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Establishment of Genetic Transformation System and Introduction of MADS Box Gene in Hot Pepper (*Capsicum annuum* L.)

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Abstract

***In vitro* plant regeneration of inbred breeding line of hot pepper (*Capsicum annuum* L.) was established using leaf and petiole segments as explants. About 28 days old plants were excised and cultured on MS medium supplemented with TDZ and NAA or in combination with Zeatin. In all of the media compositions tested, combination of TDZ 0.5 mg/L, Zeatin 0.5 mg/L, and NAA 0.1 mg/L was found to be the best medium for shoot bud initiation. Young petiole was the most appropriate explant type for the plant regeneration as well as genetic transformation in hot pepper. In this study, HpMADS1 gene isolated from hot pepper was introduced using *Agrobacterium*-mediated transformation system. Based on the analysis of Southern blot and RT-PCR, HpMADS1 gene was integrated in the hot pepper genome. It has been known that floral organ development is controlled by a group of regulatory factors containing the MADS domain. Morphological characteristics in these transgenic plants, especially flowering habit, however, were not significantly altered, indicating this MADS gene, HpMADS1 may be non-functional in this case.**

Introduction

Hot pepper (*Capsicum annuum* L.) is one of the most important vegetable crops around the world. It has been used in various food forms such as salads, sauce, and pickles. Hot pepper has been known to be a difficult crop to be regenerated under *in vitro* condition, although there were several reports on plant regenerations via somatic embryogenesis (Binzel et al., 1996), callus (Kelkar, 1996), hypocotyl and cotyledon (Attila et al., 1995; Marla and Sankhla, 1996; Rafael and Neftali, 1996), and seedling explants (Hiroshi et al., 1993; Young et al., 1997). In the genetic transformation of hot pepper using the established regeneration conditions, most of the transformed plants were F1 hybrids (Arroyo and Revilla, 1991; Kim et al., 1997; Lim et al., 1999; Liu and Parrot, 1990; Manoharan and Lakshmi, 1998) and with the parts of hypocotyl or cotyledon as materials (Virag et al., 2000). However, the inbred lines should be used as the transformation materials for further breeding system to obtain useful genes from the genetic transformation based on *Agrobacterium* in hot pepper.

Recently numerous researches have been made towards elucidating the underlying mechanisms controlling flower development in the dicot species by finding the loci such as AG (Yanofsky et al., 1990), AP1 (Mandel et al., 1992), AP3 (Jack et al., 1992) and MADS (Chung and An, 1994; Sung et al., 2000). In dicotyledonous flowering plants, MADS box genes act as homeotic selector genes that determine floral organ identity genes (Schwarz et al., 1990). Transgenic approaches including anti-sense method were undertaken to study the functional roles of the MADS box genes in plant species. Several MADS box

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genes, including hot pepper (Yu, 2001), were characterized and expressed in many plants based on genetic transformation system (Jia et al., 2000)

The objectives of this study were to establish the high efficient plant regeneration system of inbred lines in hot pepper using various explant types and plant growth regulators and to report the integration of pepper MADS box homologue and the confirmation of their expression in hot pepper.

Materials and Methods

Plant materials

Seeds of inbred lines of hot pepper were obtained from Choong Ang Seed Co., LTD (ChunAnn, Korea). Seeds were surface-sterilized with 70% ethanol for 5 min, soaked in a commercial bleach solution (100% v/v+Tween 20) for 10 min, and then washed with sterile water three times. The seeds were transferred aseptically to filter paper in petri dishes containing sterile water for the germination at 28℃. After 10-15 days, seedlings were transferred to half strength MS basal medium (Murashinge and Skoog, 1962) containing 1% sucrose and 0.8% agar. They were grown in vitro under a 16/8 day/night photoperiod at 26±1℃.

Bacterial strain and vector

The *Escherichia coli* strain JM109 was used as a host for general transformation and amplification of the plasmids. Binary plasmid λZAPII was constructed by the ligation of HpMADS1 into the *Eco*RI-*Xho*I site. The pMBPI carried the binary vector containing the T-DNA region of the HpMADS1 with CaMV 35s promoter, NOS terminator and neomycin phosphotransferase II (*NPT*II) (Figure 1). The bacteria were grown in YEP medium supplemented with 25 mg/L kanamycin at 28℃ with rotary agitation for 24 hr, and then they were spun down by centrifugation (4000×g, 1 min) and resuspended in pre-culture liquid medium for co-culturing.

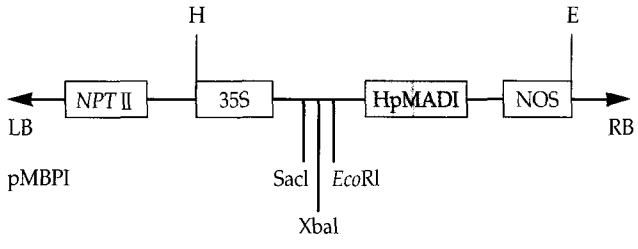

Figure 1. Schematic representation of the T-DNA region of the binary plant transformation vector pMBPI. RB: right border, LB: left border, 35S: CaMV 35S Promoter, NOS: nos terninator, H: *Hind* III restriction site, E: *Eco*RI restriction site.

Plant regeneration and transformation

Young leaf and petiole explants were excised from 4 weeks old plants, cut into 1 cm long, wounded slightly, and then placed on the regeneration medium supplemented with different concentrations of plant growth regulators such as TDZ (0.1, 0.2, 0.4, 0.6, 0.8 and 1mg/L), zeatin (0.5, 1.0 and 2.0 mg/L), and NAA (0.1, 0.5 and 1.0 mg/L). The pH of each medium was adjusted to 5.8 prior to autoclaving at 121℃ for 15 min. Plant growth regulators were added to the medium after autoclaving. *In vitro* explants were transferred to fresh media every three week. After the shoots were elongated, they were rooted on MS medium containing 0.1 mg/L NAA. When plantlets were 10 cm in height, they were transferred to pots in the green house.

Kanamycin was used as a selectable marker, and resistance levels to antibiotics were tested. The concentrations of kanamycin tested were 0, 25, 50, 75, 100, and 125 mg/L. For plant genetic transformation, explants were co-cultured with *Agrobacterium tumefacies* strain GV3101 (PMP90) harboring MADS gene (HpMADS1) and *NPT*II on the co-culture medium for 2 days. Discs were blotted on sterile filter paper and then transferred to the selection medium. After 3 weeks, shoot buds were sub-cultured.

Southern hybridization and RT-PCR analysis

Genomic DNA was isolated from young leaves, and total DNA of each sample was digested with *Hind*III, separated on a 1.2% agarose gel, and transferred to the membrane. Detection for the integration of foreign DNA was done with CDP-star system using *NPT*II gene as a probe. RT-PCR used to detect the presence of the MADS-mRNA in the putatively transgenic hot peppers, was performed using the MADS-specific primer pair (5′-GTGTGCTTTCAAAACAATTTGCA-3′ and 5′-AATACC ACG CTTTTATGAGCG-3′).

Reverse transcription was carried out at 42℃ for 1 hr in 20-100 μL vol., containing 40 unit RNase inhibitor and 2.5 μ/μL of expand reverse transcriptase. The 1 μL sample of the reaction was used as a template in the subsequent PCR amplification. PCR amplification reactions consisted of one cycle of 5 min at 94℃, followed by 40 cycles (1 min at 94℃, 30 sec at 54℃, and 1 min at 74℃), and finally by one extension cycle of 10 min at 72℃. PCR products were separated on a 0.8% agarose gel.

Results and Discussion

The effect of plant growth regulators on shoot bud initiation

Direct or indirect shoot buds were induced at the wounding site of the injured explants, and then they were differentiated into plants later on (Figure 2A and B). Regenerated buds were first observed on the leaf and petiole explants in the medium containing TDZ 1.0 mg/L with the highest level of shoot regeneration rate in this experiment (Table 1). But, the higher the level of TDZ had, the more the abnormal plants observed in the regenerated hot peppers (data not shown). The best composition of plant growth regulators for the shoot regeneration was TDZ 0.5 mg/L, zeatin 0.5 mg/L, and NAA 0.1 mg/L, and regenerationrates were 46.6% and 17.7% in petiole and leaf discs, respectively (Table 2). Also very similar result was obtained in the medium supplemented with zeatin 1.0 mg/L, TDZ 0.5 mg/L, and NAA 0.1 mg/L (Table 3). In the all hot pepper regenerated media, petiole segments of hot pepper always showed the higher regeneration rates than those of leaf discs (Table 1, 2, and 3).

Recently, new cytokinis like thidiazuron (TDZ) have been used for plant regeneration (Bretagne et al., 1994; Magioli et al., 1998), and it has been known to be more effective than BA for shoot regeneration (Tosca et al., 1996). When TDZ was added to the medium, subculture must be carried out more frequently because of the inhibitory effect of TDZ on plantlet morphology. The concentration of plant

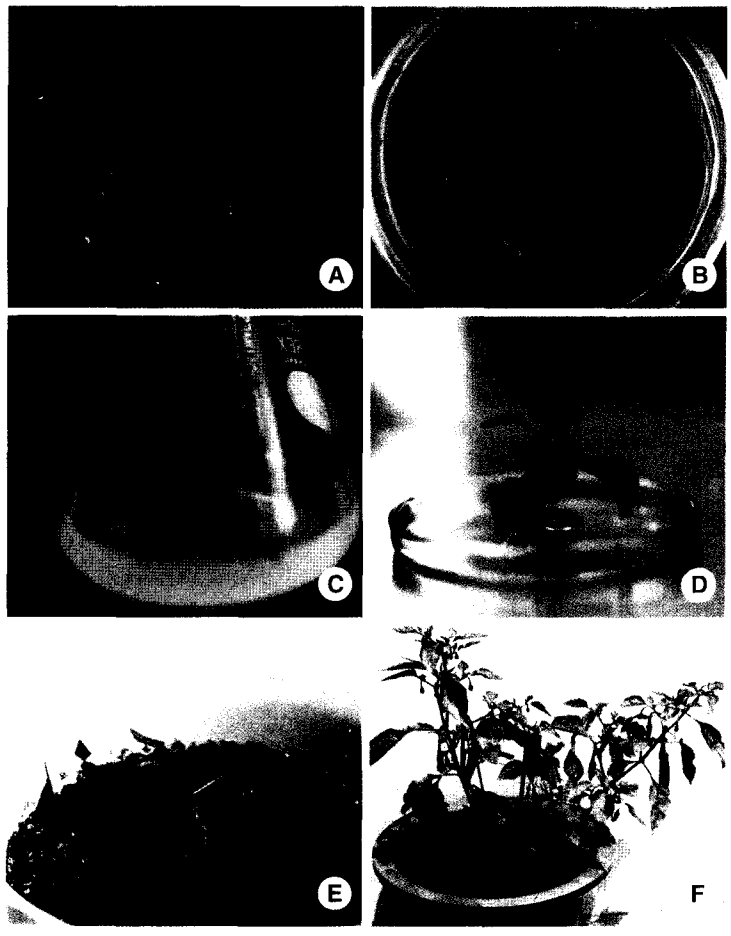

Figure 2. Production scheme of transgenic hot pepper plant, A; Callus formed on young petiole explant of inbred line of hot pepper, B; Compact, multiple shoots induced around the explants, C; Shoots transferred to rooting medium, D; Roots differentiated on regenerated shoot, E; Regenerated plantlet transferred to the pot containing horticulture soil mixture, F; Putatively transgenic plant, transformed with HpMADS1 gene, growing in the pot.

Table 1. Shoot buds differentiation from leaf and petiole explants of inbred Line in hot pepper as influenced by various levels of TDZ.

TDZ (mg/L)		0.01	0.1	0.2	0.4	0.6	0.8	1.0
Regeneration (%)	Leaf[b]	0	7.4c	8.1c	14.1b	16.3b	18.4b	43.6a
	Petioles	0	10.0d	15.0d	33.3c	63.3ab	60.0ab	76.7a[a]

[a]Mean separation with rows by Duncan's multiple range test, 5% level.
[b]Experiments were repeated three times, each time with 30 explants.

Table 2. The effect of zeatin and TDZ combination on the shoot regeneration frequency of inbred hot pepper.

Growth regulators (mg/L)	Zeatin		0.5			1.0			2.0	
	TDZ	0.1	0.3	0.5	0.1	0.3	0.5	0.1	0.3	0.5
Explant type[b]	Leaf	6.7c[a]	13.3b	17.7b	13.3b	26.7a	20.0ab	6.7c	20.0ab	-
Regeneration (%)	Petiole	-	-	46.6a	-	26.7b	46.7a	-	13.2c	6.7c

[a]Mean separation with rows by Duncan's multiple range test, 5% level.
[b]Experiments were repeated three times, each time with 30 explants. NAA (0.1 mg/L) was added to all of the media.

Table 3. The effect of TDZ with the combination of zeatin and NAA on shoot regeneration of hot pepper.

Plant growth regulators (mg/L)			Frequency (%)	
Zeatin	NAA	TDZ	Leaf	Petiole
1.0	0.1	0	-[b]	-
		0.1	13.3b[a]	-
		0.3	26.7a	26.7c
		0.5	20.0ab	46.7a
1.0	0.5	0	-	-
		0.1	6.7c	-
		0.3	6.7c	-
		0.5	6.7c	-
1.0	1.0	0	-	-
		0.1	-	-
		0.3	-	-
		0.5	6.7c	6.7c

[a]Mean separation with columns by Duncan's multiple range test, 5% level.
[b]Experiments were repeated three times, each time with 30 explants.

growth regulators should be reduced in half or more in subsequent subcultures (Huetteman and Preece, 1993). Therefore, in this experiment, induced shoots in 1-1.5 cm of height were excised from their mother explant and transferred to shoot elongation medium, consisting of MS basal salts, zeatin 1 mg/L, NAA 1.0 mg/L, GA_3 0.2 mg/L, cefotaxime 250 mg/L, kanamycin 100 mg/L, 3% sucrose, and 0.8% agar to reduce the inhibitory effects of TDZ (Figure 2 C, D, E, and F).

The results of this study showed that the use of TDZ could reduce the concentrations and numbers of plant growth regulators in the regeneration system, compared with other researches (Carl and Leung, 1996; Lim et al., 1999). Previous researches reported that combinations of BA 5 mg/L or BA 5 mg/L and PAA 2 mg/L had the best regeneration rate in pepper (Carl and Leung, 1996) and the combinations of zeatin 2 mg/L and NAA 0.1 mg/L or BAP 1 mg/L and IBA 10 mg/L were the best in the regeneration of hot pepper (Lim et al., 1999). However, in this experiment, applied TDZ reduced the concentration of zeatin to half (1 mg/L) and replaced low concentration of NAA (0.1 mg/L) with relatively high concentration of IAA (1 mg/L) to increase the regeneration rates of hot pepper.

The effect of the kanamycin levels and explant types on shoot regeneration of putatively transgenic plants

Explants of leaf and petiole of hot pepper were incubated on regeneration media containing different levels of kanamycin (0, 25, 50, 75, 100, and 125 mg/L) to determine the kanamycin concentration of selection medium. Regeneration frequency of hot pepper cultured in media containing 75 mg/L kanamycin was 16% in leaf and 21% in petiole, respectively (Table 4). No shoot regeneration took place in media containing higher than 100 mg/L of kanamycin (Table 4). Thus, 100 mg/L of kanamycin, which was two times more than that used in other research (Manoharan and Lakshmi, 1998), was used in hot pepper transformation for the cut-off point of effective selection.

The explant types had a great effect on the plant regeneration of hot pepper (Neftail et al., 1990 and Zhu et al., 1996). The experiment was carried out using hypocotyl, cotyledon, petiole, leaf, and young petiole, and the regeneration ability of putatively transgenic pepper was tested. It was shown that petiole was better than leaf (Table 1, 2, and 3) and young petiole was the best material to be used among all the tissue types, and high shoot regeneration rate (33.9%) was achieved (Table 5).

Analysis of putative transgenic plants

Southern hybridization

In order to detect the presence of HpMADS1 gene in putatively transgenic pepper plants, DNAs of 24 samples were isolated and digested with *Hin*dIII. Hybridization with the ^{32}P-labeled *NPTII* probe confirmed the presence of the 1.2 kb fragment in one of four putative transgenic plants. About 25% of the putatively transgenic plants were turned out to be transgenics based on Southern blot hybridization. It must be kept in mind that very careful

Table 4. Differences in the resistance level of hot pepper inbred line to kanamycin concentrations.

		Kanamycin (mg/L)					
		0	25	50	75	100	125
Survival	Leaf[a]	100/100	96/100	79/100	21/100	0/100	0/100
	Petiole	100/100	98/100	88/100	16/100	0/100	0/100

[a]Explant numbers for each experiment were 150.

Table 5. The effect of explant types on the rate of putative transgenic plants.

Explant types[c]	Shoot frequency (%)
Hypocotyl	21.2b[ab]
Cotyledon	1.3c
Young petiole	33.9a
Leaf	2.5c
Petiole	5.2c

[a]Mean separation with columns by Dunkan's multiple range test, 5% level.
[b]The medium composition: MS basal salts, zeatin 1 mg/L, TDZ 0.5 mg/L, NAA 1.0 mg/L, kanamycin 100 mg/L, 3% sucrose, and 0.8% agar.
[c]Explants used in the genetic transformation experiment were 150 for each explant type.

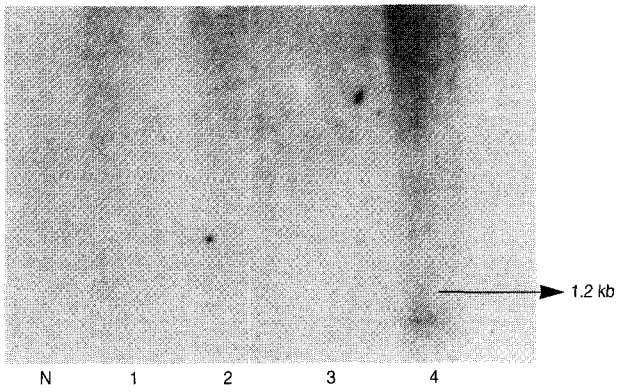

Figure 3. Southern hybridization analysis of genomic DNA derived from putative transgenic plants (HpMADS1). Total DNAs were digested with *Hin*dIII, and *NPT*III gene was used as a probe.

selection for transgenic plants during transformation procedure has to be done. No hybridization signal was observed in genomic DNA from non-transformed plants. Thus, genomic DNA blot hybridization data confirmed the introduction of *NPT*II gene in the genome of transgenic hot peppers (Figure 3).

RT-PCR analysis

In order to detect the expression of transgene in kanamycin resistant plants, RT-PCR amplification was performed with specific primers to detect sense or anti-sense transcripts. HpMADS1 transgenic plants (lane 4 and 5) showed that transgene-specific bands (Figure 4). The primers used in this experiment were designed to amplify the specific region of HpMADS1, so that other MADS genes were not detected (data not shown). HpMADS1 was cloned from hot pepper, but, overexpressed genes under

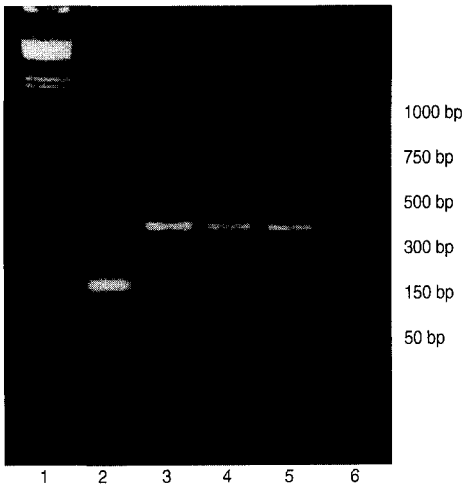

Figure 4. RT-PCR analysis for leaf tissue of transgenic pepper transformed with HpMADS1 gene. Land 1: *Hin*dIII marker; Lanes 2 and 3: negative and positive control of HpMADS1, respectively, Lanes 4 and 5: putatively transgenic plants, Lane 6: marker.

35S promoter could be detected in young leaf tissues. So, transcription of the MADS-products of this gene was demonstrated in selected transgenic lines (Figure 4).

As showed in Figure 3 and 4, integration and expression of HpMADS1 were demonstrated. But distinct morphological change was not detected in the flowers of transgenic pepper plants (data not shown). This result was corresponded with previous study (Yu, 2001) of HpMADS1 transformation into tobacco. HpMADS1 was postulated as PI group B function genes from sequence similarity. In most cases, ectopic expression of B function results in structural changes in second first and fourth whorl organ of flower except FBP1 and FBP3 of *Antirrhinum* (Schwarz-Sommer, 1990). In hot pepper, it seems that B function of floral organ identity is controlled by at least 2 PI genes with pepper DEF gene, and they interact with more complex manner (Yu, 2001).

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