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VACUUM INFILTRATION BASED AGROBACTERIUM MEDIATED GENE TRANSFER TO LENTIL (*Lens Culinaris* M.) TISSUES

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ABSTRACT

A highly efficient Agrobacterium-mediated transformation system has been developed for lentils (Lens culinaris M.) using vacuum infiltration of cotyledonary nodes and nodal segments with Agrobacterium suspension. This procedure exhibits distinct advantages over those previously reported in lentils, in that it uses vacuum infiltration enhancive force to introduce Agrobacterium suspension into the highly regenerable cotyledonary node meristems or nodal segments, which in turn rapidly produce transgenic shoots without an intermediate callus phase. The efficiency of the system has been investigated using GUS histochemical assays and PCR amplification which were evidencing integration of the transgenes and superiority of this system over conventional Agrobacterium mediated procedures that has been so far applied for lentil transformation.

Introduction

Lentil is proven to be susceptible to transformation by virulent strains of *Agrobacterium tumefaciens* via tumor induction, opine assays and Southern analysis (14). Subsequently, several explants derived from lentil seedlings were evaluated for their ability to express *uid-a* gene, after *Agrobacterium tumefaciens* inoculation, and it has been demonstrated that low but reproducible levels of GUS expression can be obtained from several types of lentil explants (15). The regeneration and transformation potential of cotyledonary nodes of lentil were also investigated using *Agrobacterium* mediated transformation, where cotyledonary node explants were shown to be responsive in plant regeneration, while the axils of cotyledonary petioles were shown to be not amenable to transformation via *Agrobacterium* (16).

Recent literature data revealed an enhanced transformation frequency in *Agrobacterium* mediated transformation systems upon mechanical reinforcement of *Agrobacterium* cells into the tissues by means of sonication (12) or vacuum infiltration (6). To our knowledge up to date these systems has not been tested in lentil transformation. In this study we aimed to investigate the efficiency of a vacuum infiltration based *Agrobacterium* mediated transformation system on highly regenerable lentil tissues.

Materials and Methods

Preparation of Plant Material

Cotyledonary nodes

Lentil seeds (cv. Sultan-I) were surface sterilized (momentarily in 70% ethanol, 8 minutes wash in 20% sodium hypochloride, followed by 3 times washes in sterile distilled water). Swollen and decolorized seeds were discarded. The seeds, from which cotyledonary node explants were to be excised, were imbibed overnight in sterile distilled water in dark at 23°C. The imbibed seeds were then germinated in dark at 23°C on MS (10) based medium supplemented with 3% sucrose and 0.8% agar, for 5 days. Cotyledonary nodes were

excised from 5 days old etiolated seedlings, the shoot and root of which were excised off at about 4-5 mm to the node, where both of the cotyledons were excised off readily with a single cut at the nodal site. The cotyledonary petioles remained intact in almost all of the explants.

Nodal segments

Seeds were surface sterilized as described above and germinated in dark at 23°C on MS based medium supplemented with 3% sucrose and 0.8% agar, for 5 days, after which the baby jars containing the already germinated seedlings were kept under 16 h photoperiod at 23°C for further 2 weeks. Nodal segments were excised from 2 weeks old seedlings, where preferably the first nodes on the epicotyl axis were excised with 5 mm length at either side (below and above) of the node. Already emerged axillary shoots, if any, were excised off from the node.

Preparation of *Agrobacterium* Culture

An *Agrobacterium tumefaciens* strain, GV2260 (4), harbouring the binary vector pGUSINT (13) was used. The binary vector pGUSINT, which is a derivative of pBI121, is containing coding sequences of neomycinphosphotransferase-II (*npt-II*), conferring resistance to kanamycin, and intron containing *uid-a* (GUS) gene.

Overnight grown culture of *Agrobacterium* (grown in YEB, pH 7.2, supplemented with 100 mg/L kanamycin and 20 mg/L rifampicin, shaken at 180 rpm, 27°C) was used for inoculation of 100 ml YEB medium supplemented with 10 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH adjusted to 5.6, antibiotics (100 mg/L kanamycin and 20 mg/L rifampicin), and 20 μM acetosyringone. The culture was grown overnight to log phase ($OD_{600} \cong 0.8$) at 27°C, 180 rpm. The *Agrobacterium* cells were then harvested and resuspended in MMA medium (MS salts,10 mM MES, 20 g/L sucrose, pH 5.6, and 200 μM acetosyringone) to a final OD_{600} of 2.4. The *Agrobacterium* suspension was kept at 22°C for 1 h, and then used for infiltrations.

Infiltration of the Explants

Cotyledonary nodes and nodal segments were immersed separately in the *Agrobacterium* suspensions in separate sterile vials, and subjected to evacuation pressure of about 650 mmHg for 20 and 30 minutes. The explants were then rinsed thrice in sterile distilled water, blot dried on sterile filter papers and then cocultivated on the nonselective regeneration medium (MSA1 medium for cotyledonary nodes, MS medium supplemented with 1 mg/L BA and MSA2 medium for nodal segments, MS medium supplemented with 0.1 mg/L GA and 10 mg/L kinetin) for 3 days in 23°C and deem light. Half of the explants of each treatment were then subjected to GUS histochemical assay (see section 2.4). The rest of the explants were left in the co-cultivation conditions up to 7 days, after which they were transferred to the selective medium (MSA1 and MSA2 medium with 500 mg/L cefotaxim and 50 mg/L kanamycin). All of the cultures were kept at 23°C, 16 h photoperiod for up to two weeks of regeneration. After 2 weeks of regenerative cultivation, the regenerated shoots were excised and assayed for GUS expression.

Analysis of Transformed Tissues

GUS Histochemical Assay

Agroinfiltrated explants and the shoots regenerated from those tissues were subjected to GUS histochemical analysis (5). In this assay, explants were placed in glass vials and covered with assay buffer composed of 100 mM potaisum phosphate buffer pH 7.0, 10 mM EDTA, 0.5 mM potassium ferriccyanide, 0.5 mM potassium ferrocyanide, 0.1% Triton X-100 and 1 mM X-Glucuronide, and a mild vacuum was applied for 1 minute to distribute the substrate equally in the cells of the explants. The reaction was allowed to proceed 48 h at 37°C. The explants were then fixed (fixative solution: 10 % formaldehyde, 20% ethanol, 5% acetic acid) for 4 h, decolorized (% 50 ethanol) for 2 h, dehy-

TABLE

Results of GUS Histochemical Assays of *Agro*-Infiltration Experiments. Results are average of two experiments with 30 explants each

Explant	Infiltration	Frequency of GUS Expression	
		GUS expression (%)[a] after 3 days	GUS expression (%)[b] after 3 weeks
Cotyledonary Nodes	20 min	73% with average # of 7.2 expression sites/explant	36% with average # of 25.4 expression sites/shoot[c]
Cotyledonary Nodes	30 min	87% with average # of 7.7 expression sites/explant	3% with average # of 13 expression sites/shoot
Nodal Segments	20 min	70% with average # of 20.7 expression sites/explant	84.6% with average # of 14.4 expression sites/shoot
Nodal Segments	30 min	75% with average # of 17.8 expression sites/explant	53% with average # of 5.1 expression sites/shoot

[a]: Number of GUS positive explants/Total number of assayed explant

[b]: Average number of GUS positive regenerated shoots/Total number of assayed regenerated shoots

[c]: Regeneration analysis under selective force (50 mg/l Kan) was conveyed for half of the explants per treatment. Randomly excised 20 regenerated shoots per treatment were analysed for GUS expression after 2 weeks of cultivation on selective media.

drated (96% ethanol) overnight and examined under dissecting microscope counting GUS expression foci.

PCR Analysis

Genomic DNA from some of the shoots regenerated from Agroinfiltrated cotyledonary node and nodal segments were isolated by Nuclospin Plant DNA isolation kit according to the instructions of the manufacturer (Macherey-Nagel, Germany). The primers 5'-GAGGCTATTCGGCTATGACTG-3' (forward) and 5'-ATCGGGAGCGGCGATACCGTA-3' (reverse) were utilized to amplify a 700 bp segment of the *npt-II* gene. Amplification was performed under following conditions: 1 min denaturation at 94°C, 45 sec min annealing at 55 °C, 30 seconds elongation at 72 °C during 25 cycles, with a final extension step of 5 minutes at 72 °C. PCR products were separated on 1% agarose gels and documented.

Results and Discussion

The efficiency of the Agro-infiltration system was compared in two different explants, cotyledonary nodes and nodal segments, as pronounced in their transient GUS expression. GUS histochemical assays were carried out after a co-cultivation period of 3 days to monitor transient gene expression. As for relatively more stable GUS expression, assays were carried out on the regenerated shoots of the explants, which had been co-cultivated for 1 week on non-selective medium, followed by further 2 weeks of regeneration in the presence of 50 mg/ml kanamycin selective medium.

The results of GUS histochemical assays are demonstrated in **Table**. As it is noticeable from the Table, in terms of their transient GUS expression, in all of the tissue types, expression frequencies (i.e. number of expressing explants / total number of infiltrated for a particular explant source) were relatively higher when Agro-infiltrated for 30 minutes rather than those treated for 20 minutes. This can be explained by the fact that, the transient gene expression is caused by both integrated and unintegrated T-DNA transcriptional events within the nucleus, and it can be suggested that due to the relatively longer time of the treatment in 30 minutes treatments, higher number of the explants had uptaken the T-DNA molecules. GUS positive nodal segments were demonstrating most of the transient expression at the bud region and the axillary shoots while the transient expres-

sion in cotyledonary nodes was occurring almost equally at nodal region with the regenerated shoots and cut end at the root side. While nodal segment derived shoots were demonstrating more scattered GUS expression, cotyledonary node derived shoots were illustrating more patchy expression mostly at the shoot apex. Furthermore, comparing the average number of transient expression sites per GUS positive explant of the nodal segments with that of cotyledonary nodes (Table), it can be suggested that, given that the number of GUS positive explants demonstrating transient GUS expression is not significantly different in two different explant sources, the nodal segments were of a higher amenability and responsiveness with respect to the T-DNA uptake, as pronounced in their average number of expression sites per GUS positive explant. This suggestion is confirmed by the evidence that, after 2 weeks of regeneration on selective medium, the regenerated shoots of nodal segments are demonstrating much higher expression frequency (number of GUS positive shoots/total number of assayed shoots) than the regenerated shoots of cotyledonary nodes, in both 20 and 30 minutes lasting treatments. Representative data from the experiments is given in **Fig. 1**.

A comparison between GUS expression frequencies of the regenerated shoots of both explant sources at 20 minutes infiltration time and that of 30 minutes (Table), may result in the conclusion that, the higher frequency of the transient GUS expression at 30 minutes treatments was partially because of the transcriptional events of the unintegrated T-DNA, since the GUS expression frequency of the regenerated shoots of 30 minutes treated explants was not any higher than that of 20 minutes treated explants. Therefore, from the latter data, it can be suggested that, 20 minutes treatment, in both of the explant sources, is more efficient in terms of rather stable transformation.

For further verification of the success of transformation, genomic DNA of shoots originated from agroinfiltrated explants were isolated and subjected to PCR analysis by using *npt-II* gene specific primers. As can be seen from **Fig. 2**, the expected DNA fragment (700 bp) was amplified in *Agrobacterium* treated cotyledonary nodes and nodal segments whereas no amplification was encountered from DNA of untransformed tissues. In addition to GUS positive results in transformed explants, this data further confirms the success of transformation experiments and integration of the transgenes in the genome.

In lentil transient gene expression has been reported using different transformation techniques and explants including cotyledonary nodes and *Agrobacterium* (15, 16), longitudinally sliced embryogenic axes and *Agrobacterium* (7) protoplast and liposomes (8) and cotyledonary nodes and particle bombardment (11). However, compared to other legumes, the success in lentil transformation is limited (1, 3). The only transgenic lentil plants reported up to date was developed by *in planta* electroporation technique as described by Chrowia et al. (2).

Compared to other tissues, cotyledonary node of lentil was shown to be one the most responsive tissue for regeneration via direct organogenesis (9,16). However previous transformation efforts on this tissue exhibited limited success. Here we report a simple and relatively high frequency *Agrobacterium* mediated gene delivery technique for lentil cotyledonary nodes and nodal segments. Due to the wide spread use of *Agrobacterium* based transformation systems in many laboratories, we believe that the application of this technique would facilitate transformation studies in lentil.

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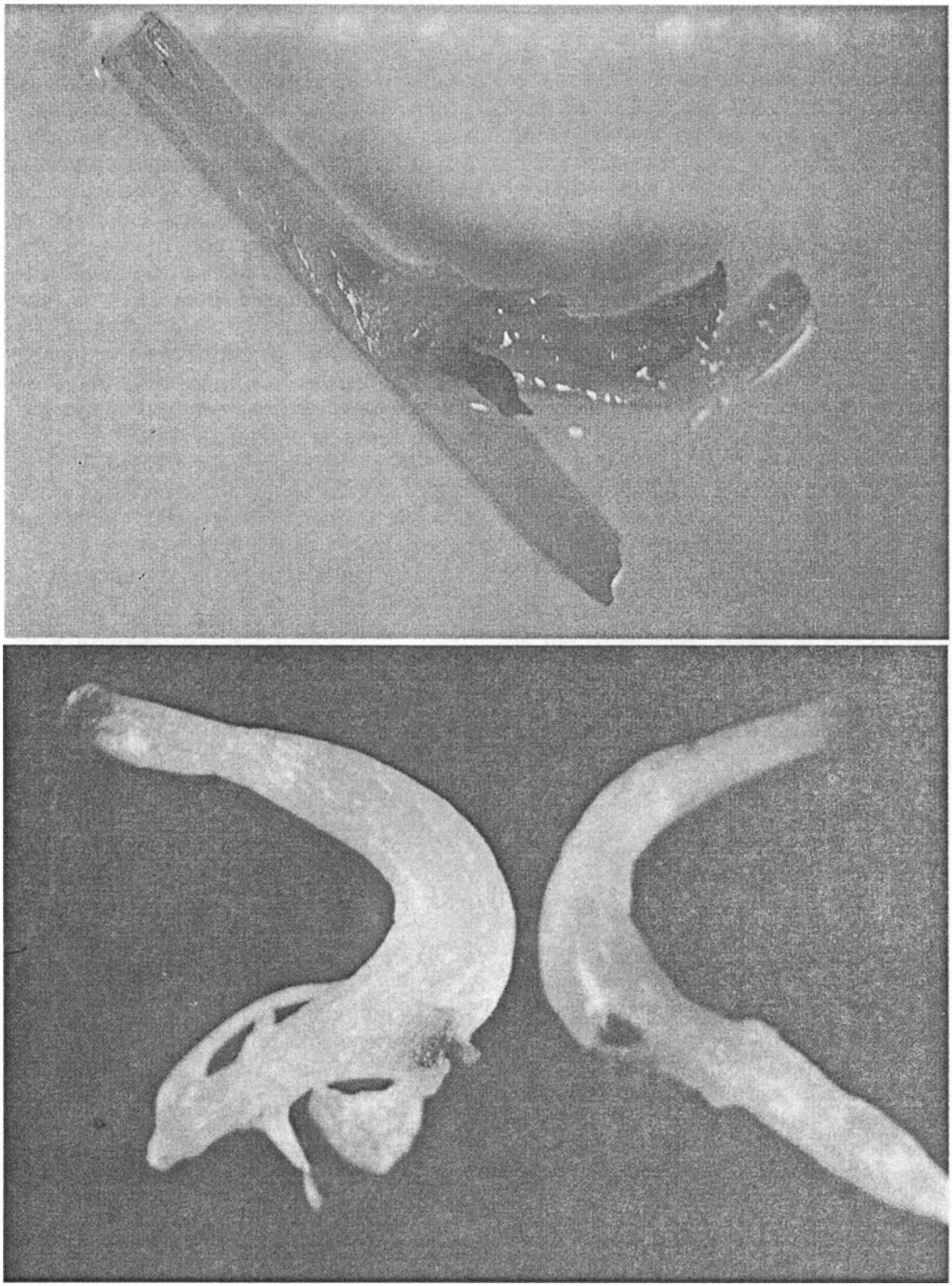

Fig. 1. Transient GUS expression in nodal segments (A) and cotyledonary nodes (B) after *Agrobacterium* infiltration at 650 mmHg for 20 minutes. Explants were co-cultivated for 3 days and stained for GUS activity as described in Materials and Methods.

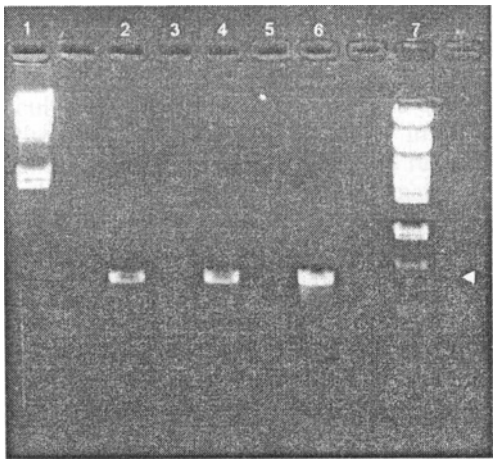

Fig. 2. PCR analysis of genomic DNA from shoots regenerated from *Agrobacterium* infiltrated cotyledonary nodes (lane 2) or nodal segments (lane 4) and untransformed cotyledonary nodes (lane 3) or nodal segments (lane 5). Lane 6 shows the amplification pattern of pGUSINT plasmid with the same npt-II specific primers. The gel is calibrated by λ-HindIII and λ-PstI markers (Lanes 1 and 7, respectively). Expected fragment size (700 bp) is shown by the arrow.

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