J. Plant Biotechnology(2003) Vol. 5(4). pp. 215~219

Journal of Plant Biotechnology

High plant regeneration and ectopic expression of *OsMADS1* gene in root chicory (*Cichorium intybus* L. var. *sativus*)

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Abstract

Optimal shoot regeneration and transformation conditions of root type chicory (*Cichorium intybus* L. var. *sativus* cv Cesare) were studied. Leaf explants were co-cultured with *Agrobacterium tumefaciens*, which contained NPTII as a selectable marker and a rice homeotic gene, *OsMADS1*, that encodes a MADS-domain-containing transcription factor. After one day of co-cultivation, explants were transferred to selection media consisting of MS basal medium supplemented with 0.5 mg/L BAP, 0.1 mg/L IAA, 70 mg/L kanamycin, and 250 mg/L cefotaxime. PCR and Southern blot analyses revealed stable integration of the *OsMADS1* gene in the chicory genome. Four-teen original transgenic plants (T_0 plants) were acclimatized in the greenhouse and examined for their morphological characters. Most of the transgenic plants showed altered morphologies, such as short, bushy, and early-flowering phenotypes with reduced apical dominance. Additionally, half of the transgenic plants exhibited altered leaf shapes, and 4 out of 14 plants were sterile. These phenotypes were inherited by the next generation. Northern blot analysis confirmed expression of the *OsMADS1* gene in both floral and vegetative organs.

Key words: *Agrobacterium tumefaciens*, MADS-box gene, transformation, transgenic plants

Introduction

Last decades, the mechanisms of flowering in monocotyledonous or dicotyledonous species have been extensively studied (Piñeiro and Coupland 1998; Hay and Ellis 1998; Davies et al. 1996; Mena et al. 1995; Kang et al. 1995). Researchers found that floral organ development was controlled by a group of regulatory genes encoding transcription factors that contain the conserved MADS-box domain (West and Sharrocks 1999; Riechmann et al. 1996; Mizukami et al. 1996). MADS-box genes have been isolated from several plant species, including *Arabidopsis* (Riechmann et al. 1996), *Antirrhinum* (West et al. 1998), petunia (Angenent et al. 1992), *Sinapis alba* (Menzel et al. 1996), potato (Kang and Hannapel 1996), hot pepper (Yu 2001), maize (Mena et al. 1995), and rice (Chung et al. 1994; Kang et al. 1995).

The rice MADS-box gene, *OsMADS1*, is expressed at a certain developing stage of the plant. Chung and his colleagues (1994) reported that the *OsMADS1* gene from rice was initially expressed uniformly in young flower primordia, and the expression is localized in the palea, lemma, and ovary at late developmental stages. However, this gene was not expressed in vegetative tissues. Other researchers (An and An 2000; Jeon et al. 2000) reported that ectopic expression of *OsMADS1* in tobacco and rice resulted in early-flowering and dwarf phenotypes.

Chicory (*Cichorium intybus* L.) is an excellent material for genetic transformation because of its great capacity to regenerate shoots from explants (Mohamed-Yassen and Splitstoesser 1991 and 1995; Park and Lim 1999; Vermeulen et al. 1992). However, most of earlier studies were limited to specific varieties, such as the witloof type and there is little information available on the genetic transformation of root type chicory (*C. intybus* L. var. *sativus*).

To establish an efficient transformation procedure of root type chicory, stable and high efficient regeneration system is necessary. Previously we regenerated plantlets from leaf and petiole of four genotypes in root-type chicory using (Park and Lim 1999). The results showed that shoot regeneration rates

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Received Nov. 10, 2002; accepted Dec. 9, 2003

varied from 20% to 95%, depending on genotype and regenerated shoots vitrified when the culture period was extended.

In this study, tissue culture system of root-type chicory was optimized not only to improve regeneration rates but also to reduce the vitrification of regenerated plantlets. Conditions to transform chicory were also examined to introduce *OsMADS1* gene.

Materials and Methods

Plant regeneration

Petiole and leaf explants were collected from five-to six-week-old plants of 'Cesare', root-type chicory (*Cichorium intybus* L. var. *sativus*), and cut into 5-to 8-mm segments. The excised explants were placed on regeneration media comprising an MS basal medium (Murashige and Skoog 1962) that included Ga-mborg B5 vitamins, 2% sucrose, and 0.8% agar (pH 5.6 to 5.8). Combinations of various concentrations of BAP (0.5, 1.0, or 2.0 mg/L) , TDZ (0.1, 0.2, or 0.5 mg/L) and NAA or IAA (0.1, 0.5, or 1.0 mg/L) were added to the medium. The explants were incubated at 22±2°C, under 80 μmol/s/m white fluorescent light. The regeneration rate was determined at three weeks after the initial culture.

Genetic transformation and morphological characterization

Two explant types, petiole and leaf, were pre-cultured for one day in a liquid MS basal medium (including Gamborg B5 vitamins, 3% sucrose, and BAP 1.0 mg/L). *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al. 1983), containing pGA1209 (Chung et al. 1994), was grown for two days, then centrifuged and resuspended in a solution with 20 mg/L acetosyringone. The pre-cultured explants were co-cultured with *Agrobacterium tumefaciens* in the liquid medium for one day.

The pGA1209 is a binary vector containing the neomycin phosphotransferase Ⅰ (NPT Ⅱ) as a selectable marker and the *OsMADS1* cDNA clone under the CaMV 35S promoter. After the co-cultivation, the explants were dried on filter paper for one to two hours, and then transferred to regeneration media (MS basal medium with Gamborg B5 vitamins, 2% sucrose, and 0.8% agar; plus 0.5 mg/L BAP and 0.1 mg/L IAA; pH 5.6 to 5.8) containing 250 mg/L cefotaxime and 70 mg/L kanamycin. Transfers were made to fresh media every three weeks. The regenerated shoots were then moved to rooting media (MS basal medium including Gamborg B5 vitamins, 2% sucrose, and 0.8% agar; plus 0.05 mg/L NAA, pH 5.6 to 5.8) that also was supplemented with 70 mg/L kanamycin and 250 mg/L cefotaxime. Plantlets with vigorous roots were propagated on rooting media with 70 mg/L kanamycin prior to being acclimatized in the greenhouse. T1 progenies, obtained by the self pollination of T0 plants, were grown in the greenhouse. Ectopic characteristics were recorded for both T0 and T1 plants.

Polymerase Chain Reaction (PCR) analysis

PCR was carried out in a 25 μL reaction containing 1x PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, and 0.1% Triton X-100; pH 8.8), 250 μM dNTPs, 20 pmol each of sense and antisense NPT Ⅱ primers, 0.5 μL (1 unit) Dynazyme DNA polymerase (Finnzyme), and 10 ng template DNA. The (amplifications of NPT Ⅱ) reaction were carried out with a Perkin Elmer 9600 thermal cycler, using the following parameters: Preheating at 94°C for 5 min; 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 sec; Post-elongation at 72°C for 10 min. Amplified PCR products were electrophoretically separated on 0.8% agarose gels, and were visualized by ethidium bromide(EtBr) staining

Southern and Northern blot analysis

For Southern analysis, 10 μg of genomic DNA isolated from the T0 plants was digested with a restriction endonuclease, *Eco*RI, fractionated on a 1.2% agarose gel, and blotted onto a nylon membrane. For Northern analysis, 20 μg of total RNA was separated on a 1.5% agarose gel in 0.5x TBE buffer and blotted onto a nylon membrane. The NPT Ⅱ probe was prepared from the PCR fragment, the *OsMADS1* probe from the cDNA clone lacking the conserved MADS-box and K-box regions (Chung et al. 1994), using a digoxygenin labeling kit (Boehringer Mannheim). The Southern hybridization was conducted at 65°C in a hybridization buffer consisted of 5x SSC, 0.1% (w/v) N-lauroylsarcosine, and 0.02% (w/v) SDS and the Northern hybridization was done at 50°C in a buffer containing 5x SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS, and 50% formamide.

Results and Discussion

Plant regeneration and transformation

Effect of auxin and cytokinin treatment on shoot regeneration from explants of 'Cesare' was examined in terms of types and concentrations of the phytohoromones(Tables 1 and 2). Since calli formed in the prensence of naphthaleneacetic acid (NAA) were very compact and non-embryogenic in all combinations of plant growth regulators tested, indoleacetic acid (IAA) was used

in the regeneration medium. Multiple shoots were observed on the medium containing 0.2 mg/L TDZ and 1.0 mg/L IAA, but they were weak and vitrified. The combination of 0.1 mg/L TDZ and 0.1 mg/L IAA allowed the most efficient regeneration from leaf explants, producing an average of seven to eight shoots per explant within a few weeks (Figure 1). Mohamed-Yassen and Splitstoesser (1991) developed a method for regenerating witloof chicory using thidiazuron (TDZ). Later, Vermeulen et al. (1992) reported that BAP was a more appropriate cytokinin for chicory regeneration. In this study, both plant growth regulators, BAP and TDZ, with IAA showed efficient regeneration rates from leaf explants of root type chicory.

Vermeulen et al. (1992) reported that 100 mg/L kanamycin was suitable for selection of transgenics in witloof chicory. In this experiment, however, 70 mg/L kanamycin was sufficient to cause necrosis of both leaf and petiole explants from 'Cesare'. After four weeks of selection on the 70 mg/L of kanamycin-containing media, transgenic shoots regenerated vigorously, whereas non-transgenic tissues became necrotic and failed to root (Figure 2A, B). The vitrification of regenerated plantlet was overcome by transferring the regenerated shoots to fresh agar medium that had no plant growth regulators. Capping the culture bottle with a micropore membrane filter encouraged the regeneration of normal plantlets, with well-developed root systems, within four weeks (Figure 2C).

Table 1. Percent rates of plant regeneration from leaf explants of 'Cesare' (*Cichorium intybus* var. *sativus*) grown on a medium containing various concentrations of plant growth regulators.

		Regeneration rates (%)					
		BAP (mg/L)			TDZ (mg/L)		
		0.5	1.0	2.0	0.1	0.2	0.5
NAA (mg/L)	0.1	*64	40	78	34	45	31
	0.5	71	10	0	57	60	0
	1.0	0	0	0	0	0	0
IAA (mg/L)	0.1	54	78	65	96	100	0
	0.5	100	46	83	90	33	78
	1.0	100	48	35	100	89	0

* More than 100 explants were examined for each treatment
* Total number of regenerated shoots / Total number of explants × 100

Table 2. Percent rates of plant regeneration from petiole explants of 'Cesare' (*Cichorium intybus* var. *sativus*) grown on a medium containing various concentrations of plant growth regulators.

		Regeneration rates (%)					
		BAP (mg/L)			TDZ (mg/L)		
		0.5	1.0	2.0	0.1	0.2	0.5
NAA (mg/L)	0.1	*50	33	55	24	33	20
	0.5	17	0	15	33	33	0
	1.0	0	0	0	0	0	0
IAA (mg/L)	0.1	33	65	43	90	100	10
	0.5	100	31	91	90	24	60
	1.0	100	15	22	100	75	0

* More than 100 explants were examined for each treatment
* Total number of regenerated shoots / Total number of explants × 100

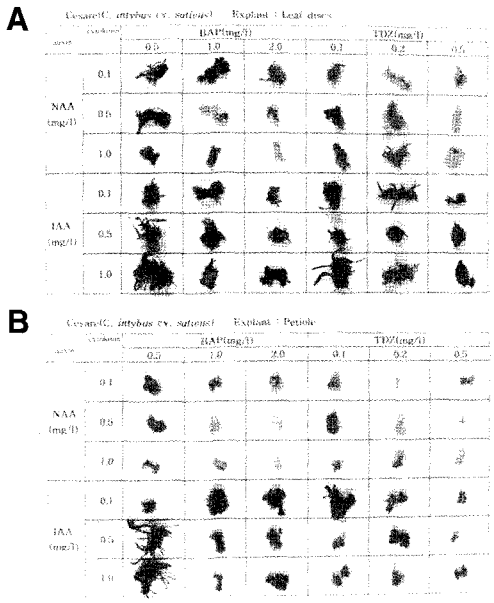

Figure 1. Effect of various plant growth regulators on the differentiation of leaf (A) and petiole (B) explants of 'Cesare' root type chicory. Photos were taken after 3 weeks of explant culture.

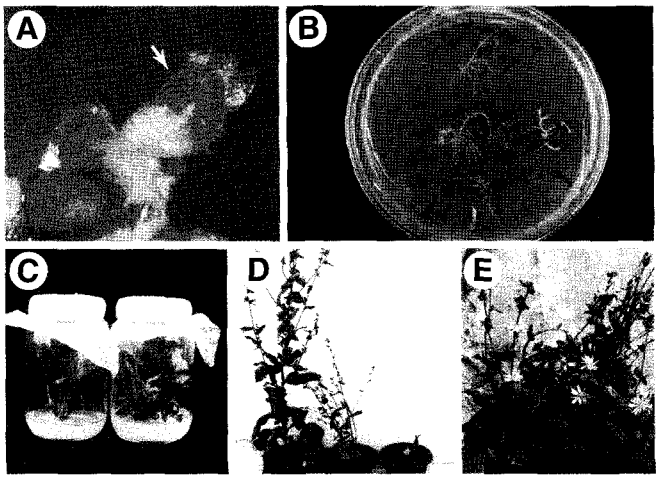

Figure 2. Plant regeneration scheme and phenotypes of transgenic chicory plants expressing the *OsMADS1* gene, A; Shoot induction from embryogenic callus, B; Plantlets with development of roots, C; Regenerated plantlets in a vessel containing microspore filter on the cap, D; Comparisons of blooming control (left) and transgenic plant at T0 (middle) and T1 (right) generations, E; Group of transgenic plants showing branchy and bushy phenotypes.

Molecular analysis of transgenic plants

To confirm that the *OsMADS1* gene was integrated in the root type chicory, all putative transgenic plants was tested by PCR analysis using NPT Ⅱ primers, and by southern blot analysis. Transgenic plants showed positive band in PCR analysis (Figure 3A) and the fragment of transgenic genomic DNA was hybridized with the NPT Ⅱ probe which was prepared from the PCR fragment (Figure 3B). Northern blot analysis showed that the *OsMADS1* transcript was present in the leaves of two randomly selected transgenic plants that had displayed an early-flowering phenotype (Figure 3C).

Ectopic expression of *OsMADS1*

Most of the transgenic chicory plants showed branchy and bushy phenotypes, and flowered at least 30 days earlier than the control plants (Figure 2D, E). Transgenic plants also had more lateral branches (Table 3). Some T1 plants exhibited extreme dwarf and early-flowering phenotypes (Figure 2D, E and Figure 4A). Such phenotypes were similar to those found with transgenic tobacco and rice plants that had ectopically expressed the *OsMADS1* gene (Chung et al. 1994; Jeon et al. 2000).

Transgenic chicory over-expressing the *OsMADS1* gene showed additional phenotypes, such as sterility and alteration of the leaf margin (Figure 4B). T1 progenies were obtained from ten lines of a total of 14 T0 plants. Four lines failed to develop fertile seeds. These results indicate that the *OsMADS1* gene might induce sterility in root chicory, although alterations in reproductive organs, e.g., sepals, petals, stamens, and carpels, were not obvious in the transgenics. However, the leaf margins on transgenic plants were remarkably changed from entire to incised shapes (Figure 4B). Early bolting is a major physiological disorder in the breeding of chicory, induced by high temperatures during root development. The anti-sense suppression of floral-promoting genes, such as the *OsMADS1* homologous, may delay bolting in root chicories.

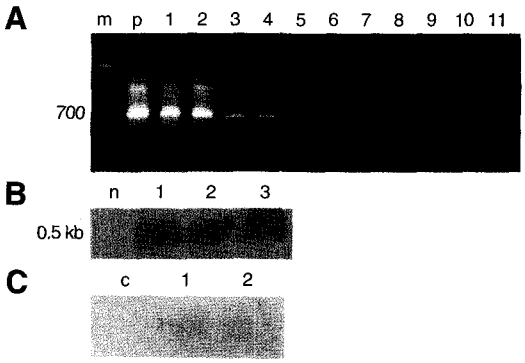

Figure 3. Molecular analysis of transgenic plants, A; PCR analysis of the NPT Ⅱ gene using NPT Ⅱ primers. Lane M: DNA size markers; Lane P: A positive control from the vector pGA1209 containing the NPT Ⅱ gene and *OsMADS1* gene; Lane 1 to 11: Putative transgenic plants. B; PCR genomic southern hybridization analysis using a NPTII probe. Lane N: A negative control plant; Lane 1 to 3: Transgenic plants. C; Northern blot analysis of the OsMADS1 transcript in young leaves of transgenic plants. Lane N: A control plant; Lanes 1 and 2, transgenic plants at T1 generation.

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Table 3. Comparison of phenotypes between transgenic and non-transgenic plants.

Plants	No. of Plants	Days to flowering[c]	Plant Height (cm)	No. of branches
T0[a]	14	90±10	60±10	6-9
T1[b]	52	70±20	40±10	5-7
Control	3	130±10	120±10	1

[a] Primary transgenic plant
[b] Next generation of T0
[c] From acclimation to first anthesis for T0 plants, and from seed germination to first anthesis for T1 progenies.

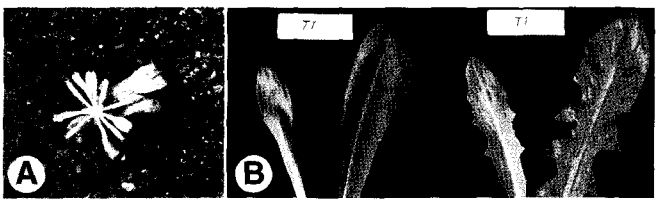

Figure 4. Morphological characteristics of T1 plants. (A) Inflorescence obtained within 40 days of sowing. (B) Change in leaf margins from entire (left) to incised shape (righ)

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