Stable Production of Transgenic Pepper Plants Mediated by *Agrobacterium tumefaciens*

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Abstract. The aim of this study was to establish a stable transformation method for hot pepper using the hygromycin phosphotransferase (*hpt*)/hygromycin selection strategy. Explants from aseptic pepper seedlings were inoculated with *Agrobacterium tumefaciens* carrying pCambia1301. A number of calli were developed on the medium containing hygromycin to discriminate the induction of “false-positive buds,” and then shoots were successfully regenerated from the hygromycin-resistant calli. Southern and Northern hybridization analysis indicated that the *hpt* gene was integrated and expressed in the transgenic pepper plants (T₀) and transmitted to the progeny (T₁) without genetic modification. Most T₁ progenies derived from self-pollination revealed a 3:1 segregation ratio for hygromycin resistance, indicating that one copy of the T-DNA was integrated into the respective transgenic lines. Both *uidA* and *hpt* genes were stably expressed in the T₁ generation and coinheritance in the progenies. Finally, homozygous progenies were identified in the T₁ generation of the transgenic peppers, and the homozygous state was maintained in all progenies tested (T₂). The results show the reliability and stability of the *hpt*/hygromycin selection protocol for pepper transformation.

Peppers are important horticultural crops worldwide, and a hot pepper is cultivated extensively in Northeast Asia. Agronomically important traits have been introduced into the pepper by conventional breeding, but the application is currently limited by the lack of genetic resources or by sexual incompatibility between species. Genetic transformation of the plant has become an important alternative for both basic and commercial plant breeding programs. However, genetic engineering of hot pepper has been hindered

by the difficulty in transforming pepper plants. Only a few papers on *Agrobacterium*-mediated transformation systems have been published (Cai et al., 2002; Lee et al., 2004; Shin et al., 2002; Zhu et al., 1996), and there have been few reports on the reliable inheritance of transformed genes. Therefore, development of more efficient procedures for *Agrobacterium*-mediated transformation could facilitate routine production of transgenic pepper lines.

In relation to tissue culture of pepper, initial efforts were concentrated on androgenesis and adventitious regeneration through organogenesis (Dumas de Vaulx et al., 1981; Ebida and Hu, 1993; Phillips and Hubstenberger, 1985). The somatic embryogenesis system was also used for in vitro regeneration in peppers but failed to develop further in some *Capsicum* varieties (Binzel et al., 1996; Steintz et al., 2003). In organogenesis, highly morphogenic tissues from seedlings exhibit a higher bud induction response, but shoot elongation was often problematic in whole-plant regeneration (Gunay and Rao, 1978). Multiple shoots

could develop directly from the explant surface or indirectly through callus derived from explant tissues (Agrawal et al., 1989). However, direct shoots were often laterally fused into leaf-like structures rather than organized into a shoot bud so that the structure was unable to elongate into a normal shoot (Liu et al., 1990; Wolf et al., 2001). Lee et al. (2004) reported that none of the regenerants from direct shooting turned out to be true transgenic plants. In indirect regeneration, shoot development from callus tissues has rarely occurred. So, despite numerous reports on tissue culture, plant regeneration from cultured pepper explants has been hampered, especially in the genetic engineering of pepper.

In transforming the pepper plant, the use of a suitable selectable marker is very important for the efficient selection of transformed event. The neomycin phosphotransferase II (*nptII*) gene has been previously used as a selection marker for pepper transformation (Cai et al., 2003; Zhu et al., 1996). However, pepper explants show some intrinsic resistance to kanamycin, as shown in some other crops (Mihalka et al., 2000). This causes poor selection of transformed cells, which results in extremely low transformation efficiency. Therefore, an alternative strategy for strict selection of transformed pepper cells is needed to avoid the growth of untransformed escapes. The hygromycin phosphotransferase (*hpt*) gene has been used in crop transformation as a marker to allow stringent selection of the transformed event (Van den Elzen et al., 1985). Thus, in the present study, the reliability of the use of hygromycin as a selection agent was examined and assessed for its efficacy in pepper transformation.

Once transgenic plants have been established, the transgenes should be stably integrated and expressed over generations. However, the expression level and patterns of transgene inheritance vary widely among transformed plants. Factors responsible for transgene instability include the site of integration in the genome, the transgene copy number, transgene rearrangement, transformation system (*Agrobacterium*-mediated, microprojectile bombardment, or PEG, and so on), the selection strategy, and the plant tissue culture system (Birch, 1997; Walden and Wingender, 1995).

In the present study, we used the *hpt* gene in pepper transformation as a selectable marker for the first time. Fertile transgenic plants were regenerated from hygromycin-resistant callus transformed by *Agrobacterium*-mediated method. Gene expression and stable inheritance of *hpt* and *uidA* genes were also analyzed in advanced generations of transgenic progenies.

Materials and Methods

Plant material. Seeds of a hot pepper (*Capsicum annuum* L. cv. Nockkwang) were obtained from a commercial source (Heung-gong Seed Co. Ltd., Ansung, Korea). The seeds were surface-sterilized in 70% EtOH

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(v/v) for 5 min and in 30% commercial bleach [Clorox (Yuhan Clorox, Incheon, Korea)] for 10 min and then rinsed three times with sterile ddH₂O. The surface-sterilized seeds were placed in a magenta box containing MS (Murashige and Skoog, 1962) medium for germination. The seeds were allowed to germinate in the dark at 25 °C. The cotyledons and hypocotyls from 7-day-old seedlings were excised and used as explants for transformation.

Plant expression vectors and preparation of *Agrobacterium tumefaciens*. The plasmid vector pCambia1301 (Cambia, Clayton Australia), which contains β -glucuronidase (*uidA*) and hygromycin phosphotransferase (*hpt*) genes under the control of the CaMV35S promoter, was transferred into *A. tumefaciens* strain GV3101. *Agrobacterium* cells harboring pCambia1301 were grown overnight in LB medium supplemented with 50 mg·L⁻¹ of kanamycin monosulfate and 50 mg·L⁻¹ of rifampicin in a shaking incubator at 28 °C. Cultured *Agrobacterium* cells were harvested and then diluted with MS medium containing 100 μ M acetosyringone (AS) to an optical density of 0.3 to 0.5 at 600 nm.

Transformation and regeneration. The explants were inoculated with *Agrobacterium* cells in suspension for 10 min and then blotted on sterile filter papers to remove excess bacterial cells. The infected explants were cocultured in semisolid MS medium containing 100 μ M AS in the dark for 48 h. After cocultivation, explants were washed two to three times with 500 mg·L⁻¹ cefotaxime and transferred onto MS medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) plant agar, 2 mg·L⁻¹ zeatin (Duchefa, Haarlem, The Netherlands), 0.2 mg·L⁻¹ indolacetic acid (IAA), 20 mg·L⁻¹ hygromycin, and 300 mg·L⁻¹ cefotaxime. Explants were subcultured onto a fresh medium at 2- to 3-week intervals. For shoot induction, the selected green calli were transferred onto regeneration medium, which was supplemented with 0.05 mg·L⁻¹ IAA, 2 mg·L⁻¹ zeatin, 10 mg·L⁻¹ hygromycin, and 300 mg·L⁻¹. Plantlets resistant to hygromycin were transferred onto rooting medium containing MS basal salts and 300 mg·L⁻¹ cefotaxime without the selectable agent. All cultures were incubated at 24 °C under a 16/8 h (light/dark) photoperiod. Plants having well-developed roots were transplanted to a pot in a greenhouse and allowed to grow until they set seeds.

Histochemical and fluorometric β -Glucuronidase (GUS) assay. β -Glucuronidase (GUS) activity was histochemically and fluorometrically assayed in plant tissues and organs from transgenic pepper plants. Histochemical staining for GUS activity was based on the method of Jefferson (1987) with a 5-bromo-4-chloro-3-indolyl- β -glucuronic acid (X-gluc) solution as a substrate. The samples were soaked overnight in X-gluc solution at 37 °C. In addition, fluorometric analysis of GUS activity was carried out in the flower, shoot apex, leaf, stem, and root from transgenic lines with the modification of Jefferson (1987). A 100 mg of plant tissue

was homogenized in 1 mL extraction buffer containing 50 mM NaPO₄ (pH 7.0), 10 μ M β -mercaptoethanol, 10 mM EDTA, 0.1% sarcosyl, and 0.1% Triton X-100. Five microliters of supernatant was reacted in the extraction buffer containing 1 mM of 4-methyl- β -D-unibelliferyl glucuronide. The enzymatic reaction was measured by a fluorometer TD-700 (Turner Designs, Sunnyvale, CA) with excitation at 365 nm and emission at 455 nm. The concentration of protein was determined by the Bradford method (Bradford, 1976).

Progeny analysis by hygromycin resistance (leaf assay in hygromycin solution). Leaf assay in hygromycin solution for the expression of the *hpt* gene was conducted to distinguish transformed plants from nontransformed plants. Leaf samples from young plants were placed in a 24-well culture plate containing 0.5 mL of 100 mg·L⁻¹ hygromycin or distilled water. Three leaf disks from a wild-type pepper plant at a similar growth stage were used as a negative control. The leaf disks were placed under light at 25 °C for 3 to 5 d. The leaf disks resistant to hygromycin remained green, indicating that the *hpt* gene was successfully integrated and expressed in pepper. In addition, primary transgenic plants (T₀) were self-pollinated and their T₁ progeny were germinated in MS medium containing 20 mg·L⁻¹ hygromycin. The data were then analyzed by χ^2 test to determine the number of functional *hpt* gene loci on the pepper genome. T₂ progeny were also tested for hygromycin resistance to identify homogeneity.

Southern and Northern blot analysis. Pepper genomic DNA was isolated by using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) as described by the manufacturer. Fifteen micrograms of each DNA sample was digested with *Hind*III in 50 μ L of the manufacturer's (NEB, Beverly, MA) buffer overnight and separated on a 0.8% (w/v) agarose gel. For blot analysis, DNA was transferred to a Hybond⁺ nylon membrane and DNA was fixed to the membrane by ultraviolet cross-linking. Prehybridization was carried out at 65 °C for 3 h in Rapid-hyb buffer (Amersham

Biosciences, Buckinghamshire, UK) and hybridization was carried out for 20 h at 65 °C in Rapid-hyb buffer with α -³²P-labeled probe. The probe DNA consisted of part of the *hpt* coding region amplified from pCambia1301 and labeled with [α -³²P]dCTP using the Random Prime labeling system (Amersham Biosciences). After hybridization, membranes were rinsed with 1X SSPE, 0.2% SDS at room temperature, and washed twice for 45 min each at 65 °C in 0.25X SSPE, 0.2% SDS. The filter was exposed at -80 °C on Kodak XAR-5 film (Kodak, Windsor, CO) with an intensifying screen.

Polymerase chain reaction analysis. Two primer sets were used on each DNA sample for amplification: 1) a 871-base pair (bp) fragment containing the *uidA* gene (forward: gaa ggt tat ctc tat gaa ctg tgc gtc; reverse: aag acg cgg tga tac ata tcc agc ca) and 2) a 412-bp fragment containing the *hpt* gene (forward: gaa gaa tct cgt gct ttc ag; reverse: gtg tgc tcc atc aca gtt t). Polymerase chain reaction (PCR) was performed in the DNA Thermal Cycler (Perkin Elmer, Norwalk, CT) programmed for 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s. PCR products were resolved by agarose-gel electrophoresis.

Results and Discussion

Selection of selectable marker gene and plant explant. To find a suitable selectable marker gene/agent for pepper transformation, the regenerability of cotyledons was compared on medium containing various concentrations of kanamycin and hygromycin (Table 1). The explants were highly resistant to kanamycin; even 150 mg·L⁻¹ did not inhibit callus induction, and, at 100 mg·L⁻¹ kanamycin, over 80% of cotyledonary explants developed shoot buds. In contrast, the explants were highly sensitive to hygromycin with complete inhibition of callus induction and bud regeneration at 10 mg·L⁻¹ and 5 mg·L⁻¹, respectively.

Several crops are known to be resistant to kanamycin so the *np11I*/kanamycin system is not applicable for the selection of the

Table 1. Determination of antibiotic sensitivity on the growth and shoot development of pepper explants on various concentration of antibiotics.

	Kanamycin (mg·L ⁻¹)					
Explants ^z	0	50	100	150	200	250
Survived (%)						
Cotyledon	25 (100) ^y	23 (92)	22 (88)	12 (48)	0 (0)	0 (0)
Hypocotyl	25 (100)	21 (84)	14 (56)	7 (28)	0 (0)	0 (0)
Regenerated (%)						
Cotyledon	25 (100) ^x	23 (92)	22 (88)	0 (0)	0 (0)	0 (0)
Hypocotyl	25 (100)	21 (84)	0 (0)	0 (0)	0 (0)	0 (0)
	Hygromycin (mg·L ⁻¹)					
Explants ^z	0	5	10	20	50	
Survived (%)						
Cotyledon	25 (100) ^y	21 (84)	0 (0)	0 (0)	0 (0)	
Hypocotyl	25 (100)	17 (68)	0 (0)	0 (0)	0 (0)	
Regenerated (%)						
Cotyledon	25 (100) ^x	0 (0)	0 (0)	0 (0)	0 (0)	
Hypocotyl	25 (100)	0 (0)	0 (0)	0 (0)	0 (0)	

^zTwenty-five explants were tested on MS medium supplemented with indolacetic acid (0.2 mg·L⁻¹) and BAP (2.0 mg L⁻¹) in each experiment.

^yNumber of explants survived (%).

^xNumber of explants regenerated (%).

transformed cells. In pepper, despite the report that the system resulted in an unacceptably high proportion of escapes (Mihalka et al., 2000), the *nptII* gene has been mainly used as a selection marker to screen transgenic pepper cells (Lee et al., 2004; Li et al., 2003; Ochoa-Alejo and Ramirez-Malagon., 2001). Our results also show that pepper cells exhibit an intrinsic resistance to kanamycin. Thus, application of kanamycin might not be enough to screen pepper cells carrying the *nptII* gene during the transformation procedure.

The *hpt*/hygromycin system has allowed stringent selection of transformed events in cassava (Zhang et al., 2000), sweetpotato (Kimura et al., 2001), cotton (Rajasekaran et al., 2000), and cucumber (Nishibayashi et al., 1996). Therefore, the *hpt*/hygromycin selection strategy was chosen for pepper transformation in a further experiment. On the basis of the present study, 20 mg·L⁻¹ hygromycin was used at the callus induction

stage and 10 mg·L⁻¹ at the regeneration stage, respectively, to select transformed callus and regenerate transgenic shoots.

Transformation of pepper explants. Cotyledonary explants were infected with *Agrobacterium* cells harboring pCambia1301 (Fig. 1A). After three to four subcultures of explants onto callus induction medium containing hygromycin, the hygromycin-resistant calli were clearly identified (Fig. 1B) and the rate of callus induction was ≈20%. The explant of the wild type was highly sensitive to hygromycin so that it was necrotized and finally died within three continuous subcultures. Only green calli survived on hygromycin were transferred to the regeneration medium containing hygromycin and within three to four continuous subcultures, adventitious buds developed from green sectors of the calli (Fig. 1D). The hygromycin-resistant shoots were transferred to rooting medium; 1 month later in the rooting medium, nine independent plants were produced (Fig. 1E).

In the greenhouse, these plants were phenotypically normal (Fig. 1F) and seeds were obtained by self-pollination.

The time required from infection to transfer of plants to the green house was 5 to 7 months. With nine transgenic plants from ≈1500 infected explants, the transformation frequency was ≈0.6%. Although the time-frame was similar to that reported by Lee et al. (2004), this transformation frequency with *hpt*/hygromycin is higher than the 0.03% to 0.19% reported for *nptII*/kanamycin in that experiment. We found that the higher transformation frequency is attributable to the use of a stringent selection system resulting in the growth of transformed cells from the early stage. Thus, a pepper transformation system using the *hpt*/hygromycin selection strategy has the potential to allow fewer escapes and require less effort in terms of time, cost, and labor. Further studies are under progress to increase transformation efficiency of pepper in combination with the *hpt*/hygromycin selection system. Development of an advanced propagation technology such as somatic embryogenesis will assist in more efficient genetic transformation of hot pepper for biotechnological purpose.

GUS expression in the transgenic peppers. GUS activity was measured histochemically as well as quantitatively in the primary transgenic pepper plants. Histochemical analysis showed that the *uidA* gene was constitutively expressed in the primary transgenic event (Fig. 1C) and in all organs, including leaves, floral organ, root, and fruit at different levels (Fig. 2A). This was also supported by fluorometric GUS assays that measured quantitatively in flower, shoot

Fig. 1. Steps for the development of transgenic pepper plants. (A) Cotyledonary explants cocultivated with *A. tumefaciens* containing pCambia 1301; (B) development of calli from the cutting edge of the infected explant on medium containing hygromycin (20 mg L⁻¹); (C) localization of histochemical GUS activity in developing calli and regenerated shoot; (D) shoot regenerated from the callus; (E) rooting of the regenerated pepper shoot; (F) pepper plants acclimated in pots.

Fig. 2. GUS activity in the primary transgenic peppers. Quantitative assays for GUS-specific activity conducted (A) in situ or (B) in extracts of various tissues from a transgenic pepper that carry the *uidA* reporter gene. (a) Flower; (b) plantlet with root; (c) leaves; (d) fruit.

apex, leaves, stem, and root (Fig. 2B). The results indicate that the *uidA* gene was stably expressed in the transgenic pepper.

To observe the GUS activity in the progenies, 21 seeds obtained from a T₀ transgenic pepper (no. 2) and the wild type were germinated on MS medium containing 20 mg·L⁻¹ hygromycin. After 2 weeks, all seedlings were evaluated and stained in X-gluc solution for GUS activity. In the transgenic line, 15 of 21 seedlings exhibited resistance to the hygromycin as well as GUS activity, whereas six seedlings did not show either hygromycin resistance or GUS activity (Fig. 3). Wild-type seedlings showed severely retarded growth in the selection medium and eventually died. This result represented that both *uidA* and *hpt* genes are transmitted to the next generation and expressed in the progeny.

Segregation analysis of transgenic pepper lines. To determine whether the transgenes were stably inherited in the next generation, self-pollinated seeds harvested from nine primary transgenic pepper lines were evaluated for the resistance to 20 mg·L⁻¹ hygromycin (Table 2). Hygromycin-resistant and susceptible seedlings were clearly identified within 1 week. A segregation ratio of 3:1 was observed in seven of the nine lines, representing a single functional *hpt* gene locus in the pepper genome. χ^2 analysis indicated a 3:1 segregation for the *hpt* gene, a ratio that suggested Mendelian segregation of a single dominant gene. The results indicate that transgenic pepper plants produced by *Agrobacterium*-mediated transformation were genetically and phenotypically stable in advanced generations. However, a non-Mendelian inheritance pattern was also observed in lines 6 and 7, of which the segregation ratio between resistant and sensitive was 10:54 and 82:12, respectively. For line 6, seed development might not be uniform or the introduced transgene might not be functionally expressed. Although we did not carry out

Fig. 3. Segregation of hygromycin resistance and GUS activity in the transgenic progeny. After germination of 21 seeds harvested from T₀ plant (no. 2), 15 seedlings were resistant to hygromycin and GUS positive; the remaining six were not hygromycin-resistant or GUS-positive. As a control, seedlings germinated from wild-type (WT) seeds showed stopping growth, no GUS activity, and no hygromycin resistance.

Table 2. Segregation of hygromycin resistance in the progeny of primary transgenic peppers.

Line	No. germinated ^z	Resistant (%) ^y	Sensitive (%) ^x	χ^2 ^{zw}	Ratio ^v
WT	60	0 (0.00)	60 (100)	—	—
1	82	64 (78.0)	18 (22.0)	0.40	3:1
2	99	76 (76.7)	23 (23.2)	0.01	3:1
3	111	81 (72.9)	30 (27.1)	0.24	3:1
4	62	48 (77.4)	14 (22.6)	0.19	3:1
5	62	50 (80.6)	12 (19.4)	1.05	3:1
6	64	10 (15.6)	54 (84.4)	120.0	—
7	94	82 (87.2)	12 (12.8)	7.50	—
8	62	48 (77.4)	14 (22.6)	0.19	3:1
9	80	58 (72.5)	22 (27.5)	0.26	3:1

^zSeeds were germinated on MS medium containing 20 mg·L⁻¹ hygromycin; germination rate >97%.

^ySeedlings [no. (%)] survived on medium containing hygromycin (20 mg·L⁻¹).

^xSeedlings [no. (%)] sensitive on medium containing hygromycin (20 mg·L⁻¹).

^{zw}Significantly different at the 5% level.

^vRatio of resistant versus sensitive seedlings on hygromycin.

Southern blot analysis with line 7, the introduced transgene might be integrated at more than two transgenic loci in the pepper genome. However, this phenomenon is commonly observed in plant transformation research (Christou et al., 1989; De Block et al., 1984).

Molecular characterization of transgenic plants. Stable integration of the transgenes was further investigated in the primary and T₁ progeny of transgenic peppers. Southern blot analysis was conducted with the genomic DNA isolated from the primary transgenic lines 2 and 3, their progenies, and the wild type as a negative control. The genomic DNAs were digested with *Hind*III and hybridized with a probe consisting of the *hpt* gene. DNA from the wild-type (nontransformed) plant showed no hybridization signal to the probe DNA (Fig. 4A). Each primary transgenic line showed a single band with a different band pattern, indicating that these two lines represented independent events. Their respective T₁ progeny showed a single band with the same band mobility as the parent plant, indicating that the introduced transgene had been successfully transmitted to the progeny without modification. Because the T-DNA of pCambia1301 has a unique *Hind*III site, the result also represents that a single copy of the transgene was integrated into the pepper genome.

To confirm that the introduced transgene was stably expressed in the transgenic peppers, Northern blot analysis was carried out with the primary transgenic lines 2 and 3, their progenies, and the wild type. The isolated total RNA was hybridized with *hpt* probe DNA. At the mRNA level, the introduced transgene was transcriptionally expressed in the two primary transgenic lines as well as their T₁ progenies, whereas no signal was detected in the wild type (Fig. 4B). This indicates that the introduced transgenes were stably expressed in the progeny. In general, a single copy of T-DNA insertion results in high levels of transgene expression, whereas multiple copies of transgene expressions may lead to suppression of the chimeric gene in some cases (Van der Krol et al., 1990).

Inheritance of transgenes in an advanced generation (T₂). PCR analysis was performed

Fig. 4. Southern and Northern blot analyses. (A) Southern blot analysis of two T₀ transgenic lines and their T₁ progenies. DNA samples from two transgenic lines and an untransformed pepper plant (WT) were digested with *Hind*III, and the resulting fragments were resolved by electrophoresis and transferred to a membrane. The membrane was hybridized with a ³²P-labeled probe DNA corresponding with the *hpt* coding region. (B) Northern blot analysis of two transgenic pepper lines. WT, wild type; lane 2, line 2 plants (T₀); lanes 3–6, T₁ progenies from line 2; lane 7, line 3 plants (T₀); lanes 8–11, T₁ progenies from line 3. The membrane was hybridized with a ³²P-labeled *hpt*.

on the two independent T₁ plants and their progenies (Fig. 5). Both the *uidA* and *hpt* genes introduced to the pepper genome were revealed in the T₁ and T₂ generations of transgenic lines 2 and 3, indicating that the transgenes were stably transmitted.

We investigated whether both of the introduced transgenes, *hpt* and *uidA*, were expressed in the T₂ generation without segregating or silencing (Table 3). Twenty T₂ seeds from lines 2 and 3 were germinated on hygromycin-containing medium and the seedlings were transplanted into pots. Leaf discs were incubated in 100 mg·L⁻¹ hygromycin to test for hygromycin resistance and in X-gluc for histochemical assay of *uidA* gene expression. All T₂ progenies were functionally resistant to hygromycin, whereas nontransgenic plants were

Fig. 5. Transmission of *uidA* (upper) and *hpt* (lower) genes to T_2 progenies determined by polymerase chain reaction analysis in two independent transgenic pepper lines. 2-1, T_1 transgenic pepper; 2-1-1, 2, 5, 6, T_2 transgenic progenies from transgenic line 2-1; 3-2, T_1 transgenic pepper; 3-2-2, 3, 8, 9, T_2 transgenic progenies from transgenic line 3-2; WT, nontransgenic pepper as a negative control; P, pCambia1301 as a positive control.

Table 3. Inheritance and functional activity of transgenes (*hpt* and *uidA*) in homozygous transgenic plants.

Line	Homozygosity ^z	Functional activity		PCR ^w
		HPT ^y	GUS ^x	
WT	—	S	—	—/—
2-1	NS	R	+	+/+
2-1-1	NS	R	+	+/+
2-1-2	NS	R	+	+/+
2-1-5	NS	R	+	+/+
2-1-6	NS	R	+	+/+
3-2	NS	R	+	+/+
3-2-2	NS	R	+	+/+
3-2-3	NS	R	+	+/+
3-2-8	NS	R	+	+/+
3-2-9	NS	R	+	+/+

Homogeneous transgenic pepper lines were confirmed by use of the hygromycin test; GUS histochemical assay and polymerase chain reaction in T_1 and their T_2 progenies.

^zHeterogeneity of hygromycin resistance of seedlings on hygromycin (20 mg·L⁻¹); NS, no segregation of hygromycin resistance in the progenies.

^yLeaf discs tested for the resistance to hygromycin (100 mg·L⁻¹) in distilled water; S, sensitive; R, resistant.

^xLeaf discs tested for GUS activity by histochemical staining.

^wTransgene inheritance in transgenic progenies was investigated by polymerase chain reaction (PCR) with *hpt* and *uidA* primer sets.

bleached or necrotized. In the histochemical GUS assay, leaf samples from all T_2 progenies, which had hygromycin resistance, showed GUS activity. Thus, the data demonstrate that *hpt* and *uidA* genes were transmitted and stably expressed in the T_2 progenies of the two independent transgenic pepper lines.

To examine the reliability of *hpt*/hygromycin system in pepper transformation, the sensitivity of pepper explants to hygromycin was compared in transgenic and nontransgenic plants by measuring fresh weight of hypocotyl explants (Fig. 6). With an increasing hygromycin concentration, the fresh weight of hypocotyls was dramatically reduced in wild type. Although untransformed pepper cells were highly sensitive to hygromycin, calli with developing buds were induced in transgenic explants. At over 10 mg·L⁻¹ hygromycin, adventitious healthy shoots were produced only in hypocotyl explants from transgenic plants.

Fig. 6. Growth of hypocotyl explants from non-transformed (WT) and transformed (T) peppers carrying the *hpt* gene on medium containing 0 to 50 mg·L⁻¹ hygromycin. (A) Callus and shoot formation. (B) Average of fresh weight of 60 explants after 3 weeks of incubation. The bars indicate SE.

In conclusion, we report the development of a reliable protocol for production of transgenic pepper plants with the *hpt*/hygromycin selection strategy. The selection strategy is critical for improving transformation efficiency of pepper. Although the *nptII* gene was successfully implemented in *Agrobacterium*-mediated transformation, the *nptII*/kanamycin selection strategy requires an extended culture in vitro and labor/cost to screen the escapes of nontransformed events. The *hpt*/hygromycin selection system allows stringent selection of transgenic pepper plants to eliminate or reduce the escape of untransformed or silenced transgenic pepper plants. Using *hpt*/hygromycin selection strategy, we confirmed stable inheritance of the introduced transgenes into subsequent generations and stable expression of the transgenes in the progenies. Therefore, the *hpt* marker system described in this study demonstrates its use for an effective transformation of pepper.

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