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Panax ginseng PgMADS1, an AP1/FUL-like MADS-box gene, is activated by hormones and is involved in inflorescence growth

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Abstract *Panax ginseng* is a perennial herbaceous plant that requires at least 3 years in the juvenile period to produce a simple umbel inflorescence in nature. Interestingly flowering can be induced in vitro from young embryos and plantlets by treatment with cytokinin and gibberellic acid. To identify the gene involved in hormone-induced flower formation, nine MADS-box genes were isolated from cDNA derived from in vitro hormone-induced flowers of *P. ginseng*. *PgMADS1, 2, 3,* and *4* were identified as homologues of the APETALA1/FRUITFULL (AP1/FUL) subfamily. *PgMADS5, 6, 7,* and *8* were identified as homologues of the *AGAMOUS* (*AG*) subfamily, and *PgMADS9* was identified as a homologue of *DEFICIENS* (*defA*). Reverse transcription polymerase chain reaction (RT-PCR) analysis revealed that *PgMADS1, 2, 3*, and *4* were highly expressed in non-reproductive floral organs. *PgMADS1* mRNA accumulation in the axillary buds was clearly enhanced after 2 weeks of benzyladenine (BA) and gibberellic acid (GA$_3$) treatment. *In situ* hybridization analysis revealed that *PgMADS1* mRNA accumulated at the periphery of the inflorescence meristems after hormone treatment. Transgenic *P. ginseng* plants overexpressing *PgMADS1* were constructed. BA and GA$_3$ treatment was necessary to induce in vitro flowering of these transgenic plants. However, transgenic plants exhibited active flower growth with long inflorescence stalks compared with that of the non-transformed plants. These results indicate that overexpression of *PgMADS1* is not sufficient to induce floral transition without hormone treatment but involved in development and growth of inflorescence in *P. ginseng*.

Keywords In vitro flowering · MADS-box gene · *Panax ginseng* · PgMADS1 · Plant hormone

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Introduction

Panax ginseng is a perennial herbaceous plant with a thickened root that contains pharmacologically active saponins, called ginsenosides (Vogler et al. 1999; Shibata 2001). In nature, *P. ginseng* plants produce an umbel inflorescence after a long juvenile period of approximately 3 years (Schlessman 1985; Nantel et al. 1996). Interestingly, flowering can also be induced by the application of exogenous plant hormones from zygotic embryos (Lee et al. 1991) and somatic embryos of *P. ginseng* (Chang and Hsing 1980). In vitro flower development occurs due to the floral change of shoot buds situated on the cotyledonary nodes of zygotic embryos (Lee et al. 1991). Combined treatment with both cytokinin and gibberellic acid is necessary to induce the floral transition of the shoot buds in *P. ginseng* (Chang and Hsing 1980; Kim et al. 1998). In vitro induced floral development is a good model for understanding the hormone-regulated floral transition from the vegetative growth phase to the reproductive phase.

The role of plant hormones in in vitro flowering has been extensively reviewed in previous reports (Scorza

1982; Rastogi and Sawheny 1989; Teixeira da Silva et al. 2014). In these reports, cytokinins and gibberellins were commonly used to stimulate in vitro flowering. Cytokinin (BA) alone promoted in vitro flowering in several plant species, including *Perilla frutescens* (Zhang 2007), orchid (Sim et al. 2007), and rose (Kanchanapoom et al. 2009). GA_3 enhanced in vitro inflorescence development in the calla lily (Naor et al. 2004). However, information concerning molecular aspects for the floral homeotic genes that respond to the hormones is quite limited in a few species, such as tobacco (Estruch et al. 1993) and *Sinapis alba* (Bonhomme et al. 2000).

The floral transition begins with the conversion of a shoot apical meristem (SAM) into a reproductive floral meristem (Bernier et al. 1993; Levy and Dean 1998). The floral transition is induced in response to endogenous and environmental signals, such as day length, low temperature, and plant hormones (Koornneef et al. 1991, 1998; Wilson et al. 1992; Bernier and Perilleux 2005). Molecular and genetic studies have shown that floral signals regulate floral integrator genes, such as *FLOWERING LOCUS T* (*FT*) (Lifschitz et al. 2006), *CONSTANTS* (*CO*) (Simon et al. 1996), *FLOWERING PROMOTIVE FACTOR1* (*FPF1*) (Kania et al. 1997), and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Yoo et al. 2005), which are expressed early in the transitioning SAM. MADS-box genes, such as *LEAFY* (*LFY*) and *APETALATA1* (*AP1*), are expressed later (Ma 1998; Pidkowich et al. 1999). Plant MADS-box genes play functionally redundant roles in the control of flower meristem identity and floral organ identity (Theissen et al. 2000). Among these genes, the *SQUAMOSA* (*SQUA*)-like genes, also referred to as the AP1/FUL clade, are characterised as floral meristem identity genes (Becker and Theißen 2003). The *AG*-like genes (Yanofsky et al. 1990; Ma et al. 1991) and the defA-like genes (Sommer et al. 1990) are characterised as floral organ identity genes.

There have been no reports describing the molecular aspects of in vitro flowering in *P. ginseng*, except for a single article reporting the isolation of the AG-like *GAG2* gene (Kim et al. 1998). In the present study, we isolated 9 MADS-box genes from *P. ginseng* flowers and investigated their transcriptional levels during in vitro flowering. We found that the transcription of *PgMADS1* gene is responsive by BA and GA_3 treatment. Constitutive expression of *PgMADS1* in transgenic *P. ginseng* resulted in active inflorescence development, which established the clear umbel inflorescence morphology.

Results

Hormone-induced in vitro flowering

Four-year old *P. ginseng* has characteristic umbel inflorescences (Fig. 1a–b). An umbel is an inflorescence that consists of several short flower stalks, termed pedicels, which spread from a common point. Umbels are characteristic of plants in the families Apiaceae, Araliaceae, and Alliaceae (Struwe 2009). *P. ginseng* belongs to Araliaceae.

In this experiment, the segments comprising the cotyledonary nodes of the seedling were cultured on medium containing BA and/or GA_3. The segments were comprised of three dormant buds: two axillary buds were situated on the base of the two cotyledons, and one bud was located on the base of the first leaf derived from the plumule (Fig. 1c). Morphologically visible flower buds were noted after 3 weeks of culture. Obvious flower buds with young umbel structures developed on the axillary buds of the cotyledonary nodes (Fig. 1d) and on the first leaf node (Fig. 1e) after 6 weeks of culture. Scanning electron microscopy revealed that the single dormant bud on the base of the leaf node (Fig. 1f) developed into an inflorescence meristem (Fig. 1g) after 3 weeks in the presence of 4.4 μM BA and 10 μM GA_3. Thereafter, these buds developed into umbel inflorescences.

Benzyladenine (BA) treatment alone induced flowers at 17.1 and 22.3 % of the cotyledonary and leaf nodes, respectively (Table 1). Treatment with GA_3 alone did not induce any flowers; however, it promoted plant elongation. Treatment with both BA and GA_3 resulted in the highest frequency of flower formation at both the cotyledon node (78.7 %) and the leaf node (87.6 %) (Table 1).

Isolation and sequence analysis of 9 MADS-box genes

We isolated 9 MADS-box genes from the cDNA of young in vitro flowers using the 5′/3′-RACE strategy. Each of the 9 sequences was classified into the subfamily of MADS-box genes with classical MIKC-type protein structures, as described by Yanofsky et al. (1990). Phylogenic sequence analysis revealed that *PgMADS1–4* belonged to the *Antirrhinum majus SQUA* subfamily, whereas *PgMADS5–8* belonged to the *Arabidopsis AG* subfamily, and *PgMADS9* resembled *Antirrhinum defA* (Fig. 2).

The deduced amino acid sequences of *PgMADS1-9* were analysed using the NCBI BLAST search program.

Fig. 1 In vitro flowering from axillary buds of *P. ginseng* leaves in the presence of 4.4 μM BA and 10 μM GA₃. **a–b** Flower (**a**) and fruit (**b**) morphology of 4-year old *P. ginseng* plants. **c** Schematic drawing of the cotyledonary node segment excised from *P. ginseng* seedlings (culture explant). **d** Two in vitro inflorescences (*arrows*) developed directly from the two cotyledonary buds after 3 weeks of culture. **e** One in vitro inflorescence (*arrows*) developed directly from a bud on the first leaf node after 3 weeks of culture. **e–f** SEM observation of in vitro flower development. **e** A young bud of a leaf node before culturing in flower induction medium. **f** A young inflorescence arising directly from a bud of the leaf node after 3 weeks of culture
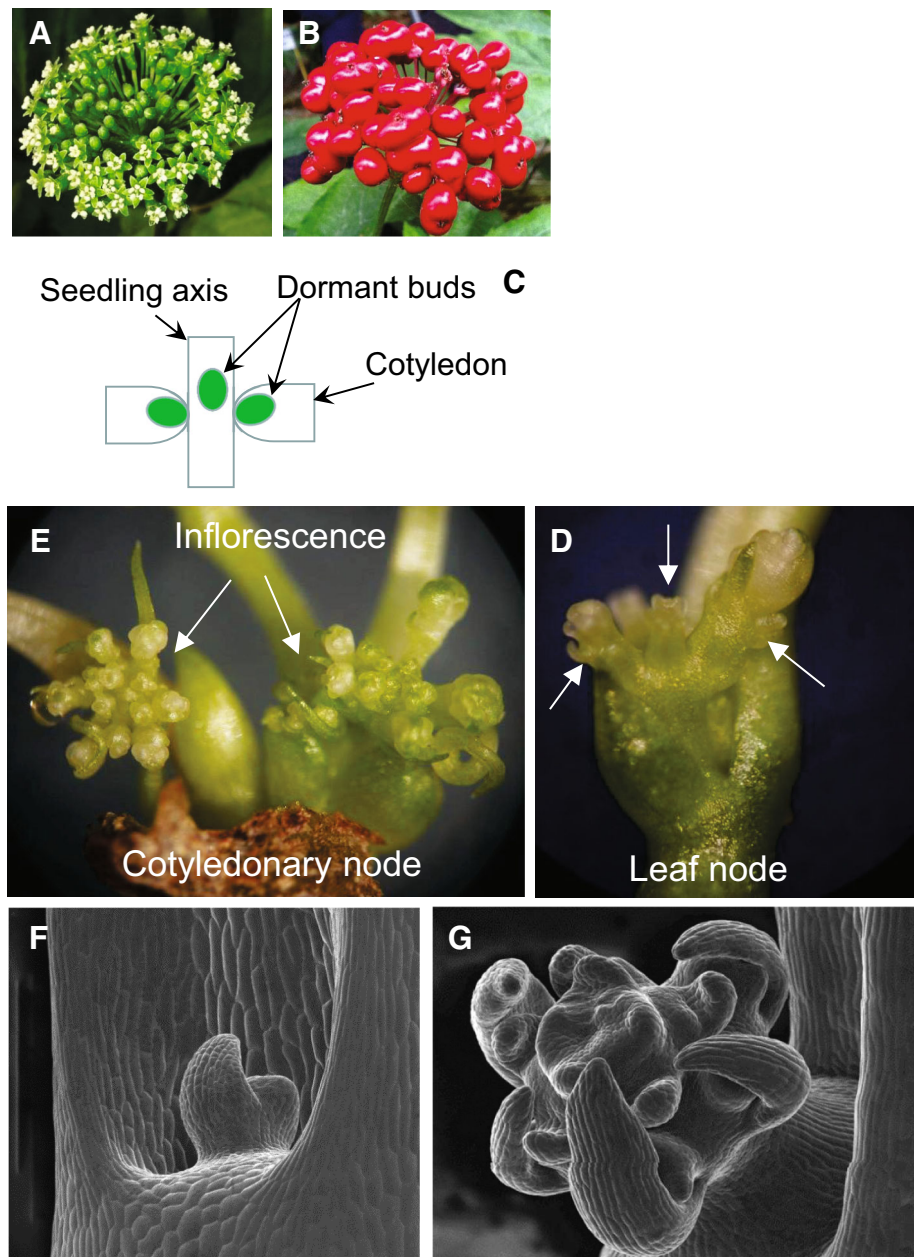
Table 1 In vitro flower formation from cotyledonary node and leaf node of *P. ginseng* seedlings on medium with BA and/or GA₃ after 6 weeks of culture

Growth regulator (μM)	Flower formation (%)	
	Cotyledonary node	Leaf node
BA 4.4	17.1 ± 2.5	22.3 ± 4.3
GA₃ 10	0	0
BA 4.4 + GA₃ 10	78.7 ± 9.7	87.6 ± 11.2

PgMADS1 (accession no. AB627121) and *PgMADS2* (AB627122) share 80 % sequence similarity. These genes are 68 % homologous with *Vitis vinifera VFUL-L*, which is involved in the floral transition (Calonje et al. 2004), and 64 % homologous with *Petunia hybrida FLORAL BINDING PROTEIN26* (*FBP26*), which is involved in the transition of the vegetative shoot apex to the reproductive phase and the maintenance of reproductive identity. *PgMADS3* (AB627123) exhibits 77 % homology with tobacco *NsMADS1* which is expressed during the flower
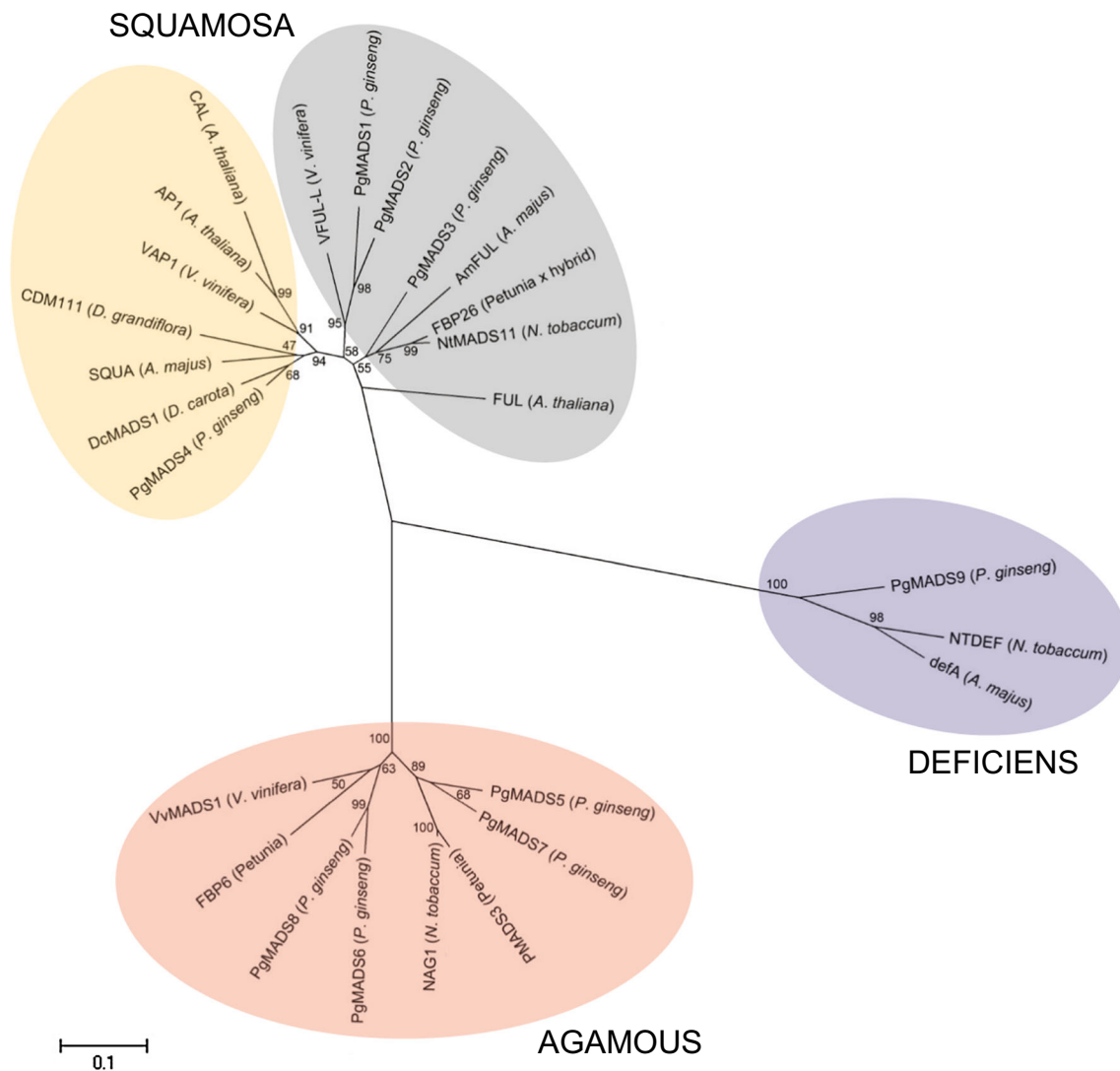
Fig. 2 Phylogenetic tree showing the relationships between the 9 MADS-box proteins and other MADS-box genes. The phylogenetic tree was generated with the distance matrix using the neighbour-joining method and TreeView software

induction process (Jang and An 1999). *PgMADS4* (AB627124) exhibits 73 % homology with *Daucus carota DcMADS1*, which is involved in the early stages of flower development (Linke et al. 2003). *PgMADS5* (AB627125) and *PgMADS7* (AB627127) share high levels of homology with tobacco *NAG1*, which participates in early flower development in the region of the floral meristem that later gives rise to the stamens and carpels (Kempin et al. 1993). *PgMADS6* (AB627126) and *PgMADS8* (AB627128) share high levels of homology (72 and 75 %, respectively) with petunia *PAGL1*. *PgMADS9* (AB627129) has 67 % homology with the *A. majus defA* gene, which controls the organogenesis of the petals and stamens (Schwarz-Sommer et al. 1992).

When the C-terminal domains of the *PgMADS1–4* and other *SQUA*-like genes were compared further, we

observed a highly conserved motif (L/MPPWML) typical of the AP1/FUL-like proteins in *PgMADS1–3* (Fig. 3) (Litt and Irish 2003). Additionally, *PgMADS4* contains a far-nesylation motif (CFAT/A) that is typical of euAP1-like proteins (Yalovsky et al. 2000).

Accumulation of *PgMADS1–9* mRNA in flower organs

Ginseng flowers have a simple umbel at the end of the peduncle and terminate in multiple hemispherical floret clusters (Fig. 4). An analysis of the MADS-box mRNA levels revealed that *PgMADS1–4* were enriched in the non-reproductive parts of the flowers, including the pedicel, involucral bract, and petal (Fig. 4), but were very weakly expressed in the reproductive parts, such as the carpel and

paleoAP1/FUL-like

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PgMADS1   QVVGVIRE-EDGYQAPPCSS-SNMPPWMLRHVNQ--
PgMADS2   QVTG------DGAQAPPCNT-SLMPPWMLRHVNR--
VFUL      VGRAVE---EDGAEARPSPN-TLMPPWMLRHVNE--
DEFH28    QTVRVE---EGGDRTRIADSRSHIPPWLLQHVNQ--
GSQUA2    GAVREE---EYSTQAHPISG-TMMPPWLFHHIYQ--
FUL       ERVGGE----NGGASSLTEPNSLLPAWMLRPTTTNE
PgMADS3   QAGEDG---EIEETLQQNQANTVMPSWMLRHLNG--
MADS11    STAGDNG--EVEGSSRQQQQNTVMPPWMLRHLNN--
PFG       PSAGDNG--EVEGSSRQQPPNTVMPPWMLRHLNG--
AmFUL     QGQRDNNGEVEGSRNQNQSSNKILPPWML------
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euAP1-like

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AP1       -------DPMAMRRNDLELTLEPVYNCNLGCFAA--
SaMADS    -------DPMEMRRNDLDLSLEPVYNCNLGCFAA--
CAL       -------DQTAMRRNNLDLTLEPIYNY-LGCYAA--
VAP1      -------EAHGARRNELDLTLEPIYPSHLGCFTT--
PgMADS4   -------GI---RRNELDLTLEPVYSCHLGCFAT--
SQUA      -------GANEDRRNELDLTLDSLYSCHLGCFAA--
CDM111    --GGGAGERADGMTNELDLSLQ--YSCHMRCFPS--
HAM75     --GGSSEGADGRTNELDLSLQPIYSCHMRCFPS--
HAM92     TSAASGGEGADGRTSQLDLSLQPIYSHHLRCFPS--
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Fig. 3 Alignment of the C-terminal ends of the AP1/FUL-like and euAP1-like proteins. The AP1/FUL-like protein motif is shown in blue. EuAP1-like proteins contain a farnesylation motif (shown in *red*) at their C termini. The *red* domain represents the euAP1 motif according to Vandenbussche et al. (2003)
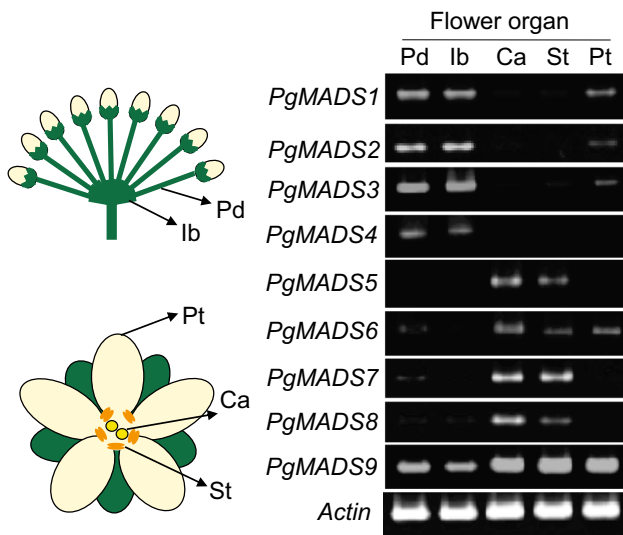

Fig. 4 Ginseng floral structure and expression patterns of the MADS-box genes in each floral organ of a 4-year-old ginseng plant. **a** (*left*) Schematic drawing of the floral structure of *P. ginseng*. Side view (*upper*) and front view (*lower*) of a flower. **a** (*right*) Expression of the 9 MADS-box genes in each floral organ as determined by RT-PCR. Se, sepal; Pd, pedicel; Ib, involucral bract; Ca, carpel; St, stamen; Pt, petal

stamen. *PgMADS5–8* mRNA was preferentially expressed in the stamen and pistil. *PgMADS9* mRNA was constitutively expressed in all parts of the flower (Fig. 4).

Accumulation of *PgMADS1–4* mRNA during in vitro flowering

The transcriptional levels of *PgMADS1–4* were analysed after 1, 2, and 3 weeks of culture in medium containing BA and/or GA$_3$. *PgMADS1* mRNA was not detected in the leaf nodes of seedlings cultured in hormone-free medium but was highly expressed in the leaf nodes of seedlings grown in medium containing BA and GA$_3$ after 2 weeks of culture (Fig. 5a). Similar expression patterns were observed for *PgMADS2*, although the transcriptional activity of this gene was also detected in the control condition. *PgMADS3* and *PgMADS4* were highly expressed in both the control and hormone-treated plants. The AG-like group (*PgMADS5–8*) did not positively respond to treatment with BA and GA$_3$; however, these genes were highly expressed after 3 weeks of culture when the flower meristems had developed (data not shown). *PgMADS9* was constitutively expressed regardless of growth regulator treatment (data not shown).

The transcriptional levels of *PgMADS1–4* were analysed after 1 week of culture were monitored by qPCR. *PgMADS1* mRNA was highly expressed in the leaf nodes of seedlings grown in medium containing BA and GA$_3$ after 1 week of culture (Fig. 5b). Similar expression patterns were observed for *PgMADS2*. Transcription levels of *PgMADS3* and *PgMADS4* were different to those of
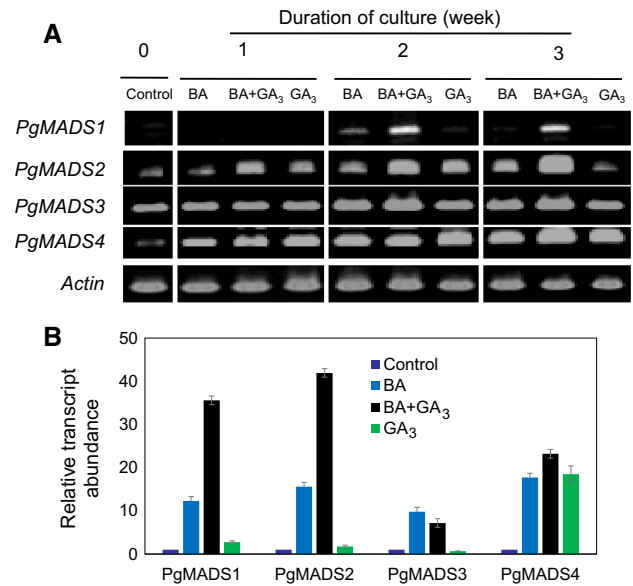
Fig. 5 Expression of the *PgMADS1–4* genes on different hormonal regimes by RT-PCR and qPCR. **a** Expression patterns of the *PgMADS1–4* genes in leaf nodes during in vitro flowering after 1, 2, and 3 weeks of culture on MS medium with 4.4 μM BA, 10 μM GA$_3$, or 4.4 μM BA and 10 μM GA$_3$ by RT-PCR. **b** Expression patterns of the *PgMADS1–4* genes in leaf nodes during in vitro flowering after 1 weeks of culture by qPCR. Control, hormone-free medium

PgMADS1–2 because the transcription of genes was not enhanced in hormone combination (BA and GA$_3$) for flowering induction.
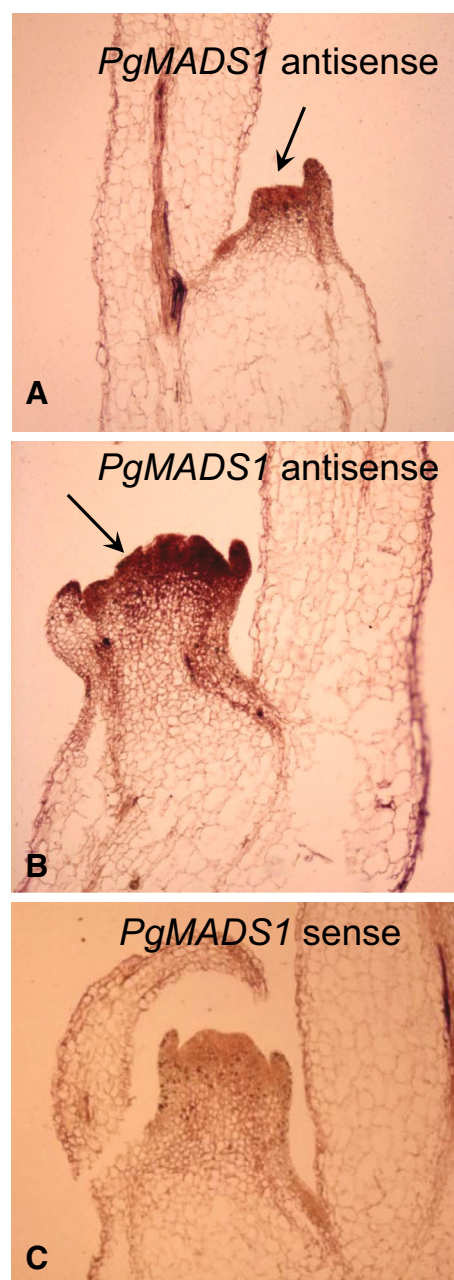
In situ hybridization of *PgMADS1*

In situ hybridization analysis revealed that *PgMADS1* was weekly expressed in the shoot apical meristems after 1 week of culture on medium containing BA and GA$_3$ (Fig. 6a). At this time, there were no obvious morphological changes indicating floral meristem development. After 2 weeks of culture, the apical meristems broadened, indicating the onset of meristem activity for inflorescence formation, and *PgMADS1* was highly expressed in these broadened inflorescence meristems (Fig. 6b). In contrast, a very weak signal was observed in the sections hybridised with the *PgMADS1* sense probe (Fig. 6c).

Transgenic *P. ginseng* overexpressing *PgMADS1*

Transgenic *P. ginseng* plants overexpressing *PgMADS1* were generated by introducing the binary plasmid vector pK2WG7 harbouring *PgMADS1* (Fig. 7a), and successful introduction was confirmed by genomic PCR of the introduced *NPTII* sequence (Fig. 7b). *PgMADS1* was highly expressed in three transgenic lines.

In vitro flower formation was not observed in either the transgenic or non-transgenic plants when they were cultured on hormone-free medium. However, there were striking differences in flower morphology between the control and transgenic plants when they were cultured in medium containing both BA and GA$_3$ (Fig. 8, Table 2). Shoot growth was severely retarded in the transgenic lines (Fig. 8a), but inflorescence development occurred rapidly, resulting in an average inflorescence length of 8.4 mm, and these plants exhibited a clear umbel inflorescence morphology with long flower stalks and pedicels after 6 weeks of culture (Fig. 8b, Table 2). Moreover, the number of flowers (flowers from a single buds) was markedly enhanced in transgenic plants compared to controls (Table 2). Abnormal flowers with irregularly high numbers of petals were frequently observed (Fig. 8c). Whereas, the shoots of the non-transgenic plants grew rapidly, reaching an average length of 22.6 mm after 6 weeks of culture (Fig. 8d), and flower development was still arrested at young stage (2.7 mm, Fig. 8e–f, arrows) although these buds showed obvious flower morphology (Fig. 8e–f).

Fig. 6 In situ hybridization analysis of *PgMADS1* mRNA accumulation during floral transition in the shoot apical meristem of *P. ginseng* cultured on medium containing BA and GA$_3$. **a** Strong hybridisation signal of the *PgMADS1* antisense probe in the meristem (*arrow*) during floral transition of the shoot bud in *P. ginseng* after 1 week of culture. **b** Strong hybridisation signal of the *PgMADS1* antisense probe in the inflorescence meristem (*arrow*) after 2 weeks of culture. **c** Weak hybridisation signal of the *PgMADS1* sense probe in the inflorescence meristem after 2 weeks of culture
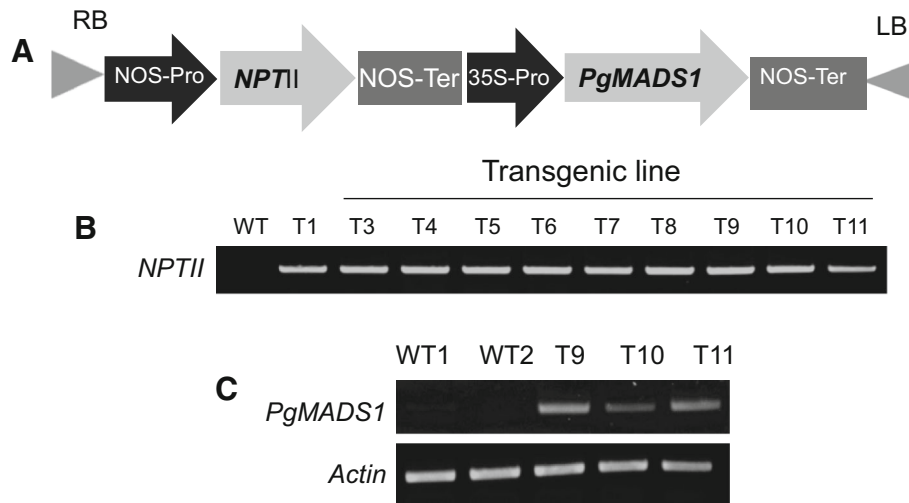
Fig. 7 Construction of the binary vector, PCR detection of the introduced gene, and analysis of the transcriptional levels of the *PgMADS1–4* genes in transgenic *P. ginseng*. **a** Schematic diagram of the T-DNA region of the binary plasmid pK2WG7. The *two-way arrows* indicate the sequences of the non-T-DNA region. NOS-Pro, nopaline synthase promoter; 35S-Pro, CaMV 35S promoter; NOS- Ter, nopaline synthase terminator. RB represents the *right border*, and LB represents the *left border* of the T-DNA region. **b** PCR detection of the *NPT*II gene (1.55 kb) in the wild-type (WT) and transgenic lines (T1 and T3–T11). **c** RT-PCR analysis of *PgMADS1* in the wild-type (WT1 and WT2) and transgenic lines (T9, T10, and T11)

Discussion

In vitro flowering of *P. ginseng* seedlings

In *P. ginseng*, the axillary buds situated on the cotyledonary nodes of the seedling are leaf primordia because they produce only leaves until the plant is 2 years old. After 2–4 years, the buds of the leaf nodes produce stems with flowers. Thus, in vitro flowering from the axillary buds of the cotyledonary nodes by hormone treatment represents the conversion of the leaf primordium directly to the flower/inflorescence.

In vitro flower formation in zygotic embryos or seedlings of *P. ginseng* tissue has been reported. The most efficient hormonal conditions for in vitro flowering include a combination of cytokinin and gibberellic acid. All types of cytokinins, such as BA, kinetin, and thidiazuron, can induce flower formation (Chang and Hsing 1980; Lee et al. 1991).

In most reports, in vitro flowering in *P. ginseng* occurred directly in intact zygotic embryos or somatic embryos (Lee et al. 1991). In these cases, most of flowers were developed from cotyledonary axillary buds but no report from the apical meristem on the base of plumule. In this report, we found that the shoot meristem on the base of first leaf node derived from plumule also produced flowers with a high frequency (Table 1). Thus, all the young leaf primordia (apical meristems) can produce in vitro flowers in the presence of the appropriate flower induction medium in *P. ginseng*.

The AP1/FUL subfamily homologues *PgMADS1* and *2* respond to hormonal treatment

MADS-box genes are involved in several developmental processes, particularly the transition from the vegetative phase to the reproductive phase, and play critical roles in flower development (Ng and Yanofsky 2001). Nine MADS-box genes were isolated from the cDNA of *ginseng* flowers. *PgMADS1, 2, 3,* and *4* were identified as AP1/FUL subfamily homologues, whereas *PgMADS5, 6, 7,* and *8* were AG subfamily homologues. *PgMADS9* was a defA homologue. *AP1/FUL* subfamily genes, also called *SQUAMOSA* (*SQUA*)-like genes, are characterized as floral meristem identity genes (Huijser et al. 1992; Gu et al. 1998; Becker and Theißen 2003). *AGAMOUS* (*AG*)-like genes (Yanofsky et al. 1990; Ma et al. 1991) and *DEFI-CIENS* (*DEF*)-like genes (Schwarz-Sommer et al. 1990; Sommer et al. 1990) are characterized as floral organ identity genes.

LFY and *AP1* are each sufficient to promote flower initiation, since ectopic expression of either gene from the CaMV35S promoter largely replaces shoots with individual flowers (Pidkowich et al. 1999). *AP1* together with *LFY* are master regulators that mark primordial meristematic cells for a floral fate (Pidkowich et al. 1999). *Arabidopsis AP1/FUL* genes are homologous to the *Antirrhinum SQUA* subfamily genes (Theißen et al. 2000; Becker and Theißen 2003). These genes play functionally redundant roles in the control of flower meristem identity. *Arabidopsis FUL* is involved in cauline leaf morphology

PgMADS1 overexpression
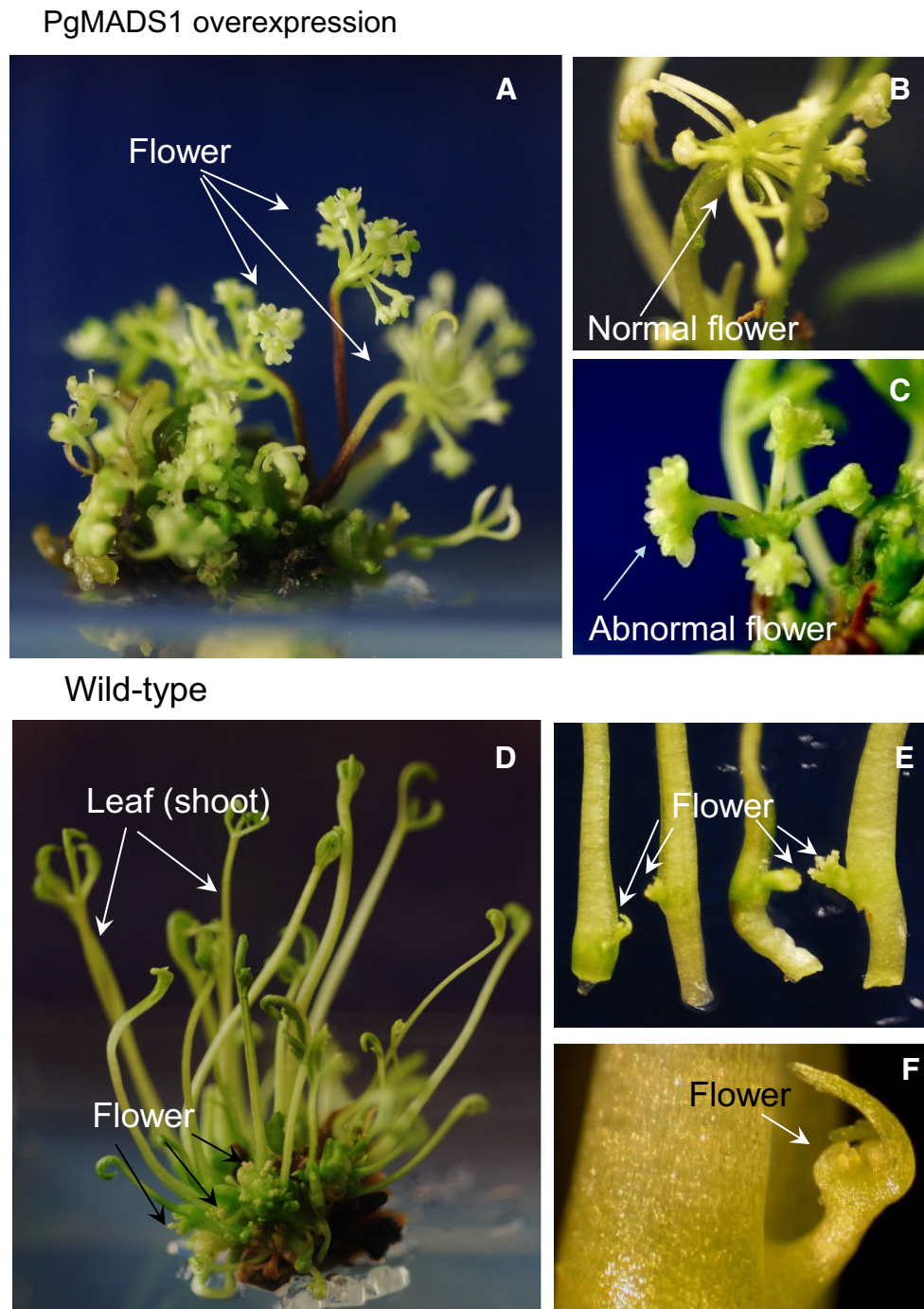
Wild-type

Fig. 8 Morphological analysis of in vitro flower development from cultured leaf nodes of wild-type and transgenic plants (T9) overexpressing *PgMADS1* grown on medium containing 4.4 μM BA and 10 μM GA₃. **a** Numerous inflorescence clusters (*arrows*) formed after 6 weeks of culture in the transgenic *P. ginseng* plants. *Note* that the leaves were only weakly developed. **b** Close-up view of an inflorescence in the transgenic plants. **c** Closed-up view of an abnormal flowers with abnormally high numbers of petals. **d** Well-developed shoot clusters in the wild-type plants after 6 weeks of culture. **e** Isolated shoots containing young flowers (*arrows*) on the leaf base of wild-type plants; the *arrows* indicate flower organs. **f** Close-up view of flower buds in the wild-type plants

and fruit development (Gu et al. 1998). In grapevine, VFUL-L and VAP1 are expressed in the lateral meristems that can give rise to inflorescences (Calonje et al. 2004). In *Gerbera hybrida*, an AP1/FUL-like gene, *GSQUA2*, plays a vital role in the meristem transition (Roukolainen et al. 2010).

RT-PCR analysis revealed that the AP1/FUL subfamily homologues (*PgMADS1–4*) were responsive by hormonal

Table 2 In vitro flower formation from leaf nodes of wild-type and transgenic *P. ginseng* overexpressing *PgMADS1* on medium with BA and GA$_3$ after 6 weeks of culture

Transgenic line	Flower length (mm)	Number of flower	Shoot length (mm)
WT	1.7 ± 0.43	2.4 ± 0.33	22.6 ± 3.64
T9	7.8 ± 1.9	3.8 ± 0.26	4.3 ± 0.95
T11	8.4 ± 0.96	3.9 ± 0.62	3.9 ± 0.83

treatment for in vitro flower induction. In particular, *PgMADS1* was clearly responsive to the hormonal treatment. Other *AG*-like genes *DEF*-like genes (*PgMADS5–9*) did not respond to hormone treatment. In situ hybridization also revealed that high levels of *PgMADS1* mRNA accumulated in the apical meristems during the early stage of inflorescence meristem growth following hormonal treatment. These results suggest that, *PgMADS1* is most responsive in hormone-induced in vitro flower formation among other *MADS* genes.

Overexpression of *PgMADS1* is not sufficient for in vitro flowering

Transgenic *P. ginseng* was constructed by overexpression of *PgMADS1*. Single gene overexpression of *PgMADS1* in transgenic *P. ginseng* is not able to induce in vitro flowering without hormone treatment and hormone-treatment requires to induce in vitro flowers in all transgenic lines. This result suggests that overexpression of *PgMADS1* alone is not sufficient to induce in vitro flowers without hormonal treatment in *P. ginseng*. However, there were striking differences in flower morphology between the control and transgenic plants when they were cultured in medium containing both BA and GA$_3$ (Fig. 8). In transgenic *P. ginseng*, inflorescence growth occurred rapidly because these transgenic plants exhibited a clear umbel inflorescence morphology with long flower stalks and pedicels compared to those of non-transgenic plants. In control plants, vegetative leaf growth was rapid and flower development was arrested at young stage although these buds showed obvious flower morphology. This result suggests that *PgMADS1* is involved in inflorescence growth in *P. ginseng*. Probably to make single umbel inflorescence with numerous florets single shoot primordium, floral transition and secondary development for inflorescence growth are necessary. *PgMADS1* may be involved in the secondary development for inflorescence growth which is also need hormones for growth stimulation.

RT-PCR analysis also revealed that enhanced transcription of *PgMADS1* was conspicuous after 2 weeks of hormone treatment but not after 1 week of hormone

treatment. *PgMADS1, 2, 3*, and *4* were highly expressed in the non-reproductive floral organs, including the pedicel and involucral bract of the umbel flower. In situ hybridization also revealed that the expression signal was evident when apical meristem showed typical flower meristem with broaden dome-shape meristem structure after 2 weeks of hormone treatment. Because *P. ginseng* flower have an umbel inflorescences which is formed from single shoot apical meristem. From this single apical meristem, numerous florets were developed from terminal of single long flower stalk as seen in Fig. 4a. Taken together, *PgMADS1* involved in umbel inflorescences development. In *Antirrhinum*, the SQUA family genes are expressed in the calyx, petal, leaf, and involucral bract (Huijser et al. 1992). Overexpression of two *Phalaenopsis* SQUA family (AP1/FUL family) genes in tobacco altered plant architecture (Chen et al. 2007), and overexpression of *GSQUA2* in *Gerbera* led to accelerated flowering (Roukolainen et al. 2010).

Panax ginseng has many type of other AP1/FUL-like genes (*PgMADS2–4*). There are many upstream genes, such as floral integrators (*FT*, *LFY*, and/or *SOC1*). Genetic evidence suggests that in *Arabidopsis*, the FT protein acts as a phloem-mobile florigen signal that moves from the leaves to the shoot apical meristem to induce flowering (Liu et al. 2013). FT acts redundantly with the floral integrator *LFY* to activate *AP1* transcription (Ruiz-Garcia et al. 1997). FT also activates another floral pathway integrator, *SOC1*, in the inflorescence meristem and activates *AP1* in the floral meristem to initiate flower development (Wigge et al. 2005; Abe et al. 2005). In *Arabidopsis*, exogenous or endogenous GAs activate the expression of *LFY* (Blazquez et al. 1997; Blazquez and Weigel 2000). Bonhomme et al. (2000) reported that cytokinins and GA$_3$ activate *Sinapis alba SaMADS A*, which shows a high degree of similarity to *SOC1* (Samach et al. 2000). Thus, to induce in vitro flower formation in transgenic *P. ginseng* without hormone treatment, floral integrators such as *FT*, *LFY*, and *SOC1* or other types of AP1/FUL-like genes or additional *PgMADS 2, 3* and *4* genes may be involved.

In conclusion, we suggest that the AP1/FUL subfamily homologues *PgMADS1* clearly respond to growth regulators, and overexpression of *PgMADS1* clearly accelerated

the inflorescence growth to make umbel flower architecture in *P. ginseng*.

Materials and methods

In vitro flower induction from the cotyledonary nodes of *P. ginseng* seedlings

Korean ginseng (*Panax ginseng* C. A. Meyer) seeds were stratified in humidified sand and allowed to mature for several months at 10 °C, as the zygotic embryos were at the immature globular stage (approximately 200 mm in length) after harvest. After stratification, the seeds were immersed in 70 % ethanol for 1 min and subsequently in 1 % sodium hypochlorite solution for 1 h. The seeds were then washed three times with sterile distilled water. After carefully dissecting the zygotic embryos from the seeds, they were placed on the surface of MS basal medium (Murashige and Skoog 1962) containing 5 % sucrose and 0.7 % agar. The medium was adjusted to pH 5.8 and then autoclaved at 120 °C for 15 min. After 5 days of germination, two cotyledons, the first leaf derived from the plumule, and the hypocotyl were removed, and only the segment comprising the cotyledonary node of the seedling was cultured on MS medium containing 3 % sucrose that was supplemented with 4.4 µM BA or 10 µM GA$_3$, or 4.4 µM BA combined with 10 µM GA$_3$. The culture room was maintained at 24 ± 2 °C under 16:8 h photoperiods of 50 µmol m^{-2} s^{-1} light provided by white fluorescent tubes. The frequency and morphogenesis of in vitro flowering were examined after 6 weeks of culture.

Isolation of MADS-box genes in *P. ginseng*

Cloning of the *P. ginseng* MADS-box genes was performed using the 5′/3′-RACE strategy (Frohman et al. 1988), following the manufacturer's instructions (5′/3′-RACE Kit; Roche Diagnostics, Indianapolis, IN, USA). 3′-RACE was performed with single-stranded cDNA synthesised from the mRNA extracted from in vitro flowers induced on MS medium containing 4.4 µM BA and 10 µM GA$_3$. PCR amplification was performed with primers anchored to the 3′-end, and degenerate primers were designed against the highly conserved regions of the MADS-box genes (Supplementary Table 1). Amplified fragments were cloned into the pGEM®-T easy vector (Promega, Madison, WI, USA). Fifty clones were selected and sequenced to analyse their sequence diversity. Among the selected clones, nine clones containing the MADS domain were identified and designated as *PgMADS1–9*. The 5′ ends of the incomplete cDNAs were recovered using 5′-RACE (5′/3′-RACE kit; Roche). The specific primers used for 5′-RACE are listed in Supplementary Table 2. The BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an automated sequencer (Model 310, Applied Biosystems) were used for sequencing.

Sequence and phylogenetic analyses

To analyse phylogenetic relationships, amino acid sequences, including the deduced sequences of the *PgMADS1–9* genes in *P. ginseng*, were obtained from EMBL, GenBank, and DDBJ. Multiple sequence alignments were generated using the ClustalX program (Thompson et al. 1994), and the phylogenetic tree was constructed using TreeView software (Page 1996). A phylogenetic analysis of the deduced amino acid alignment was performed using the neighbour-joining method with Poisson correction. A bootstrap analysis with 1000 replicates was used to assess the strength of the nodes in the tree (Felsenstein 1985).

RT-PCR analysis

Total RNA was isolated from *P. ginseng* samples and reverse transcribed with the ImProm-II^{TM} Reverse Transcription System (Promega, USA). The cDNAs were used as templates for reverse transcription (RT)-PCR analysis, which was performed using the following cycle parameters: 96 °C for 5 min followed by 30 cycles of 96 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, with a final 10-min extension at 72 °C. The primer sets used for RT-PCR of *PgMADS1–9* are listed in Supplementary Table 3. Primers were designed in regions of low degeneracy among sequences. PCR products were analysed by 1 % agarose gel electrophoresis. *β-actin* was used as a control for RNA integrity and loading accuracy.

Real-time PCR (qPCR) analysis

The RNA was isolated from cotyledonary nodes cultured on medium containing various types of growth regulators (4.4 µM BA, 10 µM GA$_3$, or 4.4 µM BA and 10 µM GA$_3$). mRNA was reverse transcribed using the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA). Real-time PCR was performed using a Qiagen Rotor Gene Q Real-time PCR detector system with a SYBR Green PCR Kit (Qiagen, Germany). The two-step amplification conditions for all real-time PCR were 95 °C for 5 min, 40 cycles of 95 °C for 5 s, and 60 °C for 10 s. The real-time PCR data shown are the average relative quantities ± SE from at least three replicates. The relative expression value of each gene was calculated using the $^{-\Delta\Delta C}$T method (Livak and Schmittgen 2001). The *P. ginseng* β-actin gene was used for normalization. All primers

used in the present study are listed in Supplementary Table 4.

In situ hybridization

A 349-bp (position 173-521) *PgMADS1* cDNA fragment was amplified using two oligonucleotide primers (5′-CAA TTC TGG AAA AGT ATG ATG GG-3′ and 5′-TCG AAC AAG GTG GAG CTT GAT AT-3′). The PCR-amplified cDNAs were cloned into the pGEM®-T easy vector (Promega, Madison, WI) and were used as templates for synthesising digoxigenin-labelled RNA probes. Sense and antisense *PgMADS1* probes were prepared using T7 and SP6 RNA polymerases after digestion with BstXI and SacI, respectively. The probes were labelled using the DIG RNA labelling kit (Roche Diagnostics GmbH, Mannheim, Germany).

Apical flower buds (5 mm in length) were fixed in FAA buffer (50 % ethanol, 5 % acetic acid, 2 % formaldehyde) for 24 h at 4 °C. The fixed tissues were dehydrated and embedded in paraffin using standard methods. Sectioning, hybridisation, and detection of hybridisation signals were performed as described by Komminoth (2002). The sections were analysed using a light microscope (Olympus BX51, Japan).

Construction of transgenic *P. ginseng* overexpressing *PgMADS1*

The open reading frame (ORF) of *PgMADS1* was amplified and subcloned into the entry vector pCR®8/GW/TOPO (Invitrogen) using the Gateway recombination system. To construct the *PgMADS1* overexpression plasmid, the entry vector harbouring *PgMADS1* was transferred to the destination vector pK2WG7 between the cauliflower mosaic virus 35S (CaMV35S) promoter and the nopaline synthase (NOS) terminator, and the final construct was transformed into *E. coli* DH5α cells according to the manufacturer's instructions (Invitrogen). The integrity of the construct was confirmed by sequence analysis. These constructs were introduced into *Agrobacterium tumefaciens* strain LBA4404.

Genetic transformation of *P. ginseng* was conducted as described in our previous report (Choi et al. 2001). Cotyledonary somatic embryos that survived on the selection medium containing 50 mg/l hygromycin and 200 mg/l cefotaxime were detached and transferred to MS medium supplemented with 20 μM GA₃ and 50 mg/l hygromycin. The embryos were then germinated and maintained on half-strength MS medium containing 2 % sucrose. To multiply the transgenic plants from each line, the upper parts of the leaves were removed, and new shoots were developed from the dormant axillary buds of the cotyledonary node and leaf node. This leaf tip removal process was repeated five times until a sufficient number of shoots were acquired.

To induce in vitro flowering, the leaf nodes of in vitro propagated wild-type and transgenic plants were cultured on MS medium with 3 % sucrose supplemented with 4.4 μM BA, 10 μM GA₃, or 4.4 μM BA and 10 μM GA₃. These plants were cultured under the conditions described above. The frequency and morphogenesis of in vitro flowering in the three different media were examined after 6 weeks of culture.

Scanning electron microscopy (SEM) observations

To observe the morphology of the flowers and shoot buds, samples were affixed to aluminium stubs and placed onto a chamber stage that had been pre-cooled to −20 °C with liquid nitrogen. These explants were then viewed using a low-vacuum scanning electron microscope (LV-SEM, S-3500 N, Hitachi, Japan) with a chamber pressure of 30 Pa and an accelerating voltage of 15 kV.

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