カーネーションの花の老化時に発現する1-アミノシクロプロパン-1-カルボン酸酸化酵素のゲノミックDNAとプロモーター活性

誌名	園藝學會雜誌
ISSN	00137626
著者	日比, 忠晴 高野, 誠 吉岡, 俊人 ほか2名,
巻/号	70巻2号
掲載ページ	p. 215-222
発行年月	2001年3月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council Secretariat

AgriKnowledge

園学雑. (J. Japan. Soc. Hort. Sci.) 70 (2) : 215-222. 2001.

Senescence Regulated Promoter Activity of a Carnation (*Dianthus caryophyllus* L.) Flower 1-Aminocyclopropane-1-carboxylate Oxidase Gene, *DC-ACO1*

Tadaharu Hibi [1], Makoto Takano [2], Toshihito Yoshioka [1], Teruyoshi Hashiba [1] and Shigeru Satoh [1]*

[1] *Laboratory of Bio-adaptation, Graduate School of Agricultural Science, Tohoku University, Tsutsumidori-amamiyamachi 1-1, Aoba-ku, Sendai 981-8555*

[2] *National Institute of Agrobiological Resources, Kannondai 2-1-2, Tsukuba 305-8602*

Summary

A genomic DNA clone, *DC-ACO1*, encoding ACC oxidase was cloned from carnation (*Dianthus caryophyllus* L. cv. Nora) plants. *DC-ACO1* encodes an amino acid sequence which, except for one amino acid, is identical to that of pSR120 obtained previously as a senescence-related ACC oxidase cDNA from carnation petals (Wang and Woodson, 1991). *DC-ACO1* contains 3 exons which are interrupted with 2 introns. Promoter activity of the 5′-flanking region up to -1170 bp, relative to the A (+1) of the translation start codon ATG, of *DC-ACO1* was analyzed by transient expression of the 5′-flanking sequence-β-glucuronidase (GUS) gene fusion after delivery by particle bombardment into carnation petals. Expression of the chimeric gene increased depending on the progress of senescence of petals, which was measured by their ethylene production rates. Truncation of the 5′-upstream sequence of -1170 bp to -247 bp increased the promoter activity by 6.5 fold, indicating that the -247 bp-long 5′-flanking sequence was sufficient for promoter activity.

Key Words: ACC oxidase, carnation, *Dianthus caryophyllus* L., flower senescence, promoter, promoter-GUS fusion.

Introduction

Ethylene plays a crucial role in the senescence of some flowers, such as carnation and sweet pea. A large amount of ethylene is produced during senescence of these flowers, and the treatment of flowers with ethylene enhances the senescence (Abeles et al., 1992; Reid and Wu, 1992). Inhibition of the synthesis or action of ethylene delays the onset of senescence symptoms and increases flower longevity. Blockage of ethylene-induced senescence in flowers is of economical importance. Although chemicals such as silver thiosulfate anionic complex (STS) or aminooxyacetic acid (AOA) are currently being used as anti-senescent preservatives in the cut-flower industry, it has been recognized that transgenic plants with suppressed production or action of ethylene will be excellent alternatives for the preservation of flower longevity.

So far, genetic transformation with transgenes related to the biosynthesis and action of ethylene has been successfully used to down-regulate ethylene production or responsiveness to ethylene in some ornamental flowers like carnation (Michael et al., 1993; Savin et al., 1995; Bovy et al., 1999), torenia (Aida et al., 1998) and petunia (Wilkinson et al., 1997). In some cases, however, negative effects of transformation have been reported for the transformants with reduced production or action of ethylene; the decrease in numbers and pigmentation of petals compared with those normally expected in carnation cv. Scania (Michael et al., 1993), the decrease in transformation-regeneration capacity and fitness that causes a loss of transformants in tissue culture and subsequent growth in carnation plants in the greenhouse (Bovy et al., 1999), and the almost complete loss of ability to form adventitious roots in petunia plants (Clark et al., 1999). These negative effects probably resulted from the disturbance of ethylene-dependent processes in plant tissues, since these transformants harbored transgenes under the control of constitutive promoters (Michael et al., 1993; Wilkinson et al., 1997; Bovy et al., 1999). Thus, to avoid future possible negative effects when using transgenic approaches to generate a wide variety of ornamental plants with decreased production of or decreased sensitivity to ethylene, it is desirable to use a flower senescence-specific promoter to drive the appropriate transgenes in plants. The promoter of carnation glutathione-*S*-transferase gene seems to be a good candidate since the gene is responsive to ethylene and expressed during petal senescence of the flower (Itzhaki and Woodson, 1993; Itzhaki et al., 1994). In addition, development of many options for such the promoter will be helpful in the future practical use.

Received; February 23, 2000. Accepted; August 7, 2000.

* Corresponding author.

During natural senescence and exogenous ethylene-induced senescence in carnation flowers, a large amount of ethylene is produced through the expression of genes for ACC oxidase and ACC synthase (Manning, 1985; Peiser, 1986; Wang and Woodson, 1989). A cDNA clone pSR120 encoding ACC oxidase was obtained from senescing carnation petals (Wang and Woodson, 1991). The transcript corresponding to pSR120 was not detected in the petals for several days after full opening of flowers, but accumulated concomitantly with the onset of ethylene production in the later period of flower senescence (Woodson et al., 1992). Thus, this gene was regarded as one of the flower senescence-related genes (Wang and Woodson, 1991). We considered that the promoter of the carnation ACC oxidase corresponding to pSR120 would serve effectively as a flower senescence-related promoter in transgenic approaches for ornamental flowers.

As the first step to test this possibility, we cloned genomic DNAs for ACC oxidase from carnation plants and analyzed their structure. Then, we analyzed the promoter activity of the 5′-flanking region of *DC-ACO1* by transient expression of 5′-upstream region-GUS fusions in carnation petals.

Materials and Methods

Plant materials

For isolation of genomic DNA, carnation (*Dianthus caryophyllus* L. cv. Nora) flowers at the full opening stage were obtained from Miyagi Horticultural Experimental Station or a local grower. Petals were detached from flowers and stored at -80℃ until isolation of DNA. In addition, carnation plants were grown under usual greenhouse conditions, and young leaves were detached from the plants and stored at -80℃ until use.

For promoter analysis, carnation cvs. Delfy or White Barbara flowers at the full opening stage were obtained from a local grower or a local market. The flowers of these cultivars have white petals. We ensured that the flowers purchased at the local market had not been treated with STS, which was also confirmed later by observing autocatalytic ethylene production during senescence. Intact petals were detached from flowers after assaying for ethylene production as described below and used for promoter analysis.

Ethylene production assay

Stems of flowers were trimmed to 2 cm long and flowers were placed with their basal end in 30 ml glass vials with 20 ml water. Ethylene production from carnation flowers was monitored daily by enclosing individual flowers in 450 ml glass containers (one flower per container) for 1 hr at 25℃. A 1 ml gas sample was taken into a syringe from the inside of the container through a rubber septum of a sampling port on the container, and analyzed for ethylene with a gas-chromatograph (263-30, Hitachi) equipped with an alumina column and a flame ionization detector.

Isolation of a genomic DNA and PCR

Genomic DNA was isolated from frozen tissue by CTAB method (Murray and Thompson, 1980). A carnation ACC oxidase genomic DNA was obtained by PCR amplification. Primers were synthesized according to the nucleotide sequence of carnation ACC oxidase cDNA clone pSR120 (Wang and Woodson, 1991) and contain in addition an *Eco*R I site or a *Hin*d III site to facilitate cloning; the upstream primer 5′-ccccgaattcCTACAAATACAAATACATTG-3′ (represents positions 1-20 bp of pSR120) and the downstream primer 5′-ggggaagcttAAGGGGATAAATTATTGTAA-3′ (represents 1217-1236 bp of pSR120). PCR was conducted in a total volume of 100 μl containing 0.5 μg of the genomic DNA, 1 μM each of both primers, 10 mM Tris (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM deoxynucleotides and 2.5 units of DNA polymerase (Expand High Fidelity PCR System, Hoffmann-La Roche). PCR was carried out with 30 cycles of 45 sec at 94℃, 1 min at 65℃, 2 min at 72℃, and supplemental incubation for 5 min at 72℃ with an automatic thermal cycler (GeneAmp PCR System 2400, Perkin-Elmer/Cetus). PCR products were separated on a 1.0% agarose gel and the band of about 1.5 kbp was recovered.

Construction of a genomic library and screening

Genomic DNA was isolated from green leaves, and partially digested with *Sau*3A I. The digests were size-fractionated by cenrifugation on a 20-40% sucrose gradient. Restriction fragments ranging in size from 7 to 10 kbp were ligated into *Bam*H I-digested λDASH II (Stratagene) arms and packaged *in vitro* using Gigapack III Gold Packaging Extract (Stratagene). The library was screened by plaque hybridization with the ACC oxidase genomic DNA as a probe. The probe was labeled with ^{32}P-dCTP by random priming using Multiprime DNA labeling systems (Amersham). Nylon membrane (Hybond N^+, Amersham) lifts were prehybridized for 3 hr at 42℃ in a solution containing 20% (v/v) formamide, 5 x Denhardt's solution (1x Denhardt's solution is 0.02% PVP, 0.02% Ficoll, 0.02% BSA), 0.5% SDS, 6 x SSPE (1 x SSPE is 0.15 M NaCl, 10mM NaH_2PO_4, 1mM EDTA, pH7.4) and 100 $\mu g \cdot ml^{-1}$ denatured salmon sperm DNA. Hybridization was carried out at 42℃ for 18 hr in the same buffer supplemented with 4.6 x 10^5 $cpm \cdot ml^{-1}$ of the ^{32}P-labeled probe. Membranes were washed for 15 min at room temperature in 2 x SSC (1x SSC is 0.15 M NaCl, 15 mM Na-citrate, pH 7.0), 1% SDS, followed by a 2nd wash for 15 min at 55℃ in the same washing solution and a final wash for 15 min at 55 ℃ in 0.1 x SSC and 0.1% SDS. The membranes were exposed to Kodak XAR-5 film with an intensifying screen at -70℃. Hybridized recombinant phages were

rescreened at a lower plaque density for plaque purity. Phage DNA was purified and restriction maps were derived from single and double restriction endonuclease digestion and Southern blot analysis.

DNA sequencing and analysis

Restriction fragments from genomic clones were digested with *Sal* I and ligated into pBluescript II SK (+) plasmid (Stratagene), and the resultant plasmids were amplified in *E. coli* XL 1-Blue (Stratagene). Plasmids were isolated by QIAprep Spin Plasmid Kit (Qiagen). For some restriction fragment subclones, a series of overlapping deletions were generated with a Deletion Kit for kilo sequence (TaKaRa). Nucleotide sequences were determined using an ABI 373A sequencer (Applied Biosystems). DNA sequence data were analyzed using DNASIS software (Hitachi Software Engineering).

Construction of chimeric DC-ACO1 promoter-GUS genes

Two DNA fragments of 5′-upstream sequence ranging from −1170 bp or −247 bp to +345 bp of the coding sequence were obtained by PCR with appropriate primers and used to construct transcriptional fusions with the β-glucuronidase (GUS) coding region in the plant transformation vector pBI101-hyg^r by inserting them at the *Sal* I/*Bam*H I site (Fig. 3). pBI101-hyg^r is pBI101 (Clontech) to which a hygromycin phosphotransferase gene was added. The resultant chimeric genes contain the 1st exon, the 1st intron and a partial sequence of the 2nd exon of *DC-ACO1*, and use the translation start codon of *DC-ACO1*. We also used pBI121 (Clonthech), which uses the cauliflower mosaic virus (CaMV) 35S promoter to drive the GUS gene, as a control.

Particle bombardment and transient gene expression assay

The transient expression of chimeric GUS genes in detached carnation (cvs. Delfy or White Barbara) petals were conducted according to Itzhaki and Woodson (1993) with modification. DNA constructs were delivered into petal cells by particle bombardment using a Biolistic PDS-1000/He particle delivery system (BioRad). Five μg of purified plasmid DNA was precipitated onto 3 mg of 1.6-μm gold particles in 1 M $CaCl_2$ and 15 mM spermidine solution according to the manufacturer's instructions. The DNA-coated gold particles were resuspended in 60 μl of ethanol and 10 μl of the suspension pipetted onto the He macrocarrier for each bombardment. Flower petals were placed on moist filter paper in a petri dish and bombarded at a distance of 2 cm from the stopping screen using 1,350 psi rupture disks. After bombardment, petals were incubated for 16 hr in a glass chamber (w 58 cm x d 27 cm x h 34 cm, 53.2 l) containing 10 $\mu l \cdot l^{-1}$ ethylene or in moist open air. The petals were subsequently subjected to fluorometric GUS assay.

GUS assay

Fluorometric GUS assay of carnation petals was done with 4-methylumbelliferyl-β-D-glucuronide (Sigma) according to Sato et al. (1996). The amount of 4-methylumbelliferone (MU), the product of the GUS reaction, was measured with a fluorescence spectrometer at 365 nm for excitation and at 455 nm for emission.

Results and Discussion

Isolation of an ACC oxidase genomic DNA clone

Using a DNA fraction obtained from carnation petals as a template a genomic DNA fragment encoding ACC oxidase was amplified by PCR with primers adopted from the known sequence of the carnation ACC oxidase cDNA pSR120 (Wang and Woodson, 1991). The PCR products were cloned into pBluescript II at the *Eco*R I/*Hin*d III site. Sequencing of 5 clones showed that they share identical nucleotide sequences of 1476 bp in size, representing one genomic DNA fragment. This DNA fragment contained a nucleotide sequence almost identical to that of pSR120, and corresponded to a region that included −39 bp to +1437 bp of the sequence shown

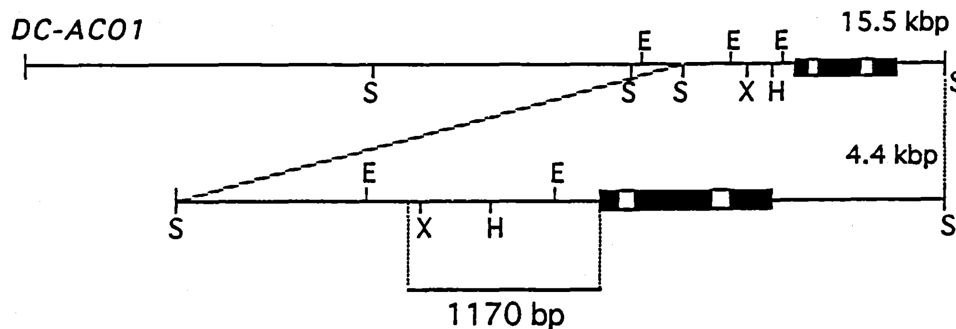

Fig. 1. Genomic DNA clone containing ACC oxidase gene, *DC-ACO1*. Boxed areas represent regions containing ACC oxidase: exons and introns are represented by closed and open boxes, respectively. Restriction sites are shown: S, *Sal* I; X, *Xba* I; E, *Eco*R I; H, *Hin*d III.

```
-1170 TCATATTTAACAATTATTCCGCAGTTATGATAATTAAATTATTAGTTAATGAATTTAGTT
-1110 AATTGAATATGTAAAATAAATTACAATGAATAATAATAAAATATATGAATGAATTACCGT
-1050 AAATAATACGGCTTGAAATAGAATGGAATATCCAACAAGAGTAACATACATCTAGAGATG
 -990 GAAGTTGGGCCGTGGGCTAATGGGCTAGCACAGCCCAGCATGGCACGCAGCCCAAGTTGG
 -930 CACGGCCCACATCAGGCACGGCCCAGCACGGTACGGCACGCAATGGGCCATGGGCCGTGC
 -870 CTGGGCCTTGCTTTTTATGAAAATGGCGTGCCTTGGCACGGCACGGCACGACCCGTCTTA
 -810 GGCACGGCCCAACTTAGGCACGCGTGCCGGCACGCTTACGGCACGTCTTAGCCCTTATTT
 -750 GTAGTGAATTATAATTATTTTTTTGTAAGTTCTCTTTCATTATACTCCATTATAATATAA
 -690 CATATTGAGATTTATGAGTGTTGATATTATACAATATATATCGGGTTCACGTTTTGTGAG
 -630 ACTAATTAACTACAATGGTTGTTGTGAGGTCAGATCGTTTCGTATTTTTAACTTTTTTTA
 -570 ATTTTTAAATGATTAATAATAAAAATTCACTTTGTATTTGCATTATTTTAAGCTTAAAAA
 -510 ATTAAATTCGTTCAAATTTTCAATCGCTCGGGTCGAAAAAAACTCAAAATATACAAATAC
 -450 TGCGGGTGGGCCGCGTGGGCTGGCACGCCAATGACCCAAGGCACGACCCAGCACGGCACG
 -390 TTGGGCCGTGGGCCACGGGCCGTGCCTGGGCCTTCATTTTGAAAACAAGGCACGGGCACG
 -330 GCACGACACGTGGCACGCTTAACCGTGCCCGGGCCATGCCAAAATTTAACTTCGGCACGC
 -270 CATGCCGGCACGTGGGCCGGCCCAGCACGGCCCACTTCCATCTCTACATACATCACATAG
 -210 GCAAAAAAGTTGAATTCAAAAGTGGGGTCTCATAATAATGTCAATTCAAGATTAAAGTTG
 -150 TCCATGTAAAACATGCATGGATTATGTATGCCGTTTGCCTATAAATAGAGACCAGTTTAT
  -90 GCAATGCTAAAATCACAACAATACTTAGCTTAATTGCCAAAACAAATACAAATACAAATA
  -30 CAAATACATTGAATTTGTTAATTAACGAACATGGCAAACATTGTCAACTTCCCTATCATT
                                 +1-M  A  N  I  V  N  F  P  I  I
   31 GACATGGAGAAGCTCAATAATTATAATGGTGTTGAGAGGAGTCTTGTTTTGGACCAAATT
       D  M  E  K  L  N  N  Y  N  G  V  E  R  S  L  V  L  D  Q  I
   91 AAGGATGCTTGTCACAACTGGGGATTCTTCCAGgtaatcatcatcatcatcatcatcatc
       K  D  A  C  H  N  W  G  F  F  Q
  151 atcatcgtcgtaataatagtaacaataccaagtaattctatataatatatgacgaattgt
  211 atgtatgattatatatgatggattattggagcatgcatgcagGTGGTGAACCATAGTTTG
                                                 V  V  N  H  S  L
  271 TCACATGAACTGATGGACAAAGTGGAGAGGATGACAAAAGAGCATTACAAGAAATTCAGG
       S  H  E  L  M  D  K  V  E  R  M  T  K  E  H  Y  K  K  F  R
  331 GAGCAAAAGTTCAAAGACATGGTTCAGACCAAAGGTTTAGTGTCTGCTGAGTCTCAAGTC
       E  Q  K  F  K  D  M  V  Q  T  K  G  L  V  S  A  E  S  Q  V
  391 AATGACATTGATTGGGAGAGCACCTTCTACCTTCGTCATCGTCCCACCTCCAACATCTCC
       N  D  I  D  W  E  S  T  F  Y  L  R  H  R  P  T  S  N  I  S
  451 GAGGTCCCTGATCTCGACGACCAATACAGGAAGTTGATGAAGGAGTTTGCAGCCCAGATT
       E  V  P  D  L  D  D  O  Y  R  K  L  M  K  E  F  A  A  O  I
  511 GAGAGGTTATCCGAGCAACTGTTGGACTTGTTATGTGAGAACCTTGGCCTTGAGAAAGGC
       E  R  L  S  E  O  L  L  D  L  L  C  E  N  L  G  L  E  K  G
  571 TACCTTAAGAATGCCTTCTATGGTGCCAATGGCCCCACTTTTGGTACCAAGGTCAGCAAC
       Y  L  K  N  A  F  Y  G  A  N  G  P  T  F  G  T  K  V  S  N
  631 TACCCGCCTTGCCCCAAACCCGACCTTATCAAAGGACTTAGGGCCCACACCGACGCTGGT
       Y  P  P  C  P  K  P  D  L  I  K  G  L  R  A  H  T  D  A  G
  691 GGCATCATTCTCTTGTTCCAGGACGACAAGGTCAGCGGCCTCCAGCTCCTCAAGGATGGT
       G  I  I  L  L  F  Q  D  D  K  V  S  G  L  Q  L  L  K  D  G
  751 CATTGGGTTGATGTTCCTCCCATGAAACACTCCATTGTTGTTAACTTGGGGGACCAACTT
       H  W  V  D  V  P  P  M  K  H  S  I  V  V  N  L  G  D  Q  L
  811 GAGgtatgacatgaatacagaataggttactccgaaattagaagccgcctccgactaata
       E
  871 tgaatgatatgaatcatacatatatgacgcatttatttatttatttacgtgtttagGTTA
                                                             V
  931 TTACAAATGGCAAGTACAAGAGTGTGATGCACCGCGTGATAGCGCAGACAGATGGTAACA
       I  T  N  G  K  Y  K  S  V  M  H  R  V  I  A  Q  T  D  G  N
  991 GGATGTCGATAGCATCATTCTACAACCCGGGAAGTGATGCCGTGATTTACCCGGCGCCAA
       R  M  S  I  A  S  F  Y  N  P  G  S  D  A  V  I  Y  P  A  P
 1051 CATTGGTGGAAAAAGAAGAGGAGAAATGCAGAGCATACCCAAAATTTGTGTTCGAGGATT
       T  L  V  E  K  E  E  E  K  C  R  A  Y  P  K  F  V  F  E  D
 1111 ACATGAATCTCTACTTAAAGCTCAAGTTCCAAGAGAAGGAGCCCAGGTTTGAAGCAATGA
       Y  M  N  L  Y  L  K  L  K  F  Q  E  K  E  P  R  F  E  A  M
 1171 AGGCCATGGAAACCACGGGTCCCATTCCAACTGCTTGAAATAATGATTTGATTTGATATA
       K  A  M  E  T  T  G  P  I  P  T  A
 1231 ATGCAATGCTTCTCATCAACCAATTTAAGTATTTCTAATATACGCCACTCTCATCTCATC
 1291 TCATATATTCATATTCATATTATTAGTGTTTGTTGAATAAGAGCTTCCTTTTAAGTATGA
 1351 TTGTTAATGTAATGTTCCATGTCCTATGGATTGTATGGTCTACACTAATACGGAGTATTC
 1411 ATTCAAATTACAATAATTTATCCCCTT
```

Fig. 2. Nucleotide sequence and translation product of carnation *DC-ACO1*. Numbering is relative to A of the translation start codon ATG. Exons and flanking regions are represented in upper case letters and introns in lower case letters. Polyadenylate addition sequence is from Wang and Woodson (1991). Accession No. AB042320.

in Fig. 2.

To isolate the 5′-flanking sequence of the genomic DNA, we constructed a library of genomic DNA fragments from cv. Nora in λ DASH II replacement vector. The genomic library was screened by hybridizing filters with the ^{32}P-labeled PCR clone. This screen identified 16 positive recombinant phages that hybridized to the probe. Their DNAs were purified, and subjected to restriction endonuclease digestion. These analyses revealed that the phage clones were classified into two distinct groups that contained ACC oxidase homologous sequences and 5′-flanking region. One group, consisting of 9 clones, was represented by a clone of 15.5 kbp, the largest in size among the 9 clones, and the other group consisted of 7 clones represented by a 14 kbp clone.

For further characterization of the 15.5 kbp genomic sequence, the 4.4 kbp restriction fragment at the right border was subjected to subcloning and nucleotide sequence analysis. This analysis showed that the genomic sequence contained an overall sequence identical to that of the PCR clone (Fig. 1). The nucleotide sequence of the 5′-flanking region was determined up to -1170 bp relative to A (+1) of the translation start codon ATG (Fig. 2). We designated this genomic DNA clone as *DC-ACO1* (Accession No. AB042320).

With respect to the 14 kbp genomic clone, we subcloned a 1069-bp fragment (Accession No. AB042321) that contained a sequence homologous ACC oxidase. This sequence located on the right border of the genomic DNA. The sequence contained 4 separate regions of 21-51 bp, each sharing high similarity (more than 90%) with corresponding regions of *DC-ACO1*, however, it lacked the region corresponding to the latter half of the coding region of *DC-ACO1*. Thus, we can conclude that the 14 kbp genomic DNA did not represent the ACC oxidase gene.

Organization and structure of exons and introns of DC-ACO1

DC-ACO1 consisted of 3 exons interrupted with 2 introns. The 3 exons were 123 bp, 561 bp and 279 bp in size in this order, and the 1st and 2nd introns were 129 bp and 113 bp in size, respectively (Fig. 2). The composite nucleotide sequence of the exons of *DC-ACO1* was identical to the nucleotide sequence of pSR120, except for 2 nucleotides at positions 569 and 570 (GC instead of CG). These nucleotide substitutions cause the substitution of Ala with Gly at position 147 in the deduced amino acid sequence. However, the replacement of these amino acids seems to have no effect on the structure and function of the enzyme. *DC-ACO1* had additional differences of 4 nucleotides in the 3′ untranslated region at positions 1253-1254 (GG was deleted), 1336 (T instead of C) and 1338 (C instead of T). These nucleotide differences probably result from genotypic differences between 'Nora' and 'White Sim' cultivars, which were used in the present and previous studies, respectively.

Previously it was demonstrated that there are 4 exons interrupted with 3 introns in ACC oxidase genomic DNAs, such as genes from tomato (*LE-ACO1*, *LE-ACO2* and *LE-ACO3*: Holdsworth et al., 1987; Barry et al., 1996), petunia (*PH-ACO1*, *PH-ACO2*, *PH-ACO3* and *PH-ACO4*: Tang et al., 1993) and banana (*MAO2*: Huang et al., 1997). However, in the case of melon, Lasserre et al. (1996) revealed the presence of two types of ACC oxidase genomic DNAs, one consisting of 3 exons/2 introns (*CM-ACO2* and *CM-ACO3*) and another consisting of 4 exons/3 introns (*CM-ACO1*). The present finding with *DC-ACO1* revealed that the structure of 3 exons/2 introns similar to that of ACC oxidase genes of melon. Lasserre et al. (1996) considered that *CM-ACO2* and *CM-ACO3* were derived from *CM-ACO1* by loss of the 3rd and the 2nd intron, respectively. *DC-ACO1* has the intron-exon splice junction nucleotide sequences (---AGgt---for the upstream junction and ---gt--- for the downstream junction) in agreement with the consensus sequence found in plants (Brown, 1986). Comparison of the nucleotide and amino acid sequences flanking respective introns of *DC-ACO1* with those of ACC oxidase genes from tomato (*LE-ACO1*: Holdsworth et al., 1987), petunia (*PH-ACO1*: Tang et al. ,1993) and melon (*CM-ACO1*: Lasserre et al., 1996) showed that *DC-ACO1* lacks the intron corresponding to the 2nd intron of the ACC oxidase gene of the 4 exons/3 introns structure (data not shown).

Promoter analysis by transient expression of the promoter-GUS fusions in carnation petals

To determine the promoter activity of the 5′-flanking sequence in the transcriptional regulation of *DC-ACO1*, we constructed a chimeric gene consisting of the coding region of GUS and 1515 bp (from -1170 bp of 5′-flanking sequence to +345 bp of the coding sequence) of the 5′-upstream region of *DC-ACO1* (Fig. 3A). This construct contains the 1st exon, the 1st intron and a partial sequence of the 2nd exon, and uses the translation start codon ATG of *DC-ACO1*.

The expression of the -1170 bp *DC-ACO1* promoter-GUS construct increased depending on the progress of senescence of petals, which correlated with their ethylene production rates. As ethylene production rates from flowers increased from 8.6 $nl \cdot hr^{-1} \cdot (g\ fresh\ weight)^{-1}$ (3 days before the onset of petal in-rolling) to 25.0 $nl \cdot hr^{-1} \cdot (g\ fresh\ weight)^{-1}$ (at the onset of marked increase in ethylene production) and 98.3 $nl \cdot hr^{-1} \cdot (g\ fresh\ weight)^{-1}$ (at the time of maximum ethylene production), the GUS activities increased from 5 ± 1 to 12 ± 2 and 49 ± 8 $pmol\ MU \cdot min^{-1} \cdot petal^{-1}$, showing similar rates in increase. These findings indicate that the 5′-flanking region of -1170 bp of *DC-ACO1* has a promoter activity and its expression is dependent on

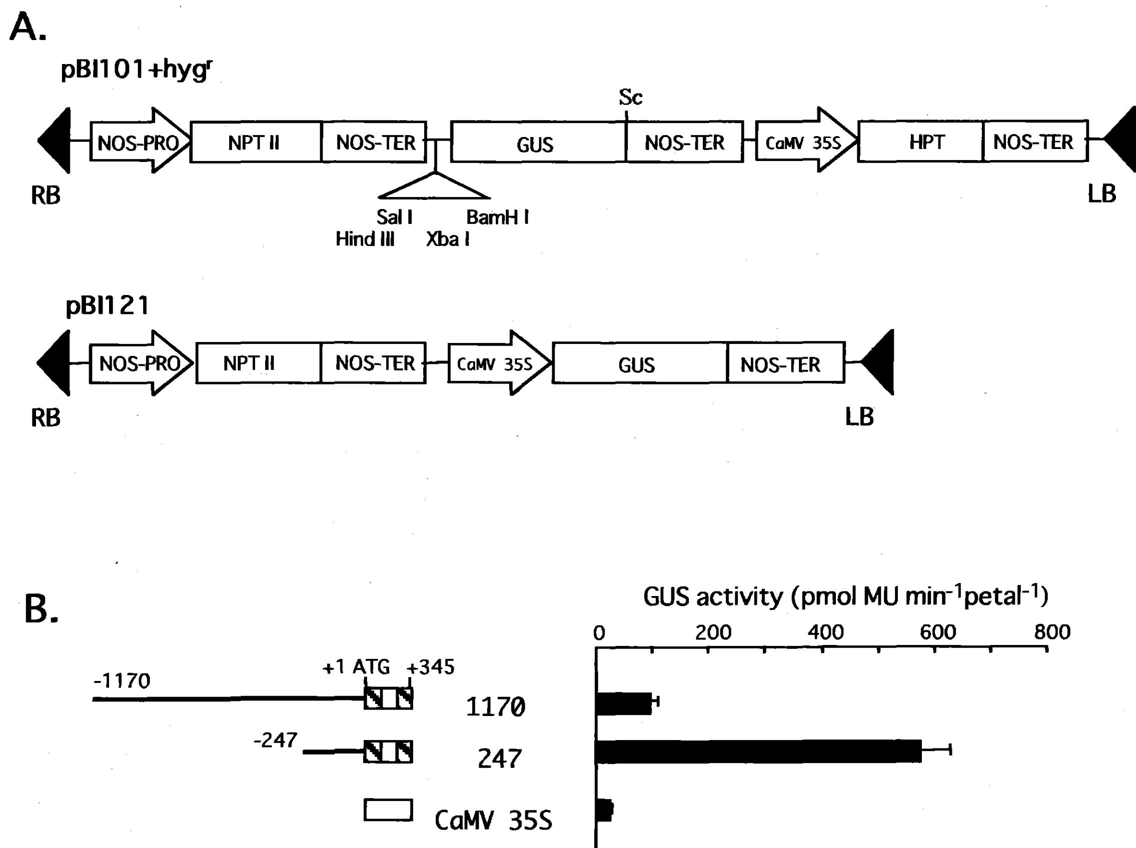

Fig. 3. Comparison of promoter activity between two 5′-flanking regions of −1170 bp or −247 bp of *DC-ACO1*.
A. Structures of *DC-ACO1* promoter-GUS fusion genes. Two 5′-upstream sequences, ranging from −1170 bp and −247 bp of the 5′-flanking sequence to +345 bp at the coding sequence of *DC-ACO1* were inserted to *Sal* I/*Bam*H I site of pBI101-hyg^r, respectively. The CaMV 35S-GUS fusion in pBI121 plasmid (Clontech) is also shown.
B. Expression of *DC-ACO1* promoter-GUS DNAs in carnation petals after particle bombardment. The CaMV 35S-GUS fusion DNA was used as a control. Petals were detached from senescing carnation (cv. White Barbara) flowers at their maximum ethylene production, and the construct DNAs were delivered by particle bombardment. After bombardment, the petals were incubated in air for 16 hr, then subjected to fluorometric GUS assay. Data are the means of 3 petals ± SE.

senescence of petals, represented by the increase in ethylene production. Woodson et al. (1992) showed that the mRNA for ACC oxidase (corresponding to pSR120) accumulated concomitantly with the increase in ethylene production during senescence of carnation petals, an indication of up-regulation of the gene expression. Thus, the present findings are in agreement with the previous results.

Finally we compared the promoter activities between two 5′-flanking sequences with the −1170 bp *DC-ACO1* promoter-GUS construct and the −247 bp *DC-ACO1* promoter-GUS construct in petals detached from senescing flowers [ethylene production rate, 45.6 nl·hr^{-1}·(g fresh weight)$^{-1}$]. The latter construct contained the 592-bp long 5′-upstream sequence ranging from −247 bp of the 5′-flanking region to +345 bp of the coding sequence (Fig. 3A). This region was chosen since it contained a sequence (AATTCAAA) at −198 bp, which is identical to an ethylene responsive element (ERE) present in the promoter region of apple ACC oxidase (Atkinson et al. 1998), carnation glutathione-*S*-transferase (Itzhaki et al., 1994) and tomato E4 (Montgomery et al., 1993). A 6.5-fold increase in the expression of the GUS DNA was observed with the −247-bp *DC-ACO1* promoter-GUS DNA as compared with the −1170-bp *DC-ACO1* promoter-GUS DNA; 569 ± 64 vs. 87 ± 22 pmol MU·min^{-1}·petal^{-1} (Fig. 3B). These results indicate that the 5′-flanking sequence up to −247 bp is sufficient for the promoter activity of *DC-ACO1*. It is speculated that the sequence ranging from −248 bp to −1170 bp in the 5′-flanking region contains an element which suppresses the expression of *DC-ACO1* in carnation petals. In addition, it may be possible that this region contains some other elements necessary for tissue-specific and/or senescence-specific expression. These speculations should be examined in future study. Meanwhile, the present findings show the possibility to increase the promoter activity by modifying the structures of the 5′-flanking region, such as deletions, as shown here, or the addition of enhancer elements (Mitsuhara et al., 1996). As the next step, we are conducting an experiment in which we introduce the *DC*

-*ACO1* promoter-GUS DNA constructs into carnation plants by *Agrobacterium*-mediated transformation to see if there are any tissue- and developmental stage-specific expression in flowers as well as vegetative tissues.

Acknowledgements

We are grateful to Associate Professor K. Toriyama, Tohoku University, for guidance on the transient assay for GUS-fusions, and to Dr. G. Yokoi for the gift of pBI101-hyg^r.

Literature Cited

Abeles, F. B., P. W. Morgan and M. E. Saltveit, Jr. 1992. Ethylene in Plant Biology. 2nd ed. p. 178-181. Academic Press, San Diego, CA, USA.

Aida, R., T. Yoshida, K. Ichimura, R. Goto and M. Shibata. 1998. Extension of flower longevity in transgenic torenia plants incorporating ACC oxidase transgene. Plant Sci. 138: 91-101.

Atkinson, R. G., K. M. Bolitho, M. A. Wright, T. Iturriagagoitia- Bueno, S. J. Reid and G. S. Ross. 1998. Apple ACC-oxidase and polygalacturonase: ripening-specific gene expression and promoter analysis in transgenic tomato. Plant Mol. Biol. 38: 449-460.

Barry, C. S., B. Blume, M. Bouzayen, W. Cooper, A. J. Hamilton and D. Grierson. 1996. Differential expression of the 1-aminocyclopropane-1-carboxylate oxidase gene family of tomato. Plant J. 9: 525-535.

Bovy, A. G., G. C. Angenent, H. J. M. Dons and A-C. van Altvorst. 1999. Heterologous expression of the *Arabidopsis etr1-1* allele inhibits the senescence of carnation flowers. Mol. Breed. 5 : 301-308.

Brown, J. W. S. 1986. A catalogue of splice junctions and putative branch point sequences from plant introns. Nuc. Acids Res. 14: 9549-9559.

Clark, D. G., E. K. Gubrium, J. E. Barrett, T. A. Nell and H. J. Klee. 1999. Root formation in ethylene-insensitive plants. Plant Physiol. 121: 53-59.

Holdsworth, M. J., C. R. Bird, J. Ray and D. Grierson. 1987. Structure and expression of an ethylene-related mRNA from tomato. Nuc. Acids Res. 15: 731-739.

Huang, P-L., Y-Y. Do, F-C. Huang, T-S. Thay and T-W. Chang. 1997. Characterization and expression analysis of a banana gene encoding 1-aminocyclopropane-1-carboxylate oxidase. Biochem. Mol. Biol. Int. 41: 941-950.

Itzhaki, H. and W. R. Woodson. 1993. Characterization of an ethylene-responsive glutathione *S*-transferase gene cluster in carnation. Plant Mol. Biol. 22: 43-58.

Itzhaki, H., J. M. Maxon and W. R. Woodson. 1994. An ethylene-responsive enhancer element is involved in the senescence-related expression of the carnation glutathione-*S*- transferase (GST1) gene. Proc. Natl. Acad. Sci. USA 91: 8925-8929.

Lasserre, E., T. Bouquin, J. A. Hernandez, J. Bull, J-C. Pech and C. Balague. 1996. Structure and expression of three genes encoding ACC oxidase homologs from melon (*Cucumis melo* L.). Mol. Gen. Genet. 251: 81-90.

Manning, K. 1985. The ethylene forming enzyme system in carnation flowers. p. 83-92. In: Roberts, J. A. and G. A. Tucker (eds.). Ethylene and plant development. Butterworths, Boston, UK.

Michael, M. Z., K. W. Savin, S. C. Baudinette, M. W. Graham, S. F. Chandler, C-Y. Lu, C. Caesar, I. Gautrais, R. Young, G. D. Nugent, K. R. Stevenson, E. L-J. O'Connor, C. S. Cobbett and E. C. Cornish. 1993. Cloning of ethylene biosynthetic genes involved in petal senescence of carnation and petunia, and their antisense expression in transgenic plants. p. 298-303. In: J-C. Pech, A. Latche and C. Balague (eds.). Cellular and molecular aspects of the plant hormone ethylene. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Mitsuhara, I., M. Ugaki, H. Hirochika, M. Ohshima, T. Murakami, Y. Gotoh, Y. Katayose, S. Nakamura, R. Honkura, S. Nishiyama, K. Ueno, A. Mochizuki, H. Tanimoto, H. Tsugawa, Y. Otsuki and Y. Ohashi. 1996. Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. Plant Cell Physiol. 37: 49-59.

Montgomery, J., S. Goldman, J. Deikman, L. Margossian and R. L. Fischer. 1993. Identification of an ethylene-responsive region in the promoter of a fruit ripening gene. Proc. Natl. Acad. Sci. USA 90: 5939-5943.

Murray, M. G. and W. F. Thompson. 1980. Rapid isolation of high molecular weight plant DNA. Nuc. Acids Res. 8: 4321-4325.

Peiser, G. 1986. Levels of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity, ACC and ACC-conjugate in cut carnation flowers during senescence. Acta Hort. 181: 99-104.

Reid, M. S. and M-J. Wu. 1992. Ethylene and flower senescence. Plant Growth Regul. 11 : 37-43.

Sato, F., S. Kitajima, T. Koyama and Y. Yamada. 1996. Ethylene- induced gene expression of osmotin-like protein, a neutral isoform of tobacco PR-5, is mediated by the AGCCGCC *cis*-sequence. Plant Cell Physiol. 37: 249-255.

Savin, K. W., S. C. Baudinette, M. W. Graham, M. Z. Michael, G. D. Nugent, C-Y. Lu, S. F. Chandler and E. D. Cornish. 1995. Antisense ACC oxidase RNA delays carnation petal senescence. HortScience 30: 970-972.

Tang, X., H. Wang, A. S. Brandt and W. R. Woodson. 1993. Organization and structure of the 1-aminocyclopropane-1-carboxylate oxidase gene family from *Petunia hybrida*. Plant Mol. Biol. 23: 1151-1164.

Wang, H. and W. R. Woodson. 1989. Reversible inhibition of ethylene action and inhibition of petal senescence in carnation flowers by norbornadiene. Plant Physiol. 89: 434-438.

Wang, H. and W. R. Woodson. 1991. A flower senescence-related mRNA from carnation shares sequence simi-

larity with fruit ripening-related mRNAs involved in ethylene biosynthesis. Plant Physiol. 96 : 1000-1001.
Wilkinson, J. Q., M. B. Lanahan, D. G. Clark, A. B. Bleecker, C. Chang, E. M. Meyerowitz and H. J. Klee. 1997. A dominat mutant receptor from *Arabidopsis* confers ethylene insensitivity in heterologous plants. Nature Biotechnol. 15: 444-447.
Woodson, W. R., K. Y. Park, A. Drory, P. B. Larsen and H. Wang. 1992. Expression of ethylene biosynthetic pathway transcripts in senescing carnation flowers. Plant Physiol. 99: 526-532.

カーネーションの花の老化時に発現する1-アミノシクロプロパン-1-カルボン酸酸化酵素のゲノミックDNAとプロモーター活性

日比忠晴[1]・高野 誠[2]・吉岡俊人[1]・羽柴輝良[1]・佐藤 茂[1]

[1] 東北大学大学院農学研究科 981-8555 仙台市青葉区堤通雨宮町 1-1
[2] 農業生物資源研究所 305-8602 茨城県つくば市観音台 2-1-2

摘 要

カーネーション'ノラ'から，PCRとライブラリースクリーニングの併用により，花の老化時に発現が増加するACC酸化酵素のcDNA, pSR-120 (Wang and Woodson, 1991)に相当するゲノミックDNA, *DC-ACO1*をクローニングした．*DC-ACO1*は，3エキソン/2イントロンの構造を有していた．5′上流域とGUS遺伝子との融合遺伝子を作成し，パーテクルガンを用いてカーネーション花弁に導入しGUS遺伝子の発現を検討した結果，-1170 bpの5′上流域が花弁の老化(エチレン生成)に依存して発現が増加するプロモーター活性を持つことが示された．上流域を-247 bpに切りつめると，プロモーター活性が6.5倍に増加した．以上の結果から，*DC-ACO1*の5′上流域が，花持ち性向上などを目指した形質転換花きの作出において，花きの老化時に発現が増加するプロモーターとして使用できる可能性が明らかになった．