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Plant regeneration and *Agrobacterium*-mediated gene transfer in brinjal (*Solanum melongena* L.)

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Abstract: The research work was conducted to standardize a protocol for plant regeneration and genetic transformation in brinjal. Plant regeneration studies were carried out using two types of explants v*iz* cotyledon and hypocotyl. The cotyledon explants showed high frequency of shoot regeneration (77.77%) on MS medium supplemented with 2.5 mg/l Kinetin and 0.4 mg/l Indole-3-acetic acid (IAA) as compared to hypocotyl (50 %) on Murashige and Skoog's (MS; 1962) supplemented with 2.5 mg/l 6-Benzylaminopurine (BAP) and 0.5 mg/l IAA. MS medium supplemented with 0.10 mg/l IAA was found to be best for root regeneration (81.81%). The brinjal plantlets were able to regenerate within 6-7 weeks. Regenerated plantlets were acclimatized. For genetic transformation, disarmed *Agrobacterium tumefaciens* LBA 4404 strain containing a reporter â-glucuronidase (*gus*) gene in binary vector pBI 121 system along with kanamycin resistance gene *(npt-II*) for selection in both bacteria and plant was used for co-cultivation experiment to transfer *gus* and *npt-II* genes in brinjal cells. The transformed cells were able to grow on selective shoot regeneration medium containing kanamycin and cefotaxime after co-cultivation only, whereas the control explants died on the selective medium. Transformation could be scored as early as 4 weeks after selection. Most of the calli obtained on the selective medium were GUS positive. Putative transgenic shoots were obtained, which were able to grow on the selective medium containing 50 mg/l Kanamycin.

Keywords: Brinjal, Plant regeneration, genetic transformation, binary vector

Introduction

Brinjal (*Solanum melongena* L.) also known as aubergine (or egg plant) is an admired vegetable crop belonging to the family *Solanaceae*; grown all over the world, though there is a heavy concentration in Asia. In 2010, China and India contributed respectively 58% and 25% of the world's production of brinjal (FAO, 2011). Eggplant has a high nutritive value (protein 1.4%, fat 0.3%, minerals 0.3%, carbohydrates 4%). It has various medicinal uses also that make it a valuable addition to diet. The crop is mainly cultivated on small family farms and is an important source of cash income for many resource poor farmers. Being an economically important crop, the application of plant tissue culture and plant genetic engineering in brinjal cultivation has special value to obtain improved or desirable traits like disease resistance, insect resistance, and to enhance the shelf life of this fruit crop. However, before working on this approach, it is important to know that whether the somatic cells of a given species/cultivar are able to regenerate and give rise complete plantlet, and are amenable for genetic transformation in brinjal cells with marker and reporter genes. Previous studies have demonstrated conventional approaches for establishing or improving a regeneration system for brinjal and its close relative tomato has been to screen a variety of explants, media and plant growth regulators regimes (Alicchio *et al.,* 1982; Sharma and Rajam, 1995; Tarre, 2004; Rai *et al*. 2012; Rai *et al*. 2103). Genetic engineering of brinjal has already been reported (Guri and Sink, 1988; Fari, 1995; Pal *et al*. 2009, Singh *et al.,*2010 ; Rai *et al*. 2103; Singh *et al*. 2013).

The present investigation describes a reproducible and efficient plant regeneration system for Pusa Purple Long cultivar of brinjal and discuss the effect of BAP and Kinetin on direct and indirect organogensis and genetic transformation and expression of neomycin phosphotransferase-II (*npt-II*) and â-glucuronidase (*gus*) genes in the transformed calli using the binary vector (pBI 121) in *Agrobacterium tumefaciens*. We are currently using these tissue culture and genetic transformation techniques for the transformation of brinjal by genetic engineered *Agrobacterium tumefaciens*.

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Material and Methods

Plant material and nutrient medium

Solanum melongena was taken as experimental material during the present investigation. The certified seeds of brinjal (*Solanum melongena* L. *cv*. Pusa Purple Long) were obtained from Indian Agricultural Research Institute, New Delhi. The seeds of brinjal were surface sterilized and inoculated on MS half strength basal medium with 0.5% sucrose, 0.8% agar and pH 5.8 for germination. After inoculation culture tubes were covered with black paper or kept under dark conditions for seed germination. Cotyledon and hypocotyl explants were excised from 10 days old *in vitro* germinated seedlings and cultured on MS medium supplemented with 100 mg/l meso-inositol, 3% sucrose, different concentrations and combinations of plant growth regulators and gelled with 0.8% agar. All the cultures were kept in the culture room at 26±2°C with 16hrs photoperiod.

Plant regeneration

Cotyledon and hypocotyl explants were taken from 10 days olds aseptically grown seedling and cut into approximately 0.5-1.0 cm long sections and cultured on MS medium supplemented with different concentrations and combinations of BAP and IAA, Kinetin and IAA, Kinetin and NAA, and BAP and NAA. For every combination, six flasks with five explants were inoculated. These explants were also evaluated for percent explant regeneration after 25-28 days in culture. The shoots regenerated from cotyledon and hypocotyl explants were transferred on MS medium supplemented with various concentrations of IAA and IBA for root regeneration to obtain complete plantlets.

Genetic Transformation

Agrobacterium strain and plasmid

Disarmed *Agrobacterium tumefaciens* LBA4404 strain containing â- glucuronidase (*gus*) gene in binary vector pBI 121 system along with a kanamycin resistance *(npt-II)* gene for selection in both bacteria and plant were used for co-cultivation experiment to transfer *npt-II* and *gus* genes in brinjal. (Fig. 1). The *Agrobacterium tumefaciens* LBA4404 strain was obtained from Dr. D.P.S Verma, Plant Biotechnology Centre, The Ohio State University, Columbus, USA.

Maintenance of Agrobacterium *strain*

Agrobacterium strain was maintained by subculturing the bacterial colonies on the YMB medium containing 50 mg/l Kanamycin. After the proper growth of the bacterial cells, the plates were kept at low temperature for storage.

Genetic transformation and selection of transformed cells

Fresh culture of *Agrobacterium* strain was prepared by inoculating a small bacterial colony into 10 ml selective liquid YMB medium. The cultures were kept overnight at 28°C in an orbital shaking incubator. Cotyledon and hypocotyl explants were cut into small pieces of 0.5-1.0 cm size and pre-cultured on the shoot regeneration medium (on which high frequency shoot regeneration was obtained) for 48 hrs. The fresh cultures of *Agrobacterium* were centrifuged for 10 minutes at 5000 rpm and the supernatant was discarded to get pellet. The pellet was resuspended in MS liquid medium to get the concentration of 10^8 cells/ml. Pre-cultured cotyledon and hypocotyl explants were infected with fresh cultures of *Agrobacterium* cells for 20 seconds, blotted on pre-sterilized filter paper and inoculated on shoot regeneration medium for co-cultivation. After 48 hrs of co-cultivation, the explants were transferred to the fresh selective shoot regeneration medium containing kanamycin (50 mg/l) and cefotaxime (500 mg/l) for the selection of transformed cells and to inhibit *Agrobacterium* growth. For the preparation of selective regeneration medium, kanamycin and cefotaxime were added by the filter sterilization. Cotyledon and hypocotyl explants were subsequently subcultured on the fresh selective regeneration medium in order to check the excessive bacterial growth and to promote further growth and differentiation of the selected transformed cells.

β-glucuronidase expression assay

Biochemical/ Spectrophotometric determination of β-glucuronidase activity in the transformed callus and control callus was conducted according to Herman and Depicker (1987). Reaction mixture in a total volume of 1 ml contained 1mM p-nitrophenyl β-D-glucuronide and suitable volume of enzyme extract. The reaction mixture was incubated at 37°C for 60 minutes and was stopped by stop buffer. The absorption was measured at 415 nm (with Perkin Elmer UV Spectrophotometer) using the reaction without enzyme extract as blank and non-transformed cells/callus as control. The molar extinction coefficient of p-nitrophenol is 14000, thus an absorbance of 0.014 represents 1 nano mole of p-nitrophenol liberated (product of the reaction). The enzyme activity is expressed in nano mole p-nitrophenol liberated/hr/g fresh weight.

Statistical Analysis

The data recorded for the different parameters were subjected to completely randomized design. The statistical analysis based on mean values per treatment was made using analysis of variance of CRD.

Results

10 days old aspectically grown cotyledon and hypocotyl explants were used for plant regeneration studies. After one week of inoculation, the size of the both explants had increased and little callus formation was seen at the cut ends of the explants (Fig.2). Of the various combinations of plant growth regulators tried in MS medium, high percentage of shoot regeneration was obtained from cotyledon explants (77.77%) (Table 1) and hypocotyl (50%) explants on MS medium containing 2.5 mg/l Kinetin and 0.4 mg/l IAA and 2.5 mg/l BAP and 0.5 mg/l IAA respectively, proved better to any other plant growth regulator combinations. The regenerated shoots elongated on the same medium.

Table 1: Effect of various combinations and concentrations of cytokinins and auxins (in MS medium) on shoot regeneration from cotyledon explants of brinjal (*Solanum melongena* L. cv. Pusa Purple Long).

Sr. No	Medium composition	Average number of shoot formed per explant	Percent shoot regeneration
1	2.0 mg/l BAP + 0.3 mg/l IAA	2.0±1.50	38.3 (35.25)±0.34
2	2.5 mg/l Kinetin + 0.4 mg/l IAA	2.8±0.30	77.77 (61.86)±0.23
3	2.0 mg/l Kinetin + 0.4 mg/l IAA	2.0±0.00	71.11 (57.49)±0.06
4	1.5 mg/l Kinetin + 0.4 mg/l IAA	1.6±0.23	66.66 (54.73)±0.33

Table 2: Effect of various combinations and concentrations of cytokinins and auxins (in MS medium) on shoot regeneration from hypocotyl explants of brinjal (*Solanum melongena* L. cv. Pusa Purple Long).

Sr. No	Medium composition	Average number of shoot formed per explant	Percent shoot regeneration
1	1.5mg/l BAP + 0.3mg/l IAA	0.4±0.00	40 (39.23)±1.15
2	2.5 mg/l BAP + 0.3 mg/l IAA	0.4±0.05	33.3 (35.25)±0.68
3	2.0 mg/l Kinetin + 0.3 mg/l IAA	0.4±0.05	46.66 (43.09)±0.15
4	2.5 mg/l Kinetin + 0.3 mg/l IAA	1.2±0.11	40.00 (39.23)±1.15

Table 3: β- glucuronidase activity (n-moles p-nitrophenol liberated/hr/g fresh weight) in non-transformed and transformed cells/callus from hypocotyl and cotyledon.

Sr. No.	Sample	B-glucuronidase activity (nano-mole p-nitro phenol liberated/hr/g fresh weight)
1	Control (Non transformed callus)	0.00
2	Transformed calli Clone-1	1114±0.88
3	Clone -2	1128±1.20
4	Clone -3	728.5±0.95
5	Clone -4	864.2±0.58
6	Clone -5	892.8±0.61
7	Clone -6	814.2±0.64
8	Clone -7	1007±1.53
S.E. ±		±0.13

0.014 OD (Absorption) = 1 nano mole p-nitrophenol liberated (product of reaction)

Elongated shoots (2-3 cm length) were excised and cultured on MS medium supplemented with 0.05, 0.10 and 0.20 mg/l of IAA. The period for induction of roots was variable among various concentration of IAA but it generally 8-10 days in culture. The number of shoots forming roots was highest on MS medium containing 0.10 mg/l IAA (81.81%). Brinjal plantlets were able to regenerate within two months and young plantlets were transferred to pots containing a mixture of sand and soil (1:1) and acclimatized.

Genetic Transformation

The cotyledon and hypocotyl explants were pre-cultured on the standardized shoot regeneration medium for 48 hrs. The pre-cultured explants were co-cultivated with *Agrobacterium tumefaciens* strain LBA 4404 containing â- glucronidase gene (*gus*) in binary vector (pBI 121) system along with disarmed helper Ti plasmid for 48 hrs and then transferred to the fresh selective shoot regeneration medium containing cefotaxime and kanamycin were added into the medium to kill the *Agrobacterium* cells and to select the transformed brinjal cells, respectively.

The genetically engineered Agrobacterium strain, used in the genetic transformation studies of brinjal had two genes viz, neomycin phosphotransferase-II (npt-II) and â-glucronidase (gus) genes under the control of NOS promoter and 35S promoters, respectively in a binary vector system. Callus formation was observed after 25-30 days at the cut edges of the cotyledon and hypocotyl segments and also at the wound site where the tissue got damaged during inoculation. The non-transformed tissue (control) did not survive on the selective medium containing kanamycin (50 mg/l). The developing calli

Fig.1. Structure of expression vector: T-DNA region of pBI 121, containing I) transcriptional fusion of NOS promoter with the coding region of *npt-II* and NOS terminator II) transcriptional fusion of CaMV 35S promoter with the coding region of *gus* and NOS terminator. *LB,* Left border of T-DNA; *RB,* Right border of T-DNA; *35S,* CaMV 35S promoter; *NOS ter,* Nopaline synthase terminator; *NOS pro,* Nopaline synthase promoter; *npt-II,* Neomycin phosphotransferase-II; *gus,* b-glucuronidase.

from the explants were tested for β-glucronidase enzyme activity.

Putative transgenic shoots were regenerated from the transformed calli obtained from cotyledon and hypocotyl explants of brinjal on the selective medium (Fig. 2). The regeneration of roots from in vitro developed shoots and their analysis are in progress.

β-glucronidase enzyme assay was carried out to confirm the transfer of *gus* gene from *Agrobacterium* into the genome of cells of brinjal. Chimeric *gus* gene was expressed in transformed calli of brinjal and the calli were also able to grow on the selective medium containing 50 mg/l kanamycin. Seven kanamycin resistant calli were obtained which showed β-glucronidase activity. The enzyme activity was not observed in the non transformed calli of brinjal. The gene expression was reliably measured from a very small transformed callus of brinjal. The transformation experiment could be scored as early as four weeks after selection. Phenotypic and enzymatic data indicated that the chimeric *npt-II* and *gus* genes were expressed in transformed brinjal cells/callus using binary vector *Agrobacterium* system.

Discussion

An efficient tissue culture system for high frequency plant regeneration from cultured tissues is a prerequisite for the success of plant transformation mediated by *Agrobacterium tumefaciens*. In this study, we have developed an efficient plant regeneration system for brinjal *cv*. Pusa Purple Long, in which 77.77% shoot regeneration from cotyledon and 50% shoot regeneration from hypocotyl were obtained on MS medium containing 2.5 mg/l Kinetin and 0.4 mg/l IAA and 2.5 mg/l BAP and 0.5 mg/l IAA, respectively. In brinjal, organogenesis had been obtained using various explants viz. leaf, petiole, cotyledon and hypocotyl explants by manipulating growth regulating substances in culture medium (Kamat and Rao, 1978; Allichio *et al.,* 1982; Sharma and Rajam, 1995; Tarre 2004; Reddy *et al.,* 2004; Neelima and Reddy, 2005; Alpsoy and Seniz, 2007; Pal *et al*. 2009, Rai *et al*. 2103; Singh *et al*. 2013).

Fig 2 Plant regeneration studies from cotyledon and hypocotyl explants of brinjal (*Solanum melongena* L. cv. Pusa Purple Long. A) Shoot regeneration from cotyledon derived callus after 25 days in culture on MS medium supplemented with 2.5 mg/l Kinetin+0.4 mg/l IAA. B) Callus proliferation and shoot regeneration obtained from cotyledon explant after 22 days of culture on MS medium supplemented with 2.5 mg/l BAP+0.5 mg/l IAA. C) A complete well developed plantlet with healthy roots after 20 days in culture on MS medium supplemented with 0.10 mg/IAA. D) *In vitro* regenerated plantlet of brinjal covered with polyethene bags to maintain humidity during hardening. E) Callus formation from cotyledon explants on selective medium (MS Basal + 2.5mg/l Kinetin+ 0.4mg/l IAA) after 29 days in culture, some explants which are not transformed turn brown. F) Shoot regeneration from hypocotyl derived callus on selective medium (MS Basal + 2.5mg/l BAP + 0.5mg/l IAA) after 35 days in culture.

The *Agrobacterium tumefaciens* LBA4404 strain was used in the genetic transformation studies of brinjal have two genes i.e. neomycin phosphotransferase-II (*npt-II*) and â- glucuronidase (*gus*) in binary vector system under the control of 'NOS' promoter and CaMV35S promoter respectively. Transformed calli were obtained from cotyledon and hypocotyl explants on the selective medium containing 50 mg/l kanamycin. The non transformed (control) callus did not survive on the selective medium. The cloned β- glucuronidase gene in binary vector has been used successfully to monitor the expression of chimeric genes introduced into brinjal cells via *Agrobacterium*- mediated gene transfer method.

To date, a number of reporter and marker gene have been used in studies of gene expression in higher plants. The two most useful reporter genes have been the bacterial genes, chloramphenicol acety transferase (*CAT*) and neomycin phosphotransferase-II (*npt-II*), which encode enzymes with specificities not normally found in plant tissues (Bevan *et al.,* 1983). However, both *CAT* and *npt-II* are relatively difficult, tedious and expensive to assay (Reiss *et al.,* 1984). Another reporter gene, i.e. the firefly luciferase gene has been used as a marker in transgenic plants, but the enzyme is liable and difficult to assay with accuracy (Ow *et al.,* 1986). â-glucruonidase is superior reporter gene system for plant transformation studies. â-glucruonidase is easily, sensitively and inexpensibly assayed spectrophotometrically, flourometrically *in vitro* and can also be assayed histochemically to localize GUS activity in transformed plant cells and tissues. GUS is very stable and tissue extracts continue to show high level of GUS activity after prolonged storage (Herman and Depicker, 1987).

Phenotypic and enzymatic data indicated that the chimeric *npt-II* and *gus* genes were expressed in the transformed cells/callus of brinjal using binary vector *Agrobacterium* system. The expression of chimeric *npt-II* and *gus* genes has recently been reported in the transformed calli of Himalayan poplar (Thakur *et al.,* 2005), strawberry (Husaini and Srivastava, 2006), *Populus deltoides* (Saraswat *et al.,* 2008), and cauliflower (Sharma *et al.,* 2013). Genetic transformation had been achieved in the various cultivars of brinjal (Guri and Sink, 1988; Fari, 1995; Zhang *et al.,* 2001; Hanur *et al.,* 2006; Rai *et al.,*2013). Though, a number of reports describe transformation protocols, a comprehensive study to optimize factors that dramatically affects transformation (explant type, genotype and cultural conditions) was lacking. Some research work has been done to standardize transformation protocol taking into account the influence of antibiotics and growth regulators (Billings *et al.,* 1997).

In the present study, protocols of plant regeneration and genetic transformation in brinjal tissues have been standardized the use of marker genes would be of great value in transfer of agronomically important genes, since the presence of an early identifiable marker linked to an agronomically desirable gene would permit efficient selection in tissue culture. Attempts are being made to regenerate plantlets form the transformed calli and analyse them for the expression of *gus* and *npt-II* genes. Advances in brinjal plant regeneration (our present data) in combination with transformation technology may eventually lead to the recovery of transgenic plants of brinjal.

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References

Alicchio R, Grosso F, Del and Roschieri F (1982) Tissue culture and plant regeneration form different cultivars of *Solanum melongena*. Experientia 38(4):449-450.

Alpsoy HC and Seniz V (2007) Researches on the *in vitro* androgenesis and obtaining haploid plants in some eggplant genotypes. Acta Horticulturae 729:137-141.

Bevan MW, Flavell RB and Chilton MD (1983) A chimeric antibiotic resistance gene as a selectable marker for plant cell transformation.Nature 304: 184-187.

Billings S, Jelenkovie G, Chin C and Eberhardt J (1997) The effect of growth regulators and antibiotics on eggplant transformation. Journal of American Society for Horticultural Science 122(2):158-162.

Fari M (1995) *Agrobacterium* mediated genetic transformation and plant regeneration via organogenesis and somatic embryogenesis from cotyledon leaves in eggplant. Plant Cell Reports 15:82-86.

Guri A and Sink KC (1988) Organelle composition in somatic hybrids between and atrazine resistant biotype of *Solanum nigrum* and *Solanum melongena.* Plant Science 58:51-58.

Hanur VS, Prakash DP, Deepali BS, Asokan R, Ramachandran Y I, Riaz M and Lalitha A (2006) Synergistic use of hypocotyls explants and high BAP preconditioning for enhanced transformation frequency in brinjal (*Solanum melongena* L.) Journal of Horticultural Sciences 1(2):116-119.

Herman L and Depicker A (1987) β-glucuronidase assay, spectrophotometric and fluorometric assay. EMBO Course on Plant Molecular Biology pp.61-66.

Husaini AM and Srivastava DK (2006) Plant regeneration and *Agrobacterium* mediated gene transfer studies in strawberry tissues (*Fragaria X ananassa* Duch.). Asian Journal of

Microbiology, Biotechnology and Environmental Sciences 8(3):671-678.

Kamat MG and Rao NA (1978) Vegetative multiplication of eggplant (*Solanum melongena)* using tissue culture techniques. Plant Science Letter 13:57-65.

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473–497.

Neelima P and Reddy KJ (2005) *In vitro* callus induction and regeneration with effect of some heavy metals in *Solanum melongena*. Advances in Plant Sciences 18:457-466.

Ow DW, Wood KV, Deluca M, Dewet JR, Helinsky DR and Howell SH (1986) Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. Science 234: 856-859.

Pal JK, Singh M, Rai M, Satpathy S, Singh DV, Kumar S (2009) Development of transgenic eggplant (*Solanum melongena* L.) resistant to shoot and fruit borer. Journal of horticulture science and biotechnology 84: 434-438.

Rai GK, Rai NP, Kumar S, Yadav A, Rathaur S and Singh M (2012) Effects of explant age, germination medium, pre-culture parameters, inoculation medium, pH, washing medium and selection regime on *Agrobacterium*-mediated transformation of tomato. In vitro cellular and developmental biology-plants 48: 565-578.

Rai NP, Rai GK, Kumar S, Kumari N and Singh M (2013) Shoot and fruit borer resistant transgenic eggplant (*Solanum melongena* L.) expressing *Cry1Aa3* gene: Development and bioassay. Crop Protection 53: 37-45.

Reddy KJ, Neelima P and Shekar CC (2004) Effect of heavy metals on somatic embryogenesis. Plant Cell Biotechnology and Molecular Biology 5:129-134.

Reiss B, Sprengel R, Will H and Schaller H (1984) A new sensitive method for qualititative and quantitative assay of neomycin phosphotransferase in crude cell extract. Gene 30: 211-218.

Saraswat A, Thakur AK, Sharma C and Srivastava DK (2008) Plant regeneration and genetic transformation in petiole tissue of *Populus deltoides* Marsh. In: Proceedings of National Symposium on Advances in Biotechnology Research for crop improvement and Food Security. Abstract, pp-92.

Sharma P and Rajam MV (1995) Genotype explant and position effects on organogenesis and somatic embryogenesis in eggplant. Journal of Experimental Botany 46(282):135-141.

Sharma P and Srivastava DK (2013) *In vitro* plant regeneration from leaf and petiole tissues of tomato (*Solanum lycopersicum* cv. Solan Vajr). Journal of Cell and Tissue Research. In Press.

Singh AK, Rai GK, Singh M and Dubey SK (2013) Bacterial community structure in the rhizosphere of a *Cry1Ac Bt*-brinjal crop and comparison to its non-transgenic counterpart in the tropical soil. Microbial Ecology 66: 927-939.

Srivastava DK (2003) Genetic transformation and crop improvement, pp.251-273. In: Biotechnological Strategies in Agro-Processing, JK Arora, SS Marwaha and RK Grover (eds.) Asiatech Publisher, India.

Tang Q, Song M, Zou J, Li Y and Liu R (2005) Study of anther culture *in vitro* ornamental eggplant (*Solanum melongena* L.). Southwest China Journal of Agricultural Sciences 18(2):175-178.

Tarre F (2004) In vitro somatic embryogenesis and adventitious root initiation have a common origin in eggplant. Revista-Brasileria-de-Botanica 27(1):79-84.

Thakur AK, Sharma S and Srivastava DK (2005) Plant regeneration and genetic transformation studies in petiole tissue of Himalayan Poplar (*Populus ciliata* Wall). Current Science 89:664-668.

Zhang XG, Liu Y, Yang Z, Suche G (2001) Establishment of a genetic transformation system in eggplant (*Solanum melongena* L. cv. Sanyuequie). Journal of Southwest Agricultural University 23(3):233-234.