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Repression of chilling-induced ACC accumulation in transgenic citrus by over-production of antisense 1-aminocyclopropane-1-carboxylate synthase RNA

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Abstract

A chilling-inducible ACC synthase gene (*CS-ACS1*) has recently been identified from *Citrus sinensis*. This *CS-ACS1* gene was constructed in an inverted orientation and placed under the control of the double 35S promoter. The antisense CS-ACS1 transgene was introduced into *Carrizo citrange*, *C. sinensis* (L.) Osbeck and *Poncirus trifoliate* by *Agrobacterium*-mediated gene transfer. The transformation efficiency in the transformation of citrus stem segment was improved significantly to 87 and 88% for *Poncirus trifoliate* (L.) Raf and *Carrizo citrange*, respectively. The transgenic citrus lines that produce higher level of antisense ACS RNA were found to repress the increase of ACC content following the chilling treatment. This work represents the first example in controlling the ethylene biosynthesis in citrus plants through the genetic engineering approach.

Keywords: Transgenic citrus; *Poncirus*; *Carrizo citrange*; Antisense; ACC synthase; Chilling

1. Introduction

Ethylene production during the degreening process of citrus fruits has been well documented [1–5], even though citrus fruit is considered to be non-climacteric and produces very low amounts of ethylene at mature green stage [6]. A diurnal low (or chilling) temperature treatment (5 °C) of attached and mature-green grapefruit (*Citrus paradisa* Macf.) as well as harvested tangerine (*C. reticulata* Blanco) fruits were found to enhance both ethylene production and the yellowing of the citrus peel [7]. Other abiotic stresses, such as fruit-waxing and high pressure washing of citrus fruits before packaging, also stimulate ethylene production [8]. Furthermore, the biotic stress of the pathogen, *Penicillium digitatum*, induced ethylene production in the infected grapefruit (*C. paradisi*, [9]).

Ethylene treatment during degreening of citrus fruit enhances chlorophyllase (35 kDa, Chlase; EC 3.1.1.14) activity [10–12] and the synthesis of carotenoids [12]. Ethylene also enhances heat damage to flavedo tissue of cured citrus fruits [13], increases the appearance of chilling injury symptoms, stem-end rot decay and the content of volatile off-flavors in the juice head space and fruit internal atmosphere during post-harvest storage [14]. Volatile compounds including ethylene from the exocarp of wounded oranges was shown to induce germination of $\approx 50\%$ of *P. digitatum* conidia on water agar compared to $< 5\%$ in the control [15]. Removal of these undesirable effects of ethylene through the ethylene action inhibitor 1-methylcyclopropane (1-MCP) treatment of citrus fruits had little effect on the weight and firmness of citrus fruits [14].

Moreover, ethylene production is known to associate with abscission. An increased ethylene production in citrus leaves elicited by infection with *Xanthomonas citri*

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caused defoliation [16]. Anatomical changes induced by ethylene have been observed in the abscission zone of citrus leaf and fruit explants [17]. Ethylene treatment enhances cellulase activities in the abscission zone of mature citrus fruit [18]. 1-MCP was found to block abscission of orange (*C. sinensis* L.) leaf explants [19]. Ethylene treatment reduced basipetal transport of IAA, an antagonist to abscission, by 70% in the midrib sections of the Cleopatra mandarin [20].

When water stress or drought induced the accumulation of ethylene precursor ACC 16-fold in citrus roots and arrested xylem flow, sharp and transitory increases in ACC (17-fold) and ethylene (10-fold) levels in leaves, as well as a high percentage of abscission (up to 47%), occurred shortly after rehydration [21]. In the roots of Cleopatra mandarin (*C. reshni* Hort. ex Tan.) plants, both the ACC synthase activity and the level of ACC increased upon rehydration after water stress [22]. It was proposed that ACC transported from roots to shoots promoted leaf abscission in these citrus seedlings [22]. Such leaf abscission induced by a drought–rehydration cycle was inhibited by aminooxyacetic acid (AOA), an inhibitor of ACC synthase, or cobalt ion, an inhibitor of ACC oxidase [22].

To breed a superior citrus plant of enhanced resistance to environmental stresses and improved post-harvest quality of fruits, ethylene production in citrus plant should be controlled. Reduction in ethylene production in the abscission zone of leaves and fruits at the onset of the natural fruit-degreening stage through genetic engineering could lead to prolonging the 'on-tree storage' time of fruits and thus, shortening the post-harvest storage time and reducing the ethylene-induced decay. Because the artificial degreening of citrus fruits by ethylene has been a commercial practice for many years [23–25], the resulting mature green genetically modified (GM) citrus fruits could also be fumigated with exogenous ethylene to elicit degreening and ripening just before being distributed to grocery stores. Success in blocking ethylene biosynthesis using either antisense ACC synthase or ACC oxidase gene [26,27] has clearly demonstrated that antisense RNA technology is a powerful and feasible way to prevent endogenous ethylene production in a specific plant organ at a specific developmental stage. In this manuscript, we report our result on the manipulation of ethylene biosynthesis in citrus plants. A chilling-induced ACS gene has been constructed in the antisense orientation and transformed into citrus plants, *Carrizo citrange* and *Poncirus trifoliate* (L.) Raf, using *Agrobacterium tumefacien*-mediated gene transfer technology [28]. Some of the resulting transgenic citrus plants were found to have both the over-expressed antisense ACS RNA and only slightly elevated ACC levels following the chilling treatments in comparison to the untransformed control plants which had greatly increased ACC levels in response to chilling.

2. Materials and methods

2.1. Plant materials

The peeled seeds of four different citrus plants, *C. microcarpa* Bunge, *Poncirus trifoliata* (L.) Raf., *Carrizo citrange* (*C. sinensis* (L.) Osbeck x. *Poncirus trifoliata* (L.) Raf.) and *C. sinensis* Osb. cv Hong Jiang, were disinfected in 75% ethanol for 2 min, followed by 0.1% $HgCl_2$ solution treatment for 15 min. The sterilized citrus seeds were then rinsed with sterile distilled water five to six times and the inner seed coats of the resulting seeds were stripped off thereafter. The seeds were then placed on 0.5 MS [29] agar medium with 3% of sucrose and maintained under darkness at 27 ± 2 °C for 2–3 weeks. The resulting seedlings were used for transformation and regeneration.

2.2. Oligonucleotides, plasmids and bacterium strain

Primers, FP_{35S} and RP_{35S}, synthesized by Gene Assembler (Pharmacia) to amplify CaMV 35S promoter using polymerase chain reaction (PCR), are: 5′ACCC AAGCTT ACTAGT CCC ACA GAT GGT TAG AGA GGC TTA CGC AGC3′ and 5′CAAA CTGCAG CCA GTC CCC CGT GTT CTC TCC AAA TGA AAT GAA3′, respectively. HindIII and SpeI restriction sites (underlined sequence) were genetically engineered into the primer FP_{35S}, whereas PstI site was engineered into primer RP_{35S} to facilitate chimeric binary vector construction. Another pair of primers, FP_{ACS} and RP_{ACS}, corresponding to nucleotides 275-306 and 1461-1433 of CS-ACS1 (AJ012551), were 5′ACGC GTCGAC AAG GGT ATA GCT ATC TTT CAG GAT TAT CAC GG3′ and 5′CGC GATCC TGA ACA AGA GGT GAT TGA GGA GAC ATG CA3′, respectively. SalI and BamHI restriction sites were genetically engineered into the two primers, respectively, to facilitate the subcloning. The primer, SPP1, used for asymmetric PCR amplification of single strand sense cDNA probe is 5′ TGC TGG CCT GTT CCT ATG GAT GGA TTT GCA3′, corresponding to the nucleotides 1105-1134 of CS-ACS1 (Accession No. AJ012551). The binary vector pBI121 was purchased from Clontech Laboratories (Palo Alto, CA). Enzymes used for DNA recombination and PCR were purchased from Novagen (Madison, WI), Clontech, Amersham-Pharmacia (Piscataway, NJ) and Invitrogen (Carlsbad, CA).

2.3. Gene transfer

The disarmed hypervirulent *A. tumefaciens* strain EHA105 [30] harboring the chimeric binary vector pBI121-aACS1 was first inoculated into 25 ml of YEP medium [31] containing 100 mg/l streptomycin and 50 mg/l kanamycin and grown at 27 ± 2 °C in darkness

on a shaker for 30–36 h with a rotation speed of 180 rpm. When the cell density reached OD values of 0.8–1.3, cells were spun down by centrifugation at $2000 \times g$. The cell pellets were then resuspended in MS medium (containing 100 μM acetosyringone). The internodal segments cut from the citrus seedlings were immersed in *Agrobacterium* strain, EHA105: pBI121-aACS1, resuspension for 10–14 min and then briefly air-dried on a filter paper, followed by co-cultivation with the strain for 2–3 days under light at 27 ± 2 °C. The co-cultivated stem explants were then transferred to a regeneration medium containing 1 mg/l BA, 400 mg/l cefotaxime and 75 mg/l kanamycin and grown under light at 27 ± 2 °C. When the regenerated shoots grew to ≈ 1 cm long, they were transferred to the regeneration medium in the absence of cefotaxime. The transfer of regenerating shoots was repeated once a week for six to eight times. The rooting was carried out on MS medium containing 0.2 mg/l NAA. When regenerated putative transgenic seedlings of *Poncirus trifoliata* (L.) Raf. were grown to a height of 5–6 cm, they were transferred to potting soil moistened with water, whereas the regenerated apical shoots of other citrus seedlings such as *Carrizo citrange* and *C. sinensis* Osb. cv Hong Jiang were grafted onto rootstocks of *Poncirus trifoliata* (L.) Raf.

2.4. Shoot tip grafting

The seedlings of *C. grandis* (L.) Osb, prepared as described in Section 2.1, were initially decapitated. The cotyledons and auxillary buds of the resulting seedlings were also removed. After shortening the roots of seedlings to 5–7 cm long, the rootstocks possess 1–1.5 cm long decapitated epicotyls. The regenerated shoot tips of *Carrizo citrange* or *Poncirus trifoliata* (L.) Raf or *C. sinensis* Osb. cv Hong Jiang were set onto the cut surfaces of the rootstock epicotyls. The vascular ring of shoot tips and that of the stems of rootstocks was placed in close contact. The two stem contacting regions were tightly wrapped with parafilm strips. The roots of the grafted seedlings were then immersed in a culture medium containing MS inorganic salts, 100 mg/l *m*-inositol, 0.2 mg/l thiamine–HCl, 1 mg/l pyridoxine–HCl, 1 mg/l nicotinic acid, 75 g/l sucrose, pH 5.7 ± 0.1. The grafted seedlings were maintained at 27 ± 2 °C with 14:10 h light:darkness regime for 4–6 weeks before they were transferred into potting soil.

2.5. Southern and Northern blot analysis

Frozen leaves of both wild type and putative transgenic citrus plants were ground with a pre-chilled mortar and pestle in liquid nitrogen. Total RNA and genomic DNA were extracted using RNasy plant mini kit (Qiagen) and DNeasy plant mini kit (Qiagen), respectively. The total RNA was first size-fractionated on a 1% formaldehyde agarose gel. The native RNA molecules were partially hydrolyzed into segments of smaller sizes by NaOH (50 mM) treatment for 20 min. The RNA gel was rinsed with DEPC-treated water and soaked in $20 \times$ SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0) for 45 min. The RNA was capillary-transferred onto a nylon membrane (Amersham-Pharmacia Biotech) and fixed on the membrane by UV cross-linking before proceeding to the hybridization step. To make DNA blot, citrus genomic DNA was digested overnight with restriction enzymes and separated on 1% agarose gel. The DNA was denatured in a denaturation buffer (0.5 M NaOH, 1 M NaCl) for 45 min and neutralized in a neutralization buffer (0.5 M Tris–HCl, 1 M NaCl, pH 7.5). The DNA was transferred onto the nylon membrane (Amersham-Pharmacia Biotech). Northern and Southern blot hybridizations were carried out as previously described [32].

2.6. Chilling treatment and ACC content determination

Branches of transgenic citrus plants were removed and kept at 4 °C for 3 days. The chilling treated citrus branches were transferred back to 25 °C for 6 h. Leaves from the chilling treated branches were harvested and immediately frozen in liquid nitrogen for later ACC measurement.

The citrus tissues that had been frozen and ground into powder were extracted with 80% ethanol. The ACC content was determined according to the previously described method [33].

3. Results

3.1. Construction of an antisense ACS gene in the binary vector pBI121

The duplication of 35S CaMV promoter has been shown to enhance the transcription strength more than that of a single 35S CaMV promoter in different types of plant cells [34]. We thus engineered a double 35S promoter from that in the pBI121 vector (Clontech). The binary vector pBI121 was double digested with *Eco*RI and *Hin*dIII and a segment of DNA containing a 35S CaMV promoter, a GUS gene and a NOS terminator was generated (Fig. 1A). This 35S CaMV gene expression cassette was inserted into a pUC18 vector to generate a recombinant plasmid pUC18-35S-GUS. An additional 35S CaMV promoter

was then generated through PCR using a pair of 35S CaMV promoter-specific primers, as described in Section 2 and the pBI121 binary vector as the template. The consequent 0.8 kb PCR amplified DNA product was ligated to the pUC18-35S-GUS after both being treated with HindIII and PstI restriction endonuclease to produce a recombinant double 35S CaMV promoter (or d35S) in tandem repeat. A citrus ACC synthase gene, *CS-ACS1*, that has been shown to be chilling-inducible and ripening-associated [33], was then inserted to replace the GUS gene in the pUC18-d35S-GUS. The antisense *CS-ACS1* gene cassette driven by the d35S CaMV promoter was then excised out of the pUC18 vector and inserted into the binary vector pBI121 to generate the recombinant binary vector pBI121-d35S-aACS (Fig. 1A).

3.2. Production of putative transformed shoots

The citrus stem segments excised as previously described [28] were infected with a supervirulent *Agrobacterium* strain, EHA105, containing the recombinant binary plasmid pBI121-d35S-aACS and then cultured on the kanamycin/cefotaxime-containing shoot regenerating medium for 10–15 days. The shooting started to initiate from the cutting edge of the segments and lasted for 2 weeks. A few calli were observed. Compared to *Carrizo citrange*, both *C. cinensis* Osb. cv Hong Jiang and *Poncirus trifoliate* (L.) Raf initiated shooting 4–5 days earlier. No shoot was observed from *C. microcarpa* Bunge except several calli (Table 1). When the shoots grew to 1 cm long, each shoot was detached from the segment and cultured on the cefotaxime-free

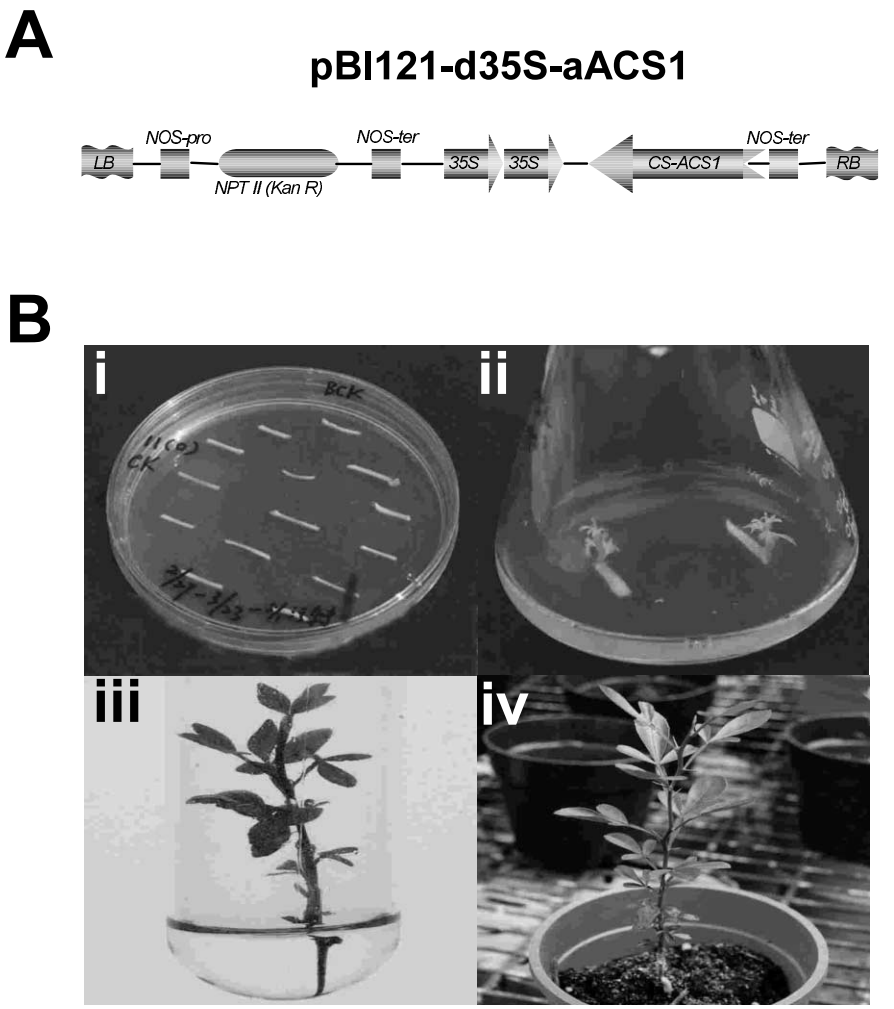

Fig. 1. (A) The recombinant antisense ACS-containing binary plasmid. The antisense CS-ACS1 (Accession No.: AJ012551) cDNA fragment corresponding to nucleotides 275 to 1461 of CS-ACS1 was digested with *Eco*RI and *Hin*dIII and the resulting antisense *CS-ACS1* gene expression cassette was inserted into the region between left boarder (LB) and right boarder (RB) of the binary vector pBI121 containing a kanamycin selectable marker. 35S, CaMV 35S promoter. NOS-ter, nopaline synthase terminator. NPTII, neomycin phosphotransferase II. (B) Regeneration of the transformed citrus plants. (i) Internodal segments co-cultured with *Agrobacterium* strain, EHA105:pBI121-aACS, followed by regeneration on a medium containing cefotaxime and kanamycin (see Section 2). (ii) Continued cultivation of shoots on medium containing kanamycin (see Section 2). (iii) Rooting of regenerated shoots was induced in medium containing NAA (for *Poncirus trifoliata* only). (iv) The pot-grown putative transgenic citrus plant (*Carrizo citrange*).

Table 1
Summary of regeneration and transformation of citrus stem segments

Genotype	Number of segments evaluated	Total number of shoots regenerated on medium for 1 month	Total number of shoots regenerated on medium for 4 months	Percentage of transformants among the shoots regenerated
Citrus microcarpa Bunge	445	0	0	–
Poncirus trifoliata (L.) Raf	1087	148	110	87%
Carrizo citrange	509	28	21	88%
Cotris sinensis Osbeck cv Hong Jiang	985	17	1	–

shoot regeneration medium for an additional 2–3 months. The surviving shoots were considered as the putative transformants. The results of transformation and regeneration of four different citrus species and cultivars are summarized in Table 1.

With each batch of transformations, two controls were also performed. One was the uninfected citrus segment directly cultured on the selective shooting medium, the other was the infected citrus segment growing on the kanamycin/cefotaxime-free medium. All of the excised segments from all four citrus cultivars died 8 weeks later, whereas the shoot generation rates for all four citrus cultivars varied from 97 to 100% (Fig. 1B). These results indicated that kanamycin at 75 μg/ml is high enough to impose the selection of the transformed citrus shoots from those untransformed ones.

Co-cultivation time with *Agrobacterium* is known to be essential to the increase of transformation efficiency [35]. In our experiments, we also examined the effect of the length of infection period. It was found that the *Poncirus trifolata* (L.) Raf segment had a transformation efficiency of 21.6 and 33.9%, respectively, if it is co-cultivated with EHA105 for 2 and 3 days. Similarly, when *Carizzo citrange* was co-cultivated with *Agrobacterium* for 2 and 3 days, the transformation efficiency increased from 4.4 to 16.5%. If the co-cultivation time exceeded 3 days, it became difficult to control the outgrowth of *Agrobacterium* in the following regeneration process. The transformation efficiency was defined as the number of survivors on the cefotaxime/kanamycin-containing medium determined 4 weeks after the infection took place over the number of the cutting ends of the infected segments. Continued cultivation of the putative citrus transformants for an additional 3 months reduced the transformation efficiency 25% (Table 1).

3.3. Molecular analysis of the transformants

The *NPTII* gene carried by pBI121 binary vector confers kanamycin resistance to the transformed citrus shoots. However, a certain number of escape shoots can also survive the kanamycin selection [36]. To distinguish the authentic transformants from the untransformed ones, the Southern blot analysis was performed on a group of randomly selected putative citrus transformants of both *Poncirus trifoliata* (L.) Raf and *Carrizo citrange*. It was found that one out of nine and two out of 15 evaluated citrus shoots were negative in Southern blot analysis for *Poncirus trifoliata* (L.) Raf and *Carrizo citrange*, respectively, indicating that 87 and 88% of the shoots that had survived on the kanamycin selection medium for 4 months were true transgenic citrus plants (Table 1). The only survived shoot of *C. cinensis* Osb cv Hong Jiang was subjected to micro-propagation and not examined by the Southern blot analysis. Because the profiles of Southern signals of each transgenic line were distinctly different, as shown in Fig. 2, it is most likely that the transgene insertion sites in these transgenic citrus lines are different and that each line derived from a single transformation event (Fig. 2). Transgenic lines 57-4a, 57-5a and 73-1c may contain two or more copies of the transgenes because two or more large size transgene-containing DNA fragments (>5 kb) were found in these transgenic citrus lines (Fig. 2, lane 4, 5 and 9).

3.4. Repression of chilling-induced ACC increase by over-expression of antisense ACS gene

Because antisense ACS gene, *CS-ACS1*, was placed under the control of double 35S CaMV promoter which is known to drive a constitutive expression in various plant tissues, it was anticipated that the expression of antisense ACS gene engineered as such should be detected in most of the citrus tissues [37]. Out of those authentic citrus transformants, nine *Carrizo citrange* and two *Poncirus trifoliata* (L.) Raf transgenic citrus plants were selected to perform the Northern blot analysis to determine if antisense ACS transcript had been expressed in vegetative tissues. A sense strand of *CS-ACS1* cDNA fragment (see Section 2) was used as a probe to determine the antisense mRNA expression level. As shown in Fig. 3, seven out of nine transgenic

Carrizo citrange plants and two *Poncirus trifoliata* (L.) Raf plants expressed the antisense CS-ACS1 mRNA to a detectable level in the transgenic citrus leaves. Two classes of antisense ACS1 transcripts were found (Fig. 3). One was 2.3 kb long, the other 1.5 kb long. The longer antisense CS-ACS1 transcript may contain 0.8 kb 35S CaMV promoter sequence because of the existence of two transcription initiation sites in the double promoter. The transgenic *Carrizo citrange* lines 67-2a, 72-2b and 73-1c expressed much higher level of 1.5 kb transcript than the rest transgenic citrus plants, whereas the antisense gene transcripts of the transgenic line 57-4a were undetectable based on the Northern blot analysis (Fig. 3B). The changes in transcript levels among these transgenic lines could be attributed to the 'position effect' of the transgene. The morphologies of the 67-2a, 72-2b and 73-1c transgenic *Carrizo citrange* young plants were indistinguishable from those of the untransformed control plant, 56-1c, (Fig. 2A).

To examine if the antisense RNA indeed suppressed the endogenous ACC synthase activity, the ACC content in the transgenic plant was determined following a chilling treatment, as described previously [33]. As expected, chilling induced 218 and 90% in the ACC level in the untransformed control (56-1c) and the transgenic line 57-5a, respectively (Fig. 4). The latter expressed a relatively lower level of antisense ACS RNA (lane 5, Fig. 3). In contrast, cold stress only induced a modest increase 23, 29 and 54% in the ACC level, respectively, in transgenic lines 72-2b, 67-2a and 73-1c (Fig. 4).

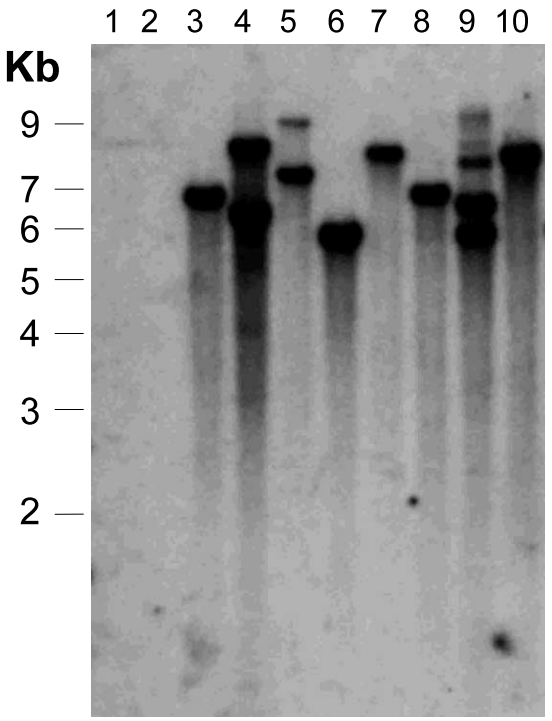

Fig. 2. Molecular analysis of transgenic *Carrizo citrange* (*Citrus sinensis* (L.) Osb x *Poncirus trifoliate* (L.) Raf). Each lane contained 3–5 μg of citrus genomic DNA. The probe was ^{32}P-labelled cauliflower mosaic virus (CaMV) 35S promoter fragment (0.8 kb) prepared by random primer labeling. Lane 1 and 2, the regenerated and untransformed citrus plants. The rest are transgenic citrus lines. Lane 3, line 56-8a; lane 4, line 57-4a; lane 5, line 57-5a; lane 6, line 63-2a; lane 7, line 67-2a; lane 8, line 72-2b; lane 9, line 73-1c; and lane 10, line 76-3a.

4. Discussion

The *Agrobacterium*-mediated transformation in citrus plant, since its establishment in 1992 (Moore et al.), has been successfully applied to many citrus species including *Carrizo citrange* [28,35,36,42], *C. sinensis* (L.) Osbeck [43,47], *C. reticulata* [28,41,47], *C. limon* [47], *C. paradisi* [47], *C. aurantium* [35,36] and *Poncirus trifoliata* [35,47]. In the past few years, the transformation efficiencies for both *Carrizo citrange* and *Poncirus trifoliata* have been improved to 41.3 and 43.1%, respectively [38,39]. In this experiment, we further increased the transformation efficiencies for both citrus species to 88 and 87% (Table 1), respectively. This high transformation efficiency is comparable to the 76–90% transformation efficiency for Mexican lime (*C. aurantifolia* (Christm) Swing) system obtained by Perez-Molphe-Balch and Ochoa-Alejo (1998). These efficient citrus transformation processes make them potentially useful systems for molecular farming in citrus fruit [40].

Although the cauliflower mosaic virus (CaMV) 35S promoter and the pea chlorophyll *a*/*b* binding protein gene (*cab*) promoter have been shown to be functional in citrus tissue [41], the level of gene expression in citrus plants was unsatisfactory [42]. To enhance the level of gene expression, we introduced a double 35S promoter arranged in a tandem array for driving the expression of antisense ACS gene (Fig. 1A). As expected, the target mRNA level in several transgenic citrus lines increased significantly (Fig. 3) compared to those previously reported with a single 35S promoter [42]. According to the Southern blot analysis, a single copy transgene was most likely introduced into citrus genome (Fig. 2, lane 7 and 8) in several transgenic lines, and such a high level expression of antisense ACS gene could result from the higher transcription activity of the double 35S promoter. The citrus line deficient in expression of antisense ACS gene (Fig. 3B, line 57-4a) may contain two copies of transgenes (Fig. 2, lane 4), which might result from spontaneous mutations occurring in the 35S promoter region or 'gene position' effect. It is also interesting to note that there exist two different sizes of antisense ACS transcripts. Because the probe used to detect the antisense ACS gene was a single strand sense ACS cDNA specific and complementary to the antisense ACS gene, the longer transcript (2.3 kb) detected by this probe could not be the endogenous ACS mRNA because the chilling-induced ACS gene

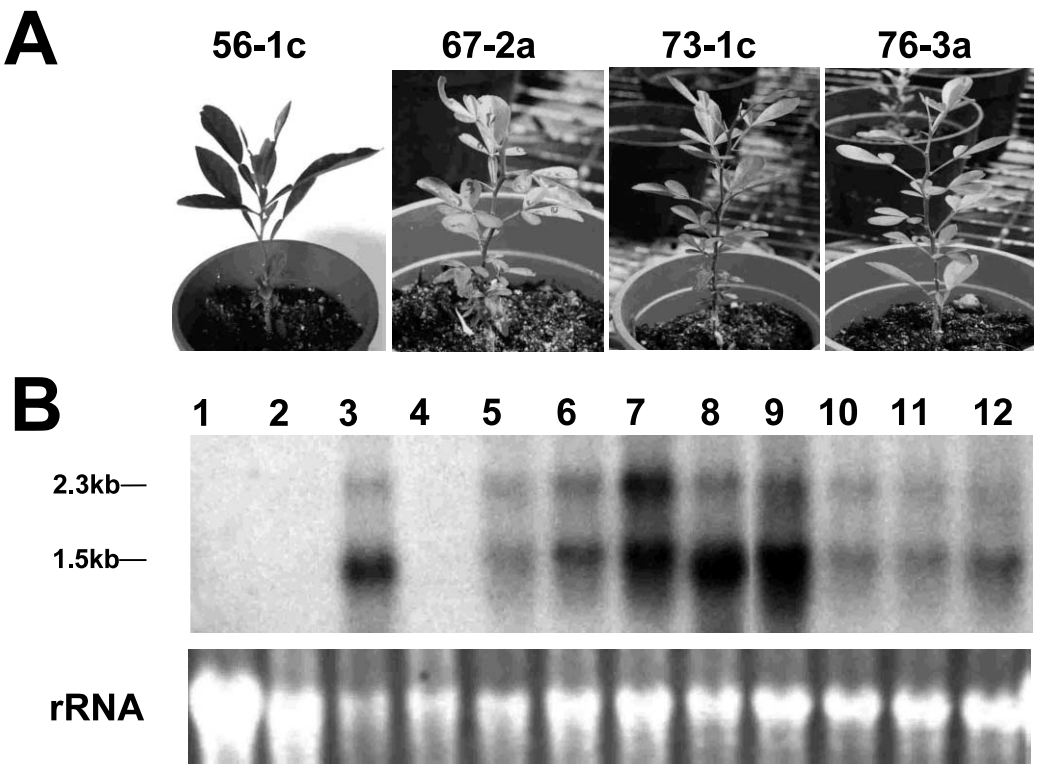

Fig. 3. Northern blot analysis of transgenic citrus plants expressing antisense ACS genes. (A) The untransformed control plant (56-1c) and transgenic *Carrizo citrange* (67-2a, 73-1c and 76-3a) grown in pots. (B) Northern blot analysis of transgene expression in citrus plants. A nylon membrane blotted with 15 μg of total RNA per lane from both untransformed and transformed citrus plants was hybridized with ^{32}P-labeled sense CS-ACS1 cDNA strand (see Section 2) prepared by asymmetric PCR using CS-ACS1 as the template and SPP1 as primer (see Section 2). The amount of RNA samples loaded into each lane was visualized by ethidium bromide-staining, as shown at the bottom of the panel B. rRNA, ribosomal RNA. Lane 1 and 2, the untransformed control plants; lane 3–10, the transgenic *Carrizo citrange* lines 56-8a, 57-4a, 57-5a, 63-2a, 67-2a, 72-2b, 73-1c and 76-3a, respectively. Lane 11 and 12 are the transgenic *Poncirus* line 20-2a and 20-2b, respectively.

transcript was determined to be 1.7 kb. The longer one might result from a 'read-through' transcription activity that transcribed both the distal 35S promoter (0.8 kb) and antisense ACS gene (1.5 kb). Our result indicated that the gene expression cassette used in this experiment should be efficient for expressing foreign genes, such as those encoding enzymes that metabolize limonin [43] in citrus plant. This is also the first example of successful expression of antisense RNA in citrus plant.

The basal levels of ACC in both the untransformed control plant and the transgenic citrus lines were found to vary from 75 to 150 pmoles per gram of fresh tissue before the chilling treatment (Fig. 4). The difference in ACC content among different plant individuals could result from the differential expression activities of other members of ACC synthase gene family, such as *CS-ACS2* [33]. The fact that the basal levels of ACC in all transgenic citrus lines were found to be higher than that of the control plants before chilling treatment (Fig. 4) suggested either that the transgenic citrus plants might have more activities of other *CS-ACS* genes or that this untransformed control plant happened to have lower level of endogenous ACC. However, the different basal levels of ACC content found among these citrus plants could not weaken the conclusion that we have drawn on the effective inhibition of ACC increase by the antisense ACS RNA in citrus. For example, although the 57-5a produced a much higher level of basal level ACC before chilling treatment, it still responded to the chilling by producing more ACC due to the fact that it

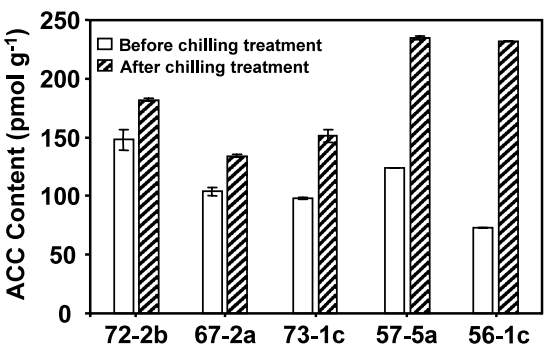

Fig. 4. Repression of ACC production in the transgenic citrus lines. The untransformed control plant is labeled as 56-1c. The transgenic citrus lines 72-2b, 67-2a and 73-1c are antisense ACS RNA over-producers. Line 57-5a is the transgenic citrus plant expressing relatively less antisense ACS RNA. The open and hatched bars represent the untreated and chilling-treated transgenic citrus plants, respectively.

is a lower antisense ACS RNA producer (Fig. 3). In contrast, the line 67-2a, which has a relatively lower basal level of ACC before chilling treatment, failed to produce more ACC due to the fact that it is a higher antisense ACS RNA producer (Fig. 3). Moreover, no detectable morphological change was found among these transgenic citrus plants compared to that of the control plant, suggesting that the constitutive production of chilling inducible antisense ACS RNA specifically affect the chilling-responsive *CS-ACS1* gene activity. It could be speculated that the reduced level of ACC in citrus tissue following the chilling exposure could at least reduce the symptom of the chilling injury of citrus plant tissues.

Citrus fruits are important agricultural commodities. Genetic engineering, or molecular breeding of citrus plants, provides an alternative approach to produce superior citrus plants of higher resistance to the biotic (such as virus) and abiotic stresses (such as chilling), leading to improved yield and enhanced post-harvest quality. Successful over-expression of antisense ACS gene in citrus adds to the repertoire of transgenic fruits [26,44–46] generated in the past years to manipulate ethylene production in these plants. Given the current trend in the use of plants as the bioreactors to produce pharmaceutical compounds and industrial materials [40], citrus fruit, being the number one fruit in terms of production volume, should be an ideal candidate for such purposes providing that the transformation protocols and gene expression system for citrus plants have been well established.

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