Control of trichome formation in *Arabidopsis* by poplar single-repeat R3 MYB transcription factors

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INTRODUCTION

Single-repeat R3 MYB transcription factors (R3 MYBs) are small proteins that typically contain ~100 amino acids, largely consist of a single MYB DNA-binding repeat, and are best characterized for their regulatory roles in trichome and root hair development (Wang and Chen, 2014). R3 MYBs are widely distributed in the plant kingdom, and they are encoded by a subset of MYB transcription factor genes (Dubos et al., 2010). In *Arabidopsis*, there are a total of seven genes encoding R3 MYBs, including *TRYPTICHLON* (*TRY*; Schnittger et al., 1999), *CAPRICE* (*CPC*; Wada et al., 1997), *TRICHOMELESS1* (*TCL1*; Wang et al., 2007), *TCL2/CAPRICE-LIKE MYB4* (*CPL4*; Gan et al., 2011), *TOMINAGA-WADA and NUKUMIZU* (2012), *ENHANCER OF TRY AND CPC1* (*ETC1*; Esch et al., 2004), *ETC2* (Kirik et al., 2004a), and *ETC3/CPL3* (Simon et al., 2007; Tominaga et al., 2008). All seven R3 MYBs contain the residues [D/E]×2[R/K]×3L×6L×3R, a conserved amino acid signature required for interaction of MYBs with R/B-like bHLH transcription factors (Zimmermann et al., 2004), and W×M, a sequence motif that has been shown to be required for cell-to-cell movement of CPC (Kurata et al., 2005).

Trichome formation in *Arabidopsis* is controlled by the interplay of R3 MYBs and several other transcription factors including the WD40-repeat protein TRANSPARENT TESTA GLABRA1 (TTG1), the R2R3 MYB transcription factor GLABRA1 (GL1), the bHLH transcription factor GLABRA3 (GL3) or ENHANCER OF GLABRA3 (EGL3), and the homeodomain protein GLABRA2 (GL2). R3 MYBs including TRICHOMELESS1 (TCL1), TCL2, TRYPTICHLON (TRY), CAPRICE (CPC), ENHANCER OF TRY AND CPC1 (ETC1), ETC2 and ETC3 negatively regulate trichome formation by competing with GL1 for binding GL3 or EGL3, thus blocking the formation of TTG1–GL3/EGL3–GL1, an activator complex required for the activation of the trichome positive regulator gene GL2. However, it is largely unknown if R3 MYBs in other plant species especially woody plants have similar functions. By BLASTing the *Populus trichocarpa* protein database using the entire amino acid sequence of TCL1, an *Arabidopsis* R3 MYB transcription factor, we identified a total of eight R3 MYB transcription factor genes in poplar, namely *P. trichocarpa TRICHOMELESS1* through *8* (*PtrTCL1–PtrTCL8*). The amino acid signature required for interacting with bHLH transcription factors and the amino acids required for cell-to-cell movement of R3 MYBs are not fully conserved in all *PtrTCLs*. When tested in *Arabidopsis* protoplasts, however, all *PtrTCLs* interacted with GL3. Expressing each of the eight *PtrTCL* genes in *Arabidopsis* resulted in either glabrous phenotypes or plants with reduced trichome numbers, and expression levels of GL2 in all transgenic plants tested were greatly reduced. Expression of *PtrTCL1* under the control of *TCL1* native promoter almost completely complemented the mutant phenotype of *tcl*. In contrast, expression of *PtrTCL1* under the control of *TRY* native promoter in the *try* mutant, or under the control of *CPC* native promoter in the *cpc* mutant resulted in glabrous phenotypes, suggesting that *PtrTCL1* functions similarly to *TCL1*, but not *TRY* and *CPC*.

Keywords: trichome formation, R3 MYBs, transcription factors, *Arabidopsis*, *Populus trichocarpa*
In addition to competing with GL1 for binding GL3, some of the R3 MYBs including TCL1 and TCL2 also directly suppress the expression of GL1 (Wang et al., 2007; Gan et al., 2011). Not all the R3 MYB genes in *Arabidopsis* are activated by the TGT1–GL3/EGL3–GL1 activator complex (Wang et al., 2008), and microRNA156 (MIR156)-targeted SQAMOSA PROMOTER BINDING PROTEIN LIKE (SPL) 9 has been shown to activate TCL1, TCL2, and TRY (Yu et al., 2010; Gan et al., 2011). These results indicate that R3 MYBs may use different mechanisms to regulate trichome formation in *Arabidopsis*.

Although single mutants of *Arabidopsis* R3 MYB genes have different phenotypes (Wada et al., 1997, 2002; Schnittger et al., 1999; Schellmann et al., 2002; Wang et al., 2007), over-expression of any of the R3 MYB genes in *Arabidopsis* resulted in glabrous phenotypes. Analysis of double, triple, and higher mutants also revealed that all the seven R3 MYB functions in a highly redundant manner to control trichome formation (Esch et al., 2004; Kirik et al., 2004a,b; Wang et al., 2007, 2008; Tominaga-Wada et al., 2008; Wester et al., 2009; Gan et al., 2011).

Functional homologs of some of these transcription factors that regulate trichome formation have been identified in other plants. For example, GaHOX1 from cotton has been identified as a functional homolog of GL2 (Guan et al., 2008) and GaMYB2 as a functional homolog of GL1 (Wang et al., 2004; Guan et al., 2011, 2014). MYB like genes from *Mimulus guttatus* and peach regulate trichome formation (Scoville et al., 2011; Vendramin et al., 2014), and expression of a tomato R3 MYB gene in *Arabidopsis* resulted in glabrous phenotypes (Tominaga-Wada et al., 2013). On the other hand, expression of *Arabidopsis* GL3 in *Brassica napus* induced ectopic trichome formation (Gruber et al., 2006). These results suggest that trichome formation in other plant species may be controlled by similar mechanisms as in *Arabidopsis*. However, trichome regulators in plants other than *Arabidopsis* remain largely unidentified.

*Populus trichocarpa* is the first tree whose genome has been fully sequenced (Tuskan et al., 2006), and it is also a good model plant for studies in areas such as wood development, ecological interactions, and other aspects of perennial plants that cannot be studied in the annual model plant *Arabidopsis* (Groover, 2005; Whitham et al., 2006; Jansson and Douglas, 2007). By using the entire amino acid sequence of TCL1 to BLAST search the *P. trichocarpa* protein database, we found there are a total of eight genes in poplar encoding R3 MYB transcription factors, namely *P. trichocarpa* TRICHOMELESSI through 8 (*PtrTCL1–PtrTCL8*).

In the study described here, we examined if R3 MYBs from poplar can functionally substitute for *Arabidopsis* R3 MYBs to regulate trichome formation in *Arabidopsis* plants.

**MATERIALS AND METHODS**

**IDENTIFICATION OF POPULAR HOMOLOGS OF Arabidopsis R3 MYB TRANSCRIPTION FACTOR TCL1**

To identify poplar homologs of *Arabidopsis* R3 MYB transcription factors, the entire amino acid sequence of *Arabidopsis* R3 MYB transcription factor TCL1 was used in BLAST searches of the *P. trichocarpa* proteome (www.phytozome.net). The entire amino acid sequences of identified poplar R3 MYB transcription factors were then used in BLAST searches until no more poplar R3 MYBs were identifiable. Full-length amino acid sequences of *Arabidopsis* and poplar R3 MYBs were subjected to phylogenetic analysis using “One Click” mode of Phylogeny (www.phylogeny.fr) with default settings.

**PLANT MATERIALS AND GROWTH CONDITIONS**

Poplar xylem tissue from *P. trichocarpa* was collected as described previously (Geraldes et al., 2011; Liu et al., 2013; Wang et al., 2014), and used for RNA isolation and poplar R3 MYB gene cloning. The *tcl1* and *try* mutants, and the 35S:*HA-TCL1* transgenic plant were in the Columbia-0 (Col-0) background (Esch et al., 2003; Wang et al., 2007). The *cpc* mutant was in theWs background (Wada et al., 1997).

Unless specified otherwise, *Arabidopsis* ecotype Col-0 was used for plant transformation. Seedlings used for RNA isolation were obtained by growing sterilized seeds on 1/2 Murashige and Skoog (MS) basal medium with vitamins (Plantmedia) and 1% (w/v) sucrose. Seedlings used for phenotypic analysis were obtained either by growing seeds on 1/2 MS medium or by directly sowing seeds into soil. All plants were grown in growth rooms at 22°C with 14/10 h photoperiod, and light density of approximately 120 μmol m−2 s−1.

**RNA ISOLATION AND RT-PCR**

Total RNA from poplar samples was isolated using PureLink Plant RNA Reagent (Invitrogen), and cleaned with RNeasy Plant Mini Kit (Qiagen) as described previously (Geraldes et al., 2011; Wang et al., 2014). Total RNA from *Arabidopsis* seedlings was isolated using EasyPure™ Plant RNA Kit (Transgene) according to the manufacturer’s instructions. All RNA samples were treated with RNase-Free DNase set (Qiagen) to eliminate possible DNA contamination.

cDNA was synthesized using 2 μg total RNA by Oligo(dT)-primed reverse transcription using Omniscript RT Kit (Qiagen). Some of the primers used for cloning or examining the expression of corresponding genes have been described previously (Wang et al., 2007, 2008, 2010; Gan et al., 2011), and poplar R3 MYB gene-specific primers are shown in Table 1.

**CONSTRUCTS**

To generate HA (human influenza hemagglutinin)- or GD (Gal4 DNA binding domain)-tagged constructs for poplar R3 MYB genes, the full-length, open-reading frames (ORF) of corresponding poplar R3 MYB genes were amplified by RT-PCR using RNA isolated from poplar xylem samples, and the PCR products were then cloned in-frame with an N-terminal HA or GD tag into the pUC19 vector under the control of the double 35S enhancer promoter of *CaMV* (Wang et al., 2005).

The 35S:*PtrTCL1-GFP* construct was cloned by fusing *PtrTCL1* in frame with GFP (Green fluorescent protein) and then cloned into the pUC19 vector under the control of the 35S promoter. The *TCL1* promoter was replaced with the *PtrTCL1* promoter in the 35S:HA-*PtrTCL1* with TCL1, TRY, and CPC promoters, respectively (Wada et al., 1997; Esch et al., 2003; Wang et al., 2007).
For plant transformation, corresponding constructs in the pUC19 vector were digested with EcoRI and subcloned into the binary vector pPZP211 or pPZP221 (Hajdukiewicz et al., 1994).

PLANT TRANSFORMATION AND TRANSGENIC PLANT SELECTION
About five-week-old plants with several mature flowers on the main inflorescence were used for plant transformation. Plants were transformed by using the floral dip method via Agrobacterium tumefaciens GV3101 (Clough and Bent, 1998). T1 seeds were germinated on plates containing antibiotics to select transgenic plants. Effector and reporter plasmids were prepared using the GoldHi manufacturer's instructions. The procedures for protoplast isolation, transfection, and GUS activity assay have been described previously (Tiwari et al., 2003; Wang et al., 2005, 2008; Wang and Chen, 2008). Briefly, protoplasts were isolated from rosette leaves collected from ~4-week-old Arabidopsis plants. Effector and reporter plasmids were co-transfected into protoplasts and incubated at room temperature for 20–22 h under darkness. GUS activities were measured using a Synergy™ HT microplate reader (BioTEK).

| Primers | Sequences |
|---------|-----------|
| PttTCL1-Nde1F | 5′-CAACATATGGATAGCGTCCAGG-3′ |
| PttTCL1-Sac1R | 5′-CAAGAGCTCTCTACAGATGAGTATAC-3′ |
| PttTCL2-Nde1F | 5′-CAACATATGGATAGCGTCCAGG-3′ |
| PttTCL2-Sac1R | 5′-CAAGAGCTCTCTACAGATGAGTATAC-3′ |
| PttTCL3-Nde1F | 5′-CAACATATGGATAGCGTCCAGG-3′ |
| PttTCL3-Sac1R | 5′-CAAGAGCTCTCTACAGATGAGTATAC-3′ |
| PttTCL4-Nde1F | 5′-CAACATATGGATAGCGTCCAGG-3′ |
| PttTCL4-Sac1R | 5′-CAAGAGCTCTCTACAGATGAGTATAC-3′ |
| PttTCL5-Nde1F | 5′-CAACATATGGATAGCGTCCAGG-3′ |
| PttTCL5-Sac1R | 5′-CAAGAGCTCTCTACAGATGAGTATAC-3′ |
| PttTCL6-Nde1F | 5′-CAACATATGGATAGCGTCCAGG-3′ |
| PttTCL6-Sac1R | 5′-CAAGAGCTCTCTACAGATGAGTATAC-3′ |
| PttTCL7-Nde1F | 5′-CAACATATGGATAGCGTCCAGG-3′ |
| PttTCL7-Sac1R | 5′-CAAGAGCTCTCTACAGATGAGTATAC-3′ |
| PttTCL8-Nde1F | 5′-CAACATATGGATAGCGTCCAGG-3′ |
| PttTCL8-Sac1R | 5′-CAAGAGCTCTCTACAGATGAGTATAC-3′ |

### MICROSCOPIY
Trichomes were analyzed and photographed using a Motic K microscope equipped with a Canon digital camera. Localization of PttTCL1-GFP proteins in transgenic plants expressing PttTCL1–GFP under the control of the 35S promoter was examined under an Olympus FV1000 confocal microscope. Protoplast cells isolated from the PttTCL1–GFP transgenic plants were stained with DAPI and then examined under an Olympus FV1000 microscope.

### RESULTS
IDENTIFICATION OF R3 MYB TRANSCRIPTION FACTORS IN POPLAR
A total of eight poplar R3 MYB transcription factors were identified, and collectively named as PttTCL1 to PttTCL8. Corresponding gene names for the PttTCLs identified are as follows: PttTCL1, Potri.002G168900; PttTCL2, Potri.014G096300; PttTCL3, Potri.015G022000; PttTCL4, Potri.007G122800; PttTCL5, Potri.017G03700; PttTCL6, Potri.011G026300; PttTCL7, Potri.004G021300; and PttTCL8, Potri.004G015100.

Similar to their homologs in Arabidopsis, nearly the entire protein of poplar R3 MYBs is made up of the single MYB domain (Figure 1A). The amino acid signature [D/E]L×2[R/K]×3L×6L×3R, that is required for the interaction with R/B-like bHLH transcription factors (Zimmermann et al., 2004), is fully conserved in all seven R3 MYB transcription factors in Arabidopsis (Wang et al., 2007, 2008; Gan et al., 2011), but is found in only three poplar R3 MYBs including PttTCL1, PttTCL2, and PttTCL3 (Figure 1A). In the other four poplar R3 MYBs, D/E, the first amino acid in the amino acid signature is replaced by T/N (Figure 1A). Similarly, the amino acid motif W×M, that has been shown to be required for the cell-to-cell movement of CPC (Kurata et al., 2005), is conserved in all seven Arabidopsis R3 MYBs (Wang et al., 2007, 2008; Gan et al., 2011), but is found in only PttTCL1, PttTCL2, and PttTCL3 (Figure 1A). In the other four poplar R3 MYBs, the M in the motif is replaced by S/T (Figure 1A).

Phylogenetic analysis using full-length protein sequences of poplar R3 MYBs and TCL1 showed that the clade of PttTCL1–PttTCL3 is most closely related to the clade of TCL1, TCL2, ETC2, and TRY (Figure 1B). Together, PttTCL1–PttTCL3 and seven Arabidopsis R3 MYBs formed one subgroup, and PttTCL4–PttTCL8 formed another subgroup.
fused PtrTCLs (GD–PtrTCLs; Figure 2), were co-transfected into Arabidopsis protoplasts. GD and GD–TCL1 were used as negative and positive controls, respectively. As expected, neither GD nor GD–TCL1 activated the reporter gene in the absence of GL3. In the presence of GL3, GD–TCL1 but not GD activated the reporter gene. Similarly, none of the eight poplar R3 MYBs activated the reporter gene in the absence of GL3; however, all of them activated the reporter gene in the presence of GL3 (Figure 2).

PtrTCLs NEGATIVELY REGULATE TRICHOME FORMATION WHEN EXPRESSED IN Arabidopsis

Having shown that all the eight poplar R3 MYBs interact with GL3 in plant cells, we further analyzed if PtrTCLs regulate trichome formation by generating transgenic Arabidopsis plants expressing HA-tagged PtrTCLs under the control of 35S promoter (35S:HA-PrtTCLs). As shown in Figure 3A, expression of PtrTCL1, PtrTCL2, PtrTCL3, PtrTCL5, PtrTCL7 and PtrTCL8 in Arabidopsis resulted in glabrous phenotypes, similar to the phenotype observed in
plants over-expressing TCL1 (Wang et al., 2007). In contrast, transgenic plants expressing PtrTCL4 and PtrTCL6 had greatly reduced number of trichomes on rosette leaves, but none of the transgenic plants obtained showed glabrous phenotypes (Figure 3). RT-PCR analysis showed that poplar R3 MYB genes were highly expressed in their corresponding transgenic lines (Figure 4), indicating that the phenotypes observed in PtrTCL4 and PtrTCL6 transgenic plants were not due to relative lower expression levels of the corresponding genes.

GL2 is one of the target genes of the activator complex TTG1–GL3/EGL3–GL1, and it positively regulates trichome formation in Arabidopsis. Because binding of PtrTCLs to GL3 indicated that expression of PtrTCLs in Arabidopsis resulted in the inhibition of the formation of TTG1–GL3/EGL3–GL1 activator complex, we examined the expression of GL2 in Arabidopsis transgenic plants expressing poplar R3 MYB genes. As shown in Figure 4, expression of GL2 was dramatically reduced in transgenic plants expressing any of the poplar R3 MYB genes.

SUBCELLULAR LOCALIZATION OF PtrTCL1

Among the eight poplar R3 MYB transcription factors, PtrTCL1 has highest amino acid similarity with TCL1, and phylogenetic analysis also showed that the clade of PtrTCL1–PtrTCL3 is most closely related to TCL1 (Figure 1). We wanted to further explore the functions of poplar R3 MYB transcription factors in the regulation of trichome formation in Arabidopsis by taking PtrTCL1 as an example. We first examined the subcellular localization of the PtrTCL1 protein by generating transgenic plants expressing PtrTCL1–GFP under the control of the 35S promoter. As shown in Figure 5A, expression of PtrTCL1–GFP in Arabidopsis resulted in a glabrous phenotype, a phenotype similar to transgenic plants expressing PtrTCL1 (Figure 3). This indicated that the PtrTCL1–GFP fusion protein is likely functional. By examining the transgenic plants obtained, we found that GFP fluorescence was mainly observed in the nucleus of epidermal cells (Figure 5B); examination of DAPI stained protoplast cells isolated from the PtrTCL1–GFP transgenic plants confirmed that the GFP was mainly observed in the nucleus (Figure 5C), indicating that PtrTCL1 is a nuclear localized protein.
Transgenic plants were generated in a tcl1 background to express PtrTCL1 under the control of the TCL1 native promoter (TCL1p:HA-PtrTCL1/tcl1). Previously, we showed that expression of TCL1-GFP under the control of the TCL1 promoter fully rescued the tcl1 trichome phenotype (Wang et al., 2007), indicating that the TCL1 promoter used is functional in Arabidopsis. As shown in Figure 6, expression of PtrTCL1 under the control of TCL1 native promoter almost completely rescued tcl1 mutant phenotype, i.e., tcl1 mutant forms ectopic trichomes in both stem internodes above the first flower and pedicels, while TCL1p:HA-PtrTCL1/tcl1 plants do not have trichomes on stem internodes after the first flowers, but have a few trichomes on first pedicel only. These results indicate that PtrTCL1 is likely the functional equivalent of TCL1 in controlling trichome formation on the inflorescence stems and pedicels.

In addition to tcl1, single mutants try and cpc showed trichome phenotypes; try mutants have trichome clusters, and cpc mutants have increased numbers of trichome on leaves (Wada et al., 1997; Schnittger et al., 1999), so we also examined if PtrTCL1 is functionally equivalent to TRY or CPC. Transgenic plants were generated to express PtrTCL1 under the control of TRY native promoter (TRYp:HA-PtrTCL1/try) in the try background, and under the control of CPC native promoter (CPCp:HA-PtrTCL1/cpc) in the cpc background. As shown in Figure 7, expression of PtrTCL1 under the control of TRY native promoter in a try mutant background, or under the control of CPC native promoter in a cpc mutant background resulted in glabrous phenotypes.

**DISCUSSION**

In this study we identified poplar homologs of Arabidopsis R3 MYB transcription factors, and analyzed their function in trichome formation in Arabidopsis. We showed that expression of any of the eight poplar R3 MYB genes under the control of the 35S promoter in Arabidopsis resulted in either a glabrous phenotype or a great reduction in trichome numbers (Figure 3). These results suggest that poplar R3 MYBs act as negative regulators for trichome formation and may have overlapping functions, similar to their Arabidopsis R3 MYB homologs.

Among the eight poplar R3 MYB transcription factors, PtrTCL1 is in the most closely related clade to TCL1 (Figure 1B), is localized in the nucleus (Figure 5), and expression of PtrTCL1 under the control of the TCL1 promoter in the tcl1 mutant background (TCL1p:HA-PtrTCL1/tcl1) almost fully restored the trichome phenotype of the tcl1 mutant (Figure 6). Expression of PtrTCL1 under the control of the TRY native promoter in the try background (TRYp:HA-PtrTCL1/try), or under the control of the CPC native promoter in the cpc background (CPCp:HA-PtrTCL1/cpc) resulted in glabrous phenotypes (Figure 7), suggesting that PtrTCL1 may be the functional equivalent of TCL1, rather than TRY and CPC. It should be noted that ETC2 and TCL2 are also in the clade that is sister to the clade of PtrTCL1–PtrTCL3; however, because no mutant available for TCL2 and etc2 mutant does not have any trichome phenotype, we could not test whether PtrTCL1 might be functionally equivalent to TCL2 or ETC2.

In Arabidopsis, R3 MYBs inhibit trichome formation by competing with GL1 for binding of GL3 or EGL3, thus inhibiting...
the formation of the TTG1–GL3/EGL3–GL1 activator complex (Schellmann et al., 2002; Esch et al., 2003; Schiefelbein, 2003; Pesch and Hülskamp, 2004; Ishida et al., 2008). In accordance with these results, all seven Arabidopsis R3 MYBs have the conserved [D/E]L×2[R/K]×3L×6L×3R amino acid signature that is required for interaction with R/B-like bHLH transcription factors (Zimmermann et al., 2004), and protoplast transfection assays showed that they all interact with GL3 in plant cells (Wang et al., 2008; Gan et al., 2011). Sequence alignment results showed that only three of the poplar R3 MYBs, namely PtrTCL1, PtrTCL2, and PtrTCL3, have the fully conserved amino acid signature required for interacting with bHLH transcription factors (Figure 1A). When tested in protoplasts, however, all the eight poplar R3 MYBs interacted with GL3 (Figure 2), and the interaction with GL3 may be stronger for those R3 MYBs without the fully conserved amino acid signature as judged by the GUS activities (Figure 2). Our results indicate that either the conserved amino acid signature is not required for the interaction of poplar R3 MYBs with GL3, or a single-amino-acid substitution (D/E > T/N) in poplar R3 MYBs does not affect their interaction with GL3. It is also possible that previous designations of conserved residues may be biased due to a smaller set of proteins analyzed. Furthermore, interaction of poplar R3 MYBs with GL3 in plant cells suggests that poplar R3 MYBs can block the formation of the activator complex required for trichome formation. In accordance with this, RT-PCR results showed that expression of GL2 is dramatically reduced in the transgenic plants expressing poplar R3 MYB genes (Figure 4). Since our previous results showed that R3 MYBs in Arabidopsis may regulate
trichome formation in a GL2 independent manner (Wang et al., 2010), we cannot rule out the possibility that poplar R3 MYBs may also regulate trichome formation through other mechanisms.

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