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Leaf Disk Transformation of *Lactuca sativa* Using *Agrobacterium tumefaciens*

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Abstract

Reliable methods of transferring cloned genes into plants are essential for engineering crops with desired traits. In order to induce sap-sucking insect resistance *Agrobacterium tumefaciens* LBA 4404 (1065) strain containing the binary vector pMOG 23 and hypervirulent pTOK 47 plasmid was used. *pta* (*Pinellia ternata* agglutinin), salmon *ct* (calcitonin) and *cgrp (calcitonin gene related protein)* genes were successfully integrated into *Lactuca sativa* plants. This genetic modification conferred lettuce resistance to orthopteran and homopteran insects like *Nilaparvata lugens* Stl or *Myzus persicae* Sulzer. *Lactuca sativa* could be routinely transformed using Ti plasmids of *A. tumefaciens* containing a chimeric kanamycin resistance gene (*nos nptII. nos*).

Keywords: lettuce transformation, *Agrobacterium tumefaciens*, lectins, insects resistance

Introduction

Cloning of genes selected from different wild plants which belong to wild flora and introducing them into cultivated plant species stands for one of the most studied areas in actual molecular biology (McCabe *et al.*, 2001; Garratt *et al.*, 2002; Davey *et al.* 2003a.,b.; Garratt *et al.*, 2003). This is how a better genetical quality of cultivated vegetal species is obtained. In order to improve the culture characteristics of economical interest plants this method provides resistance to attacking insects and to a wide range of diseases as well.

Insects and diseases seriously damage crops every year. Biotechnological practices try to reduce wastage by transferring different insect resistance genes to cultivated vegetal species. Wild plants include many of these agronomically useful genes like insect resistance or disease eradication. They were progressively transferred to other plant species by using *Agrobacterium tumefaciens* (Van Lijsebettens *et al.*, 1991; Holford *et al.*, 1992; Anthony *et al.*, 2000; Yao *et al.*, 2003; Komari *et al.*, 2006; Curtis, 2010). This paper presents the method of obtaining genetically transformed lettuce (*Lactuca sativa*) resistant to sap-sucking insects of Orthoptera and Homoptera groups (locusts and aphids). The subjected insects were the brown plant hopper (*Nilaparvata lugens* Stl), the peach-potato aphid, *Myzus persicae* Sulzer (Sun *et al.*, 2001) and *Lipaphis erysimi* (Ahmed *et al.*, 2007), all of them seriously damaging lettuce cultures.

Pta (*Pinellia ternata* agglutinin) gene and two other genes responsible for calcitonone synthesis like *ct* and *cgrp* from salmon were introduced into the genome of lettuce plants.

Lectins act like a defence system against viruses or damaging insects. They are binding proteins between glycans and glycoproteins, glycolipids or high affinity polysaccharides having a carbohydrate structure. Previous studies revealed their strong lethal effect on aphids and sap-sucking insects (Higgins *et al.*, 1983; Raikhel and Wilkins, 1987; Van Damme *et al.*, 1991).

Galanthus nivalis agglutinin (*GNA*, or *gna*) from snowdrop was the only lectin gene reported until now to have a significant lethal effect on homopteran and orthopteran insects (Rao *et al.*, 1998; Yao *et al.*, 2003). Other traditionally used medicinal species of Chinese flora contain lectins which eradicate damaging insects in cereal cultures.

Lectins were purified from these species and they were analyzed leading to the evidence that they had biochemical characteristics similar to those of *gna* gene (Wilkins and Raikhel, 1989; Yang and Czapla, 1993). As a conclusion of these studies it was commonly accepted that lectins from *Pinellia* group had common sequences with some fragments of GNA protein. On the basis of the information provided by GNA analysis purification of lectin genes from this group of plants became possible.

Materials and methods

Plant material

Seeds of *Lactuca sativa* cvs. 'Evola', 'Lake Nyah', 'Cortina', 'Luxor', were sterilized with Domestos (Leve Bros.) 10% (v/v) 30 min., *in vitro* cultivated on ½ MS medium (Murashige and Skoog, 1962) containing agar, salts and vitamines, 10 g/l sucrose, 8 g/l agar (Sigma), pH 5.9. The Petri dishes containing sterilized lettuce seeds (40 seeds/dish) were incubated at 23±2°C, 16 h photoperiod, with a light intensity of 18 μmol $m^{-2} s^{-1}$ (daylight fluorescent tubes) for 7 days, until cotyledons fully expanded.

Bacterial strain

A. tumefaciens strain 1065 was grown on LB agar culture medium containing 10 g/l bacto tryptone, 5 g/l yeast extract, 10 g/l NaCl, 18 g/l agar (Sigma, St. Louis, MO),

pH 7.0., for 2-3 days. One loopful of bacteria was taken and transferred to 10 ml of LB liquid culture medium (with antibiotics) and it was grown overnight at 28°C, in the dark, on an orbital shaker (180 rpm).

A. tumefaciens strain LBA4404 used included the binary vector pMOG23 (Sijmons *et al.*, 1990) and hypervirulent pTOK47 (Jin *et al.*, 1987). The binary vector had both the *nptII* (neomycin phosphotransferase II) and β–glucuronidase (*gus*) intron (Janssen and Gardner, 1989; Vancanneyt *et al.*, 1990) coding sequences under the control of *nos* (nopalin synthetase) and CAMV–35S promoters, also a *Nos* terminator (Curtis *et al.*, 1999; McCabe *et al.*, 1999). This strain was described as 1065. *nptII* was used as a selectable marker gene, coding for kanamycin resistance of transformed lettuce plants (Vancanneyt *et al.*, 1990).

The binary vector included *pta* as well as *ct* and *cgrp* (calcitonin gene related protein) genes of economical interest, to be transferred to lettuce plants. These genes were subjected to confer resistance to sap-sucking homopteran and orthopteran insects, aphids and locusts. Apart of these target genes, the biary cloning vector had origins of DNA replication (*ori*) for both *E. coli* and *A. tumefaciens*, together with a marker selectable gene for these bacterial strains (Zambrysky *et al.*, 1983; Shahin and Simpsons, 1986; McInnes *et al.*, 1989; Komari *et al.*, 2006; Curtis, 2010).

Constructs used in this transformation were presented in Tab.1.

Tab. 1. Genes, source and constructs used for transformation of *Lactuca sativa* plants

No.	Genes	Source	Vectors
1.	*pta* (*Pinellia ternata* agglutin)	*Pinellia ternata*	pBI 121
2.	*ct* (calcitonin)	salmon	pCAMBIA 2301
3.	*cgrp* (calcitonin gene related protein)	salmon	pCAMBIA 2301

Selection of A. tumefaciens strains including the binary vector

Selection of *A. tumefaciens* strains including the binary vector was performed on LB medium. Culture media variants used included antibiotics: liquid LB medium with 50 mg/l kanamycin sulfate, 40 mg/l rifampicin, 2 mg/l tetracycline-HCl. Agar culture medium: LB medium with 50 mg/l kanamycin sulfate, 100 mg/l rifampicin, 5 mg/l tetracycline-HCl.

Infection of cotyledons

Cotyledons from 7-8-day-old seedlings were excised, keeping intact their petiole, removing their apices in order to increase the wounded surface. The abaxial surface was scored, making shallow cuts (1 mm apart) with a scapel, across the surface of the leaf. Cotyledons were floated 10 min. with their wound-surface in contact with an overnight liquid culture of Agrobacterium, diluted 1:10 (v:v) and 5:10 (v:v) with liquid UM medium (Horsch *et al.*, 1985; Beck and Camper, 1991; Komari *et al.*, 2006). Control cotyledons floated on liquid UM without Agrobacterium were prepared as well.

Test plates (Petri dishes) were set up as following:

a. Control explants without *Agrobacterium* inoculation on:

- SI (Shoot Inducing) medium
- SI+50 mg/l kanamycin sulfate
- SI+100 mg/l kanamycin sulfate.

b. Explants inoculated with *Agrobacterium* on:

- SI+50 mg/l kanamycin sulfate
- SI+100 mg/l kanamycin sulfate.

In order to improve transformation efficiency nurse cultures were also used.

Shoot Induction

SI medium: 4.71 mg/l MS salts and vitamins, 30 g/l sucrose, 0.04 mg/l NAA, 0.5 mg/l BAP, 500 mg/l carbenicillin, 100 mg/l cefotaxime, 50 or 100 mg/l kanamycin sulfate, 8 g/l agar (Sigma), pH 5.8. Explants were transferred on solid SI medium, submerging their petiolar ends into the medium. They were incubated as for germinating seeds and subcultured to fresh SI agar medium every 14 days. After 49 days of culture, those explants which callused and generated shoots were transferred to 175 ml capacity glass jars (Davey *et al.*, 2002), containing 0.11% (w/v) MES (2[N-morpholino] ethanesulfonic acid) each, one explant/jar.

When shoots grew 1cm high they were transferred to glass jars each containing 40 ml of rooting induction medium (RI) and were incubated at 18 μmol m^{-2} s^{-1}, 16 h photoperiod, 23±2°C.

Standardization of plant selection

Explants were cultured on MS medium supplemented with different concentrations of NAA (0.04, 0.05 and 0.1 mg/l) and BAP (0.1, 0.5 and 1 mg/l).

Tab. 2. PCR sample composition used for testing *Lactuca sativa* transformed plants

Substance	Volume	Concentration
Sterile ultra pure water (pH 7.0)	9.5 μl	
10X PCR buffer	2 μl	2x
dNTPs mix (2 mM)	2 μl	0.2 mM
$MgCl_2$ (25 mM)	2 μl	2.5 mM
Primer 5` (20 pm/μl)	1 μl	1 pm/μl
Primer 3` (20 pm/μl)	1 μl	1 pm/μl
genomic DNA of *Lactuca sativa* (50 ng/ul)	1 μl	50 ng/20μl
DNA Taq polimerase Sigma (5u/μl)	0.5 μl	2.5 units/20μl
PVP (*polyvinyl pyrrolidone*) 30%	1 μl	

Tab. 3. PCR programs used to analyze transformed *Lactuca sativa* plants

No	Heating of *Taq* polymerase	DNA Denaturation temp.	Annealing	Extension	Final step	Storage	No. of cycles	Construct used
1.	94°C 5 min	94°C 50 sec	58°C 1 min	72°C 2 min	72°C 2 min	4°C	35	pBI 121 pta EHA 105
2.	94°C 5 min	94°C 30 sec	56°C 50 sec	72°C 30 sec	72°C 30 sec	4°C	35	pCAMBIA 2301 CGRP
3.	94°C 30 min	94°C 1 min	65°C 1 min	72°C 1:30 min	72°C 2 min	4°C	35	pCAMBIA 2301 CT

Tab. 4. Structure of primers used to amplify the three genes used as an insert

Primers	PCR product of Pinellia (approx. 800 bp) gene	PCR product of CGRP - salmon (approx. 120 bp) gene	PCR product of CT – salmon (approx. 301 bp) gene
Primers structure	PXF1: atg gcc tcc aag ctc ctc ct	cgrpF1: atg gct tgc gat act gct acc	Primer F1 (p35S): cgt tcc aac cac gtc tta aa
	PRR1: gct tat taa ttc acc ttc tcc gtc	cgrpR1: tta tta gaa agc ctt aga tcc	Primer R1 (Nos): caa gac cgg caa cag gat tc

Ex vitro transfer

When rooted plants were removed from the jars, the culture medium was washed from their roots, and they were individually transferred on pots containing a mixture of Levington M3 compost (Fisons), John Innes No.3 (J. Bentley Ltd., Barrow-on-Humber, UK) compost and Perlite (Silvaperl, Gainsborough, UK) (3:3:2 [v:v:v]). Plants were enclosed in polythene bags and incubated at a light intensity of 29 μmol m^{-2} s^{-1}, 16 h photoperiod, 23±2°C. Every 7 days one corner of the bag was removed. After 21 days plants were transferred to glasshouse and they were grown to maturity.

Three distinct constructs were used in *Lactuca sativa* transformation as following: pBI 121 pta EHA 105, pCAMBIA 2301 CGRP și pCAMBIA 2301 CT**.** Plasmid DNA purification was performed using a Sigma miniprep kit. Samples were migrated in 1.2% Sea Kem agarose gel, 50 V for 40 min.

In vitro regenerated plants were used as plant material for transformation tests assessment. Leaves were collected from transformed lettuce plants and genomic DNA was purified using a DNA isolation kit (Sigma 70 prep G2N-70) and liquid nitrogen for grinding the leaves.

Samples were amplified by PCR using specific primers (Tab. 4) and migrated in 1.6% agarose gel, 100V for one hour. PCR sample composition was presented in Tab. 2.

Each sample was amplified in a reaction volume of 20 μl. PCR program used to amplify each of the three genes introduced into *Lactuca sativa* genome (Tab. 3) was different in accordance with the primers used. The structure of the primers was presented in Tab. 4.

Results and discussion

Effect of kanamycin concentration

In order to develop a rapid and efficient *Agrobacterium*-mediated transformation method for lettuce there was assayed the action of different concentrations of antibiotics which were currently used as selectable markers. Kanamycin sulfate at a concentration of 50 mg/l was the lowest concentration required to inhibit growth of control cotyledons. Control cotyledons cultured without antibiotics produced many adventitious shoots (20-30 shoots/explant). Kanamycin sulfate at 100 mg/l was more efficient for selection of transformed shoots compared to a concentration of 50 mg/l.

The number of adventitious shoots and the proportion of transgenic plants varied with every cultivar. A percent ranging between 45-98% represented the transgenic shoots out of total shoot population.

Standardization of plant selection and regeneration of lettuce shoots

An experiment of standardization of hormonal combinations in regeneration medium *in vitro* grown leaf explants was setup. The highest percentage (65%) of shoot regeneration was noticed for MS + 0.04 mg/l NAA + 0.5 mg/l BAP.

Molecular analysis of transgenic plants

Leaves were collected from *in vitro* grown transformed lettuce plants. DNA was extracted using liquid nitrogen and a Sigma 70 prep G2N-70 purification kit. Samples obtained were amplified by PCR, using specific primers and programs, and electrophoretically migrated in a 1.6% agarose gel at 100V, for one hour.

PCR amplification products of all three plasmidial constructs (obtained with distinct PCR programs) were between 120-800 bp. Samples were migrated comparatively in a single agarose gel (Fig. 1). In position P_1 the amplification product of pCAMBIA 2301 CGRP vector, having 120 bp, was represented. Position P_2 was represented by the amplicon of pBI 121 pta EHA 105 vector, a band of 800 bp. P_3 position figured the amplification product for pCAMBIA 2301 CT vector, having a molecular weight of 301 bp. PCR programes used to amplify each of these three vectors were presented in Tab. 3. In position S on

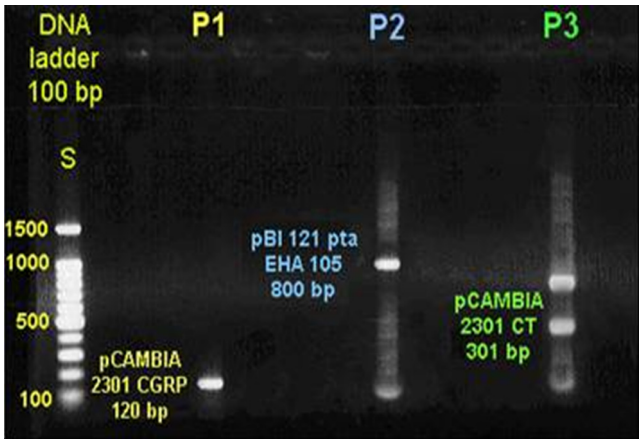

Fig. 1. PCR amplification products of three plasmidial constructs: P_1 pCAMBIA 2301 CGRP - 120 bp, P_2 pBI 121 pta EHA 105 - 800 bp, P_3 pCAMBIA 2301 CT - 301 bp. Each amplification was processed using a distinct PCR program

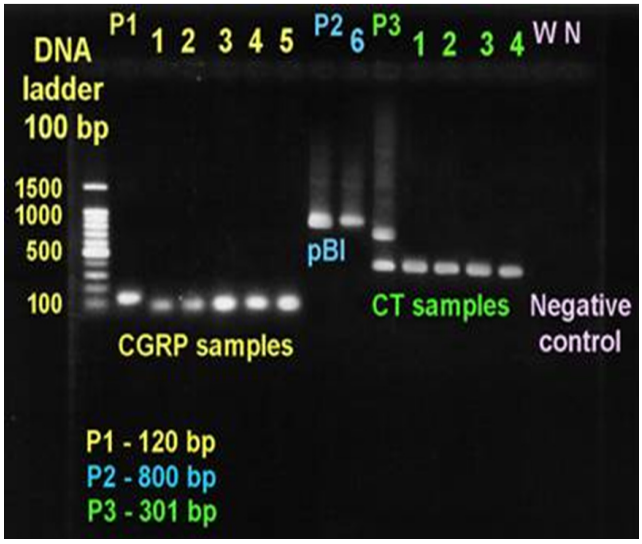

Fig. 2. PCR products of three cloning constructs used to induce lettuce transformation together with several samples extracted from plants transformed with these cloning vectors. P1 - amplicon of pCAMBIA 2301 CGRP vector, 120 bp. Pos. 1-5, lettuce plants transformed with this construct. P2 - amplicon of pBI 121 pta EHA 105 vector, 800 bp. Pos. 6, lettuce plant transformed with this construct. P3 - amplicon of pCAMBIA 2301 CT vector, 301 bp. The following 1-4 positions, lettuce plants transformed with this construct. W, N pos. - negative (water, no transformed plant) controls. S - 100 bp DNA molecular weight marker (Promega)

the agarose gel DNA molecular weight marker was represented (Promega 100 bp DNA ladder).

Fig. 2 is a representation of a comparative electrophoretic migration of PCR amplification products of three plasmidic cloning vectors together with samples extracted from lettuce plants infected with these constructs. Position P_1 pictures the pCAMBIA 2301 CGRP cloning vector amplicon, having 120 bp; positions 1-5 represent samples extracted from plants infected with this construct. These plants were genetically transformed by including the *cgrp* gene into their genomes. Migration bands were situated at the same level with the 120 bp band of pCAMBIA 2301 CGRP vector in pos. P1, used to induce transformation. Vector pBI 121 pta EHA 105 was represented in position P_2. Its amplicon weighed 800 bp. In pos. 6 the amplicon of a sample extracted from a plant transformed with this construct was represented, wich was another confirmation of a transformed plant. Position P_3 stands for the pCAMBIA 2301 CT cloning vector, having 301 bp. We can also see some unspecific amplification bands on this position, due to the PCR program used. The next four positions stand for samples extracted from plants which were transformed by infection with pCAMBIA 2301 CT vector, at the level of 301 bp. Position W (water) and pos. N (No transformation)-were samples extracted from plants not transformed, used as negative controls. Position S - DNA 100 pb (Promega) molecular weight.

Several samples extracted from genetically transformed lettuce plants using pCAMBIA 2301 CT vector were presented in Fig. 3. Samples 1-12 were processed starting from *Lactuca sativa* plants being previously infected with a strain of *Agrobacterium tumefaciens* including the plasmid vector pCAMBIA 2301 CT. The PCR amplification products of these samples presented a migration band at the same level of 301 bp of the cloning vector (in position

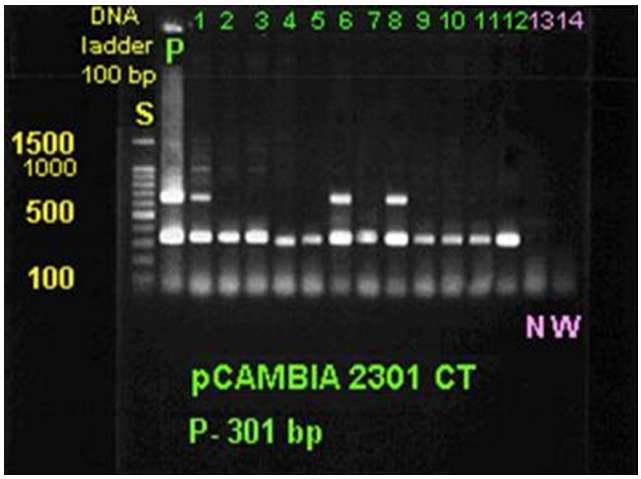

Fig. 3. Confirmation of several lettuce plants transformed with pCAMBIA 2301 CT vector. P - 301 bp band of pCAMBIA 2301 CT. Positions 1-12, lettuce plants transformed with this construct. N, W, negative controls. S - Promega 100 bp DNA molecular weight marker

P). Pos. 13, 14 – stand for N (not transformed plant) and W (water), and they were used as negative control.

In position S, DNA molecular weight (Promega, 100 bp) was represented. In some of the cases unspecific migration bands were obtained, related to the PCR program used for amplification.

The transgenic plants developped very fast, 45 days after cotyledons were transferred to SI medium and they grew rapidly in a few weeks after transferring them on rooting medium. The foliar mass was well represented. No

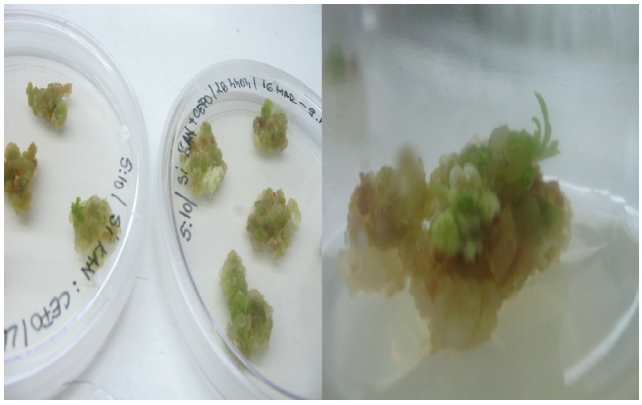

Fig. 4. Somatic embryogenesis - selection and plant regeneration of lettuce plantlets with *pta* (*Pinellia ternata* agglutinin) gene. Leaves infected with pBIP 121 vector containing *pta* gene under the control of *35S* promoter and *Nos* terminator (5-6 weeks after culture)-lettuce callus on SI medium supplemented with NAA, BAP, kanamycin and rifampicin

significant differences related to the level of pigmentation between untransformed and genetically modified lettuce leaves were observed. The root system of transgenic plants was well developed starting with the early weeks, after transferring shoots individually to 175 ml jars (Fig. 4, 5).

The phenotype of the genetically transformed lettuce plants did not change very much compared to original plants. The leaves of these plants looked similar to those untransformed. The edge of the leaves remained uncurly

Fig. 5. Aspect of in vitro *Lactuca sativa* transformed plants on root inducing (RI) culture medium supplemented with kanamycin

and the surface of the leaf was slightly waved, similar to the original phenotype (Fig. 5). The transformed lettuce leaves contained much chlorophyll and they had a highly developped turgescence, parameters which stimulated *in vitro* regeneration of these plants on culture media.

Conclusions

In summary, genetically transformed lettuce plants were successfully obtained with all three vectorial constructs, pBI 121 pta EHA 105, pCAMBIA 2301 cgrp and pCAMBIA 2301 CT. The transformation process followed the usual protocol; using LBA4404 *A. tumefaciens* strain and the mentioned vectors plants were transformed in a high number. Plants were selected on culture media with antibiotics and transferred *ex vitro* until they flowered and produced seeds.

The number of transgenic shoots from total shoot population varied between 45-50% in pCAMBIA 2301 CGRP and 96-98% in pCAMBIA 2301 CT experiments.

Strains of *A. tumefaciens* including the pTOK 47 hypervirulent plasmid induced the obtaining of more transformed lettuce shoots compared to those lacking this plasmid.

Nurse cultures improved transformation efficiency, even if it was not sure whether they were essential or not for transformation.

A concentration of 100 mg/l kanamycin sulfate was proper to selection of transformed shoots, more efficient than 50 mg/l or less.

Dilution of *Agrobacterium* strain was also important, most transformation events happened at a 5:10 (v/v) dilution of an overnight culture of bacterial strain.

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