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## **Genotype Independent Regeneration and *Agrobacterium*-mediated Genetic Transformation of Sweet Potato (*Ipomoea batatas* L.)**

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### **Abstract**

Development of an efficient genotype independent regeneration and genetic transformation system in sweet potato continues to be of great interest. *Agrobacterium*-mediated genetic transformation protocol was established in two different cultivars of sweet potato using *Agrobacterium* strain EHA105 harbouring binary plasmid pBI121 containing *GUS* and *nptII* genes. The internodal stem segments from 30-day-old micropropagated plants were used as explant with different combinations of media and hormones. MS and LS media with various concentrations of growth regulators proved to be non-responsive and the infecundity was severe with the addition of cytokinins. Nonetheless, MS with 2,4-D and TDZ gave a good percentage of callusing but with low differentiation. In different concentrations of NAA, significant amount of callusing was observed but percentage of rooting remained low in both the genotypes. Gamborg's B5 supplemented with NAA proved to be the most suitable media and hormone combination, which yielded shoot formation after 8 - 10 weeks with a regeneration efficiency of 40 - 70%. Stable integration of transgene was confirmed by PCR analysis. Furthermore, qRT-PCR analysis was performed to assess the transcript accumulation in addition to the *GUS* enzymatic assay in the transgenic lines.

### **Introduction**

Sweet potato is the world's seventh most important food crop and is grown in more than hundred countries world-wide covering tropical, subtropical and temperate zones. As an efficient biomass-producing plant for starch, especially

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for bio-alcohol, sweet potato plays a crucial role and has become an attracted target for genetic improvements (Newell et al. 1995, Noh et al. 2010, Chen et al. 2010). Sweet potato ranks fifth after rice, wheat, maize and white potato, in terms of total global food production (Janson and Raman 1991). Asia ranks first in sweet potato production followed by Africa and South America, and it is one of the main food sources for the poorest class of population in underdeveloped and developing countries (Peirce 1987).

Highly efficient and reproducible transformation technologies are essential for genetic improvement program(s) for major food and fodder crops including the tuber species. An efficient plant regeneration and transformation system is highly desirable for the successful application of genetic engineering in sweet potato as it is a hexaploid, highly male sterile and self incompatible crop (Dhir et al. 1998). The demand for efficient transformation systems to facilitate transgene expression or RNAi-mediated silencing of target genes has been greatly increasing in sweet potato. The use of such technology in sweet potato may have future applications in improving the ability of this tuberous crop to withstand different adverse environmental factors, in increasing yield, and enhancing nutritional quality. Sweet potato is found to be relatively easy to micropropagate; however, it is quite recalcitrant to genetic transformation (Sihachakr and Ducreux 1993). In addition, the regeneration frequency in sweet potato seems to be genotype-dependent (Jarret et al. 1984, Wang et al. 1998, Aloufa 2002, Santa-Maria et al. 2009). In most of the cases, regeneration at a high frequency has been restricted to one or a few genotypes. The critical hurdle for genetic transformation systems in sweet potato is to overcome genotype dependence. Therefore, an ideal transformation system would be genotype independent but this remains one of the key challenges for sweet potato. In this study, we made an attempt to establish a simple, robust and efficient regeneration and genetic transformation system in sweet potato via somatic embryogenesis from culture of internodal explants.

## Materials and Methods

Two cultivars of sweet potato (*Ipomoea batatas* L., cv. SP-6 and SP-17) were obtained from the Central Tuber Crop Research Institute (CTCRI), India. Plants were micropropogated in MS supplemented with IAA and maintained at  $22 \pm 2^\circ\text{C}$  with  $270 \mu\text{mol/m}^2/\text{s}$  light intensity under 16 hrs photoperiod. The internodal stem segments (3 - 5 mm) from four-week-old micropropogated plants were used as explants.

*Agrobacterium tumefaciens* strain EHA105 harboring binary plasmid pBI121 (Fig. 1) containing the *uidA* and *nptII* genes was used for genetic transformation. *Agrobacterium* strain containing the gene of interest was first grown in YEP plates supplemented with kanamycin (50 mg/l) and rifampicin (50 mg/l). The *Agrobacterium* suspension culture was prepared by picking a single colony and inoculated in YEP liquid medium containing the same antibiotics and allowed to grow at 28°C for 16 - 18 hrs. One-fiftieth volume of primary culture was inoculated and allowed to grow at 28°C until the optical density (OD600) reached 0.6 - 0.8. The *Agrobacterium* culture was pelleted at 5000 g for 5 min at ambient temperature (~25°C) by centrifugation and resuspended in same volume of MS liquid medium. About 50 explants were incubated for 45 min in a saturated culture of *Agrobacterium* with occasional swirling. The explants were retrieved from the culture and blotted on sterile Whatman filter paper to drain away extra inoculum. The explants were then co-cultivated on Gamborg's B5 basal medium supplemented with NAA (0.4 mg/l) and 0.8% agar for 48 hrs. The media was supplemented with bacteriostatic agent cefotaxime (250 mg/l) and selectable antibiotic kanamycin (100 mg/l). Growth room conditions maintained throughout were 22 ± 2°C and 16 hrs photoperiod. All cultures were examined periodically and morphological changes were recorded. The regenerated putative transformants were transferred to rooting media i.e., MS containing IAA (0.1 mg/l) and requisite antibiotics.

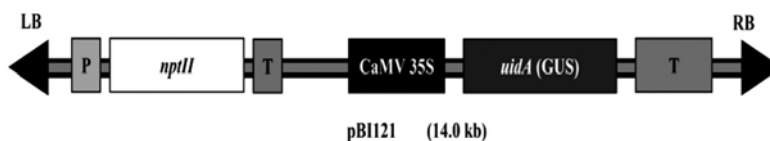


Fig. 1. Schematic representation of the plant expression vector pBI121 containing *nptII* and *uidA*, (encoding neomycin phosphotransferase II and *GUS*, respectively). LB, T-DNA left border; P, NOS promoter; T, NOS terminator; RB, T-DNA right border.

Integrity of the selectable marker gene *nptII* was assessed at the genome level by PCR analysis using genomic DNA isolated from the putative transformants and the wild-type plants as described earlier (Dellaporta et al. 1983). The presence of transgene was confirmed by PCR using gene specific primers (*nptIIF* 5' - ATGATTGAACAAGATGGATTGCACGCAGG -3' and *nptIIR* 5'- GAAGAA CTCGTCAAGAAGGCCGATA -3'), which delimits 0.8 kb fragment from the *nptII* coding region. PCR analysis was performed in 20 µl reaction mix containing 1X PCR buffer, 2 mM MgCl<sub>2</sub>, 200 µM dNTP mix, 0.5 µM each primer and 0.5 units of Taq polymerase. The cycling conditions employed were 3 min at 94°C denaturation and 30 amplification cycles using 94°C for 1 min, 58°C for 45 sec annealing and 72°C for 45 sec followed by 5 min extension at 72°C.

For qRT-PCR analysis of *GUS* expression, total RNA was isolated by phenol and guanidine thiocyanate method using the monophasic TriPure Isolation Reagent (Roche) as per the manufacturer's instructions. After determining RNA concentration by nanodrop, cDNA were prepared using 3'-RACE kit (Invitrogen). The *GUS*-specific primers (*GUSF* 5'-TGGTAATTACCGACGAAAACGGC-3' and *GUSR* 5'-ACGCGTGGTTACAGTCTTGCG-3') and the primers for housekeeping gene actin (*ActinF* 5'-CTCCCCTAATGAGTGTGATGTGAT-3' and *ActinR* 5'-GAGCCCCATGAGAACATTACCA-3') were designed using the primer express software. The quantitative real-time RT-PCR was performed with the ABI PRISM 7700 sequence detection system (Applied Biosystems) using SYBR green dye (Applied Biosystems). The analyses were done with two biological and three technical replicates. Mean of Ct values for target and endogenous control was considered for calculating the relative quantitation (RQ) value using comparative Ct ( $2^{-\Delta\Delta Ct}$ ) method. Two putative transformants and the wild-type counterparts from both the genotypes were selected for quantitative enzyme assay. *GUS* ( $\beta$ -glucuronidase, EC 3.2.1.31) activity was determined using the fluorometric method described earlier (Jefferson 1987) with few modifications. The tissues were ground and homogenized with extraction buffer (50 mM sodium phosphate, pH 7.0, 10 mM beta-mercaptoethanol, 10 mM EDTA, 0.5 mM PMSF, 0.1% sodium lauryl sarcosine and 0.1% Triton X-100). The homogenates were then centrifuged (10000 g for 5 min at 4°C) and the supernatants were further used for assay. The reaction mixture in triplicate, consisted of 50 mM sodium phosphate, pH 7.0, 1 mM 4-methylumbelliferyl- $\beta$ -D-glucuronide (MUG) and the tissue extract was then incubated at 37°C for appropriate time intervals (5 - 60 min). The reaction was terminated by the addition of 0.2 M Na<sub>2</sub>CO<sub>3</sub>. Fluorescence was measured at 455 nm using a Varian Cary Eclipse Fluorescence Spectrophotometer (Agilent) set at an excitation wavelength of 365 nm.

## Results and Discussion

In the present investigation *in vitro* regeneration experiments were carried out using internodal explants from two different genotypes of sweet potato. In the past, there have been many attempts to produce transgenic sweet potato especially using the electroporation of protoplasts (Nishiguchi et al. 1992, Garcia et al. 2000) and particle bombardment (Prakash and Varadarajan 1992, Yang et al. 2005). In particular, the *A. tumefaciens* mediated transformation system has been widely used because of its efficiency, simplicity and stability of the introduced gene. The first successful such transformation protocol for sweet potato was based on the formation of hairy roots using leaf-discs explants by *A. rhizogenes*

(Dodds et al. 1991, Otani et al. 1993). However, morphological abnormalities shown by regenerated transgenic plants were a big question mark for the method. *A. tumefaciens* mediated transformation in sweet potato was well established by several workers (Al-Juboory and Skirvin 1991, Carelli et al. 1991, Prakash and Varadarajan 1991, Lowe et al. 1994, Otani et al. 2001). In general, these procedures have been mostly genotype-dependent with lower transformation efficiency (Otani et al. 2003, Song et al. 2004, Shimada et al. 2006, Luo et al. 2006), and often difficult to reproduce (Lowe et al. 1994). *Agrobacterium*-mediated transformation in sweet potato has also been applied for regeneration via somatic embryogenesis using somatic embryos or organs as explants by several workers (Pido et al. 1995, Newell et al. 1995, Gama et al. 1996, Otani et al. 1998, Luo et al. 2006).

To develop a simple regeneration and genetic transformation protocol, two different genotypes of sweet potato were considered as explant source. Over the years, *E. coli uidA* gene encoding *GUS* has been the most chosen reporter gene and widely used to assess the transient and stable transformation in plants. The *GUS* gene fusion system has found extensive application in plant gene expression studies due to the enzyme stability and high sensitivity (Fior and Gerola 2009). One of the most critical factors to develop a high efficiency transformation protocol is the use of a hypervirulent strain of *A. tumefaciens* and an appropriate medium for optimum infection of explants. Therefore, to mobilize the binary expression plasmid pBI121 harbouring *uidA* and *nptIII* genes into *A. tumefaciens* strain EHA105, triparental mating strategy was used.

In plant cell culture, growth and morphogenesis are significantly affected by the type(s) of media and the concentrations of growth regulators. Plant growth regulators are conceivably the most important components affecting shoot regeneration capacity of explants. Thus, optimization of the correct combinations of auxins and cytokinins is indispensable for high frequency shoot regeneration. Also, the genotype of the transformed explants is a deciding factor for their growth and development. To introduce the expression plasmid into sweet potato, diverse media and hormone combinations were examined. Some hormones and media combinations showed only callus formation, while some showed both shooting and rooting. During the first week of inoculation, little morphological differentiation was observed. The cut-ends began to swell and slight increase in tissue volume was observed. Callus formation took place from both side of the explants and began to increase in size in the following weeks. Root formation was evident from the third week depending on the plant genotypes and the concentrations of the hormone used. Use of MS with 2, 4-D and TDZ yielded significantly good percentage of calli, but showed no shoot or

root development. Similar results were observed when BAP and/or Zeatin were used alone or with combination of NAA and GA<sub>3</sub> (Table 1). The inhibitory action of cytokinin on regeneration of sweet potato has been well documented and contradicts the common practice of using cytokinin in combination with an auxin in regeneration trials. Several co-workers had previously reported inhibitory effect of cytokinin in shoot regeneration (Carswell and Locy 1984, Ozias-Akins and Perera 1992, Otani and Shimada 1996, Pido et al. 1995). Addition of either of these cytokinins inhibited root and shoot development in such a way that higher the cytokinin concentration, greater was the inhibitory effect; however, a corresponding increase in callus growth was also observed. Roots are the primary organ for the synthesis of cytokinins and thus rooted explants should conceivably be able to synthesize a little amount of cytokinins (Pido et al 1995). In addition, several tissues are considered to be cytokinin independent, and the findings of the present study also suggest that it is not necessary to supplement the regeneration medium with cytokinin for the regeneration of sweet potato. A similar response was observed in LS media in both the genotypes. However, overall performance of cv. SP-6 was found to be better when compared with SP-17, suggesting its better adaptability and efficacy. In different concentration of NAA, fair amount of callusing was observed in the explants in four weeks duration, but percentage of root development remained low in both the genotypes suggesting that media containing NAA alone might induce regeneration (Table 1). An alternate regeneration procedure with a single hormone was then employed wherein explants were first kept on callus inducing media (CIM) [3.2 g/l Gamborg's B5 with minimal organics, 30 g/l sucrose, 0.8% agar type A and 0.4 mg/l NAA, pH 5.6-5.8]. Within 3 weeks, callusing was observed from both the corners of the explants followed by initiation of roots. Once the complete rooting took place, the transformed explants were transferred to low auxin shoot induction media (SIM) [3.2 g/l Gamborg's B5 with minimal organics, 30 g/l sucrose, 0.8% agar type A and 0.2 mg/l NAA, pH 5.6-5.8]. Microshoots were produced within 8 - 10 weeks of subculturing in SIM (Fig. 2A-D). Regenerating shoots were harvested and transferred to root induction media (RIM) [4.4 g/l MS with vitamins (M5519, Sigma), 30 g/l sucrose, 0.8% agar type A and 0.1 mg/l IAA, pH 5.6-5.8) with cefotaxime (250 mg/l) and kanamycin (100 mg/l) (Table 2).

In *Agrobacterium*-mediated transformation system, activation of the virulence genes in the Ti plasmid is modulated by molecular signals provided by wounded tissue that is mimicked by phenolic compounds such as acetosyringone or hydroxyacetosyringone. The use of acetosyringone has been reported earlier in various sweet potato transformation systems (Luo et al. 2006, Yu et al. 2007).

Table 1. Differential response of intermodal explants in various culture conditions.

Genotype	Callusing media	No. of explants	Callus formation	% callus formation	Subculturing media	Root formation	% root formation
SP-6	BNG, MS	170	119	70	BNG (combination of different conc. of BAP, NAA, and GA <sub>3</sub> ), MS	-	-
	2,4 D-BAP, MS	42	35	83.3	2,4 D-BAP, MS	-	-
	2,4 D-TDZ, MS	80	69	86.25	2,4 D-TDZ, MS	-	-
	Different conc. of NAA (0.1-0.8 mg/l), MS	70	65	92.8	MS, NAA (in different conc.)	15	21.42
SP-17	BNG, LS	65	11	16.9	BNG, LS	-	-
	Different conc. of NAA (0.1-0.8 mg/l), LS	60	27	45	LS, NAA (in different conc.)	12	20
	BNG, MS	42	5	11.9	BNG, MS, BAP (in different conc.)	-	-
	2,4 D-BAP, MS	40	24	60	BNG, MS	-	-
	2,4 D-TDZ, MS	60	30	50	BNG, BAP (in different conc.)	-	-
	Different conc. of NAA(0.1-0.8 mg/l), MS	45	36	80.0	NAA (in different conc.), MS	8	17.7
	BNG, LS	50	9	18	BNG, LS	-	-
	Different conc. of NAA(0.1-0.8 mg/l), LS	62	21	35	LS, NAA (in different conc.)	10	16.12

However, we developed the stable transformation system without the addition of exogenous signaling compound, considering the predominant presence of phenolic compounds in sweet potato. The regeneration efficiency was calculated as the number of stable transgenic plants obtained from the total number of explants that responded to rooting and callusing. The overall regeneration efficiency ranged between 40 - 70%, which appeared to be the best for sweet potato. The regeneration efficiency of cv. SP-6 was significantly better than that

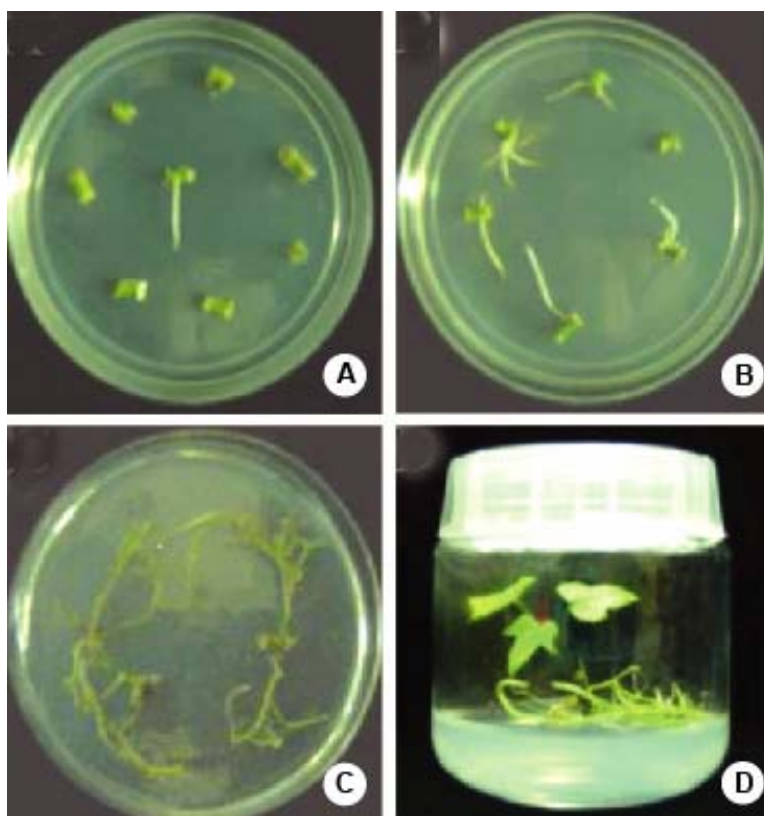


Fig. 2. Regeneration stages of putative transformants. The internodal explants infected by *A. tumefaciens* (strain EHA105) harbouring the expression plasmid pBI121 responded in a stage wise manner *viz.* callusing (A) followed by rooting (B and C) and shooting (D) in CIM and SIM, respectively.

of SP-17. Intriguingly, the induction of somatic embryogenic tissues at high frequencies has been found to be limited to a few genotypes, and when attempts were made to extend this into a wide range of genotypes, the majority was found to be recalcitrant or to respond at low frequencies. Al-Mazrooei et al. (1997) had optimized the culture condition for somatic embryogenesis in 14 genotypes and obtained the regeneration frequency up to 68%. It is evident that there has been a



gap between the regeneration frequency and the transformation efficiencies. However, transformation efficiencies of up to 20% reported by Luo et al. (2006) from leaf petioles were higher than those of several reports (Newell et al. 1995, Otani et al. 1998, Moran et al. 1998). A relatively rapid regeneration system (12 - 16 weeks) with a higher transformation frequency of 30.8% was reported by Song et al. (2004) from stem explants. In comparison with prolonged regeneration steps required in the somatic embryogenesis (Newell et al. 1995, Otani et al. 1998, Zhang et al. 2000), the rapid *de novo* organogenesis is likely to lower somaclonal variations (Luo et al. 2006). We observed the regeneration in time span of 8-12 weeks, starting from the day of infection with *Agrobacterium* which also strengthen the claim for the development of a relatively more rapid regeneration system for sweet potato.

**Table 2. Combination of various media used for the induction of calli, roots and shoots from internodal explants.**

No. of variants	Media code	Media composition
1	CIM	3.2 g/l Gamborg's B5, 30 g/l sucrose, 0.8% agar, and 0.4 mg/l NAA, pH 5.6 - 5.8.
2	SIM	3.2 g/l Gamborg's B5, 30 g/l sucrose, 0.8% agar, and 0.2 mg/l NAA, pH 5.6 - 5.8.
3	RIM	4.4 g/l MS, 30 g/l sucrose, 0.8% agar, and 0.1 mg/l IAA, pH 5.6 - 5.8 and supplemented with 250 mg/l cefotaxime and 100 mg/l kanamycin.

Selectable marker genes have a pivotal role in validating the plant transformation technologies as the marker genes allow to distinguish between the transformed and untransformed plant(s). While untransformed escapes are the common phenomena in transgenic technology of plants (Zang et al. 2000), a suitable concentration of test antibiotic can reduce such risk. To screen the true transformation events, high concentration of kanamycin at 100 mg/l was used and the kanamycin-resistant plants were considered for downstream analysis. While 18 out of 25 regenerated plants in cv. SP-6 were found to be positive, 9 out of 15 regenerated plants were positive in cv. SP-17. As stated above, morphological abnormality is one of the major constrains in the development of a successful regeneration and transformation system. We observed no detectable morphological difference between the wild-type and the transgenic plants.

Successful integration of the transgene at genome level was examined by PCR analysis in 12 putative transformants using *nptII* gene specific primers. The presence of 0.8 kb amplicons in the putative transformants confirmed the successful integration of the transgene (Fig. 3A, B).

Out of these 12 transgenic plants, 4 (2 from both the genotypes) were further selected to assess the transcript accumulation and relative *GUS* expression. The transcript accumulation of *GUS* showed 3 to 12-fold expression compared to the wild-type. Accumulation pattern also revealed more *GUS* transcript in the transformants of cv. SP-6 when compared with that of SP-17 transformants (Fig. 4A). The *GUS* activity of each transgenic plant was significantly higher than that of wild-type and untransformed plant. In addition, there was a positive correlation between the transcript accumulation and enzyme activity (Fig. 4B).

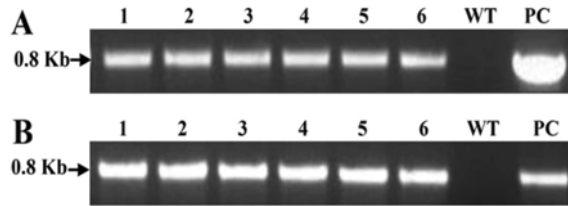


Fig. 3. Confirmation of transgene integration. Successful integration of the transgene was confirmed by PCR analysis in 6 putative transformants, each from cv. SP-6 (A) and SP-17 (B), respectively. An amplicon of 0.8 Kb confirmed the successful transgene integration in putative transformants which was absent in wild-type (WT) plants. Expression plasmid pBI121 was used as positive control (PC).

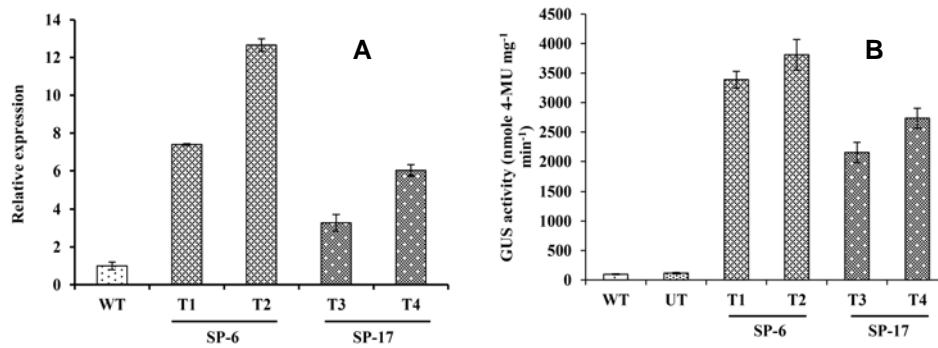


Fig. 4. Transcript accumulation and enzymatic activity of *GUS*. The transcript accumulation of *GUS* showed a 3 to 12-fold increase in expression than that of wild-type (WT) plant (A). Higher *GUS* activity was observed in the transgenic plants than WT and UT (untransformed plant) (B). Each analysis was performed in 4 transgenic plants (2 from each of the genotypes).

The genetic transformation protocol reported here may be used to incorporate agriculturally and/or industrially important candidate gene(s) in sweet potato. However, it is likely that, by manipulating the regeneration process or better susceptibility to *Agrobacterium*, it will be possible to address issues of

genotype-dependence and to improve transformation efficiencies in sweet potato further.

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