JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. 2003, 51, 2569−2575 2569

Novel Broccoli 1-Aminocyclopropane-1-carboxylate Oxidase Gene (*Bo-ACO3*) Associated with the Late Stage of Postharvest Floret Senescence

CHIH-YUAN YANG,[†] FANG HUA CHU,[‡] YUH TAI WANG,[†] YU-TING CHEN,[‡] SHANG FA YANG,[‡] AND JEI-FU SHAW*,[‡]

Life Science Center, Hsing Wu College, No. 11-2 Fen-Liao Road, Lin-Kou, Taipei, Taiwan 11244, and Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan 11529

A novel 1-aminocyclopropane-1-carboxylate (ACC) oxidase gene (*Bo-ACO3*) was first isolated from senescing broccoli florets by subtractive hybridization. The cDNA clone comprised a 963 bp open reading frame encoding a protein of 321 amino acids. The predicted molecular mass and p*I* were 36 kDa and 5.42, respectively. *Bo-ACO3* shares 68% identity in the coding region with *Bo-ACO1* (*ACC Ox1*) and *Bo-ACO2* (*ACC Ox2*) and is quite divergent from the 3′ untranslated regions. *Bo-ACO3* transcript was accumulated to high levels only at the late stage of senescence after harvest. Southern blot hybridization using full-length cDNA as a probe suggested that the *Bo-ACO3* gene is a single-copy gene in the broccoli genome. The deduced 321 amino acid sequence of Bo-ACO3 shares 70% identity with either Bo-ACO1 or Bo-ACO2. The *BO-ACO3* gene was expressed in *Escherichia coli* as a 38 kDa active ACO enzyme. It was concluded that *Bo-ACO3* is a senescence-associated gene involved in the late-phase senescence of postharvest broccoli.

KEYWORDS: 1-Aminocyclopropane-1-carboxylate oxidase; senescence; postharvest; broccoli

INTRODUCTION

In higher plants, 1-aminocyclopropane-1-carboxylate (ACC) oxidase (ACO) catalyzes the final step of ethylene formation and is established as the critical metabolite for fruit ripening, seed germination, leaf senescence, abscission, and flower senescence (*1*−*3*). Multigene families that are differentially expressed encode ACO genes, including petunia (*4*), mung bean (*5*), tobacco (*6*), rice (*7*), tomato (*8*), melon (*9*), and broccoli (*10*).

The ACO gene family is highly regulated and differentially expressed with different functions in different tissues and stages having distinct development and physical conditions (*6*−*14*). Its transcripts were shown to increase greatly following the pollination of orchid flowers (*15*), mechanical wounding (*5*, *16*−*18*), ripening of climacteric fruits (*17*, *18*), and vegetative senescence of tomato and broccoli (*10*, *19*).

The numbers of ACO gene family members and regulation patterns vary greatly across different plant species. In tomato (*8*, *20*), both *Le-ACO1* and *Le-ACO3* transcripts accumulated during the senescence of leaves, fruit, and flowers, but *Le-ACO2* expression was anther cone specific. *Le-ACO4* was identified as a ripening-associated gene. Only *Le-ACO1* was inducible in wounded leaves. Petunia *Ph-ACO1* expressed specifically in senescing corollas and *Ph-ACO3* and *Ph-ACO4* in developing pistil tissue, and all of them could be enhanced by ethylene treatment (*14*). In melon, *CM-ACO1* was induced in ripe fruit and in response to wounding and ethylene treatment in leaves (*9*). However, neither *CM-ACO2* (etiolated hypocotyls specific) nor *CM-ACO3* (mainly expressed in flower) was affected by wounding or ethylene treatment.

Because ethylene is usually induced by stress in plants, ACO gene family numbers and regulation patterns are plant dependent. Thus, ACO isoforms might be necessary in plants to ensure that ACO is always available for ethylene production under various conditions. Moreover, it is interesting to study the regulation mechanism of ACO genes in response to their growth environments.

Broccoli (*Brassica oleracea*), a floral vegetable with rapid differentiation and growth before harvest, has an individual floret unpollinated at harvest and senesces rapidly afterward. The first visual sign of broccoli senescence is chlorophyll loss, and its yellowing was mediated by ethylene action (*10*, *21*). Investigation of the ACO genes will give us insight into shelf life improvement of broccoli. Two ACO cDNA clones (*Bo-ACO1* and *Bo-ACO2*) were isolated from the senescing broccoli florets by Pogson et al. (*10*). In this study, we further cloned and characterized a new broccoli ACO gene, *Bo-ACO3*, in unpollinated florets after harvest and propose that the novel *Bo-ACO3* gene might be involved in the late-stage senescence of broccoli

* Author to whom correspondence should be addressed (telephone +886-2-27899590, ext. 108; fax +886-2-27827954; e-mail boplshaw@gate.sinica.edu.tw).
[†] Hsing Wu College.
[‡] Academia Sinica.

10.1021/jf034007m CCC: $25.00 © 2003 American Chemical Society
Published on Web 03/26/2003

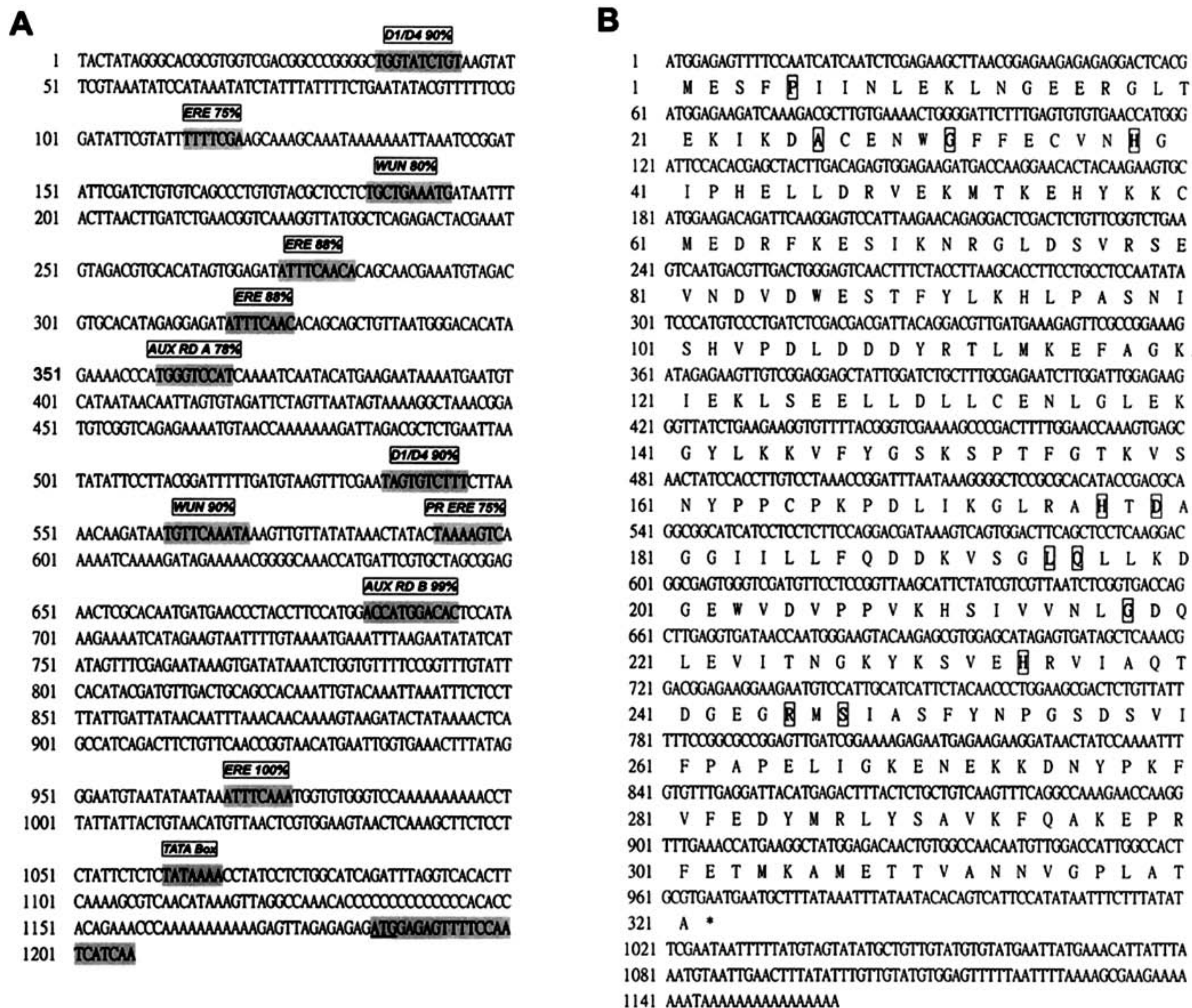

Figure 1. Upstream untranslated promoter region and nucleotide and deduced amino acid sequences of the *Bo-ACO3* cDNA. (A) The 5′ upstream untranslation region of promoter sequence contained 1207 bp. Several motifs with significant homology to known *cis*-acting elements are boxed and labeled as follows: *WUN* (wound responsive elements), *D1*/*D4* (auxin responsive elements; *35*), *AUX RD A* and *AUX RD B* (auxin responsive elements; *34*), *PR ERE* and ERE (ethylene responsive elements). (B) Nucleotide and deduced amino acid sequence represented *Bo-ACO3* derived from broccoli florets cDNA clone of ACO. The *BO-ACO3* cDNA clone comprised a complete open reading frame coding for 321 amino acid residues. Twelve amino acid residues (P5, A27, G32, H39, H177, D179, L195, Q196, G218, H234, R244, and S246), which are conserved among all enzymes that require ferrous ion and ascorbate for activity, are indicated with a full box. The asterisk denotes the stop signal.

florets for ethylene production to trigger the irreversible phase of senescence.

MATERIALS AND METHODS

Plant Materials and Chemicals. Green broccoli florets (*Brassica oleracea* L. cv. Green King) harvested from the field were kept at 20 °C in the dark for 0−4 days and then immediately frozen in liquid nitrogen and stored at −70 °C until use.

mRNA Preparation. About 10 g of frozen broccoli floret tissues fresh or stored for 4 days at 20 °C in the dark was ground to a powder using a ceramic mortar with liquid nitrogen. The basic procedures were performed according to the method of Ecker and Vavis (*22*). PolyA$^+$ mRNAs were isolated using an RNA isolation system IV kit (Promega). cDNAs were synthesized and used for cloning the differentially expressed cDNA using a PCR-based subtractive hybridization kit (Clontech).

PCR-Based Subtractive Hybridization and CapFinder PCR. Broccoli cDNAs derived from fresh and dark-treated florets (4 days at 20 °C) were used in a subtractive hybridization (Clontech, PCR-Select cDNA subtraction kit) according to the procedure developed by Wang and Brown (*23*). The procedure consisted of a series of long and short hybridizations carried out between driver and tester cDNAs, followed by PCR amplification of the enriched cDNA fragments. cDNAs from the dark-treated sample served as the driver, and those from the fresh sample served as the tester. The subtractive hybridization was intended to enrich cDNAs that are up-regulated in dark-treated broccoli florets. The CapFinder PCR method (Clontech) was used to isolate the 5′ and 3′ ends of important cDNA inserts, according to the protocols provided by the manufacturer.

Subcloning and Sequence Analysis of *Bo-ACO3* cDNA. ACO3-1 primer (5′ATGGAGAGTTTTCCAATCATCAATCT3′) and ACO3-2 primer (5′TCACGCAGTGGCCAATGGTCCAACAT 3′) were synthesized according to the sequence of a broccoli ACO derived from the subtractive hybridization and with the CapFinder PCR. One 0.96 kb DNA was amplified by using the PCR technique. This fragment was subcloned into pGEM-T easy vector (Promega) using *Escherichia coli* DH5α as host. The full-length nucleotide sequence was determined in autosequencing (ABI PRIZM DigDye Terminator Cycle Sequencing

Figure 2. Optimal alignment of ACO from broccoli (*Bo-ACO1*, *Bo-ACO2*, and *Bo-ACO3*) (*B. oleraceae*). Numbers refer to amino acid residues of broccoli. A dash denotes deletion. Shaded region refers to identities. Amino acid residues that are conserved for enzymes requiring ferrous ion and ascorbate are indicated with stars. Box 2 is a putative leucine zipper (potential dimerization), and box 3 is a predicted ferrous ion binding domain.

Ready Reaction kits) with an ABI PRIZM 377 DNA sequencer. The *Bo-ACO3* promoter was isolated according to the Universal Genome Walker kit (Clontech Laboratories, Inc., Palo Alto, CA). Adaptor primer1 and ACOP1 (5′-CTCTCTCTTCTTTGAAAGTATTTGGT-3′) were used for the first-time PCR amplification; adaptor primer 2 and ACOP2 (5′-CTCTAGACCCTCTCTATGTAAACTTA-3′) were used for the second. A 1207 bp PCR product was amplified.

Recombinant DNA Preparation and Transformation. Twenty nanograms of *Bo-ACO3* cDNA was used as a template. Ten micromolar 5′-primer (5′ATTCCATATGGAGAGTTTTTCAATCATCAA3′) containing an *Nde*I site and 3′-primer (5′CCGGAATTCCGCAGTGGCCAATGGTCCAA3′) containing an *Eco*RI site were added. A 0.96 kb DNA fragment amplified by the PCR technique was ligated with pGEM-T easy (Promega) and then transformed into *E. coli* DH5α host. Plasmid DNA was digested with *Nde*I and *Eco*RI and then run as 1% agarose. A 0.96 kb insert DNA containing *Nde*I and *Eco*RI sites was recovered and ligated to pET 23a (+) (pretreated with *Nde*I and *Eco*RI) from Novagen. The recombinant gene was transformed into *E. coli* AD494.

Culture and Enzyme Extraction. The transformed *E. coli* cells were grown at 37 °C in 4 mL of Luria Bertani (LB) medium containing 50 μg mL^{-1} ampicillin until A_{600} reached 0.6. Isopropyl β-D-thiogalactopyranoside (IPTG) was then added to a concentration of 1 mM. The culture was incubated at 25 °C for 5 h at 150 rpm. The bacterial cells were harvested by centrifugation at 1000*g* for 10 min, and the cell pellet was frozen at −20 °C until use. The bacterial cells were suspended in 0.1 mL of 100 mM MOPS buffer (pH 7.5) containing 2 mM DTT and 10 μM 1.10-phenanthroline and then sonicated in an ice bath for eight bursts of 30 s at maximum output. The homogenate was centrifuged at 12000*g* for 5 min. The supernatant fraction contained active ACO.

Enzyme Activity Assay of ACO. ACO activity was assayed according to a modified method (*24*). The reaction mixture was prepared as follows: To a 250 μL sample was added 400 μL of reaction buffer (250 mM MOPS pH 6.5, 5 mM DTT, 25% glycerol, 0.5 mg mL^{-1} catalase, and 0.05 μg mL^{-1} BSA), 200 μL of 0.5 M $NaHCO_3$, 50 μL of 0.2 mM $FeSO_4$, and 50 μL of 20 mM ACC. The reaction was initiated by the addition of 50 μL of ascorbate. The test tube was sealed and incubated for 10 min at 30 °C. Gas sample (1 mL) was withdrawn with a syringe from the headspace of the tubes, and ethylene production was determined by gas chromatography (Hitachi G-3000).

Western Blot Immunoanalysis. The bacterial cells were suspended in 10 μL of MOPS buffer. Then 2.5 μL of 4× SDS-PAGE sample buffer, containing 240 mM Tris buffer (pH 6.8), 20% glycerol, 1.6% SDS, 12 mM 2-mercaptoethanol, and 0.08% bromophenol blue, was added and heated for 10 min before centrifugation at 12000*g* for 10 min. After centrifugation, the total supernatant was electrophoresed on 12% SDS-PAGE (*25*) and analyzed by Western blot immunoanalysis (*1*). The primary antibody employed was rabbit antisera prepared against the apple ACO (provided by Dr. Shang-Fa Yang), and the secondary antibody was goat anti-rabbit IgG horseradish peroxidase (IgG-HRP) (Durham, NC).

Northern Blot Hybridization. For Northern hybridization, 30 μg of total RNA isolated from various postharvest florets was applied to a formaldehyde denaturing gel and then transferred to an Immobilon Ny^{+} nylon membrane (Millipore) after electrophoresis according to the method of *Molecular Cloning* (*26*). The *Bo-ACO3* 3′ end partial cDNA

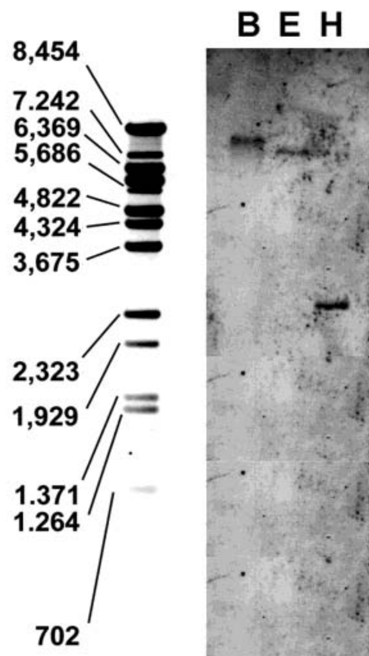

Figure 3. Genomic DNA analysis of *Bo-ACO3* in broccoli. Genomic DNA (0.03 g) was digested with *Bam*HI (B), *Eco*RI (E), or *Hin*dIII(H) and hybridized with *Bo-ACO3* DIG-labeled probe adn washed at high stringency. Marker represents λDNA digested with *Bst*II (Promega) and is indicated on the left.

was labeled with digoxigenin-11-dUP nucleotide as probe for Northern blots with CSPD substrate (Roche).

Southern Blot Hybridization. Young leaves of broccoli were harvested and ground in liquid N_2. The powder was transferred to a centrifuge tube and mixed gently and thoroughly with *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB) methods (*27*). Genome DNA (30 μg) was digested with restriction enzymes (*Eco*RI, *Hin*dIII, and *Bam*HI), and separated on 0.8% agarose gels. The method for DNA transfer onto a NytranN nylon membrane (Schleicher and Schuell) basically followed the protocol of *Molecular Cloning* (*26*). The *Bo-ACO3* full-length cDNA was labeled with digoxigenin-11-dUP nucleotide as probe for Southern blots with CSPD substrate (Roche).

RESULTS

Isolation and Structural Analysis of the *Bo-ACO3* Gene. Broccoli is a very important economic dietary vegetable crop; its floral tissues differentiate rapidly before harvest and then senesce rapidly after harvest. Harvest disrupts energy, nutrient, and hormone supplies and leadd to rapid senescence in vegetables (*28*). Associated with this postharvest deterioration is an increase in the ethylene produced by florets. Understanding the ethylene synthesis of associated genes, such as ACC synthase and ACC oxidase, is therefore necessary. To fully understand the senescence-associated genes of broccoli florets, PCR-based subtractive hybridization was used. After subtractive hybridization, CapFinder PCR (Clontech) methods, and DNA sequence comparison, a full-length *Bo-ACO3* cDNA of broccoli senescent florets was cloned and sequenced (GenBank accession no. AF252853). The *Bo-ACO3* cDNA clone comprised a 963 bp coding region of 321 amino acids, a 159 bp 3′-untranslated region (**Figure 1B**), and a 1207 bp 5′-upstream untranslated promoter region (**Figure 1A**). The deduced protein exhibited high amino acid sequence identities with plant ACC oxdiase of broccoli (*B. oleracea*, GenBank accession no. X81628, 70%, and GenBank accession no. X81629, 70%), papaya (*Carica papaya*, GenBank accession no. L76283, 76%), petunia (*Petunia hybrida*, GenBank accession no. L21976, 77%), sunflower (*Helianthus annuus*, GenBank accession no. L29405, 79%), kiwifruit (*Actinidia deliciosa*, GenBank accession no. M97961, 77%), apple (*Malus syluestris*, GenBank accession no. M81794, 76%), mung bean (*Vigna radiata*, GenBank accession no. U06046, 74%), red sim (*Dianthus caryophyllus*, GenBank accession no. L35152, 76%), bridger (*Brassica napus*, GenBank accession no. L27664, 70%), and orchid (*Doritaenopsis* sp., GenBank accession no. L07912, 72%, and GenBank accession no. L37103, 72%).

The predicted molecular mass and p*I* value of polypeptide encoded by *Bo-ACO3* were 36 kDa and 5.42, respectively. Twelve amino acid residues (P5, A27, G32, H39, H177, D179, L195, Q196, G218, H234, R244, and S246) were conserved as other ferrous ion and ascorbate required enzymes (*4*, *24*) (**Figures 1B** and **2**). H177 and A179, as well as H234, were demonstrated as essential ligands for Fe^{2+} binding in apple ACO (*29*). Structural comparison of Bo-ACO3 with isopenicillin N

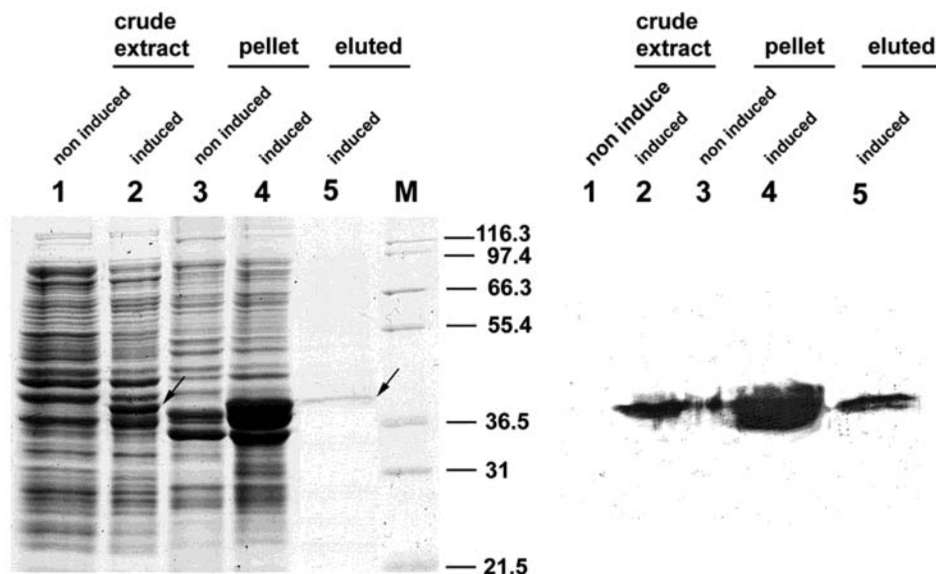

Figure 4. Coomassie blue stained SDS-PAGE (A) and Western blot immunoanalysis (B) of the crude extract, pellet, and His-tag eluted recombinant of broccoli ACC oxidase (Bo-ACO3); (M) protein molecular weight marker. Arrows indicate 38 kDa Bo-ACO3.

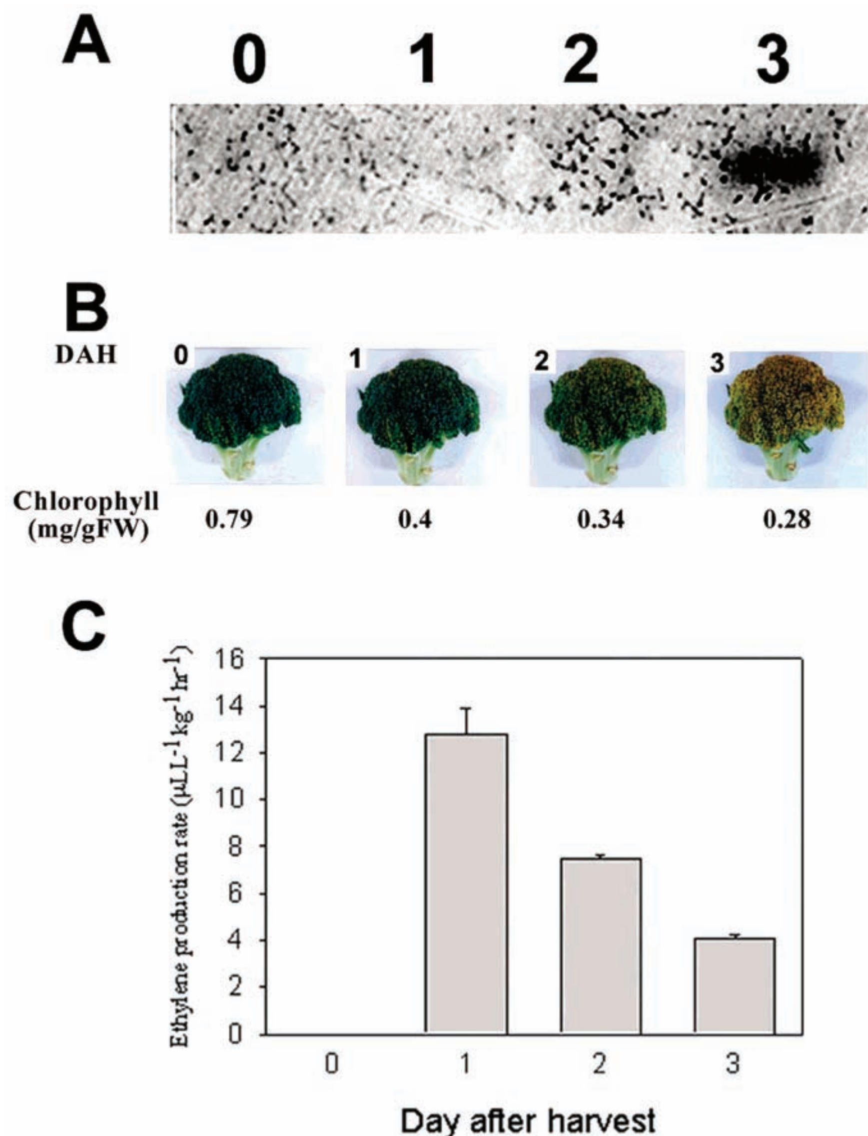

Figure 5. Northern blot analysis of the *Bo-ACO3* gene (A), floret morphology changes (chlorophyll content, mg g^{-1} of FW) (B), and ethylene production in florets (pmol g^{-1} of FW) (C). (A) Total RNAs isolated in different stages after harvest of broccoli florets were resolved on 1% agarose–formaldehyde gel. Gel was blotted onto membrane, and the blots were hybridized to a DIG-labeled probe for *Bo-ACO3*. 0, 1, 2, and 3 indicate 0, 1, 2, and 3 days after harvest (DAH), respectively.

synthase (IPNS) (*30*, *31*) as a template indicated that all three broccoli ACO isoforms showed sequence conservation for the 14 β-strands and 9 α-helices (**Figure 2**). However, Bo-ACO3 protein contained amino acid variation within α-helix 6, where the glycine at position 118 was replaced by alanine and the lysine at position 119 was replaced by glycine. Furthermore, the glycine and alanine at positions 118 and 119, in the direct vicinity of α-helix 6, might affect the α-helix structure and change its protein function. On the other hand, the other α-helix and β-strand of IPNS was conserved in the three ACC oxdiase proteins (**Figure 2**). Otherwise, Bo-ACO3 contained three amino acid deletions between positions 3 and 5 of Bo-ACO1 and four amino acid insertions between positions 274 and 277 of Bo-ACO1. Biochemical variations obviously exist among ACO isozymes, which might perform different functions in broccoli development.

Motif Analysis of the *Bo-ACO3* Promoter Region Sequence. A 1207 bp *Bo-ACO3* promoter of broccoli genomic DNA was isolated by using the genome walker kit (Clontech). Several *Bo-ACO3* promoter regions showed high homology to previously described *cis-acting* elements (**Figure 1A**), including wound-inducible elements (WUN) homologous to WUN1 (*32*), ethylene-responsive elements (ERE) homologous to the ERE sequence and PR ERE (*33*), and auxin response elements (AUX) homologus to AUX RDA, AUX RD B, and D1/D4 (*34*, *35*). This revealed that *Bo-ACO3* might be under the regulation of ethylene, wounding, and auxin.

Genomic DNA Blot Analysis. *Bo-ACO1* and *Bo-ACO2* are single copy under high-stringency Southern blot analysis (*10*). Southern blot analysis of *Bo-ACO3* on broccoli genomic DNA showed that *Bo-ACO3* hybridized to a single band of ~6.0 kb in *Bam*HI-digested genomic DNA fragments, 5.6 kb in *Eco*RI-digested genomic DNA fragments, and 2.3 kb in *Hin*dIII-digested genomic DNA fragments under high-stringency conditions (**Figure 3**), suggesting that the *Bo-ACO3* transcript originates from a single-copy gene.

Western Blot Immunoanalysis and Enzyme Activity Assay of ACO. The coding sequence of *Bo-ACO3* was cloned into the pET-23a(+) expression vector, transformed into *E. coli* D494, and cultured at 37 °C in LB medium containing 50 μg mL^{-1} ampicillin until A_{600} reached 0.9. In a typical enzyme activity assay, a 50 μL enzyme crude extract from 4 mL of

culture produced high enzyme activity [115 ± 22 nmol h^{-1} (mg of $protein^{-1}$)] under the assay conditions. The recombinant Bo-ACO3 protein was detected with apple anti-ACO polyclonal antibodies as a single band at 38 kDa (**Figure 4B**) and as a distinctive protein by SDS-PAGE using Coomassie blue stain (**Figure 4A**). From our observation, it is obvious that *Bo-ACO3* is a functional gene and contributes to ethylene production after harvest in senescent broccoli florets.

***Bo-ACO3* Is a Senescence-Associated Gene in Florets.** Florets lost chlorophyll following harvest. The majority was lost within 24 h after harvest (**Figure 5B**), and loss continued sequentially from 2 to 5 days after harvest (DAH) (data not shown), but morphology change appeared at 3 DAH. The ethylene production rate of postharvest broccoli florets exhibited high-level peaks on day 1 and then decreased on days 2 and 3 after harvest (**Figure 5C**). Northern analysis of the *Bo-ACO3* gene was performed in florets of broccoli during postharvest storage at 20 °C. A partial fragment of the *Bo-ACO3* 3′ untranslated region labeled with digoxigenin-11-dUTP was used as specific probe for hybridization of total RNA samples. A high density of single band was detected only in a floret, which was stored in darkness for 3 days after harvest (**Figure 5A**). Thus, *Bo-ACO3* was not likely associated with the triggering of chlorophyll loss in broccoli, because chlorophyll began decreasing at 1 DAH and then sequentially decreased from 2 DAH to the late stages of postharvest senescence (**Figure 5B**). These results suggest that *Bo-ACO3* is a late-stage senescence-associated gene and that its transcript, accumulated only at a late stage of postharvest, contributes to ethylene production and mediates the late-stage senescence of postharvest florets.

DISCUSSION

In this study, we successfully cloned and characterized a new broccoli *Bo-ACO3* gene. Expression of the *Bo-ACO3* gene in *E. coli* as an active recombinant protein was confirmed by both ACC oxidase activity assay and Western blot immunoanalysis (**Figure 4**). Our results indicated that *Bo-ACO3* was a functional gene and played a role in ethylene production of broccoli florets. Expression and regulation of *Bo-ACO3* were quite different from those of ACO genes reported in other plants during development and senescence and in response to ethylene (*8*, *9*, *14*, *36*−*43*). *Bo-ACO3* was detected only in the florets at a late stage of broccoli senescence (**Figure 5A**), whereas chlorophyll was lost from florets at an earlier senescence stage, particularly from the petal, and the color turned yellow at 3 DAH (**Figure 5B**). In a previous study, *Bo-ACO1* transcripts increased markedly at the floret and late senescence stage of leaf frozen immediately after harvest (168 h). Appearance of these transcripts also initiated chlorophyll loss in the sepal. In contrast, *Bo-ACO2* transcript was detected in florets at 2 h after harvest (*10*). Therefore, *Bo-ACO1* might be responsible for the senescence of vegetative tissues; *Bo-ACO2* preceded the increase in ethylene production at postharvest and played a major role in sepal yellowing. *Bo-ACO3* was detected only at the later senescence stage of broccoli florets. Thus, it was a late senescence stage associated gene and might be responsible for triggering of the irreversible phase in postharvest floret senescence. Thus, we propose that the marked accumulation of *Bo-ACO3* transcript at the late stage of postharvest senescence contributes to the increase of ethylene production in florets and then triggers the irreversible phase of postharvest senescence, including antibiotic accumulation, release of free radical, elimination of remaining metabolites, and irreversible loss of cell integrity and viability.

In broccoli, changes in respiration activity, carbon metabolism, and wounding-induced ethylene occur during the first 3 h after harvest (*10*). The transcript level of *Bo-ACO1* increased markedly after harvest; *Bo-ACO2* transcript began within 2 h, and *Bo-ACO3* was detected at 3 DAH. Broccoli sepal yellowing commences between 24 and 48 h after harvest and is essentially complete by 96 h (*44*). Hence, ACO isoforms might contribute to ethylene production at different senescence stages and cooperate in ethylene synthesis to regulate aspects of postharvest senescence in broccoli florets.

It becomes increasingly clear that ethylene production in various tissues at different stages of development has different physiological functions. However, the biological roles of the different ACO isoforms, which regulate ethylene production, are not yet known. The differential expression patterns of *Bo-ACO3* in this work and *Bo-ACO1* as well as *Bo-ACO2* in a previous paper (*10*) reveal that ACO gene families are not simply a gene redundancy but could be cases of overlapping gene functions. This is also supported by Mulligan et al. (*45*), who concluded that signal perception by phytochrome, the light receptor, is transmitted to the nucleus by complex interactions, multiple signal input from various types, and members of receptor families inducing the expression of regulated genes and then initiating the changes in development and morphology.

Consequently, the differential expression of ACO genes in broccoli and other plants suggests that developmental and tissue-specific ACO isoforms cooperate through different regulatory pathways and allow for the optimal production of ethylene for physiological changes in plants. Our future work will focus on ACO isoform mutant screening and double-stranded RNA mediated gene silencing in order to elucidate the biological roles of different ACO isoforms.

ACKNOWLEDGMENT

We thank May-chih Fen, Institute of Botany, Academia Sinica, for DNA sequencing.

LITERATURE CITED

(1) Brady, C. J.; Speirs, J. Immunoblotting. In *Protein Methods*; Bollag, D. M., Edelstein, S. J., Rozycki, M. D., Eds.; Wiley-Liss: New York, 1991; pp 181−208.
(2) Yang, S. F.; Dong, J. G. Recent progress in research of ethylene biosynthesis. *Bot. Bull. Acad. Sin.* **1993**, *34*, 89−101.
(3) Yang, S. F.; Hoffman, N. E. Ethylene biosynthesis and its regulation in higher plants. *Annu. Rev. Plant Physiol.* **1984**, *35*, 155−189.
(4) Tang, X.; Wang, H.; Brandt, A. S.; Woodson, W. R. Organization and structure of the 1-aminocyclopropane-1-carboxylic acid oxidase gene family from petunia hybrida. *Plant Mol. Biol.* **1993**, *23*, 1151−1164.
(5) Kim, W. T.; Yang, S. F. Structure and expression of 1-amino cyclopropane-1-carboxylate oxidase homologs isolated from excised mung bean hypocotyls. *Planta* **1994**, *194*, 223−229.
(6) Kim, Y. S.; Choi, D.; Lee, M. M.; Lee, S. H.; Kim, W. T. Biotic and abiotic stress-related expression of 1-aminocyclopropane-1-carboxylate oxidase gene family in *Nicotiana glutinosa* L. *Plant Cell Physiol.* **1998**, *39*, 565−573.
(7) Chae, H. S.; Cho, Y. G.; Park, M. Y.; Lee, M. C.; Eun, M. Y.; Kang, B. G.; Kim, W. T. Hormonal cross-talk between auxin and ethylene differentially regulates the expression of two members of the 1-aminocyclopropane-1-carboxylate oxidase gene family in rice (*Oryza sativa* L.). *Plant Cell Physiol.* **2000**, *41*, 354−362.
(8) Barry, C. S.; Blume, B.; Bouzayen, M.; Cooper, W.; Hamilton, A. J.; Grierson, D. Differential expression of the 1-aminocyclo-propane-1-carboxylate oxidase gene family of tomato. *Plant J.* **1996**, *9*, 525−535.

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

(10) Pogson, B. J.; Downs, C. G.; Davies, K. M. Differential expression of two 1-aminocyclopropane-1-carboxylic acid oxidase genes in broccoli after harvest. *Plant Physiol.* **1995**, *108*, 651−657.

(11) Blume, B.; Grierson, D. Expression of ACC oxidase promoter-GUS fusions in tomato and *Nicotiana plumbaginifolia* regulated by developmental and environmental stimuli. *Plant J.* **1997**, *12*, 731−746.

(12) Hunter, D. A.; Yoo, S. D.; Butcher, S. M.; McManus, M. T. Expression of 1-aminocyclopropane-1-carboxylate oxidase during leaf ontogene in white clover. *Plant Physiol.* **1999**, *120*, 131−141.

(13) Kend, J. H.; Zeevaart, J. A. D. The five "classical" plant hormones. *Plant Cell* **1997**, *9*, 1197−1210.

(14) Tang, X.; Gomes, A. M. T. R.; Bhatia, A.; Woodson, W. R. Pistil-specific and ethylene-regulated expression of 1-aminocyclopropane-1-carboxylate oxidase genes in petunia flowers. *Plant Cell* **1994**, *6*, 1227−1239.

(15) Bui, A. Q.; O'Neill, S. D. Three 1-aminocyclopropane-1-carboxylate synthase genes regulated by primary and secondary pollination signals in orchid flowers. *Plant Physiol.* **1998**, *116*, 419−428.

(16) Yu, S. J.; Kim, S.; Lee, J. S.; Lee, D. H. Differential accumulation of transcripts for ACC synthase and ACC oxidase homologs in etiolated mung bean hypocotyls in response to various stimuli. *Mol. Cells* **1998**, *8*, 350−358.

(17) Holdsworth, M. J.; Schuch, W.; Grierson, D. Nucleotide sequence of an ethylene-related gene from tomato. *Nucleic Acids Res.* **1987**, *15*, 10600.

(18) Balague, C.; Watson, C. F.; Turner, A. J.; Rouge, P.; Picton, S.; Pech, J. C.; Grierson, D. Isolation of a ripening and wound-induced cDNA from *Cucumis melo* L. encoding a protein with homology to the ethylene-forming enzyme. *Eur. J. Biochem.* **1993**, *212*, 27−34.

(19) Davies, K. M.; Grierson, D. Identification of cDNA clones for tomato (*Lycopersicon esculentum* Mill.) mRNAs that accumulate during fruit ripening and leaf senescence in response to ethylene. *Planta* **1989**, *179*, 73−80.

(20) Nakatsuka, A.; Murachi, S.; Okunishi, H.; Shiomi, S.; Nakano, R.; Kubo, Y.; Inaba, A. Differential expression and internal feedback regulation of 1-aminocyclopropane-1-acarboxylate oxidase, and ethylene receptor genes in tomota fruit during development and ripening. *Plant Physiol.* **1998**, *118*, 1295−1305.

(21) Wang, C. Y. Effect of aminoethoxy analog of rhizobitoxine and sodium benzoate on senescence of broccoli. *HortScience* **1977**, *12*, 54−56.

(22) Ecker, J. R.; Davis, R. W. Plant defense genes are regulated by ethylene. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 5202−5206.

(23) Wang, Z.; Brown, D. D. A gene expression screen. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 11505−11509.

(24) Lin, C. T.; Lin, M. T.; Shaw, J. F. Cloning and characterization of a cDNA for 1-aminocyclopropane-1-carboxylic acid oxidase. *J. Agric. Food Chem.* **1997**, *45*, 526−530.

(25) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(26) Sambrook, J.; Fritsch, E.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989.

(27) Rogers, S. O.; Bendich, A. J. Exrtaction of total cellular DNA from plants, algae and fungi. *Plant Mol. Biol. Man.* **1994**, *D1*, 1−8.

(28) King, G. A.; Woollard, D. C.; Irving, D. E.; Borst, W. M. Physiological changes in asparagus spear tips after harvest. *Physiol. Plant.* **1990**, *80*, 393−400.

(29) Shaw, J. F.; Chou, Y. S.; Chang, R. C.; Yang, S. F. Characterization of the ferrous ion binding sits of apple 1-aminocyclopropane-1-carboxylate oxidase by site-directed mutagenesis. *Biochem. Biophys. Res. Commun.* **1996**, *225*, 697−700.

(30) Roach, P. L.; Schofield, C. J.; Baldwin, J. E.; Clifton, I. J.; Hajdu, J. Crystallization and preliminary X-ray diffraction studies on recombinant isopenicillin N synthase from *Aspergillus nidulans*. *Protein Sci.* **1995**, *4*, 1007−1009.

(31) Feig, A. L.; Lippard, S. J. Mechanistic studies of the reaction of dioxygen with dinuclear iron(II) compounds to form (mu-peroxo)diiron(III) complexes. *J. Am. Chem. Soc.* **1994**, *116*, 8410−8411.

(32) Siebertz, B.; Logemann, J.; Willmitzer, L.; Schell, J. *cis*-analysis of the wound-inducible promoter *wun1* in transgenic tobacco plants and histochemical localization of its expression. *Plant Cell* **1989**, *1*, 961−968.

(33) Itzkaki, H.; Maxson, J. M.; Woodson, W. R. An ethylene-responsive enhancer element is involved in the senescence-related expression of the carnation glutathione-*S*-transferase (GST1) gene. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 8925−8929.

(34) Ballus, N.; Wong, L. M.; Theologis, A. Identification of the auxin-responsive element, *Aux*RE, in the primary indoleacetic acid-inducible gene, PS-IAA 4/5, of pea (*Pisum sativum*). *J. Mol. Biol.* **1993**, *233*, 580−596.

(35) Nagao, R. T.; Goekjian, V. H.; Hong, J. C.; Key, J. L. Identification of protein-binding DNA sequence in an auxin-regulated gene of sobybean. *Plant Mol. Biol.* **1993**, *21*, 1147−1162.

(36) ten Have, A.; Woltering, E. J. Ethylene biosynthetic genes are differentially expressed during carnation (*Dianthus caryophyllus* L.) flower senescence. *Plant Mol. Biol.* **1997**, *34*, 89−97.

(37) Liop-Tous, I.; Barry, C. S.; Grierson, D. Regulation of ethylene biosynthesis in response to pollination in tomato flowers. *Plant Physiol.* **2000**, *123*, 971−978.

(38) Kahana, A.; Silberstein, L.; Kessler, N.; Goldstein, R. S.; Perl-Treves, R. Expression of ACC oxidase genes differs among sex genotypes and sex phases in cucumber. *Plant Mol. Biol.* **1999**, *41*, 517−528.

(39) Nadeau, J. A.; Zhang, X. S.; Nair, H.; O′Neill, S. D. Temporal and spatial regulation of 1-amino-cyclopropane-1-carboxylate oxdiase in the pollination-induced senescence of orchid flowers. *Plant Physiol.* **1993**, *103*, 31−39.

(40) Wilson, I. D.; Zhu, Y.; Burmeister, D. M.; Dilley, D. R. Apple ripening-related cDNA clone pAP4 confers ethylene-forming ability in transformed *Saccharomyces cerevisiae*. *Plant Physiol.* **1993**, *102*, 783−788.

(41) Liu, X.; Shiomi, S.; Nakatasuka, A.; Yasutaka, K.; Nakamura, R.; Inaba, A. Characterization of ethylene biosynthesis associated with ripening in banana fruit. *Plant Physiol.* **1999**, *121*, 1257−1265.

(42) Mita, S.; Kawamura, S.; Yamawaki, K.; Nakamura, K.; Hyodo, H. Differential expression of genes involved in the biosynthesis and perception of ethylene during ripening of passion fruit (*Passiflora edulis* sims). *Plant Cell Physiol.* **1998**, *39*, 1209−1217.

(43) Ruperti, B.; Bonghi, C.; Rasori, A.; Ramina, A.; Tonutti, P. Characterization and expression of two members of the peach 1-aminocyclopropane-1-carboxylate oxidase gene family. *Physiol. Plant.* **2001**, *111*, 336−344.

(44) Tian, M. S.; Downs, C. G.; Lill, R. E.; King, G. A. A role for ethylene in the yellowing of broccoli after harvest. *J. Am. Soc. Hortic. Sci.* **1994**, *119*, 276−281.

(45) Mulligan, R. M.; Chory, J.; Ecker, J. R. Signaling in plant. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2793−2795.

Received for review January 6, 2003. Revised manuscript received February 23, 2003. Accepted February 27, 2003. This study was supported by Grant NSC-89-2313-B001-002 from the National Science Council, Taiwan, to J.-F.S.

JF034007M