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Overriding Photoperiod Sensitivity of Flowering Time by Constitutive Expression of a MADS Box Gene

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Most plants sense changes in environmental signals, such as day length or temperature. Here, we report the identification of a regulatory gene, OsMADS1, which controls the photoperiod sensitivity of flowering time. Constitutive expression of OsMADS1 in a long-day flowering plant, *Nicotiana sylvestris*, resulted in flowering under both short-day and long-day conditions. Similarly, ectopic expression of the gene in a short-day flowering plant, *Nicotiana tabacum* cv. Maryland Mammoth, also induced flowering, regardless of day length. Transition time depended on the level of the OsMADS1 transcript in transgenic plants. These suggest that OsMADS1 is a key regulatory factor that determines the transition from shoot apex to floral meristem, and that it may be used for controlling flowering time in a variety of plant species.

Keywords: floral initiation, MADS box gene, *Nicotiana*, photoperiod

Plants differ widely in their response to environmental factors (Halevy, 1985-1989; Bernier et al., 1993; Meeks-Wagner, 1993). A short-day plant flowers when day length is less than its critical length; long-day plants flower when day length is longer than its critical length. Day length has no effect on floral induction until the plant has attained a certain amount of growth (Coen and Carpenter, 1993; Meeks-Wagner, 1993). After the basic vegetative phase is completed, flower initiation is probably controlled by transmissible signals that are transported to the shoot apex. Lang et al. (1977) have postulated that the leaves of photoperiodic plants produce either flowering promoters when exposed to favorable day-length regimes or flowering inhibitors when exposed to unfavorable day-length conditions. The nature of these transmissible signals is still a controversial issue (O'Neill, 1992). Efforts have been unsuccessful in isolating the signaling substances and their target genes in the shoot apex.

We have previously demonstrated that transgenic tobacco plants constitutively expressing a rice gene, OsMADS1, flowered earlier than untransformed controls (Chung et al., 1994). A similar gene, AP1, from *Arabidopsis thaliana* also exhibited identical phenomena when it was overexpressed in *A. thaliana* (Mandel and Yanofsky, 1995). These genes are important members of the MADS box gene family that is involved in a variety of developmental regulations in plants, animals, and yeast (Norman et al., 1988; Passmore et al., 1988; Schwarz-Sommer et al., 1990; Sommer et al., 1990; Yanofsky et al., 1990; Ma, 1994; Weigel and Meyerowitz, 1994; Kang and An, 1997; Kang et al., 1997; Nam et al., 1999). Some MADS box genes are involved in development of the floral meristem. AP1 in *A. thaliana* and SQUA in *Antirrhinum majus* are essential for the transition of an inflorescence meristem into a floral meristem (Huijser et al., 1992; Mandel et al., 1992). MADS box genes also play important roles in controlling floral organ development. They are members of key regulatory elements that determine floral organ identity. These include AG, AP3, PI in *A. thaliana* and PLE, DEF A, and GLO in *A. majus* (Yanofsky et al., 1990; Jack et al., 1992; Goto and Meyerowitz, 1994). Mutations in these genes result in homeotic conversion of floral organs (Sommer et al., 1990; Trobner et al., 1992; Bradley et al., 1993). More recently, some MADS box genes were found to be specifically expressed in vegetative tissues or embryos (Heck et al., 1995; Rounsley et al., 1995; Zhang and Forde, 1998).

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MATERIALS AND METHODS

Plant Materials and Growth

Seeds of long-day flowering *Nicotiana sylvestris* and short-day flowering *Nicotiana tabacum* cv. Maryland Mammoth. were kindly provided by Richard Ama-

sino. The plants were propagated in a growth chamber at 26°C, under either short-day conditions (10 h light per day) or long-day conditions (16 h light per day).

Bacterial Strains and Plant Transformation

Escherichia coli MC1000 (*ara, leu, lac, gal, str*) was the recipient in routine cloning experiments. *Agrobacterium tumefaciens* LBA4404 (Hoekema et al., 1983), containing the Ach5 chromosomal background and a disarmed helper-Ti plasmid pAL4404, was used in transforming *N. sylvestris* and *N. tabacum* via cocultivation (An et al., 1988).

RNA Blot Analysis

Total RNA was isolated from young, fully expanded leaves by the guanidium thiocyanate method (Sambrook et al., 1989). Twenty microgram of total RNA were fractionated on a 1.3% agarose gel. Equal amounts of RNA loading was confirmed by ethidium bromide staining of rRNAs. After RNA transfer onto a nylon membrane, the blot was hybridized in a solution containing 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, 1% BSA, and 7% SDS, for 20 h at 60°C (Jang and An, 1999). After hybridization, the blot was washed twice with a solution containing 0.1 × SSPE and 0.1% SDS for five min at room temperature, followed by two washes with the same solution at 55°C for five min each.

RESULTS

OsMADS1 Causes Early Flowering of a Long-Day Flowering Plant, *N. sylvestris*, under Permissive Conditions

We have previously shown that expression of the *OsMADS1* gene with the cauliflower mosaic virus (CaMV) 35S promoter induced early flowering and dwarf phenotypes in transgenic tobacco plants. In this study, we investigated the role of this MADS box gene in photoperiod-sensitive plant species. *N. sylvestris* is a long-day flowering plant that flowers only when plants are grown under long-day conditions. When these plants are grown under short-day conditions, they exhibit a pronounced rosette growth habit and form very short shoot axes (Lang et al., 1977).

For constitutive expression of the gene, *OsMADS1* cDNA was placed under the 35S promoter that functions in most plant cells (Benfey and Chua, 1990). This chimeric molecule was linked to a kanamycin-resistant marker and introduced to *N. sylvestris* using an Agrobacterium-mediated Ti-plasmid vector system (An et al., 1988). Transgenic plants were regenerated on a kanamycin-containing culture medium and grown under long-day conditions. Among the 20 independently transformed plants, most flowered earlier than untransformed controls under permissive flowering conditions.

To confirm whether the phenotypes were inherited, five independently transformed transgenic plants were chosen for further studies. T2 offspring were selected on a kanamycin-containing medium and the seedlings were grown under long days. *N. sylvestris* plants that were transformed with the kanamycin-resistant marker alone were used as the control. Under long-day (permissive) conditions, the transgenic plants flowered 7 to 11 days earlier than the controls, which flowered 106 days after seed germination (Table 1). Transgenic plants were branched at the top with crusted flowers and were shorter than the controls (Fig. 1A). These phenotypes were similar to the day-neutral transgenic tobacco plants expressing the *OsMADS1* gene (Chung et al., 1994).

OsMADS1 Causes Flowering of *N. sylvestris* under Non-Permissive Conditions

Untransformed *N. sylvestris* and transgenic plants carrying the kanamycin marker alone did not develop floral organs under short days. In contrast, when the T2 offspring of transgenic *N. sylvestris* plants were grown under the same short-day conditions, they

Table 1. Ectopic expression of *OsMADS1* in *N. Sylvestris*.

Transgenic line	Short-day condition		Long-day condition	
	Days to flowering	Height (cm)	Days to flowering	Height (cm)
1	102	62	98	68
2	85	35	95	45
3	146	65	99	72
4	84	36	96	46
5	97	52	97	52
control	ND	ND	106	85

Six T2 plants, which were selected on a kanamycin-containing medium from five independent transgenic lines and a control, were grown in both short-day (10 h light) and long-day (16 h light) conditions. This experiment was done twice under the same conditions and the results were an average of the two sets of experiments. Heights were measured on the first day of anthesis. ND, not determined because the control plants did not flower within 200 days.

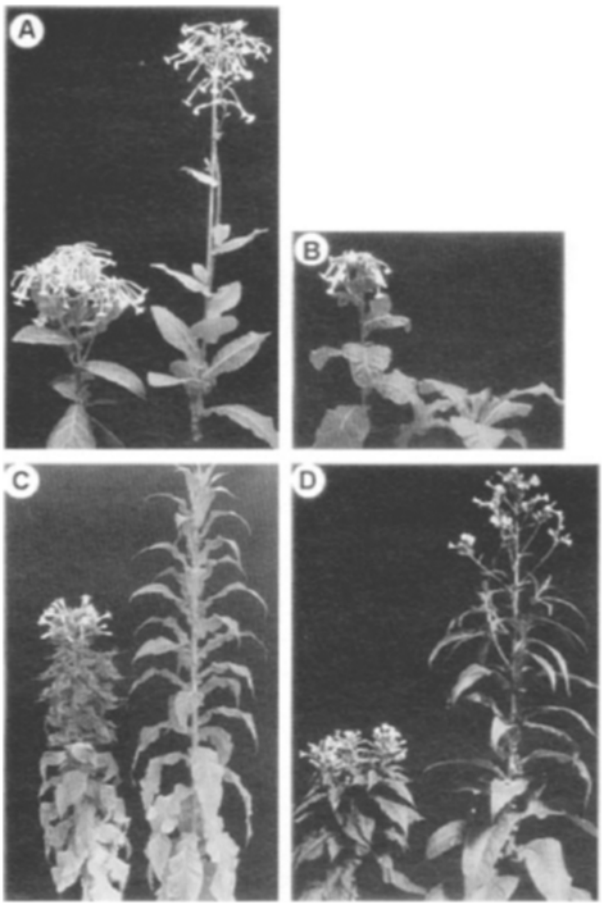

Figure 1. Transgenic *N. sylvestris* and *N. tabacum* cv. Maryland Mammoth plants expressing the *OsMADS1* gene. Long-day flowering plants, *N. sylvestris* (A, B) and short-day flowering plants, *N. tabacum* cv. Maryland Mammoth (C, D) were transformed with pGA1209, which contains a kanamycin-selectable marker and a chimeric fusion between the CaMV 35S promoter and OsMADS1-coding region. Transgenic plants were selfed, and kanamycin-resistant T2 offspring were grown under either long-day (permissive for *N. sylvestris*, non-permissive for *N. tabacum* cv. Maryland Mammoth) conditions (16 h light) (A, C) or short-day (non-permissive for *N. sylvestris*, permissive for *N. tabacum* cv. Maryland Mammoth) conditions (10 h light) (B, D) at 26°C, 70% humidity.

flowered in 85 to 146 days (Table 1, Fig. 1B). The phenotypes of these plants were similar to those grown under long-days. Transgenic Lines 2 and 4, grown under short days, flowered much earlier than plants grown under long days, but plants from transgenic Line 3 flowered much later under short-day conditions.

OsMADS1 Transcript Level Controls Flowering Time

We used RNA blot analysis to confirm whether the

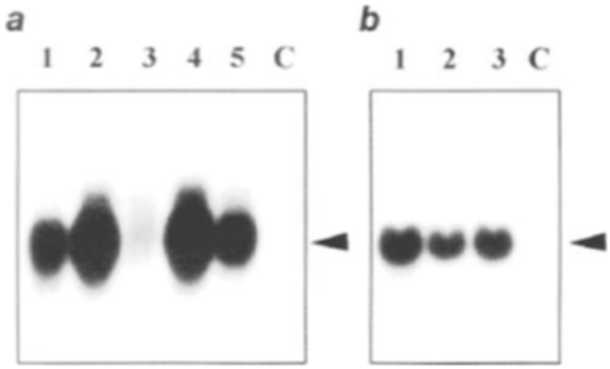

Figure 2. RNA blot analysis of transgenic *N. sylvestris* and *N. tabacum* cv. Maryland Mammoth plants. Twenty microgram of total RNA was prepared from separated *N. sylvestris* (a) and *N. tabacum* cv. Maryland Mammoth (b) transgenic plants on a 1.3% agarose gel, blotted onto a nylon membrane, and hybridized with a ^{32}P-labeled gene-specific probe. Numbers indicate independent transgenic plant lines of T2 generation. Transgenic plant transformed with the kanamycin marker alone was used as a control (C). An arrowhead indicates 1.2-kb *OsMADS1* transcripts.

phenotypes of the transgenic plants were due to expression of the *OsMADS1* gene. Because a constitutive promoter was used for expression of the gene, we expected the transcript to be present in all plant parts. We have previously shown that the 35S promoter-driven *OsMADS1* transcript is almost equally expressed in both leaves and flowers (Chung et al., 1994). Total RNA was prepared from young, fully expanded leaves of the five transgenic lines at the same age. The level of the *OsMADS1* transcript was measured using a gene-specific cDNA probe that hybridized specifically to the *OsMADS1* gene (Chung et al., 1994). All the transgenic plants accumulated the *OsMADS1* transcript; the amount of this mRNA was directly correlated with the number of days from germination to flowering (Fig. 2a). Transgenic Lines 2 and 4, which flowered earliest among the five transgenic lines, expressed the highest level of the *OsMADS1* mRNA, whereas Line 3, flowering latest, contained the lowest level of the transcript. Those transgenic lines with intermediate phenotypes carried intermediate levels of the transcript.

OsMADS1 Overrides the Day-Length Requirement of a Short-Day Flowering Plant, *N. tabacum* cv. Maryland Mammoth

We tested whether expression of the *OsMADS1* gene could also cause overriding of the day-length requirement of short-day *N. tabacum*. Fifteen inde-

Table 2. Ectopic expression of *OsMADS1* in *N. tabacum* cv. Maryland Mammoth.

Transgenic line	Short-day condition		Long-day condition	
	Days to flowering	Height (cm)	Days to flowering	Height (cm)
1	98	61	202	102
2	103	65	206	105
3	99	63	203	104
control	119	143	ND	ND

Six T2 plants, which were selected on a kanamycin-containing medium from three independent transgenic lines and a control, were grown in both short-day (10 h light) and long-day (16 h light) conditions. This experiment was done twice under the same conditions and the results were an average of the two sets of experiments. The height was measured on the first day of anthesis. ND, not de- termined because the control plants did not flower within 250 days.

pendently transformed plants were obtained with the *OsMADS1* chimeric molecule. As observed with day-neutral or long-day plants, transformation of the *OsMADS1* chimeric gene into the short-day plant also resulted in early flowering and dwarf phenotypes in most of the transgenic plants. Three independently transformed lines were further studied. T2 offspring were selected on kanamycin-containing me- dium and grown under short-day (permissive) or long-day (non-permissive) conditions.

Under the permissive condition, the T2 transgenic lines flowered 16 to 21 days earlier than untransformed controls, which flowered in 119 days (Table 2, Fig. 1D). The transgenic plants were not even half as tall as the control plants. Under the non-permissive condition, transgenic plants flowered in 202-206 days, whereas the controls did not flower (Table 2, Fig. 1C). In the RNA blot analysis, all three lines expressed the transgene; the degree of the phenotype characterization was correlated with the level of the transcript (Fig. 2b). Therefore, expression of the *OsMADS1* gene also overcomes the day-length requirement of a short-day flowering plant.

DISCUSSION

We have demonstrated here that ectopic expression of *OsMADS1* overrides the day-length dependence of flowering in both long-day and short-day plants. Under permissive conditions, transgenic plants flowered earlier than controls. Under non-permissive conditions, expression of the transgene overrode the day-length requirement for flowering. The effect was more evident when the gene was highly expressed. Therefore, under natural conditions, expression of the *OsMADS1* gene probably is tightly controlled by environmental factors, and the flowering process is initiated by triggering the gene expression. The fact that *OsMADS1* overrides the day-length dependence of both short-day and long-day plants suggests that the regulatory gene controlling flowering time may be conserved between the two plant types.

Transgenic *N. sylvestris* plants that strongly expressed the *OsMADS1* gene flowered earlier than control plants, regardless of day length. This indicates that the regulatory gene alone is sufficient for overriding day-length sensitivity. However, in *N. tabacum* cv. Maryland Mammoth, expression of the gene did not completely suppress day-length sensitivity because transgenic plants flowered much later under long-day (non-permissive) than under short-day (permissive) conditions. Additional factors apparently are required for flowering to occur in short-day plants.

Some plant species are extremely sensitive to day-length whereas others are less so. In nature, different ecotypes within a single species can also respond differently to day length. The difference between a day length-sensitive variety and one not so sensitive to day length is probably controlled by one or several regulatory genes. Our study results indicate that *OsMADS1* is probably one of these regulatory genes that determines day-length sensitivity. We observed that, especially under non-permissive conditions, flowering time in transgenic *N. sylvestris* was directly correlated with the expression level of the *OsMADS1* transcript. This supports the hypothesis that expression of this regulatory gene plays a key role in determining sensitivity to day length. It will be interesting to study whether, in some plants, expression of the *OsMADS1* homolog is controlled mainly by environmental factors, while in others it is regulated by developmental factors.

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