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An Efficient *Agrobacterium*-mediated Genetic Transformation Method of Lettuce (*Lactuca sativa* L.) With an Aphidicidal Gene, *Pta* (*Pinellia ternata* Agglutinin)

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Abstract: *Lactuca sativa* is widely used leafy vegetable belonging to the asteraceae family, which is adopted as a model plant for transgenic research. We established an *Agrobacterium tumifaciens*- mediated transformation procedure for *L. sativa.* Leaf discs of *L. sativa* were incubated with *A. tumefaciens* strain LBA4404 containing pBIPTA plasmid with the *npt* gene as a selectable marker for kanamycin resistance and intron containing *pta* gene as an aphicidal gene. Following co-cultivation, leaf discs were cultured on selective medium containing 50 mg l^{-1} kanamycin + 50 mg l^{-1} cefotaxime. Kanamycin resistant shoots were induced from the leaf discs after four weeks. Shoot regeneration was achieved after transferring the tissues onto fresh medium of the same combination. Finally, the shoots were rooted on MS medium containing 50 mg l^{-1} kanamycin. Incorporation and expression of the transgenes were confirmed by PCR and RT-PCR analysis. Using this protocol, transgenic lettuce plants can be obtained in approximately 6 months with a high transformation frequency.

Key words: Agrobacterium · *Pinellia ternata* agglutinin · transformation method

INTRODUCTION

Lettuce is a widely used and freshly consumed leafy vegetable and has been cultivated all over the world. Lettuce belongs to the Asteraceae family, which has an estimated genome size of ca, 2.5 Gb [1], is amenable to classical and molecular genetic analysis. To date, genetic improvement of lettuce has been achieved mainly by conventional plant breeding methods, but recent advancement in genetic transformation techniques have opened new avenues for crop improvement. Lettuce was chosen as a model plant for its fast growth, agronomic value and short genome size.

Successful genetic transformation has been reported through *Agrobacterium tumefaciens* in many plant species, such as, aphid resistance transgenic tobacco expressing *pta* gene [2]; freezing resistance lettuce [3]; brown planthopper and green leafhopper resistance indica rice [4] and aphid resistance transgenic cotton with *aca* gene [5].

Aphids (*Lipaphis erysimi*) not only damage lettuce seedlings by sucking cell saps from the phloem tissues but also carry different viral inoculums. Toxic compounds, which is released from insecticides used by farmers to shield aphids from the field contaminates fauna including human and flora. *Pinellia ternata* is a Chinese medicinal plant species, belonging to the Araceae family. Tropical insect bioassay studies showed that agglutinins of *P. ternata* (PTA) purified from the tubers had significant insecticidal activities towards cotton aphids (*Aphis gossypii* Glover) and peach potato aphids (*M. persicae*) when incorporated into artificial diets [6,7]. The full-length cDNA of *P. ternata* agglutinin gene (*pta*) was cloned recently using the RACE-PCR protocol from inflorescences of *P. ternata* in the State Key Laboratory of Genetic Engineering, China [8]. Its size is 1191 bp and contained a 810 bp open reading frame encoding 269 amino acids with a calculated molecular mass of 29.4 kDa (p*I* 6.58).

The aim of the present study reported here was to establish an efficient *Agrobacterium*-mediated genetic transformation method for lettuce with an aphidicidal gene, *pta*. This will provide an alternative method for the molecular analysis of gene functions in this plant.

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MATERIALS AND METHODS

Plant material: Seeds of lettuce cultivar ‘Evola’ were provided by Plant Breeding and Gene Engineering Lab., Dept. of Botany, University of Rajshahi, Bangladesh for transformation.

Agrobacterium strain and plasmid: cDNA sequence of *pta* gene was inserted into pBI 121 Ti plasmid under the control of 35S promoter and Nos terminator to develop pBIPTA plasmid. The recombinant vector pBIPTA containing 0.81 kb fragment of *pta* was transferred from *Escherichia coli* DH5α into *A. tumefaciens* LBA4404 by triparentalmating [12] and was received from Professor M. Monzur Hossain of the Plant Breeding and Gene Engineering Lab., Dept. of Botany, University of Rajshahi, Bangladesh.

Tissue culture conditions: Seeds of lettuce were surface sterilized for 30 min with Clorox followed by washing with double distilled water and then transferred to agar gelled hormone free MS medium in 9 cm petridishs to germinate. Seedlings were grown at 20°C in a 16 h light (60 μE m^{-2} s^{-1}; cool white fluorescent tubes). The same growth conditions were used for tissue culture procedures. All plant media were adjusted with 1N NaOH to pH 5.7, solidified with 8 g l^{-1} agar and autoclaved at 121°C for 20 min.

Sensitivity test of explants to kamamycin: The sensitivity of lettuce leaf to kanamycin was assayed by culturing the leaves without co-cultivation with *A. tumefaciens* on selection medium contained different concentrations of kanamycin (0, 25, 50, 75, 100 mg l^{-1}).

Transformation of lettuce leaf explants: Leaves from 14-days old seedlings were cut into 0.5×0.5 cm and inoculated with the *Agrobacterium* cells harbouring LBA4404 having *pta* gene in liquid MS medium supplemented with 50 mg l^{-1} kanamycin for 1-10 min. Subsequently, the density of *Agrobacterium* inoculums of 0.50-2.50 at 600 nm and co-cultivation for 12-72 h on agar gelled MS_0 medium. After co-cultivation, the explants were transferred and placed upside down onto selection and regeneration medium (MS + 0.1 mg l^{-1} NAA + 0.01 mg l^{-1} BA + 50 mg l^{-1} kanamycin + 50 mg l^{-1} cefotaxime) and inoculated under light. After 4 weeks shoots formed at the cut end of the leaf discs and transferred onto same fresh medium for shoot induction. Kanamycin resistant shoots were separated and transferred to MS basal medium supplemented with 50 mg l^{-1} kanamycin for shooting.

PCR and RT-PCR analysis: The presence of transgenes in transformed and control (non-transformed) plants was analysed by the polymerase chain reaction (PCR) and its transcripts by RT-PCR (reverse transcriptase polymerase chain reaction). Genomic DNA of lettuce was extracted from young leaves following the protocol of Edwards *et al*. [10]. PCR analyses to detect the presence of the *pta* gene were carried out using the PCR Screening Kit (Sigma Chemicals Ltd., USA) in the presence of following pair of primers: forward primer (5-ATGGCCTCCAAGCTCCTCCT-3) and reverse primer (5-ATTCAGTAGCCAGCCAGCAG-3). The PCR mixtures were denatured at 94ºC for 4 min followed by 35 cycles for 1 min at 94°C, 1 min at 58°C for annealing, 1.30 min at 72°C for extension and finally incubated at 72°C for 10 min. Expected PCR product size was about 120 bps. PTA transcripts analyses were carried out to confirm the transgenic status of plants showing a positive reaction in RT-PCR analysis. Total RNA was isolated from the transgenic plants according to the manufacturer’s instructions (Quagene, UK).

First strand cDNA synthesis with poly-T primers and subsequent cDNA amplification with the target gene specific primers were done with RT-PCR kits (Applied Biosystem, UK) according to supplier’s manual. Amplified cDNAs were resolved on 1.5-2% agarose gel, stained with ethidium bromide (EtBr) and documented.

RESULTS AND DISCUSSION

Effect of kanamycin concentration: To develop a rapid and efficient *Agrobacterium*-mediated transformation method for lettuce, the action of different concentrations of antibiotics that are currently used as selectable markers in plant transformation procedures was assayed. Kanamycin 50 mg l^{-1} completely blocked regeneration from untransformed explants and, therefore, could be used to select for transformed cells (Fig. 1). Preliminary transformation experiments indicated that cefotaxime, an antibiotic commonly used to kill *Agrobacterium* after co-cultivation with plant material, severely inhibited regeneration from *Agrobacterium* leaf explants and 50 mg l^{-1} cefotaxime noticed to be optimum. This result is in consistent with the findings of indian mulberry, *Morus indica* cv. K2 [11] and grasspea (*Lathyrus sativus* L.) [12].

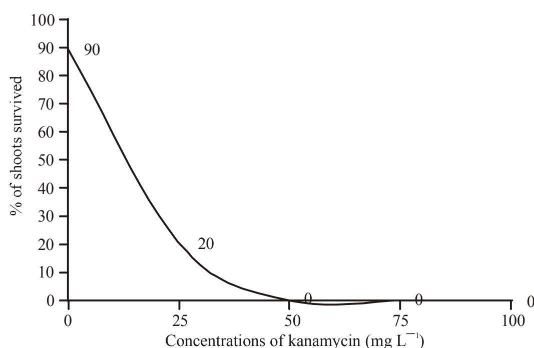

Fig. 1: Determination of kanamycin sensitivity for the selection of kanamycin resistant tissue. Among different concentrations of kanamycin on leaf explants 50 mg l^{-1} showed the best performance

Table 1: Effects of incubation period on survival percentage of leaf explants of lettuce cv. Evola. The regeneration medium contained 50 mg l^{-1} kanamycin

Incubation Period (Min)	No. of explants inoculated	No. of explants survived	Surviving % of explants	Mean (X±SE)
1	15	5	33.33±0.29	35.33±0.13
2		10	66.66±0.12	
3		12	80±0.18	
4		11	73.33±0.09	
5		7	46.66±0.22	
6		4	26.67±0.14	
7		3	20±0.12	
8		1	6.67±0.08	
9		-	-	
10		-	-	

Table 2: Effects of co-cultivation period on survival percentage of leaf explants of lettuce cv. Evola. The regeneration medium containing 50 mg l^{-1} kanamycin

Explant type	Co-cultivation period (h)	Percentage of surviving explants
Leaf	12	57.78±0.65
	24	71.00±1.06
	36	46.66±0.86
	48	37.78±0.68
	72	22.22±0.92

Standardization of plant selection and regeneration from lettuce leaf explants: Initial experiment was setup to standardize hormonal combinations in regeneration medium from aseptically grown leaf explants and cultured on MS medium supplemented with different concentrations of NAA (0.1, 0.5 and 1.0 mg l^{-1}) and BA (0.01, 0.05, 0.1, 0.5 and 1.0 mg l^{-1}). The highest percentage (70%) of explants showing regeneration was noticed with MS + 0.1 mg l^{-1} NAA + 0.01 mg l^{-1} BA. Similar results have already been reported in peanut (*Arachis hypogaea* L.) [13].

Genetic transformation of leaf explants: Leaf discs were inoculated with the *Agrobacterium* cells harbouring LBA4404 for different intervals (1-10 min). Different densities of *Agrobacterium* inoculums were examined and highest 2.96% of kanamycin resistance explants were observed at 1.50 $OD_{600\ nm}$. Considering the post infection process, an incubation period of 3 min (Table 1) was selected in the subsequent transformation experiments. After inoculation, 24 h of co-cultivation period was optimized (Table 2). The leaf explants were washed with distilled water and transferred onto the selection medium containing 50 mg l^{-1} kanamycin + 50 mg l^{-1} cefotaxime. A very similar result in accordance with this experiment was noticed in *Arabidopsis thaliana* [14-17], in peanut [12], in raspberry [18] and in sweet orange [11]. Four weeks later, green shoots grew out from the cut surface of the leaf discs. The experiment was repeated three times and 51 explants showed kanamycin resistance shoots and highest 20% transformation efficiency was observed in the experiment no. 2. Subsequently, 51 kanamycin resistance shoots (one shoot from one individual resistant leaf explants) rooted on MS medium containing 50 mg l^{-1} kanamycin. Similar findings were observed in pear [19] and in sweet orange [20]. Findings are shown in Figure 4 (A, B, C and D).The successfully rooted plants were assumed as T_0 line.

Molecular analysis of transgenic plants: PCR and RT-PCR analysis were further used to confirm the presence and the integration of T-DNA in lettuce genome, as well as the copy number integrated. PCR analysis of T_0 plants revealed that 45 out of 51 plants were positive for the *pta* gene. PCR profile of the putatively transformed T_0 plants exhibited the presence of 0.81 kb fragment of *pta* gene (Fig. 2). T_1 progeny of five independently derived T_0 transgenic plants (PT1A, PT11B, PT13B, PT19A and PT26B) were analysed for the presence of *pta* gene by PCR. Results showed that except PT19A, the *pta* gene in all four lines was inherited at a segregation ratio of 3:1, indicating integration of the *pta* transgene as a single copy in these transgenic lettuce progeny, which was commonly observed in other the transgene segregation studies [21-24,2]. Among 45 *pta*-PCR positive plants, randomly selected 29 independent *pta*-PCR positive plants were further analyzed by RT-PCR (Fig. 3) and

Fig. 2: PCR analysis of genomic DNA of lettuce (cv. Evola) to detect the presence of *pta* gene. The PCR products were separated on a 2% agarose gel and stained with ethidium bromide. M: 100-bp size marker (Promega). P: positive control. The arrow indicates the expected PCR prtoduct of the *pta* gene.

Fig. 3: RT-PCR analysis of total RNA of T_0 plants to detect the transcript of *pta* gene. M: 100-bp size marker (Promega). Lanes *1 -16*: T_0 plants showing 120 bps PTA cDNA; lane *17*: RNA of T_0 plant used as template without RT-reaction and lane *18*: control plant regenerated without infection

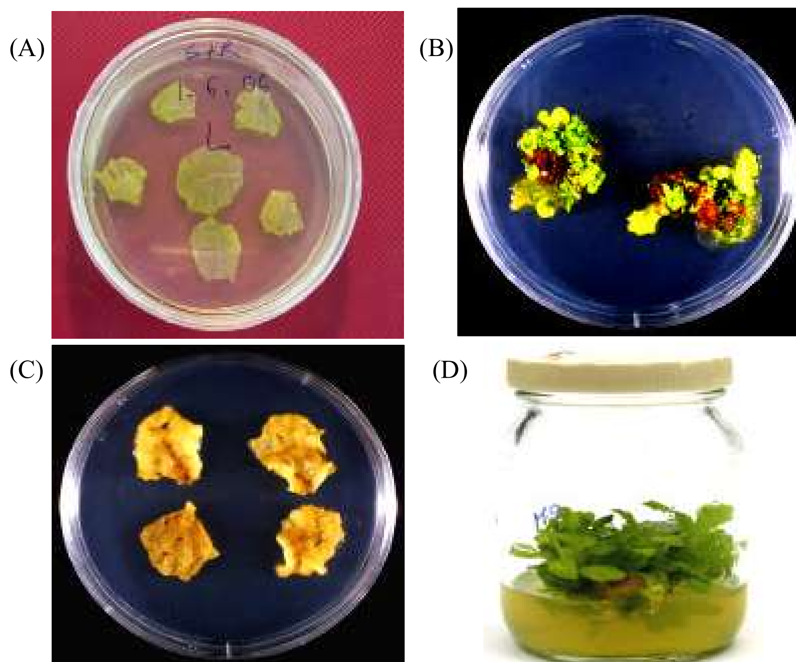

Fig. 4: Selection and plant regeneration of lettuce with *pta* (*Pinellia ternata* agglutinin) gene. (A) Leaves infected with pBIPTA vector containing *pta* gene under the control of *35S* promoter and *Nos* terminator (6 weeks after culture in regeneration medium with Km 50mg l^{-1}). (B) Regenerated plantlets in the regeneration medium. (C) Uninfected leaves in regeneration medium with Kn 50 mg l^{-1} (6 weeks after culture). (D) Transgenic T_0 plant regenerated from infected leaf

22 were found RT-PCR positive. In the transformation experiment homozygous plants were selected and *pta* inheritance pattern was observed. A very similar PCR and RT-PCR result was obtained in salt tolerant transenic buck wheat [25].

In summary, a highly efficient *Agrobacterium*-mediated leaf disc transformation method was established for lettuce and this method might be used as a accompanying method to the widely used *Agrobacterium*-mediated genetic transformation method in different plants like lettuce.

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