

Alteration of floral organ identity by over-expression of *IbMADS3-1* in tobacco

Mi-Rae Shin · Sang-Gyu Seo · Ji-Seoung Kim ·
Seo-Bum Joen · Seung-Won Kang · Gung-Pyo Lee ·
Suk-Yoon Kwon · Sun-Hyung Kim

Received: 31 January 2010 / Accepted: 8 June 2010 / Published online: 22 June 2010
© Springer Science+Business Media B.V. 2010

Abstract The MADS-box genes have been studied mainly in flower development by researching flower homeotic mutants. Most of the MADS-box genes isolated from plants are expressed exclusively in floral tissues, and some of their transcripts have been found in various vegetative tissues. The genes in the STMADS subfamily are important in the development of whole plants including roots, stems, leaves, and the plant vascular system. *IbMADS3-1*, which is in the STMADS subfamily, and which has been cloned in *Ipomoea batatas* (L.) Lam., is expressed in all vegetative tissues of the plant, particularly in white fibrous roots. Sequence similarity, besides the spatial and

temporal expression patterns, enabled the definition of a novel MADS-box subfamily comprising *STMADS16* and the other MADS-box genes in STMADS subfamily expressed specifically in vegetative tissues. Expression of *IbMADS3-1* was manifest by the appearance of chlorophyll-containing petals and production of characteristic changes in organ identity carpel structure alterations and sepaldoidy of the petals. In reverse transcription-polymerase chain reaction analysis with a number of genes known to be key regulators of floral organ development, the flowering promoter *NFL1* was clearly reduced at the RNA level compared with wild type in transgenic line backgrounds. Moreover, *NtMADS5* showed slight down-regulation compared with wild-type plants in transgenic lines. These results suggest that *IbMADS3-1* could be a repressor of *NFL1* located upstream of *NtMADS5*. *IbMADS3-1* ectopic expression is suggested as a possible means during vegetative development by which the *IbMADS3-1* gene may interfere with the floral developmental pathway.

Mi-Rae Shin and Sang-Gyu Seo should be regarded co-first authors.

M.-R. Shin · S.-G. Seo · J.-S. Kim · S.-B. Joen ·
S.-H. Kim (✉)
Department of Environmental Horticulture,
University of Seoul, Seoul 130-743, Korea
e-mail: mongdal@uos.ac.kr

S.-W. Kang · G.-P. Lee
Department of Applied Plant Science, College
of Industrial Science, Chung-ang University,
Anseong-si, Gyeonggi-do 456-756, Korea

S.-Y. Kwon
Systemic Engineering Research Center, Korea Research
Institute of Bioscience and Biotechnology,
52 Eoeun-dong, Yuseong-gu, Taejeon 305-806, Korea

Keywords *IbMADS3-1* · MADS-box gene ·
STMADS subfamily · Sweet potato (*Ipomoea*
batatas L. Lam) · Vegetative development

Introduction

During the last decade, a huge amount of genetic and molecular information about *Arabidopsis*, *Antirrhinum*,

and rice has accumulated. These collective research efforts have led to increased understanding of the complexity of development in higher plants (Aswath and Kim 2005). The initiation of organized development is a complex morphogenetic phenomenon in which extrinsic and intrinsic factors are important (Aswath and Kim 2005; Prakash and Kumar 2002). The MADS-box genes, which encode transcription factors, have been studied mainly in flower development by identifying and characterizing flower homeotic mutants (Jack 2001; Kaufmann et al. 2005; Theissen et al. 2000). Although some of these genes have also been studied in vegetative tissues with regard to plant growth and development, a few genes expressed in vegetative tissues, specifically, have been identified in *Arabidopsis* (*AGL24* and *SVP*) and in potato (*STMADS11* and *STMADS16*) (Kim et al. 2002). In rice plants, a large number of MADS box genes were expressed in vegetative tissues and seed along with floral tissue (Arora et al. 2007), but it was still necessary to understand their function in plant growth and development.

Phylogenetic analyses performed for the MADS-box multigene family have distributed these genes into distinct groups or subfamilies (Munster et al. 1997; Purugganan et al. 1995; Theissen et al. 1996). Phylogenetic analyses have shown that the *StMADS11* subfamily of the MADS-box gene family (Becker and Theissen 2003; Garcia-Maroto et al. 2000) includes *JOINTLESS* in tomato (which controls the formation of pedicel abscission zones; Mao et al. 2000), *BMI* in barley (transcripts of which accumulate at high levels in both internodes and nodes; Schmitz et al. 2000), and *IbMADS4* from sweet potato (which causes a highly branched phenotype in transgenic chrysanthemum; Aswath et al. 2004). Ectopic expression of the *STMADS11* subfamily members in the development of the reproductive structure is a feature shared by genes that are not normally expressed, and which may have a vegetative role.

AGL24 and *SVP* are two *Arabidopsis* MADS-box genes in the *STMADS* subfamily. These genes also control floral transition. Despite their extensive sequence similarity, *AGL24* acts as a promoter of this process (Michaels et al. 2003; Yu et al. 2002). However, *SVP* has a role as a repressor of flowering (Hartmann et al. 2000; Lee et al. 2007). Expression of *AGL24* with the CaMV 35S promoter accelerates

flowering, but accentuates floral abnormalities including alterations in floral organ number and the appearance of chlorophyll-containing petals (Michaels et al. 2003; Yu et al. 2004). Ectopic expression of the *SVP* gene is associated with a late-flowering phenotype (Masiero et al. 2004) and conversion of sepals and petals into leaf-like structures in a manner similar to that of *35S::AGL24* plants (Liu et al. 2007; Masiero et al. 2004).

Over-expression of *OsMADS22* and *OsMADS47*, the orthologues of *SVP* and *AGL24*, respectively, in *Arabidopsis* produces the severe floral abnormalities similar to those observed in plants that over-express *SVP* or *AGL24*, and delays senescence. When *StMADS16* from *Solanum tuberosum* is ectopically expressed in tobacco, the plants have a deferred flowering time, greater node numbers under short-day conditions only, and flowers with vegetative features (Garcia-Maroto et al. 2000). These results indicate that the *STMADS* gene subfamily might be involved in the regulatory network that directs vegetative organogenesis during plant growth (Garcia-Maroto et al. 2000) and may indirectly control the flowering process.

Three criteria can help evaluate whether orthologous genes of different species share a common function:

- conservation of amino acid sequence;
- similarity of mRNA transcription patterns during development; and
- phylogenetic relationships.

The *IbMADS3-1* protein shares 98% amino acid identity with *STMADS16* (Garcia-Maroto et al. 2000), and 81 and 91% amino acid identity with *STMADS11* (Carmona et al. 1998) and *SVP* (Hartmann et al. 2000), respectively, in the MADS-box sequence. *STMADS11* and *STMADS16* are found in vegetative and floral tissues (Carmona et al. 1998; Garcia-Maroto et al. 2000). Taken together, these observations suggest that the *IbMADS3-1* genes from sweet potato and *STMADS16* from potato might share functional similarity.

In this paper, we report integration of identification of the *IbMADS3-1* gene isolated from the sweet potato and its functional characterization in tobacco. The results indicate that *IbMADS3-1* may interfere with floral development and superimpose a vegetative program in tobacco.

Materials and methods

Plant materials

Sweet potato plants (*Ipomoea batatas* L. “Yulmi”, “White star”, “Elegantsummer”, and “Koganesengan”) and tobacco plants (*Nicotiana tabacum* “Xanthi”) were used in this study. Plants were grown in a climate chamber at $25 \pm 3^\circ\text{C}$ under a 16 h light/8 h dark photoperiod. In particular, tobacco transgenic plants T₂ lines were grown under 460 nm blue and 630 nm red LED light (DYLED 45, Dynebio, Korea).

Cloning of the full length *IbMADS3-1* cDNA

Total RNA was isolated from white fibrous roots and pigmented roots of sweet potato (*Ipomoea batatas* L. “Yulmi”) using the modified CTAB method (Kim and Hamada 2005). cDNA synthesis was performed with the 3'-RACE (Rapid Amplification of cDNA Ends) system (TaKaRa, Japan) in accordance with the manufacturer's instructions. 3'-RACE adaptor primer and degenerate primer (VLCDAADV (forward primer: GTI (C/T) TI TG(C/T) GA(C/T) GCI GA (A/G) GT)) (Kim et al. 2002) were used for isolation of the *IbMADS3-1* gene. Moreover, detailed sequence comparison and alignment were performed with the CLC Free workbench program (CLC Bio, Denmark), the protein BLAST program in the NCBI database, and the ClustalW program (<http://www.ebi.ac.uk/clustalw>).

Structure analysis of the *IbMADS3-1* gene

Specific primers for the open reading frame (ORF) were used in polymerase chain reaction (PCR) analysis. PCR amplification was done as follows: a total volume of 50 μl PCR reaction mixture assembled with 30 ng DNA as template, 5 μl 10 \times Buffer, 4 μl 2.5 mM dNTPs, 1 μl 10 pmol primers, and 2.5 U ExTaq polymerase (Takara). PCR reaction mixtures were heated at 94°C for 5 min, and then 35 cycles of amplification were performed as follows: denaturation for 30 s at 94°C , annealing for 30 s at 60°C , extension for 1 min at 72°C , with a 5 min final extension at 72°C . The amplified PCR products were assayed using 1% agarose gel electrophoresis. The purified PCR fragments were cloned into a T&A cloning vector (Real Biotech, Taiwan). Sequence analyses

including sequence alignment and comparison were executed with the CLC Free workbench program (CLC Bio).

Plant transformation and molecular analysis

For *A. tumefaciens*-mediated tobacco transformation, tobacco leaves from in-vitro-grown plants were used. The coding region of *IbMADS3-1* was fused to the cauliflower mosaic virus (CaMV) 35S promoter at the *Bam*HI and *Xba*I sites of the pCAMLA vector (pCAMBIA 1300 + P35S-Tnos cassette) (Lee et al. 2005). The pCAMLA vector was transformed into *A. tumefaciens* strain EHA 105 by a freeze-thaw method (An 1987). Tobacco leaf discs inoculated with bacterial suspension at $\text{OD}_{600} = 0.6\text{--}1.0$ for 5 min were washed with distilled water (dH_2O) and dried on sterile filter paper. The inoculated leaf discs were incubated in the dark for 2 days and then transferred on MS medium supplemented with 0.1 mg l^{-1} 1-naphthaleneacetic acid (NAA), 1.0 mg l^{-1} benzylaminopurine (BAP), 50 mg l^{-1} hygromycin, and 500 mg l^{-1} cefotaxime for shoot induction. Regenerated shoots were rooted on half-strength MS medium containing 50 mg l^{-1} hygromycin and 500 mg l^{-1} cefotaxime. Transgenic lines selected on the basis of hygromycin resistance were further identified by *IbMADS3-1* specific PCR and Southern hybridization analysis. Verified transgenic T₂ generation seedlings were transplanted into pots and grown in the growth room for further experiments.

Southern hybridization analysis and RT-PCR analysis

Genomic DNA was isolated from tobacco leaves by a modified CTAB method (Kim and Hamada 2005). Twenty micrograms each of DNA samples was digested overnight with *Xba*I. Also, genomic DNAs were extracted from sweet potato root of the “White star”, “Elegantsummer”, “Koganesengan”, and “Yulmi” cultivars. These genomic DNAs were digested with *Eco*RI and *Bam*HI. Digested products were purified and separated by 0.8% agarose gel electrophoresis. After electrophoresis, the gel was agitated in a tray containing 0.25 M HCl for 15 min and rinsed briefly with dH_2O , and again agitated in a tray containing 0.4 M NaOH for 30 min. The DNAs were then transferred on to a positively charged

Nylon membrane (Roche Molecular Biochemicals, Germany) by the capillary transfer method (Sambrook and Russell 2001). The membrane was rinsed in $2\times$ SSC for 5 min, and then allowed to dry in a dry-oven set to 80°C . A 248 bp *IbMADS3-1* fragment was labeled as a probe by two primers (5'-CAG GAC TGA GCC GTG TTA TAG AG-3', 5'-GTC CTC CAC AAG ACT ACG ATG AC-3') using a PCR DIG probe synthesis kit (Roche Molecular Biochemicals, Germany). Hybridization, washing, and detection were conducted according to the guidelines established by the manufacturer.

Total RNA was isolated from tobacco leaves and flower buds of *IbMADS3-1* T₂ lines using the modified cetyl trimethylammonium bromide (CTAB) method (Kim and Hamada 2005), and total RNAs were also isolated from six organs of sweet potato (*Ipomoea batatas* L. "Yulmi"): leaf, petiole, stem, tuberous root, thick pigmented root, and white fibrous root.

Each total RNA was treated with DNase to remove genomic DNA using the PrimeScript first strand cDNA Synthesis kit (Takara). One microgram of total RNA was synthesized to first-strand cDNA using Prime Script reverse transcriptase (Takara) and oligo dT primer. Then, reverse transcription (RT) was performed for 90 min at 42°C . The 1 μl RT reaction mixture was used as template in a 30 μl PCR-amplification reaction comprising $10\times$ buffer, 200 mM dNTPs, 10 pmol primers, and 2.5 U exTaq polymerase (Takara, Japan).

PCR was run for 35 cycles of 30 s at 94°C , 30 s at 60°C and for 1 min at 72°C , with a 5 min final extension at 72°C . As an internal positive control of tobacco, a primer set (5'-GGA CTC TGG TGA TGG TGT C-3', 5'-CCT CCA ATC CAA ACA CTG TA-3') designed on the basis of the tobacco actin gene sequence was used in the experiment with tobacco. The primer set of sweet potato tubulin gene (5'-CAA CTA CCA GCC ACC AAC TGT-3', 5'-CAA GAT CCT CAC GAG CTT CAC-3') was used as the positive control in the sweet potato experiments. The amplified PCR products were visualized by electrophoresis on a 1% agarose gel.

To confirm the expression level of the floral organ identity genes, the following seven specific primer sets were used in the PCR: *NAG1* (5'-CTG AAA CTG AGA GAG CTC AGC A-3', 5'-GTA CAC TGC CAG ACA AGG AAC A-3'), *NFL1* (5'-AAG GAG CGA GGA GAG AAT GTT G-3', 5'-AGG AGG TAG ATA

TAG GGG CAA GC-3'), *NtGLO* (5'-CAC AGA GAG CTC ATG ATG TTG G-3', 5'-CTG AAC TCG GAA GGC AAA AG-3'), *NtAP2* (5'-CAC CCT ACT ACC CCA TGG AAA T-3', 5'-CTC ATC AGA AAA CGC ACA CC-3'), *NtPLE36* (5'-CTT GAG AAG GCC ATT GGT AGA G-3', 5'-GCG AGA GTA ATG GGG ATT AGG T-3'), *NtDEF* (5'-GGG GAC TAT AAC TCT GTG CTT GG-3', 5'-GCT AAA GTC CGA CCG ATT CAC T-3'), and *NtMADS5* (5'-GCA TCT TTC CTC CTA CAA CCA C-3', 5'-GGG CAT AAC ATT GTA TTG AAC-3').

Chlorophyll contents

Tobacco petals of *IbMADS3-1* transgenic plants were collected after complete flowering. Petals (0.2 g) were immersed in and extracted with 80% acetone solution for 7 days at 4°C before measurement of absorbance. This experiment was repeated three times for each *IbMADS3* transgenic line. The contents (*Ca*, chlorophyll a; *Cb*, chlorophyll b; *Ca + b*, total chlorophyll; and *Cx + c*, total carotenoids) were determined by use of Lichtenthaler's equation (1982):

$$Ca(\mu\text{g ml}^{-1}) = 12.25 A_{663.2} - 2.79 A_{646.8}$$

$$Cb(\mu\text{g ml}^{-1}) = 21.50 A_{646.8} - 5.10 A_{663.2}$$

$$Ca + b(\mu\text{g ml}^{-1}) = 7.15 A_{663.2} + 18.71 A_{646.8}$$

$$Cx + c(\mu\text{g ml}^{-1}) = (1,000 A_{470} - 1.82 Ca - 85.02 Cb)/198.$$

Scanning electron microscopy

Observation of tobacco T₂ plants petal surface using scanning electron microscopy (SEM) was performed as previously described (Kim 2008). Pieces of petals were immersed in modified Karnovsky's fixative consisting of 2% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) at 4°C overnight and washed with the same buffer three times each for 10 min. The specimens were fixed in second fixative containing 1% (w/v) osmium tetroxide in the same buffer at 4°C for 2 h. They were washed briefly two times with dH₂O and dehydrated in a graded ethanol series at room temperature. Dehydration was performed using 10 min exposure to 30, 50, 70, 80, 95, and three times in 100% ethanol. The specimens were treated with isoamyl acetate two times each for 10 min, and dried

in a CPD 030 critical point drier (BAL-TEC, Liechtenstein). Tobacco petal specimens were individually mounted on metal stubs, sputter-coated with gold, and observed by SEM.

Results and discussion

Sequence analysis of *IbMADS3-1*

To isolate *IbMADS3-1* mRNA, a PCR amplification primer was designed for a highly conserved sequence in the MADS-domain of the STMADS family

(*IbMADS4*, *STMADS16*, *STMADS11*, *SVP*, and *AGL24*) (Kim et al. 2002). Sequence analysis showed that the full length *IbMADS3-1* cDNA was 1,194 bp long and consisted of a 279 bp 5'-untranslated region (UTR), 231 bp 3'-UTR, and a 684 bp ORF encoding 227 amino acids (Fig. 1a). The whole *IbMADS3-1* gene was further amplified from sweet potato genomic DNA, using specific primers for the ORF. Comparison of the genomic DNA sequence with the *IbMADS3-1* cDNA revealed that it consisted of seven exons, which perfectly matched the cDNA sequence and first intron of 1,399-bp length and six introns (Fig. 1b). Sequence analysis of *IbMADS3-1* grouped the gene as a member

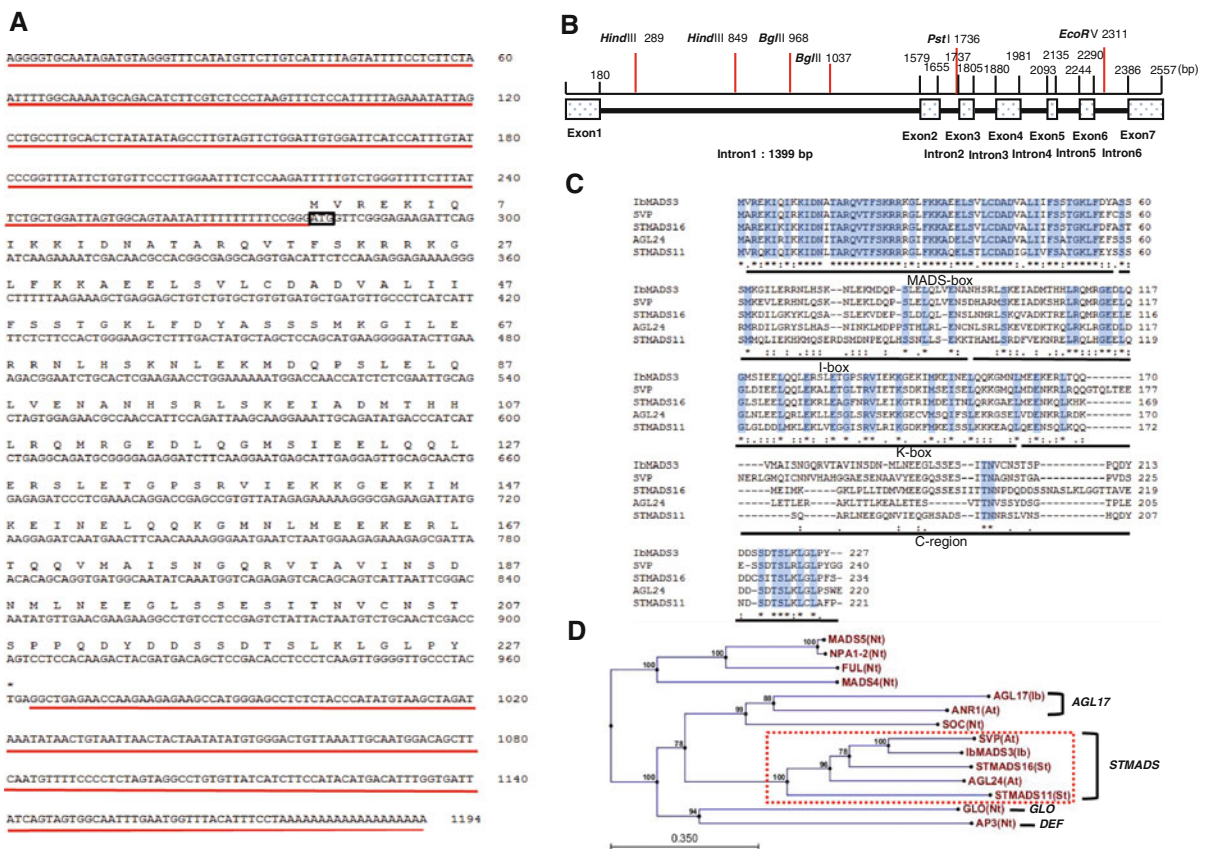


Fig. 1 Structure and sequence analysis of the *IbMADS3-1* gene. **a** Numbers on the right represent nucleotide and amino acid positions. The deduced amino acid sequence is shown in single-letter code above the nucleotide sequence. The start codon is indicated by a box and the termination codon is marked with an asterisk. The underlined nucleotide sequences are 5'-UTR and 3'-UTR. **b** *IbMADS3-1* consists of seven exons and six introns. Especially, the first intron of *IbMADS3-1* is much longer (about 12–19-fold) than the other introns. Filled boxes indicate the ORF, and lines between boxes indicate introns. The numbers

represent nucleotides. **c** Comparison of the amino acid sequences of *IbMADS3-1* and other homologous proteins. **d** Alignment of deduced amino acid sequences of *IbMADS3-1* with high homologues including *SVP* (AAG24508), *STMADS16* (AAB94005), *AGL24* (AAC63140), and *STMADS11* (AAB94006). The sequences were aligned using the ClustalW program. Identical amino acid residues in all five sequences are indicated by asterisks. Colons and dots indicate that amino acid residues are conserved in most sequences. Analysis was performed using the CLC workbench program

of the MADS-box transcription factor family. Its deduced amino acid sequence displayed high identity with those of SVP (AAG24508), STMADS16 (AAB94005), AGL24 (AAC63140), and STMADS11 (AAB94006) (Fig. 1c). The MADS-domain sequence of *IbMADS3-1* was 90% identical with those of SVP, STMADS11, and STMADS16, and was 83% identical with that of AGL24. Phylogenetic analysis of the five MADS-box proteins—SVP and AGL24 from *Arabidopsis*, STMADS11 and STMADS16 from potato, and *IbMADS3* from sweet potato—classified this group into the STMADS family (Kim et al. 2002) (Fig. 1d).

STMADS11 and *STMADS16* from potato are expressed during vegetative growth (Carmona et al. 1998). Although *SVP* and *AGL24* are expressed in inflorescence meristems, stem, leaves, and roots, *SVP* transcripts disappear before emergence of the sepal primordia during flower development, and may positively regulate a repressor of flowering (Alvarez-Buylla et al. 2000). Subfamily members tend to share highly similar sequences and related functions (Doyle 1994; Purugganan et al. 1995; Theissen et al. 1996), so that *IbMADS3-1* seems to be exerting its function in the vegetative phase.

These results were expected, considering that *IbMADS3-1* is a sister gene to the *STMADS*-related genes that are specific for vegetative tissues, and that most previously characterized MADS-box genes cluster in phylogenetic clades of genes with similar functions and expression patterns (Alvarez-Buylla et al. 2000; Rounsley et al. 1995).

Southern hybridization and expression pattern analysis of *IbMADS3-1*

Southern hybridization suggested the presence of at least two copies of *IbMADS3-1* in each cultivar of “Koganesengan”. The band patterns in *EcoRI* digestion within the “White star”, “Elegant summer”, and “Yulmi” cultivars were different. In the case of digested *BamHI* restrict enzyme, only “White star” exhibited a different band pattern among the cultivars (Fig. 2a). “Elegant summer”, “Koganesengan”, and “Yulmi” displayed similar band patterns. It is unclear at present whether this result was because of the hexaploid and heterozygous nature of sweet potato.

Total RNA was isolated from leaf, petiole, stem, and root tissues, and analyzed with the *IbMADS3-1* specific primer. To determine the local expression

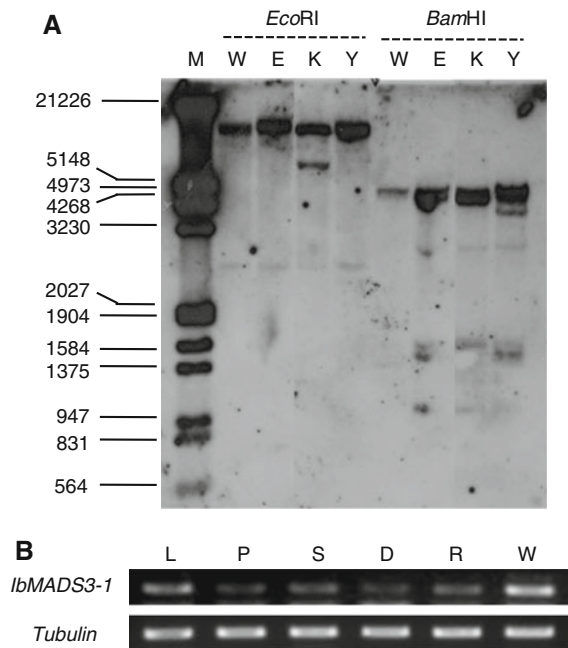


Fig. 2 Southern blot and RT-PCR analysis of the *IbMADS3-1* gene activity in sweet potato plants. **a** Genomic DNA of “White star” (W), “Elegant summer” (E), “Koganesengan” (K), and “Yulmi” (Y) cultivars was digested with *EcoRI* and *BamHI*. The genomic DNAs were separated on a 0.8% agarose gel, blotted, and probed with a DIG-labeled *IbMADS3-1* fragment. The size marker (M) fragments of DNA are indicated on the left. **b** Total RNA was isolated from leaf (L), petiole (P), and stem (S), developing tuberous root (D), thick pigmented root (R), and white fibrous root (W). *Tubulin* was used as a control

pattern of *IbMADS3-1*, expression of the RNA from each organ was examined. RT-PCR revealed that each gene was expressed in all organs. In particular, the *IbMADS3-1* gene was strongly expressed in white fibrous root (Fig. 2b). This result demonstrated that *IbMADS3-1* transcripts are constitutively and differently expressed in various organs, suggesting a constitutive and/or specific function of *IbMADS3-1* in vegetative tissue development. Additionally, the preferential expression of *IbMADS3-1* more actively in root tissues (especially white fibrous root) implies that *IbMADS3-1* may play an important role in root formation or development.

Nonetheless, previous studies of *IbMADS3-1* had already suggested that this gene could function in vegetative organ development because of its high and apparently specific expression in roots (Kim et al. 2002). In this study, we confirmed that *IbMADS3-1* is

indeed expressed in vegetative tissues. In accordance with this pattern of expression, *IbMADS3-1* is important for vegetative organ development.

Phenotype of *IbMADS3* transgenic plants

Because of the minimum amount of plant tissue needed for PCR analysis, all the putative transformed leaves that exhibited hygromycin resistance were analyzed by PCR. A predicted 248-bp internal fragment for the *IbMADS3-1* was amplified in all the leaves, consistent with integration of the transgene into the genomes. The genomic DNA from putative transgenic plants was subjected to gel blot analysis under high-stringency conditions. Hybridization with an *IbMADS3-1* specific probe confirmed foreign DNA integration in the transgenic lines analyzed (Fig. 3a). Southern blot results indicated that the transformants selected had a single-copy insertion of T-DNA at different sites in the tobacco plant chromosome (Fig. 3b). Genomic DNA from the wild-type did not give any signal.

The *IbMADS3-1* gene is expressed in vegetative tissue only, and *IbMADS3-1* protein is a member of the STMADS subfamily (Kim et al. 2002). In general, gene expression correlates with the place where the gene exerts its function. In order to test this possibility, we created transgenic tobacco plants that

constitutively transcribed *IbMADS3-1* cDNA from the CaMV 35S promoter. The transgenic plants showed no morphological or growth differences in vegetative tissues, as compared with Wt plants. On the other hand, *IbMADS3-1* over-expression lines exhibited remarkable morphological alterations in floral organs including sepal, petal, carpel, ovary, and peduncle, and the appearance of chlorophyll-containing petals (Fig. 4e). Only the stamen was unchanged to the naked eye (Fig. 4c). Sepals of *IbMADS3-1* transgenic lines decreased compared with those of wild type. Furthermore, the wild-type sepal was connected with a pedicel of approximately 1-cm and transgenic plants pedicels were nearly degenerate (Fig. 4b).

The petal was altered in both shape and color. Petals of *IbMADS3-1* transgenic lines grew longer than normal petal and color was yellowish green generally (Fig. 4b). Most of the transgenic plants retained pink color at the petal tip (Figs. 4f, 5d). By contrast, at the top of some petal tips of transgenic plants the white color disappeared, and just below the top of the petal tip was a yellowish color (Fig. 4f). The carpel length of wild-type flowers was the same as the petal length. The carpel length of transgenic plants was always reduced, but the extent of the decrease varied. Moreover, the stigma became smaller and had a reduced viscosity compared with

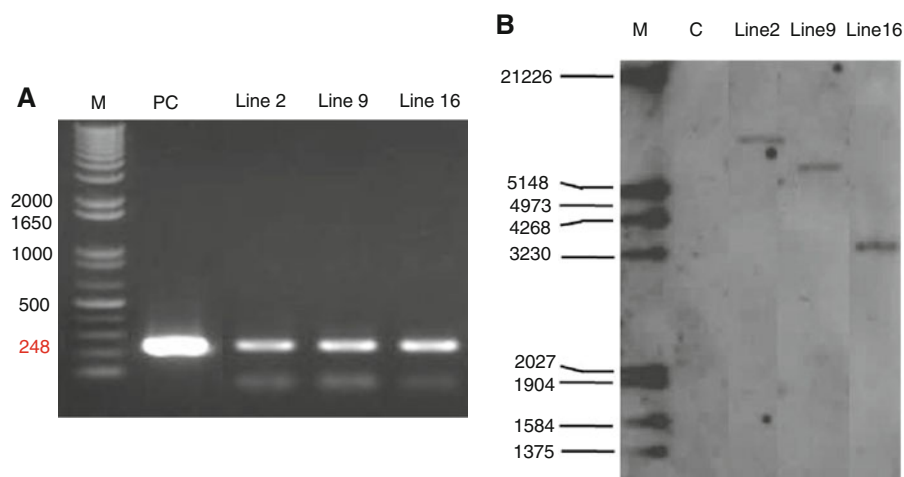


Fig. 3 Confirmation of the *IbMADS3-1* gene integration in transgenic plants. **a** PCR analysis of genomic DNA isolated from transgenic T₂ lines. Abbreviations: *M*, marker; *PC*, Positive control (*lines 2, 9, and 16* represent individual lines of transgenic tobacco plants). **b** Southern blot analysis of genomic

DNA from transgenic T₂ tobacco plants. Genomic DNA was digested with *Xba*I. Abbreviations: *M*, DIG-labeled DNA molecular weight marker. *C*, control (wild-type plant). *Lines 2, 9, and 16* are individual lines of transgenic tobacco plants

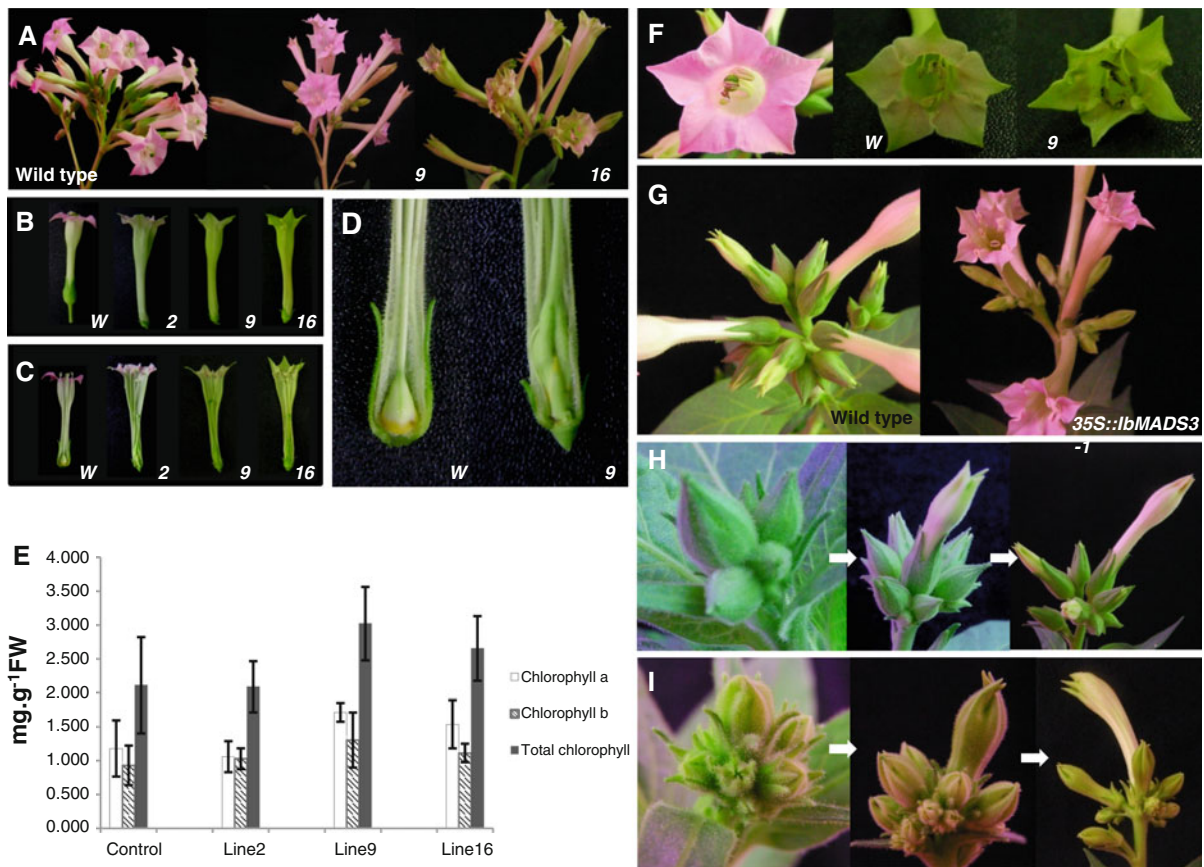


Fig. 4 Phenotypic alterations of the transgenic tobacco plants over-expressing *IbMADS3-1* and arrangement of flower buds in the wild-type and over-expressing *IbMADS3-1* tobacco plant. **a** Inflorescences of the tobacco wild type and *IbMADS3-1* over-expressing line 9 and line 16. **b** Flower of the tobacco wild type and *IbMADS3-1* over-expression lines. **c** Longitudinal sections of the tobacco flowers. The order is the same as in **b**. **d** The tip of petal of the tobacco wild type and *IbMADS3-1* over-expression lines. **e** Chlorophyll content of wild type and

the wild type (Fig. 4c). The ovary had an extended structure with a central furrow and a hardened internal structure, compared with the normal ovary. The nectaries, which normally appeared as an orange ring at the base, were lacking in the transgenic flowers (Fig. 4c, d). Such ovary shape alterations may reflect a lack of coordination in placental development at anthesis (stage 12 according to Garcia-Maroto et al. 2000; Koltunow et al. 1990). Past and previous observations support the notion that deformations of the ovary are produced by the absence of control in placental development. The arrangement of flower buds in the wild-type plant was very regular

IbMADS3-1 over-expression lines. *Control* is wild-type tobacco petal and *lines 2, 9, and 16* are transgenic tobacco petals. Contents were similar in all lines. The density seemed to be higher for line 9 than for the other lines. **f** Flower bud arrangement of normal plant and transgenic plant. **g** Flower bud arrangement of wild-type plants was regular. **h** In contrast, the morphology of the flower bud arrangement of transgenic plants was altered and irregular

and displayed a circular pattern (Fig. 4g, h), while the arrangement in transgenic plants was slightly altered; both pedicel and sepal connecting with pedicel were shorter than those of normal plants (Fig. 4g). These observations bolster the view that the *IbMADS3-1* gene product is concerned with suppression of pedicel and sepal development.

Authentic surface cell types can be distinguished by SEM. Specified parts of tobacco flowers were used for SEM analysis. Cells from the petal tip were characterized by the presence of globular-tipped trichomes, stomata, and irregularly shaped cells. The trichomes of *IbMADS3-1* transgenic line were altered in terms of an

irregular size and increased density. In the petal tips of transgenic flowers, the epidermal cells were a dark color, rendering the stomata more clearly visible (Fig. 5a–c). The stomatal density was increased, as was the density of trichomes. Meanwhile, the surface of the corolla tube was similar to the petal tip surface in the density of trichomes and stomata, and the epidermal cells were elongated (Fig. 5d–f). In particular, *IbMADS3-1* transgenic line 16 had remarkable visible changes in floral organ as contrasted with the other line and the wild-type plant. (Fig. 5c, f). This result showed that the petal tip surface morphology of transgenic flowers more closely mirrors that of the normal corolla tube (Fig. 5b–d).

Ectopic expression of *IbMADS3-1* produced characteristic changes in organ identity, carpel structure, alterations and sepaldoidy of the petals. One possible explanation of this observation is that the *IbMADS3-1* product alone is capable of partially inducing the developmental pathways leading to petal and carpel formation. The *IbMADS3-1* gene is exclusively expressed in the vegetative organs of normal plants, and phenotypic alterations are exclusively observed in the reproductive structures. It may be that overexpression of *IbMADS3-1* in tobacco is able to trigger developmental events that are appropriate for vegetative tissues. In agreement with this suggestion, *IbMADS3-1* expression is more abundant in

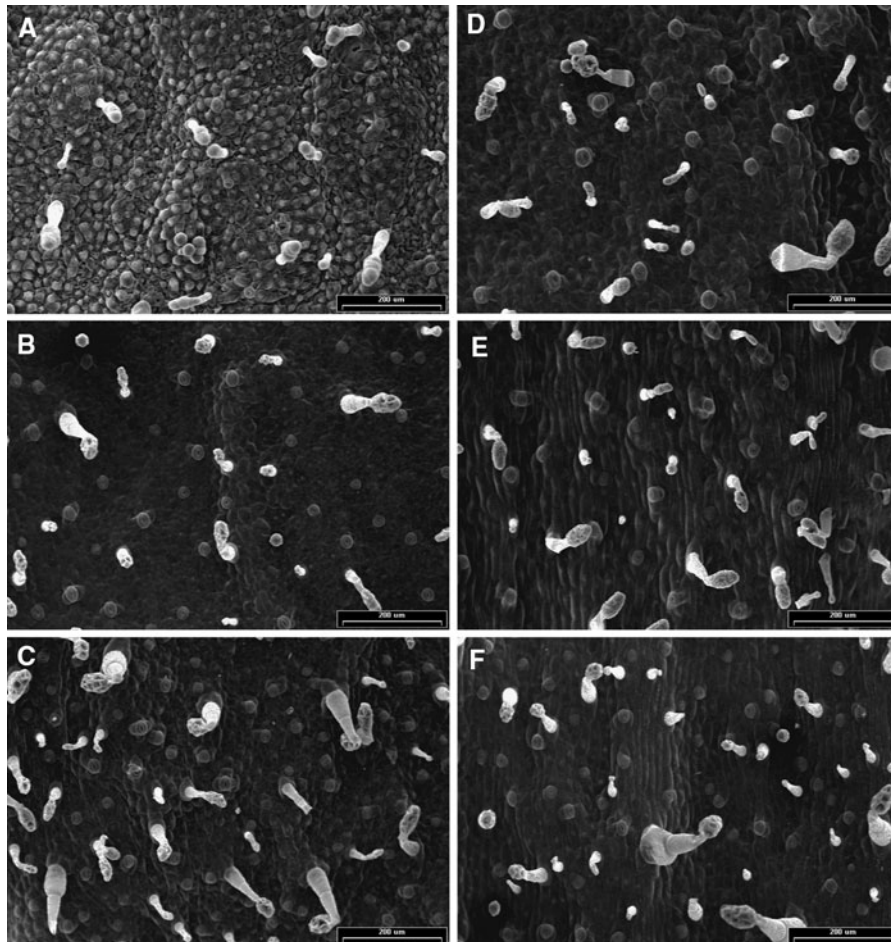


Fig. 5 SEM analysis of characteristic cell surface morphology in wild-type tobacco petal and transgenic tobacco petals. Regions of petals of tobacco flowers sampled for SEM analysis from top of the tobacco petal tip (*left*) and middle of tobacco corolla tube (*right*). Surface cells of regions of each part of

wild-type and transgenic petals were taken. **a** A wild-type petal tip. **b** A petal tip of *IbMADS3-1* line 9. **c** A petal tip of *IbMADS3* line 16. **d** A wild-type corolla tube. **e** A corolla tube of *IbMADS3* line 9. **f** A corolla tube of *IbMADS3* line 16

vegetative tissues such as the white fibrous roots but not in the flower (Kim et al. 2002). It has previously been suggested that ectopic expression of *STMADS16* (Garcia-Maroto et al. 2000), and *OsMADS22* and *OsMADS47* (Fornara et al. 2008) show vegetative characteristics, and similar alterations in tobacco and Arabidopsis flower development, respectively. These phenotypes could indicate that failure to establish floral identity results in enhancement of the vegetative traits of the flower.

Expression levels of the floral organ identity genes in flower buds of plants over-expressing *IbMADS3-1*

To confirm a possible genetic interaction between *IbMADS3-1* and previously characterized genetic components of floral organ formation, mRNA expression of a number of genes known to be key regulators of floral organ development was assessed. It was confirmed that *IbMADS3-1* had drastically increased levels of expression in transgenic lines (Fig. 6). Interestingly, the flowering promoter, *NFL1*, was clearly reduced at the RNA level compared with wild type in the background of transgenic lines 2, 9, and 16 (Fig. 6). In contrast, *NAG1*, *NtGLO*, *NtPLE36*, and *NtDEF*, which are upstream regulators in floral organ development (Davies et al. 1996; Hansen et al. 1993; Kang et al. 1995; Kempin et al. 1993; Theissen 2001), did not show significant alterations in mRNA expression in the transgenic lines. On the other hand, *NtMADS5*, which is principally required to direct a primordium to develop into a flower in tobacco (Smykal et al. 2007), showed a slight down-regulation compared with wild-type plants in transgenic lines. The results correlate with the abnormal shoot-like flower phenotypes. The transition from vegetative to reproductive development involves the coordination of several endogenous and environmental factors. Central to the transition to flowering is the increased expression of the *LFY* gene at the site of floral meristem formation in *Arabidopsis*, followed by the transcription of *API* in the developing floral meristem (Mandel et al. 1992). The expression of *API* is delayed and reduced in *lfy* mutant plants (Liljegren et al. 1999). In agreement with this, *NtMADS5* is down-regulated by *NFL1* in transgenic line backgrounds (Calonje et al. 2004). Therefore, our data suggest that *IbMADS3-1* could be a repressor of *NFL1*

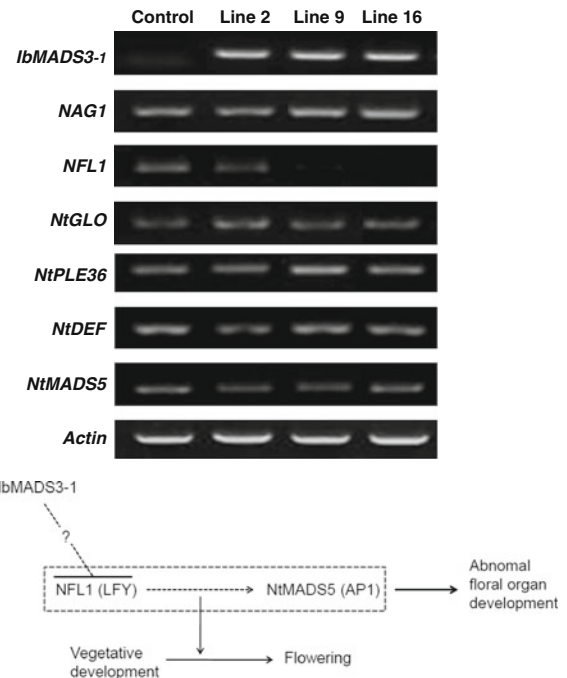


Fig. 6 Expression pattern of the floral organ identity genes in flower buds of over-expressed *IbMADS3-1* and schematic representation of the regulation of flowering transition. RNA was isolated from flower buds of a wild-type and three transgenic plants. The specific primer sets of six genes, *NAG1* (L23925), *NFL* (U15798), *NtGLO* (X67959), *AP2* (AJ299252), *NtPLE36* (U63163), *NtDEF* (X96428), and *NtMADS5* (AF068724) were used in the analysis

upstream of *NtMADS5*. However, complementation of *ntmads5* and *nfl1* mutants with *IbMADS3-1* over-expression constructs should be pursued to confirm this proposed role of *IbMADS3-1*.

There is one possibility of reconciling abnormal shoot-like flower phenotypes in the *IbMADS3-1* over-expression line. Ectopic expression of *IbMADS3-1* may interfere with floral development, especially repressed *NFL1*, and superimpose a vegetative program in tobacco.

Acknowledgments This work was supported by the University of Seoul 2009 Research Fund.

References

- Alvarez-Buylla ER, Liljegren SJ, Pealz S, Gold SE, Ditta GS, Vergara-Silva F, Yanofsky MF (2000) MADS-box gene evolution beyond flowers: expression in pollen, endosperm, guard cells, roots and trichomes. *Plant J* 24: 457–466

- An G (1987) Binary Ti vectors for plant transformation and promoter analysis. *Methods Enzymol* 153:292–305
- Arora R, Agarwal P, Ray S, Singh AK, Singh VP, Tyagi AK, Kapoor S (2007) MADS-box gene family in rice: genome-wide identification, organization and expression profiling during reproductive development and stress. *BMC Genom* 8:242–263
- Aswath CR, Kim SH (2005) Another story of MADS-box genes-their potential in plant biotechnology. *Plant Growth Regul* 46:177–188
- Aswath CR, Moa SY, Kim SH, Kim DH (2004) IbMADS4 regulates the vegetative shoot development in transgenic chrysanthemum (*Dendrathera grandiflora* (Ramat.) Kitamura). *Plant Sci* 166:847–854
- Becker A, Theissen G (2003) The major clades of MADS-box genes and their role in the development and evolution of flowering plants. *Mol Phys Evol* 29:464–489
- Calonje M, Cubas P, Martinez-Zapater JM, Carmona MJ (2004) Floral meristem identity genes are expressed during tendrill development in grapevine. *Plant Physiol* 135:1491–1501
- Carmona MJ, Grtega N, Garcia-Maroto F (1998) Isolation and molecular characterization of a new vegetative MADS-box gene from *Solanum tuberosum* L. *Planta* 207:181–188
- Davies B, Rosa AD, Eneva T, Saedler H, Sommer H (1996) Alteration of tobacco floral organ identity by expression of combinations of *Antirrhinum* MADS-box genes. *Plant J* 10:663–677
- Doyle JJ (1994) Evolution of a plant homeotic multigene family: toward connecting molecular systematics and molecular developmental genetics. *Syst Biol* 43:307–328
- Fornara F, Gregis V, Pelucchi N, Colombo L, Kater M (2008) The rice *StMADS11*-like genes *OsMADS22* and *OsMADS47* cause floral reversions in *Arabidopsis* without complementing the *svp* and *agl24* mutants. *J Exp Bot* 59:2181–2190
- Garcia-Maroto F, Ortega N, Loxana R, Carmona MJ (2000) Characterization of the potato MADS-box gene *STMADS16* and expression analysis in tobacco transgenic plants. *Plant Mol Biol* 42:499–513
- Hansen G, Estruch JJ, Sommer H, Spena A (1993) NT-GLO: a tobacco homolog of the GLOBOSA floral homeotic gene of *Antirrhinum majus*: cDNA sequence and expression pattern. *Mol Gen Genet* 239:310–312
- Hartmann U, Hohmann S, Nettesheim K, Wisman E, Saedler H, Huijser P (2000) Molecular cloning of SVP: a negative regulator of the floral transition in *Arabidopsis*. *Plant J* 21:351–360
- Jack T (2001) Plant development going MADS. *Plant Mol Biol* 46:515–520
- Kang HG, Nho YS, Chung YY, Costa MA, An K, An G (1995) Phenotypic alterations of petal and sepal by ectopic expression of a rice MADS box gene in tobacco. *Plant Mol Biol* 29:1–10
- Kaufmann K, Melzer R, Theissen G (2005) MICK-type MADS-domain proteins: structural modularity, protein interactions and network evolution in land plants. *Gene* 347:183–198
- Kempin SA, Mandel MA, Yanofsky MF (1993) Conversion of perianth organs by ectopic expression of the tobacco floral homeotic gene *NAG1*. *Plant Physiol* 103:1041–1046
- Kim KW (2008) Visualization of micromorphology of leaf epicuticular waxes of the rubber tree *Ficus elastica* by electron microscopy. *Micron* 39:976–984
- Kim SH, Hamada T (2005) Rapid and reliable method of extracting DNA and RNA from sweetpotato, *Ipomoea batatas* (L.) Lam. *Biotech Lett* 27:1841–1845
- Kim SH, Mizuno K, Fujimura T (2002) Isolation of MADS-box genes from sweet potato (*Ipomoea batatas* (L.) Lam.) expressed specifically in vegetative tissues. *Plant Cell Physiol* 43:314–322
- Koltunow AM, Truettner J, Cax KH, Wallroth M, Goldberg RB (1990) Different temporal and spatial gene expression patterns occur during anther development. *Plant cell* 2:1201–1224
- Lee JH, Kim SH, Jung YH, Kim JA, Lee MO, Choi PG, Choi W, Kim KN, Jwa NS (2005) Molecular cloning and functional analysis of rice (*Oryza sativa* L.) *OsNDR1* on defense signaling pathway. *Plant Pathol J* 21:149–157
- Lee JH, Yoo SJ, Park SH, Hwang I, Lee JS, Ahn JH (2007) Role of SVP in the control of flowering time by ambient temperature in *Arabidopsis*. *Genes Dev* 2:397–402
- Lichtenthaler HK (1982) Synthesis of prenyllipids in vascular plants (including chlorophylls, carotenoids, prenylquinones). In: Matsui A, Black CC (eds) CRC handbook of biosolar resources, vol I part I: basic principles. CRC Press, Boca Raton, pp 405–421
- Liljegren SJ, Gustafson-Brown C, Pinyopich A, Ditta GS, Yanofsky MF (1999) Interactions among *APETALA1*, *LEAFY*, and *TERMINALFLOWER1* specify meristem fate. *Plant Cell* 11:1007–1018
- Liu C, Zhou J, Bracha-Drori K, Yalovsky S, Ito T, Yu H (2007) Specification of *Arabidopsis* floral meristem identity by repression of flowering time genes. *Development* 134:1901–1910
- Mandel MA, Gustafson-Brown C, Savidge B, Yanofsky MF (1992) Molecular characterization of the *Arabidopsis* floral homeotic gene *APETALA1*. *Nature* 360:273–277
- Mao L, Begum D, Chuang HW, Budiman MA, Szymkowiak EJ, Irish EE, Wing RA (2000) *JOINTLESS* is a MADS-box gene controlling tomato flower abscission zone development. *Nature* 406:910–913
- Masiero S, Li MA, Will I, Hartmann U, Saedler H, Huijser P, Schwarz-Sommer Z, Sommer H (2004) *INCOMPOSITA*: a MADS-box gene controlling prophyll development and floral meristem identity in *Antirrhinum*. *Development* 131:5981–5990
- Michaels SD, Ditta G, Gustafson-Brown C, Pelaz S, Yanofsky M, Amasino RM (2003) *AGL24* acts as a promoter of flowering in *Arabidopsis* and is positively regulated by vernalization. *Plant J* 33:867–874
- Munster T, Pahnke J, Rosa AD, Kim JT, Martin W, Saedler H, Theissen G (1997) Floral homeotic genes were recruited from homologous MADS-box genes preexisting in the common ancestor of ferns and seed plants. *Proc Natl Acad Sci USA* 94:2415–2420
- Prakash AP, Kumar PP (2002) *PkMADS1* is a novel MADS box gene regulating adventitious shoot induction and vegetative shoot development in *Paulownia kawakamii*. *Plant J* 29:141–151
- Purugganan MD, Rounsley SD, Schmidt RJ, Yanofsky MF (1995) Molecular evolution of flower development:

- diversification of the plant MADS-box regulatory gene family. *Genetics* 140:345–356
- Rounsley SD, Ditta GS, Yanofsky MF (1995) Diverse roles for MADS box genes in Arabidopsis development. *Plant Cell* 7:1259–1269
- Sambrook J, Russell DW (2001) *Molecular cloning. A laboratory manual*, 3rd edn. Cold Spring Harbor, New York
- Schmitz J, Franzen R, Ngyuen TH, Garcia-Maroto F, Pozzi C, Salamini F, Rohde F (2000) Cloning, mapping and expression analysis of barley MADS-box genes. *Plant Mol Biol* 42:899–913
- Smykal P, Gennen J, Bodt SD, Ranganath V, Melzer S (2007) Flowering of strict photoperiodic Nicotiana varieties in non-induction conditions by transgenic approaches. *Plant Mol Biol* 65:233–242
- Theissen G (2001) Development of floral organ identity: stories from the MADS house. *Curr Opin Plant Biol* 4:75–85
- Theissen G, Kim JT, Saedler H (1996) Classification and phylogeny of the MADS-box multigene family suggest defined roles of MADS-box gene subfamilies in the morphological evolution of eukaryotes. *J Mol Evol* 43:484–516
- Theissen G, Becker A, Di Rosa A, Kanno A, Kim JT, Munster T, Sinter KU, Saedler H (2000) A short history of MADS-box genes in plant. *Plant Mol Biol* 42:115–149
- Yu H, Xu Y, Tan EL, Kumar PP (2002) *AGAMOUS-LIKE 24*, a dosage-dependent mediator of the flowering signals. *Proc Natl Acad Sci USA* 99:16336–16341
- Yu H, Ito T, Wellmer F, Meyerowitz EM (2004) Repression of *AGAMOUS-LIKE 24* is a crucial step in promoting flower development. *Nat Genet* 36:157–161