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Ectopic expression of *TrPI*, a *Taihangia rupestris* (Rosaceae) *PI* ortholog, causes modifications of vegetative architecture in *Arabidopsis*

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SUMMARY

In eudicotyledonous model plants, the B-function genes encode a pair of partner MADS-domain proteins, APETALA3 (AP3) and PISTILLATA (PI) in *Arabidopsis* and DEFICIENS (DEF) and GLOBOSA (GLO) in *Antirrhinum*. These proteins, which must form heterodimers to function, are required to specify petal and stamen identity during flower development. Here, we report cloning and characterization of *TrPI* (*Taihangia rupestris PISTILLATA*), a *PI*/*GLO*-like gene from the core eudicot species *Taihangia rupestris* (Rosaceae). DNA gel blot analysis showed that *TrPI* is a single copy gene in the *T. rupestris* genome. Quantitative RT-PCR and *in situ* hybridization analyses revealed that *TrPI* is transcribed in both the vegetative and reproductive organs at different levels. Ectopic expression of *TrPI* in *Arabidopsis* caused severe modifications in vegetative plant architecture, including rosette leaves and cauline leaves arranged in a non-spiral phyllotaxy, and a flattened primary inflorescence stem that produced two or three offshoots at the base, middle or top. Moreover, we show that the *TrPI* gene is capable of rescuing *pi-1* mutant phenotypes. Yeast two-hybrid assays showed that *TrPI* forms homodimers. Taken together, these results show that *TrPI* might function in regulating plant architecture in addition to its function as a floral organ identity gene in *T. rupestris*, suggesting that the TrPI protein has biochemical features that distinguish it from the well-studied orthologs, PI and GLO.

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1. Introduction

The MADS-box gene family encoding transcription factors has been subjected to extensive gene duplication events and subsequent functional divergence during plant evolution (Theissen et al., 2000; Becker and Theissen, 2003; Irish and Litt, 2005). These transcription factors control diverse developmental processes, such as regulation of flowering time, flower meristem and organ identity, fruit ripening and dehiscence, fertility, leaf development, and root architecture (Alvarez-Buylla et al., 2000; Theissen et al., 2000; Causier et al., 2002).

A large number of MADS-box genes have been isolated from angiosperms to date. These represent 13 different gene groups, namely, the *APETALA3*- (*AP3*-)/*DEFICIENS*- (*DEF*-), *PISTILLATA*- (*PI*-)/*GLOBOSA*- (*GLO*-), *AGAMOUS*- (*AG*-), *AGL6*-, *AGL12*-, *GGM13*- (*Bsister*), *TM3*-, *STMADS11*-, *AGL2*-, *AGL17*-, *APETALA1* (*AP1*)/*SQUA*-, *AGL15*- and *FLC*-like genes (Becker and Theissen, 2003). In *Arabidopsis thaliana* and *Antirrhinum majus*, the *AP3*/*DEF*-like genes (*AP3* and *DEF*, respectively) and the *PI*/*GLO*-like genes (*PI* and *GLO*, respectively) (Goto and Meyerowitz, 1994; Yang et al., 2003), encode the B-function proteins of the classic ABC model of flower development (Coen and Meyerowitz, 1991). B-function combines with A-function to specify the development of petals, and with C-function to determine the development of stamens. Mutations in either of the B-function genes result in homeotic transformations of petals into sepals and stamens into carpels. Therefore, *AP3* and *PI* in *Arabidopsis*, and *DEF* and *GLO* in *Antirrhinum* are obligate heterodimers required to specify petal and stamen identities (Sommer et al., 1990; Jack et al., 1992; Trobner et al., 1992; Goto and Meyerowitz, 1994; Jack et al., 1994). *AP3*/*DEF*- and *PI*/*GLO*-like genes appear to be highly conserved in number and function in *Arabidopsis* and *Antirrhinum*. However, in *Petunia*, another model plant in the core eudicots, two *AP3*/*DEF*-like genes, *PhDEF* (previously *Green Petals*, *GP*) and *PhTM6*, and two *PI*/*GLO*-like genes, *PhGLO1* (previously *FBP1*) and *PhGLO2* (formerly *PMADS2*) have been identified as being produced by gene duplication events (Angenent et al., 1992; Angenent et al., 1993; van der Krol et al., 1993; Tsuchimoto et al., 2000). These paralogous genes are partially redundant, but have diverged in functionality, as reflected by whorl-specific

Abbreviations: aa, amino acids; Ade, Adenine; CaMV, cauliflower mosaic virus; His, histidine; LacZ, β-Galactosidase activity; Leu, leucine; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase polymerase chain reaction; Trp, Tryptophan; 3-AT, 3′-aminotriazole.

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functions and partner specificity. Thus far, numerous B class genes have been isolated and characterized from other core eudicots, basal eudicots and basal angiosperms. They show expression patterns ranging from vegetative tissues (e.g. leaves, stems, root nodules, young seedlings) to floral organs and fruits (e.g. sepals, petals, stamens, carpels, ovules) (Zahn et al., 2005). However, there are no genetic data demonstrating that potential B class genes are involved in regulating vegetative development. To date, data about MADS-box genes in the plant family Rosaceae, including plants of significant horticultural and agronomic importance, such as roses, apples, pears, peaches, apricots, almonds, plums, strawberries and many more, are scarce (Yao et al., 1999; Kitahara et al., 2001; Linden, 2002; van der Linden et al., 2002; Tani et al., 2009).

To parse the extent to which MADS-box gene functions have diversified in angiosperms, we present here a characterization of the *PI/GLO*-like MADS-box gene, *TrPI*, from *Taihangia rupestris* (*T. rupestris*). *T. rupestris* represents a clade that is evolutionarily distant from the Asterids *Antirrhinum* and *Petunia*, and more closely related to the Rosid *Arabidopsis* (Soltis et al., 1999; APGII., 2003). *T. rupestris* is a rosette perennial polycarpic herb species endemic to China. It belongs to the Dryadeae, Rosoideae, Rosaceae. Within the Dryadeae tribe, *T. rupestris* is a distinctive species because it is diploid, whereas other genera, such as *Dryas*, *Geum*, *Coluria* and *Waldsteinia* are polyploids. Leaf primordia are initiated from the flanks of the shoot apical meristem. The rosette leaves are arranged in a spiral phyllotaxy around the very short stem. Its flowers have both advanced and primitive features. Five sepals and petals arranged in actinomorphic morphology, numerous and apostemonous androecia and gynoecia, stamen filaments with same length, and marginal placentation represent primitive characters, whereas sepals and petals arranged alternately, partial synsepaly at the base, carpels with differentiated ovary, stigma and style are advanced. Its inflorescence stems are round. The determinate inflorescences are unusually composed of two or three florets born in the axils of each leaf (Lü et al., 2007; Du et al., 2008). Expression analyses indicate that *TrPI* is transcribed in both leaves and floral organs. Ectopic expression of *TrPI* in *Arabidopsis* causes modifications in plant architecture. The *TrPI* gene, driven under control of a double Cauliflower Mosaic Virus (CaMV) 35S promoter, can complement the floral defects of *pi-1* mutants. Yeast two-hybrid assays show that the interaction pattern of TrPI is distinct from those of PI in *Arabidopsis* and GLO in *Antirrhinum*. Based on these results, we hypothesized that *TrPI* might function in regulating plant architecture in addition to its putative function as a floral organ identity gene in *T. rupestris*.

2. Materials and Methods

2.1. Plant material

T. rupestris plants were cultivated in growth chambers under the temperature condition of 10-12 °C. Inflorescence stems, floral buds across different developmental stages, and leaves were harvested and frozen in liquid nitrogen for DNA extraction and RNA isolation, or fixed and embedded in paraplast for *in situ* hybridization

2.2. Cloning of TrPI

Total RNA was isolated using Plant RNA Purity Reagent (Invitrogen) from the inflorescences of *T. rupestris* at the floret differentiation stages. Poly $(A)^{\pm}$ mRNA was purified from total RNA using the Oligotex mRNA Mini Kit (Qiagen). First strand cDNA was synthesized by SuperScript™ III Reverse Transcriptase (Invitrogen) with a polyT primer PTA with a 5′ adapter sequence. Hemi-nested PCR amplification was performed with the degenerate MADS-box sequence forward primer B2 and the adapter primer AP. The amplified fragments of about 1.0 kb (kilobase pair) were cloned into the pGEM-T vector (Promega) and sequenced. To obtain the 5′ partial cDNA ends of *TrPI*, 5′ rapid amplification of cDNA ends (5′ RACE) was used. Internal gene-specific primers P3PI1, P3PI2 and P3PI3 were designed for *TrPI*. The full-length cDNA of *TrPI* was obtained by 3′ RACE with primers 3N72 and AP. Sequencing of cDNAs was performed with the ABI PRISM dye terminator kit (PE Applied Biosystems, Foster City, CA). The final sequence has been deposited in GenBank under accession number DQ248947. All the primer sequences discussed in this study are shown in Table S1.

2.3. Genomic DNA extraction and DNA gel blot analysis

Genomic DNA was extracted and DNA gel blot hybridization was performed as published previously (Lü et al., 2007). A 3′ end fragment lacking of the MADS domain and partial I domain of *TrPI* (nucleotides 211-839 counted from the start codon ATG) was labeled with $[^{a-32}P]$ dCTP using Prime-a-Gene® label as a probe.

2.4. Sequence analysis of TrPI and construction of phylogenetic tree

Protein sequences were aligned using CLUSTALW1.81 under default settings and refined by hand. We generated neighbor-joining (NJ) trees based on the aligned full-length protein sequences comprising the MADS, I, K, and C domains using the MEGA4 (Molecular Evolutionary Genetic Analyses, version1.1, Pennsylvania State University) package. Bootstrap values were based on 1,000 replicates. Gaps were encoded as missing data.

2.5. Vectors construction and Arabidopsis transformation

The full-length *TrPI* cDNAs were amplified using Ex-Taq DNA polymerase (TaKaRa) with a forward primer TrPIBam and a reverse primer TrPISal harboring *Bam*HI and *Sal*I site, respectively. The PCR products were cloned between the *Bam*HI and *Sal*I restriction site into pFP101 vector, which placed *TrPI* transcription under the control of a 2 × 35S promoter in the sense orientation. The 35S::*TrPI* construct described above was introduced into wild-type Columbia and heterozygous *PI/pi-1 A. thaliana* plants by the floral dip method (Clough and Bent, 1998). The Homozygous *pi-1* plants were identified and the transgenic plants were analyzed as published previously (Lamb and Irish, 2003; Su et al., 2008).

2.6. RNA in situ hybridization and RNA gel blot hybridization

In situ hybridization and RNA gel blot hybridization methods used here have been reported previously (Li et al., 2005; Lü et al., 2007). The less-conserved sequences (nucleotides 568-899 counted from the start codon ATG) were used as the template to synthesize the probes for the *in situ* hybridization and RNA gel blot analyses.

2.7. Yeast two-hybrid assays

The two-hybrid system, *Saccharomyces cerevisiae* strain, activation domain (AD) cloning vector, and DNA-binding domain (DNA-BD) cloning vector used here, as well as the methods of constructing the fusion protein vectors, yeast transformation and interactions analysis were performed as described previously (Lü et al., 2007). Yeast two-hybrid assays were performed using the GAL4-based MATCHMAKER Two-Hybrid System (Clontech). *Saccharomyces cerevisiae* strain AH109, GAL4 activation domain (AD) cloning vector pACT2 and GAL4 DNA-binding domain (DNA-BD) cloning vector pAS2 were used. Full-length cDNA of *TrFUL*, *TrAP1*,

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