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RESEARCH ARTICLE

AGROBACTERIUM-MEDIATED GENETIC TRANSFORMATION AND REGENERATION FROM LEAF EXPLANTS OF *SOLANUM THORVUM* (SWARTZ) A MEDICINALLY IMPORTANT PLANT.

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Abstract

Solanum torvum commonly known as turkey berry, devil's-fig, or prickly *Solanum* is a plant of great economic importance. Its extract, rich in Solanine and Solasodine (steroidal alkaloids) have beneficial effect on bronchial asthma. It is cultivated in tropics for its immature edible fruits. In India the *S. torvum is* used in medicines to cure diabetes, asthma, cholera, bronchitis and dysuria. The fresh or dry leaf and fruit are said to reduce blood cholesterol level. In this plant is susceptible to a number of diseases and pests capable of causing serious crop losses. This problem has been addressed by hybridizing *S. torvum* plant with wild resistant *Solanum* species, which present a wild genetic diversity and are source of useful agronomic traits. *S. torvum* plant tissues present a high morphogenetic potential that is useful for developmental studies as well as for establishing biotechnological approaches to produce improved varieties such as embryo rescue, *in vitro* selection, somatic hybridization and genetic transformation, In order to generate *S. torvum* leaf explants were cultured on Murashige and Skoog medium containing (0.5mg/L) IAA supplemented with BAP, in addition to either (100 mg/L) Kanamycin and (200mg/L) cefotaxime after co-cultivation with disarmed *Agrobacterium tumefaciens* harboring a plant expression binary vector. Explants co-cultivated with *A. tumefaciens* LBA 4404 produced putative transgenic adventitious shoots at (2.3±0.4) shots/explants, respectively. The transgenic plants were grown to maturity after vernalization in a greenhouse and appeared morphologically normal. Progeny analysis of independent transgenic plants demonstrated that the *gus* gene was transmitted in a Mendelian pattern in 3 lines, indicating a single copied gene was incorporated into the genome.

Introduction:-

Solanum torvum commonly known as turkey berry, devil's-fig, or prickly *Solanum* (Pier 2003) is a plant of great economic importance. Its extract, rich in Solanine and Solasodine (steroidal alkaloids) have beneficial effect on

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bronchial asthma. It is cultivated in tropics for its immature edible fruits (Langeland and Burks 1998). Alkaloids are a class of nitrogen compounds structurally diverse, found in all plant groups, curring most of them in angiosperms (Henriques *et al.,* 2002; Hughes and Shanks, 2002). Many species of the Solanaceae have been regenerated by shoot organogenesis using young leaf explants. *Solanum surattense* (Gupta and Handra, 1982), *Solanum candidum, S.quitoense, Solanum sessiliflorum* (Hendrix *et al.,* 1987), *Solanum melongena* (Mukherjee *et al.,* 1991) and *Solanum commersonii* (Cardi *et al.,* 1993). Arulmozhi and Ramanujam (1997) conducted *in vitro* culture studies on *Solanum trilobatum* L. with foliar and stem explants on MS medium containing IAA, BAP and KIN combinations. Madhavan *et al.,* (1999) induced high frequency of shoot regeneration from mature seeds of *Solanum trilobatum* L. Callus was induced from root and shoot apical region and hypocotyls on MS medium supplemented with 2,4-D. the plant tissue culture methods also provide base for the improvement of crop to induce somaclonal variations, *in vitro* mutations, herbicide tolerance, di-haploid induction, genetic transformation of economically important genes and development of somatic hybrids, efficient plant regeneration protocol is required. Such advance tech-niques in combination with conventional breeding give a momentum to the improvement of a crop thus, realizing the prospects for future research, relevant literature to attempt has been made to formulate a suitable Protocol for efficient micropropagation from seedling shoot tips explants of *S. torvum*.

For genetic improvement of plant, we usually use selection method as well as *in vitro* molecular breeding technique. Plant breeders showing great interest on molecular breeding technique for plant modification genetically because conventional selection method takes long time, tedious and occurs large variation within clones. For molecular breeding based genetic transformation, we know, efficient regeneration systems are prime requirement. Stem segments are used as important explant for genetic transformation system, described in many plant species (e.g., Rastogi and Dwivedi, 2006).

In Some countries, the highest percentage of the acreage of some economically important crops is transgenic; an increasing number of these transgenic varieties are or will soon be generated by *Agrobacterium-mediated* transformation (Gelvin, 2003). Due to the wide host range *Agrobacterium* mediated genetic transformation is very popular method for introducing gene of interest into plant. *A. tumefaciens* as a gene vector is limited to soybean (Hinchee *et al.,* 1988, broad bean (Jelenic *et al.,* 2000), sesame (George *et al.,* 1987) and sunflower (Weber *et al.,* 2003). *Brassica* is also a suitable host for *Agrohacterium* spp. (Godwin *et al.,* l991; Toriyama *et.al.,* 1991). So, the non-oncogenic *Agrobacterium* strain as a vector (Lin dsey, 1992) can make possible to transfer desired gene in *Brassicu.* Still now, a little success in genetic transformation has been reported in oil crop due to their recalcitrant *in vitro* condition (Nisbet and Webb, 1990). The main problem about the *Brassica* is that, the transformed tissues (callus) are not regenerable and the regenerable tissues (meristematic tissues) arc not transformable. Considering all issues, the objectives of the present study was to develop a reproducible any efficient protocol for the insertion of molecular genes into *S. torvum* through *A. tumefaciens* vectors and to standardize the periods of pre culture and co-cultivation required for transformation and to analyze the putative transgenic plants using histochemical *GUS* assay.

Material and Methods:-

Seeds of *S. torvum* collected from the plant grown in the research field. Department of Botany S.R.R. Govt. Arts & Science College Karimnagar. Dried mature seeds were soaked in sterile distilled water for 24 hours and sterilized with 0.1% (w/v) aqueous $HgCl_2$ for 3-5 minutes followed by washing 3 times with sterile distilled water. Later these were dried on sterile on sterile tissue paper under laminar-flow hood 20 seeds per culture bottle were germinated aseptically on Ms basal medium containing 3%(w/v) sucrose and 0.8%(w/v) agar. These culture bottles were incubated at 25 ± 10 C under 16 h photoperiod. Light was provided by cool white fluorescent tubes with an intensity of 50-60 Leaf explants from 30 day old seedlings were used for transformation experiments.

Bacterial Strain:-

The *Agrobacterium* strain used was LBA 4404 harbon ring a binary plasmid PBIN 19 which has a npt II (Neomycin photo transferase II) gene and a vid A (gus gene). The *Agrobacterium* strain was grown on Lury and Bertani (LB) medium plates containing 5.0 gm/L NaCl, 10 gm/L Bactotryptone, 5 gm/L Yeast Extract and 100 mg/L Kanamycin and the pH was adjusted to 7.0 and solidified with 7 gm/L Difco/Bacto Agar.

Transformation and Plant Regeneration:-

For co-cultivation two colonies from a freshly streaked plate were transferred to 10 ml of Liquid LB medium. *Agrobacterium* strain LBA 4404 was grown at 28^{o} C overnight in LB liquid medium containing 100 mg/L Kanamycin(KM} with shaking (approx. 250 rpm). Kanamycin was added since the binary vectors are not

completely stable in *Agrobacterium* in the absence of antibiotic selection for transformation, the hypocotyls explants were submerged and gently shaken in the *A. tumefaciens* suspension for about 10 minutes and blotted dry on a sterile filter paper. Afterwards, they were transferred to shoot regeneration (SR) medium containing MS salts (0.5 mg/L) IAA+(3.0 mg/L) BAP for leaf explants and co cultivated under 16 hr. photo period of 50-60 µmol m-2 S-1 For 3 days at 25 ± 2°C. After co-culture, the explants were washed in the MS liquid medium blotted dry on a sterile filter paper and transferred to the freshly prepared selective SR medium (MS1) supplemented with antibiotics 200 mg/L Cefotoxime and 100 mg/L Kanamycin.(Table-1) Simultaneously a control was also maintained. After 4 weeks, the growing shoots were excised from the primary explants and sub cultured in fresh proliferation selective medium containing 100 mg/L KM (MS2). The green healthy shoots from explants were subjected to 2-3 passages of selection by repeated excision of branches and their exposure to selective elongation medium (MS2).The green shoots were transferred to MS medium containing (0.1 mg/L) with Kanamycin (100 mg/L) for root induction (Table-2). (Fig I B, C and D)

Culture conditions and Data Analysis:-
All the cultures were incubated at 25± 2°C and 16 hr/8 hr photoperiod under 50-60 µ mol m-2 S-1 white fluorescent light. All the experiments were carried out in 10 replicates. The experiments were replaced at least 3 times, keeping all the Parameters unchanged.

Histrochemical Gus Assay:-
The Histochemical GUS assay was carried out according to Staining was done by placing the tissue into X-gluc, staining buffer in a small vessel, X-Gluc stock was prepared by dissolving X-Gluc 20 mg/ml in Dimethyl Sulphoxide (DMSO). To make 1 ml of staining buffer 0.85 µl sterile distilled water was mixed with 100 µl monosodium PO4 (pH-7), 5 ml of X-Gluc stock and 5 µl Triton X-100 in an eppendoraff tubes. The sample was incubated overnight at 37°C. Later these explants were treated with aceto alcohol (1:3 v/v) mixture to remove chlorophyll and then fixed in 70 % ethanol. The tissues were examined under stereomicroscope for the evidence of blue cells. X-glucuronide (5-bromo-4- chloro-3-indolyl glucuronide) is colorless but the indoxy product derived after glucoronidase activity undergoes oxidative dimerisation to from an
Insoluble indigo blue (Fig I A).

Results:-

We have standardization the plant regeneration in *S. torvum* from leaf explants using. MS medium supplemented with 0.5 mg/L IAA+3.0 mg/L BAP. The combination was routinely used for the present transformation experiments. The explant (Leaf) co-cultivated with *A. tumefaciens* formed shoots 6 weeks of culture on selective shoot regeneration medium (MS1). The control explants which were not co-cultivated did not produce when cultured on MS1 medium indicating the effective level of Kanamycin (100 mg/L) (Table-2). Kanamycin sensitivity of leaf explants was assessed prior to *Agrobacterium* transformation to determine the concentration of Kanamycin needed for effective growth of transgenic plants. At (50 mg/L) Kanamycin caused chlorosis and eventual necrosis in all explants by the end of the fourth week. Whereas concentrations of (75 mg/L) and (100 mg/L) Kanamycin completely inhibited the formation of shoot buds. In the present study higher concentration of Kanamycin (100 mg/L) was used for selection of transformants to prevent possible escapes. High percentage of cultures producing green shoots was observed in leaf explants (Table- 2). After four weeks the growing green shoots (Fig I B) from MS1 medium were excised and transferred into MS2 medium for proliferation.

The transformed shoots were multiplied by culturing on MS2 medium containing 100mg/L Kanamycin to confirm the stability of the transgenic shoots. Leaf explants from transgenic plants when cultured on shoot regeneration medium containing Kanamycin showed the plant regeneration thus the stability was also achieved by leaf strip assay. Most of the transgenic clones appeared morphologically normal in comparison with the untransformed plants. The putative transformed shoots which attained 2-3 cm in length were excised and then transferred to the MS3 medium for rooting (Fig I D).

The leaves from transgenic shoots were subjected to in situ GUS assay (Fig I C). The expression of uid a gene was verified by histochemical staining of the leaf of the transgenic plants. The npt II positive regenerants showed the typical indigo blue colouration of X-Glue treatment while the untransformed ones didn't show GUS activity. Also, more than 33% and 27% of the regenerants from leaf explants respectively were Gus positives. These results clearly demonstrate the stability of the transformed plants.

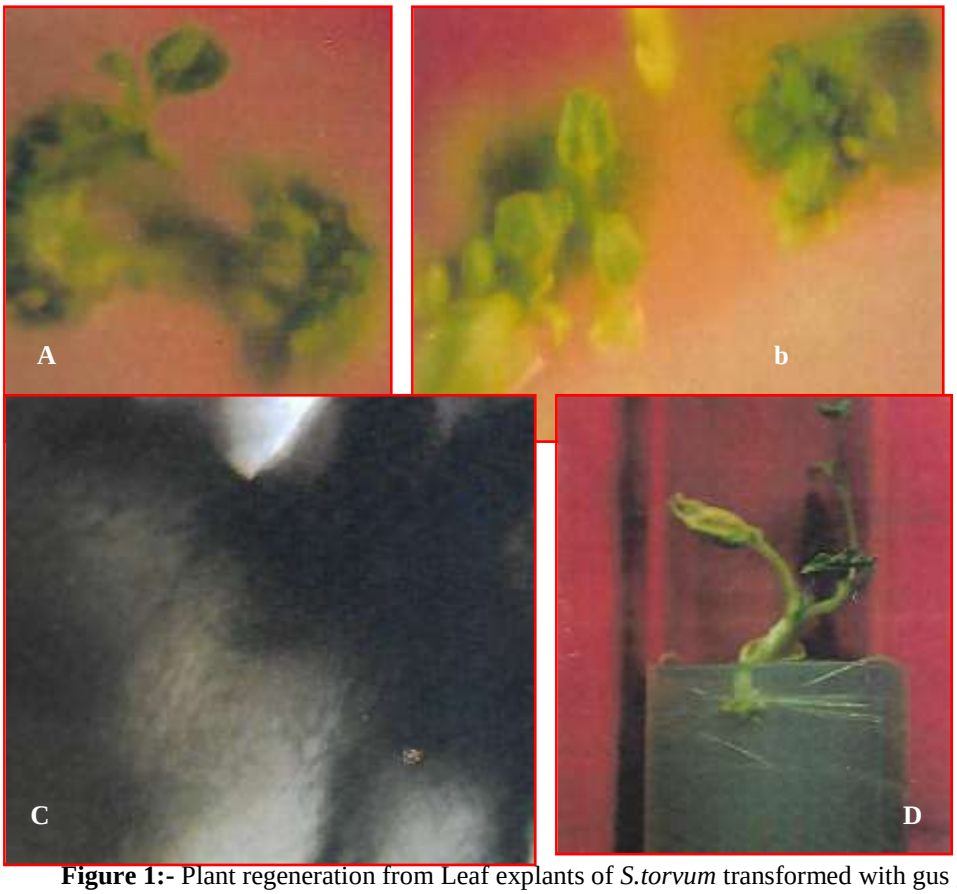

Figure 1:- Plant regeneration from Leaf explants of *S.torvum* transformed with gus gene

A. Blue colour formation on selection medium with 100 mg/ L Kanamycin
B. Shoots elongated from the Kanamycin resistant from leaf explants after 4 weeks of Culture.
C. GUSexpression in transformed tissue (developing shoots after 6 weeks of culture.
D. *In vitro* rooting from Kanamycin resistant micro shoots after 8 weeks of culture.

Table 1:- Successive growth media used for the transformation and Selection of Transgenic Shoots from Leaf Explants of *S. torvum*

Sl No	Steps and Components of Media	MS 1 Medium	MS 2 Medium
1	Time for *A. tumefacience* Inoculation	8 minutes	----------
2	Co-cultivation with *A. tumefacience*	2 minutes	----------
3	Proliferation of shoots	----------	4-5 Weeks
4	Kanamycine selection and shoot regeneration	----------	6-7 Weeks
5	MS salts gm / L.	4.5	4.3
6	Sucrose gm / L	25.0	30.0
7	Hormone mg/L.	IAA+BAP (0.5)+(3.0)	IAA+BAP (0.5)+(3.0)
8	Cefotaxime mg/L.	----------	200
9	Kanamycine mg / L.	----------	100
10	pH	5.8	5.8
11	Agar-Agar gm/L	8.0	8.0

Table 2:- Successive growth media used for the transformation and selection of Transgenic shoots from Leaf explants of *S. torvum*

Explant	Strain	No of Explants Culture	Explants Bleached	Explants with callus Shoots	Mean No of Shoots/Explants (S.E}*
Leaf**	-----	40	----------	3.0±1.0	7.3±0.3
Leaf	LBA4404	40	15	15±03	2.3±2.0

*** Mean ± Standard Error, ** on Normal Regeneration Medium**

Discussion:-

We have achieved the successful genetic transformation mediated by *A. tumefaciens* strain LBA-4404 which has a binary vector pBIN 19 derivative with an intron containing GUS gene. The successful transformation was also reported in a number of *Solanaceous* species *Solanum melongena* (Flippone and Lurquin, 1989: Rotino and Gleddie, 1990; Leon *et al.,* 1993; Fari *et. al.,* 1995) *S. sismbrifolium* (Rao *et al.,* 1997) *S. muricatum* (Atkinson and Gardner, 1991), *S. tuberosum* (Sheerman and Bevan, 1988); *Lycoperiscon esculentum* (Hood *et.al.,* 1986a) *Capsicum annum* (Liu *et.al.,* 1990); *Nicotina tobacum* (Hood *et.al.,* 1986) and produced transgenic plants. Transformation efficiency was found to be higher in other *Solanaceous* plants. This transformation efficiency is dependent on various factors type of explants, size, explants orientation on selective regeneration medium, gelling agent and plate sealed and the frequency of transfer to fresh selective medium. Frary and Earle (1996) have examined the effect of various factors on efficiency of *Agrobacterium*–mediated transformation in *L. esculentum* cv. Money maker using cotyledon and hypocotyls explants.

Mc Cormick (1991) has reported that the leaf explants were more efficient in generating transgenic shoots as observed in the present investigations. Whereas Liu *et al.,* (1990) has reported that the transformation efficiency was higher in leaf followed by hypocotyls and cotyledon explants and also noted the same differential response between the *A. tumefaciens* strains C58 and A281 used in bell pepper.

The strain C58 showed more transformation efficiency compared to A 281 in all the explants co-cultivated. Although most published protocols report the use of whole cotyledons as explants (Davis *et. al.,* 1991; Mc Cormick, 1991), cutting cotyledons into two or three pieces (depending) on their size is recommended as a way to maximize the number of transformants obtained from a minimum number of seedlings (Fray and Earle, 1996). Armstead and Webb (1987) found that cotyledons of *Lotus corniculatus* were more readily transformed than leaves from seedlings grown *in vitro.* Young leaves were transformed more frequently than old leaves.

However, leaves from old papaya plants were found to be more easily transformed than cotyledons or leaves from young plants (Pang and Sanford, 1988). *Agrobacterium*- mediated genetic transformation efficiency is not only genotype dependent but also varies with the strains used. The stable integration of GUS and NPT II genes in *Mentha arvensis* and *Mentha spicata* has been achieved by *A. tumefacies* mediated gene transfer. Differences in transformation efficiency between *M. spicata* and *M. arvensis* became apparent in the percentage of explants producing Kanamycin resistant (Km R) calli for the two *Agrobacterium* strains used. *M. spicata* explants produced 53% KmR calli with GV 2260/GL and 71% with EHA 105/MOG whereas 5% and 1.2% *M. arvensis KmR* calli were obtained respectively with GV 2260/GI and EHA 105/MOG (Diemer *et. al.,* 1991) reported the difference in degree of transformation caused by three different *A. tumefaciens* strains, pTi-A6 related plasmids (i.e., those in A6 and A66) have less expression of *vir* genes than pTiBO 542 plasmids (i.e., those in strain A 281).

Hussain *et.al.,* (1997) also reported in two varieties of Chick pea (6153 and CM72), *Agrobacterium* strain A281 was found to be more efficient in transformation than C58 Strain A281 was found to be more efficient in transformation than C58. Schroeder *et. al.,* (1993) reported that presence of growth regulators in the co-cultivation medium enhanced transformation frequency in *Pisum.* Venkatachalam *et.al.,* (2000) have also found an important factor for efficient transformation in *Arachis hypogaea* was the 2-day pre culture of the cotyledon explants, which probably served to reduce wound stress and increased the number of competent cells at the wound site.

Similar results were also reported in other species by Muthukumar *et. al.,* (1996). Davis *et. al.,* 1991 have studied the effect of tomato cultivar, leaf age and bacterial strain and density of bacterial inoculum on transformation by *A. tumefaciens* and reported the variation in transformation frequencies based on those factors. Plant cultivar was also found to have an 85-fold higher transformation rather than another (Eapen-Kohler *et. al.,* 1987). Petunia nurse cell

culture technique also enhances the efficiency of *A. tumefaciens* mediated transformation. Recently, Rama Swamy *et. al.,* (2001) have reported the high transformation efficiency (96%) with petunia nurse cell suspension feeder layer culture compared to without feeder layers in leaf discs of *Nicotiana tobacum* cv. Sunsun. Acetosyringone (AS) is a phenolic compound released by wounded cells and it plays an important role in the natural infection of plants by *A. tumefaciens* as it activates the virulence genes of the Ti-plasmid and initiates the transfer of the T-DNA region to the plant DNA. Exogenous addition of AS in the medium has shown to increase Ti transformation frequencies with *Allium cepa, Antirrhinum majus, Arabidopsis thaliana, Atropa belladonna, Brassica compestris, Glycine mad, Nicotiana tabacum* and pickling cucumber (Mathews *et. al.,* 1990; Godwin *et. al.,* 1991: Sarmento *et. al.,* 1992). Frary and Earle (1996) have also reported the enhanced transformation in tomato when the explants (cotyledons and hypocotyls segments) co-cultivated in the presence of AS showed the npt II gene more efficient expression than control transformants. Furthermore, regeneration efficiency from transformed explants of *Solanum melongena* was enhanced by using growth regulators, such as TDZ and antibiotics like augmentin (300 µ g/ml) (Billings *et. al.,* 1997).

Agronomically important characters have been genetically engineered in major crop plants using *Agrobacterium* mediated genetic transformation. Hinchee (1988) first time achieved the successful recovery of transformed Soyabean plants for engineering herbicide resistance. After words, this transformation technology was used for introducing agronomically important traits for improvement of the crop in the following species using *Agrobacterium:* sugar beet (*Herbicide tolerance* –D'Halluin *et. al.,* 1995), cotton (Insect resistance, Herbicide tolerance Umbeck 1987), Papaya (virus resistance – Fitsch *et. al.,* 1993), poplar (Herbicide resitance – Filatti, 1988), Potato (Insect resistance, virus resistance, herbicide tolerance, - Van den Elzen *et. al.,* 1995), and tomato (Delayed ripening (increased shelf life), virus resistance – (Sanders *et. al.,* 1992; Redenbaugh *et. al.,* 1993; Reed *et. al.,* 1995). Kemper *et. al.,* (1992) have developed the transgenic *Arabidopsis thaliana which* are methatrexate resistant due to integration of T-DNA vectors containing a Chimeric dihydrofolate reduductase gene.

Lawrence and Koundal (2001) have developed the transgenic pigeonpea resistant to chewing insects mainly pod borers using *A. tumefaciens* strain GV 2260 containing the construct of isolated cowpea protease inhibitor gene (pCPI). The Indian Council of Agricultural Research (ICAR) has developed biotech plant types of cotton, Brinjal and tomato and now working on evolving similar plants of rice, chickpea an pigeon and thus, over 35 genetically improved plants created this way. Trials have also been going on in various laboratories to introduce important traits such as herbicide tolerance, virus, abiotic stress and disease resistance including "nif" genes in cereals viz., maize, sorghum and oryza using this *A. tumefaciens.*

After transfer of the gene of interest using *A. tumefaciens* the transgene expression is also an important one. The trasgene expression in transgenic populations can vary due to dominant effect exerted by neighbouring plant sequences such as enhancers and silencers may also influence the activity of the introduced genes. Breyene *et. al.,* (1992a) have studied the influence of the T-DNA configuration on inter–transformant expression variability of a reporter gene. The transcriptional interference can diminish the activity of a gene located downstream in opposite orientation (Ingelbrecht *et. al.,* 1991).

Breyene *et. al.,* (1992a) have introduced an additional *3'nos* region between the transgene and the RB (right border) in such an orientation that it would stop possible trancscripts coming from the flanking plant DNA. The presence of *3'nos* resulted in transgenic population with 1.5 to 2-fold higher mean *gus 'A'* proximinity of the '35S' enhancer sequences to 'Pnos' results in an increase of *Gus'A'* expression. Other molecular causes such as the local DNA structure and / or the higher- order chromatin arrangement (Breyene *et. al.,* 1992 b) possibly also have an important role in the overall level of gene expression.

In view of the importance of *A. tumefaciens* mediated genetic transformation; the protocol which was developed during the present studies can be utilized to transfer genes of interest for genetic improvement of medicinally important herb *S. torvum*

Conclusion:-

We demonstrated that *Agrobacterium*-mediated genetic transformation in *S. torvum* for the first time. The frequency of genetic transformation conducted by the system established in this study was lower than the reported frequency by floral-dip method (Curtis and Nam 2001; Curtis *et al.* 2002). However, *Agrobacterium* method is more popular than floral-dip method for higher plants so as to enable this method to be more rapidly improved. Therefore, genetic

transformation of *S. torvum* by *Agrobacterium* method could also be further developed. *Agrobacterium* mediated genetic transformation would be applicable to improvement of this crop including development of pathogen-resistant cultivars.

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