoresed on a 1% agarose gel, extracted with a gel extraction kit (GeneAll Expin™ Gel SV, Seoul, Korea), and sequenced (Macrogen http://dna.macrogen.com/kor/).

Vector construction and production of transgenic plants

The full-length genomic DNA encoding PtrMYB012 (Potr001G224500) was amplified from hybrid poplar (clone BH) by PCR and inserted downstream of the 35S promoter in the pK2GW7 vector (Karimi et al. 2002) using the Gateway cloning system to produce 35S:PtrMYB012 constructs. The resulting constructs were verified by DNA sequencing. Vector constructs were introduced into Agrobacterium tumefaciens strain C58, which was used to transform Arabidopsis and hybrid poplar by the floral-dip method (Clough and Bent 1998) and leaf disk transformation-regeneration method (Horsch et al. 1985, Choi et al. 2005), respectively. The transgenic plants were used for all experiments described in this study.
respectively. Transformed cells from poplar were selected on MS medium containing 1.0 mg l\(^{-1}\) 2,4-dichlorophenoxyacetic acid, 0.1 mg l\(^{-1}\) benzylaminopurine, 0.01 mg l\(^{-1}\) 1-naphthalacetic acid (NAA), 500 mg l\(^{-1}\) cefotaxime and 50 mg l\(^{-1}\) kanamycin. Shoots were regenerated from calli by transferring them to woody plant medium containing 1.0 mg l\(^{-1}\) zeatin, 0.1 mg l\(^{-1}\) benzyladeneine and 0.01 mg l\(^{-1}\) NAA. Throughout the experiments, cultures were maintained in a culture room at 25 ± 2 °C under cool white fluorescent light (150 μmol m\(^{-2}\) s\(^{-1}\), 14 h photoperiod).

**Histological analysis**

For microtome sectioning, leaves were collected from 22-day-old soil-grown WT and 35 S::PtrMYB012 Arabidopsis plants, fixed with formaldehyde–acetic acid–ethanol. After fixation, the tissue was dehydrated with increasing concentrations of ethanol. The ethanol was then replaced with increasing xylene concentrations, and paraffin was dissolved in the tissue. After embedding, the paraffin-containing tissue was cut and cross-sectioned using a microtome (JUNG RM 2045, Leica, Wetzlar, Germany). The sectioned samples were stained with 0.05% toluidine blue O for 1 min and observed under a light microscope (CHB-213, Olympus, Tokyo, Japan).

**RNA extraction and semi-quantitative RT-PCR**

Total RNA was extracted using TRIZOL reagent (Ambion, Auxin, TX, USA) according to the suggested method with slight modifications. One microgram of total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in a 20 μl reaction. RT-PCR was carried out using 1 μl of the twofold diluted reaction product as a template. Amplified DNA fragments were separated on 1% agarose gel and visualized with ethidium bromide staining.

**Quantitative real-time PCR (qRT-PCR) validation**

The expression patterns of genes were analyzed by quantitative real-time PCR (qRT-PCR) (Song et al. 2010). All primer sequences were generated using the Primer3 program (http://fokker.wi.mit.edu). As a quantitative control, primers were also designed for the actin gene (An et al. 1996). Primer sequences for the selected transcripts are provided in Table S1 available as Supplementary Data at Tree Physiology Online. Data were analyzed with two-tailed, unpaired t-test by using GRAPHPAD PRISM 7.03 (Graphpad Software, San Diego, CA, USA). P-values of <0.05 were considered significant.

**Modified 5’-Rapid Amplification of cDNA Ends (5’-RACE)**

The GeneRacer Kit (Invitrogen) was used according to the manufacturer’s instructions with some modifications. Briefly, two μg of the purified total RNA was ligated directly to 250 ng of the provided RNA oligo adaptor, without the de-capping step described in the manufacturer’s protocol. Gene-specific 5’-RACE reactions were performed with a GeneRacer 5’-Nested Primer and a PtrMYB012 gene-specific nested primer (see Table S1 available as Supplementary Data at Tree Physiology Online). PCR was performed using the following settings: 1 cycle at 94 °C per 5 min; 32 cycles at 95 °C per 30 s, 60 °C per 30 s, and 72 °C per 2 min; 1 cycle at 72 °C for 5 min. A 5’-RACE product of the expected size (~450 bp) was extracted from the gel, purified, cloned into a pGEM-T vector (Promega, Madison, WI, USA) and sequenced.

**Results**

**Identification of a poplar MYB gene responsible for transgenic Arabidopsis plants with upwardly curled rosette leaves and dwarfism**

During phenotypic characterization of a transgenic Arabidopsis population transformed by overexpression constructs of the entire poplar MYB TF family (data not shown), two plants (#290 and #326) showed upwardly curled rosette leaves with dwarfism (Figure 1). To confirm which poplar MYB genes were inserted in the genome of these plants, we performed genomic DNA PCR using 35 S forward primer (35 S_pF) and attB2 reverse primer (attB2) (Figure 1b) and sequenced the resulting PCR products. Sequencing results indicated that the responsible

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**Figure 1.** Identification of a poplar MYB gene responsible for transgenic Arabidopsis plants with upwardly curled rosette leaves and dwarfism. (a) Twenty-day-old soil-grown transgenic Arabidopsis plants showing upwardly curled rosette leaves with dwarfism (#290 and #326) compared with wild type (WT). Size bars indicate 1 cm. (b) Diagram indicating the Gateway vector constructs cloned PtrMYB012 gene for overexpression by 35 S promoter. (c) Genomic DNA-PCR to confirm the introduction of the PtrMYB012 vector construct in both transgenic Arabidopsis plants (#290 and #326). The plasmid vector construct of the PtrMYB012 (i.e., vector) was used as a positive control. 35 S_pF is a forward primer for the 3’ region of the 35 S promoter, and attB2 is a reverse primer for the attB2 region of the pK2GW7 vector (Karimi et al. 2002). PtrMYB012_R is the PtrMYB012 gene-specific reverse primer.
gene was PtrMYB012 (Potri.001G224500) in both plants (#290 and #326) (data not shown) (see Figure S1 available as Supplementary Data at Tree Physiology Online). This finding was further confirmed by another genomic DNA PCR using 35 S forward primer (35_S_pF) and PtrMYB012 gene-specific reverse primer (PtrMYB012_R), resulting in identical PCR products from both plants (#290 and #326), as well as a vector construct containing the PtrMYB012 gene (Figure 1b and c).

Sequence analysis showed that the PtrMYB012 gene is similar to Arabidopsis AtMYB65 (AT3G11440.1) and AtMYB33 (AT5G06100.1), which are known as GAMYB-like genes (Figure 2a; Alonso-Peral et al. 2010). The amino acid sequence of PtrMYB012 was highly conserved with several other GAMYB genes, especially the MYB DNA binding domain and miR159 target sequence (Figure 2b). Previously, it was reported that the AtMYB33/65 transgenic Arabidopsis plant mutated at the miR159 binding site showed upwardly curled leaves with dwarf phenotype (Allen et al. 2007), which is very similar to the phenotype of our transgenic plants (#290 and #326). However, our transgenic plants have overexpression of an intact PtrMYB012 gene rather than a mutation in the miR159 target sequence.

Tissue-specific expression of PtrMYB012 in the poplar tree was examined using qRT-PCR. Our result showed that PtrMYB012 is specifically expressed in flower (i.e., male catkin) tissue (Figure 3a). Further in silico analysis using the poplar
eFP browser (http://bar.utoronto.ca/efppop/cgi-bin/efpWeb.cgi) showed that PtrMYB012 has the strongest expression in male catkins (see Figure S2a available as Supplementary Data at Tree Physiology Online), which is consistent with our result. This expression pattern of PtrMYB012 is similar to that of AtMYB65, which has the highest expression in anthers (see Figure S2b available as Supplementary Data at Tree Physiology Online). In addition, we found that the expression of PtrMYB012 is upregulated by GA, which was confirmed by exogenous GA3 treatment on poplar leaves (Figure 3b). To verify this experiment, we checked the expressions of PtrGA20ox (Potri.015G134600.1) and PtrGA2ox (Potri.010G149700.1). As expectedly, PtrGA20ox, a GA biosynthetic gene, was down-regulated while PtrGA2ox, a GA catalyzing gene, was upregulated by GA3 treatment (Figure 3c and d; see Figure S2c available as Supplementary Data at Tree Physiology Online). These results suggest that PtrMYB012 is orthologous to AtMYB65, a GAMYB-like gene in Arabidopsis.

**Confirmation of PtrMYB012 as the gene responsible for the phenotypic consequences**

To confirm whether PtrMYB012 is the gene responsible for the phenotypic consequences in the transgenic plants (#290 and #326), we produced transgenic plants overexpressing an intact PtrMYB012 gene (35S::PtrMYB012) in both Arabidopsis and poplar. Among a total of 19 independent T1 35S::PtrMYB012 Arabidopsis plants, 12 of them showed a similar phenotype to #290 and #326 plants (data not shown). To perform additional detailed analyses, we selected two T3 homozygous transgenic plants (i.e., 15-2 and 16-4 lines of 35S::PtrMYB012) (Figure 4). For example, the 35S::PtrMYB012 plants showed upwardly curled rosette leaves (Figure 4a) and dwarfing statures compared with WT (Figure 4c and d). These phenotypic changes were correlated with expression level of the introduced PtrMYB012 gene (Figure 4b).

To understand the upwardly curled rosette leaf phenotype of the 35S::PtrMYB012 plants, we examined the cellular structures of the upwardly curled leaves by microtome-sectioning (see Materials and methods) (Figure 5). In WT plants, the numbers of epidermal cells on the adaxial and abaxial sides were not significantly different (around 15.5% more cells on the abaxial side). However, the number of adaxial cells was significantly reduced in the 35S::PtrMYB012 plants (#16-4), which had 30% less cells on the adaxial than abaxial side (Figure 5b). Next, we measured the sizes of the epidermal cells on both sides. Interestingly, in WT plants, cells on the adaxial side were significantly bigger (~31%) than cells on the abaxial side, but there were no significant differences when comparing the cell sizes in 35S::PtrMYB012 plants (#16-4), although the overall cell size was much smaller than WT (Figure 5c). Thus, we hypothesize that the differences in cell growth and proliferation on both sides of the epidermis in 35S::PtrMYB012 plants causes the upwardly curled leaf phenotype.

**35S::PtrMYB012 plants showed reduced fertility**

35S::PtrMYB012 plants showed reduced fertility with smaller flowers than WT plants in the maturation stage (Figure 6). Based on observation of the anthers of 35S::PtrMYB012 plants (#16-4), these plants seem to have a defect in anther dehiscence because most of the pollen could not be released (Figure 6b). Next, we measured the length of both the carpels and stamens of 35S::PtrMYB012 plants in comparison with WT plants. In 35S::PtrMYB012 plants (#16-4), the stamens were drastically shorter than the carpels (~40%), while there were no significant differences between stamen and carpel length in WT plants (Figure 6c).

These phenotypes (e.g., defects in anther dehiscence and stamen elongation), which are consistent with HvGAMYB overexpression (Murray et al. 2003), could explain the reduced fertility of 35S::PtrMYB012 plants. Thus, 35S::PtrMYB012 plants also have defects in silique development (Figure 6d-f). The length of the siliques in 35S::PtrMYB012 #16-4 plants was only 50.7% compared with WT (Figure 6d and e). In addition, the percentage of normal siliques in WT was 98%, whereas it was only 20% in 35S::PtrMYB012 #16-4 (Figure 6f).

**Expression changes of GAMYB-regulated genes in 35S::PtrMYB012 plants**

Previously, Alonso-Peral et al. (2010) reported a list of GAMYB regulated genes in Arabidopsis identified using whole transcriptome analysis. They found 121 up-regulated and 45 down-regulated genes in the miR159ab mutant compared with WT. To determine if 35S::PtrMYB012 plants have a similar transcription phenotype as a GAMYB over-expressor, we performed a qRT-PCR analysis by selecting four up-regulated genes, CYSTEINE PROTEINASE1 (AtCP1, AT4G36880), beta-ZYLOSIDASE1 (AtBXL1, AT5G49360), DISURUPTED MEIOTIC CONTROL1 (AtDMC1, AT3G22880) and Nodulin MtN21 transporter (AtNMT, AT4G28040), and one down-regulated gene, ACC OXIDASE1 (AtACO1, AT2G19590) (Figure 7). Our result revealed that the expression of AtCP1, AtBXL1, AtDMC1 and AtNMT was clearly increased, while AtACO1 expression was decreased in 35S::PtrMYB012 plants compared with WT (Figure 7), which is consistent with the previous result (Alonso-Peral et al. 2010).

Interestingly, the expression of endogenous AtMYB33 and AtMYB65 genes were significantly down-regulated in 35S::PtrMYB012 plants compared with WT (Figure 8a and b). We hypothesized that these results come from the increase of endogenous miR159. Subsequent qRT-PCR analysis of both miR159a and miR159b in 35S::PtrMYB012 plants provided support for our hypothesis. Expressions of miR159a and miR159b were up-regulated significantly in 35S::PtrMYB012 #16-4 compared with WT (Figure 8c and d).
Figure 3. *PtrMYB012* is specifically expressed in flower tissues and upregulated by GA in poplar. (a) Quantitative real-time PCR (qRT-PCR) analysis of *PtrMYB012* expression using various poplar tissues (e.g., petiole, SAM (shoot apical meristem), stem, flower (i.e., male catkin), leaf and root). (b–d) qRT-PCR analyses of *PtrMYB012* (Potri.001G224500.1) (b), *PtrGA20ox* (Potri.015G134600.1) (c) and *PtrGA2ox* (Potri.010G149700.1) (d) expressions in response to GA3 treatment in poplar leaf tissues. Treatment time and concentrations of GA3 are indicated. Error bars indicate SE of three biological replications (*P*-value < 0.05, **P*-value < 0.01, ***P*-value < 0.001, unpaired t-test).

Figure 4. Confirmation of *PtrMYB012* as the gene responsible for the phenotypic consequences. (a) Upwardly curled rosette leaves were observed in #290 and T3 homozygotes (#15-2 and #16-4) of 35S::*PtrMYB012* Arabidopsis plants grown on soil for 20 days. (b) Up-regulation of *PtrMYB012* expression in transgenic Arabidopsis plants using semi-quantitative RT-PCR. (c) Strong dwarfsisms in 60-day-old transgenic Arabidopsis plants compared with WT. (d) Measurement of stem heights shown in (c). Error bars indicate S.E. (n = 12) (**P*-value < 0.01, ****P*-value < 0.0001, unpaired t-test). Scale bars in (a) and (c) indicate 1 and 10 cm, respectively.
Overall, these data indicate that 35 S::PtrMYB012 plants phenocopied the GAMYB over-expressor (e.g., 35 S::AtMYB33m or miR159ab) not only phenotypically, but also transcriptionally. Thus, we conclude that PtrMYB012 is orthologous to AtMYB33/65 as a positive regulator of GA signaling.

35 S::PtrMYB012 transgenic poplars showed no significant phenotypes

To assess the phenotypic consequences of PtrMYB012 overexpression in poplar, we produced 13 independent transgenic poplars containing the same vector construct (35 S::PtrMYB012) that was used to produce the transgenic Arabidopsis plants. Interestingly, our transgenic poplar plants have no significant phenotypic changes in leaf shape or stem elongation (Figure 9).

PtrMYB012 transcripts were only partially degraded in Arabidopsis

PtrMYB012 has a conserved miR159 target sequence similar to Arabidopsis GAMYBs (Figures 2 and 10a) (Achard et al. 2004). To confirm that miR159 regulates PtrMYB012 transcripts, we performed a 5′-RACE cleavage assay using total RNA extracted from the leaf tissue of each 35 S::PtrMYB012 Arabidopsis and poplar transgenic plant. As a result, a digested PtrMYB012 transcripts of expected size (~0.45 kb), probably by miR159, was found in both transgenic plants and sequence analysis of this band revealed that the cleavage of the target sequence occurred at the 10th nucleotide of the miR159 locus (Figure 10b). However, intact PtrMYB012 transcripts (~1.2 kb) were strongly amplified in 35 S::PtrMYB012 transgenic Arabidopsis, while no such products were detected in poplar transgenic plants (Figure 10b). This result suggests that there might be problems in degrading PtrMYB012 transcripts in Arabidopsis. Therefore, the intact PtrMYB012 transcripts still exist in 35 S::PtrMYB012 Arabidopsis plants and, thus, exhibit the typical phenotypes. However, in the poplar, all of the PtrMYB012 transcripts were degraded completely, so the phenotypes did not appear.

Discussion

Despite the recent research on the R2R3 MYB transcription factors in diverse organisms, still many of these genes have not been characterized yet. During phenotype-based screening, we found two transgenic Arabidopsis plants that have upwardly curled rosette leaves with a dwarfish phenotype, and subsequent molecular analysis identified the responsible gene as PtrMYB012 (Figure 1). Using the amino acid sequence of PtrMYB012, we found that the closest Arabidopsis gene was ATMYB65 (Figure 2), one of the GAMYB genes related to GA signaling (Alonso-Peral et al. 2010).

The most prominent phenotype of 35 S::PtrMYB012 Arabidopsis plants was upwardly curled rosette leaves (Figures 1a and 4a). From our leaf anatomy experiment, we found that cell proliferation
of the adaxial epidermis was significantly reduced compared with the abaxial epidermis (Figure 5b), but cell size was similar on both sides of 35 S::PtrMYB012 Arabidopsis leaves (Figure 5c). However, the cell size was significantly smaller than that of WT leaves (Figure 5c). Thus, we hypothesize that these discrepancies in cell growth and proliferation cause the upwardly curled leaf phenotype. In support of this notion, the expression of AtCP1 and AtBXL1, known to be associated with autolysis and cell death (Fath et al. 2000), was significantly increased in leaf tissues of 35 S::PtrMYB012 Arabidopsis plants compared with WT (Figure 7). Previously, it was reported that the CP1 gene from rice was involved in programmed cell death in anthers (Lee et al. 2004, Lee et al. 2007). Indeed, these genes were down-regulated in the myb33myb65myb101 mutant aleurone (Alonso-Peral et al. 2010). The dwarving phenotype of 35 S::PtrMYB012 Arabidopsis plants could also be affected by these genes. However, the reason for the differential inhibition of cell proliferation on both sides of the epidermis of 35 S::PtrMYB012 Arabidopsis plants is currently unknown.

GAMYB regulates flowering time in Arabidopsis (Blazquez et al. 1998, Blazquez and Weigel 1999) and is associated with anther development (Murray et al. 2003, Kaneko et al. 2004, Tsuji et al. 2006, Aya et al. 2009). Consistent with HvGAMYB overexpression (Murray et al. 2003), 35 S::PtrMYB012 Arabidopsis plants showed defects in anther development (e.g., defects in anther dehiscence and stamen elongation) (Figure 6). Expression of the DMC1 gene is restricted to pollen mother cells in anthers and to
megaspore mother cells in ovules (Couteau et al. 1999), and CP1 is important in the fertility of anthers in rice (Lee et al. 2004). Up-regulation of these GA-inducible genes in 35 S::PtrMYB012 Arabidopsis plants (Figure 7) might be involved in the reduced fertility phenotype (Figure 6).

Through comparison of the amino acid sequence of PtrMYB012 with GAMYB genes in various species, we found that their amino acid sequences were very similar and have a conserved miR159 target sequence (Figures 2 and 10a). Previously, transgenic Arabidopsis plants overexpressing either AtMYB33 or AtMYB65 show no obvious pheno-type consequences on plant growth because miR159 cleaved AtMYB65/33 genes in vegetative tissues (Palatnik et al. 2003, Li et al. 2016). However, transgenic Arabidopsis plants overexpressing either AtMYB33 or AtMYB65 with a mutated miR159 target sequence showed the typical phenotypes of dwarfism, upwardly curled leaves and male sterility (Achard et al. 2004, Allen et al. 2007, 2010, Li et al. 2014).

Interestingly, even though we did not mutate the miR159 target sequence, 35 S::PtrMYB012 Arabidopsis showed the typical phenotypes (Figures 1, 4 and 6). However, 35 S::PtrMYB012 poplar plants did not show any conspicuous phenotypes compared to WT (Figure 9). To understand the molecular mechanism underlying this finding, we performed 5’-RACE using each transgenic plant (Figure 10). In the 35 S::PtrMYB012 Arabidopsis plant, PtrMYB012 transcripts were only partially degraded, probably by miR159. However, in the poplar transgenic plant, the PtrMYB012 transcripts were completely degraded. Although PtrMYB012 has a conserved miR159 target sequence, the sequence in poplar differs slightly from the Arabidopsis sequence in that two nucleotides are different (Figure 10a). Thus, we hypothesize that the binding efficiency of Arabidopsis miR159 to PtrMYB012 transcript is lower than that of AtMYB33/65, causing the incomplete degradation of PtrMYB012 transcripts in 35 S::PtrMYB012 Arabidopsis plants. In support of this hypothesis, the poplar has a more diverse miR159 family consisting of six members while there are only three miR159 family members in Arabidopsis (Figure 10a). Among poplar miR159 family members, Ptc-miR159c, Ptc-miR159e and Ptc-miR159f have a different nucleotide corresponding to miR159 target sequence of PtrMYB012. Interestingly, other plant species such as grape (Vitis vinifera), soybean (Glycine max) and maize (Zea mays) also have miR159 members similar to those of poplar (see Figure S3 available as Supplementary Data at Tree Physiology Online). To address this hypothesis, genetic complementation experiments in both Arabidopsis and poplar will be required by using Arabidopsis and poplar miR159 members and with mutated miR159 target sequence of PtrMYB012.
The miR159 family has been found to regulate the expression of GAMYBs or GAMYB-like genes by binding to the conserved miR159 target sequence in plant species separated by considerable evolutionary distance, including monocots, dicots and gymnosperms (Tsuji et al. 2006, Palatnik et al. 2007, Csukasi et al. 2012, Li et al. 2013, Yang et al. 2014, Zheng et al. 2017). This indicates that the miR159–MYB relationship is critical in plant growth and development. In loss-of-function mutations of all three Arabidopsis miR159 family members with differences in only one or two nucleotides, it was found that each mutant did not function properly in a similar way (Rajagopalan et al. 2006, Allen et al. 2007, 2010). Recently, Li et al. (2014) reported that although high miRNA:target complementarity may be a prerequisite for a strong silencing of target transcripts, many additional factors including flanking nucleotide sequences of targets are important for complete silencing. Indeed, Zheng et al. (2017) suggested that the distinct RNA secondary structure of flanking sequence of the target miR159 binding site is a major determinant of miR159 efficacy. Since PtrMYB012 has the conserved two stem-loops structure predicted in AtMYB33/65 (see Figure S4 available as Supplementary Data at Tree Physiology Online), PtrMYB012 is probably a sensitive targets of miR159 (Zheng et al. 2017).

Conclusively, we found a poplar PtrMYB012 gene that is a GAMYB ortholog, suggesting that the GA-GAMYB signaling pathway is probably conserved in this woody plant species. In

Figure 8. Expression of endogenous AtMYB33 and AtMYB65 genes were down-regulated in 35 S::PtrMYB012 plants. (a) AtMYB33 (ATSG00100.2), (b) AtMYB65 (AT3G11440.1), (c) miR159a (miRNA159a, AT1G73687) and (d) miR159b (miRNA159b, AT1G18075). *P-value <0.05, unpaired t-test. Quantitative real-time PCRs were performed using the first-stand cDNA synthesized from leaf total RNA extracted from 40-day-old plants. Error bars indicate SE of three biological replications (*P-value <0.05, **P-value <0.01, ***P-value <0.001, unpaired t-test).

Figure 9. Phenotypic characterization of 35 S::PtrMYB012 poplar transgenic plants. (a) Overall growth of 35 S::PtrMYB012 poplar plants compared with non-transformed control plants (WT). (b) Leaf morphology of WT and 35 S::PtrMYB012 poplar plants. (c) Stem height of 35 S::PtrMYB012 poplar plants compared with WT. Error bars indicate SE (n = 6).

The miR159 family has been found to regulate the expression of GAMYBs or GAMYB-like genes by binding to the conserved miR159 target sequence in plant species separated by considerable evolutionary distance, including monocots, dicots and gymnosperms (Tsuji et al. 2006, Palatnik et al. 2007, Csukasi et al. 2012, Li et al. 2013, Yang et al. 2014, Zheng et al. 2017). This indicates that the miR159–MYB relationship is critical in plant growth and development. In loss-of-function mutations of all three Arabidopsis miR159 family members with differences in only one or two nucleotides, it was found that each mutant did not function properly in a similar way (Rajagopalan et al. 2006, Allen et al. 2007, 2010). Recently, Li et al. (2014) reported that although high miRNA:target complementarity may be a prerequisite for a strong silencing of target transcripts, many additional factors including flanking nucleotide sequences of targets are important for complete silencing. Indeed, Zheng et al. (2017) suggested that the distinct RNA secondary structure of flanking sequence of the target miR159 binding site is a major determinant of miR159 efficacy. Since PtrMYB012 has the conserved two stem-loops structure predicted in AtMYB33/65 (see Figure S4 available as Supplementary Data at Tree Physiology Online), PtrMYB012 is probably a sensitive targets of miR159 (Zheng et al. 2017).

Conclusively, we found a poplar PtrMYB012 gene that is a GAMYB ortholog, suggesting that the GA-GAMYB signaling pathway is probably conserved in this woody plant species. In
addition, we suggested a hypothesis that species-specific family members of miR159 may be important for normal growth and development in plants.

Supplementary Data
Supplementary Data for this article are available at Tree Physiology Online.

Conflict of interest
None declared.

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