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Original Paper

In vitro flowering and viable seed setting of transgenic lettuce cultures

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Abstract Production of transgenic lettuce seeds *via in vitro* flowering and fruit setting is reported here. Six days old cotyledons were co-cultivated with *Agrobacterium tumefaciens* strain EHA105 harbouring the binary vector pCAMBIA2301 carrying the reporter gene α-glucuronidase intron (GUS-INT) and the marker gene neomycin phosphotransferase (NPTII). Transgenic calluses and shoot buds were induced on MS medium augmented with 0.1 mg l^{-1} benzyladenine (BA), 0.1 mg l^{-1} α-naphtaleneacetic acid (NAA), 100 mg l^{-1} kanamycin and 500 mg l^{-1} timentin (ticarcillin clavulanate). After transferring the cultures onto MS basal medium augmented or not with kanamycin, *in vitro* flower induction was observed within 90 days and eventually matured and produced seed pods. Pollen grains obtained from flowers induced on basal medium without kanamycin were heterogeneous containing both GUS positive and negative pollen grains. In the corresponding seed population, a Mendelian ratio (3 : 1) of *gusA* transgene segregation was observed. On the other hand, flowers that were induced on basal medium under kanamycin selection, all the pollen grains were GUS positives.

Key words: *Agrobacterium* transformation, GUS, *Lactuca sativa*, *in vitro* flowering, *in vitro* seed setting, transgene segregation.

Flowering is an important phase of plant's reproduction where the vegetative meristem is converted into a flowering meristem due to physiological, physical or chemical stimuli. Often, flowering occurs in tissue cultures under *in vitro* conditions, and is known as "*in vitro* flowering". Incidence of *in vitro* flowering has been reported in several plant species (Asawaphan et al. 2005; Chang and Chang 2003; Franklin et al. 2000; Galoch et al. 2002; Hee et al. 2007; Ignacimuthu et al. 1997; Lin et al. 2004; Ochatt and Sangwan 2008; Sivanesan and Jeong 2007; Vu et al. 2006; Wang et al. 2001). Commonly, flowering occurs more quickly under *in vitro* condition than the field condition in many species (Franklin et al. 2000; Hee et al. 2007; Ignacimuthu et al. 1997; Nadgauda et al. 1990; Ochatt and Sangwan 2008; Trinh et al. 1987), which would be extremely useful for studies related to flowering of plants that have a long juvenile period under natural conditions (Hee et al. 2007; Nadgauda et al. 1990). *In vitro* flowering could also be used in breeding of plant species that show irregular pollen development or asynchronous flowering between and within varieties, which can greatly compromise the efficiency of breeding. Moreover, the chemical and physiological parameters affecting flowering and floral organ development can be studied under precisely controlled conditions.

In many species, *in vitro* flowers could produce seeds similar to the field-grown plants (Asawaphan et al. 2005; Bodhipadma and Leung 2003; Franklin et al. 2000; Ignacimuthu et al. 1997; Ochatt and Sangwan 2008; Rao et al. 2005; Saritha and Naidu 2007; Zhang 2007). Hence, *in vitro* flowering can also be used as a tool to reduce the seed generation time in functional genomics research, if viable seed setting is possible. For example, this phenomenon was used to reduce the seed generation time drastically in the model plant *Arabidopsis thaliana* (Ochatt and Sangwan 2008) and also utilized to produce transgenic seeds quickly in *Nicotiana plumbaginifolia* (Trinh et al. 1987). *In vitro* flowering of transgenic cultures might be a useful tool for obtaining spores uniformly containing the transgenes. For e.g. due to the chromosome segregation upon sporogenesis, the resulting seed population will be generally heterogenic for the transgene. Since the differentiation and development of pollen partly depends on its haploid genome (Mascarenhas 1988), it is possible to eliminate susceptible spores by antibiotic selection and in turn, to produce all the first generation seeds with transgene and even with homozygocity.

In spite of the availability of reports on the *in vitro* flowering and seed setting of many plant species, there is no report on the *in vitro* production of transgenic seeds other than *N. plumbaginifolia* (Trinh et al. 1987). In this communication, we report on the production of

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transgenic lettuce seeds through *in vitro* flowering and seed setting. In addition, we have also studied the transgene segregation pattern of the seeds obtained *in vitro*. Production of transgenic seeds under *in vitro* conditions has several advantages, especially in functional genomics related to sexual reproduction, flower and seed development and seed-specific metabolic engineering. Additionally, if this *in vitro* flowering and seed setting strategy could be combined with co-transformation, marker-free transgenic seeds can be produced *in vitro*.

Materials and methods

Media and culture conditions

MS (Murashige and Skoog 1962) basal salt mixture including vitamins (Duchefa Biochemie, The Netherlands) containing a combination of 3% (w/v) sucrose and 0.8% (w/v) bacteriological grade agar (Sigma, Barcelona) was suitably supplemented with plant growth regulators (PGRs) as per requirement. The pH of the media was adjusted to 5.8 with 1 M NaOH or 1 M HCl before autoclaving at 1.0 kg cm^2 for 15 min. When required, antibiotics were added after cooling down the media to about 60°C. Each Petri plate containing 25 ml medium was inoculated with 10–15 explants. All the cultures were incubated at 25°C under photoperiodic (16 h) conditions under fluorescent bulbs (Fluora, Germany) at the light intensity of 25 μmol s^{-1} m^{-2} unless otherwise mentioned.

Aseptic seed germination

Approximately 50–100 lettuce (Batavia blonde) seeds were taken in an Eppendorf tube and surface sterilized in 70% ethanol for 5 min. After thoroughly washing the seeds with sterilized distilled water, they were further treated with a commercial bleach solution (Lixivia, Famalicão, Portugal) containing 1% active chlorine, for 5 min. Decontaminated seeds were again washed two times with sterile distilled water and transferred to Petri dishes containing half strength MS basal medium and incubated in darkness for germination. Once the emergence of radicle was noticed (after 2 days), culture plates were transferred to photoperiodic conditions.

Explant preparation and preculture

Cotyledonary leaves of 6 d old seedlings were cut removed and collected in a sterile Petri dish containing sterile distilled water. Gentle wounds were made near the proximal end using surgical blade. Explants were then transferred to MS medium augmented with 0.1 mg l^{-1} BA and 0.1 mg l^{-1} NAA (regeneration medium) in such a way that their abaxial surface touching the medium. Plates were sealed with parafilm and incubated under photoperiod for the preculture of explants.

Plasmid vector, Agrobacterium strain and culture

The plant expression vector pCAMBIA2301 (CAMBIA, Australia) was used in the present study. T-DNA region of this plasmid harbours *nptII* gene encoding neomycin phosphotransferase as selectable marker and *gus*A gene encoding glucuronidase as reporter. Both of these genes are driven by CaMV 35S promoter, which favour the constitutive expression of transgenes in plants. Since the *gus*A gene is disrupted by a catalase intron, it can express only in cells that possess intron splicing mechanism. Hence, the integration and expression of the T-DNA in the plant tissues can be followed by GUS assay. This plasmid was transferred into the disarmed *A. tumefaciens* strain EHA105 and used for lettuce transformation.

Single *A. tumefaciens* colony was inoculated into 5.0 ml of liquid LB medium augmented with 25 mg l^{-1} rifampicin and 50 mg l^{-1} kanamycin and incubated at 28ºC in a rotary shaker at 200 rpm for 12–16 h. Subsequently, 0.5 ml of grown bacterial broth was subcultured to 250 ml Erlenmeyer flask containing 100 ml of fresh medium and maintained under similar conditions. When the culture broth reached an optical density 0.5–0.8 at 660 nm, bacteria were spun down using a tabletop centrifuge (Eppendorf, USA) at 2,415 *g* and re-suspended in sterilized distilled water and used for infecting the explants.

Agrobacterium- mediated lettuce transformation

After 1–2 days of preculture, explants were collected in a sterile Petri dish and infected with *A. tumefaciens* suspension for 5 min with gentle shaking. Infected explants were blot-dried on a sterile filter paper disc and transferred back to the same plates, for co-cultivation. After 5–7 days (visible *A. tumefaciens* growth), explants were thoroughly washed with sterilized distilled water and transferred to selection medium (regeneration medium + 75 mg l^{-1} kanamycin + 500 mg l^{-1} ticarcillin clavulanate). Explants were subcultured to fresh medium as required or at least once in every 15 days until the induction of kanamycin resistant calluses and shoot buds.

In vitro flowering and seed setting of transgenic shoots

Transgenic calluses with or without shoot initials were transferred to MS basal medium with antibiotics. After 15–30 days, cultures with 2–3 elongated shoots were transferred to fresh medium augmented or not with kanamycin and subcultured regularly. Once flowering induction was noticed, the flowering shoots were cut removed and subcultured to fresh medium and maintained in the same medium until the maturity of flower(s). Matured heads were excised from the cultures for seed collection. Dry seeds from each head were stored separately in small tubes at room temperature for further analyses.

Transgene segregation analysis

To analyze the transgene segregation pattern of pollen grains, three healthy flowers from each selection and non-selection were incubated overnight in GUS solution (Jefferson et al. 1987). The number of pollens stained blue or not were counted under a light microscope and the ratio was calculated. The segregation pattern of transgene in seeds was studied by germinating them on half strength MS medium augmented with 75 mg l^{-1} kanamycin. The ratio between resistant and susceptible seedlings was calculated. To further confirm the segregation ratio, one of the cotyledonary leaves from each seedling was analysed by GUS assay.

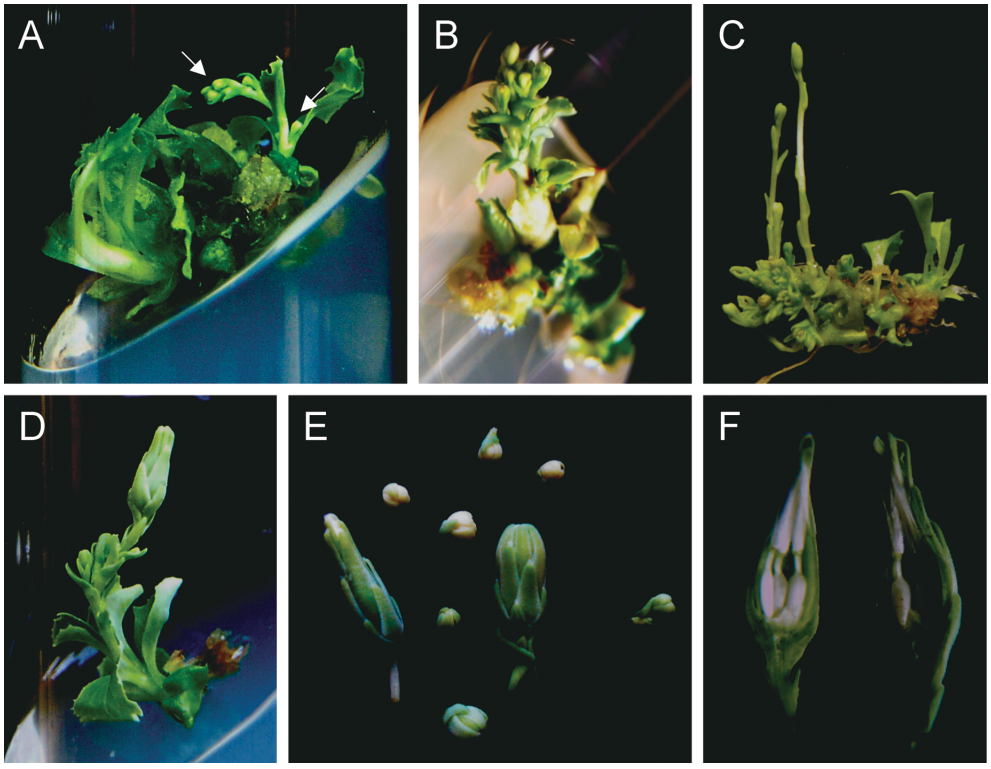

Figure 1. *In vitro* flowering and seed setting of lettuce (cv. Batavia blonde) after *A. tumefaciens* mediated transformation. (A) *In vitro* inflorescence development of a culture on MS basal medium; single culture containing both vegetative as well as flowering shoots (arrow) indicating asynchrony of floral induction. (B) Many flower heads induced synchronously from a shoot. (C) A culture with many developing flower heads showing stalk elongation of few heads after 10 days culture in liquid medium. (D) A shoot with single mature flower head. (E) Well developed as well as tiny flower heads could be seen in the same culture at a time. (F) A dissected lettuce flower head showing seeds developed *in vitro* as a result of self-fertilization.

Results and discussion

A. tumefaciens mediated transformation of lettuce

Profuse callusing of the infected explants along the wounds was noticed within 7 days of selection. Subsequently, shoot buds initiated from the calluses. GUS staining of leaves taken from these cultures has demonstrated 100% positive and that the selection medium used in the present study is efficient in eliminating escapes. Subculture of explants with transgenic calluses onto fresh medium, promoted shoot bud differentiation further. Although some of these buds elongated into shoots in the same medium, most of them did not elongate. Therefore, we transferred all the cultures to MS basal medium to synchronize shoot elongation.

In vitro flowering of transgenic shoots

On the basal medium, cultures showed different morphogenetic responses. *In vitro* flowering was noticed in 25% of the cultures, in addition to shoot elongation. *In vitro* flowering of transgenic shoots was noticed as early as in 90 days of their transfer to basal medium. In the same culture, both vegetative as well as flowering shoots could be seen, indicating asynchronous flowering induction (Figure 1A). This might be due to the fact that the explant is composed of different tissue types, which might have lead to the variation between shoots arising from different tissues of the same explant. Different segments (midrib and leaf blade) of single sugarcane leaf varied in their morphogenetic potential significantly (Franklin et al. 2006). All the more, *in vitro* flowering of explants depends on complex and co-ordinated interactions of several internal and external factors (Teixeira da Silva and Nhut 2003).

Two types of flowering shoots were observed in the cultures. In one type, several flowers were induced from a shoot synchronously at a time (Figure 1B) which was observed in 7.6% cultures. In the second one, single flower was induced from one shoot at a time and the flowering efficiency of these cultures was 24.2%. Although the number of flower buds per culture was higher in the former (6.5 ± 1.3/culture), very few could attain maturity and none of them set seed. Transferring these cultures to liquid medium in order to provide continuous supply of nutrients also did not help them to attain maturity, but resulted in the elongation of flower stalk (Figure 1C). However, in the later type, only one flower developed well at a time and attained maturity

(Figure 1D). Both matured as well as tiny flower buds could be seen in the same culture/shoot at a time (Figure 1E).

In the present study, lettuce cultures flowered in a medium without plant growth regulator (PGR) supplementation as reported earlier in *Capsicum fruitescens* (Bodhipadma and Leung 2003). Nevertheless, the type and concentration of PGRs critically affected the induction of *in vitro* flowering in many other plant species (Franklin et al. 2000; Galoch et al. 2002; Lin et al. 2004; Taylor et al. 2005; Vu et al. 2006; Wang et al. 2001). As the pattern of inflorescence development is differentially regulated by PGRs (Nadgauda et al. 1990), which may result in the malformation of flower (Vu et al. 2006), *in vitro* flowering in the absence of PGR is advantageous in breeding and to study the hormonal regulation of flowering and floral organ development.

Transgenic seed setting in vitro

Lettuce is an obligate self-pollinating species and its flowers shed their pollen before the emergence of stigma, assuring 100% self-fertilization. In many self-pollinating crops, seed setting followed by *in vitro* flowering has been previously reported (Asawaphan et al. 2005; Bodhipadma and Leung 2003; Franklin et al. 2000; Ignacimuthu et al. 1997; Nadgauda et al. 1990; Rao et al. 2005; Saritha and Naidu 2007; Zhang 2007). Correspondingly, in the present study also mature lettuce seeds have developed *in vitro* (Figure 1F). Using similar *in vitro* approach, seed generation time has been reduced significantly in *Vigna mungo* (Ignacimuthu et al. 1997), *Pisum sativum* (Franklin et al. 2000), *Dendrobium* (Hee et al. 2007) and *Arabidopsis thaliana* (Ochatt and Sangwan 2008). Even though some flowers induced under selection seemed to be matured, when collected, there were no fertile seeds. *In vitro* flowers of different maturity periods stably expressed the transgene, as revealed by GUS assay (Figure 2A). *Gus*A gene expression could be seen in individual florets (Figure 2B) and their reproductive organs like stigma (Figure 2C), anther tube (Figure 2C) and gynoecium (Figure 2D).

Transgene segregation analysis of pollen grains

In transgenic plants, the gametes (n) derived from meiotically divided sporophytic cells (2n) still represents the T0 generation. Hence, we analyzed the segregation pattern of pollen grains by GUS assay. The ratio between pollens with and without *gus*A gene expression was 1 : 1 in those flowers that were induced under non-selective condition (Figure 2E), as usual for the transgene segregation after meiosis of single locus insert vegetative cells.

On the other hand, all the pollen grains of flowers those were induced under selection were positive in GUS assay (Figure 2F, G). Previous evidences suggest that this may be due to the fact that the haploid cells which contain the *nptII* gene were only capable of normal growth and maturation under kanamycin selection. For e.g. *in vitro* maturation of transgenic (T0) tobacco pollen grains for three days in selection medium reduced successive pollen germination to 50%, as expected for

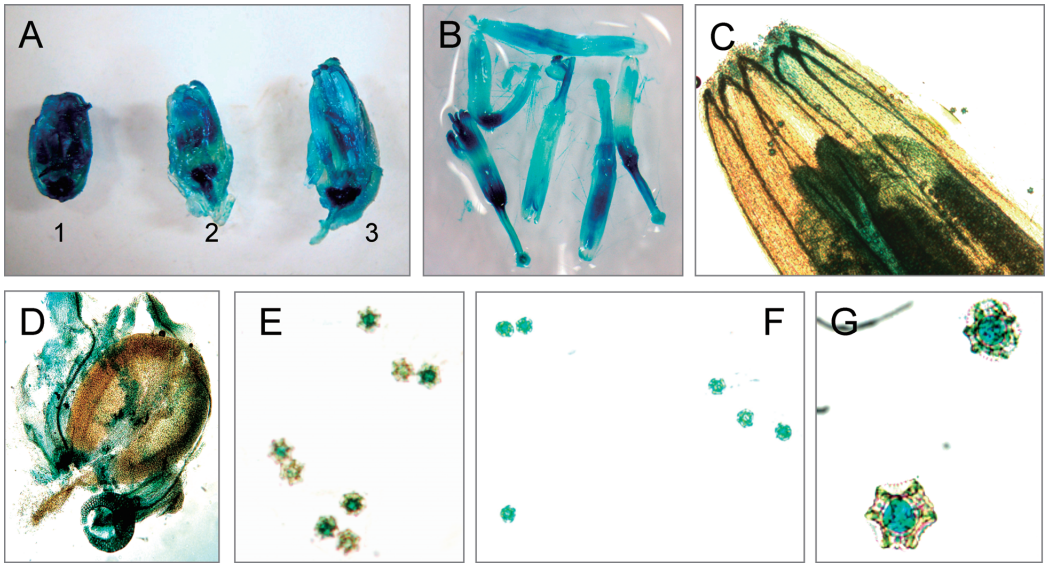

Figure 2. GUS analyses of transgene expression in transgenic lettuce flowers induced *in vitro*. (A) Flower heads collected after different periods (1–10 days, 2–15 days, 3–20 days) of induction stably expressing the *gus*A gene. (B) Expression of *gus*A gene in florets. (C) Close-up view of a floret showing *gus*A gene expression in the reproductive organs like stigma and anther tube. (D) Close-up view of lower part of a floret showing *gus*A gene expression in the gynoecium. (E) Pollen grains of flowers induced under non-selection showing segregation of *gus*A gene. (F) All the pollen grains of flowers induced under kanamycin selection stably expressing *gus*A gene. (G) Close-up view of pollen grains stably expressing *gus*A gene.

meiotic segregation of single locus insert (Touraev et al. 1995). Subsequently, pollination of wild-type plants with these selected pollen yielded 100% transgenic offspring. In another study, tomato pollen grains containing *nptII* gene readily germinated in a medium containing kanamycin, whereas, normal pollen grains without this gene did not germinate under similar condition (Chesnokov et al. 2000). Collectively, these findings underscores that the fertilization events by antibiotic susceptible pollen grains can be avoided by selection.

Transgene segregation in the progeny

Seeds produced under *in vitro* conditions have been reported to germinate normally (Franklin et al. 2000; Hee et al. 2007; Ignacimuthu et al. 1997; Ochatt and Sangwan 2008; Trinh et al. 1987). Same way, all the mature seeds collected from the flower heads germinated on 1/2-strength MS medium supplemented with kanamycin. After germination, resistant and susceptible seedlings could be distinguished based on their morphology. A set of 15 seedlings had white roots with lateral branches and a healthy shoot system with greenish primary leaves (Figure 3A) and resembled the control seedlings germinated without kanamycin. The other set of 6 seedlings (Figure 3A, arrows) had stunted roots with no lateral branches and primary leaves or chloretic primary leaves, resembling the control seedlings germinated in the presence of kanamycin (Figure 3B). As expected, the normal phenotypes were GUS positive, whereas the sensitive seedlings were GUS negative which further confirmed the segregation of *gus*A gene in 3 : 1 ratio (Figure 3C). Similar to our finding, other authors have also reported a 3 : 1 segregation of kanamycin resistance to sensitivity amongst T1 plants (Dinant et al 1997; Enomoto et al. 1990). Polymerase chain reaction analysis of genomic DNA isolated from resistant seedling showed the

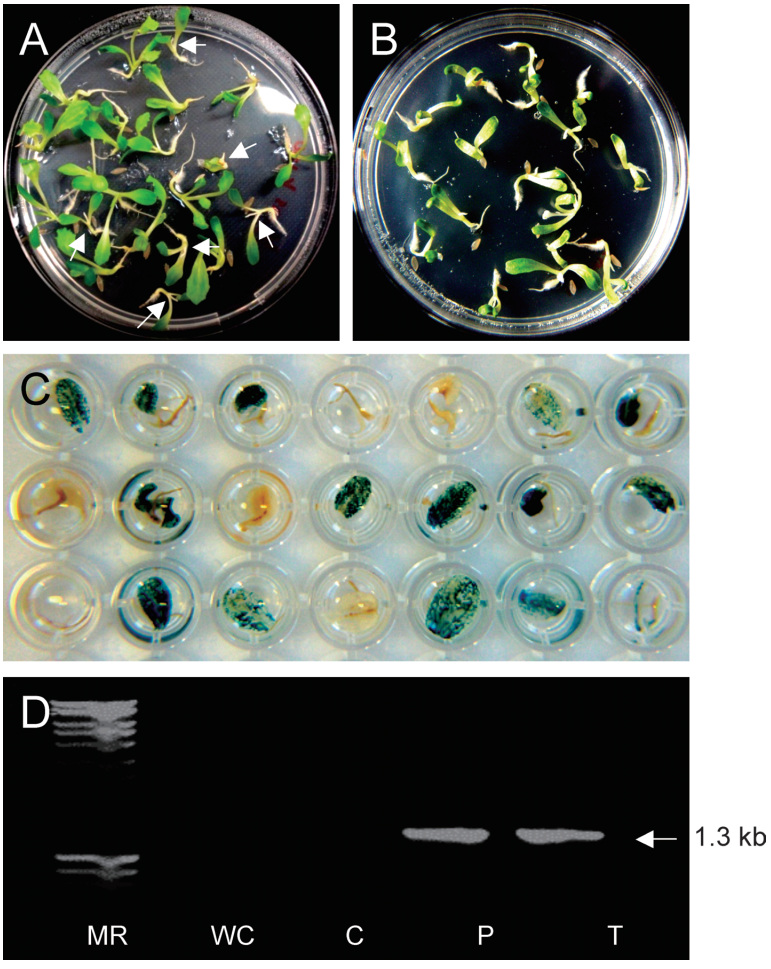

Figure 3. Analyses of T1 seedlings. (A) Seedlings derived from T1 seeds showing resistant and susceptible (arrows) phenotypes upon germination in the presence of kanamycin. (B) Control seedling germinated in the presence of kanamycin showing stunted growth. (C) Cotyledonary leaves of seedlings showing 3 : 1 ratio of *gus*A gene segregation as revealed by GUS assay. (D) PCR amplification of *gus*A gene using forward primer sequence 5′GATCGCGAAAACTGTGGAAT3′ and reverse primer sequence 5′TGAGCGTCGCAGAACATTAC3′ in the genomic DNA of a T1 seedling (Lanes 1- mass ruler, 2- water control, 3- control seedling genomic DNA, 4- plasmid DNA and 5- transgenic plant genomic DNA).

presence of *gus*A gene (Figure 3D).

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