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

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**The majority of plants sense environmental signals, such as day length or temperature, to select their transition timing from vegetative growth to flowering. Here, we report the identification of a regulatory gene, OsMADS1, that controls the photoperiod sensitivity of flowering time. Constitutive expression of OsMADS1 in a long-day flowering plant, *Nicotiana sylvestris*, resulted in flowering in both short-day and long-day conditions. Similarly, ectopic expression of the gene in a short-day flowering plant, *N. tabacum* cv. Maryland Mammoth, also induced flowering regardless of the day length. The transition time was dependent 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 can be used for controlling flowering time in a variety of plant species.**

Plants differ widely in their response to environmental factors (Bernier et al., 1993: Halevy, 1985-1989: Meeks-Wagner, 1993). A short-day plant flowers when the day length is less than its critical length and a long-day plant flowers when the 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 (Meeks-Wagner, 1993: Coen and Carpenter, 1993). After completion of the basic vegetative phase, initiation of flowers is believed to be controlled by transmissible signals that are transported to the shoot apex. It was postulated that the leaves of photoperiodic plants produce flowering promoters when exposed to favorable day-length regimes or flowering inhibitors when exposed to unfavorable day-length conditions (Lang et al., 1977). The nature of these transmissible signals is still a controversial issue (O'Neill, 1992). Efforts for isolation of the signaling substances and their target genes in shoot apex have been unsuccessful.

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, API, from *Arabidopsis thaliana* also exhibited identical phenomena when it was overexpressed in *A. thaliana* (Mandel and Yanofsky, 1995). These genes are members of the MADS box gene family that plays an important role in a variety of developmental regulations in plants, animals, and yeast (Ma, 1994: Norman et al., 1988: Passmore et al., 1988: Schwarz-Sommer et al., 1990: Sommer et al., 1990: Weigel and Meyerowitz, 1994: Yanofsky et al., 1990). Some MADS box genes involve 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 (Mandel et al., 1992: Huijser 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* (Goto and Meyerowitz, 1994: Jack et al., 1992: Yanofsky et al., 1990). Mutations in these genes result in homeotic conversion of floral organs (Bladley et al., 1993: Sommer et al., 1990: Trobnet et al., 1992). More recently, it was found that some MADS box genes are specifically expressed in vegetative tissues or embryos (Rounsley et al., 1995: Heck et al., 1995).

## RESULTS

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

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 have 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 were grown under long-day conditions (16 h of light per day). When the plants were grown under short-day conditions (under 10 h of light per day), they exhibited a pronounced rosette habit of growth and formed a very short shoot axis (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

**Table 1.** Ectopic expression of OsMADS1 in *Nicotiana 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. The height was measused on the first anthesis day. ND, not determined since the control plants did not flower within 200 days.

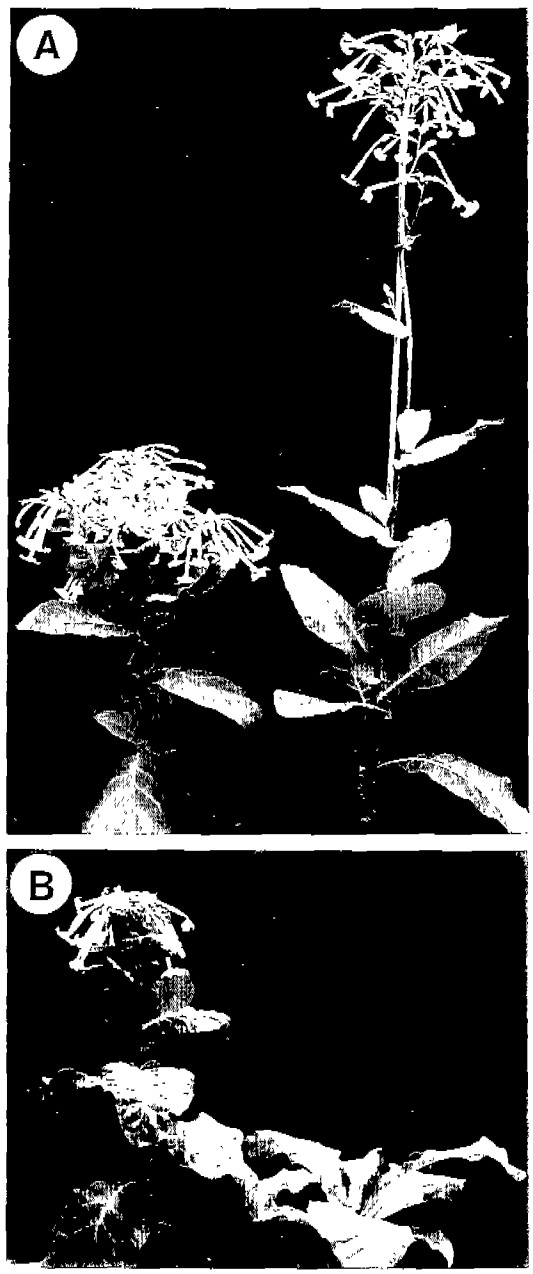


**Figure 1.** Transgenic *N. sylvestris* plants expressing the OsMADS1 gene. Long-day flowering plants, *N. sylvestris*, were transformed with pGA1209, which contains a kanamycin selectable marker and a chimeric fusion between the CaMV 35S promoter and OsMADS1-coding region. Transgenic plants were selfed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

plants were regenerated on a kanamycin-containing culture medium and were grown under long-day conditions. Among 20 independently transformed plants, most transgenic *N. sylvestris* flowered earlier than untransformed controls under the permissive flowering conditions. In order 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 the long-day condition (16 h light per day). *N. sylvestris* plants that were transferred with the kanamycin resistant marker alone were used as a control. Under the long-day (permissive) condition, the transgenic plants flowered 7 – 11 days earlier than the controls which flowered in 106 days after seed germination (Table 1). Transgenic plants were branched at the top with crusted flowers and shorter compared to 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 a Non-Permissive Condition

Untransformed *N. sylvestris* and transgenic plants carrying the kanamycin marker alone did not develop floral organs under the short-day condition (10 h light per day). Whereas, when the T2 offspring of transgenic *N. sylvestris* plants were grown under the same short-day condition, they flowered in 85 – 146 days (Table 1, Fig. 1B). The phenotypes of these plants were similar to those grown under the long-day condition. The transgenic lines 2 and 4 grown under the short-day condition flowered much earlier compared to the plants grown under the long-day condition. Whereas, the transgenic line 3 flowered much later under the short-day condition.

## OsMADS1 Transcript Level Controls Flowering Time

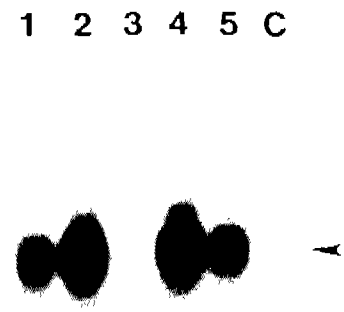


Figure 2. RNA blot analysis of transgenic *N. sylvestris plants.* Twenty mg of total RNA was separated on a 1.3% agarose gel, blotted onto a nylon membrane, and hybridized with a $^{32}P$-labeled gene-specific probe. Numbers indicate independent transgenic plant lines of T2 generation. *N. sylvestris*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

In order to confirm whether the phenotypes of the transgenic plants were due to the expression of the OsMADS1 gene, RNA blot analysis was performed. Since a constitutive promoter was used for expression of the gene, it was expected that the transcript was present in all the plant parts. We have previously shown that the 35S promoter-driven OsMADS1 transcript was almost equally expressed in both leaves and flowers (Chung et al., 1994). Total RNA was prepared from fully expended leaves of the five transgenic lines at the same age and the level of the OsMADS1 transcript was measured using a gene-specific cDNA probe which hybridized specifically to the OsMADS1 gene (Chung et al., 1994). This experiment showed that all the transgenic plants accumulated the OsMADS1 transcript and that the amount of this mRNA is in direct correlation with the number of days from germination to flowering (Fig. 2). The transgenic lines 2 and 4, which flowered earliest among the five transgenic lines, expressed the highest level of the OsMADS1 mRNA whereas the line 3, which flowered latest, contained the lowest level of the transcript. Those transgenic lines with intermediate phenotypes carried intermediate levels of the transcript.


Figure 3. Transgenic *N. tabacum* cv. Maryland Mammoth plants expressing the OsMADS1 gene. Short-day flowering plants, *N. tabacum* cv. Maryland Mammoth were transformed with pGA1209, which contains a chimeric fusion between the CaMV 35S promoter and OsMADS1-coding region. T2 offspring were grown under either a long-day (non-permissive) condition (16 h light) (A) or a short day (permissive) condition (10 h light) (B) at 26C, 70% humidity.

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

We, then, tested whether expression of the OsMADS1 gene would also result in overriding the day-length requirement of a short-day flowering plant, *N. tabacum* cv. Maryland Mammoth. Fifteen independently transformed plants were obtained with the OsMADS1 chimeric molecule. As observed with the day-neutral or long-day plant, 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 medium and grown under the short-day (permissive) or long-day (non-permissive) condition.

Under the permissive condition, the T2 transgenic lines flowered 16 – 21 days earlier than untransformed controls which flowered in 119 days (Table 2, Fig. 3B). The height of the transgenic plants was less than a half of the control plants. Under the non-permissive condition, transgenic plants flowered in 202-206 days, whereas the control did not flower (Table 2, Fig. 3A). RNA blot analysis exhibited that all three lines expressed the transgene and that degrees of the phenotypes correlated with the level of the transcript (Fig. 4). This indicates that expression of the OsMADS1 gene also overcomes the day-length requirement of a short-day flowering plant.

Table 2. Ectopic expression of *OsMADS1* in *Nicotiana 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 measused on the first anthesis day. ND, not determined since the control plants did not flower within 250 days.

## DISCUSSION

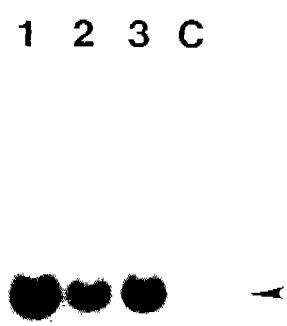


Figure 4. RNA blot analysis of *N. tabacum* cv. Maryland Mammoth. Twenty μg of total RNA was separated on a 1.3% agarose gel, blotted onto a nylon membrane, and hybridized with a $^{32}$P-labeled gene-specific probe. Numbers indicate independent transgenic plant lines of T2 generation. *N. tabacum* cv. Maryland Mammoth plant transformed with the kanamycin marker alone was used as a control (C). An arrowhead indicates 1.2-kb OsMADS1 transcripts.

In this study, we have demonstrated 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. Our results suggest that, in natural conditions, expression of the OsMADS1 gene is under the tight control of 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 types of plant species.

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

Some plant species are extremely sensitive to day-length whereas others are less sensitive. It was often found in the nature that different ecotypes within a single species have different responses to day length. It is believed that the difference between a day length-sensitive variety and one that is not so sensitive to day length is controlled by a single or several regulatory genes. Our study indicates that OsMADS1 is probably one of these regulatory genes that determine sensitivity to the day length. The observation that, especially under non-permissive condition, the flowering time of the transgenic *N. sylvestris* directly correlates with the expression level of the OsMADS1 transcript level supports the hypothesis that the 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 mainly controlled by the environmental factors whereas in others it is regulated by developmental factors.

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