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Agrobacterium Tumefaciens Based Transformation of Pelargonium x Hortrum cv. 'Samba' with Anti-1-aminocyclopropane-1-carboxylate Synthase cDNA to Regulate Ethylene Biosynthesis

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ABSTRACT

Phytohormone, ethylene plays an important role in plant growth and development including fruit ripening and flower senescence. The synthesis of 1-aminocylo-propane-1- carboxylate (ACC), the immediate precursor of ethylene, from S-adenosyl-methionine is catalyzed by ACC synthase; and which is also a rate limiting step in the ethylene biosynthetic pathway. Therefore, it plays a key role in ethylene biosynthesis and the genes that code for ACC synthase are of special interest. Moreover, in zonal geraniums, ethylene bursts released from cuttings can have profound impact on the viability of explants for plant propagation. Biotechnological approach involving genetic modification that may reduce ethylene levels has potential for increasing the shelf-life of cuttings for plant propagation. These considerations have led us to clone several cDNA of ACC synthase genes from Pelargonium x hortorum cv. 'Sincerity'. To transform geranium cells with Agrobacterium tumefaciens an in vitro regeneration system was developed using very young petiole explants. An Antisense construct of ACC synthase cDNA (PHSacc41) ligated into binary vector pAM696 was introduced into A. tumefaciens EHA 105 cells. Petiole explants were incubated with the Agrobacterium for 15 min and then co-cultivated for several days on MS medium containing 5 mM BAP and 1 mM IAA in the dark without the antibiotics. Selection for transformants was carried out in the presence of kanamycin and timentin. Transgenic plantlets generated were examined for inserted gene cassette by PCR and Southern blotting. Recovery of positive transformants that survived selection suggested that it is possible to transform and introduce genes via transformation in hybrid geraniums for genetic modification.

Keywords: ACC Synthase; Ethylene Biosynthesis; Transformation; Geranium Regeneration; Agrobacterium Tumefaciens

1. Introduction

Pelargonium sp., popularly known as geraniums are grown for their colorful flowers and to a limited extent for their scented foliage. They are important ornamental plants that are widely grown in North America and Europe [1-3]. Because of their spectacular flowers *P. zonale* hybrids are some the most important varieties and command a substantial portion of the market share of over $300 millions. Unlike seed geraniums, hybrid varieties are vegetatively propagated [2,3], therefore, improvements through conventional breeding are difficult and time-consuming [4]. Contemporary plant biotechnology provides a potential alternative to conventional breeding through genetic modification for plant improvement with potential for savings in time and cost to the growers.

The phytohormone, ethylene plays an important role in plant growth and development including fruit ripening and flower senescence [5-10]. In geraniums ethylene bursts released by plants during shipping (stress) and when cuttings are prepared for vegetative propagation are a major source of losses to growers (1). Genetic modification may provide a potential means by which ethylene bursts in the plants may be regulated either through antigene technology or through modification of a key gene(s) promoter(s). In the present investigation we have attempted to develop an *Agrobacterium tumefaciens* based transformation system in the vegetatively propagated geranium with an anti-1-aminocyclopropane-1-carboxylate (ACC) synthase cDNA construct in a *Agrobacterium* binary vector [11,12] using a plant regeneration system developed for vegetatively propagated geraniums. Our rationale being, that ACC synthase catalyses the rate limiting step in the ethylene biosynthetic pathway [6] and modulation of this step may result in regulation of ethylene biosynthesis. These considerations had previously led us to the cloning of several cDNA of ACC synthase genes from *P* x *hortorum* cv. 'Sincerity' [13,14]. Results from the present studies show that transformants which survived kanamycin selection show integration of *NPT II* gene in transgenic plants both by PCR analysis and by southern hybridization.

2. Materials and Methods

2.1. Explants and Bacterial Strains

Petioles of very young leaves were used as explants from *P.* x *hortorum* cv. 'Samba' (provided by Pelfi Fisher Geranium USA, Boulder Colorado). The bacterial strains of *Agrobacte- rium tumafeciens*, LBA 4404 or LBG 66 or EHA 105 were used.

2.2. Growth of A. Tumefaciens

A single colony of *A. tumefaciens* was grown in YEP medium containing 25 µg/ml of kanamycin and 10 µg/ml of tetracycline containing pAM-41 plasmid and allowed to grow overnight at 28º. The next day one of the cultures was transferred to a fresh YEP medium (25 ml) and allowed to grow on a shaker to an O·D· of about O.8-1.0. The cells were harvested by centrifugation at 6000 rpm at 28º and suspended in 20 ml of sterile distilled water.

2.3. Preparation of Explants

Young, healthy petiole explants were harvested and sterilized with 15% Clorox for 15 min with frequent agitation and then washed three times with sterile distilled water. The explants were cut into 2-3 mm size and then transferred to a Petri plate containing suspension of *Agrobacterium* (prepared previously) with gentle shaking. The explants were removed and blotted dry over a sterile paper towel and then transferred to a Mureshige and Skoog (MS) medium (15) containing 5 µM benzylaminopurine (BAP) and 1 µM indole-3-acetic acid (IAA); and were incubated at 25º in the dark for 2-3 days. The explants were removed and wiped clean with a sterile paper towel. They were then transferred to fresh MS medium containing 5 µM BAP and 1 µM IAA; and 100 µg/ml of kanamycin and 200 µg/ml of timentin. After three weeks, petiole explants that showed growth of green buds or shoots were transferred to MS medium containing 0.44 µM BAP and 0.1 µM IAA; and 100 µg/ml of kanamycin and 200 µg/ml of timentin. After three weeks, well grown shoots were separated and transferred to fresh medium containing 0.44 µM BAP and 0.1 µM IAA; and 100 µg/ml of kanamycin and 200 µg/ml of timentin. Shoots that attained a height of about 1 cm were transferred to culture tubes containing fresh MS medium and 0.1 µg/ml IAA but without the antibiotics to allow for rooting to take place. The rooted plants were moved into pots containing 1:1 ratio of vermiculite and perlite.

2.4. Preparation of Vector Construction Containing Anti-ACC synthase cDNA

ACC synthase cDNA (PHSacc41) (13,14) maintained in pBK-CMV plasmid was at first digested with *Not* I. The ends were filled with dCTP and dGTP using Klenow DNA polymerase in buffer containing 0.2 mM Tris-HCl (pH 7.6), 0.2 mM dCTP and dGTP and 30 units of Klenow DNA polymerase. Samples were incubated at 30º for 30 minutes. The DNA was extracted using phenol/chloroform (1:1) and again with chloroform. DNA was precipitated with ethanol in the presence of 50 mM sodium acetate and finally washed with 70% ethanol. PHSaac 41 cDNA was then released from the vector by digestion with *BamH* I and then gel purified. The binary vector DNA was digested with *Hpa* I and *Bgl* II and PHSacc41 was ligated into the vector and used to transform *Escherichia coli* cells. Transformants were isolated and checked for the ACC synthase cDNA insert by PCR. Results showed successful insertion both by PCR as well as from the size of the DNA inserted. The vector was named pAM 696-PHSacc-41.

2.5. Mobilization of binary vector pAM 696-PHSacc-41into A. tumefaciens

A. tumefaciens strain LB 4044 or EHA 103 was gown in YEP medium at 28º overnight; next day 1 ml aliquot from it was transferred to fresh medium (25 ml) and allowed to grow to an OD of 0.2-0.4. The cells were harvested by centrifugation and resuspended in 1/10 the volume of fresh YEP medium and 0.1 ml aliquots were transferred to eppendorf tubes and 1 ml of binary vector construct was added to the solution and it was then frozen in liquid nitrogen. The sample was allowed to thaw to room temperature and then platted on YEP agar containing 20 µg/ml of tetracycline. Transformant colonies were identified and further confirmed to carry plasmid AM-696-PHSacc41 by PCR using primers designed for *NPT II* and PHSacc41 genes. These bacteria were used for plant transformation. The same procedure was used to introduce plasmid constructs into other strains of *A. tumefaciens* (11).

2.6. Polymerase Chain Reaction and Southern Hybridization

Polymerase chain reaction (PCR) was used to detect the integration of cassette containing *NPT II* gene and ACC synthase into the plant genome. DNA from young leaves of transgenic and control plants was isolated according to Taylor (16). The *NPT II* primer sequences used were: 5'-CTG AAT GAA CTG CAG GAC GAGG-3' and 5'-GCC AAC GCT ATG TCC TGA TAGC-3' which is expected to yield a fragment of 500 bp from the *NTP II* coding sequence. The primers used to detect integrated anti ACC synthase genes were: 5'-CCC AAA TTT GGG GGG GGG from 3'end and GGG CCC AAA TTT GGGGG from the 5'end which is part of the 35's CaMV promoter and is expected to amplify a DNA fragment of 600 bp. The DNA from transgenics and control plants were also used for southern hybridization. Southern transfers were prepared by digestion of DNA (10 µg) with *Bam H*I and electorphoresed in 0.8% agarose gel. Hybridizations were carried out with [32p] labeled *NTP II* probe developed using the random primer labeling Kit from Amersham according to manufacturer's directions.

3. Results and Discussion

In order to transform geranium cells with *A. umefaciens* an *in vitro* regeneration system has been developed using very young petiole explants in the presence of zeatin or BAP and IAA (**Figures 1** and **2**). These studies showed that the regeneration efficiencies vary considerably with different cultivars; varying from 10-60% (see also17) but with cultivar Samba nearly 90-100% of the explants regenerated. It should be noted that with zeatin regeneration efficiencies in general were a little better than BAP but differences were not that significant. Samba with nearly 100% regeneration efficiency thus provides a system that could be used to determine whether genetic transformation of hybrid geraniums with *Agrobacterium* is feasible.

Antisense constructs of ACC synthase cDNA (pPHSacc41) (14) were ligated into binary vector pAM696 (**Figure 3**) and Lanes from left to right are: Lane 1, DNA ladder; lane 2, DNA from control plant and lanes 3-11 DNA from transformants. introduced into *A. tumefaciens* EHA105 cells. Petiole explants

were incubated with *Agrobacterium* for15 min and then co-cultivated for several days on MS medium containing 5 mM BAP and 1mM IAA in the dark without the antibiotics. Selection for transformants was carried out in the presence of kanamycin and timentin. After about three weeks, petioles with

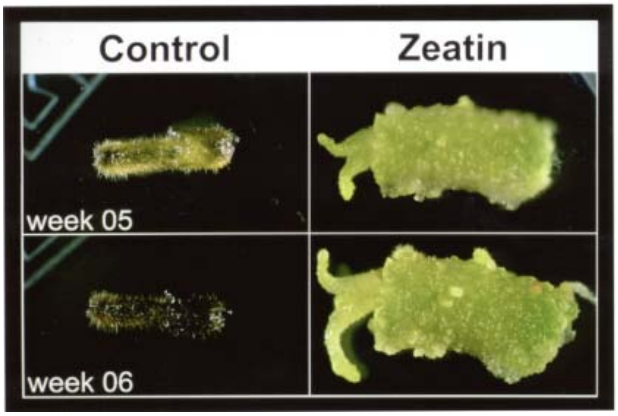

Figure 1. Growth and expansion of shoots from petiole explants in the presence and absence of hormone zeatin.

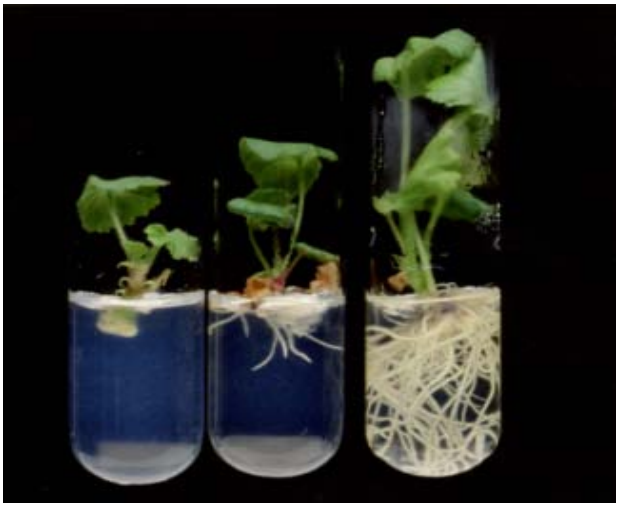

Figure 2. Rooting and growth of plantlets in the presence of IAA. Shoot buds separated from mother explants were cultured individually. Within 3-4 weeks it developed a healthy root system.

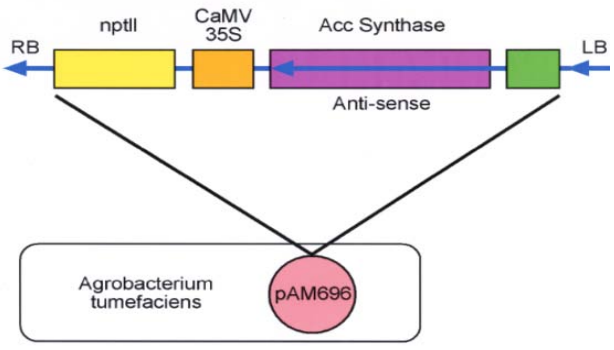

Figure 3. A diagrammatic representation of antisense of ACC synthase cDNA (pPHSacc41) construct in plasmid vector.

Figure 4. PCP amplification of *NTP II* gene from DNA of control and transformed plantlets.

green buds or shoots were transferred to fresh MS medium containing 0.44 mM BAP and 0.1 mM IAA and finally on medium containing 0.1 mM IAA for rooting (**Figure 2**). Plantlets generated after transformation were examined for the insertion of gene cassette into the plant by PCR and Southern hybridization. Results showed nearly 50% of the tranformants that survived selection were positive by PCR (**Figure 4**) as well as by Southern hybridization for the *NTP II* gene (results not shown); suggesting that a successful transformation of geranium in culture has been established. These plants are being evaluated further in the greenhouse.

Previously, transformation of other types of geraniums has been carried out with variable success (18-20). Our findings on the development of a regeneration and *Agrobacterium* based transformation system with hybrid geraniums (sometimes also called zonale geraniums) one of the most desirable of the geranium species, now provides a system for further genetic modifications of these ornamental plants. Future modification may include flower color change, introduction of desirable flower scents and enhanced disease resistance to bacterial or viral pathogens and others, limited only by imagination.

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