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Transformation of Rice *OsMADS1* Gene Causes Homeotic Mutations in Floral Organs of Chinese Cabbage (*Brassica campestris*)

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We introduced *OsMADS1*, a MADS box gene from rice, into Chinese cabbage using the *Agrobacterium*-mediated method. Among the 30 regenerated transformants, 7 transgenic plants were confirmed to carry *OsMADS1* transcript as the transgene. This gene had been put under the 35S promoter by RT-PCR and RT-PCR northern hybridization. The *NPT II* and *OsMADS1* primers detected 0.7-kb and 0.6-kb PCR products, respectively, that corresponded with the expected size from their transcripts. Our results suggest that those transgenes are expressed in the transgenic plants. We also observed the plants at the flowering stage in order to identify any possible morphological alteration caused by the transgene during flowering. One notable mutation involved the homeotic replacement of a carpel with another flower instead.

Keywords: Chinese cabbage, flower development, homeotic mutation, *OsMADS1*

Chinese cabbage (*Brassica campestris* ssp. *napus* var. *pekinensis*) is a cultivated crop in Asia, particularly in China, Japan, and Korea. Members of the *Brassica* genus have been regenerated via the culturing of pollen (Sato et al., 1989), protoplasts (Han and Kim, 1990), and somatic embryos (Choi et al., 1994). In addition, *Agrobacterium*-mediated transformation has been successful with *B. napus* (Radke et al., 1988; de Block et al., 1989; Moloney et al., 1989), *B. oleracea* (de Block et al., 1989), *B. juncea* (Mathews et al., 1990), *B. carinata* (Babic et al., 1998), and *B. campestris* (Mukhopadhyay et al., 1992; Radke et al., 1992; Takasaki et al., 1997). However, Chinese cabbage remains recalcitrant to tissue culture, with the production of transgenic plants only first being reported by Jun et al. (1995).

Plant development occurs in two phases: vegetative and reproductive. These stages are regulated by a number of genes in the MADS box family (Mandel et al., 1992; Weigel et al., 1992; Hempel et al., 1997; Jang and An, 1999). Members of this gene family are classified into four subfamilies according to their phylogenetic relationships -- AP3/PI, AG, AP1/AGL9, and orphan gene clades (Purugganan et al., 1995; Theissen et al., 1996; Alvarez-Buylla et al., 2000). Within the AP1/AGL9 subfamily, genes specify floral-meristem identity and control organ development in the three inner whorls, i.e., petals, stamens, and carpels (Jeon et al., 2000; Prasad et al., 2001). For example, the *OsMADS1* gene used in the current study has been isolated from rice. Its products share 68.4% identity with AGL9, 56.2% identity with AGL2, and 44.4% identity with the AP1 proteins, all found in *Arabidopsis* (Chung et al., 1994). In fact, when *OsMADS1* is introduced into *Arabidopsis*, the resulting transformants flower earlier than the controls and exhibit dwarfism. This gene also demonstrates functions and expression patterns distinct from others in the AP1/AGL9 subfamily for dicotyledonous species (Jeon et al., 2000; Prasad et al., 2001).

In this study, we used the *OsMADS1* gene, driven by the 35S promoter, as the transgene to transform Chinese cabbage. Our objective was to determine whether cotyledon explants were more effective than other tissue types (e.g., hypocotyls) for use in transformation. We also investigated transformation efficiency and possible phenotypic alterations to floral organs.

MATERIALS AND METHODS

Plant Material and Germination Condition

Seeds of the Samjin and Seoul cultivars of Chinese cabbage (*B. campestris* ssp. *napus* var. *pekinensis*) were disinfected with 1% sodium hypochlorite for 15 min. They were then rinsed three or four times with sterile-distilled water and placed on an MS medium for 4 d. Germination conditions included a temperature of 25 ± 2°C and a 16-h photoperiod.

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Plant Transformation and Regeneration

pGA1209, comprising *OsMADS1* transcript designed to be driven by the 35S promoter, was introduced into *Agrobacterium tumefaciens*. Afterward, the *A. tumefaciens* was cultivated at 28°C in a YEP medium containing 50 mg/L kanamycin (Km). Cotyledon explants of Chinese cabbage were inoculated into the suspension medium and co-cultivated for 2 d. The explants were then transferred to a shoot-inducing medium (SIM) that contained 5 mg/L Km, 250 mg/L Cefotaxime, and NAA and BA with various concentrations (Table 1) . Following a four-week screening period, the induced shoots were transferred to a shoot elongation medium (SEM) containing 0.0025 mg/L BA, and were cultivated for two to four weeks. After roots formed in a root-inducing medium (RIM, containing 0.1 mg/L NAA), the plantlets were transferred to soil for the hardening procedure. All procedures were performed at 25 ± 2°C under a 16-h day length.

RT-PCR and RT-PCR Northern Blot Hybridization

Total RNA was isolated from the leaves of our transgenic plants by the guanidium thiocyanate method. PCR was performed after reverse transcription at 42°C for 90 min. The primer sequences for *NPT II* and *OsMADS1* were (5'-GAGGCTATTCGGCTATGACTG-3',5'-ATCG GGAGCGGCG ATACCGTA-3') and (5'-AGTTCTCCAG CTCATCATGC-3',5'-AGCCGGATGGGATGTGTTCA-3'), respectively. PCR reactions in the presence of each primer were subjected to 36 cycles at 94°C (45 s), 54°C (45 s), and 72°C (1 min 30 s), for denaturation, annealation, and extension, respectively. The PCR products were separated and confirmed on a 0.8% agarose gel, then transferred onto a nylon membrane (Hybond-N+, Amersham). A probe was prepared from the *OsMADS1* transcript in which the MADS-box region was removed to avoid non-specific hybridization via the random-primer method, using non-radioactive digoxigenin (Feinberg and Vogelstein, 1983). The membrane was fixed by UV light for 2 min, prehybridized at 42°C for 2 h, and hybridized at 42°C for 16 h. Afterward, the membrane was washed with a solution containing 2.0× SSC/0.1% SDS and 0.1× SSC/0.1% SDS, and was visualized for 30 min with a 1:10,000-diluted anti-digoxigenin-alkaline phosphatase-conjugated antibody (Boehringer Mannheim). The membrane was finally washed with a buffer containing 100 mM Tris-HCl and 150 mM NaCl for 15 min, then reacted with fluorescent CSPD (Boehringer Mannheim).

Table 1. Efficiency of shoot regeneration from Chinese cabbage (*B. campestris* ssp. *napus* var. *pekinensis* cv. Samjin and Seoul) using various concentrations of NAA and BA.

Cultivar	NAA (mg/L)	BA (mg/L)	Shoot regeneration (%)
Samjin	0	1	64.8
		2	84.2
		3	57.8
		4	55.2
		5	80.2
	0.5	1	63.1
		2	60.5
		3	71.0
		4	52.6
		5	63.1
	1	1	60.5
		2	52.6
		3	57.8
		4	50.5
		5	42.0
Seoul	0.5	4	69.2
		5	60.0
		6	62.1
		7	43.8
	1	4	56.3
		5	69.2
		6	55.3
		7	51.3

RESULTS AND DISCUSSION

Transformation of *Brassica* with *OsMADS1*

Cotyledon explants of Chinese cabbage that were placed on the SIM showed multiple shoot primordia after 10 to 14 d of cultivation (Fig. 1A). After transfer to the SEM, however, shoot elongation was delayed approximately one week, perhaps because of the stress caused by the *Agrobacterium* and antibiotics (Gupta et al., 1993; Jun et al., 1995). Nevertheless, after four weeks of shoot elongation (Fig. 1B), the tissues were transferred into the RIM for root induction (Fig. 1C). Within two to four weeks, healthy roots had formed, and the plants were then transferred to soil (Fig. 1D).

Overall efficiency, for both regeneration and transformation, was much higher from Samjin than from Seoul, and their preferences for NAA and BA concentrations also differed (Table 1). Compared with our previous experience using hypocotyls from Chinese cabbage, we were able to transform cotyledons at a higher frequency of regeneration (Table 2). Moreover, the time required for culturing with hypocotyls was longer, with the callus-induction period itself lasting about four weeks. These results demonstrate that the

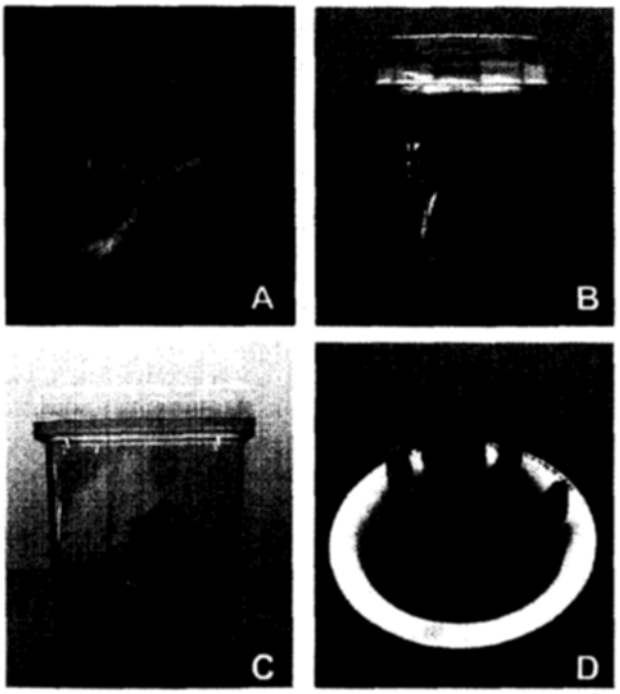

Figure 1. Regeneration procedures for transformants. **(A)** Multiple shoots formed from the explant. **(B)** Shoot transferred to SEM. **(C)** Shoot after transferred to RIM. **(D)** Transformant potted in soil.

Table 2. Comparison of transformation efficiencies between cotyledon (cv. Samjin) and hypocotyl explants of Chinese cabbage (*B. campestris* ssp. *napus* var. *pekinensis*).

Explant type	Treatment (mg/L)	Transformation efficiency (%)
cotyledon	0.0 NAA + 2.0 BA	29.2
hypocotyl	1.0 NAA + 7.0 BA	16.7

use of cotyledon explants is more effective than that of other tissue types in transforming Chinese cabbage. Putative transformation efficiencies (based on the number of Km-resistant shoots) were 29.2% and 23.8% from 'Samjin' and 'Seoul', respectively. These rates are higher than the12.5% frequency reported by Zhang et al. (2000).

RT-PCR Analysis

Following transformation with the T-DNA, we were able to obtain 30 regenerated plants, which were transferred to soil. RT-PCR was then used to verify them as true transgenics. In 7 of our 30 plants, the *NPT II* and the *OsMADS1* primers detected 0.7-kb and 0.6-kb PCR products, respectively, that corresponded to the expected size from their transcripts (Fig. 2A). RT-PCR

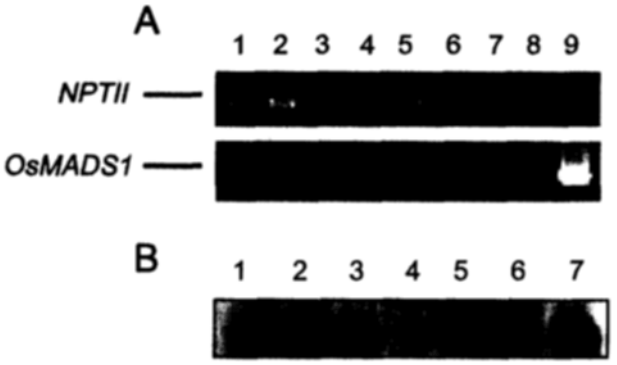

Figure 2. (A) Products of RT-PCR analysis, obtained by using *NPT II* primer and *OsMADS1* primer that detect 0.7-kb band and 0.6-kb band, respectively. Lanes 1-7, transformants; Lane 8, non-transformant; Lane 9, pGA1209 vector. **(B)** RT-PCR northern blot hybridized with *OsMADS1* specific probe. Lanes 1-7, transformants.

northern blot hybridization was also performed to confirm that the PCR products of the *OsMADS1* primer had indeed resulted from the expression of *OsMADS1* in those transgenic plants (Fig. 2B).

Homeotic Mutation of Floral Organs

Chung et al. (1994) have shown that ectopic expression of *OsMADS1* in transgenic tobacco plants causes reduced apical dominance, dwarfism, and early flowering. This early-flowering phenotype has been confirmed when the gene is ectopically expressed in an homologous species, thereby suggesting it has a functional role in floral-meristem determination (Jeon et al., 2000). In addition, Jeon et al. (2000) and Prasad et al. (2001) have proposed that this gene functions in the development of floral organs. In the current study, we also observed that the transgenic plants showed reduced height growth compared with the wild types (data not shown). However, we cannot conclude that this phenotype results from reduced apical dominance. More careful analysis with both progenies is required at both the molecular and the genetic level.

Our transformed plants also showed mutations in their floral organs during flowering (Fig. 3). The transgenic flowers contained sepals, petals, and stamens that corresponded to Whorls 1, 2, and 3, respectively, as well as the homeotic replacement of the carpel organ with one or more flowers (Fig. 3, B, C, and D). Formation of sepals and petals varied among the transgenic plants, with the size and number of organs being inconsistent. We also observed shorter stamens, which probably reflected the dorsal effect of the transgene in our transformed plants.

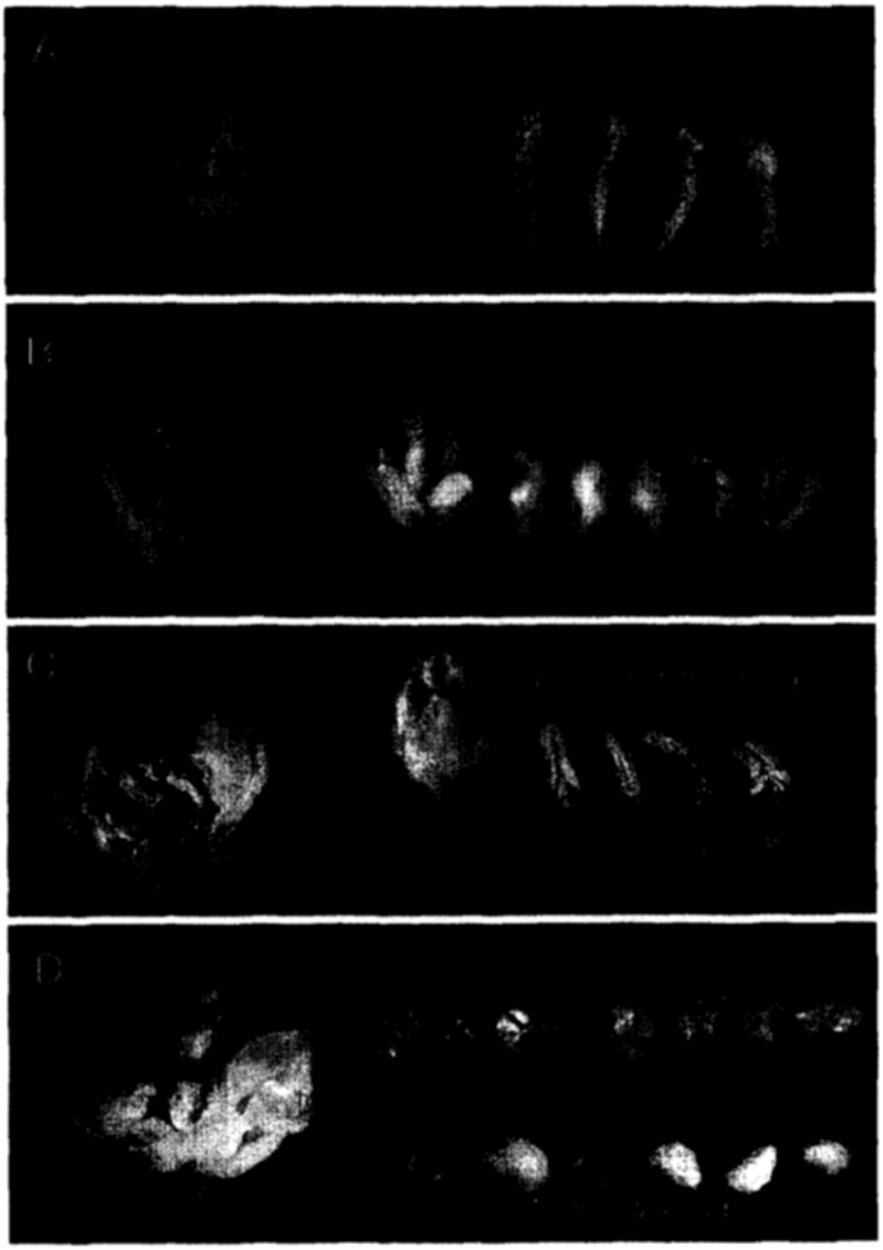

Figure 3. Phenotypes of *35S::OsMADS1* transgenic flowers. **(A)** A wild-type flower. **(B)** - **(D)** Abnormal flowers obtained from transgenic plants. **(B)** Flower consisting of three petals, two sepals, shortened stamens, and a carpel. **(C)** Flower with shortened stamens and the carpel replaced by another flower. **(D)** Transgenic flower with six new flower buds inside. S, sepal; P, petal; St, stamen; C, carpel.

Chung et al. (1994) have reported that the *OsMADS1* gene in rice is expressed mainly in the organs of the palea/lemma and the carpel, which are considered Whorl-1 and Whorl-4 organs in dicotyledonous plants. This may explain why, in Whorl 4, the carpel was homeotically replaced with another flower instead. The mis-sense mutation of the *OsMADS1* gene in rice also exhibits phenotypic alterations during the formation of spikelets, including an elongated leafy palea/lemma, fewer stamens, and more carpels (Jeon et al., 2000). That, however, does not explain the changes in our transgenic plants. MADS box genes actively promote dimers in either the homo or the hetero form. In fact, genes that lack the MADS domain will fail to dimerize and bind to DNA (Mizukami et al., 1996). Thus, the likelihood that different phenotypes will appear in a

particular transgenic plant system, especially one that is heterologous, may depend on having the appropriate environment for gene activation.

Based on its sequence homology, *OsMADS1* can be classified in the *AP1/AGL9* subfamily, a group believed to be active in determining floral identity (Purugganan et al., 1995). Other MADS box genes, e.g., *TM5* and *FBP2*, also reportedly control organ identity as well as floral-meristem development (Angenent et al., 1994; Pnueli et al., 1994). *OsMADS1* shares some similarity with *TM5* and *FBP2* with respect to their determination of floral meristems (Jeon et al., 2000). Therefore, it may also play a pivotal role during early flower development. However, careful analysis of the progeny from transgenic plants is required if we are to better understand how *OsMADS1* influences floral meristems in Chinese cabbage.

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