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RESEARCH ARTICLE

Comparing the efficiency of sonication assisted *Agrobacterium* -mediated and particle bombardment for the production of transgenic canola plants

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Abstract

In order to achieve higher transformation rate in canola plants, the efficiency of sonication assisted *Agrobacterium* mediated and particle bombardment was compared. The hypocotyls explants isolated from 4 days old seedlings of two canola cultivars (Serw-3 and Serw-4) were transformed using both methods. The explants were treated by sonication for different time (5,10,20 and 30 sec.) before co-cultivation with *Agrobacterium tumefaciens* strain LBA4404 harboring the binary vector (*PBI-121-gfp*) that carried genes coding for kanamycin sulphate, and green fluorescence protein (*gfp*) under the genetic control of *35-S CMV* promoter and *nos* terminator. The data indicate that 5 sec. sconication treatment is enough to get higher transformation rate (55 %). Based on our data, we could draw the following conclusions: the transformation frequency of the two tested canola cultivars after biolistic gun was 25% and 16%, while the sonication assisted *Agrobacterium* gave 33% and 55% for the cultivars Serw-4 and Serw-3, respectively. The stable integration of the T-DNA in the T_0 transgenic plant genomes was confirmed by PCR and dot blot analyses. The PCR analysis using primers specific to the 35 S-promoter showed a clear band (250 bp) corresponding to the expected size of the 35-S promoter only in transgenic plants however; no such bands were seen in the non-transformed plants. The dot blot analysis data indicate that clear dots specifically hybridized with the probe prepared from the *gfp* gene. The data of the present study reveal that 5 sec. sonication followed by *Agrobacterium* transformation resulted in high frequency of transgenic shoot production comparing to biolistic mediated transformation. The improved transformation protocol reported here is repeatable and can replace the ordinary *Agrobcaterium* transformation method and could be used to express the genes of interest in economically important crop plants.

Introduction

The brassicaceae (Cruciferae) or mustard family includes many economically important edible and industrial oilseed, vegetable, condiment, and fodder crop species. The most important edible oil crop is canola or oilseed rape (*Brassica napus* (Cardoza and Stewart, 2004; Warwick, 2011), vegetable oils remain one of the most difficult problems of the Egyptian agriculture, so expansion of the new oil crop (canola production) cultivation in the new reclaimed lands is probably the most promising approaches (Dawood *et al*. 2013).

To enhance the quality and quantity of canola products, one solution is the transfer of useful properties such as insect resistance and herbicide resistance via genetic transfer techniques (Tarinejad *et al*. 2013).Several biotic and abiotic stress factors known as to limit plant productivity worldwide. Improvement of canola plants through traditional methods is labor and time consuming process therefore the genetic engineering techniques combined to the traditional methods will solve the problem (Lichtenstein and Draper, 1985, Moghaieb *et al*. 2006).

It is well documented that regeneration and gene transfer rate to canola varieties are very variable and is highly dependent on genotype (Keara and Mathias, 1992; Cardoza and Stewart, 2004; Poulsen, 1996 and Mashayekhi *et al.* 2008).

The *B. napus* is an important model for crops; it is among the first genetically transformed crop plants in the history of genetically modified crops. Several reports dealing with the transformation of canola with *Agrobacterium tumefaciens* have been published (Fry *et al.* 1987, Bhalla and Singh 2008). Various techniques including PEG-mediated DNA uptake (Golz*et al*. 1990), microprojectile bombardment (Chen and Beversdorf 1994), electroporation (Chapel and Glimelius 1990), microinjection (Jones-Villeneuve *et al.* 1995), protoplast transfection (Hu *et al*. 1999) and microspore transfection (Huang 1992, Palmer and Keller 1997) have been tested for the transformation of canola. The success of transformation in canola depends on genotype, so the method of transformation should be selected depend on the available genotype.

Green fluorescent protein (GFP) can replace conventional methods and serve as an *in vivo* real-time marker for the presence of transgenes (Stewart *et al.* 1996a, b). GFP was cloned from the jellyfish, *Aequorea victoria*, and has been expressed in many different organisms (Prasher *et al.* 1992; Chalfie *et al.* 1994; Leffel *et al.* 1997). When coupled to another transgene, GFP can indicate the presence of transgenic material by whole-plant fluorescence without destructive tissue sampling or laboratory analysis (Leffel*et al.* 1997). GFP could facilitate the monitoring of gene flow from agriculture to the environment, which would be intractable by conventional molecular techniques. For optimizing plant transformation protocols, early and strong expression of a reporter gene can be useful, and GFP has become a widely tested reporter (Niedz *et al.*, 1995; Elliott *et al.*, 1999; Jordan, 2000).

In this study the efficiency of expressing *gfp* in transgenic canola using biolistic gun and sonication assisted *Agrobacterium*-mediated gene delivery was compared. The stable integration and expression of *gfp* in the transgenic plant genome was confirmed using different molecular approaches.

Materials and methods

Plant materials

Two canola genotypes, namely Serw-3, Serw-4 were kindly provided by Field Crop Institute, Agricultural Research Center, Ministry of Agriculture – Egypt.

Canola cultivars seeds namely, Serw-3,Serw-4 were surface sterilized by immersion in 70% ethanol followed by immersion in 3% (v/v) sodium hypochlorite, and rinsed in sterile distilled water. The sterilized seeds were germinated in flasks on 0.7% agar (w/v). The cultures were incubated at 25°C under a 16/8 h day/night photoperiod (1000-Lux).

Hypocotyl segments (0.5 cm in length) were excised from 5-day-old canola seedlings. The explants were transferred to the callus induction medium containing MS salt, 3% (w/v) sucrose, 1% (w/v) agar in addition to 1 mg/l 2,4-D. Two weeks later the explants were transferred onto shoot induction (MS) medium (Murashige and Skoog, 1962), supplemented with BA 4.50 mg/l, pH 5.8 according to Moghaieb *et al.* (2006).

Genetic transformation in canola

Bacterial strain

Agrobacterium tumefaciens strain LBA4404 harbouring the binary vector (*p35S-gfp*) (kindly provided by Professor N. Tanaka, Hiroshima University, Japan) was grown overnight in 30 ml of LB medium (Luria and

Burrow, 1955) containing 50 µg/ml Kanamycin at 37°C fig (1) . The plasmid preparation was carried out according to the standard protocol described in Sambrook *et al.* (1989).

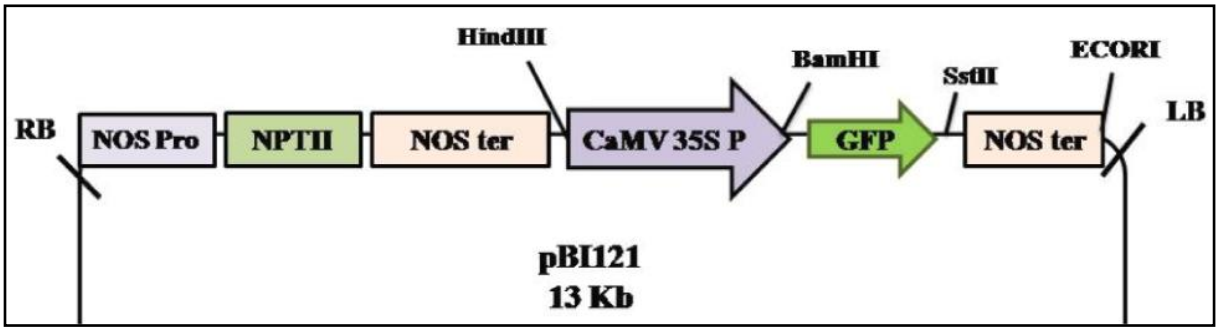

Fig. 1.Schematic representation of the *PBI-121* plasmid harboring the *gfp* gene under the control of 35-S promoter and *nos*terminator.

Transformation methods:

Two different transformation methods were used to express *gfp* in transgenic canola plants as follows:

biolistic gun mediated transformation:

The hypocotyl of two canola cultivars (serw-3, sewr-4) were transformed with *gfp* using gene gun mediated transformation. Microprojectile bombardment was carried out using (model: PDS- 1000/HeBiolistic® Particle Delivery System, BIORAD) and previously published procedures for adsorption of DNA onto gold particles (Singrsit*et al*., 1997). Bombardment pressures were adjusted to (1350 psi).

Sonication assisted Agrobacterium mediated transformation (SAAT)

The *A. tumifaciens*strain *LBA4404* harboring the plasmid *P35s- gfp* (Jefferson *et al.*, 1987) was grown overnight in 30 ml of LB medium containing 50 mgl/l kanamycin sulfate (Sigma-Aldrich, Japan) at 28°C. The hypocotyl segments (0.5 cm in length) were prepared from 5-day-old canola seedlings (cultivars: Serw-4 and Serw-3) and treated with sonication for different periods of time (5, 10, 20 and 30 seconds)using Sonicor (model No./SC/152, N.Y) water bath, then immersed in the bacterial suspension for 5 min. Thereafter, the segments were blotted on sterilized filter paper, placed onto a co-cultivation medium, which consisted of MS medium supplemented with 1mg/l 2, 4-D and then were incubated under dark conditions. Then the explants were transferred onto shoot induction medium supplemented with 4.5 mg/l BA in addition to 50 mg/l kanamycin sulfate. The plates were sealed with a parafilm and incubated at 26°C under a 16/8-h light/dark photoperiodic regime (1000-Lux).

Molecular conformation of the transformation events:

In order to confirm the stable integration and transformation of the transgenic plantlet, total genomic DNA was isolated from both of *gfp* transformed and non-transformed plants according to the method described previously by Rogers and Bendich (1989).Genomic DNA subjected to PCR analysis using 35S promoter specific primers. The sequence of forward and reverse primers for 35S promoter 5'-AAA GGA AGG TGG CTC CTA CAA AT-3' and 5'-CCT AGT AAA GTA AAC CTC TCC-3', respectively.

The reaction mixture (20 µl) contained 10 ng DNA, 200 µM dNTPs, 1 µM of each primer, 0.5 units of Taq polymerase (AB gene House, UK) and 10-X Taq polymerase buffer (AB gene House, UK). Samples were heated to 94°C for 5 min, then subjected to 35 cycles of 1 min at 94°C; 1 min at 56°C and 1 min at 72°C. The PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide. Plants which showed positive result for 35S promoter were acclimatized and transferred in to the growth chamber to collect the T_1 seeds.

Dot blot. Total genomic DNA was isolated from both *gfp* expressing (T_0) and control plants as mentioned above to show the integration of the *gfp* gene, then denatured for 10 min, and spotted to a nylon membrane followed standard protocols. Labeling of the probes, hybridization and detection was carried out using the Biotin Chromogenic Detection kit #K0661, #K0662 according to instructions provided by the manufacturer (Ferments Life Sciences, USA).

Detection of GFP illumination:

GFP fluorescence was detected during the transformation experiment and canola life cycle. Developing callus was screened within 48- 72 hours after bombardment using UV light (Spectroline high-intensity long wave UV lamp,

BIB-150P model, 350 nm). Also seedlings plants at the 4–8 leaf stage and canola pods were viewed to visualize green fluorescence of transgenic plants.

Result and Discussion

Several plant transformation methods have been reported and can be applied to generate transgenic plant species, among these methods *Agrobacterium* and biolistic gun are widely used. Biolistic transformation was initially used as an alternative method for generating transgenic plant species. This method offers a mean to bypass genetic barriers to genotype independent transformation, direct transformation of organized tissues and relatively rapid recovery of transgenics (Christou, 1996a, b; John, 1997). Particle gun bombardment requires the identification and selection of stable germline events among a population of chimeric transformants and is yet another long, drawn out process that requires the maintenance of a large number of plants and requires considerably more plants than *Agrobacterium* mediated transformation. Biolistic also is inefficient in terms of penetration to germline layers and secondary damage to target tissue (Wilkins et al., 2000).

Plant transformation using *Agrobacterium* is affected by both host specificity and the inability of *Agrobacterium* to reach the right cells in the target tissue so a new and efficient *Agrobacterium* based transformation method that overcomes these limitations and enhances DNA transfer was required (Hussain *et al.,* 2007).

In order to improve the production of transgenic canola plants, the transformation efficiency of the biolistic gun and the sonication assisted Agrobacterium mediated transformation (SAAT) was compared.

Before the co-cultivation with *A. tumefaciens* strain LBA4404, the hypocotyls explants prepared from 5 days-old seedlings of the canola cultivars Serw-3 and Serw-4 were subjected to sonication treatment for 5, 10, 20 and 30 sec. respectively. The transformation percentage calculated based on *gfp* gene expression and PCR analysis data indicate that, the *Agrobacterium* transformation efficiency reached 55% when the explants was exposed to 5 sec. of sonication and then it was remarkably decreased with increasing the exposure time (Fig.2). These results agreed with Hussain *et al.,(*2007) who reported a new procedure for cotton transformation based on cavitations caused by sonication which results in thousands of micro wounds on and below the surface of plant tissue and allow *Agrobacterium* to travel deeper and completely throughout the tissue. According to their data, this wounding fashion increases the probability of infecting plant cells lying deeper in tissue. On the other hand our results disagree with Bakshi *et al* (2011) who found that the *A. tumefaciens* transformation efficiency in cowpea is increased by increasing the time of sonication and they reported that, sonication for 20 s resulted in highest transient GUS expression efficiency.

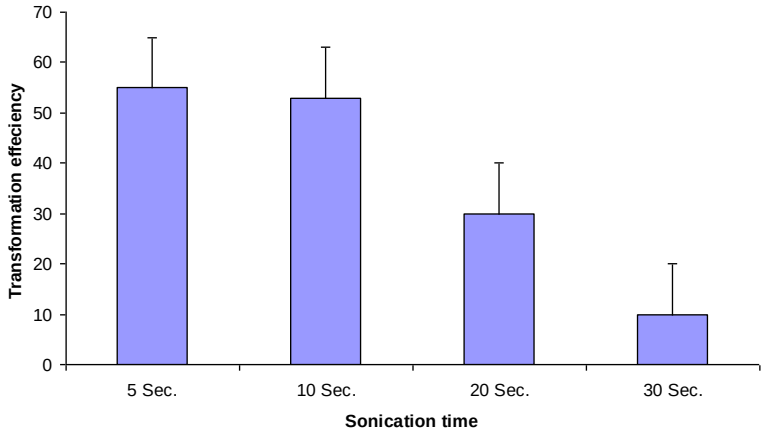

Fig. 2. The effect of sonication time on *Agrobacterium* transformation efficiency in canola

The hypocotyl explants isolated from both cultivars were transformed by two different methods, the first method mediated by A. *tumefaciens* strain LBA4404 harboring the pBI 121-gfp plasmid after exposure to 5 sec. of sonication and the second method mediated by biolistic gun.

The explants were transfer to callus induction medium MS medium containing 1mg/l 2,4-D (Moghaieb *et al*., 2006). It was noted that callus proliferation started from cut ends of the transformed hypocotyls from both transformation methods a similar result was obtained by Maheshwari *et al*. (2011).

Hypocotyl explants-derived calli were placed on regenerating media containing 4.5 mg/l BA for two weeks during the selection culture; the explants were sub-cultured into fresh medium containing 50 µg/ml kanamycin which greatly reduced the number of escapes (Fig.3). During the selection process successfully transformed calli continued to grow vigorously to produce shoot initiations, whereas the non-transformed ones failed to form shoots and eventually bleached and became necrotic within 3 weeks. The present results agree with Mashayekhi *et al.* (2008) who reported that, the highest frequency of shoot regeneration was achieved on the 1 mg/l 2,4-D and 4.5 mg/l BAP using hypocotyl-derived calli. Transformation efficiency of both methods was calculated as No. of kanamycin resistant plants / No. of shoots obtained and also as the No. of plants that show positive PCR results with 35-S promoter/No. of shoots.

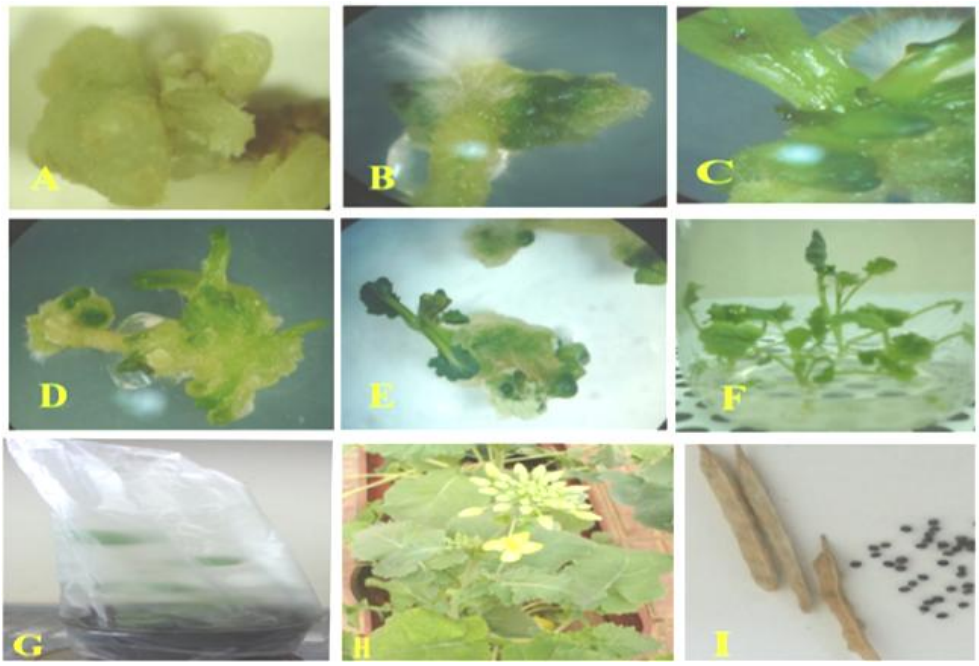

Fig 3. The recovery of fertile transgenic canola plants, A: embryonic callus, (B- C) Germination of somatic embryos in shoot regeneration medium, D: shoot buds, E: Shoot initiation F: regenerated plants, G: Acclimatization process , H: Flowering plants and I: Harvested pods.

To confirm the stable integration of the T-DNA in the putative transgenic plantlet genomes, all T_0 plants that can survive at 50 µg/ml kanamycin containing medium were subjected to dot blot hybridization and to PCR analysis using primers specific to the 35 S-promoter. The dot blot analysis data indicate that clear dots specifically hybridized with the probe prepared from the *gfp* gene (Fig. 4-a). Fig (4-b) showed a clear band at 250 bp was detected only in transgenic plants however, no such bands were seen in the non-transformed controls under identical conditions. The *gfp* gene expression was detected in transgenic plants only, Fig. (5) shows that, the exposure of transgenic callus, putative transgenic plants and seeds to UV light resulted in green light illumination in transgenic plant only (*gfp* gene expression).

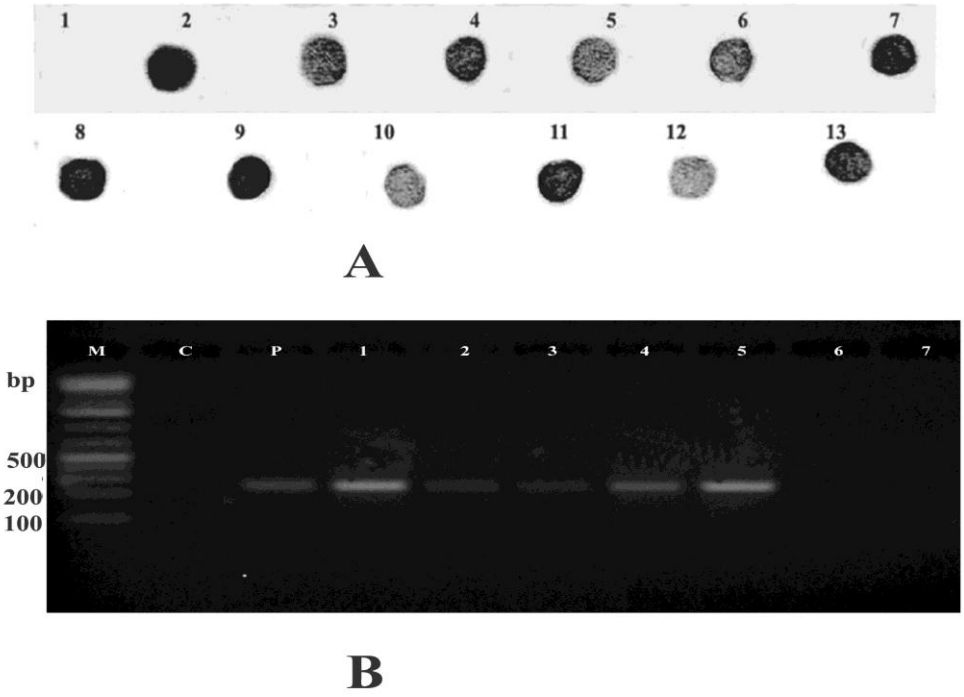

Fig 5 . Molecular analyses of transgenic canola plants: A : Dot blot analysis of transgenic plants. 1: represent Negative control, 2: represent Positive control, and 3-13: show the transgenic canola plants. B: PCR analysis using 35-S promoter specific primer. C: Negative control, P: positive control (plasmid), 1-7: transgenic plants.

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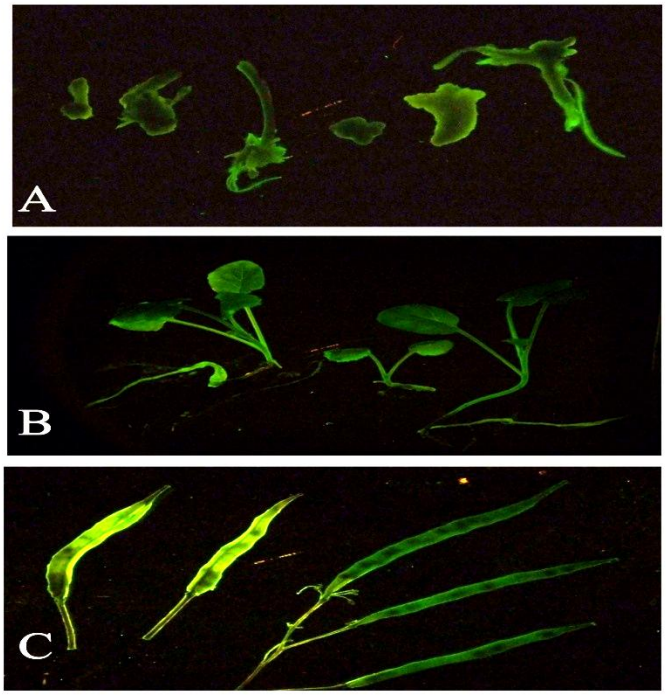

Fig 7. Detection of GFP fluorescence in the life cycle of canola.. A- Canola calli under UV light. B- GFP canola stems and roots under ultraviolet light. C- GFP canola pods under ultraviolet light from T1 plants, 12 weeks post-germination

The transformation efficiency of the two tested canola cultivars after biolistic gun and selection in the presence of 50 mg/l kanamycin sulfate was 25 and 16%, with a total of 20 and 10 transgenic plants produced form the cultivars Serw-4 and Serw-3, respectively. While the sonication assisted *Agrobacterium* mediated transformation (SAAT) method results in a higher transformation rate (55% and 33%) with a total of 55 and 30 transgenic plants produced for the cultivars Serw-4 and Serw-3, respectively (Table 2).

Table 2. The transformation frequencies of the two canola cultivars transformed with *gfp* gene using Biolistic gun and sonication assisted *Agrobacterium* mediated transformation (SAAT)

Transformation method	Cultivar	No. of calli	No. of regenerated shoots	No. of Kan.+ regenerated plants	PCR primer specific to 35S promoter	Transformation %
Biolistic gun	**Srew-3**	200	60	10	10	16
	Serw-4	200	80	20	20	25
SAAT	**Srew-3**	200	90	30	30	33
	Serw-4	200	100	55	33	55

The data of the present study reveal that 5 sec. sonication followed by *Agrobacterium* transformation resulted in high frequency of transgenic plants production comparing to biolistic mediated transformation (Table-2).The improved *Agrobacterium* mediated transformation method described in this investigation showed higher transformation efficiency (55%) for the cultivar Serw-4 compared to 19% transformation using *Agrobacterium* without sonication previously reported by Moghaieb *et al*. (2006).

Based on the data of the present study we can concluded that, treatment of explants by sonication improves *Agrobacterium* transformation efficiency in both cultivars by increasing the wounding sites and as a consequence increase phenolic compounds production from these sites that increase the transformation events. The improved transformation protocol reported here is repeatable and can replace the ordinary *Agrobcaterium* transformation method and could be used to express the gene of interest in economically important crop plants.

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