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## A new selection method for pepper transformation: callus-mediated shoot formation

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**Abstract** We used two genes, *TMV-CP* and *PPII* (pepper-PMMV interaction 1 transcription factor), to transform commercially important chili pepper (*Capsicum annuum*) inbred lines (P915, P409) by means of *Agrobacterium* co-culture. Eighteen independently transformed T<sub>0</sub> plants were obtained. The most critical point in the pepper transformation protocol was the selection of shoots growing on calli—referred to as callus-mediated shoot formation (indirect shooting)—because shoots not grown from the callus (direct shooting from the wounded surface) developed into non-transformants. Selection of the correct right callus type also proved to be an important requirement for obtaining transformed peppers. Six different types of callus developed during the selection process. Shoots regenerated from two of these types, while one type regenerated significantly more shoots than the other types, suggesting that the capacity for shoot formation is callus type-specific. Although the transformation rate was low, transformation via callus-mediated shoot formation proved to be reproducible and was confirmed by Southern and Northern blot analyses. Based on the experimental data, we have succeeded in developing a

new protocol for the selection and transformation of pepper and expect that it will be used in the future for pepper transformation.

**Keywords** Pepper transformation · Callus-mediated shoot formation · *TMV-CP* · *PPII* · Resistance

**Abbreviations** *AS*: Acetosyringone · *CGMMV*: Cucumber green mottle mosaic virus · *CMV*: Cucumber mosaic virus · *CP*: Coat protein · *IAA*: Indole-3-acetic acid · *NAA*:  $\alpha$ -Naphthaleneacetic acid · *PPII*: Pepper-PMMV interaction 1 transcription factor gene · *TMV*: Tobacco mosaic virus · *ToMV*: Tomato mosaic virus

### Introduction

Chili pepper (*Capsicum annuum*) is one of world's staple vegetables. Variable genetic sources have been developed, and classical breeding programs for pepper cultivation have been well established. However, approaches aimed at the genetic engineering of pepper have been protracted due to the difficulty in transforming pepper by *Agrobacterium*. While pepper regeneration itself is not an obstacle and is performed routinely (Valera-Montero and Ochoa-Alejo 1992; Ebida and Hu-C 1993; Harini and Sita 1993; Lee et al. 1993), the high level of effort invested into transferring genes into chili pepper explants has not yet yielded a successful and reproducible transformation method. Consequently, relatively few studies have been undertaken to explore methodology (Lee et al. 1993; Kim et al. 1997; Manoharan et al. 1998; S.H. Kim et al. 2001; Cai et al. 2002; Li et al. 2003). Cotyledons or hypocotyls have been the most common source of explants, and the composition of the selection media and culture conditions are similar. Cai et al. (2002) showed the successful transformation of *CP* genes from *CMV* and *TMV* in the same vector and tested resistance against the virus with T<sub>3</sub> progeny. The most successful group in the areas of chili pepper transformation, led by K.H. Paek, has transferred several different genes over the past 10 years into pepper

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plants (CMV satellite RNA: Lee et al. 1993; Kim et al. 1997; CP of CMV and ToMV: Shin et al. 2002a; Tsi 1: Shin et al. 2002b). Although transgenic plants have been tested for functional phenotypes, transformation efficiencies have been very low and the transformation itself was neither consistent nor repeatable (K.H. Paek, personal communication). In addition, successful transformation studies have been reported in several short meeting proceedings (Dong et al. 1995; Szasz et al. 1997; Arous et al. 2001; Y.H. Kim et al. 2001), but no follow-up papers were ever published.

Taken together, the procedures described in the literature for chili pepper transformation are not very helpful in terms of achieving routine transformation. To the contrary, successful cases are regarded as rare events. Two major factors clearly inhibit efficient transformation of chili pepper: (1) the shoot regeneration rate of peppers is genotype-dependent (Christopher and Rajam 1996; Kim et al. 2002) and genotype specificity affects the transformation rate; (2) the very low efficiency of transformation indicates that gene transfer via *Agrobacterium* infection into cut or injured cotyledon and hypocotyl tissues hardly ever occurs for reasons that are not well understood.

We report here a protocol useful for pepper transformation with a suitable selection method. As the transformed pepper shoots can be obtained from callus-mediated shoots, a strong selection pressure should be exerted so that shoots or multi-shoots growing directly from wounded tissue are eliminated during the selection process.

## Materials and methods

### Plant materials

Seeds from commercially important pepper (*Capsicum annuum*) inbred lines (P915, P409, P410, and P101 from the Nong Woo Bio Co, Yeosu, South Korea) were used. Of the 30 inbred lines tested by Kim et al. (2002), these four lines showed a very high rate of regeneration. The seeds were surface-disinfected in 95% EtOH for 30 s and in 50% bleach (Yuhanox) for 10 min and then rinsed three times with sterilized water. The sterilized seeds were then placed in half-strength MS medium (Murashige and Skoog 1962) and allowed to germinate in under light or dark conditions at 25°C. The cotyledons and hypocotyls from 8- to 10-day-old plants were excised and used as explants.

### Pre-culture and inoculation with *Agrobacterium*

Explants were transferred to a pre-culture medium consisting of MS medium supplemented with 2 mg/l zeatin and 0.05 mg/l NAA or 0.1 mg/l IAA and placed in a growth chamber under light conditions at 25°C for 2–36 h. For transformation, *Agrobacterium* strain EHA105 or LBA4404 and pCAMBIA 2300 vectors harboring coding regions for the *TMV-CP* gene (L35074; Park et al. 1997) and the *PPII* gene (AF430372; Lee et al. 2002) were used. *Agrobacterium* was grown in YEP media supplemented with 50 mg/l kanamycin, 50 mg/l rifampicin and 100  $\mu$ M AS. The *Agrobacterium* culture was centrifuged and then diluted with MS to an optical density (O.D.) of 0.3–0.5. This bacterial suspension was then mixed with MS liquid containing 100  $\mu$ M AS and inoculated into the

explants for 10–20 min, co-cultured in the dark for 38–96 h and then washed three times with either 500–800 mg/l cefotaxime or 500–800 mg/l lilacilline.

### Shoot formation and regeneration

Explants were transferred to a regeneration medium consisting of a modified MS medium supplemented with 2 mg/l zeatin and 0.05 mg/l NAA or 0.1 mg/l IAA. The hormone compositions and antibiotic concentrations used to select transgenic shoots are described in Table 5. The shoot formation rate was measured by comparing the number of shoots transferred to elongation medium to the total number of explants. Explants were incubated on selection medium (2.0 mg/l zeatin + 0.05 mg/l NAA or 0.1 mg/l IAA) for 6–8 weeks, on elongation medium (2.0 mg/l zeatin + 0.01 mg/l NAA or 0.01 mg/l IAA) for 7–10 weeks, and on rooting medium (hormone-free) for 6–8 weeks. The regenerated plants were acclimated for 2 weeks in zippy pot soil at 25°C under a 16/8-h (light/dark) photoperiod.

### PCR, Southern, and Northern blot analysis

Pepper genomic DNA was isolated as previously described (Lee et al. 2002). The primer sequences used in the PCR analyses for detecting the *TMV-CP* gene insertion were 5'-ATGACGCA-C AATCCCCTAT-3' (sense: 35S promoter region at 3,185–3,204 bp of accession no.X84105) and 5'-CGAACCCCTGAA-AATAAT-3' (antisense: *TMV-CP* gene at 648–631 bp of accession no. L35074); for detecting the *PPII* gene insertion the primer sequences were 5'-ATGACGCACAATCCCCTAT-3' (sense: 35S promoter region) and 5'-GTACCACTTGAAGAAGC-3' (antisense: *PPII* gene at 587–570 bp of accession no. AF430372). PCR analysis were carried out using 0.65  $\mu$ M primers, 299  $\mu$ M dNTP, 1 U/ $\mu$ M *Taq* DNA polymerase (New England Biolabs, Beverly, Mass.) in 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, and 10 mM Tris-HCl, pH 8.3. The PCR program consisted of 35 cycles of 94°C, 55°C, and 72°C, each for 1 min. For Southern blot analysis, DNA samples from transgenic peppers (T<sub>0</sub>) were isolated and 30  $\mu$ g DNA was digested to completion with restriction enzymes. For Northern blot analysis, total RNA was extracted from pepper leaves (Choi et al. 1996) and 30  $\mu$ g RNA was used.

Southern and Northern blots were performed as previously described (Church and Gilbert 1984) using Hybond N membranes (Amersham Biosciences, Piscataway, N.J.) and hybridized to random-primed probes following the manufacturer's instructions (Amersham Biosciences). The probes labeled with [<sup>32</sup>P]-dCTP were a 678-bp fragment of the *TMV-CP* gene and a 1,211-bp fragment of the *PPII* gene.

### Resistance test for TMV

A total of 408 T<sub>1</sub> pepper seedlings (four-leaf stage) segregated from two different T<sub>0</sub> plants were initially exposed to TMV by carborundum and then re-exposed to TMV 2 weeks later. The TMV strain (isolated from Korean peppers) was provided by the KT&G Central Research Institute (Daejeon, Korea) (Park et al. 1997). A leaf disk from each T<sub>1</sub> plant was taken, and ELISA was performed using the indirect ELISA test as described by Shin et al. (2002b). A reading was done at an absorbance of 405 nm using an ELISA Thermo Max Microplate Reader (Molecular Devices, Sunnyvalley, Calif.).

## Results

### Direct shoot versus indirect shoot formation

In order to develop a protocol of general use for pepper transformation, we used about 190,000 explants from four different inbred lines and carefully studied the shoot formation process. Two general patterns of pepper shoot formation were identified. First, a shoot or multi-shoots formed directly from the wound or cut region of the explants (direct regeneration) (Fig. 1). This pattern was observed in many cases, and we expected to find transformed shoots among these grown shoots. Five stages of shoot development were observed: direct shoot formation, multi-shoot, elongation, single-shoot elongation, and root formation. In general, 19–26 weeks of shoot development after co-culture was required before that acclimation treatment. Second, shoots were selected from callus tissues that had formed around the cut after 4–5 weeks of culture on the shoot selection medium (indirect regeneration) (Fig. 2). These cases are unusual because calli are not easily formed from the wounded region of cotyledons. Five stages of shoot development were determined: callus formation, callus development, shoot formation, single-shoot elongation and root formation.

### Callus types for shoot formation

We identified six different types of callus that developed from the explants. Shoot regeneration capability was callus-type-dependent. Those able to form shoots were designated type A (white, hard surface and green tissue inside) and type B (dark-green callus with hard surface, but a little bit moist) (Fig. 3). The regeneration rate of type A was about 90% higher than that of type B (data not

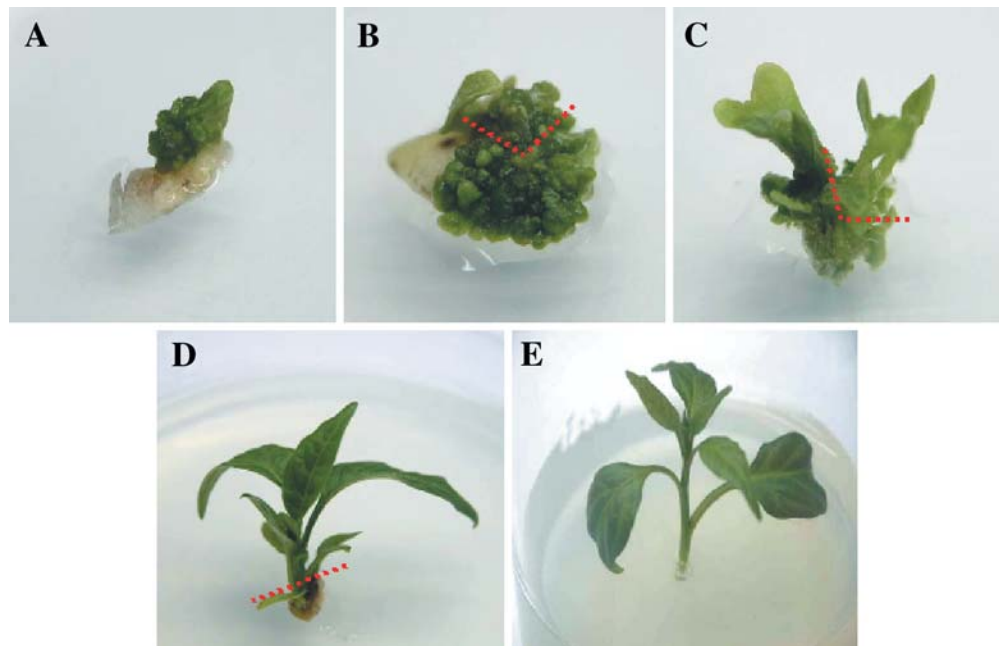
shown). The callus types that were not able to produce shoots were designated types C (yellow and brittle), D (yellow and hard surface), E (moist and a little transparent), and F (green, moist, and brittle). All six types appeared in the four pepper lines.

### Frequencies of direct shoot and indirect (callus-mediated) shoot formation following co-culture

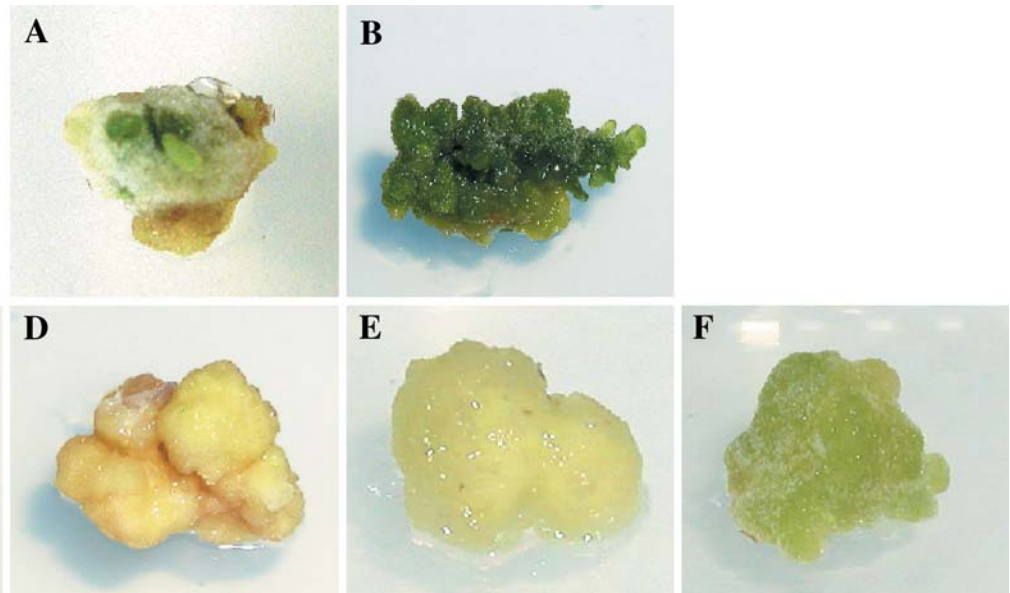
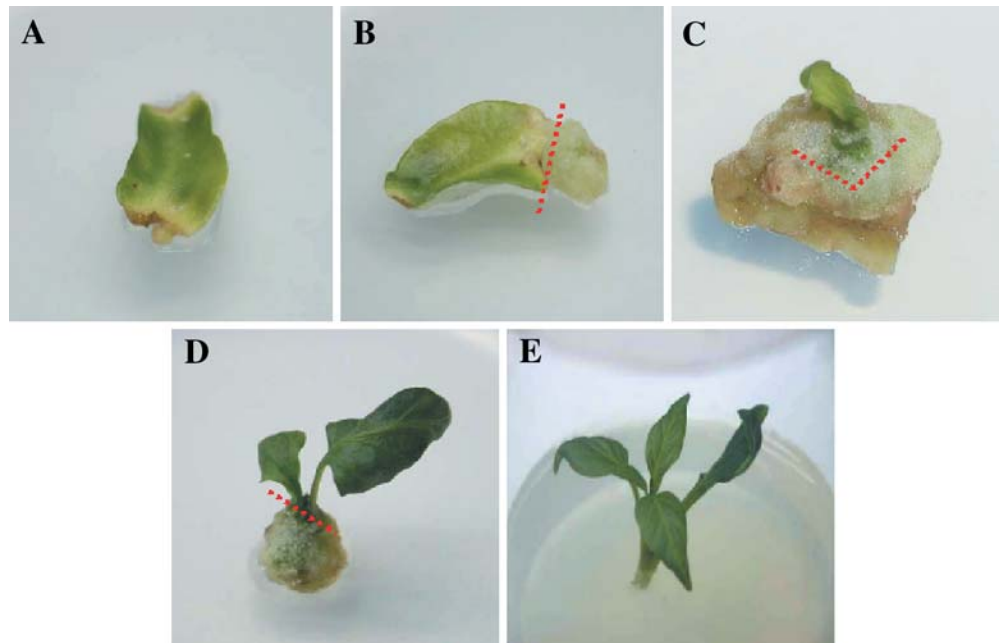
In order to determine the direct shoot formation rate after transformation, 151,700 explants from the four different lines were transformed with the *TMV-CP* and *PPII* genes. The rate of developing a direct shoot in the shooting medium after co-culture was 5.3% (8,089/151,700) (Table 1). The number of shoots surviving in the rooting medium decreased by a factor of 6 (0.93%: 1,407/151,700). Among the four lines, P915 line showed the highest rate of shoot development.

To find the indirect shoot formation rate, we transformed 37,500 explants from four different lines with the *TMV-CP* and *PPII* genes. The frequency of callus generation from the explants was 1.2% (459/37,500) (Fig. 4, Table 2). However, not all calli produced a shoot. The frequency of shoot development from callus was 11.6% (53/459). The frequency of root formation from the callus-mediated shoots was 52.8% (28/53). Of the four lines, P915 produced 68% (19/28) of the total shoots with root development in the rooting medium, suggesting a genotype preference of pepper regeneration after transformation treatment.

**Fig. 1a–e** Development of direct shoot formation following co-culture. **a** Shoot formation (5 weeks on selection medium), **b** multi-shoot formation (7 weeks old), **c** multi-shoot elongation (9 weeks old), **d** single-shoot elongation from a multi-shoot (11 weeks old), **e** root formation (14 weeks old). The red dotted line indicates placement of the cut for removing the single shoot to the next culture



**Fig. 2a–e** Development of indirect shoot formation following co-culture. **a** Callus formation (5 weeks on selection medium), **b** callus development (7 weeks old), **c** shoot formation (9 weeks old), **d** shoot elongation (11 weeks old), **e** root formation (14 weeks old). The *red dotted line* indicates placement of the cut for removing the tissue to the next culture



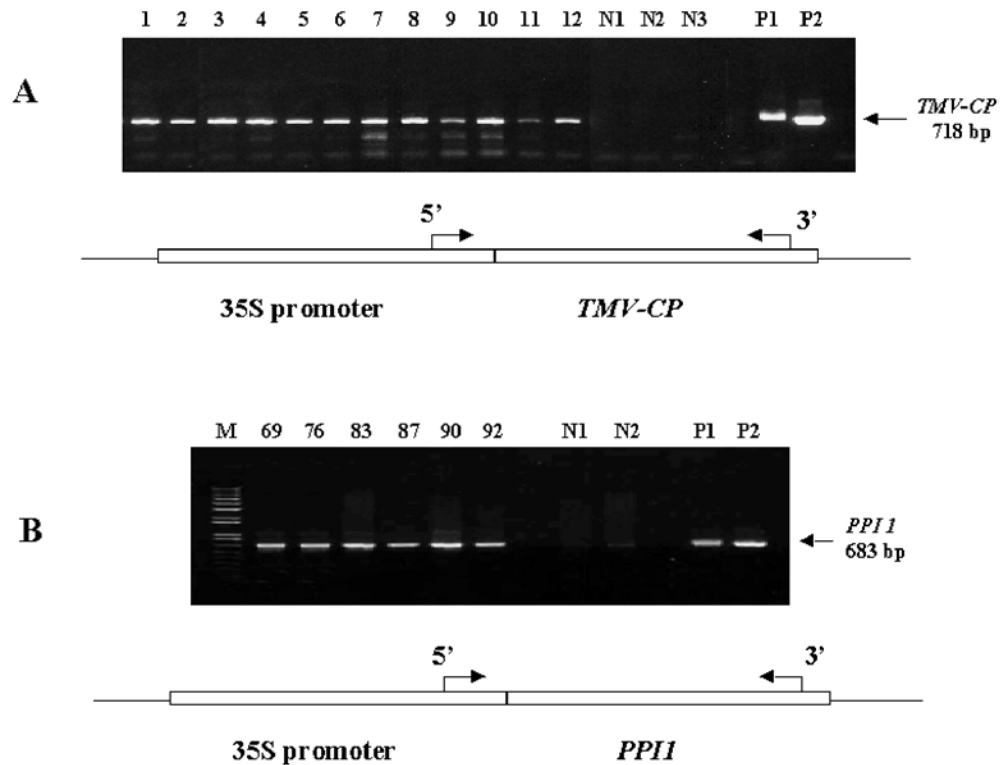
**Fig. 3** Callus types for shoot formation (**a, b**) and for non-shoot formation (**c–f**). **a** Type A, white hard surface and green tissue inside; **b** type B, dark-green callus with a hard surface but a little bit

moist; **c** type C, yellow and very brittle; **d** type D, yellow and with a hard surface; **e** type E, moist and slightly transparent; **f** type F, green, moist and very brittle

**Table 1** Frequency of direct shoot formation

Gene	Number of explants				Number of shoots				Number of shoots with roots			
	P915	P409	P410	P101	P915	P409	P410	P101	P915	P409	P410	P101
<i>TMV-CP</i>	30,512	26,039	24,107	21,983	2,106	1,017	1,697	628	392	156	311	49
<i>PPII</i>	14,413	14,080	7,488	13,078	1,024	587	720	310	186	103	176	34
Sub-total	44,925	40,119	31,595	35,061	3,130	1,604	2,417	938	578	259	487	83
Total	151,700				8,089				1,407			

**Fig. 4 a** PCR analysis of peppers transformed with the *TMV-CP* gene ( $T_0$ ). Lanes: 1–12 transformed, N1–3 non-transformed, P1–2 positive control (cloned bacterial cell and pTMV-CP, respectively). **b** PCR analysis of peppers transformed with the *PPII* gene ( $T_0$ ). Lanes: 69, 76, 83, 87, 90, 92 transformed, N1–2 non-transformed, P1–2 positive control (cloned bacterial cell and pPPII, respectively)



#### Transformation rate

A total of 1,407 direct shoots grown in the rooting media were tested by PCR to identify transformed pepper plants; none of the shoots contained *TMV-CP* or *PPII* inserts (Table 3). To test the transformation rate of indirect callus-mediated shoots, we analyzed 28 shoots in the rooting medium by PCR (Table 3). The transformation rate was 0.19% (15/37,500) for the P915 line and 0.03% (3/37,500) for the P409 line. However, the transformation rate as determined by the number of PCR-positive versus the number of callus-mediated shoots was 34% (18/53), indicating that shoots grown from the callus could be transformed at a higher probability. Therefore, the selection of a callus-mediated shoot from among a large number of shoots growing on selection medium tended to discriminate transformed plants from non-transformed plants.

#### Southern and Northern analysis of $T_0$ peppers

DNA samples from transformed peppers ( $T_0$ ) randomly chosen from the PCR-positive peppers were isolated, and 30  $\mu$ g DNA was digested to completion with *Xba*I for *TMV-CP* and with *Bgl*II for the *PPII* gene. Figure 5a shows a Southern blot analysis of *TMV-CP*-transformed peppers digested by *Xba*I. The transformed peppers showed different *TMV-CP* insertion sites and those had one copy of the *CP* gene inserted. Northern blot analysis showed that  $T_0$  plants produced *TMV-CP* transcripts at

678 bp (Fig. 5b) but that non-transformed plants did not synthesize the transcript

The *PPII* insert digested with *Bgl*II was also localized at different sites in *PPII* transformed peppers (Fig. 6). The 5 kb band on the Southern blot was present in all lanes, and represented that the pepper *PPII* gene internally embedded in the genome, whereas the other inserts represented newly transformed loci. Interestingly, all of the transformed peppers had only one copy of the *PPII* gene inserted.

#### Resistance against TMV infection

To examine the resistance efficiency of *TMV-CP*-transformed pepper against infection by pepper-isolated TMV,  $T_0$  plants were self-crossed and a total of 408  $T_1$  plants were exposed to TMV. The leaves were inoculated twice at a 2-week intervals. Two weeks after the second inoculation, ELISA was performed. Leaf patches with a mosaic pattern identified susceptible plants, whereas no mosaic spot was developed on resistant plants. Twenty-eight  $T_1$  plants resistant to TMV infection were obtained (Table 4) and 380  $T_1$  plants were susceptible. PCR analysis showed that all of the 28 resistant  $T_1$  plants contained the *TMV-CP* insert (data not shown). The non-transformed control plants that were susceptible did not possess the *TMV-CP* insert.

**Table 2** Frequency of callus development and shoot formation from callus

Gene	Number of explants				Number of calli formed				Number of shoots from callus				Number of shoots with roots			
	P915	P409	P410	P101	P915	P409	P410	P101	P915	P409	P410	P101	P915	P409	P410	P101
<i>TMV-CP</i>	5,188	6,917	5,491	7,210	81	30	25	94	14	5	1	7	12	3	0	2
<i>PPII</i>	2,903	3,056	3,798	2,937	107	46	17	59	15	4	2	5	7	2	1	1
Sub-total	8,091	9,973	9,289	10,147	188	76	42	153	29	9	3	12	19	5	1	3
Total	37,500				459			53					28			

**Table 3** Transformation efficiency of direct and indirect shoot formation. Percentages were obtained by dividing the number of PCR-positive (+) with the number of explants used

Gene	PCR (+)			
	P915	P409	P410	P101
Direct shoot formation				
<i>TMV-CP</i>	0	0	0	0
<i>PPII</i>	0	0	0	0
Total	0	0	0	0
Transformation rate (%)	0	0	0	0
Indirect shoot formation				
<i>TMV-CP</i>	10	2	0	0
<i>PPII</i>	5	1	0	0
Total	15	3	0	0
Transformation rate (%)	0.19	0.03	0	0%

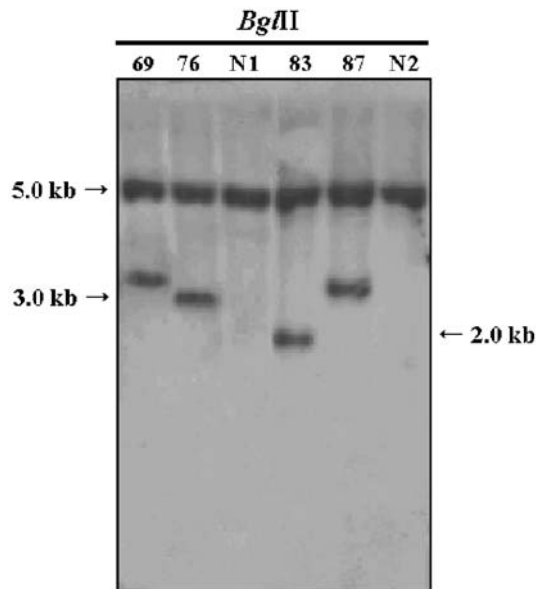
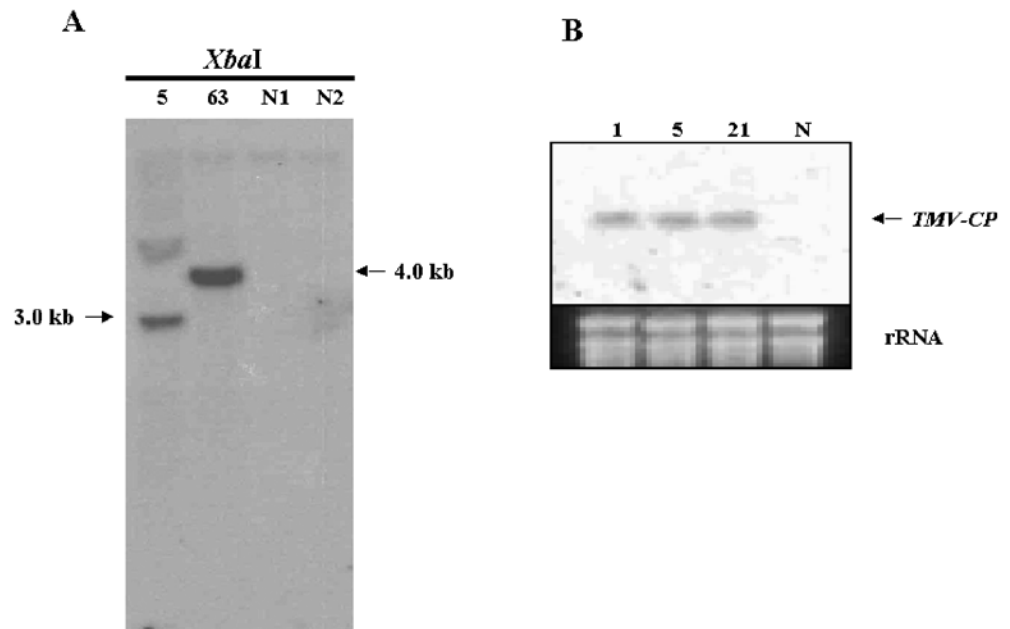
### A protocol leading to successful transformation

Several cases of successful transformation protocols are summarized in Table 5. The one we report here is not significantly different from the others previously reported. Our successes were obtained using two lines (P915 and P409) and two different genes (*PPII* and *TMV-CP*), indicating a recurrence of transformation. Two *Agrobacterium* strains, EHA105 and LBA 4404, were used preferentially. The duration of pre-culture and co-culture was not necessarily fixed because the transformation was successful as long as the experiments were performed under the conditions described in Table 5. As an auxin source, NAA or IAA could be used in the medium, but the protocol does require the use of zeatin as a cytokinin source. The most important factor in our protocol for pepper transformation is the identification of shoots generated from callus in the selection medium and the elimination of the shoots grown directly. This selection pressure saves time, labor, and cost.

### Discussion

Pepper is known to be a difficult plant to transform and has resisted the efforts of many laboratories for many years. To obtain a successful transformation system for pepper plants, we developed a protocol based on the selection of shoots grown from calli that had developed from cotyledon or hypocotyl tissue. Most of the shoots we obtained seemed to grow well directly near or on the wounded surface of explants on selection medium at a rate that depended on the conditions used. However, we never obtained a transformed pepper plant from shoots grown directly from the explants (Table 3). In contrast, some shoots grew from callus tissues that had formed around the cuts we made on the explants. Shoot formation from callus started 2–3 weeks after callus development (Table 5) and was an unusual phenomenon as callus is normally not easily formed from the wounded epidermis. These indirect shoots that grew from the callus showed a high probability of being transformed (Table 3). This difference between the origin of the shoots provides a

**Fig. 5** **a** Southern blot analysis of peppers transformed with the *TMV-CP* gene ( $T_0$ ). Lanes: 5, 63 transformed, N1–2 non-transformed. **b** Northern blot of peppers transformed with the *TMV-CP* gene ( $T_0$ ). Lanes: 1, 5, 21 transformed, N non-transformed



**Fig. 6** Southern blot analysis of transformed peppers ( $T_0$ ) with *PPII* gene. Lanes: 69, 76, 83, 87 transformed, N1–2 non-transformed. The 5.0-kb band belongs to the endogenous gene of *PPII*

**Table 4** Resistance test was performed by exposing  $T_1$  plants twice to TMV at 2-week intervals. Resistance was determined by ELISA using the absorbance threshold of the control (non-treated) ( $0.39 \pm 0.01$ )

	Number of plants tested	Susceptible	Resistant
$T_1$	408	380	28
Absorbance		$0.79 \pm 0.01$	$0.31 \pm 0.05$
Non-transformed	66	66	0
Absorbance		$0.86 \pm 0.02$	

means of selection that avoids unnecessary testing of non-transformed shoots.

Another important factor for selection was to determine the right callus type because not all calli produced shoots (Table 2). We observed six different callus types (Fig. 3) and of these, type A generated the most shoots—for some yet unknown reason. Consequently, we can state here that a researcher would have the most likelihood of finding putative transformed shoots from a pepper transformation process by looking for a shoot growing on callus type A.

Why were the callus-mediated or indirect shoots transformed while the direct shoots were not? It seems that the infection of *Agrobacterium* into pepper tissues or cell layers on the wounded surface of explants is not possible. However, the callus cell layer is not the same as the differentiated cell layer, rather it is a non-differentiated cell mass, and it is the altered tissue specificity of this non-differentiated cell mass that may help *Agrobacterium* invade the cells. This initial callus cell layer would have developed during the pre-culture period and could have provided a place for *Agrobacterium* to penetrate during co-culture. This hypothesis is reasonable because the transformation of monocot plants such as rice and grass, which initially presented difficulties, was successful with shoots generated from induced calli (Hiei et al. 1994; Rashid et al. 1996; Kusano et al. 2003).

The majority of earlier investigations reported the occasional successful transformation of one or two plants but not continuous success with different genes and lines and the production of many transformed plants (Lee et al. 1993; Kim et al. 1997; Manoharan et al. 1998; S.H. Kim et al. 2001; Shin et al. 2002a,b). However, Cai et al.'s review (2002) describes a large number of T generation progenies that stably expressed *CMV-CP* or the *TMV-CP* gene or both *CP* genes. Since this review does not provide

**Table 5** A protocol for pepper transformation and selection

Line	P915 or P409	Duration
Germination	Germination under light or dark conditions did not influence the transformation (1/2MS + 1.5% sucrose + 0.8% agar, pH 5.8)	8–10 days
Explant	Sample source—cotyledon or hypocotyl—did not make a large difference on induction transformation	
Pre-culture	Basic media (MS + 3.0% sucrose + 0.8% agar, pH 5.72) 2.0 mg/l zeatin + 0.05 mg/l NAA or 2.0 mg/l zeatin + 0.1 mg/l IAA	2–36 h
cDNA insert	<i>PPII</i> or <i>TMV-CP</i>	
<i>Agrobacterium</i>	EHA105 or LBA4404	
Inoculation	Basic media Presence of absence of acetosyringone in medium did not matter O.D. <sub>600</sub> : 0.3–0.5	10–20 min
Co-culture	Basic media 2.0 mg/l zeatin + 0.05 mg/l NAA or 2.0 mg/l zeatin + 0.1 mg/l IAA Washing buffer (1/2MS+1.5% sucrose, pH 5.8 with 500–800 mg/l cefotaxime or 500–800 mg/l lilacilline)	38–96 h in the dark
Selection	Basic media 80–100 mg/l kanamycin + 300 mg/l cefotaxime or lilacilline with 2.0 mg/l zeatin + 0.05 mg/l NAA Or 2.0 mg/l zeatin + 0.1 mg/l IAA	Callus formation: 4–5 weeks Callus development: 2–3 weeks
Shooting	Basic media 60–100 mg/l kanamycin + 300 mg/l cefotaxime with 2.0 mg/l zeatin + 0.01 mg/l NAA or 2.0 mg/l zeatin + 0.01 mg/l IAA	Shoot formation: 1–2 weeks Shoot elongation: 6–8 weeks
Rooting	Basic media 20–30 mg/l kanamycin + 200 mg/l cefotaxime without hormone	Root formation: 4–5 weeks 10-cm height: 2–3 weeks

details of transformation efficiency nor describe the factors critical for generating  $T_0$  plants, our results cannot be easily compared. However, the general procedure for transformation and culture that is described in this review (Cai et al. 2002) is similar to the data we show in Table 5, although the pH of the MS medium in the former was 7.0 whereas that is our investigation was 5.8.

Our transformation efficiency was low. From the 37,500 explants, 18 peppers were transformed independently with two different genes, *TMV-CP* and *PPII*, into two different pepper lines (Table 3). However, the present report is the first to demonstrate pepper transformation using a new selection tool—i.e. the isolation of a callus-mediated shoot (indirect shoot formation). The total number of  $T_0$  peppers originally obtained was 45, but 27 of these were developed by propagating calli (data not shown). Therefore, transformed callus was able to be sub-cultured and the shoots could be grown from the propagated calli. This indicates that a certain  $T_0$  plant can be maintained and preserved as a callus form for multiplication. Of the 45  $T_0$  plants 18 were obtained from independent calli by transforming independent explants.

The selection of genotypes for the transformation is also an important factor. Four lines (P915, P409, P410, and P101) that are known to have high regeneration rates (Kim et al. 2002) were used for the transformation, and only two of these lines (P915 and P409) were successfully transformed. The P915 proved to be by far the best line for transformation, as 68% of the transformed peppers were developed from this line (data not shown).

TMV-resistant peppers were selected from among  $T_1$  plants after a sequential but time-delayed double inoculation with TMV followed by ELISA (Table 4). Phenotypically no HR (hypersensitive response) or mosaic was found in the resistant peppers, whereas susceptible plants

had mosaic leaves. All 28 resistant peppers possessed the insert, and the resistance rate was about 7% (28/408). PCR analysis was carried out with 408 self-crossed  $T_1$  plants after TMV inoculation. Of these, 380 plants were susceptible, and of these 380 plants, 315 were PCR-positive (data not shown). However, many transformed peppers that contained the *TMV-CP* insert were susceptible, whereas non-transformed peppers did not contain the insert. We do not know why so many PCR-positive plants were susceptible, but several hypotheses can be made. First, the gene silencing caused by the inserted gene transcription (RNA-mediated resistance) would not occur: the Northern blot of  $T_0$  (Fig. 5b) and some  $T_1$  peppers (data not shown) showed a detectable steady-state accumulation of the *CP* gene. Second, the coat protein that was supposed to interact with a viral RNA to prevent replication (protein-mediated resistance) was not expressed in  $T_1$  peppers. For this, coat-protein levels should be examined in TMV-resistant peppers. Third, transgenic plants are generally somaclonal variants. Therefore, the resistance levels might be dependent just on how the *TMV-CP* gene product segregating in the  $T_1$  plants was influenced by somaclonal variation. Very recently, our laboratory observed similar phenomena from transformation experiments with CGMMV and WMV coat-protein genes to watermelon. Many  $T_1$  plants contained the *CP* gene insert, but only some plants of  $T_1$  with the *CP* gene insert were identified as resistant plants (4–5%, data not shown). Further studies with T-generation peppers could support any of these conjectures.

We present here a protocol and a selection method for pepper transformation which is successful and dependable (Table 5). The most important selection pressure for pepper transformation is to maintain the callus-mediated shoots and to eliminate the direct shoots during culture.



Recently, our laboratory has been involved in the development of a callus induction system using hormones to monitor the transformation efficiency of *Agrobacterium* using green fluorescent protein expression. Preliminary data show that the transformation rate of induced calli is dramatically increased. Studies on just how to enhance the regeneration rate from the induced calli are underway.

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