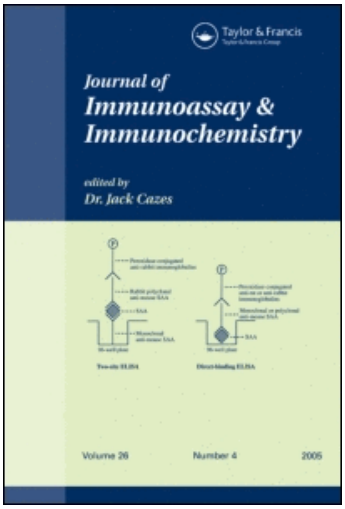

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ipt Gene Transformation in Petunia by an *Agrobacterium* Mediated Method

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Abstract: To prevent leaf senescence of petunia, the cytokinin biosynthetic gene isopentenyl transferase (*ipt*) was placed under the control of 35S promoter and introduced into petunia. PCR analysis showed an expected 0.5 Kb fragment of *ipt* gene in transgenic petunia. RT-PCR analysis indicated the expression of *ipt* gene in the transgenic lines. Leaves from transgenic plants remained green and healthy in normal culture condition, while the non-transformed plants turned to yellow. Transgenic plants showed a reduction in height and smaller leaf sizes. In transgenic lines, the internodes were shorter, and the roots grew slower than the non-transformed plants.

Keywords: Genetic transformation, Isopentenyl transferase gene (*ipt*), Petunia, RACE, Senescence, TAIL-PCR

INTRODUCTION

Leaf senescence is a type of programmed cell death (PCD) characterized by loss of chlorophyll, lipids, total protein, and RNA.[1] Plant growth regulators including auxins, gibberellins, ethylene, abscisic acid, and cytokinins, are believed to play important roles in regulating senescence.[2]

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Cytokinins belonging to a class of plant hormones were firstly noted with the ability to promote cell division.[3] Since then, many evidences indicate that cytokines are active throughout the plant developmental stages, controlling processes as diverse as apical dominance, root formation, leaf senescence, stomatal behavior, and chloroplast development.[4] In practice, exogenous cytokinin applications are often not efficacious in commercial horticulture because they are expensive to use and are not readily assimilated.[5]

The *ipt* gene encodes the enzyme isopentenyl transferase, which catalyses the rate-limiting step in cytokinin biosynthesis.[6] The *ipt* gene transformed plants demonstrate the potential ability in extending the storage life of ornamental plants, green vegetables, and fruits. But, most *ipt*-transgenic plants exhibit morphological abnormalities, which restrict the potential for use in commercial production.[1,7]

In this paper, we report the transformation of isopentenyl transferase gene (*ipt*) by *Agrobacterium tumefaciens* mediated transformation and its expression in petunia.

EXPERIMENTAL

Transformation and Regeneration of Transgenic Plants

Petunia was grown at 25°C in the green house in pots containing a peat-base substrate. Leaf and stem tissues from young newly developed shoots were used as explant tissue for plant transformation. Young, fully expanded leaves of petunia were sterilized with 0.6% sodium hypochlorite for 20 min and then rinsed four times with sterilized water.

The bacterial suspension was cultured in YEB medium with 50 mg/L hygromycin. The suspension was incubated at 28°C on a rotary shaker (180 rpm) until the optical density value of OD_{600} reached to 0.5–0.6. The suspension was then centrifuged and the pellet was re-suspended in fresh liquid MS medium.

Leaf explant of petunia was soaked in the infection medium for 15 min, blotted dry, and kept for 3d in the dark at 25°C on plates with MS medium containing 2 mg/L N^6-bebzyladenine (ba), 0.01 mg/L NAA. After days, explants were transferred to the selection media containing 5 mg/L of hygromycin and 100 mg/L cefotaxime.

Explants were transferred to fresh medium every 2 weeks, until shoots developed. Shoots were excised and transferred to MS medium containing 0.5 mg/L hygromycin and 100 mg/L cefotaxime until root induction was evident. Rooted explants were transferred to a peat-based medium and acclimated to the green house environment.

Plant DNA Extraction and Polymerase Chain Reaction (PCR) Analysis

Total DNA was isolated from leaf tissue and 250 ng DNA was subjected to the PCR reaction. The primers used to detect the *ipt* gene were as follows: forward primer 5′-GGTCCAACTTGCACAGGAAA-3′, and reverse primer 5′-GAGATCGATGGATATCGATAT-3′. PCR amplification was performed in a thermocycler (T-Gradient Whatman Biometra). The total volume of the reaction mixture was 25 μL, which contained 200 ng DNA template, 100 μM of each dNTPs, 0.5 μM of each primer, 2 mM of Mg^{2+}, 2.5 μL of 10 × Taq PCR buffer and 1 U Taq DNA polymerase, finally adjusted to 25 μL with double-distilled water. PCR was performed as follows: the samples were denatured at 94°C for 5 min, then amplification was performed for 40 cycles programmed as follows: 94°C for 1 min, 60°C for 1 min, 72°C for 1 min; finally, the reaction mixture was kept at 72°C for 10 min for extension. After the amplification, a 10 μL aliquot was analyzed on a 0.8% agarose gel and visualized under UV light. A 100 bp (Sangon, Shaihai, China) molecular marker ladder was used as a reference to determine DNA fragment size.

Analysis of *ipt* Gene Expression in the Leaves of Petunia

Total RNA was isolated for RT-PCR analysis from the wild type plant and two *ipt*-transgenic plants with an RNA isolation kit. Plant samples were frozen with liquid nitrogen and then ground in a mortar to a fine powder. The isolated RNA was treated with DNase (RNase free) to eliminate DNA contamination. Then, the first strand cDNA was synthesized from 1 μg of total RNA method. For RT-PCR analysis, 0.5 μL of RT-mix was used in a final volume of 25 μL. PCR for the amplification of *ipt* gene fragment was carried out as described above. PCR products (8 μL PCR reaction mixture) were run on a 0.8% agarose gel.

Senescence of Excised Leaves

To determine whether the transgenic plant delayed senescence or not, excised leaves from petunia were sterilized by surface with 0.8% sodium hypochlorite for 1 min. The sterilized leaves were rinsed five times and then placed on moist filter paper in a 10 cm Petri dish. Leaves were selected from an individual transgenic line and non-transformed wild type plant, respectively. Each Petri dish contained two excised leaves from both an individual transgenic plant and a non-transformed wild

type plant. Four transgenic lines were tested. The plants were exposed to 25°C, coincided with a photoperiod of 16 h (8 h dark period).

RESULTS

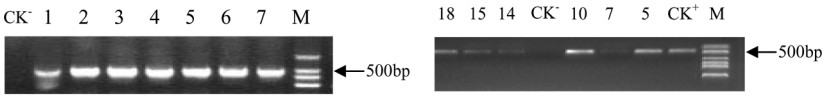
Plant Transformation

Antibiotic selection and PCR analysis confirmed that 20 transgenic T_0 plants were obtained. In PCR analysis, the plasmid DNA of the expression vector (control) and genomic DNAs from transgenic plants produced the expected 0.5 Kb fragment of the *ipt* gene; however, no amplification product was detected in non-transformed plants (Fig. 1). PCR positive lines of transgenic plants were used for subsequent experiments.

Reverse transcription-PCR (RT-PCR) analysis was used to confirm *ipt* expression in transgenic lines. Total RNAs were extracted from the leaves of non-transformed plants and selected transgenic lines. RT-PCR analysis showed that the specific 0.5 Kb fragment of the *ipt* gene was found in transgenic lines, but not in non-transformed plant (Fig. 2). The above results indicate that the donor *ipt* gene has integrated into the genomic DNA of the transgenic plants and expressed in transgenic plants, at least at the transcription level.

Leaf Senescence Response

The leaf senescence response of transgenic plants and non-transformed plants under normal culture conditions was different. Overall, leaves from transgenic plants remained green and healthy in such conditions,



(a) (b)

Figure 1. PCR analysis of DNA from the plasmid pCAMBIA1300 that contains the *ipt* gene and putative transgenic petunia lines. (a) PCR analysis showing the presence of the expected 0.5 Kb fragment in the plasmid pCAMBIA1300, which was used in the subsequent transformation. CK^-: water; 1–7: bacteria clone number; M: DNA marker DL2000. (b) PCR analysis showing the presence of expected 0.5 Kb fragment of *ipt* gene from putative-transgenic petunia lines. CK^+: plasmid pCAMBIA1300 that contains the *ipt* gene; CK^-: water; Numbers 5, 7, 10, 14, 15, 18: transgenic plant number; M: 100 bp DNA ladder.

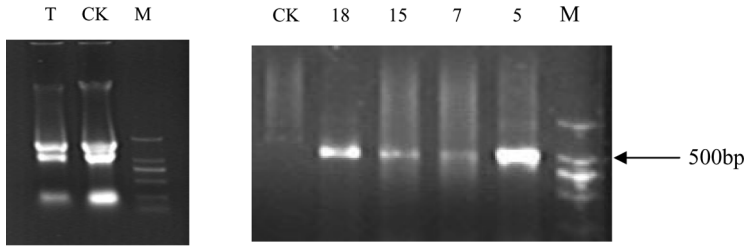


Figure 2. RT-PCR analysis of *ipt* gene expression in transgenic plants and non-transformed plant. (a) Total RNA prepared from transgenic plant and the non-transformed plant, which were used in the subsequent experiment. T: transgenic plant; CK: the non-transformed plant; M: 100 bp DNA ladder. (b) RT-PCR analysis to check the expression of *ipt* gene in the transgenic plants (5, 7, 15, 18). CK: non-transformed plant. M: 200 bp DNA ladder.

while the wild–type plants turned to yellow and sick (Fig. 3). Results indicated that the leaf senescence was really delayed to some extent in transgenic plants compared with non-transformed plants.

Plant Morphology

Transgenic plants showed a reduction in height. The leaf size in the transgenic plants was also reduced. The internodes in the transgenic plants were shorter than those in the non-transformed plants. Roots of transgenic plants grew slower than in the non-transformed plants.

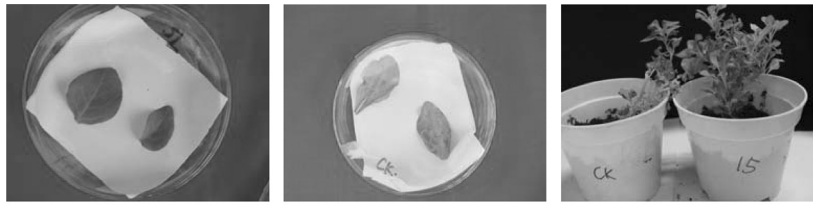


Figure 3. Senescence in excised leaves and whole plants. (a) Excised transgenic petunia leaves were exposed to normal culture conditions. After 25d, the leaves remain green and healthy. (b) Leaves from non-transformed plants in the same culture condition. After 25d culture, the leaves turned to yellow and sick. (c) Whole plants in the same culture condition. The transgenic plants remained green, while the non-transformed plants were yellow.

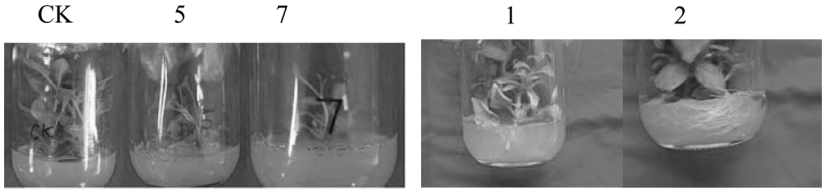


Figure 4. Morphology of transgenic plants and non-transformed plants. (a) The main differences between transgenic lines and the non-transformed plant included reduction in plant height and leaf size. CK: non-transformed plant; 5, 7: transgenic plants. (b) The roots of transgenic plants grew slower than the non-transformed plants. 1: Transgenic plant; 2: Non-transformed plant.

Commonly, the roots of non-transformed plants grew to maturity in 10–15 d, but the transgenic plants reached maturation in 28–35 d (Fig. 4).

DISCUSSION

Cytokinins are thought to play regulatory roles in two processes that describe plant development: growth and differentiation.[8] But, externally applied cytokinins exhibit a wide variety of effects. These include shoot initiation from callus cultures, promotion of axillary bud growth, directed transport of nutrients, stimulation of pigment synthesis, inhibition of root growth, and delay of senescence. But, in the horticulture industry, billions of vegetative cuttings are produced annually for sale to commercial growers. However, long term storage can only be successful if the majority of the plants survive and remain vigorous. Because of the reason mentioned above, external application of cytokinin is unpractical. So, we started the study of transfering the *ipt* gene into the cut petunia. Through improving the plant regeneration media, the frequency of the abnormality in transgenic plants was reduced, and the frequency of rooting was increased to 100% within 18–25d. In tobacco plants, when the *ipt* gene was transferred into the host, the root growth of the transgenic line was inhibited.[9] In our experiments, the roots of the transgenic lines were not inhibited, simply delayed.

In our experiments, PCR analytical results confirmed the *ipt* gene was integrated into the host genome. RT-PCR results confirmed that the *ipt* expressed in the transgenic lines. And, the transgenic plants do show delayed leaf senescence. It was reported that the *ipt* gene transgenic plant showed growth abnormality, so the promoters were changed.[10]

In our case, the plant morphology of transgenic lines was just different in the early phase of culture, but there were no obvious differences. Therefore, we think this method could be used in the commercial cut flower fields.

ABBREVIATIONS

CK, cytokinin; BLAST, basic local alignment search tool; NAA, naphthalene acetic acid; ABA, abscisic acid; EST, expressed sequence tag; GA, gibberellic acid; GUS, α-glucuronidase; SA, salicylic acid; PLACE, plant cis-acting regulatory DNA elements; PlantCARE, plant cis-acting regulatory element database; T-DNA, transferred DNA; ORF, open reading frame; RACE, rapid amplification of cDNA ends; TAIL-PCR, thermal asymmetrical interlaced polymerase chain reaction; iPA, isopentenyladenosine; DMAPP, dimethylallyl pyrophosphate; iP, isopentenyladenine; 2iP, N^6-(Δ^2-isopentenyl) adenine; iPMP, isopentenyladenosine monophosphate; IPT, adenylate isopentenyltransferase; tRNA IPT, tRNA isopentenyltransferase; 35S promoter, cauliflower mosaic virus 35S promoter.

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