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Flower Power: Genetic Engineering of hybrid geraniums (*Pelargonium x hortorum*) with Antisense Gene(s) of 1-Aminocyclopropane-1-carboxylate (ACC) Synthase to Regulate Ethylene Synthesis

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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-aminocylopropane-1-carboxylate (ACC), the immediate precursor of ethylene, from S-adenosyl-methionine is catalyzed by ACC synthase which is also the rate limiting step, therefore, this enzyme plays a key role in ethylene biosynthesis. In zonal geraniums, ethylene bursts released from cuttings can have profound impact on the viability of explants for plant propagation. Genetic modification that may reduce ethylene level has potential of increasing the shelf-life of cuttings for plant propagation. These considerations have led us to clone five full length and several partial length but different cDNA of ACC synthase genes from Pelargonium x hortorum cv Sincerity (PHS-ACS25, PHS-ACS41, PHS-ACS44,PHS-ACS45, PHS-ACS49, PHS-ACS71 and PHS-ACS72) . Based on gene expression analysis, PHS-ACS41 appears to be induced by wounding stress. For this purpose, an in vitro regeneration system has been developed from very young petiole and leaf explants suggesting that tissue culture may be used as an alternative to propagate these hybrid geraniums (beside vegetative propagation from cuttings). The regeneration efficiencies varied and were cultivar dependent (20 to 100 %). Finally, to transform geranium cells with Agrobacterium tumefaciens an antisense construct of ACC synthase cDNA (PHS-ACS41) ligated into binary vector pAM696 was introduced into A. tumefaciens EHA103 cells. Petiole explants were incubated with the Agrobacterium for 15 min and then co-cultivated for several days on MS medium containing 5 mM BA and 1 mM IAA in the dark without antibiotics. Selection for transformants was carried out in the presence of kanamycin and timentin. Transgenic plantlets generated were examined for inserted gene cassette by Southern blotting. Positive results from more than 50% to 75 % of the transformants that survived selection suggested that it is possible to transform and introduce genes via Agrobacterium based transformation into hybrid geraniums for genetic modification. The technology that has been developed here has future potential of introducing desirable trails such as flower color change, introduction of flower scent and enhancing disease resistance to bacterial or viral pathogens and others limited only by imagination.*

Keywords: *ACC synthases, Agrobacterium, gene expression, genetic engineering, geranium and in vitro transformation.*

Abbreviations: *ACC, 1-aminocyclopropane-1-carboxylate and ACS; ACC Synthase*

Geraniums are grown worldwide as bedding plants because of their spectacular flowers [1]. Their large flower with bright colors intricate petal and leaf morphology; and profuse flowering bring delight to consumers. They are found in a variety of colors from white to dark purple (Figure 1) and new shades of colors are created all the time through breeding programs. In general bright red color (Figure 2) to crimson red are widely more popular. Conservatively, the market value of geranium sales in the United States of America and Europe are in the range of $300 to 400 million [1,2]. If one takes into consideration contributions of sales in China, India and South America, these number may be in the range of $500 million. These large numbers suggest there is considerable room for value addition which can enhance earning potential of geranium growers. Still relatively unknown and unappreciated by public at-large, is contribution of geraniums to herbal medicine. South Africa, the native country of geraniums, the role of certain varieties of geranium root extracts as a source of herbal medicine has been part of the cultural folklore which has found its way into the market place in Western countries. Geranium root extracts of the

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Figure 1: A number of geranium varieties of varied colors are shown

variety *Pelargonium sidoides* is marketed under trade name "Umcka" in the United States of America (by Natures Way Products, LLC, green Way, Wisconsin) to elevate severity of symptoms of common cold and flu.

Figure 2: *P. hortorum* cv Samba with bright red color flowers

In this article no attempt is being made to describe all that is known about the classification of geranium verities. Only a broad description of common varieties is provided. In general, four to five varieties of geraniums are widely recognized: It should be pointed out that over 10,000 different varieties are known [1]. The original geranium brought to the USA is exemplified by *Pelargonium x domesticum* or also called regal geranium or zonal geraniums [1]. This variety includes both seed and vegetatively propagated material. Second variety is called *Pelargonium x hortorum* or hybrid geraniums also known as the common geraniums [1]. Their genetic origin is uncertain but they have become a major contributor to the geranium market. They are vegetatively propagated and are the most desirable of all the geranium varieties as bedding plants, at least in the USA. The third variety is called *Pelargonium x peltatum* or also called the Ivy geraniums. Ivy geranium once considered as a novelty is now a mature component of the market. Finally, there are the **novelty** geraniums such as rose bud or tulip flower geraniums including the scant geraniums or smell good geraniums such as *P. tomentosum* with peppermint scant; *P. crispum* with lemon sacnt; *P. nervesum* with lime scant and *P. graveolens* with rose scant.

The role of phytohormone, ethylene in fruit ripening and flower senescence is widely known [3]; ethylene also plays an important role in plant growth and development and stress related processes [reviewed in 4-9]. Ethylene synthesis in plants (Figure 3) is initiated by the conversion of L-methionine into S-adenosyl- L-methionine (AdoMet) by the enzyme S-adenosyl-methionine synthase and AdoMet is transformed into 1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase. ACC is then converted into ethylene by ACC oxidase [10]. ACC synthase catalyzes the rate limiting step in the biosynthesis of ethylene [4-8] and is transcriptionally regulated and induced by a variety of factors that include germination, seeding growth, flowering, fruit ripening and during organ senescence and is also

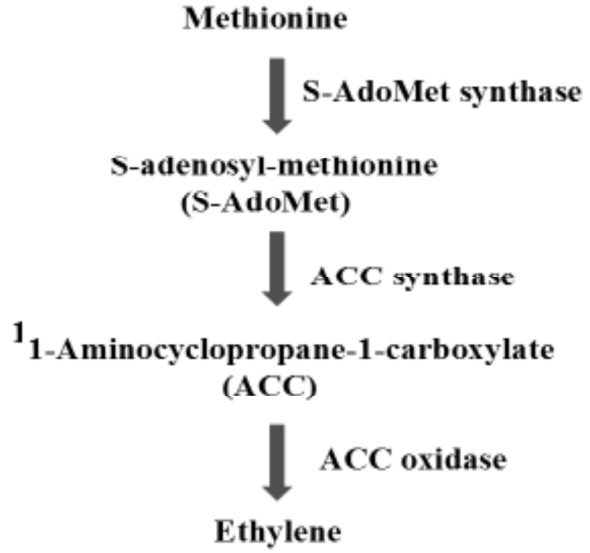

Figure 3: Ethylene Biosynthetic Pathway

induced by a variety of external factors, including wounding, hormone, viral or fungal infection, chilling injury, and exposure to some chemicals [4-26]. Induction of ethylene production requires *de novo* synthesis of ACC synthase [11,22,23]. Due to the critical role of ACC synthase in ethylene biosynthesis, ACC synthase gene(s) have received considerable attention and have been cloned from variety plant sources [4-26] and reviewed in [9].

These studies have led to the conclusion that ACC synthases are encoded by a multigene family [8-26], the expression of which appears to be differentially regulated by a variety of internal and external cues [reviewed in 9, 4-26].

Geranium is one of the most ethylene sensitive flowering plants [27]. The hybrid geraniums are vegetatively propagated; the process involves growing a elite mother stock from which cuttings are prepared for rooting. During the preparation of cuttings, stress-induced ethylene bursts [19, 24-27] cause premature loss of rooting potential which is a major source of losses to growers. Understanding of the genes involved in the stress-induced ethylene burst is important and may provide an opportunity, and means to address this problem. This review provides a complete cycle of research starting with cloning of ACC synthase genes; and development of an *in vitro* regeneration system from *P. hortorum* varieties that are vegetatively propagated; a process that remained elusive to researchers for sometime but is essential for any genetic modification(s) in geraniums. Finally, the last step involving the development an *in vitro Agrobacterium tumefaciins* based transformation system. The results show that it is possible to genetically modify geraniums *in vitro;* making it possible for future enhancement of market value of the geraniums for growers in desirable traits and even in herbal medicine through genetic manipulation of biochemical pathway(s) for biochemicals that are useful for human health.

MATERIALS AND METHODS

Plant Materials: Three different varieties of geraniums were use in this study: they are *Pelargonium x hortorum* cv Samba; *Pelargonium x hortorum* cv Sincerity and *Pelargonium x hortorum* cv Yours Truly. Plants were grown in greenhouse. For cDNA cloning senescing flowers from *P. hortorum* cv Sincerity were collected and frozen in liquid nitrogen and used immediately for DNA or RNA extractions.

Procedures for the cloning and sequencing of ACC Synthases cDNA: Following procedures have been described previously (24,25, 28-30) Preparation of polyadenylated mRNA, preparation of cDNA and ligation of size selected mRNA into lambda ZAP Express Vector; its package with Gigapack II to creat a primary library (with a titer of $3.5x10^6$ pfu). These recombinant phages were screened for ACC synthase cloned using a[^{32}P] labeled probe [26,27). After three cycles of screening of putative positive clones, a total of seven positive clones were isolated and sequenced [25, 29,30].

The details of *in vitro* transcription and translation of cDNA and tissue specific expression of ACC synthase genes have also been described [24].

Tissue specific expression of ACC synthase genes: The expression pattern of ACC synthase genes PHS-ACS41 in plant tissues was determined by RT-PCR using gene specific primers generated from the ACC synthase clone. For these studies, flower buds are defined as stage 1; just open flowers are defined as stage 2; and, mature, fully open flowers are defined as stage 3. For the expression pattern from leaf material, leaf buds collected from the apical region of major stems are defined as stage 1; the first leaves below the apical are defined as stage 2, they are green, thick and partially folded; and, fully expanded leaves are defined as stage 3. Tissues defined as wounded were treated as follows. Initially plant tissue (1 g) was treated by placing the base of the tissue in 10 ml of 1 mM sodium citrate, 1 mM Pipes, 1 mM KCl, and 50 ìg/ ml-1 chloramphenicol solution (pH 6.5) for 100 min at room temperature. Tissues defined as chemically treated were treated with the same buffer but in addition contained 0.5 mM IAA, 0.1 mM BA,

and 50 mM LiCl. After treatment, total RNA was isolated (200 ìg) and polyadenylated mRNA (2 ìg) was prepared to synthesize the first strand cDNA using Ready-To-Go TPrimed First-Strand Kit.Other details are described [24]

Procedures for *in vitro* regenrarion of planlets from explants. Briefly very young explants from petioles or unfolded leaves were used from geranium (*P. hortorum*) cultivars Samba, Sincerity and Yours Truly. The plant material was obtained from Tagawa Greenhouses, Inc, Brighton, Colorado and cultivar Samba from Pelpi Fisher Geranium USA, Boulder, Colorado. The explant material was surface sterilized by periodic agitation in 15% Clorox solution (0.79% NaOCl without surfactant) for 15 min, followed by several rinses with sterile distilled water. Leaf explants were cut into four pieces and petioles were cut into lengths ca. 1.5±2.5 mm. About 12 explants were placed on a 90 x 15mm disposable plastic petri dish containing ca. 25 ml MS medium [Murashige and Skoog, 1962] with 3% sucrose, 0.8% general purpose plant agar [31], pH 5.8. Leaf segments were placed abaxial side down. The concentrations of zeatin in the medium varied from 1.5, 3.0, 5.0, 7.5 and 10.0 mM along with either 1 or 2 mM indole-3-acetic acid (IAA). N6benzyladenine (BA) concentrations in the culture medium ranged from 1, 2.5, 5.0, 10.0, and 12.5 mM along with either 1 or 2 mM IAA. Controls without zeatin or BA in the medium were also included.

Petri dishes were sealed with parafilm, wrapped in aluminum foil and maintained in darkness for 5 days to prevent phenolic oxidation. Cultures were placed under cool white fluorescent light (GE trimline T8, USA) with a 16 h light photoperiod at 24 ^{0}C: Explants that exhibited browning were subcultured to fresh medium. After about four weeks explants showed initiation of shoot formation. Other procedures have been described [32].

Procedures for transformation of explants with *Agrobacterium tumefaciens*: Following procedures have described in full: Growth of *Agrobacterium* strains (LB 4044 or EHA 103; preparation of explants; vector construction containing anti- ACC synthase cDNA into binary vector (pAM 696); mobilization of binary vector pAM 696- PHS-ACS-41 into *A. tumefaciens,* and Southern hybridization (2,28).

RESULTS AND DISCUSSION

Seven different full-length or partial length putative positive cDNA clones were isolated. Based on DNA sequence analysis they all belong to ACC synthase mutigene family and are characterized by ACC synthase signature conserved eleven invariant amino acids (SLSKDLGMPGFRV) and characteristic substrate and pyridoxyl-5′-phosphate binding site [24]. An additional sequence analysis revealed that genes can be classified into two groups based on the position of termination codon in 5′-untranslated region. In group I (PHS-ACS41, 44 and 45), the termination codon (TAG) is located just before the initiation codon (ATG). The group II, typified by gene PHS-ACS49, the termination codon (TGA) is located twenty one bases upstream from the initiation codon (ATG) (Figure 4). It should be noted that we were also able to clone a full –length genomic clone of PHS-ACS49 which has been fully characterized including the promoter region (results in press) [33]

ctcagtacgtacgtgttgtacgtgtttacaTAGATG (PHS-ACS41)

ttttcttTGAgcaaaacaacatcgatcaaaaATG (PHS-ACS49)

Figure 4: The figure shows the position of termination codon (Caps and underlined) in Group I and Group II PHS-ACS genes in the 5′-untranslated region. Initiation codon (ATG) is shown in capital letters

Gene specific primer (PHS-ACS41-1200F- 5′ GGGGTCGTCGTTTCATIGC and PHS-ACS41R- 5′ CACTGCATATATATCTACGG) were used to determine the expression pattern of these genes *in vivo* by RT-PCR (resulting in amplification of a 385 bp fragment) and under various conditions such as wounding or chemical stress. Data for gene PHS-ACS41 is shown in Figure 5 and the others are presented in a separate paper [24]. Results show that low level of PHS-ACS41 gene is expressed regardless of the stages of flower or leaf development but this expression is dramatically increased upon wounding or treatment with chemicals (Figure 5; lanes 5 and 6). Whereas the expression of other genes were not influenced by wounding or chemical treatment [see results 24]. For this reason gene PHS-ACS41 was selected for genetic transformation study in the antisense orientation.

Studies on the *in vitro* plant regeneration potential of vegetatively propagated geraniums (*Pelargonium x hortorum*) were carried out and showed that by using various combinations of growth regulators it is possible to regenerate plantlets from very young petioles or leaf explants (Figure 6, left panel). In all, the three cultivars were examined (Samba, Yours Truly and Sincerity); very young petiole explants exhibited a higher regeneration potential than leaf explants. Regeneration efficiencies were found to be highly dependent on the cultivar, with cv. Samba

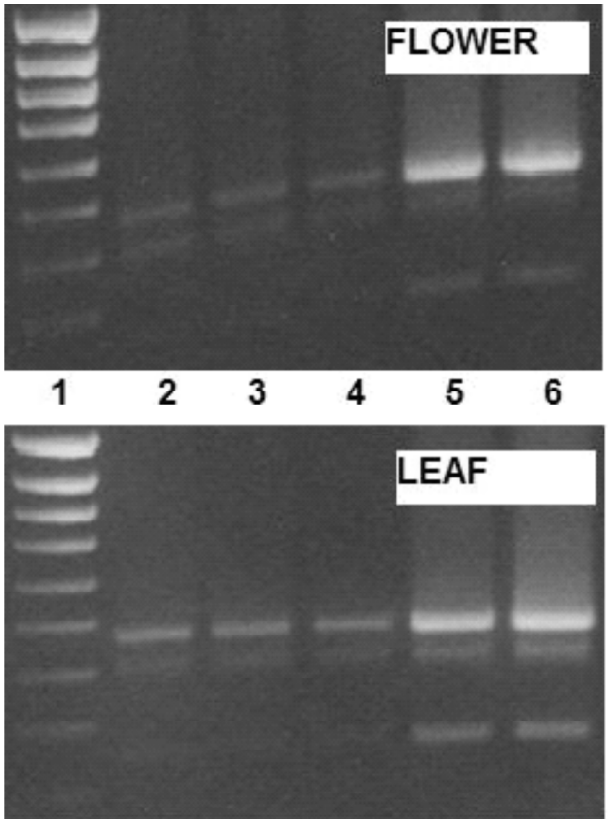

Figure 5: Details are described in the text. Lane 1, shows DNA leader; lanes 2-4 various stages of flower or leaf; lanes 5 and 6, wounded and chemically treated samples respectively

showing the highest regeneration potential (100 %), followed by cv Yours Truly (20-60 %) and then Sincerity (12-25 %). Samba also showed the highest number of shoots from both the petiole (57 shoot buds per petiole explants) and leaf explants (43 shoots per leaf explants). Shoot buds on transfer to Murashige and Skoog (MS) medium supplemented with 0.44 mM N6-benzyladenine and 0.11 mM IAA grew vigorously and attained 1±2 cm in length in 3±4 wk. The shoots rooted with 100% efficiency (Figure 6, right panel) and plants showed normal growth and flowering under greenhouse conditions.

An important take home message from this study is that concentration of growth factors (zeatin or BA) should be titrated to determine the optimum concentration for regeneration and in general zeatin gave better results than BA. More complete details are described in a separate publication [32].

With the development a regeneration system it was now possible to move forward with the next step in genetic modification studies. Based on our results on the expression pattern of various ACC synthases, we selected PHS-ACS41 for transformation work since it appears to be activated by wounding stress or chemical treatment. Antisense constructs of ACC synthase cDNA (pPHS-ACS41) [24] were ligated into binary vector pAM696 (Figure 7, left panel) and introduced into *A. tumefaciens* EHA103 cells.

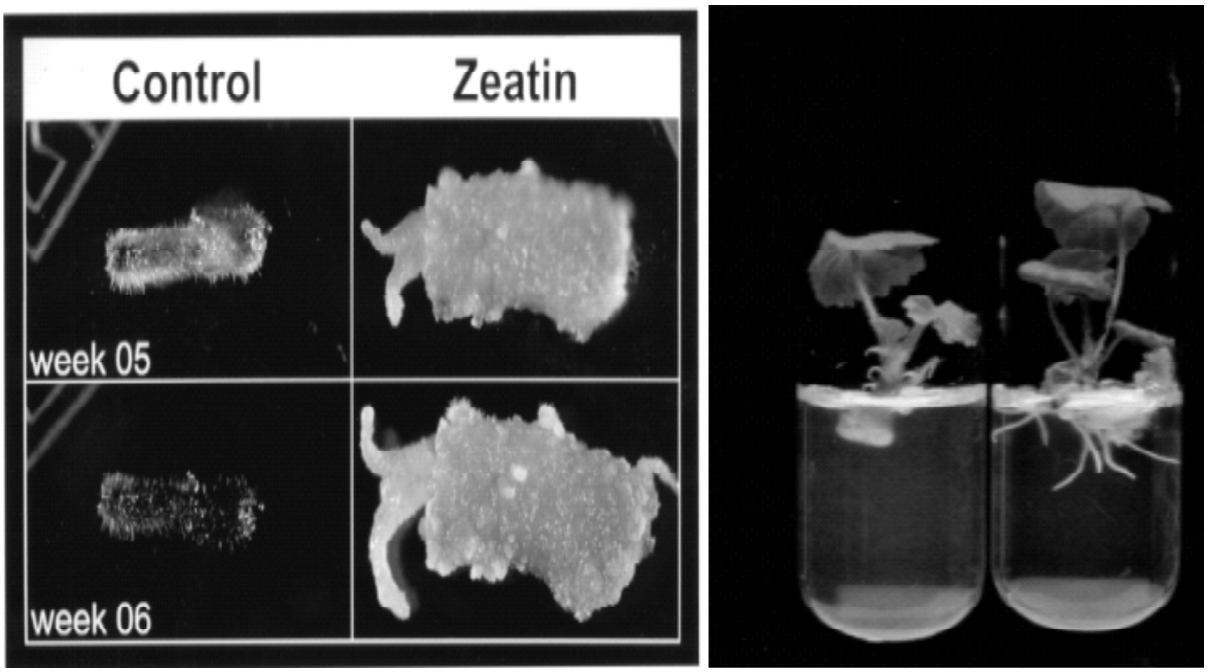

Figure 6: Left panel; Growth and expansion of shoots from petiole explants in the presence and absence of zeatin. Right Panel; Rooting and growth of plantlets in the presence of IAA. Shoot buds separated from mother explants were cultured individually. Within 3-4 weeks it developed a healthy root system [2]

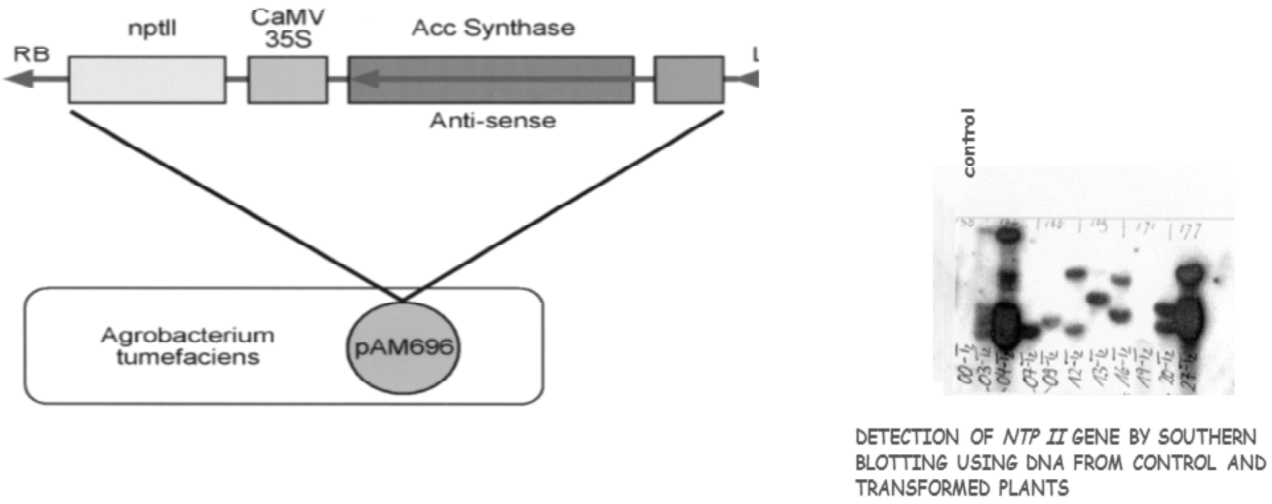

Figure 7: Left Panel; A diagrammatic representation of antisense of ACC synthase cDNA (pPHS-ACS41) construct in plasmid vector pAM696. Right Panel; Southern blott of *NTP II* gene from DNA of control and transformed plantlets. Lanes from left to right are: Lane 1, DNA from control plantlet; the rest from transformants

Petiole explants were incubated with *Agrobacterium* for 15 min and then co-cultivated for several days on MS medium containing 5 mM BA 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 green buds or shoots were transferred to fresh MS medium containing 0.44 mM BA and 0.1 mM IAA and finally on medium containing 0.1 mM IAA for rooting. Plantlets generated after transformation were examined for the insertion of gene cassette into the plant by Southern blotting (Figure 7, right panel). Results showed nearly 50 to 75 % of the tranformants that survived selection were positive by Southern hybridization [28] for the *NTP* gene or by PCR (results not shown); suggesting that a successful transformation of geranium in culture has been established. Based on the intensity of signal and position of DNA bands, it appears that some of the transformants have multiple insertions and insertion position varies. These plants are being evaluated in the greenhouse.

In the present research a full cycle of processes and protocols for genetic modification have been developed for this high value bedding plant, thus opening up an entire area for future basic research and applied areas for the growers. Since these plants are propagated vegetatively the present system of genetic modification is not influenced through fertilization and segregation of inserted genes. Focus in this case would be on the selection process for the best performing plants.

An unexpected and really rewarding outcome of this research is that growers are not entirely dependent on cuttings for vegetative propagation. Tissues culture based regeneration system is a better alternative and far more economical. A single petiole explants can yield 50-60 planlets (each petiole yields 4-5 explants). These plants are healthy and disease free and show no difference under greenhouse conditions to those developed from cuttings.

In addition, this research has contributed to our understanding to some of the unique features of the gene structure and gene expression. It does not appear likely that we have cloned all the ACC synthase genes of this multigene family since this research has focused on flower tissue [24, 34,35]. In future, additional ACC synthase genes are likely to be identified and knowledge of their differential expression may contribute to better understanding of the growth and development of these plants for the economical benefit of the growers. As the saying goes, sky is the limit.

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