Study of a Tobacco MADS-Box Gene Triggering Flower Formation

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Recently, we have reported a rice MADS-box gene, OsMADS1, as a molecular factor triggering flower formation; this has been well studied in a heterologous system (Chung et al., 1994). In order to study whether the OsMADS1 homolog exists in other plant species, the OsMADS1 cDNA was used as a probe to screen a tobacco cDNA library, and a potential homolog, NtMADS3, was isolated. Sequence analysis revealed that the gene shares 56.1% identity in whole amino acids with OsMADS1. Like OsMADS1, the NtMADS3 gene starts to express at a very early stage of flower development, and the expression continues up to flower maturation. In the tobacco flower, the gene is expressed in whorl 2, 3, and 4, corresponding to the petal, stamen, and carpel, respectively. Upon ectopic expression in the homologous system, NtMADS3 caused a transition from inflorescence shoot meristem into floral meristem, reducing flowering time dramatically. These phenotypes strongly suggest the NtMADS3 gene is the OsMADS1 homolog of tobacco. Hybrids between the OsMADS1 and the NtMADS3 plants were also generated. The hybrids flowered even earlier than these two transgenic plants. The detailed studies are discussed here.

Flowering is an attractive system for the study of development in plants. In recent years, tremendous progress, especially on the molecular level, has been made in understanding flower development of plants, and the molecular analyses have identified the genes involved in both flower induction and its organ development. These genes, called MADS-box genes, are now known to encode the transcription factor (Yanofsky et al., 1990; Gasser, 1991).

Several MADS-box genes from both dicotyledonous and monocotyledonous plant species have been identified. In dicotyledonous plant species, AGL1-AGL6 (Ma et al., 1991), APETALA1 (AP1) (Mandel et al., 1992), APETALA3 (AP3) (Jack et al., 1992), and PISTILATA (PI) (Goto et al., 1994) in Arabidopsis thaliana and SQUAMOSA (SQUA) (Huijser et al., 1992), GLOBOSA (GLO) (Trobner et al., 1992), and PLENA (PLE) (Sommer et al., 1990) in Antirrhinum majus are known to contain the conserved domains. Other MADS-box genes are NAG1 in tobacco (Kempin et al., 1993), TAG1, TM3-TM6, and TM8 in tomato (Pnueli et al., 1991, 1994a, and 1994b), and FBP1, FBP2, FBP6, and pMADS3 in petunia (Angenent et al., 1992; van der Krol et al., 1993; Tsuchimoto et al., 1993), and ST-Deficiens (St-Def) in potato (Garcia-Maroto et al., 1993). Whereas in monocotyledonous plant species, ZAG1 and ZAG2 in maize, om1 in orchid, and four rice MADS-box genes, OsMADS1 – OsMADS4, were reported to contain the MADS domain (Schmidt et al., 1993; Lu et al., 1993; Chung et al., 1994 and 1995; Kang et al., 1995).

Recently, a rice MADS-box gene, OsMADS1 which is involved in flower induction has been reported (Chung et al., 1994). DNA sequence analysis revealed that the rice clone encodes a putative protein of 257 amino acid residues containing the conserved MADS-box and K-box domains. The OsMADS1 amino acid sequence is homologous to other MADS-box genes especially to APETALA1 (44.4% identity) and SQUAMOSA (42.6% identity) that are involved in floral initiation (Mandel et al., 1992 and Huijser et al., 1992). In addition, OsMADS1 shows extensive similarity to the functionally anonymous Arabidopsis MADS box genes, AGL2 (56.2% identity) and AGL4 (55.4% identity) (Ma et al., 1991). Transgenic tobacco plant that undergoes an ectopic expression condition of the

OsMADS1 showed severe phenotypes of dwarfism and early flowering.

In this study, a OsMADS1 homolog of tobacco, NtMADS3, was isolated and characterized. Interestingly, an ectopic expression of the NtMADS3 gene in transgenic tobacco plant also caused a transition from inflorescence to florescence resulting in early flowering and dwarfism, suggesting that the gene is involved in flower induction. The detailed studies of the tobacco MADS-box gene are discussed.

MATERIALS AND METHODS

Plant Materials and Bacterial Strains

Tobacco floral organ libraries were constructed from mRNA isolated from *Nicotiana tabacum* cv. Xanthi. Samples for RNA and DNA studies of NtMADS3 were harvested from the same variety. For transformation, both *Nicotiana tabacum* cv. Petite Havana SR1 and *Nicotiana tabacum* cv. Xanthi were used.

Escherichia coli strains MC1000 [*F-, araD139, Δ (araABC-leu)7679, galU, galK, ΔlacX74, thi, rpsL(Str^r)] and JM83 [F-, ara, Δ(lac-proAB), rpsL(Str^r), φ0d lacZΔM15*] were used as hosts for molecular cloning. The f1 helper phage R408 and E. coli strain XL-1Blue [*Fi::Tn10 proA+B+, lacIq, lacZΔM15/recA1, endA1, gyrA96(Nal^r), thi, hsdR17(rk-mk+), supE44, relA1, lac*] were used for in vivo excision of pBluescript plasmid vector from the λZapII phage (Stratagene, California). *Agrobacterium tumefaciens* LBA4404 (Hoekema et al., 1983) containing the Ach5 chromosomal background and a disarmed helper-Ti plasmid pAL4404, was used for transformation of tobacco plants by the cocultivation method (An et al., 1988).

Construction of cDNA Libraries and Isolation of *NtMADS1*

cDNA libraries were constructed using the λZAPII vector (Stratagene) and poly (A)+RNA isolated from the tobacco floral organs, petals, anthers, and carpels. An adapter containing EcoRI and NotI sites (Pharmacia Biotech) was used to ligate the vector and cDNAs. Plaque hybridization using OsMADS1 cDNA as a probe was performed to screen tobacco cDNA library. 5 × 10^4 plaques from petals cDNA library was lifted to nitrocellulose membranes and hybridized at 60°C with the probe which was radioactively labeled using [α-32P]dCTP by the random priming method (Sambrook et al. 1989).

RNA Blot Analysis

Total RNAs were isolated by the guanidium thiocyanate-CsCl method. Fifty μg RNAs were separated by running an 1.3% agarose gel and were transferred onto a nylon membrane with 10 × SSPE transferring buffer. The RNA blot was then hybridized with radioactively labeled probes at 60°C in the solution containing 0.5 M NaPO4 (pH 7.2), 1 mM EDTA, 1% BSA, and 7% SDS for 20 h at 60°C (Church, 1984). After hybridization, the blot was washed first with 1 × SSPE and 0.5% SDS three times for 5 min each at 45°C. The blots were autoradiographed with an X-ray film.

In Situ Hybridization

The procedure for in situ hybridization using a radioactive material [a-^{35}S]-dUTP is essentially same as the protocol described by Cox and Goldberg (1988) except with some modifications. Flower samples were fixed in a solution containing 1.4% glutaraldehyde, 2% paraformaldehyde, and 50 mM PIPES, pH 7.0 for overnight at 4°C. The fixed tissues were dehydrated with a series of ethanol solutions from 10% to 100% and embedded in paraffin (m.p. 60 – 62°C, Polyscience). The paraffin-embedded tissues were sliced into 8 μm sections with a rotary microtome. The sections were then attached to glass slides, and were prehybridized with a

hybridization solution containing 50% formamide, 300 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 × Denhardt's reagent, 10% dextran sulfate, 70 mM DTT, 25 units/mL RNase inhibitor, 500 mg/mL poly A, 150 mg/mL tRNA for 2 hr at room temperature. The antisense and sense RNA probes were prepared using 35S-dUTP with following the procedure as suggested by the manufacture (Stratagene, RNA Transcription) and added to the hybridization solution. Hybridization was performed at 48°C for overnight in a humid chamber. The slides were washed in 4 × SSPE, 5 mM DTT for 5 min at 50°C, treated with 25 μg/mL RNase A in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 500 mM NaCl at 37°C for 30 min, then washed in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 500 mM NaCl, 5 mM DTT two times for 15 min each at 37°C. The slides were coated with liquid emulsion X-ray film (Kodak) and exposed for 5 days. The sections were stained with 0.05% toluidine blue and examined under a light microscope.

Genomic DNA Blot Analysis

Genomic DNA was isolated from tobacco leaves with CTAB (Cetyltrimethylammonium bromide) method. Thirty μg of the genomic DNA was digested with 200 units of EcoRI, HindIII, and PstI restriction enzymes for overnight and run on a 0.7 % agarose gel. The DNAs were then blotted onto nylon membranes with 20 × SSC as a transferring buffer and hybridized with radioactively labeled probes.

RESULTS

Isolation and Sequence Analysis f *NtMADS3*

Tobacco petals cDNA library was screened with OsMADS1 cDNA as a probe. 5 × 10^4 plaques from the cDNA library were hybridized with radioactively labeled OsMADS1 cDNA probe at 60°C. Of the 25 positive clones that are strongly hybridized with the probe, *NtMADS3* was selected and further characterized. Sequence analysis showed that *NtMADS3* is 945 bp-long and contains an open reading frame of 242 amino acid residues (Fig. 1). The amino acid residues contained the conserved MADS-box between the amino acids 2 and 57 and the K-box domain between 95 and 159, indicating that the gene is a member of the MADS-box gene family in tobacco. The NtMADS3 was extensively homologous to OsMADS1 with 56.1% identity (70% similarity) in the whole amino acids, suggesting that the gene may be the OsMADS1 homolog of tobacco.

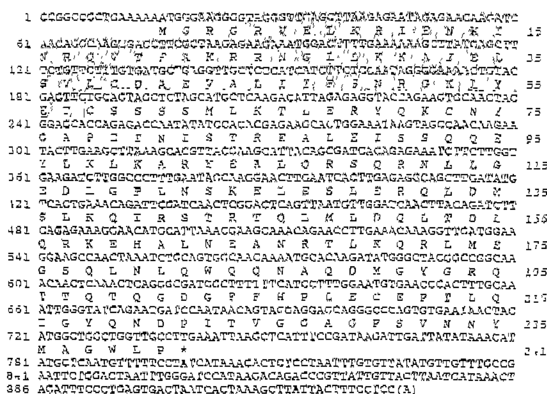

Figure 1. Nucleotide sequences and deduced amino acid sequences of NtMADS3 clone. MADS-box and K-box are indicated by purple and green colors, respectively. Nuleotides and amino acid residues are numbered on the left and on the right of each figure, respectively.

Expression Pattern of *NtMADS3*

RNA blot analyses were conducted to study the expression patterns of the MADS-box gene. In order to avoid any cross-hybridization with other MADS-box genes, the probes were developed to avoid both MADS-domain and K-domain regions (Fig. 2A). Figure 2B shows spatial expression pattern of the MADS-box gene. *NtMADS3* was expressed in whorl 2, 3, and 4 that correspond to petals, anthers, and carpels, respectively. The gene was not detectable in

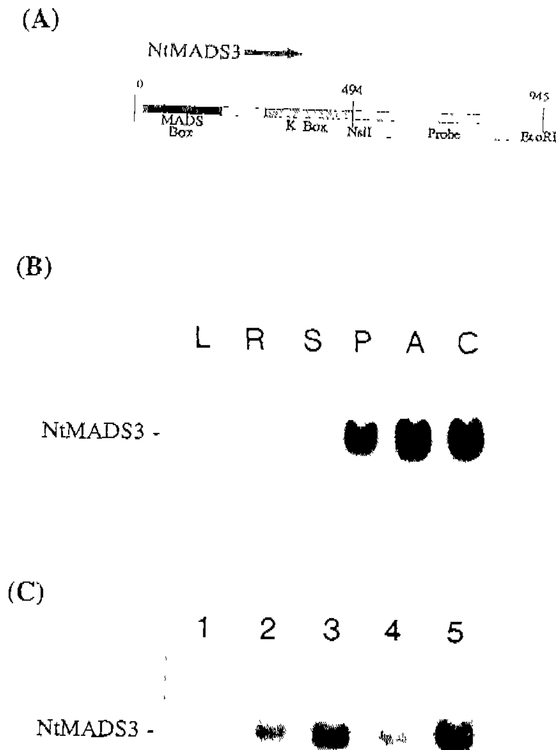

Figure 2. RNA blot analyses. Fifty μg of each RNA sample was hybridized with radioactively labeled NtMADS3 probe lacking MADS- and K-box regions. (A) shows the area used for preparing the probe. (B) Spatial expression pattern of *NtMADS3* transcript. L, leaf; R, root; S, sepals; petals, A, anthers; C, carpels. (C) Temporal expression pattern of the NtMADS3 transcript. 1, floral buds size less than 3 mm; 2, floral buds between 3-5mm; 3, floral buds between 0.5-1 cm; 4, floral buds between 1-3 cm; 5, flowers at anthesis.

other vegetative organs, leaf and root.

Temporal expression pattern of the gene was also studied (Fig. 2C). Total RNA was isolated from five different developmental stages of tobacco flower: stage 1, floral primodia; stage 2, flower at pollen mother cell development; stage 3, pollen meiosis; stage 4, pollen vacuolation; and stage 5, mature flower at anthesis (see figure legend for floral bud size of each stage). The expression pattern of the gene during flower development at each stage was very similar to the temporal expression pattern of OsMADS1 in rice (Chung et al., 1994). Like

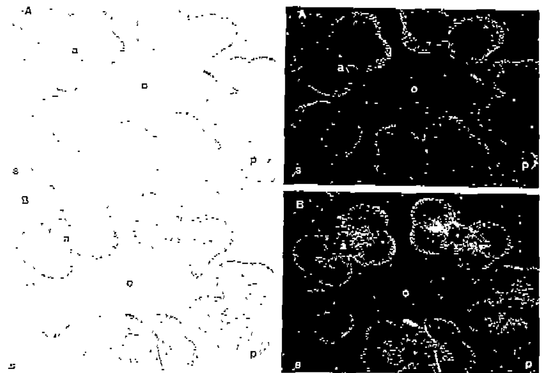

Figure 3. *In situ* localization of *NtMADS3* transcript. Eight μm of cross sections of tobacco flower at stage 2 was hybridized with sense (A) and antisense (B) probes. The hybridized sectins were visualized with 0.05% toluidine blue and photographed under bright-field (left) and dark-field (right) conditions. a, anther; o, ovary; p, petal; s, sepal.

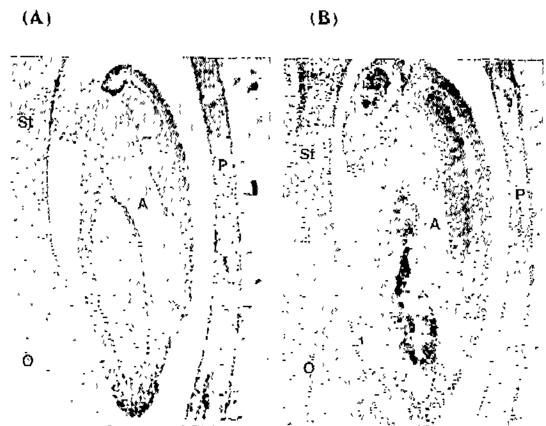

Figure 4. Tissue-specific localization of *NtMADS3* transcript in a developing anther. (A) control section hybridized with sense probe. (B) hybridized section with antisense probe. A, anther; O, ovary; P, petal; St, style.

OsMADS1 gene, the *NtMADS3* gene was expressed during all stages of flower development, and the expression increased reaching its peak level when the flower become mature. These mRNA studies suggest that the gene may be involved both in floral meristem identity and in floral organ identity. In situ hybridization was performed in order to study the tissue specific localization of the transcript (Fig. 3 and 4). At stage 2, *NtMADS3* transcript was

found to be expressed in anther, carpel, and petal (Fig. 3). In the anther, a tissue specific localization pattern of the transcript that occurs in the connective tissue was found. At later stage of the anther development, however, more transcripts were accumulated in pollens, indicating that the gene is very active during the anther development (Fig. 4). No tissue-specific localization pattern of the gene in the petal and the carpel were observed although the gene existed uniformly in the organs.

DNA Blot Analyses

In order to study the genomic complexities of the tabacco MADS-box gene, DNA blot hybridization was conducted (Fig. 5).

When whole cDNA clone was used as probes, the gene was shown up with several bands on the DNA blot, indicating that the gene exists as a high copy number in the genome.

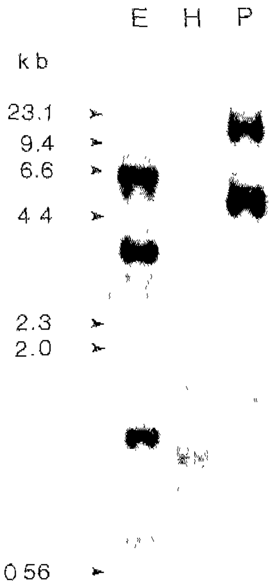

Figure 5. DNA blot analysis of *NtMADS3*. Thirty mg of genomic DNA was loaded in each lane and hybridized with *NtMADS3* probe. E, EcoRI: H, HindIII: P, PstI.

Transgenic Plants and Hybrids

In order to study whether the NtMADS3 is indeed the OsMADS1 homolog of tobacco, the entire NtMADS3-coding region was placed under the cauliflower mosaic virus (CaMV) 35S promoter, and the chimeric molecule was transferred to tobacco plant (*Nicotiana tabacum* cv. Petite Havana SR1) using the *Agrobacterium*-mediated Ti plasmid vector system (An et al., 1988). Interestingly, the transgenic plants showed phenotypic changes that involve early flowering and dwarfism as observed in the OsMADS1 transgenic plant (Chung et al., 1994) (Fig. 6). Like OsMADS1 transgenic plant, these plants were very short, and contained several lateral branches (Fig. 6B). The phenotypes of the NtMADS3 transgenic plants were also inherited to the next generation, and the progenies flowered about 10 days earlier than wild-type (Table 1). The results strongly suggest that the NtMADS3 is the OsMADS1 homolog of tobacco.

Hybrids between the NtMADS3 and the OsMADS1 transgenic plants were also generated. RNA blot hybridization using the total RNA isolated from the leaves of the hybrid plants was conducted to test whether both transgenes are expressed. The RNA blot revealed that both transgenes are present in the hybrid plants (data not shown). Surprisingly, the hybrids flowered even earlier than those two transgenic plants: the plants flowered about 10 days earlier than NtMADS3 and OsMADS1 transgenic plants (Fig. 7). The hybrids were also much shorter than the two transgenic plants (Table 1).

Table 1. Comparison of phenotypes of transgenic plants and hybrid with wild-type control. Seeds which were collected from selfed fruits of the primary transgenic plants were germinated in MS media, and were grown under greenhouse conditions.

	Days to flower open	Height (cm)
Wild-type	62	119.5
NtMADS3	52	27.5
OsMADS1	53	3.4
Hybrids	42	17.3

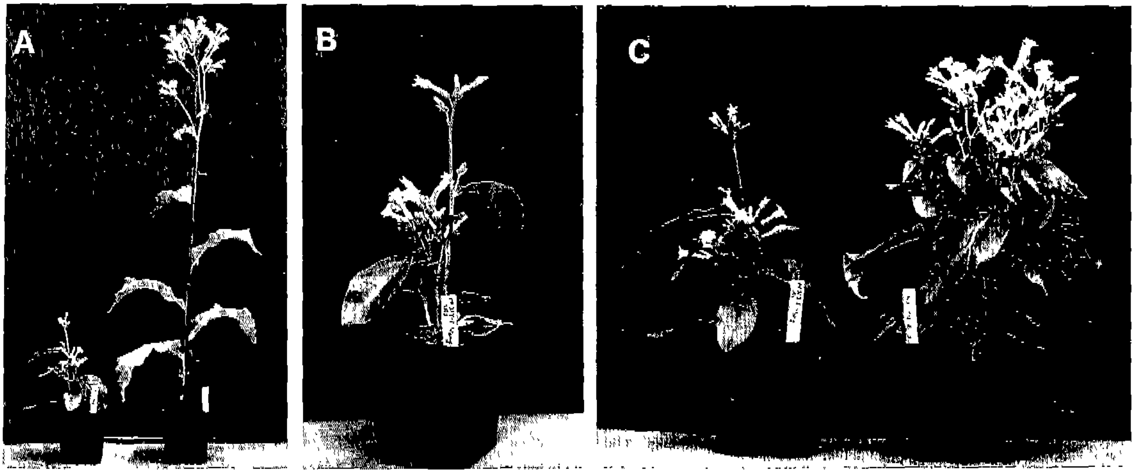

Figure 6. NtMADS3 transgenic plants. (A) Comparison of NtMADS3 transgenic plant (left) and wild-type plant (right). (B) A close-up view of the transgenic plant. (C) Comparison of NtMADS3 transgenic plant (left) and OsMADS1 transgenic plant (right).

Figure 7. Comparison of NtMADS3 transgenic plant (right) and hybrid (left) between OsMADS1 female and NtMADS3 male. The hybrid flowered 10 days earlier than the NtMADS3 transgenic plant which flowers at 52 days after seedling.

DISCUSSION

We have reported recently OsMADS1 as a molecular factor that triggers flower induction in rice (Chung et al., 1994).

The OsMADS1 gene is flower specific and does not exist in vegetative organs of rice. The gene is continuously expressed during the entire flower development from floral identity to floral maturation. Functional analysis of the gene in transgenic plant at an ectopic expression condition revealed that the gene induces a transition from vegetative growth to reproductive growth. Eventually, the transition caused early flowering and dwarfing phenotypes with reduced apical dominance.

In order to study whether the OsMADS1 homolog exists in other plant species, especially in dicot plants, we chose tobacco plant as a homologous model system. In order to clone out the homolog, tobacco petal cDNA library was screened with OsMADS1 cDNA as a probe at a high-stringent hybridization condition (60°C). Of the 25 positive clones that are strongly hybridized with the probe, *NtMADS3* was selected and further characterized. Sequence analysis revealed that *NtMADS3* was extensively homologous to OsMADS1 in the whole amino acids (56.1% identity and 70% similarity). The homology search, however, showed that the *NtMADS3* is more homologous to FBP2 of petunia (Angenent et al., 1994) with 82% identity (data not shown). In fact, one of the most homologous proteins in dicot with OsMADS1 protein was FBP2 (55% identity and 68% similarity), suggesting that the proteins may be functionally related. The MADS-box domain of the NtMADS3 shares a

significant homology (89.5% identity and 96.5% similarity) to the one of OsMADS1. Similar pattern of homology was also found in K-box domain between these proteins. The results suggest that the genes are closely related in functional motif so that they functionally overlap in flower development.

mRNA expression patterns of *NtMADS3* were studied. RNA blot hybridization with *NtMADS3* probe showed that the *NtMADS3* is expressed in three different floral whorl organs, petal, anther, and carpel, suggesting that the gene belongs to a new class of MADS-box gene. The spatial expression pattern of the tobacco MADS-box gene was different from that of *OsMADS1*, which lacks in anther. Rather, the gene expression pattern was more similar to that of FBP2 of petunia (Angenent et al., 1994). It is possible that the organelle expression of the *NtMADS3* is different from the *OsMADS1* due to morphological differences between monocot and dicot species. However, the temporal expression pattern of the *NtMADS3* during flower development of tobacco was very similar to that of OsMADS1 (Fig. 2C). Like *OsMADS1* gene, the gene expression persisted throughout the development of tobacco flower. The *NtMADS3* gene started to express at a very early stage of floral identity, and the expression level reached at the peak level when the flower get mature. This suggests that the *NtMADS3* gene is involved in floral identity as *OsMADS1* gene does in rice.

In order to study whether the tobacco MADS-box gene indeed behaves functionally same with OsMADS1 in triggering flower formation, the transgenic plants which undergo an ectopic-expression condition of the *NtMADS3* gene were generated. As observed in the OsMADS1 transgenic plants, the NtMADS3 transgenic plants showed phenotypes of early flowering and reduced apical dominance with several lateral branching caused by a transition from infloral shoot meristem into floral meristem, indicating that the NtMADS3 triggers flower formation in tobacco (Fig. 6). The flowering time of the NtMADS3 transgenic plant was very similar to OsMADS1 (Table 1). These results strongly suggest that the NtMADS3 is indeed the OsMADS1 homolog of tobacco. The phenotypes of the *NtMADS3* transgenic plants were inherited to the next generation with a dominant Mendelian trait. Antisense transgenic plants by placing an antisense orientation of the NtMADS3 coding region under the control of the 35S promoter were also generated. The inhibition of NtMADS3, however, did not cause any phenotypic alteration. No homeotic conversion or morphological alteration during flower development was observed. It is possible that the NtMADS3 mediates two unrelated secondary regulatory systems. One system is the early function of the floral meristem identity so that in the sense transgenic plants, overexpression of the gene causes a transition of the inflorescence to the florescence meristem. The other system is the function of the major floral organ formation. One possible explanation that the inhibition of the gene did not induce any phenotype in the transgenic plants is that because more than one copy of the NtMADS3 genes exist in the plant genome as shown in the DNA blot (Fig. 5), partial mRNA blocking is made in the plants so that the blocking is not enough to cause any phenotypic alteration. Another possibility is that because the tobacco plant used in the transgenic analysis were *Nicotiana tabacum* which is a hetero-tetraploids generated from two different parent, in the antisense transgenic plants only one parental mRNA is blocked, and the other half works for the normal growth.

Hybrids between the NtMADS3 and the OsMADS1 transgenic plants were generated. It is possible that if both genes are active or interact each other in a same plant, the phenotypes of the early flowering and the dwarfism could be more significant. The hybrids between OsMADS1 female and NtMADS3 male, or between OsMADS1 male and NtMADS3 female were generated. RNA blot hybridization using the total RNA isolated from the leaves of the hybrid plants was conducted to test whether both transgenes are expressed. The RNA

blot revealed that both transgenes are present in the hybrid plants (data not shown). Surprisingly, the hybrids showed the two phenotypes more significant than the two transgenic plants: the flowering time of the hybrid was reduced about 10 days earlier than those, and the height of the hybrid was much shorter (Table 1).

Not only for investigating the factor which triggers flower formation in plant, but also for using the early flowering trait that are important for agricultural crop plants, the two MADS-box genes, *NtMADS3* and *OsMADS1*, should be proven to be useful molecular tools. Therefore, more detailed analyses on these genes are on the way.

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