AGROBACTERIAL TRANSFORMATION AND TRANSFER OF THE ANTIFREEZE PROTEIN GENE OF WINTER FLOUNDER TO THE STRAWBERRY

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Abstract

A protocol for agrobacterial transformation of garden strawberry leaf explants was developed. The strain *A. tumefaciens* A 281 (vector pPCV730) is the most virulent for strawberries giving a transformation frequency of 2.7%. Strain GV3101 with the plasmid-helper pMP90RK (vector series pPCV) is less efficient, the transformation frequency being 1.0-1.7%. On the basis of this protocol, transgenic plants were obtained with the antifreeze protein of winter flounder (AFP). The integration of sequences of genes NPT II and AFP was confirmed by PCR analysis.

1. Introduction

Strawberry (*Fragaria ananassa* Duch.) is one of the major berry crops in countries with a temperate climate. Refinement of regeneration methods of whole plants from various somatic tissues and progress in the technology of recombinant DNA permits the transfer of foreign genes to plant genomes with the minimum disturbance of a cultivar genotype. Development of a method for the genetic transformation of strawberry opens new prospects for the improvement of cultivars.

The most important problem is the production of cultivars with resistance to different abiotic stresses, particularly to early spring frosts. Early spring frost damage of the flowers and ovary lead later to poor yields and erratic fruiting. The transfer of genes determining antifreeze proteins from arctic fish (AFP) is one means of improving frost resistance. In this research the AFP gene from the winter flounder *Pseudopleuronectes americanus* was used.

The AFP gene from winter flounder codes for a protein of 91 amino-acids composed of prepro-peptide and mature protein (53 amino-acids) (Gourlie *et al.*, 1984). The mode of action of this gene product has been studied in detail (Davies and Hew, 1990) and is based on delaying the growth of ice crystals within tissue through binding AFP molecules with growing ice crystals at low temperatures. Lee *et al.* (1990) obtained transgenic plants of tobacco with the AFP gene. The transgenic plants were indeed more resistant to frost.

The goal of our research was to develop an efficient protocol for agrobacterial transformations of strawberry leaf explants and to obtain transgenic plants with AFP.

2. Materials and methods

2.1. Plant material

In our experiments we used *in vitro* strawberry plants of the cultivar 'Firework'. Micropropagation was done in the medium of Boxus *et al.* (1977) containing 1.0 mg/l BAP. The strawberry shoots rooted on this medium without hormones. The plants were grown for 4-5 weeks in 500 ml Ehrlenmaier flasks containing 100 ml of hormone-free enhancing medium. The youngest, fully unfolded leaves were taken for transformation and cut into explants of about 1 cm^2. Cultivation conditions were as follows: a photoperiod of 16/8 hours, illumination 3500 lux, temperature 26/22°C.

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2.2. Agrobacterial strains, plasmids

To transform the strawberries pPCV631 and pPCV702 vectors were used with a cloned AFP gene (clone IIA7) (Lin and Gross, 1981; Gourlie *et al.*, 1984) and pPCV730 with the selective genes of NPT II and HPT (provided by Dr N. Strizhov, MPI, Cologne). In the pPCV702 plasmid the cloned sequence of the AFP gene corresponding to the pro-peptide was used, while in the pPCV631 it was the mature form. In both vectors the AFP gene sequences were under the control of the 35S promoter CaMV and flanked on the 3' end terminator sequence of the *A. tumefaciens* nos gene. The pPCV702 vector contained the NPT II plant selective gene under the control of the nos promoter and the pPCV631 vector contains the hygromycinphosphotransferase (HPT) gene under the control of the same promotor. The plasmid pPCV631 contains the promoter free sequence of gene NPT II. The pPCV730 plasmid contained gene NPT II under the control of the nos promoter and the HPT gene under control of the mannopin synthase promotor. Vectors pPCV631, 702 and 730 were carried over to *A. tumefaciens* GV3101 (plasmid-helper of pMP90RK) with the aid of three-parental crossing; vector pPCV730 was also carried over to the supervirulent strain A 281 (pTiBo 542). Before transformation, bacteria were grown on a shaker overnight on the LB medium with 100 g/ml carbenicillin and 50 g/ml rifampicin to A600, equal to 0.6-0.9. Before transformation the bacteria were thrice washed with liquid medium MS without antibiotics.

2.3. Transformation, selection and regeneration of transformants

Leaf explants were incubated in a bacterial suspension for 20-40 min, then transferred to a co-cultivation medium with agrobacteria. Co-cultivation was done in a MS medium containing 2.0 mg/l IAA on filtration paper for 2-3 days at a temperature of 26°C.

After co-cultivation the explants were transferred into a regeneration and selection medium (Murashige-Skoog) containing 5.0 mg/l BAP, 0.3 mg/l IBA, 500 mg/l cefotaxime, 50 mg/l kanamycin (vectors pPCV702 and pPCV730) or 10 mg/l hygromycin (vector pPCV631). The explants were passed on to fresh medium at 3-week intervals. When the calluses formed at the cut edges of the explant reached 2-3 mm, they were separated from the explant and their cultivation was continued in a similar medium. From the fourth passage when the calluses reached 5-6 mm the concentration of selective antibiotics was decreased by half. Regeneration of the transformants was done in the dark at 26°C.

The regenerated shoots on the selective media were separated from the callus, and transferred onto the multiplication media indicated in section 2.1, containing 50 mg/l kanamycin or 10 mg/l hygromycin. Further multiplication of the transformants was done only in media with antibiotics for selection. Transformants were rooted on hormone-free medium containing 50 mg/l kanamycin or 10 mg/l hygromycin.

Culture of the transformants *in vitro* was done at the same temperature and illumination conditions as indicated in section 2.1.

The rooted transformants were adapted to conditions *in vivo* at relative humidity 92-95%, temperature 22/24°C, daylength 16 h and illumination of 2,500 lux; they were planted into a mixture of loam and perlite (1:1) and transferred for further growth to a greenhouse.

2.4. Isolation of genomic DNA, PCR analysis

One gram of fresh leaves collected from the greenhouse plants was ground in liquid nitrogen and extracted three times in acetone, pre-cooled to -70°C (Schneiderbauer *et al.*, 1991). The genomic DNA was isolated from the extracted material (Dellaporta *et al.*, 1985). Then the DNA preparation was refined by means of CTAB extraction.

PCR analysis was used to confirm the integration of genes NPT II and AFP sequences in the genome of transgene plants. The amplification reaction was done in a buffer containing 7mM Tris-HCl (pH 8.8), 15 mM $(NH_4)_2SO_4$, 6 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 mg/ml BSA, 0.5 Nonidet NP40, 2 units of Taq-polymerase, 1.0 µg DNA, 25 pkM of each

primer in a volume of 30 µl. A 40 µl amount of mineral oil was layered onto the reaction mixture. The amplification regime was: preliminary denaturation (100°C) 2 min; then 35 cycles of annealing primers (58°C) 1 min, completion of chain reaction (72°C) 1.5 min, denaturation (94.5°C) 1 min. The PCR products were analysed using electrophoresis in 2% agarose gel. The length of the amplified fragment was 269 bp for AFP gene and 423 bp for NPT II.

3. Results and discussion

The results of the strawberry genetic transformation experiments are presented in Table 1.

The maximum transformation frequency, 2.69%, was obtained by the use of the supervirulent strain A281 with plasmid pTi Bo542. Strain GV3101 with plasmid pMP90RK and various vectors of the pPCV series yielded a less effective transformation, 1.04-1.69%.

As is shown in our preliminary experiments, the constant cultivation of transgenic calluses on the medium with high concentration of selective antibiotics lowered the number of regenerated shoots. The first regenerants arose 2-3 weeks after transfer to a lower concentration of the selective antibiotics, regeneration continued in the following 3-5 weeks, and calluses tended to lose morphogenetic potential.

On the evidence of various authors (Janssen and Gardner, 1989; Mathews *et al.*, 1995; Renou *et al.*, 1993; Firoozabady *et al.*, 1994), transgenic calluses often have a chimerical nature and consist of transformed and non-transformed cells surviving on the medium with selective antibiotics. Therefore, to eliminate possible chimeric or non-transformed plants, regenerated shoots are further propagated and rooted on the medium with 50.0 mg/l kanamycin or 10 mg/l hygromycin. We did not see dead regenerated plants at that concentration of antibiotics. Three lines of transgenic plants were obtained after transformation by the pPCV702 vector and five lines with the pPCV631 vector which correspond to a transformation frequency of 1.04% and 1.55% respectively. (The transformation frequency is determined as the relationship between the number of transgenic plants regenerated in the presence of selective antibiotics and the number of regenerants obtained in the non-transformed control without selection.) Analysis of genomic DNA of transgenic plants by the PCR method confirmed the presence of the AFP and NPT II genes in the transformant genomes. An amplification fragment of the expected length (Figs. 1 and 2) was present in all the transgenic lines.

The transgenic plants of the strawberry easily adapted to *in vivo* conditions and growth in the greenhouse. The morphology of the transgenic plants was not different from the initial form.

4. References

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Figure 1 – Polymerase chain reaction (PCR) analysis of strawberry transformants. DNA was amplified using primers specific to the NPTII gene. Amplification produced a 423 bp product. Lanes 1 and 8 correspond to the molecular size marker pBR322/Alu 1, lane 2 – non-transformed plant, lanes 3-7 – DNA transformed plants, 631-04, 631-05, 631-06, 2036-02 and 2036-06.

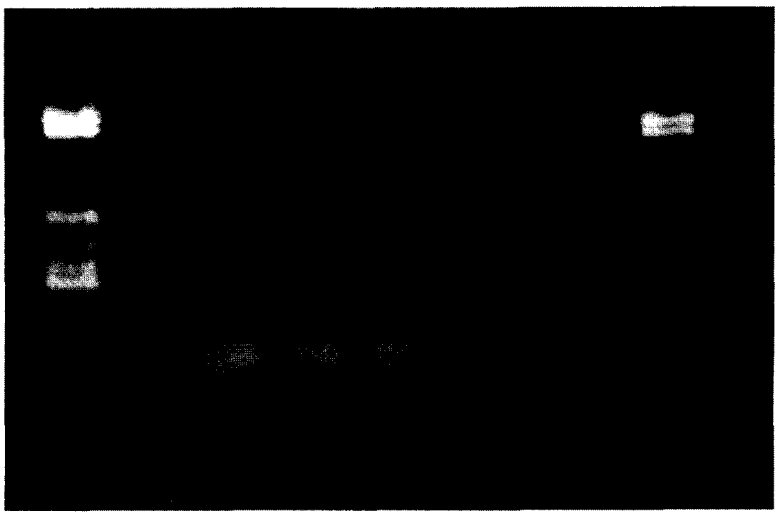

Figure 2 – Polymerase chain reaction (PCR) analysis of strawberry transformants. DNA was amplified using primers specific to the AFP gene. Amplification produced a 269 bp product. Lanes 1 and 8 correspond to the molecular size marker pBR322/Taq 1. Lane 2 – non-transformed plant, lanes 3-7 – DNA from transformed plants, 631-04, 631-05, 631-06, 2036-02 and 2036-06.

Table 1 – Effect of various *A. tumefaciens* strains on the transformation frequency of strawberry leaf explants

Strains of *Agrobacterium*	Vir-region	Vector	Selective antibiotic	Leaf discs with calluses growing in selective media %	Transformation frequency %	Number of transgenic lines analysed by PCR	Number of lines with NPT II gene
GV3101	pMP90RK	pPCV730	Km	45.5	1.69	2	2
GV3101	pMP90RK	pPCV702	Km	15.7	1.04	3	3
GV3101	pMP90RK	pPCV631	Hyg	30.2	1.55	5	5
A281	pTiBo542	pPCV730	Km	55.6	2.69	4	4