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EXPRESSION OF *HBs Ag* IN TOMATOES RESULTED IN ABNORMAL SHOOT REGENERATION *IN VITRO*

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Abstract

HBsAg M gene–modified tomatoes (*Lycopersicon esculentum* var. *cerasifarm*) had been constructed by *Agrobacterium tumefaciens*–mediated transformation. We obtained one line (N244) of transgenic mutant. During the *In vitro* micropropagation of N244, we found abnormal shoot regeneration as to compared with the non-genetically modified tomatoes (control) under MS medium containing 10 μM 6-benzyladenine (BA) and 1.0 μM α-naphthalene acetic acid (NAA). Histological studies identified two distinct pathways in the regeneration of the HBsAg M gene–modified tomato via callus tissues, somatic embryogenesis, and *de novo* shoot organogenesis, whereas only shoot organogenesis was detected in the non-genetically modified tomatoes (control). However, the bud regeneration frequency and the bud number per leaf explant in the HBsAg M gene–modified tomato were same as those in control. Furthermore, the abnormal shoot regeneration did not change the stability of HbsAg M gene in regenerated plant. Flow cytometric analysis suggested that the regenerated transgenic tomato was triploid (n=36) whereas the control plant was the normal diploid (n=24). The change in chromosome number may account for the abnormal *In vitro* shoot regeneration. The study reveals the effects of gene insertion, and provides insights into the possible variance mechanisms of the HBsAg M gene–modified tomato.

Introduction

Many researchers have examined the tissue culturing of tomatoes and the key factors in plant regeneration, such as hormone levels (Muhammad Sarwar *et al.*, 2006; Afroz *et al.*, 2009; chaudhry *et al.*, 2010), explant types (Niedz *et al*., 1985), and culture conditions (Ichimura, 1995). The regeneration of transgenic tomatoes was also reported in several papers (Lima *et al*., 2004; Hasan *et al.*, 2008; Praveen *et al*., 2005). However, the characterization of shoot development and the establishment of the plant regeneration process are yet to be examined. Furthermore, information regarding bud morphological differentiation during the regeneration of tomato is scarce. Understanding regarding the regeneration pathways of transgenic tomatoes is limited.

Previous studies regarding plant species regeneration via somatic embryogenesis and shoot organogenesis, such as those in *Catharanthus roseus* (Dhandapani *et al*., 2008), *Guizotia abyssinica.* (Sarvesh *et al*., 1993), *Astragalus melilotoides* (Hou *et al*., 2004), *Panax ginseng* (Tang 2000), *Physalis ixocarpa* Brot. (Rocío *et al*., 2009), *Echinacea* (Choffe *et al*., 2000), *Leucaena leucocephala* (Rastogi *et al*., 2008), and *Hypericum perforatum* (Franklin & Dias, 2006) have been reported. Although the factors determining regenerative competence and the regeneration pathway remain largely undefined, the interaction of biochemical, nutritional, cultural, and environmental factors can apparently determine the developmental pathway of competent cells (Gorecka *et al*., 2005; Cistue *et al*., 2006). Skoog & Miller (1957) hypothesized that the route of morphogenesis is determined mainly by the relative ratios of auxins and cytokinins. Furthermore, researchers have hypothesized that auxin and cytokinin are required for regeneration in cultured plant tissues (Steward *et al*., 1964). Different types and combinations of auxins and cytokinins regulate the corresponding regeneration pathways (Murthy *et al*., 1996). Despite advancements in research, the mechanism determining regenerative competence and the regeneration pathway is still unclear.

In our research group, HBsAg M gene–modified *Lycopersicon esculentum* var. *cerasifarm* plants were produced by *Agrobacterium tumefaciens*–mediated transformation (Zhang *et al*., 2005). Southern blots and inheritance analysis of the foreign genes indicated that the T-DNA was stably integrated into the plant genome. ELISA and western blotting showed that HbsAg M, a 24 kDa gene, was expressed in the transformed plant (Hao *et al*., 2007). However, the resulting transgenic tomato was conserved through the propagation of the axillary bud, which is time consuming and results in a low number of propagules. Therefore, we developed an effective regeneration protocol for HbsAg M gene-modified tomatoes *In vitro*. Histological studies identified the different shoot morphogenesis pathways in the regeneration of the HBsAg M gene–modified tomato compared with the non-genetically modified tomato. We reveal the effects of HBsAg M gene insertion, and provide insights into the possible variance mechanisms in HBsAg M gene–modified tomatoes.

Materials and Methods

Materials and *In vitro* regeneration: Seeds of wild cherry tomato (*Lycopersicon esculentum* var. *cerasiforme*) were kindly provided by Deng Dr. (Shannxi, Xi'an, Vegetable Research Institute). The seeds were immersed in water for two hours and sterilized with 70% alcohol for one minite and were washed several times with sterile distilled water. Then they were sterilized with 10% NaClO for five minites and rinsed several times. At last the seeds were placed to germinate on 1/2 Murashige–Skoog medium without any hormone. Cotyledonary segments from 10-day-old seedlings were excised and used for *Agrobacterium*-mediated

transformation. Seedlings were subcultured on 1/2 MS medium and used as the wild (non-transformed) control.

A transgenic mutant with HBsAg gene named N244 was obtained after co-cultivating explants with *Agrobacterium tumefaciens* strain LBA4404 harbouring the vector pCAMBIA1301/HB. The T-DNA of pCAMBIA1301/HB contains the HBsAg gene (approx. 0.7kb). Transformation procedure was used to obtain the mutant N244 as described in Hao *et al*., (2007). Both non-transformed plant control and transformed mutant N244 were conserved though the propagation of the axillary bud with a 16 h photoperiod under cool white light (30-40 μmol $m^{-2}s^{-1}$) at 25°C the plantlets of N244 and control were used as the material for next experiment.

Leaf explants (approximately 0.5 cm × 0.5 cm in size) were obtained by sectioning from the *In vitro* plantlets (about 6.0 cm height, Fig. 1A) though the axillary bud propagation, and incubated in MS solid medium supplemented with 10 μM 6-benzyladenine (BA) and 1.0 μM α-naphthalene acetic acid (NAA). They were then moved into a 25°C growth chamber with a 16 h photoperiod under cool white light (30-40 μmol m^{-2} s^{-1}). Green and healthy regenerated shoots larger than 40 mm were excised from their mother tissue and cultured in half-strength MS solid medium supplemented with 5 μM indole acetic acid (IAA) for root induction. All media were adjusted to pH 5.8, and then 0.6% agar and 30 g l^{-1} sucrose were added before autoclaving at 121°C for 18 min.

Fig. 1. Plant regeneration from leaf explants of HBsAg(M)-gene-modified tomato. (A) Germinated intact seedling of transgenic tomato though propagation of axillary bud. *Bar* 1.5 cm. (B) Shoot primordia appeared on the surface of callus (*Bar* 5.0 mm) after 14 days of culture on MS medium with 10.0 μM BA and 2.5 μM NAA. (C) Regenerated shoots cultivated on MS medium wit 10.0 μM BA and 2.5 μM NAA after 35 days. *Bar* 4.0 mm. (D) Rooting of regenerated shoots on half-strength MS medium supplemented with 5

μM IAA after 28 days. *Bar* 3.0 cm. (E) Micropropagated plants transplanted in soil after 30 days. *Bar* 6.0 cm. (F) Transgenic tomatos after transfer to soil. (G) The fruit of transgenic tomato. (H) The internal structure of transgenic tomato. (I) The internal structure of non-transgenic tomato.

Upon maturation, the rooted plantlets were removed from the *In vitro* culture, rinsed with water, and then transferred into a potting soil mixture in the greenhouse. Each plantlet was covered with polyethylene bags to maintain a high level of humidity (~80%). After 21 days, the polyethylene covers were removed, and the plants were gradually exposed to greenhouse conditions. The mean day-time and night-time temperatures in the greenhouse were 27.5 and 15.5°C., respectively. No supplemental lighting was provided for the greenhouse and the average light level on the benches at the time of collection was 244 µmol m^{-2} s^{-1}.

Light microscopy: For histological examination, tissues (4-6 mm) from the regenerated cultures were excised and fixed in a formalin: acetic acid: ethanol solution (5:5:90 by volume). The tissues were dehydrated using an ethanol–tertiary butyl alcohol series, and embedded in paraffin blocks, as described by Sharma & Sharma (1980). Sections (10 µm thickness) were cut using an ultramicrotome (Porter-blum ultramicrotome MT-1, Ivan Sorvall, Newtown, Conn.), stained with safranine-fast green, and then observed under a compound microscope (Nikon, Japan).

PCR analysis: The regenerative transgenic tomato plants were screened by PCR amplification of the HbsAg(M) gene fragments (700 bp) using designed primers P_1 (5-AACGGGATCCCGCACCATGGAGAACACAACAT CA-3) and P_2 (5-CCCGGAATTCCGGCTTAAATGTA TACCCA AAGAC-3). The young leaves were picked from mature tomato plants in the cultivated base, and DNA extraction was carried out according to the method of Saunders *et al*., (1993). PCR amplification was conducted as follows: 5 min at 94°C, followed by 35 cycles at 94°C for 30 s, 59°C for 40 s, and 72°C for 60 s, and then final extension at 72°C for 10 min. All reagents were from Dingguo, Beijing.

Southern blotting: For Southern blotting analysis, the total RNA was extracted from the young leaves of transgenic and non-transgenic tomatoes using a Tri-Reagent Kit (Molecular Research Center Inc., USA), electrophoresed on 1% agarose gel with 2% formaldehyde, transferred onto nylon membranes, hybridized with a probe, and labeled and detected using a digoxigenin labeling and detection kit (Boehringer, Mannheim, Germany).

Flow cytometric analysis: Flow cytometry was used to determine the ploidy of the HBsAg M gene–modified tomato and the control. Leaf samples (1 cm^2) were chopped with a razor blade and suspended in ice-cold neutral Otto I buffer (100 mM citric acid; 0.5% (V/V) Tween-20, pH 2–3). The samples were filtered through a 75 µm nylon mesh and stained with nucleic acid [(5% (W/V) propidium iodide; 5% (W/V) RNase]. The amount of nuclear DNA was measured in a FACStar PLUS flow cytometer (Becton Dickinson, USA) according to Shiba and Mii (2005). Data were subjected to analysis using Cell Quest software (Becton Dickinson, USA).

Results

The regenerative pathway of the HbsAg M gene–modified tomato: The *In vitro* propagation of the HBsAg M gene–modified *L. esculentum* is described in Fig. 1. Histological observations regarding the leaf explants treated with BA combined with IAA revealed that the regenerated plants were derived from adventive organogenesis or somatic embryogenesis. Bud primordial zones were observed on the surface of the calli derived from 3–7 layers of subepidermal cells within two weeks of cultivation. These meristematic cells were smaller, isodiametric, and exhibited a dense cytoplasm, which stained darkly (Fig. 2A). Adventitious shoots emerged from the tissues on the proximal cut surface of the explants. After a few days, the shoot buds had well-developed shoot meristems surrounded by a pair of leaf primordia, and had developed vascular connections with the leaf explants (Fig. 2B).

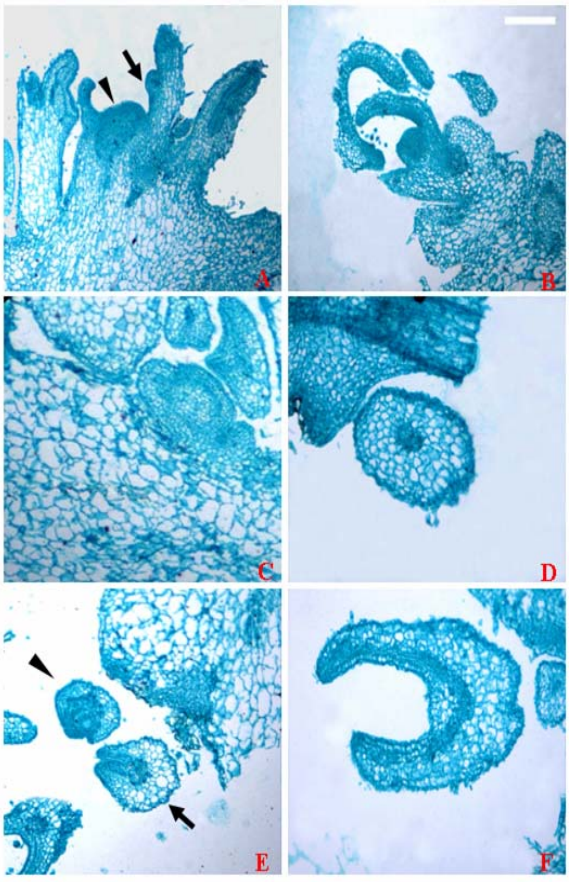

Fig. 2. Histological evidence of transgenic tomato regenerative process (A) A shoot bud consists of an apparent shoot meristem (arrowhead) and leaf primordia (arrows) (*B*ar 200 µm). (B) An intact shoot with visible leaf primordial. (C) A globular-stage somatic embryo consisting of a mass of compactly arranged actively dividing cells surrounded by a well-defined epidermis (*B*ar 200 µm). (D) Globular somatic embryo induced direct from hypocotyl epiderm after 10 days of cultivation (*B*ar 250 µm). (E) Torpedo somatic embryo (arrowhead) and early hypocotyl somatic embryos stage (arrow) (*B*ar 400 µm). (F) Hypocotyl somatic embryos with a well-defined epidermis (*B*ar 400 µm).

Histological observations also confirmed the other regenerative pathway, somatic embryogenesis, under the same culture conditions. After 2 weeks of culturing, the proembryos, which consisted of many actively dividing cells, were visible (Fig. 2C). Subsequently, the proembryos further developed into globular somatic embryos (Fig. 2D). These globular somatic embryos then developed into heart-shaped somatic embryos, which also had well-defined epidermises and distinct growth centers (Fig. 2E). The heart-shaped somatic embryos developed into mature cotyledon-stage somatic embryos that had well-defined epidermises (Fig. 2F, indicated by arrowhead) and pairs of cotyledons.

PCR and Southern blotting of the HbsAg M gene–modified tomato DNA: After the transformation of the tomato and the regeneration of mature plants, genomic DNA extracts from the leaves of selected transformants were screened by PCR and then further evaluated by Southern blotting. The PCR results show that the 700 bp fragment was amplified in the transgenic tomato sample, which was the expected size of the HbsAg M gene fragment (Fig. 3A). The same 700 bp fragment was absent in the non-transformed plants (Fig. 3A). Southern blotting analysis also confirmed transgenic inheritance (Fig. 3B). Furthermore, the PCR mapping and Southern blotting suggested that the regeneration pathway (shoot organogenesis or somatic embryogenesis) had no effect on HbsAg M gene replication.

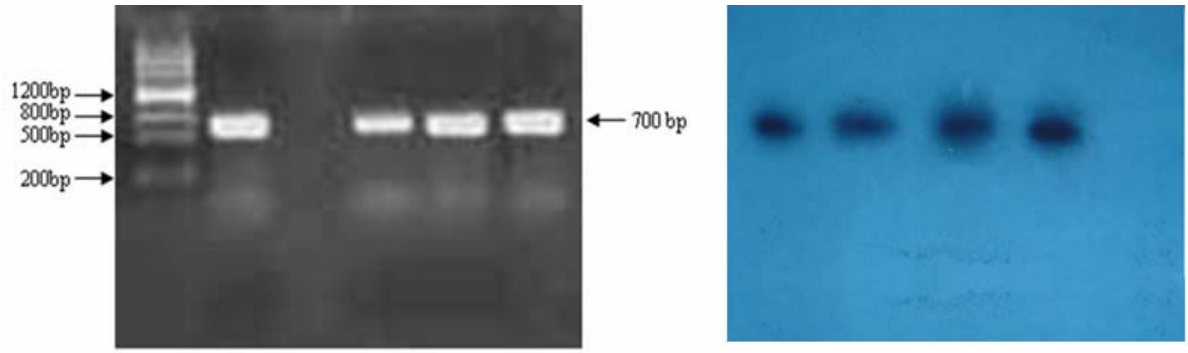

Fig. 3. The stability analysis of *HBsAg(M)* gene in regenerative tomato. A: PCR analysis. B: Southern bloting analysis. Genomic DNA digested with *Eco*RI/*Hind*III released a 2.1 Kb fragment from the ransformed plants. The gels were loaded as follows: M: Molecular weight markers; 1: the vector (pCAMBIA1301/HB) as positive control; 2 and 10: non-transformed; 3, 6 and 7: regenerative transgenic tomato through shoot organogenesis; 4, 5, 8 and 9: regenrative transgenic tomato through somatic embryogenesis.

Ploidy of the transgenic tomato: The ploidy of the transgenic plants was analyzed by flow cytometry. Under the same conditions, the DNA content of the samples was measured by comparing fluorescence peaks. A typical flow cytometric profile is shown in Fig. 4. The G1 peak could be statistically calculated from thousands of nuclei. The transformed plants had a different ploidy number from that of the control (Figs. 4A and 4B). The G1 peak value of the control was 63475 whereas it was 100138for the transformed tomato, which indicates that the 2C DNA content of the control was 1.96 pg whereas it was 2.99 pg for the transgenic plants. Thus, the DNA content of the transgenic tomato is about 1.5 times of the control. Based on chromosome number analysis and flow cytometry, the transgenic tomato was triploid.

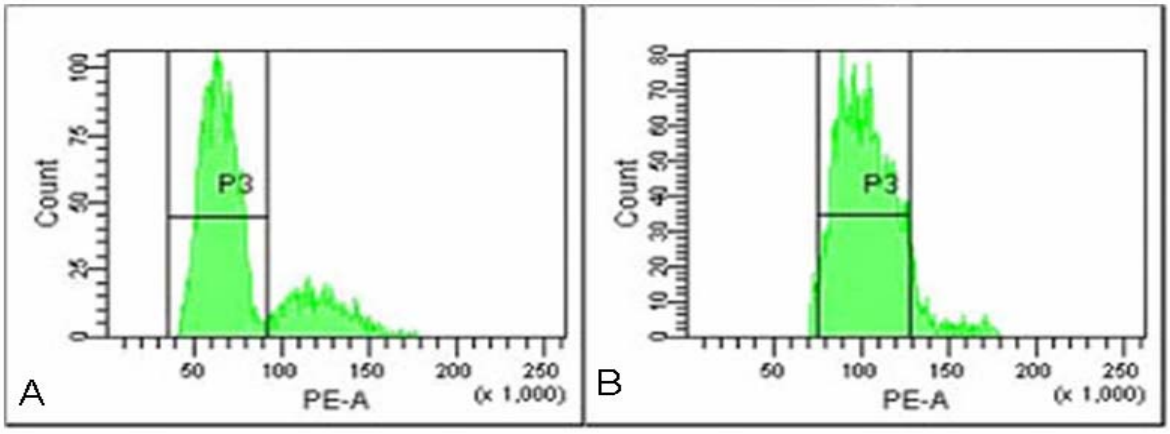

Fig. 4. Flow cytometric profiles of transgenic tomato and the control. The peaks of the horizontal axis correspond to relative nuclear DNA content, which is expressed as the fluorescence intensity. The number of nuclei is shown on the vertical axis. (A) DNA content of leaves of control plant (diploid = 2×); (B) DNA content of leaves of transgenic plants (diploid = 3×).

Discussions

Transgenic technology involves the transfer of one or more useful genes into plants to express them in the recipient organism. Eventually the recipient organism is expected to acquire new genetic traits if the plant genetic background does not produce any changes. In certain cases, some transgenic plants showed irrelevant phenotypic and agronomic traits, even the occurrence of something similar to mutations, which are accompanied by changes in genetic basis (Cuzzoni *et al*., 1990). According to the literature, variations in transgenic plants are shown by growth traits, leaf shape, leaf color, and fertility among others. Singh *et al*., (1998) reported that transgenic soybean plants produced by bombarding embryogenic suspension cultures with DNA-coated particles showed morphological variations, including plant growth retardation, dark green leaves, and sterility of seeds. Transgenic rice, expressing *Arabidopsis* phytochrome A, was cultivated up to the T3 generation in paddies exhibited dwarfing, higher chlorophyll content and low grain fertility as compared to wild-type plants (Kong *et al*., 2004). Transgenic tobacco plants carrying cDNA of *CYP11A1* encoding cytochrome P450 of bovine adrenal cortex had higher soluble protein content in the leaves compared with control plants (Spivak *et al*., 2009). However, the mechanisms of changes in the genetic basis are unclear.

In our experiment, we found abnormal shoot regeneration in HBsAg M gene–modified tomato *In vitro*. Two distinct pathways were identified in the regeneration of HBsAg M gene–modified tomato via callus tissues, namely, somatic embryogenesis and *de novo* shoot organogenesis. Only the shoot organogenesis pathway was detected in the control. However, the bud regeneration frequency and the bud number (not shown here) per leaf explant in the transgenic tomato were the same as those in the control. The abnormal shoot regeneration did not change the stability of the HbsAg M gene in the regenerated plant (Fig. 3).

The phenotypic alterations in the transgenic plants (including plant morphology, chloroplast number, and fruit fertility) are probably a consequence of the ploidy change. Choi *et al*., (2000) reported that cytological variations in transgenic plants mainly presented as significant increases in the proportion of aneuploidy. The aneuploidy reached 58% in transgenic oat plants generated by gene gun bombardment. In our study, through flow cytometry analysis, it was found that the HBsAg M gene–modified tomato plants and other transgenic lines presented triploids, but the control plants were normal diploids. Three carpels were observed in the HBsAg M gene–modified *L. esculentum*, whereas only two carpels were seen in the control (Figs. 1H and 1I), which are consistent with the result of the flow cytometry analysis (Fig. 4). As growth and ploidy analysis of both the transgenic and control plants were carried out under the same conditions, the ploidy change was caused only by the transformation event and not by the *In vitro* culture during plant regeneration. The ploidy change in the HBsAg M gene–modified tomato plant is the key reason for the abnormal *In vitro* shoot regeneration.

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