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Agrobacterium-mediated Transformation of Lentil (*Lens culinaris* Medik.)

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Abstract

Agrobacterium-mediated transformation system was developed for two microsperma varieties of lentil (*Lens culinaris* Medik.) namely, Barimasur-2 and Barimasur-4. Transformation ability of different explants like cotyledonary node, decapitated embryo, immature embryo and epicotyl were tested with *Agrobacterium tumefaciens* strain LBA4404 harbouring binary plasmid pBI121, containing the GUS and *nptII* genes. Highest percentage of GUS positive regions were found in epicotyl explants followed by decapitated embryo as detected by transient assays. Decapitated embryo was found to be more effective in formation of multiple shoots on MS medium supplemented with 0.5 mg/l BAP, 0.5 mg/l Kn, 0.1 mg/l GA_3 and 5.5 mg/l tyrosine following *Agrobacterium* infection and selection. Selection of the transformed shoots was carried out by gradually increasing the concentration of kanamycin to 200 mg/l. Stable expression of the GUS gene was observed in various parts of the transformed shoots. Genomic DNA was isolated from these shoots and stable integration of GUS and *nptII* genes was confirmed by polymerase chain reaction (PCR) analysis.

Introduction

In many developing countries of the world, pulses have gained much importance in view of the wide prevalence of protein malnutrition. Pulses are, therefore considered as the "meat of the poor" as well as the main source of protein for livestock feed and inland fish production. Moreover, these crops have the unique ability to improve soil fertility by fixing atmospheric nitrogen symbio-tically.

In Bangladesh, a number of pulses are being cultivated, these include lentil, chickpea, blackgram, mungbean, grasspea, field pea and cowpea. However, lentil is the most important in terms of preference (Gowda and Kaul 1982). In India and Bangladesh, there is an ever increasing annual demand for human consumption, but lentil production in most countries is characterized by low yield potential. Several factors such as susceptibility to diseases and pests, massive flower drop and post harvest losses are responsible for the low yield. Diseases of lentil at various stages of growth are caused by fungi, bacteria, viruses and nematodes. Among these constraints fungal diseases produce the maximum damage. The major fungal diseases in the Indian subcontinent are *Stemphylium sarciniformae, Uromyces fabae, Ascochyta fabae* and root rot caused by *Fusarium oxysporum/Sclerotium rolfsii*. These diseases cause vari-able degrees of yield loss depending on the severity of infection (Bakr and Rahman 1998).

A number of attempts were made in the past to improve lentil varieties using conventional breeding techniques, but failed to obtain desired results due to the narrow genetic base and non-availability of resistant genes against the major fungal diseases in the germplasms. Under these circumstances, *Agrobacterium*-mediated gene transfer can be one of the methods of choice to introduce gene/genes of interest into lentil varieties. Success of transformation in leguminous crops is limited because of their recalcitrant nature in *in vitro* condition (Nisbet and Webb 1990). Nevertheless, *Agrobacterium*-mediated transformation has been demonstrated in a number of leguminous crops such as soybean (Hinchee et al. 1988; Jacobsen 1992), pea (Schroeder et al. 1993), subterranean clover (Khan et al. 1994) and chickpea (Kar et al. 1996; Krishna-murthy et al. 2000).

To date only a few reports are available on attempts to transform lentil. The tumor producing ability of four strains of *Agrobacterium* in stem and shoot apices of lentil under field conditions was first reported by Warkentin and McHughen (1991, 1992, 1993). They used a number of explants such as shoot apices, epicotyl, root and cotyledonary nodal segments for transformation experiments. Transient GUS expression was observed at all wound sites on the explants except at the cut end of root explants, proximal to the cotyledonary node. They also reported that the axils of the cotyledonary petioles were not responsive to transformation. Co-electroporation of plasmid DNA in lentil root protoplasts was reported by Maccarrone et al. (1995). Chowrira et al. (1995) performed *in planta* transformation, using intact nodal meristems. They used GUS reporter gene for this experiment and could recover transgenes from the offspring of electroporated individuals. The above authors are also reported to

have recovered chimeric branches from the R1 seeds developed through electroporation of GUS reporter gene in the intact plant tissue of lentil (Chowrira et al. 1996). Öktem et al. (1999) performed transformation experiments by subjecting cotyledonary nodes of lentil using particle gun bombardment. They observed 50% GUS expression in the bombarded explants. However, none of the workers has reported the development of a complete plantlet expressing foreign genes.

For the present set of experiments *Agrobacterium*-mediated transforma-tion was carried out using various explants from two local varieties of lentil (Barimasur-2 and Barimasur-4). Here, a simple protocol for multiple shoot regeneration from different explants following *Agrobacterium*-mediated gene transfer is reported. Expression of the GUS gene was detected by histochemical assay. Integration of GUS and *nptll* genes was confirmed by PCR analysis.

Materials and Methods

Seeds of microsperma type of two lentil (*Lens culinaris* Medik.) varieties, namely Barimasur-2 (BM-2) and Barimasur-4 (BM-4) collected from Bangladesh Agriculture Research Institute (BARI), Joydebpur, Gazipur were used in the present investigation. Experiments were conducted on MS medium supplemented with different concentration and combinations of auxins, cytokinins, GA_3 and additives for regeneration of shoots. The cultures were maintained under fluorescent illumination on a 16 h photoperiod at 25 ± 2_C.

For the preparation of explants the seeds were first washed three times in distilled water and surface sterilized in the laminar flow with 0.1% $HgCl_2$ for 15 minutes. The seeds were then placed in the dark on 3% sugar - agar medium for germination. Decapitated embryo explants were collected from overnight germinated seeds by splitting open the cotyledons and cutting root and shoot tips of the embryo. Explants of cotyledonary node and epicotyl were collected from three-day old germinated seedlings. Immature embryo explants were collected from 14 - 16 days old seeds developed in field grown plants.

Agrobacterium tumifaciens strain LBA4404 with the binary plasmid pBI121 was used for transformation experiments. The binary vector pBI121 has the background of pBIN19. It contains a scoreable reporter gene GUS (β-glucurunidase) driven by a CaMV35S promoter and NOS terminator and a selectable marker gene *nptII* fused between NOS promoter and NOS terminator encoding for the enzyme neomycin phosphotransferase conferring kanamycin resistance (Herrera-Estrella et al. 1983). Fifty ml of liquid YMB (Hooykaas 1988) containing 50 mg/l kanamycin was inoculated with *Agrobacterium* from a

fresh bacterial plate and grown at 200 rpm on a rotary shaker at 28_C for 16 h. The culture was subsequently spun at 5000 rpm at 20_C for 10 min in a centrifuge and the pellet resuspended in 10 ml liquid MS medium maintaining the optical density of 1.0 - 1.2.

The explants were prepared with a scalpel while submerged in the *Agrobacterium* suspension. The cut explants were kept incubated in the *Agrobacterium* suspension in a small Petri dish for an additional 30 minutes. They were then blotted dry on a sterilized Whatman filter paper and co-cultured in Petri plates on MS with 0.5 mg/l BAP + 0.5 mg/l Kn + 0.1 mg/l GA_3 + 5.5 mg/l tyrosine (regeneration medium) for three days in the dark. Following co-culture the explants were washed several times in liquid MS with gentle shaking until no opaque suspension was seen. The infected explants were finally washed for 10 min in MS with 300 mg/l ticarcillin (Duchefa, Nether-lands), dried with a sterile Whatman filter paper and placed on regeneration medium with 50 mg/l combactum (Pfizer, Germany) and 50 mg/l ticarcillin to kill the bacteria. The explants were then placed in the growth room for regeneration under 16/8 hours light/ dark cycle at 25 ± 2_C.

To eliminate untransformed tissues, the regenerating explants were subcultured on fresh regeneration medium initially with 50 mg/l kanamycin after three weeks. The selection antibiotic was increased with each subculture at 14 days intervels up to 200 mg/l kanamycin. During each subculture the dead and deep brown tissues were discarded and green shoots and shoot buds were subcultured to fresh medium containing the next higher concentration of kanamycin.

Transformation ability of the explants was monitored by GUS histochemical assay (Jeffereson et al. 1987) by submerging them in the substrate X-gluc (5-bromo, 4-chloro, 3-indolyl α-D glucuronide) and incubating them at 37_C for two - three days. They were then washed in 70% alcohol and scored for GUS expression. Shoots developing from uninfected lentil explants were used as negative control and transgenic tobacco tissues were used as positive control. X-gluc was prepared by dissolving in 100 µl dimethyl-formamide and made up to a final concentration of 1.0 mg/ml with 50 mM phosphate buffer at pH 7.0. Cultured explants were subjected to transient GUS assay after antibiotic wash (see above). Tissues and shoots under selection pressure of around two months were monitored for stable GUS expression.

The presence of the GUS and *nptII* genes in the lentil genomic DNA was analysed by polymerase chain reaction. DNA was isolated from non-transformed plant and transformants using the CTAB method (Doyle and

Doyle 1990). For the detection of the *nptII* coding sequence, DNA was subjected to PCR using the following primers and conditions: forward 5′-TGA TTG AAC AAG ATG GAT TG-3′ and reverse 5′-CAT TTT CCA CCA TGA TAT TC-3′. For the GUS gene the primers were : forward 5′-CCT GTA GAA ACC CCA ACC CG-3′ and reverse 5′-TGG CTG TGA CGC ACA GTT CA-3′ (MGW-Biotech, AG, Germany). All primers were used at a concentration of 100 pmol/µl. The plasmid pBI121 isolated from *Agrobacterium tumefaciens* was used as the positive control. PCR reaction mix of 25 µl contained 2.5 µl of 10 ∇ PCR buffer with 15 mM $MgCl_2$ (Gene Craft, Germany), 1 µl of 5 mM of the dNTP mix, 1 µl of Red Taq polymerase (Natutech, Germany), 1 µl of each of the respective primers, and 1 µl (50 - 80 ng/µl) of the sample DNA and 17.5 µl ultra pure water. For PCR amplification of the GUS gene, DNA was denatured at 94_C for 3 min and then amplified in 30 cycles using 94_C for 1 min, 64_C for 1 min (annealing) and 72_C for 1 min followed by 5 min at 72_C. For *nptII* gene the cycling conditions were 3 min at 94_ denaturtion and 30 amplification cycles using 94_C for 1 min, 55_C for 1 min (annealing) and 72_C for 1 min followed by 5 min at 72_C. The amplified DNA was run on 1.0% agarose gel and stained with ethidium bromide (0.05 µg/ml).

Results and Discussion

Transformation capability of various explants of the two lentil varieties after *Agrobacterium*-mediated transformation was monitored by expression of the GUS (β-glucuronidase) gene. GUS expression was detected by histochemical assay and such expression was characterized by the formation of indigo blue colour within the transformed cells of the infected explants.

Results of the transient GUS assay for the various explants are presented in Table 1. A good number of explants co-cultured with *Agrobacterium* showed positive GUS staining. GUS positive regions were visualized at the peripheral area of the cut surfaces as well as within the internal tissues of various explants (Figs. 1, 2 and 3). From Table 1 it was evident that the highest percentage of GUS positive explants was found in case of epicotyl which was followed by decapitated embryo explants. Transient expression of GUS gene was relatively low in case of immature embryo explants.

The percentage of cells transformed by *Agrobacterium* is low and therefore it is important that there is a coincidence of regeneration with transformed cells. The results obtained here suggest that the cotyledonary nodes are not amenable to *Agrobacterium*-mediated transformation due to the fact that multiple shoot regeneration occurs from pre-existing meristems in the inner

layer of the explants which are beyond the reach of *Agrobacterium.* Regeneration responses from epicotyl were also found to be very low and therefore these two explants are not suitable for further transformation experiments. Öktem et al. (1999) reported the presence of GUS positive areas in cotyledonary nodes of lentil but did not report regeneration of transformed shoots from these areas. A. Gulati (personal communication) tried to develop transformed plantlets from the pre-existing meristems of the cotyledonary node by injuring the nodal regions by pricking it with needles but failed to get any positive result.

Table 1. Responses of various explants of lentil (BM-2 and BM-4) towards GUS histochemical assay following three - four days of co-cultivation.

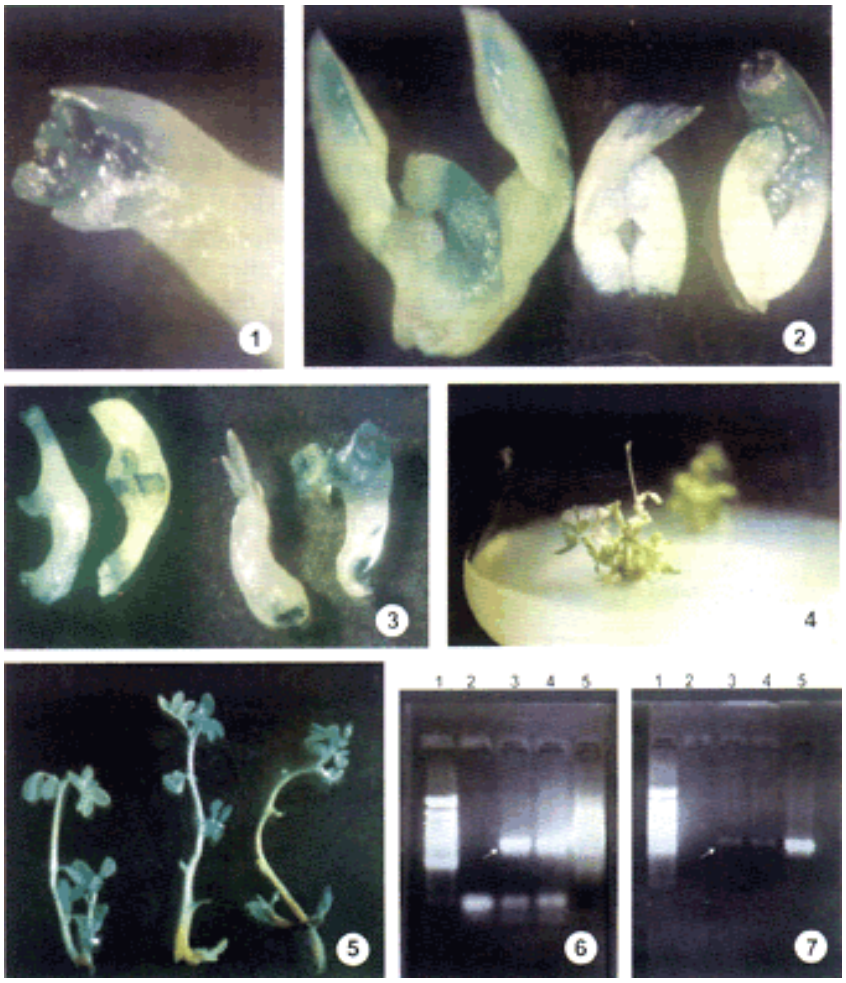
Explants	No. of explants assayed for GUS	No. of GUS +ve explants	% of GUS +ve explants
Cotyledonary node	95	53	55.79
Decapitated embryo	90	70	77.7
Immature embryo	65	30	46.15
Epicotyl	85	72	84.7

Among the different explants used in the present investigation the decapitated zygotic embryo showed high transient as well as stable GUS expression as confirmed by histochemical GUS assay (Fig. 3). In this explant the point of regeneration was also found to coincide with the transformed cells. The use of such embryo-derived explants has previously been reported in grain legumes leading to the recovery of transgenic plants (Schroeder et al. 1993, 1995).

Following co-cultivation the explants were transferred to suitable medium where regeneration of shoots were obtained through organogenesis. The most effective medium was found to be MS supplemented with 0.5 mg/l BAP, 0.5 mg/l Kn, 0.1 mg/GA_3 and 5.5 mg/l tyrosine. This regeneration medium was slightly different from the one previously reported for lentil by Khanam et al. (1995) where GA_3 and tyrosine were not included.

Regeneration experiments were carried out with and without selection pressure of kanamycin in the medium. Initiation of multiple shoots was found to be retarded in the presence of kanamycin. Therefore, following their initiation in absence of kanamycin, regenerating shoots were transferred to medium containing kanamycin. Similar observations were reported in other plant species including alfalfa, chickpea and peanut (Pezzotti et al. 1991; Kar et al.

1996; McHughen et al. 1989; Sarker et al. 2000) where a preculture period and a delayed selection with kanamycin were used in obtaining regeneration from explants with high transformation frequency.



Figs. 1 - 7: Transformation of lentil using GUS and *nptII* gene. 1. Transient GUS activity in epicotyl. 2. Same as Fig. 1 but in cotyladonary node. 3. Same as Fig.1 but in case of decapitated embryo. 4. Selection of regenerating shoots in presence of 150 mg/l kanamycin (note that the non transformed shoots became albino). 5. Stable expression of GUS gene in shoots regenerated from decapitated embryo explants after two months in selection media with 150 mg/l kanamycin (note the development of blue colour all over the transformed shoot). 6. PCR amplification of transformed shoots for GUS gene (lane-1. 100bp ladder; lane-2, water control; lanes-3&4 genomic DNA of lentil and lane-5, plasmid DNA of pIBGUS as positive control (arrow indicates the position of GUS positive band). 7. Same as Fig. 6 with premers for the *nptII* gene.

Initially 50 mg/l kanamycin was used as the selection pressure. Subsequently, the concentration of kanamycin was increased up to 200 mg/l. The effect of gradual increase in kanamycin selection pressure on the regenerating shoots from various infected explants and the recovery of transformants is presented in Table 2. Due to the effect of kanamycin, the *in vitro* grown shoots first became albino suggesting that the elimination of non-transformed tissue was taking place (Fig. 4). It was observed that with the increase of kanamycin concentration the number of surviving shoots decreased and most of the non-transformed shoots failed to survive in presence of 200 mg/l kanamycin. None of the cotyledonary node-derived shoots were able to continue their growth in presence of higher concentration of kanamycin, whereas a few decapitated embryo-derived shoots were recovered after such selection (Table 2). Almost identical observations were made for both the varieties of lentil.

Table 2. Effect of kanamycin on shoots.

Name of the variety	Name of the explant	No. of infected explants	No. of shoots in selection (50 mg/l kanamycin)	No. of shoots in selection (100 mg/l kanamycin)	No. of shoots in selection (150 mg/l kanamycin)	No. of shoots in selection (200 mg/l kanamycin)
BM-2	Cotyledonary node	980	1564	470	11	00
	Decapitated embryo	1275	1310	615	45	24
BM-4	Cotyledonary node	1055	1585	425	8	00
	Decapitated embryo	1588	1705	912	38	23

When organogenesis is used for transformation and subsequent regeneration of transgenic plants, the chance of getting chimeric shoots exists. We have observed *nptII* to be an efficient selectable marker for lentil. Similar findings were reported for peanut and chickpea during the selection of transformed plantlets (Eapen and Gerge 1994; Kar et al. 1996) which does not agree with the findings of Pounti-Kaerlas et al. (1990) who earlier reported that the *nptII* gene is not a suitable marker for legumes.

Stable expression of GUS gene was visualized through histochemical staining in the regenerating shoots from decapitated embryo developed in presence of selection pressure of 150 mg/l kanamycin (Fig. 5).

During the present set of experiment no induction of roots from the base of the regenerated shoot was observed, although occasional induction of roots was observed from the stem region following the culture of excised shoots on

medium containing various concentrations of auxins. However, such roots are ineffective for the transfer of plantlets to soil. As an alternative method the technique of *in vitro* grafting (Pickardt et al. 1991; Gulati et al. 2001) can be applied to develop an efficient root system for this crop.

The transgenic nature of the shoots was confirmed by PCR amplification of the GUS and *nptII* gene present within the genomic DNA (Figs. 6 and 7) of ten randomly selected transformants. Specific primers were used for this purpose as detailed in the Materials and Methods. Such observation primarily indicates the presence of transgenes within the DNA of the host plant shoots. Analysis of the transformants by Southern hybridization would have confirmed the nature, copy number and sites of integration of the transgenes, which will be done.

Following the results of the present study the decapitated embryo was selected as a suitable explant for transformation in lentil. The most successful transformation systems in grain legumes leading to the recovery of transgenic plants are those which have exploited embryonic axes (Schroeder et al. 1993, 1995), stem nodal segments or cotyledon-hypocotyl sections (de Kathen and Jacobsen 1990; Nauerby et al. 1991; Davies et al. 1993) and apical explants (Pickardt et al. 1991; Russell et al. 1993). All such explants have terminal or axillary meristems and hence, showed a high shoot regeneration capacity with high transformation efficiency.

The protocol reported here is reproducible for the *Agrobacterium*-mediated transformation of microsperma types of lentil. For this method dry seeds are used as the starting material and regeneration is obtained without an intervening callus phase. Therefore, the transformation method established during this study could be applied to transfer useful candidate genes conferring disease, insect and pest resistance.

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