

Histocytological examination on organogenesis and somatic embryogenesis of HBsAg-transgenic cherry tomato mutant

Evaluación histológica de la organogénesis y embriogénesis somática del tomate cereza mutante transgénico-HBsAg

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Abstract. The initiation and development of organogenic buds and somatic embryos in HBsAg-transgenic cherry tomato (*Lycopersicon esculentum* var. *cerasiforme*) mutant were studied histologically. The leaf explants of the mutant were cultured on Murashige & Skoog (MS) basal medium supplemented with 6-BA 1.0 mg/L and IAA 0.05 mg/L for callus induction. Histological studies on the leaf explants of the mutant at various developmental stages revealed that organogenic buds first appeared in the axillary position of explants on the 14th cultured day, and then somatic embryos formed in the same mutant explants after 35 days of culture. Transmission electron microscopy and scanning electron microscopy indicated that there were significant changes in morphology and quantity of some organelles in the mutant callus cells compared with the control. On the 7th day of culture, embryogenic callus cells of the control showed dense cytoplasm and abundant organelles; at the same time, there was little cytoplasm or less organelles, except for a great number of dense lipid bodies in the mutant callus cells. In the later stage, the chloroplasts, Golgi bodies and mitochondria in the mutant cells also had obvious differences. These findings demonstrated that the regeneration pathway *in vitro* of the HBsAg-transgenic cherry tomato mutant showed variation, which was useful for the regeneration and gene transfer protocol.

Keywords: Histocytological; Organogenesis; Somatic embryogenesis; HBsAg; Transgenic; Cherry tomato; Mutant.

Abbreviations: HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; MS: Murashige–Skoog; SEM: Scanning electron microscopy; TEM: Transmission electron microscopy.

Resumen. La iniciación y desarrollo de yemas organogénicas y embriones somáticos en el mutante *Lycopersicon esculentum* var. *cerasiforme* transgénico-HBsAg fueron estudiadas histológicamente. Los explantos foliares del mutante se cultivaron en medio basal Murashige y Skoog suplementado con 6-BA 1,0 mg/L y IAA 0,05 mg/L para inducir la formación de callos. Estudios histológicos en los explantos foliares del mutante en varios estados del desarrollo revelaron que las yemas organogénicas aparecieron primero en la posición axilar de los explantos después de 14 días de iniciado el estudio, y los embriones somáticos se formaron en los mismos explantos del mutante después de 35 días de cultivo. Microscopía electrónica de transmisión y de barrido indicaron que hubo cambios significativos en la morfología y cantidad de algunas organelas en las células del callo del mutante comparado con el control. Luego de 7 días de cultivo, las células del callo embriogénico del control mostraron un citoplasma denso y organelas abundantes; al mismo tiempo, hubo poco citoplasma o menos organelas, excepto por un gran número de cuerpos lípidos densos en las células del callo del mutante. En estados más tardíos, los cloroplastos, cuerpos de Golgi y mitocondria en las células mutantes también tuvieron diferencias obvias. Estos resultados demostraron que la vía de regeneración *in vitro* del mutante de tomate *Lycopersicon esculentum* var. *cerasiforme* transgénico-HBsAg mostró variabilidad, lo cual fue útil para la regeneración y el protocolo de transferencia de genes.

Palabras clave: Histocitológica; Organogénesis; Embriogénesis somática; HBsAg; Transgénico; Tomate cherry; Mutante.

Abreviaturas: HBsAg: Antígeno de superficie de Hepatitis B; HBV: Virus de Hepatitis B; MS: Murashige–Skoog; SEM: Microscopía electrónica de barrido; TEM: Microscopía electrónica de transmisión.

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INTRODUCTION

Cherry tomato (*Lycopersicon esculentum* var. *cerasiforme*) is a subvariety of common tomato, an annual or perennial herb of the nightshade (*Solanaceae*), tomato genus (*Lycopersicon*). Being a delicious, nutritious food, it is one of the most extensive and in-depth materials used in research fields such as genetics, breeding, physiology and molecular biology. In our previous work, a transgenic plant expression vector containing HBsAg gene was constructed and an HBsAg-genetically-modified cherry tomato mutant was obtained by *Agrobacterium* mediation (Hao et al., 2007; Guan et al., 2010). The transgenic mutant of cherry tomato had a good expression of hepatitis B surface antigen. The transfer of HBsAg gene caused a series of complex changes of genetic basis, and a profound influence on biochemical and physiological growth and development of the transformed mutant. The mutant exhibited robust growth, dark green leaves, less obvious leaf margin notch, hypertrophic leaves, short and thick stems, more adventitious roots, round fruits, and abortive seeds (Guan et al., 2011a).

In recent years, the follow-up reports of transgenic tomato focused on some physiological changes (Luo et al., 2000), post-harvest physiological characteristics (Tian et al., 2003; Huang et al., 2006), endogenous hormone levels, fruit cell structures, resistance (Delannay et al., 1989; McGurl et al., 1994) and nutritive qualities (Romer et al., 2000; Fraser et al., 2002). There were no detailed reports on morphogenesis during *in vitro* regeneration of transgenic tomato plants.

Somatic embryogenesis and organogenesis are two ways that can lead to the formation of plantlets *in vitro*. There were some reports on cherry tomatoes cultured *in vitro* (Sun, 2000; Lu, 2001; Chen et al., 2002; Zhou et al., 2002; Bartley & Ishida, 2007). The leaves of cherry tomato as explants were cultured on MS medium added with 6-BA and IAA to form embryogenic callus. Some of the mesophyll cells started to form embryonic cells at the cultured 5th day and could continue to produce somatic embryos (Guan et al., 2011b). However, the research on *in vitro* culture of transgenic cherry tomato has not been reported. Also, no detailed microstructural and ultrastructural studies have been carried out in order to clarify the morphogenesis variation of genetically transformed plants from callus culture.

Further information on developmental stages of embryogenesis and organogenesis are needed to study the developmental pattern of *in vitro* morphogenesis in transgenic mutant. This could lead to a better understanding of *in vitro* development of cherry tomato, and consequently result in higher regeneration rates that should benefit clonal propagation and transformation works. Leaf explants of HBsAg-transgenic cherry tomato mutant were induced *in vitro* and the changes of morphogenesis were processed for TEM and SEM during its callus development to characterize the embryonic development in the transgenic plants. This paper

described the developmental pattern of the mutant, embryogenesis and organogenesis, which were induced from the leaf explant source at a cellular level scale. This research might have important theoretical significance on the use of transgenic plant mutants, and help to understand further the role of the HBsAg gene.

MATERIALS AND METHODS

The control (wild) plant. Seeds of cherry tomato (*Lycopersicon esculentum* var. *cerasiforme* Cherry No.1) were kindly provided by DENG JJ (Shannxi, xi'an, Vegetable Research Institute). After immersed in water for two hours, the seeds were sterilized with 70% alcohol for one minute, and were washed with sterile distilled water several times. Then, they were sterilized with 10% NaClO for five minutes and were rinsed several times. Finally, the seeds were placed to germinate on 1/2 half strength Murashige–Skoog (MS) (Murashige & Skoog, 1962) medium without any hormone. Seedlings of non-transformed cherry tomato plants were used as controls NK.

The HBsAg-transgenic mutant. The transgenic mutant was obtained after co-cultivating leaf explants with *Agrobacterium tumefaciens*, strain LBA4404, harbouring the vector pCAMBIA1301/HB. The T-DNA of pCAMBIA1301/HB contains the HBsAg gene (approx. 0.7 kb). Transformation procedures used to obtain the mutant were described in Hao et al. (2007). To allow sterile seeds of the existing transgenic mutant, the transformed plants were subcultured for follow-up research.

Callus induction and culture conditions. Leaves were excised from 3-week-old seedlings of the transgenic mutant and the control for callus induction. The explants were grown on MS medium, supplemented with 6-BA 1.0 mg/L and IAA 0.05 mg/L, under a 14-h photoperiod, irradiance of 2500 lx, and temperature of 25 ± 2 °C.

Cytological observations. *Light microscopy:* Leaf explants were harvested at 0, 7, 14, 21, 28 and 35 days from culture on both the control and the mutant cultured on medium. Samples were immediately fixed in formalin/glacial acetic acid (FAA) and 50% ethanol mixture (5:5:90: v/v/v) in 24 h, followed by dehydration in a graded ethanol series (30%, 50%, 70%, 85%, 95%, 100%) and embedded in paraffin wax. An 8-mm thick section was cut from each paraffin block containing the corresponding sample with a rotary Microtome (Laica, Germany) and then stained with 1% safranin, and counterstained with 0.2% fast green, for general histological examinations. The sections were observed and digitally recorded using a Leica microscope (DMLB) equipped with a video camera (Leica, DC 300F; Leica Microscopy and Systems GmbH, Wetzlar, Germany).

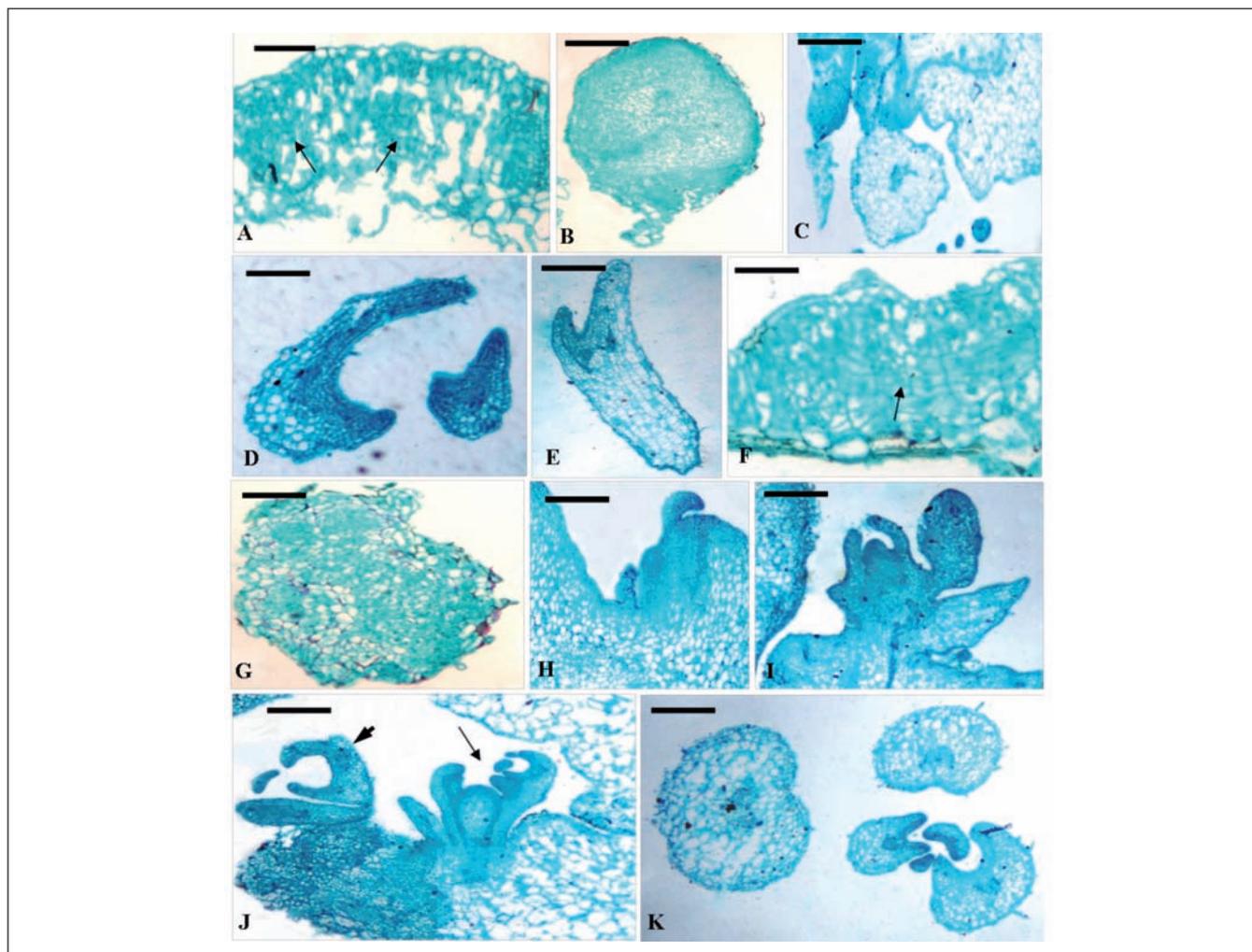


Fig. 1 Cytological analysis of the leaf explant development of both HBsAg-transgenic mutant and the control. A~F. Histological observation of somatic embryo development of the control. A. Initiation of rapid cell division (arrows) in the leaf surface after 7 d on MS medium (bar = 400 μ m); B. Initiation of many globular embryos with a suspensor after 14 d on MS medium (bar = 400 μ m); C. The heart-shaped globular embryo further developed (bar = 400 μ m), torpedo-shaped embryo (D, bar = 400 μ m) and finally formed cotyledon embryo (E, bar = 400 μ m) after 35 days of culture (F, bar = 400 μ m). F~K. Histological observations of organogenetic and somatic embryo development of the transgenic mutant. F. Cells generally started to divide strong in periclinal or anticlinal way in the local area, or cortex, epidermis outside the vascular tissue of the mutant leaf explants after 7 d on MS medium (bar = 400 μ m); G. General view of an independent callus produced at the proximal part of the 14-day-old explant (bar = 250 μ m); H. Emergence of adventitious shoot primordia in the axillary position (arrows) (bar = 250 μ m); I. Well-developed bud originating from the peripheral cell layers of a shoot primordium (Bar = 400 μ m); J. Shoot organogenesis (arrows) and somatic embryo (arrowheads) happened simultaneously after 35 days on MS medium (bar = 250 μ m); K. Various embryo appeared after 35 days of culture (bar = 250 μ m).

Fig. 1. Análisis citológico del desarrollo de explantos foliares tanto para el mutante transgénico-HBsAg como para el control. A~F. Observación histológica del desarrollo de embriones somáticos del control. A. Iniciación de división celular rápida (flechas) en la superficie foliar después de 7 días en medio MS (barra = 400 μ m); B. Iniciación de muchos embriones globulares con un suspensor después de 14 días en medio MS (barra = 400 μ m); C. El embrión más desarrollado globular, con forma de corazón (barra = 400 μ m), embriode con forma de torpedo (D, barra = 400 μ m) y finalmente la formación del cotiledón embriode (E, barra = 400 μ m) después de 35 días de cultivo (F, barra = 400 μ m). F~K. Observaciones histológicas del desarrollo del embrión somático y organogenético del mutante transgénico. F. Las células comenzaron a dividirse fuertemente en forma anticlinal o periclinal en el área local, o corteza, epidermis fuera de los tejidos vasculares de los explantos foliares del mutante después de 7 días en medio MS (barra = 400 μ m); G. Vista general de un callo independiente producido en la parte proximal del explanto de 14 días de edad (barra = 250 μ m); H. Emergencia de primordios de tallos adventicios en la posición axilar (flechas) (barra = 250 μ m); I. Yema bien desarrollada originada de las capas periféricas de un primordio de tallo (barra = 400 μ m). J. La organogénesis del tallo (flechas) y del embrión somático (punta de flechas) sucedieron simultáneamente después de 35 días en medio MS (barra = 250 μ m); K. Varios embriones aparecieron luego de 35 días de cultivo (barra = 250 μ m).

Transmission electron microscopy: Leaf explants at various development stages were pre-fixed at 4 °C overnight in 2.5% glutaraldehyde (v/v) in 0.1M PBS (Phosphate Buffered Saline) at pH 7.2, and then they were washed three times with the same buffer. The samples were post-fixed in 2% OsO₄ in 0.1M PBS for 2 hour, then washed three times in 0.1M PBS, with 10 minutes intervals between each washing. Then they were dehydrated through a graded ethanol series from 50% to 100%, and then infiltrated and embedded in Spurr's resin. Ultrathin sections (40 nm), obtained by a Reichert OM-U3 ultramicrotome, were stained with uranyl-acetate (Gibbons & Grimstone, 1960) and lead citrate (Reynolds, 1963), and observed under a transmission electron microscope (JEOL TEM-1230EX).

Scanning Electron Microscopy: Calli at different developmental stages were fixed in 2.5% buffered glutaraldehyde, washed in 0.05 mol/L potassium phosphate buffer (pH 6.8), dehydrated in ethanol series from 30% to 100%, critical point dried in liquid CO₂ and coated with gold-palladium. The mounted samples were observed with a JEOL6100 Scanning Electron Microscope.

RESULTS AND DISCUSSION

We analyzed the callus of both the mutant and the control using safranin/fast green as stains. Using a light microscope, histological examination showed that the mutant had a different regeneration pattern from the control during the callus formation and differentiation. To study more closely the morphogenesis process of the HBsAg-transformed mutant tissues, we performed microscopic analysis at different developmental stages. Meristematic areas were seen on the surface of the leaf explants as early as 7 days after culture (Fig. 1).

In the 7-day-old control callus, many spherical cell clusters appeared, which had clear boundaries with the surrounding parenchyma cells. Some cells of large-nuclear and small in size, especially multinucleated cells could be observed (Fig. 1A). The subsequent developmental process showed that these peculiar cells in the control callus, what were called embryonic cells, were the original source of somatic embryogenesis (Fig. 1B). Then their development progressed through typical globular, heart and torpedo-shapes at cotyledonary stages (Fig. 1C~E). However, the formation periods of the majority of the embryos did not possess a clearly defined boundary. Various types of embryos all appeared simultaneously after 35 days of cultivation. These results indicated that regeneration of the control occurred via embryogenesis on MS medium containing 6-BA and IAA. The presence of somatic embryos at different developmental stages at the same time showed the asynchrony of this process, which has been related to the strong trend of scutellum of somatic embryos to secondary proliferation, so that two or more embryoid generations could appear simultaneously (Wang & Vasil, 1982).

At the cortex, epidermis outside the vascular tissue of 7-day-old mutant leaf explants, cells generally started to divide strong in periclinal or anticlinal ways (Fig. 1F), and further developed into an independent callus so as to get the whole explant swelling, and the surface falling apart at 14 days of culture (Fig. 1G). Subsequently, adventitious shoot primordia emerged in the axillary position of callus mass (Fig. 1H) and developed into a complete bud (Fig. 1I). Cultured after 35 days, many embryomatic cells formed near the buds of N244 callus (Fig. 1J~K).

We demonstrated that shoot organogenesis and somatic embryogenesis occurred together during the *in vitro* regeneration of transgenic cherry tomato mutant leaf explants treated by 6-BA combined with IAA. However, only somatic embryogenesis pathway was observed during the regeneration of non-transformed cherry tomato plants under the same culture condition. In addition, after dedifferentiation, the cells of the control explants directly differentiate into embryonic cells and further formed somatic embryos; nevertheless, the complete callus was firstly produced on the surface of the mutant leaf explants, and then indirectly formed organ primordia and somatic embryos.

To study further the variation of callus development in the transgenic mutant, we performed an observation at the electron microscopy scale. The 7-day-old control callus was mostly composed of well-developed meristematic tissues containing (1) small, uniform, actively dividing meristem-like cells with large nucleus, (2) prominent stained nucleoli, (3) lack of intercellular spaces, (4) few small vacuoles, (5) dense cytoplasm, (6) abundant organelles, and (7) a small number of lipid bodies (Fig. 2A~C). These cellular masses could be easily fragmented resembling young globular somatic embryos (Fig. 2D). This indicated that these somatic cells changed to embryonic cells. In general, in these structures and in the embryogenic calli, a large number of amyloplasts were present in almost all cells. Moreover, normal chloroplasts were never found (Fig. 2E). With the further development of embryonic cells, nuclear shifting to the end, cell wall thickening (Fig. 2F), and lipid bodies increased significantly; to a later stage, cells were very rich in organelles (e.g., Golgi bodies) (Fig. 2G), enlarged lipid bodies, particularly with respect to an increased number of active mitochondria (Fig. 2H). At the stage of cotyledonary embryo, cells with large central vacuole appeared, the nucleus was close to the cell wall, numerous chloroplasts began to form and were affixed to the cell wall, and other organelles decreased (Fig. 2I).

Compared with the control, there were dramatic differences in the cellular ultrastructure of the mutant callus (Fig. 3). On the 7th day of culture, the most outer mesophyll cells of the incision died, the disrupted layer appeared, and some elongated and disorganized cells formed on the surface of the mutant callus (Fig. 3A, B). There were little cytoplasm or other organelles, except a great number of dense lipid bodies.

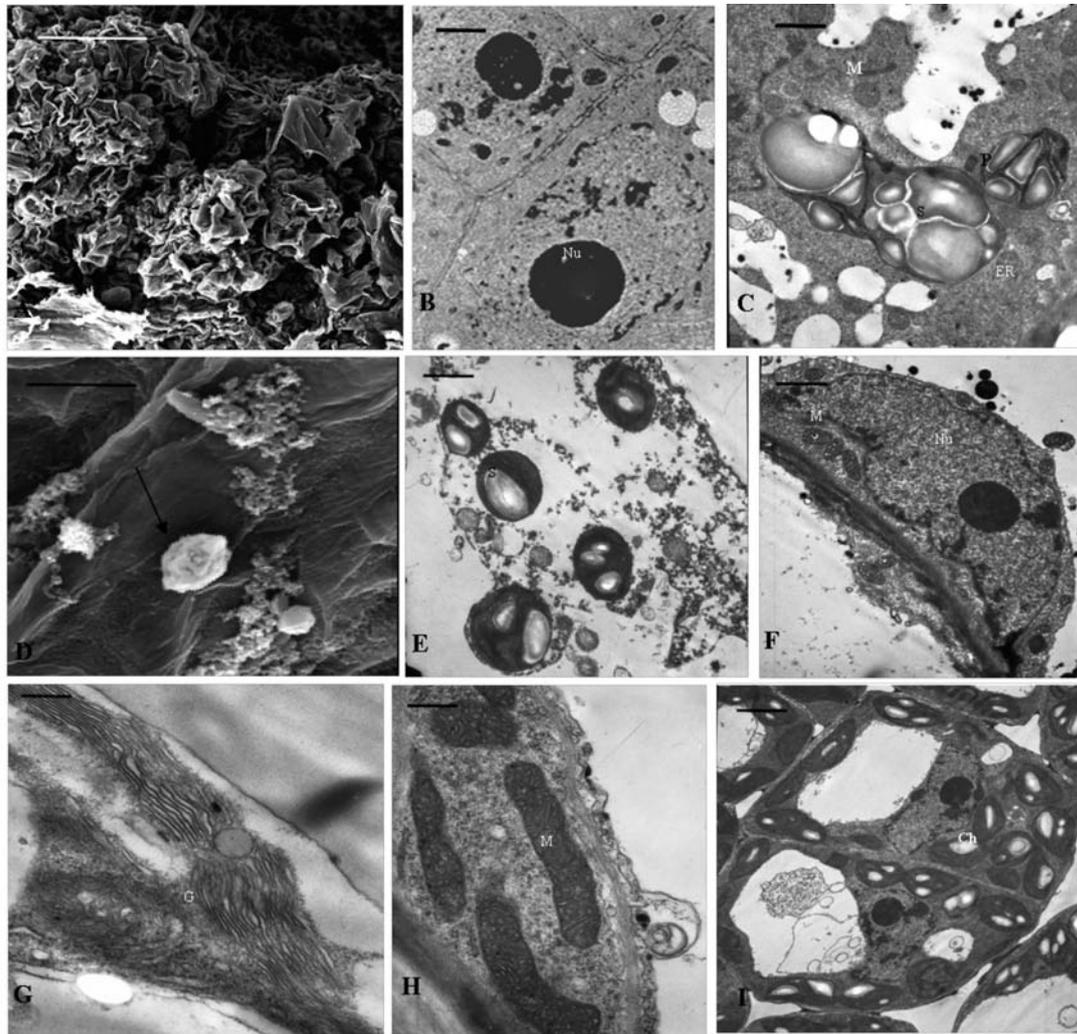


Fig. 2 The somatic embryogenetic ultrastructure of the control plant. A. SEM observation of callus surface after 7 days of culture (bar = 20 μ m). Its traits included prominent nucleoli (B, bar = 2 μ m), few small vacuoles, abundant organelles, a small number of the lipid bodies (LB) and dense cytoplasm (C, bar = 2 μ m). D. SEM evidence of young globular somatic embryos (bar = 10 μ m). E. A great quantity of amyloplasts were present in the somatic cells (bar = 2 μ m). With the further development of embryonic cells, nuclear shifting to the end, cell wall thickening (F, bar = 2 μ m); to a later stage, cells were very rich in Golgi bodies (G, bar = 0.5 μ m), particularly in the increased number of active mitochondria (H, bar = 0.5 μ m). At the stage of cotyledonary embryo, cells with large central vacuoles appeared, the nucleus was close to the cell wall, numerous chloroplast began to form and were affixed to the cell wall (I, bar = 1 μ m).

Fig. 2. Ultraestructura embriogenética somática de la planta control. A. Observación SEM de la superficie del callo después de 7 días de cultivo (barra = 20 μ m). Sus características incluyeron nucleolo prominente (B, barra = 2 μ m), unas pocas vacuolas pequeñas, organelas abundantes, un pequeño número de los cuerpos lipídicos (LB) y citoplasma denso (C, barra = 2 μ m). D. Evidencia SEM de embriones somáticos globulares jóvenes (barra = 10 μ m). E. Un gran número de amiloplastos estuvieron presentes en las células somáticas (barra = 2 μ m). Con el desarrollo adicional de células embriónicas, cambio nuclear hacia el final, espesamiento de la pared celular (F, barra = 2 μ m); en un estado más tardío, las células contuvieron un gran número de cuerpos de Golgi (G, barra = 0,5 μ m), particularmente en un mayor número de mitocondrias activas (H, barra = 0,5 μ m). En el estado de embrión cotiledonar, aparecieron células con grandes vacuolas centrales, el núcleo estuvo cerca de la pared celular, se comenzaron a formar numerosos cloroplastos y se fijaron a la pared celular (I, barra = 1 μ m).

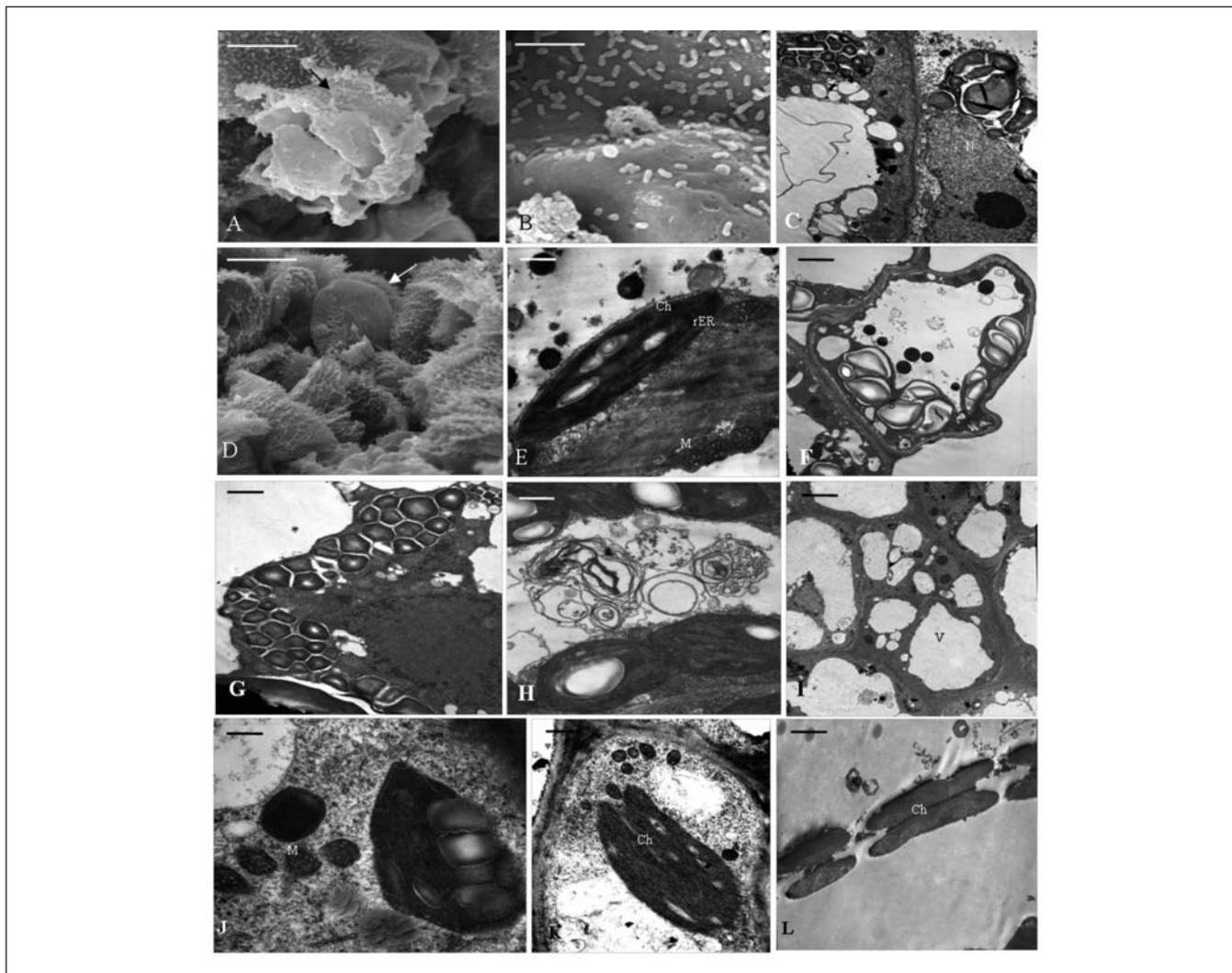


Fig. 3 The organogenetic and embryonic ultrastructure of the transgenic mutant. A. After 7 days of culture, cut edges of leaf explants showed dead cellular layer (bar = 20 μ m). B. Elongated and disorganized cells in the mutant callus (bar = 10 μ m). C. A great number of dense plastids appeared, and some small vacuoles of different size were around the central vacuole (bar = 2 μ m). D. After 13 days of culture, the meristematic cell clusters near the surface of the callus differentiated into normal bud primordia (arrow) (bar = 20 μ m). E. After 18 days of culture, intracellular organelles significantly increased (e.g., mitochondria and rough endoplasmic reticulum), and the double-membrane structure of chloroplast was visible (bar = 1 μ m). F-G. After 26 days of culture, the plastids with some starch grains of varying sizes changed obviously (bar = 1 μ m). In addition, a mass of digestive bubble was present within the central vacuole (H, bar = 1 μ m). After the formation of organs began, intracellular starch grains gradually disappeared (I, bar = 2 μ m), plastids gradually changed to chloroplasts (J, bar = 1 μ m), chloroplast lamellar system was reduced (K, bar = 1 μ m), until it finally disappeared completely (L, bar = 1 μ m). Ch: chloroplast; ER: endoplasmic reticulum; G: Golgi body; M: mitochondria; N: Nuclear; Nu: nucleolus; P: plastid; S: starch grains; rER: rough endoplasmic reticulum; V: vacuole.

Fig. 3. La ultraestructura embrionaria y organogenética del mutante transgénico. A. Después de 7 días de cultivo, cortes de explantos foliares, mostraron capas de células muertas (barra = 20 μ m). B. Células desorganizadas y alargadas en el callo mutante (barra = 10 μ m). C. Aparece un gran número de plástidos densos, y algunas vacuolas pequeñas de diferente tamaño estuvieron alrededor de la vacuola central (barra = 2 μ m). D. Después de 13 días de cultivo, los grupos de células meristemáticas cerca de la superficie del callo se diferenciaron en primordios de yemas normales (flecha) (barra = 20 μ m). E. Después de 18 días de cultivo, las organelas intracelulares se incrementaron significativamente (ej. mitocondria y retículo endoplasmático en etapas iniciales), y fue visible la estructura de doble membrana de los cloroplastos (barra = 1 μ m). F-G. Después de 26 días de cultivo, los plástidos con algunos granos de almidón de tamaño variado cambiaron obviamente (barra = 1 μ m). Después que comenzó la formación de órganos, los granos de almidón intracelulares desaparecieron gradualmente (I, barra = 2 μ m), los plástidos cambiaron gradualmente a cloroplastos (J, barra = 1 μ m), el sistema lamelar de los cloroplastos fue reducido (K, barra = 1 μ m) hasta que desapareció completamente (L, barra = 1 μ m). Ch: Cloroplasto; ER: Retículo endoplasmático; G: Cuerpos de Golgi; M: Mitocondria; N: Núcleo; Nu: Nucleolo; P: Plástido; S: granos de almidón; rER: retículo endoplasmático en sus inicios; V: vacuola.

Some small vacuoles of different size were around the central vacuole (Fig. 3C). After 21 days from culture, the meristematic cell clusters near the surface of the mutant callus differentiated into normal bud primordia (Fig. 3D). At this time, intracellular organelles increased significantly, especially mitochondria and rough endoplasmic reticulum. The lipid bodies obviously changed, and chloroplast grana lamellae formed interspersed with some starch grains of varying sizes. Also the double-membrane structure of the chloroplast, and other organelles such as mitochondria and plastids were clearly visible (Fig. 3E~G). In addition, a mass of digestive bubbles were presented within the central vacuole (Fig. 3H). After the formation of organs began, intracellular starch grains gradually disappeared, and the chloroplast lamellar system also became smaller until it disappeared completely (Fig. 3J~L). These differences in cellular ultra-microstructure probably determined the distinct morphogenesis pathway of the mutant, and that the shoot organogenesis and somatic embryogenesis occurred together.

This paper mainly described organogenesis and somatic embryogenesis *in vitro* in transgenic mutant, especially in transformed cherry tomato (*Lycopersicon esculentum* var. *cerasiforme*). The organogenesis of transgenic cherry tomato developed indirectly via callus formation and further differentiation to adventitious buds. That was to say, for introduction of foreign gene, the regeneration *in vitro* of the wild cherry tomato added to new pattern - organogenesis. And adventitious shoots and somatic embryos were simultaneously induced on the same explant for the transgenic mutant.

Somatic embryogenesis and organogenesis *in vitro* are a direct result of hereditary, developmental, and environmental conditions, as well as growth regulators, and other physical and chemical components of the culture media (Finstad et al., 1993). Initial genetic dissections of the organogenesis process in plant tissue culture were performed through the characterization of the mutants in terms of their morphogenic responses (Ozawa et al., 1998; Yancheva et al., 2003). The present study examined shoot organogenesis and somatic embryos from the mutant leaf explants. It was done from a developmental point of view on the basis of the current understanding of the concept of tissue competence, determination and differentiation. Considerable attention was given to the development of the regeneration process using detailed histology, SEM and TEM.

In conclusion, the present work elucidated the temporal framework and certain aspects of the developmental states of cell determination and morphological differentiation that are associated with shoots and somatic embryos development in transgenic cherry tomato mutant leaf explants. Histological and ultrastructural observations of the early events in the induction and development of shoot meristems in transgenic mutant could be useful for the regeneration and gene transfer protocol. This study contributed to the basis for a further

molecular analysis of the genes involved in the *in vitro* cherry tomato explant regeneration. Further research on the physiological, biochemistry variation mechanisms of the regeneration pathway of the *in vitro* transformed mutant is ongoing.

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