

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 *viz* cotyledon and hypocotyl. The cotyledon explants showed high frequency of shoot regeneration (77.77%) on MS medium supplemented with 2.5 mg/l Kn and 0.4 mg/l IAA compared to hypocotyl (50%) on MS medium supplemented with 2.5 mg/l 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 β -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. After co-cultivation only the transformed cells were able to grow on selective shoot regeneration medium containing kanamycin and cefotaxime, whereas control explants died on the selective medium. Transformation experiment 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, binary vector, gene expression, genetic transformation, β -glucuronidase, *npt-II* genes, plant regeneration.

Introduction

Brinjal (*Solanum melongena* L.) also known as aubergine (or eggplant) 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 (FAOSTAT, 2011). The crop is mainly cultivated on small family farms and is an important source of cash income for many resource poor farmers. Eggplant has a high nutritive value (protein 1.4%, fat 0.3%, minerals 0.3%, carbohydrates 4%). It also has various medicinal uses that make it a valuable addition to diet. Being an economically important crop, the application of tissue culture and genetic engineering in brinjal cultivation is of special value to obtain improved or desirable traits like disease and insect resistance and to enhance the shelf life of this crop. However, before undertaking this approach, it is important to know that whether the somatic cells of this species/cultivation are able to regenerate in such a way as to give rise to whole plantlet and to standardize the technique of genetic transformation in brinjal cells with marker and reporter genes. Previous studies have demonstrated that conventional approach for establishing or improving a regeneration system for brinjal has been to screen a variety of explants, media and plant growth regulators regimes (Alicchio *et al.*, 1982; Sharma and Rajam, 1995; Picoli *et al.*, 2002; Tarre, 2004). Genetic engineering of brinjal has already been reported (Guri and Sink, 1988; Fari, 1995; Franklin *et al.*, 2004; Soniya *et al.*, 2005; Narendran *et al.*, 2007).

The present investigation describes a reproducible and efficient plant regeneration system for Pusa Purple Long cultivar of brinjal and discuss the effect of BA and Kn on direct and indirect organogenesis and genetic transformation and expression of neomycin phosphotransferase-II (*npt-II*) and β -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*.

Material and Methods

Plant material and nutrient medium

S. melongena L. was taken as experimental material during the present investigation. The certified seeds of brinjal (*S. 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 old 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, Kn and IAA, Kn 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* LBA 4404 strain containing β -glucuronidase (*gus*) gene in binary vector pBI 121 system alongwith 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 *A. tumefaciens* LBA 4404 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 sub-culturing the bacterial colonies on the YMB medium containing

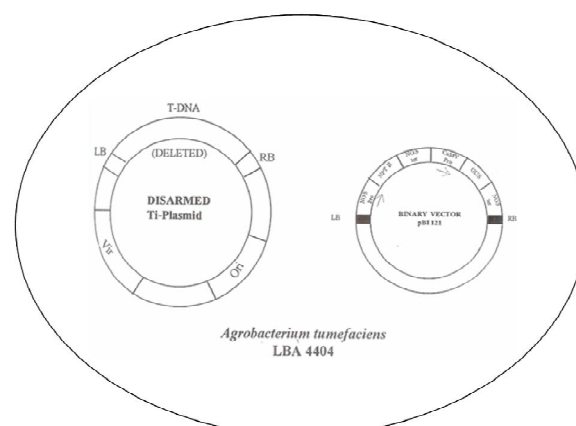


Fig.1a. The outline diagram of the disarmed *Agrobacterium tumefaciens* (LBA 4404) containing reporter b-glucuronidase (*gus*) gene in binary vector pBI 121 along with kanamycin resistance gene (*npt-II*) for selection in both bacteria and plant and pTi as helper plasmid

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 re-suspended in MS liquid medium to get the concentration of 10⁸ cells/ml. Pre-cultured cotyledon and hypocotyl explants were infected with fresh cultures of agrobacterial 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 agrobacterial 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 nontransformed 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 (Gomez and Gomez, 1984). The statistical analysis based on mean values per treatment was made using analysis of variance of CRD.

Results

Ten 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. 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 Kn 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 (Table 2). The regenerated

shoots elongated on the same medium.

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 0.10mg/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 *A. tumefaciens* strain LBA 4404 containing β -glucuronidase 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 agrobacterial 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 β -glucuronidase (*gus*) genes under the control of NOS promoter and 35S promoters, respectively in a binary vector system (Jefferson *et al.*, 1987). 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 nontransformed tissue (control) did not survive on the selective medium containing kanamycin (50 mg/l). The developing calli from the explants were tested for β -glucuronidase enzyme activity.

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	MS basal medium + 2.0 mg/l BAP + 0.3 mg/l IAA	2.0±1.50	38.3 (35.25)±0.34
2	MS basal medium + 2.5 mg/l Kn + 0.4 mg/l IAA	2.8±0.30	77.77 (61.86)±0.23
3	MS basal medium + 2.0 mg/l Kn + 0.4 mg/l IAA	2.0±0.00	71.11 (57.49)±0.06
4	MS basal medium + 1.5 mg/l Kn + 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	MS basal medium + 1.5mg/l BAP + 0.3mg/l IAA	0.4±0.00	40 (39.23)±1.15
2	MS basal medium + 2.5 mg/l BAP + 0.3 mg/l IAA	0.4±0.05	33.3 (35.25)±0.68
3	MS basal medium + 2.0 mg/l Kn + 0.3 mg/l IAA	0.4±0.05	46.66 (43.09)±0.15
4	MS basal medium + 2.5 mg/l Kn + 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	1114 \pm 0.88
3	Clone -1	1128 \pm 1.20
4	Clone -3	728.5 \pm 0.95
5	Clone -4	864.2 \pm 0.58
6	Clone -5	892.8 \pm 0.61
7	Clone -6	814.2 \pm 0.64
8	Clone -7	1007 \pm 1.53
S.E. \pm		\pm 0.13

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

Putative transgenic shoots were regenerated from the transformed calli obtained from cotyledon and hypocotyl explants of brinjal on the selective medium.

β -glucuronidase 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 -glucuronidase 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 agrobacterial 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 Kn 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; Matsuoaka and Hinata, 1979; Fassiliotis *et al.*, 1981; Allichio *et al.*, 1982; Magioli *et al.*, 1998; Sharma and Rajam, 1995; Picoli *et al.*, 2002; Tarre 2004; Reddy *et al.*, 2004; Neelima and Reddy, 2005; Tang *et al.*, 2005; Khatun *et al.*, 2006 and Alpsy and Seniz, 2007).

The *Agrobacterium tumefaciens* LBA 4404 strain was used in the genetic transformation studies of brinjal have two genes i.e. neomycin phosphotransferase-II (*npt-II*) and β -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 (Srivastava, 2003). The two most useful reporter gene have been the bacterial genes, chloramphenicol acetyl transferase (*CAT*) and neomycin phosphotransferase-II (*npt-II*), which encode enzymes with specificities not normally found in plant tissues (Bevan *et al.*, 1983, Herrera-Estrella *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



Fig.1b. 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
<i>npt-II</i>	Neomycin phosphotransferase-II
<i>gus</i>	β -glucuronidase

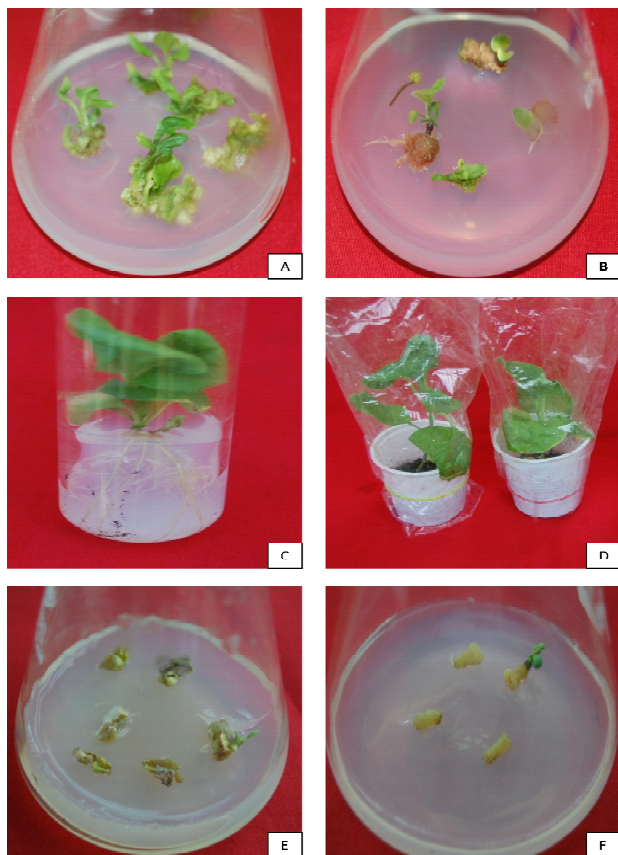


Fig 1(A-F)

Fig 1 (A-F) 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 Kn+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/l 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 Kn+ 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.

transgenic plants, but the enzyme is liable and difficult to assay with accuracy (Ow *et al.*, 1986). β -glucuronidase is superior reporter gene system for plant transformation studies. β -glucuronidase is easily, sensitively and inexpensively assayed spectrophotometrically, fluorometrically *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 (Jefferson *et al.*, 1987; 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 agrobacterial 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), bell pepper (Sharma *et al.*, 2006), strawberry (Husaini and Srivastava, 2006), *Populus deltoides* (Saraswat *et al.*, 2008), cucumber (Amita *et al.*, 2012) and cauliflower (Sharma and Srivastava, 2013). Genetic transformation had been achieved in the various cultivars of brinjal (Guri and Sink, 1988; Rotino and Gleddie, 1990; Fari, 1995; Zhang *et al.*, 2001; Franklin *et al.*, 2003; Soniya *et al.*, 2005; Hanur *et al.*, 2006). 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, protocol of plant regeneration and genetic transformation in brinjal tissues have been standardized using marker genes, This 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 from 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.

Acknowledgement

We emphatically express our worthy and venerable thanks to Dr. D.P.S Verma, Plant Biotechnology Centre, The Ohio State University, Columbus, USA for providing the *Agrobacterium* strain used in the study.

सारांश

बैंगन में पौध पुनर्जनन एवं अनुवांशिक रूपांतरण मानक तैयार करने के लिए शोध किया गया। पौध पुनर्जनन कार्य के लिए दो प्रकार के कर्त्तव्य यानी अंकुर तथा हाइपोकोटाइल लिए गये। अंकुर में सबसे ज्यादा बारम्बरता में प्ररोह का पुनर्जनन (77.77 प्रतिशत) हुआ जो एमएस माध्यम 2.5 मिग्रा./ली. बीएपी तथा 0.5 मिग्रा./ली. आईएए

के साथ से तैयार थे। एम.एस. माध्यम पूरक जो 0.01 मिग्रा./ली. आईएए (81.81 प्रतिशत) जड़ पुनर्जनन के लिए उत्तम था। बैंगन के पौधों में पुनर्जनन 6-7 सप्ताह में पाया गया। पुनर्जनन पौधों जलवायु अभ्यस्त किये गये। अनुवांशिक रूपांतरण निरस्त एग्रोबैक्टिरियम एलबीए -4404 एक रंग धारक सूचक बीटा-ग्लुकुरोनीडेस (जीएसयू) जीन द्विआधारी संवाहक पीबीआई 121 तंत्र के साथ कानामायसिन प्रति रोधी जीन (एनपीटी-८) जीवाणु व पौध दोनों के चयन में सह-कृषि प्रयोग करके बैंगन की कोशिकाओं में स्थानान्तरण जीयूएस तथा एनपीटी-८ जीन, का करते हैं। सह-कृषि उपरान्त केवल परिवर्तित कोशिका ही चयनित प्ररोह पुनर्जनन माध्यम जिनमें कानामायसिन व सेफोटाक्सीम मौजूद थे, जबकि नियन्त्रित कर्तातक चयनित माध्यम पर मृत हो गये। रूपांतरण प्रयोग को चयन उपरान्त 4 सप्ताह के अन्दर अंकित किया जा सकता है। सबसे अधिक कल्ली की प्रति चयनित माध्यम पर पाये गये जिनमें जीयूएस घनात्मक था। चयनित माध्यम जिनमें 50 मिग्रा./ली. कानामायसीन था उनमें ख्यात ट्रान्सजेनिक प्ररोह प्राप्त हुए एवं वृद्धि किए।

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