

***MdMADS2* – transgenic chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura) showing the reduction of the days to flowering**

Bong-Hee Han · Su Young Lee · Seong Youl Choi

Received: 10 August 2009 / Accepted: 17 December 2009
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Abstract This study was conducted to develop new lines expressing the characteristic of early flowering by introducing *MdMADS2* gene in chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura) ‘Zinba’. Transformation of chrysanthemum was conducted by *Agrobacterium tumefaciens* LBA4404 harboring the binary vector containing *MdMADS2* controlled by double CaMV 35S promoters. Ninety three shoots were regenerated from 1,463 leaf segment explants cultured on the first selection medium (MS basal salts + 1.0 mg/L BA + 0.5 mg/L IAA + 10 mg/L kanamycin + 400 mg/L cefotaxime, pH 5.8) after co-cultivation, and 20 out of the 93 shoots rooted on the second selection medium containing 20 mg/L kanamycin and 400 mg/L cefotaxime. Many escapes (98.6%) were removed on the selection stage for rooting. Nineteen lines were confirmed as transgenic plant with transgene by PCR analysis. Six transgenic plants flowered 2–11 days earlier than non-transgenic plant without big change of phenotype, and especially, 3 (Mo-7, Mo-11, Mo-17) out of 6 transgenic lines showed a significant reduction in days to flowering compared to non-transgenic plant. Introduction and expression of *MdMADS2* gene in them were confirmed by Southern and real-time PCR analyses, respectively.

Keywords *Agrobacterium tumefaciens*, Kanamycin, *MdMADS2*, rooting selection, transformation

Introduction

Chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura) is one of the major cut flowers in the world, and its many characteristics have been improved through conven-

tional breeding methods. Recently, the molecular transformation technique has an alternative approach to improve specific characteristics of chrysanthemum making use of its advantage as ‘one point’ crop improvement (Ledger et al. 1991; Han et al. 2003; Han et al. 2007 Khodakovskaya and Vaňková 2009; Tong et al. 2009). In addition, because chrysanthemum is propagated mainly by cutting and suckering, any desirable lines obtained through transformation can be easily developed as useful cultivars (Aswath et al. 2004).

The life cycle of chrysanthemum is very important for year round cut flower production. In the Netherlands, flowers of chrysanthemum are harvested 3.5 to 4 times a year, whereas only two times in Korea due to environmental and cultural restricting factors found in greenhouse such as propagation of plantlets, temperature extremes during summer and winter, and long life cycle. Therefore, the shortening of its life cycle is needed to increase the number of its harvesting in a year (Han et al. 2003).

Flower formation in higher plants is a complex process controlled by genetic and environmental factors (Bernier 1988; Yanofsky 1995; Amasino 1996; Levy and Dean 1998). Much has been learned from genetic and molecular studies of floral meristem and floral organ formation in *Arabidopsis* and *Snapdragon* (Sung et al. 1999). It has been known that MADS-box genes are important regulators of meristem and floral organ identity. Recent research demonstrated that MADS-box genes in *Arabidopsis* are important regulators of meristem and floral organ identity as well as flowering timing and cell-type specification in floral organs (Jack 2002). *MdMADS2*, which shows high sequence similarities to the *SQUAMOSA* subfamily genes isolated from Fuji apple, was introduced in tobacco. The transgenic tobacco expressing the *MdMADS2* gene from the CaMV 35S promoter showed early flowering and shorter bolts without any homeotic changes in the floral organs (Sung et al. 1999). These results suggest that *MdMADS2* plays an important role during early stages of flower development.

B.-H. Han · S.-Y. Lee (✉) · S.-Y. Choi
(Floriculture Research Division, National Institute of Horticultural & Herbal Science, 540-41 Tap-dong, Gwonseon-gu, Suwon, 441-440, Korea)
e-mail: lsy8542224@korea.kr

Based on this information, this study was conducted to develop new lines showing the reduction of its life cycle through expressing the characteristic of early flowering by introducing *MdMADS2* gene into chrysanthemum cv. Zinba, which has a big white flower as a standard cut flower cultivar, has been cultivated in most parts of Korea since 2000 because of its vigorous growth, and has late flowering time and long life cycle flowering.

Materials and methods

Plant material

Chrysanthemum cv. Zinba applicable for improving a characteristics using genetic transformation technique was used as plant material because of its high regeneration ability (Han et al. 2009). Young shoots were taken from the mother plants growing in green house at National Institute of Horticultural & Herbal Science (NIHHS), and the surface of the shoots was sterilized with 70% ethyl alcohol for 30 s and 1.0% sodium hypochlorite for 15 min, and then rinsed three times with sterilized water. The shoots were incubated on MS basal medium (Murashige and Skoog 1962) and subcultured at eight-week intervals.

Agrobacterium-mediated transformation

MdMADS2 gene (NCBI No. U78948), which was cloned from apple (*Malus × domestica*) and subcloned into pCAMBIA 2301 (containing double CaMV 35S promoter digested with *NcoI* and *EcoRI*) by Soon-Kee Sung in Dongbu Technology Institute, was used (Fig. 1). The whole process of *Agrobacterium*-mediated transformation from co-cultivation to the first selection was conducted as described by Han et al. (2007). A number of studies reported that chrysanthemum is very susceptible to kanamycin (the lethal concentrations are 10–50 mg/L depending on the cultivars), and many ‘escape’ (non-transgenic) shoots were obtained through transformation process (Ledger et al. 1991; Lowe et al. 1993; Renou et al. 1993; De Jong et al. 1994; Urban et al. 1994; Fukai et al. 1995 Taka-

tsu et al. 1998). Due to this sensitivity to kanamycin of chrysanthemum, we performed the two-stage selection. At first, we let shoots from leaf explants regenerated on shoot regeneration medium containing 1.0 mg/L BA, 0.5 mg/L IAA, 10 mg/L kanamycin and 400 mg/L cefotaxime. And then, we let the regenerated shoots rooted on rooting selection medium (hormone-free MS medium, the second selection medium) containing 20 mg/L kanamycin and 400 mg/L cefotaxime. After the four weeks culture in the second selection medium, the rooted plantlets were selected for PCR analysis and continuously maintained on MS medium containing 2 g/L activated charcoal as putative transformants.

PCR analysis

Putative transgenic plantlets, which regenerated in the first selection medium and rooted in the second selection medium, respectively, were subjected to PCR analysis. Genomic DNA was isolated from leaves of in vitro-grown plantlets according to the manufacturer’s instructions of the DNeasy Plant Mini Kit (Qiagen, Germany). PCR was conducted using both neomycin phosphotransferase II (*nptII*) gene specific primers (Han et al. 2007) and transgene specific primers (forward 5'-AAG GGT GCA GCT GAA GAG AA-3', and reverse 5'-TGG AGT TGG GGT TTC ATC TC-3'). The reaction mixture composition and amplification condition for *nptII* gene PCR were conducted as described by Han et al. (2007). The reaction mixture of *MdMADS2* transgene PCR was composed of 5 µL of extracted DNA, 10 pmol each of the forward and reverse primers, and 45 µL of Platinum Blue PCR Super Mix (Invitrogen, USA). The amplification was carried out with 35 cycles of 30 s at 98°C, 1 min at 58°C, 1 min at 72°C in an automatic thermal cycler (iCycler, Bio-Rad, USA).

Investigation of early flowering trait in transgenic chrysanthemum

The transgenic lines confirmed by PCR analysis, and non-transgenic plant (for control) were propagated on basal MS medium. Six plants per line were acclimatized on the cultural medium mixed with perlite and vermiculite in the ratio of 1:1

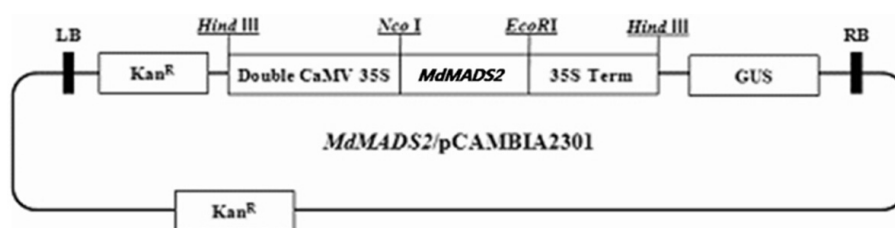


Fig. 1 A map of pCAMBIA 2301 with *MdMADS2* gene used for plant transformation in this study

in green house and treated with cold temperature (5 °C) in cold room for 1 month. The cuttings excised from each line were planted into the pots (30 cm in diameter) in green house on July 2, 2008 after rooting. The trial included 3 replicates of pots per line. The characteristic of early flowering in the transgenic lines was investigated whenever the transgenic lines flowered.

Southern analysis

Southern blot analysis was performed on 6 transgenic lines showing the trait of early flowering. Genomic DNA was isolated from their leaves grown in greenhouse by using the CT AB method (Doyle and Dickson 1987). Twenty mg of DNA digested with *EcoRI* was followed by electrophoresis on 1.0% agarose gel in TBE buffer (45 mM Tris-borate, 45 mM boric acid, 1 mM EDTA (pH 8.0) for 12 h. Following electrophoresis, the DNA was transferred to Nylon HybondTM-N⁺ membrane (Amersham Life Science, UK). Hybridization was carried out with Church's buffer for 12 h at 65 °C with *MdMADS2* gene specific probe labeled with (α -³²P) dCTP by random primed labeling (Foster et al. 2002).

Real-time qPCR

Total RNA was isolated from 100 mg leaves of 6 transgenic lines, respectively, and control with the RNeasy Plant Mini Kit (QIAGEN, Germany) according to the manufacturer's recommendations. A quality and concentration of the isolated RNA were done by gel electrophoresis (1%, RNase-free) and spectrophotometry (260/280 nm). The two-step qPCR was performed. First strand cDNA synthesis was performed with 1 μ g of total RNA treated with DNase I (Promega, USA) using PrimeScriptTM reverse transcription (RT) reagent kit (TAKARA, Japan) according to the supplier's recommendation. Primers were designed using the program primer 3 (Rozen and Skaletsky 2000). Sequence of *MdMADS2* gene specific primers and chrysanthemum *actin* cDNA (GenBank accession No. AB205087) gene (used as a

constitutive control gene) specific ones was as follows: forward of the one, 5'-GGGTGGAGGTTTTACAAAGAAA-3'; reverse of the one, 5'-TACTGCGTTCTCCTTCTCCTTC-3'; forward of the other, 5'-ACATGCTATCTTGCCTTTGG-3'; reverse of the other, 5'-CTCTACAATTTCCCGTTCA-3'. The real-time PCR was carried out using the MJ OPTICON (BioRad, USA). The mixture (20 μ L final volumes) containing 10 μ L of a 2 \times SYBR Premix Ex TaqII (TAKARA, Japan), 2 μ L of primer-pair mix (primers concentration: 500/500 nM), and 2 μ L cDNA first strand template was reacted with 40 cycles of 30 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C after 10 min at 95 °C and following by a final step at 72 °C for 10 min. Subsequently, melting curve was recorded between 65 and 95 °C with the hold every 1 s. The 2^{- Δ Ct} method (Livak and Schmittgen 2001) was used to calculate the relative expression of transgene. Each assay was repeated three times.

Results and discussion

Agrobacterium-mediated transformation of *MdMADS2*

A total of 1,463 leaf segments were co-cultivated with *Agrobacterium tumefaciens* LBA4404 harboring the binary vector pCAMBIA2301 containing the *MdMADS2* gene. Shoot regeneration occurred on the edges of leaf segments. After 6 weeks culture on the first selection medium, 93 shoots from 1,463 leaf explants were regenerated. After that, when these greenish shoots were cultured in the second selection medium for 4 weeks in light condition, only 20 out of 93 shoots rooted in the second selection medium. From PCR analysis using *nptII* gene-specific primers, the 20 rooted plantlets were confirmed to contain *nptII* gene. However, from PCR analysis using *MdMADS2* gene-specific primers, 19 out of them were confirmed to have transgene, whereas, 1 of them not to have transgene (Table 1, Fig. 2). As a result, 74 out of 93 regenerated on the 1st selection medium were found as 'escape'. Seventy three (98.6%) out of these 74 escapes was removed on the process of rooting selection medium with 20

Table 1 PCR analysis of shoots regenerated or plantlets rooted on the selection medium after co-cultivation with *Agrobacterium tumefaciens* LBA4404 harboring a binary vector pCAMBIA 2301 containing *MdMADS2* gene in chrysanthemum (*Dendranthema \times grandiflorum* (Ramat.) Kitamura) cv. Zinba

No. of explants	No. of shoots regenerated on the 1 st selection medium ^z	No. of shoots rooted on the 2 nd selection medium ^y	No. of transgenic shoots with <i>nptII</i> gene	No. of transgenic shoots with <i>MdMADS2</i> gene
1,463	93	20	20	19

^z MS medium was containing 1.0 mg/L BA, 0.5 mg/L IAA, 400 mg/L cefotaxime, and 10 mg/L kanamycin.

^y MS medium was containing 400 mg/L cefotaxime, and 20 mg/L kanamycin.

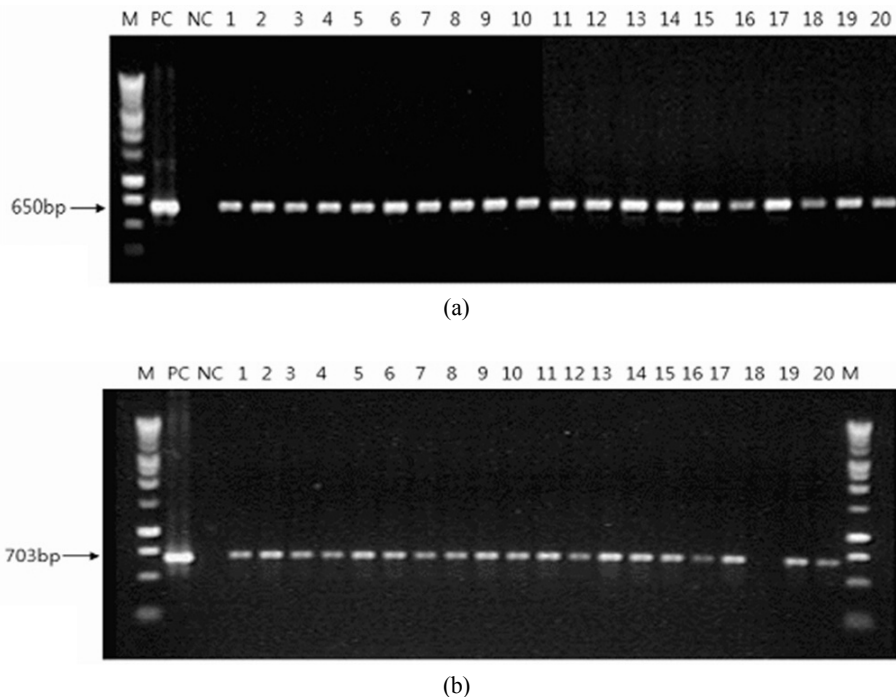


Fig. 2 PCR analysis of 20 pseudo-transgenic chrysanthemum plants rooted on the 2nd selection medium after co-cultivation with *Agrobacterium tumefaciens* LBA4404 harboring a binary vector pCAMBIA 2301 containing *MdMADS2* gene. PCR amplifications were performed with primers for *nptII* (A) and *MdMADS2* (B). M: molecular size(1 kb ladder), PC: pCAMBIA 2301 vector DNA, NC: non-transgenic plant, 1-20: putative transgenic plants

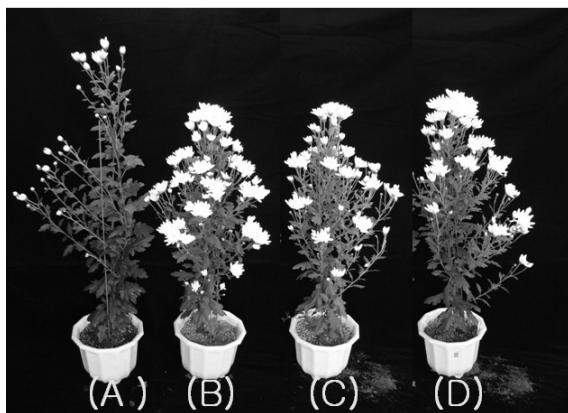


Fig. 3 *MdMADS2*-transgenic chrysanthemum lines showing early-flowering. (A) non-transgenic plant. (B)–(D) transgenic lines; (B) MO-7, (C) MO-11, (D) MO-17

mg/L kanamycin. It could be said that this two-stage selection system, in which most of shoots regenerated without transgene in the first selection medium containing 10 mg/L kanamycin and 400 mg/L cefotaxime were not rooted on rooting medium containing 20 mg/L kanamycin and 400 mg/L cefotaxime, was very desirable in removing escapes for genetic transformation of chrysanthemum. It is known that sensitivity to kanamycin varies depending on tissues. Root formation is generally more sensitive than shoot formation or callus growth (Draper et al. 1988). Saini et al.

(2003) reported that a second round of selection at the rooting stage was critical for transformation of *Vigna mungo*. The escape shoots from selection of regeneration stage were dramatically reduced by the rooting selection. The elimination of escapes by the rooting selection was reported in many studies (Boase et al. 1998; Yan and Wang 2007; Zou et al. 2008), too.

Field selection of *MdMADS2* transgenic lines expressing traits of early flowering

Six out of 19 transgenic lines showed earlier flowering than non-transgenic plants (control). They flowered 2.3 to 11.3 days earlier than control, and three ones (MO-7, MO-11, and MO-17) (Fig. 3) appeared to have a significant reduction in days to flowering. They did not show differences in the number of branches on average compared to control. Whereas, their plant height was significantly reduced by about 30 cm at flowering time compared to control, and they showed a few differences in the number of nodes compared to the latter. The number of nodes of them was 3-7 fewer on average than that of control (Table 2). These results were the same as Sung et al. (1999)' report that let *MdMADS2* gene introduced into tobacco. The several transgenic tobaccos showed to have shortened the number of flowering days by 8 to 10 days. At flowering time, the plant height of the transgenic tobaccos

Table 2 Growth characteristics of 6 *MdMADS2*-transgenic lines from chrysanthemum (*Dendranthema × grandiflorum* (Ramat.) Kitamura) cv. Zinba

Lines ^z	Plant height (cm)	No of nodes	No of branches	Days to flowering
Control	95.3±3.5 ^y a ^x	63.3±3.0 a	46.3±0.3 a	128.3±1.9 a
MO-2	79.7±2.3 cd	55.3±2.3 b	43.0±2.9 a	126.0±0.6 a
MO-7	73.3±1.8 d	52.0±1.5 b	39.0±2.1 a	117.0±0.6 c
MO-11	78.3±1.8 cd	57.7±0.9 ab	39.3±2.8 a	117.0±1.2 c
MO-14	87.7±1.8 b	57.3±2.3 ab	38.3±3.4 a	122.7±0.3 b
MO-17	76.3±2.2 cd	55.7±2.0 b	43.3±3.5 a	118.3±0.9 c
MO-20	82.3±1.7 bc	57.7±0.9 ab	44.3±0.3 a	119.0±0.6 b

^z The plant lines were planted in greenhouse on July 2nd, 2008, and the data was investigated from October 25th to November 15th, 2008.

^y Mean±standard error

^x Different letters in same column represent significant differences by the Duncan's multiple range test, $P \leq 0.05$

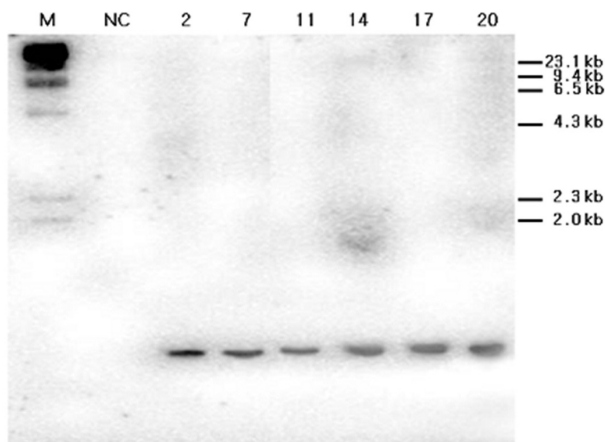


Fig. 4 Southern analysis of *MdMADS2*-transgenic chrysanthemum lines showing early-flowering. Total genomic DNA of non-transgenic and transgenic plants was digested with *EcoR* I. M: Lambda-*Hind*III digested marker DNA, NC: non-transgenic plant, 2, 7, 11, 14, 17, and 20: transgenic lines

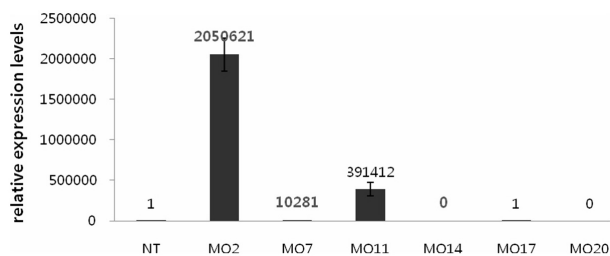


Fig. 5 Real time PCR analysis of *MdMADS2*-transgenic chrysanthemum lines showing early-flowering

was reduced by about 13 to 30 cm. This reduction seemed to coincide with the decrease in node number.

Confirmation of introduction and transcription level of transgene in *MdMADS2*-transgenic lines by Southern and real-time PCR analyses

Southern analysis was done to confirm introduction of *MdMADS2* gene in 6 transgenic lines showing the trait of early-flowering. All six transgenic lines were confirmed to be integrated *MdMADS2* gene (Fig. 4).

Also, real-time PCR analysis was performed to identify transcription level of *MdMADS2* gene in them. Relative transcription levels of the transgene in 6 transgenic lines and control are shown in Fig. 5. Whereas the transgene was transcribed in 4 lines (Mo-2, Mo-7, MO-11, and Mo17), it was not in two lines (Mo-14 and Mo-20). Its transcription level in three (Mo-2, Mo-7, and Mo17) lines was very high compared to that in control, and Mo-2 line, of which the period to flowering was little shorter than other 5 transgenic lines, exhibited the highest transcription level. Mo-17 line showing reduction of 10 days to flowering on average compared to non-transgenic plant exhibited the same transcription level as control. Flachowsky et al. (2007) also reported that overexpression of *BPMADS4* from silver birch induces early-flowering in apple, but no correlation between expression level of transgene and development of *in vitro* flowers of transgenic clones was found. It is known that flowering of a facultative short-day plant including chrysanthemum cv. Zinba, which flowers from August to mid-October under natural field condition in Korea, is mainly controlled by photoperiod. Though, endogenous factor cannot rule out in controlling of flowering. In other words, it is said that many genes controlling environmental and endogenous factor for flowering need to control it. So, it believed that no correlation between expression level of the transgene and the reduction of days to flowering in *MdMADS2*-transgenic lines obtained in this study was found, because another gene except *MdMADS2* gene is certainly involved for the control of flowering in chrysanthemum cv. Zinba.

In conclusion, *MdMADS2* gene from apple could induce an

early flowering without big change of phenotype in transgenic lines from chrysanthemum cv. ‘Zinba’. *MdMADS2* transgenic lines obtained in this study will be key fundamental genetic materials for breeding early-flowering chrysanthemum new cultivar.

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