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AGROBACTERIUM TUMEFACIENS MEDIATED TRANSFORMATION OF TWO IMPORTANT INDIAN VARIETIES OF POTATO *(SOLANUM TUBEROSUM* L.)

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Stable regeneration and transformation protocols were standardized for the two important Indian varieties of potato (*Solanum tuberosum) viz.,* Kufri Swarna and Kufri Jyoti. The transformation protocol was standardized using *Agrobacterium tumefaciens* strain EHA105 carrying the reporter gene β-glucuronidase (GUS) and neomycin phosphotransferase (*nptII*). After infection and co-culture with *Agrobacterium* the internodal explants were selected on callusing media supplemented with 50mg/L kanamycin. The calli formed after 4 weeks were transferred to the shooting media containing 50mg/L kanamycin. The regenerated shoots were excised and grown in MS media for rooting. Molecular analysis of the putative transformants by PCR, Southern blot analysis and RT-PCR confirmed the stable integration and expression of foreign genes.

Keywords : *Agrobacterium;* β-glucuronidase; Gibberellic acid; Neomycin phosphotransferase; 6-benzyl adenine; Naphthalene acetic acid.

Introduction

Potato is one of the most agronomically important plants in the world due to its high productivity and high starch, vitamin and protein content. Sterility and tetraploidy along with a high level of heterozygosity greatly reduce the efficiency of traditional methods for potato breeding. An alternative approach for improvement of commercial potato varieties uses *in vitro* techniques, including somatic hybridization, mutagenesis and genetic transformation.

Of the various techniques employed, *Agrobacterium* mediated genetic transformation is the most commonly used method due to its simplicity and efficiency. Further, potato is amenable to this method and hence it is widely used for the production of transgenic potato plants[1-3]. Potato shows a great deal of variations among the varieties and cultivars regarding regeneration and transformation efficiencies. Thus, though transformation protocols have been standardized for major varieties like Desiree, Bintje, Kaptah Vandel etc., there are only a few reports regarding the Indian varieties. Thus, we selected the two popular Indian varieties *viz.,* Kufri Jyoti and Kufri Swarna and standardized the regeneration and *Agrobacterium* mediated transformation protocols for them.

Material and Methods

Agrobacterium strain and plasmids- Agrobacterium tumefaciens strain EHA105[4] was used in the present study. The binary vector pBE2113[5] has β-glucuronidase (GUS) gene driven by the chimeric combined promoter cassette consisting of enhancer of Cauliflower mosaic virus (CaMV) 35S RNA promoter and 5' un-translated region (Ω) of tobacco mosaic virus for enhanced expression. Neomycin phosphotransferase II gene *(npt*II) that confers kanamycin resistance was used for the selection of putative transformants.

Plant materials-In vitro plantlets of *Solanum tuberosum var.* Kufri Jyoti and Kufri Swarna were established by *in vitro* germination of tubers in solidified medium containing quarter the strength of Murashige-Skoog (MS) basal media[6] without sucrose. The germinated plantlets were transferred and maintained in MS basal medium supplemented with sucrose (30g/L).

Plant transformation and selection-Internodal explants from *in vitro* grown plants were cut into small pieces and preincubated for two days in callusing medium [MS medium supplemented with 4mg/L 6-benzyl adenine (BA) and 1mg/L naphthalene acetic acid (NAA)]. *A. tumefaciens* EHA105 (pBE2113) was grown overnight at 28°C in liquid AB minimal medium containing 50mg/L kanamycin. The preincubated explants were infected with *Agrobacterium* solution for 15 min. and co-cultivated on the callusing medium for 48 h. Following co-cultivation, the explants were washed with sterile water several times and

transferred to selection medium comprised of callusing medium supplemented with 50mg/L kanamycin and 300mg/L cefotaxime. After four weeks the calli formed were transferred to shoot induction medium [MS medium supplemented with 5mg/L BA and 0.3mg/L gibberellic acid (GA_3)] containing 50mg/L kanamycin. The putative transformed shoots were rooted in MS medium supplemented with 50mg/L kanamycin.

GUS assay-Histochemical GUS staining of explants was performed according to Jefferson *et al.*[7]. The shoots from putative transformants were immersed in 100mM phosphate buffer (pH 7.0) containing 1mM 5-bromo-4-chloro-3-indolyl-β-Dglucronide (X-Gluc), 4mM $K_3Fe(CN)_6$, 4mM $K_4Fe(CN)_6$ and 0.1% Triton X-100. They were incubated overnight at 37°C. The chlorophyll content was removed by ethanol wash and the staining pattern was observed.

PCR and Southern blot analysis- Duplex PCR analysis was conducted to screen transformed plants using primers for both GUS and *nptII* genes. Reactions were subjected to 25 cycles with an annealing temperature of 55°C. The primers used were 5' ATA CCG TAA AGC ACG AGG 3' *(nptF)* and 5' ATC TCA CCT TGC TCC TGC 3' *(nptR)* for *nptII* and 5' GTA TCA CCG TTT GTG TGA ACA ACG 3' (GUSF) and 5' GTA TCG GTG TGA GCG TCG CAG AAC 3' (GUSR) for GUS gene.

Southern blot analysis was conducted to confirm stable integration of transgenes as well as to detect its copy number. 20µg of total DNA was digested independently with the restriction enzymes, *Eco*RI and *Hind*III, and digested DNAs were separated by electrophoresis in a 0.8% agarose gel and transferred onto HybondN$^+$ membrane (Amersham Pharmacia Biotech). Probe was labeled with αP^{32}dCTP using NEblot kit and hybridization was done according to the standard protocol[8]. DNA fragments containing the GUS gene and *npt*II gene were amplified by the same primer sets for the PCR analysis, and used as hybridization probes on Southern blot membranes. The blot was hybridized with GUS probe and the pattern was recorded. The membrane was deprobed using 20% SDS and 20X SSC in boiling water and reprobed with *npt*II gene.

RT-PCR analysis-The expression of the transferred genes was studied by reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from a control plant as well as two PCR positive plants and first strand cDNA was synthesized using M-MuLV Reverse Transcriptase (M-MuLV RT) and Oligo dT_{18} primer. The first strand cDNA was amplified by PCR using the primers for GUS and housekeeping Actin1 gene (as control) independently. The PCR amplification was carried out with an initial denaturation at 94°C for 5 min followed by 30 thermal cycles consisting of denaturation (30s at 94°C), annealing (30s at 50°C for Actin /60°C for GUS) and extension (1 min at 72°C). Final extension was performed at 72°C for 7 min.

Results and Discussion

Regeneration from internodal explants- Indirect regeneration was obtained from both internodal and leaf explants by inducing callusing in solid MS basal medium supplemented with BA (4mg/L), NAA (1mg/L) and sucrose (30g/L). The calli obtained were then transferred to MS basal media supplemented with BA (5mg/L), GA_3 (0.3mg/L) and sucrose (30g/L) for shoot induction. The regenerated plantlets were rooted and maintained in MS basal media supplemented with sucrose (30g/L).

The sensitivity of the potato plants to kanamycin was tested by checking the ability of stem explants to root in MS basal medium containing varying concentrations of kanamycin. The control shoots inoculated in MS basal medium without any kanamycin showed good rooting while the explants in MS medium supplemented with kanamycin 50mg/L (1) and kanamycin 100mg/L (2) failed to root (data not shown). Thus, kanamycin at a concentration of 50mg/L was used for the screening of transformants carrying *npt*II gene for selection.

Regeneration efficiency was calculated as percentage of explants showing shoot regeneration. Kufri Jyoti showed a regeneration efficiency of 59.8% while, Kufri Swarna showed only 43.7% efficiency (Table 1).

Agrobacterium mediated transformation and Histochemical GUS Assay-Agrobacterium tumefaciens strain EHA105 carrying the binary vector pBE2113(GUS) was used for the standardization of transformation protocol in the potato cultivars Kufri Jyoti and Kufri Swarna. Internodal explants were used for transformation and kanamycin (50mg/L) was used for selection. The putative GUS transformants were initially screened by histochemical GUS assay. The putative transformants showed intense GUS staining in the leaves, while the wild plant did not show any staining. Intense blue staining was found in the leaves as well as in the conducting tissue of the tubers of transformants and a cross section of the stem revealed intense staining in the conducting tissues while, the wild plant did not show any staining. Transformation efficiency calculated based on GUS staining showed 42% efficiency for Kufri Jyoti, while Kufri Swarna had only 36% efficiency (Table 2).

PCR analysis -The presence of GUS and *npt*II genes in the genome was confirmed by duplex PCR using primers

Table 1. Regeneration efficiency of potato *cv.* Kufri Jyoti and Kufri Swarna.

Variety	No. of explants	No..qf shwoing callusing	% of callusing	No. of shoots per explant	% of shoot regeneration
KS	34	28	82.4	15	44.1
	30	25	83.3	13	43.3
	Average		82.6		43.3
KJ	34	30	88.2	16	58.8
	28	25	89.3	17	60.7
	Average		88.75		59.8

Table 2. Transformation efficiency of potato *cv.* Kufri Jyoti and Kufri Swarna

Variety	No. of explants	No. of GUS +ve shoots	% of transformation
KS	34	12	35.3
	30	11	36.7
	Average		36
KJ	34	14	41.2
	28	12	42.9
	Average		42.05

specific for GUS and *npt*II genes together. The presence of a 600bp fragment confirmed the presence of *npt*II gene and the presence of a 1kb fragment confirmed the presence of GUS gene in all the putative transformants. Both the bands were absent in the wild plant (Fig. 1).

Southern blot analysis-Transgene integration was confirmed by Southern blot analysis. All transgenic plants yielded 1 to 3 bands hybridized with GUS or *npt*II gene, but non-transgenic plants did not yield any bands (Fig. 2). Based on banding patterns on Southern blots, transgenic plants were classified into at least 5 independent lines.

RT-PCR analysis using Actin and GUS specific primers-With GUS gene specific primers, amplification of 1kb fragment was seen in both the transformants in Kufri Jyoti and Kufri Swarna, while control plant showed no amplification. With Actin gene specific primers, all plants showed the amplification of 250bp fragment (Fig. 3). The amplification of the GUS transcript from the cDNA of the transformants proved the incorporation of the gene into the genomic DNA and its stable expression.

An efficient transformation protocol has been deve'oped for the two important Indian varieties, Kufri Swarna and Kufri Jyoti using internodal explants. Use of internodal explants reduced the problems of somaclonal variations and polyploidy[9]. All the transformants obtained were phenotypically normal and showed no variations compared to the control wild plants.

An average transformation efficiency of about 42% was obtained with Kufri Jyoti, while Kufri Swarna had about 36% efficiency. Relatively recalcitrant cultivars like Russet Burbank shows a transformation efficiency of about 18% only[10], while, for the cultivars Bintje, Desiree and Kaptah Vandel show an average transformation efficiency of about 90%[9].

Several protocols for genetic transformation of potato have been successfully employed to generate transgenic plants resistant to drought and diseases, as well as to increase its nutritive value. However, most of these protocols are genotype dependent[10], accompanied by high variability in the transformation efficiency. The

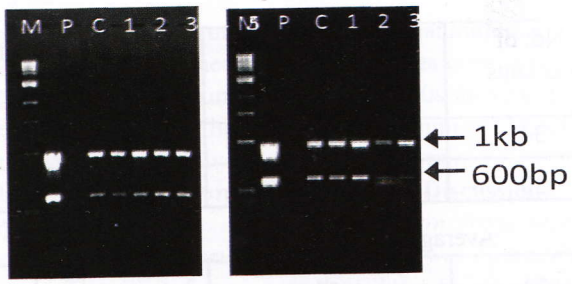

Fig.1. Duplex PCR using GUS and *npt*II gene specific primers in the wild (C) and putative transformants (1-5) in both Kufri jyoti (A) and Kufri Swarna (B). [M - 1kb ladder, P - positive control (Plasmid)].

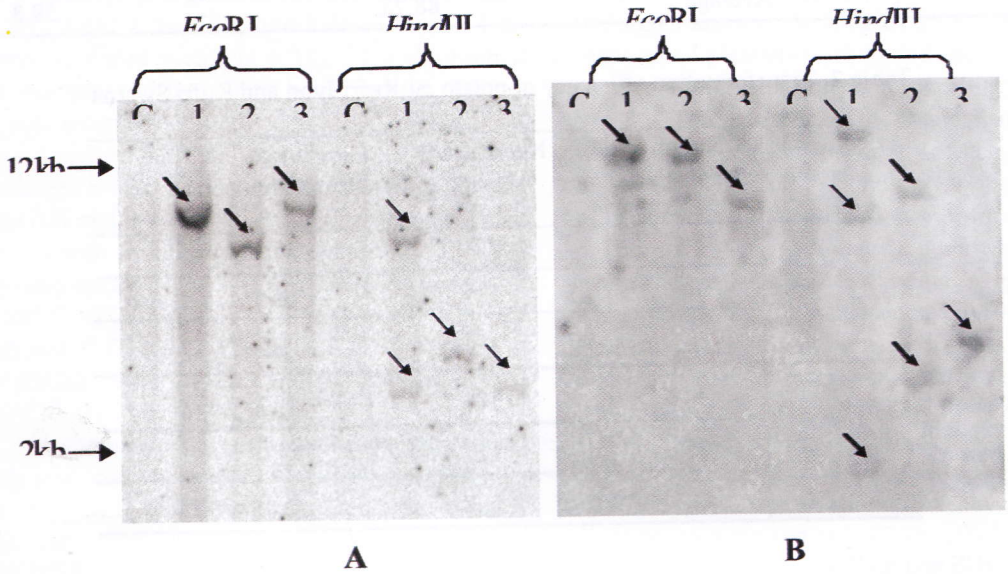

Fig.2. Southern hybridization of genomic DNA isolated from the wild control (C) and *gus* transformants (1 -4) of Kufri Swarna (A) and Kufri Jyoti (B) to confirm the integration of insert GUS gene into genomic DNA.

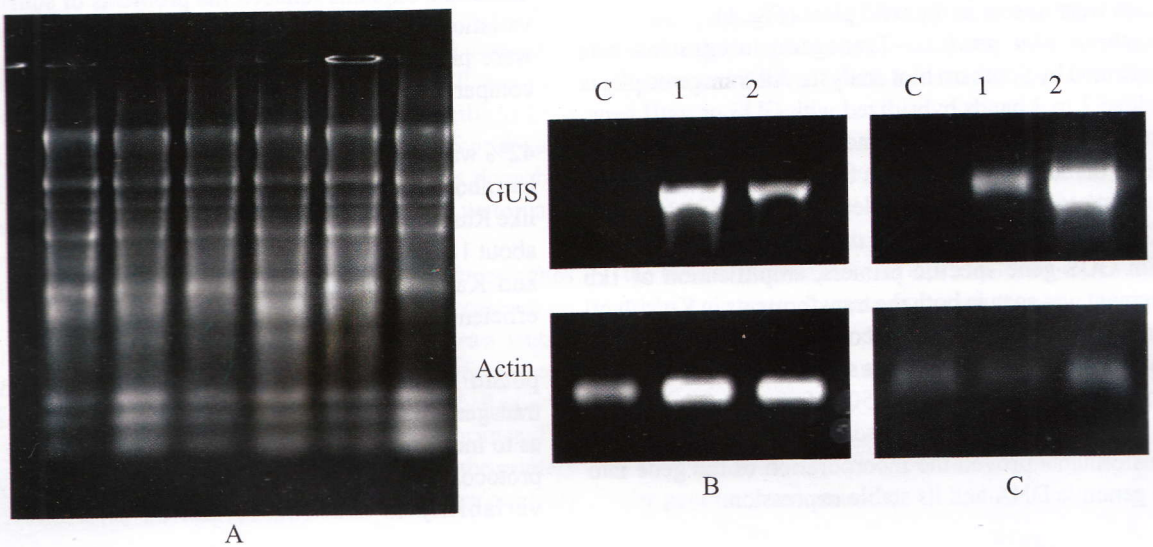

Fig.3. Total RNA isolated from the leaves of wild type and two putative transformants of Kufri Jyoti and Kufri Swarna plants (A). RT-PCR analysis using Actin1 and GUS gene specific primers (C - wild type, 1 & 2 - GUS transformants) in Kufri Jyoti (B) and Kufri Swarna (C).

development of efficient transformation protocols for the popular varieties is a pre-requisite for any genetic improvement program. We have developed efficient transformation protocols for two important varieties of India *viz.*, Kufri Jyoti and Kufri Swarna with mean transformation efficiency of 42% and 36% successively. The reproducible system described may be useful for the introduction of agronomically important traits in the Indian varieties of potato.

References

1. Chakraborty S, Chakraborty N and Datta A 2000, Increased nutritive value of transgenic potato by expressing a nonallergenic seed albumin gene from *Amaranthus hypochondriacus*. *PNAS* **97**(7) 3724-3729
2. Kreft O, Hoefgen R and Hesse H 2003, Functional analysis of cystathionine γ-synthase in genetically engineered potato plants. *Plant Physiol.* **131** 1843-1854
3. Ducreux L J M, Morris W L, Hedley P E, Shepherd T, Davies H V, Millam S and Taylor M A 2005, Metabolic engineering of high carotenoid potato tubers containing enhanced levels of β-carotene and lutein. *J. Exp. Bot.* **56**(409) 81-89
4. Hood E E, Gelvin S B, Melchers L S and Hoekema A 1993, New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgen. Res.* **2** 201-218
5. Mitsuhara I, Ugaki M, Hirochika H, Ohshima M, Murakami T, Gotoh Y, Katayose Y, Nakamura S, Honkura R, Nishimiya S, Ueno K, Mochizuki A, Tanimoto H, Tsugawa H, Otsuki Y and Ohashi Y 1996, Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. *Plant and Cell Physiol.* **37** 49-59
6. Murashige T and Skoog F 1962, A revised medium for the rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* **15** 473- 497
7. Jefferson R A, Kavanagh T A and Bevan M W 1987, GUS fusions: β-glucuronidase as a sensitive and versatile gene. *Embo J.* **6** 3901-3907
8. Sambrook J, Fritsch E F and Maniatis T 1989, Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. Beaujean A, Sangwan R S, Lecardonnel A and Sangwan-Norreel B S 1998, *Agrobacterium* mediated transformation of three economically important potato cultivars using sliced internodal explants: an efficient protocol of transformation. *J. Exp. Bot.* **49**(326) 1589-1595
10. Newell C A, Rozman R, Hinchee M A, Lawson E C, Haley L, Sanders P, Kaniewski W, Tumer N E, Horsch R B and Fraley R T 1991, *Agrobacterium* mediated transformation of Solanum tuberosum L. *cv.* "Russet Burbank". *Plant Cell Rep.* **10** 30-34