Expression of arginine decarboxylase and ornithine decarboxylase genes in apple cells and stressed shoots

Yu-Jin Hao1,*, Hiroyasu Kitashiba1, Chikako Honda1, Kazuyoshi Nada2 and Takaya Moriguchi1,†

1 Department of Plant, Cell and Environment, National Institute of Fruit Tree Science, Tsukuba, Ibaraki 305-8605, Japan
2 Faculty of Bioresources, Mie University, Tsu, Mie 514-8507, Japan

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Abstract
Arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) are two important enzymes responsible for putrescine biosynthesis. In this study, a full-length ADC cDNA (MdADC) was isolated from apple [Malus sylvestris (L.) Mill. var. domestica (Borkh.) Mansf.]. Meanwhile, a partial ODC (pMdODC) could be amplified only by a second RCR from the RT-PCR products, whereas a full-length ODC could not be obtained by either cDNA library screening or 5'- and 3'-RACEs, suggesting quite low expression. Moreover, α-arginine, an ADC inhibitor, caused a decrease in ADC activity and severely inhibited the growth of apple callus, which could be partially resumed by exogenous addition of putrescine, whereas α-difluoromethylornithine (DFMO), an inhibitor for ODC, caused the incomplete repression of callus growth without changing ODC activity. RNA gel blot showed that the expression level of MdADC was high in young tissues/organs with rapid cell division and was positively induced by chilling, salt, and dehydration, implying its involvement in both cell growth and stress responses. By contrast, the transcript of ODC could not be detected by RNA gel blot analysis. Based on the present study, it is possible to conclude that (i) the ODC pathway is active in apple, although the expression level of the pMdODC gene homologous with its counterparts found in other plant species is quite low; and (ii) MdADC expression correlates with cell growth and stress responses to chilling, salt, and dehydration, suggesting that ADC is a primary biosynthetic pathway for putrescine biosynthesis in apple.

Key words: Apple (Malus sylvestris var. domestica), arginine decarboxylase, cell growth, gene expression, ornithine decarboxylase, stress response.

Introduction
Polyamines, such as putrescine, spermidine, and spermine, are ubiquitously present in plants and are involved in a number of physiological processes (Evans and Malmberg, 1989). Generally, putrescine is synthesized from ornithine by ornithine decarboxylase (ODC; EC 4.1.1.19), and then converted into spermidine and spermine by spermidine synthase (SPDS; EC 2.5.1.16) and spermine synthase (SPMS; EC 2.5.1.22), respectively, with addition of aminopropyl groups generated from S-adenosylmethionine by S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50). In plants, putrescine is also produced through an alternative arginine decarboxylase (ADC; EC 4.1.1.17) pathway, involving two intermediates, namely agmatine and N-carbamoylputrescine (Malmberg et al., 1998). It is well known that the activities of ADC and ODC are regulated separately depending on physiological and developmental conditions. ADC appears to be the primary enzyme for cell extension, secondary metabolic processes and stress responses, whereas ODC activity seems to regulate cell division in actively growing tissues (Slocum et al., 1984; Tabor and Tabor, 1984; Evans and Malmberg, 1989).

Recently, many genes encoding polyamine biosynthetic enzymes were isolated and characterized in different organisms, which makes it possible to elucidate the molecular mechanisms of polyamine biosynthesis. In plants, ADC genes have been isolated from many species including oat...
Arabidopsis another two enzymes in the ADC pathway, namely agmatine iminohydrolase and N-carbamoylputrescine amidohydrolase, were cloned in Arabidopsis (Janowitz et al., 2003; Piotrowski et al., 2003). Several lines of evidence indicate the involvement of ADC in the response of plants to environmental stresses. For example, ADC activity increases upon exposure to acid stress and is positively correlated with the ADC mRNA level in soybean (Nam et al., 1997). Under salt stress, ADC activity and the accumulation of ADC transcripts are enhanced in a salt-tolerant rice cultivar, but are down-regulated in a salt-sensitive rice cultivar (Chattopadhyay et al., 1997). In mustard, ADC transcripts are up-regulated in response to some stresses such as chilling, salt, and mannitol (Mo and Pua, 2002). More recently, Urano et al. (2004) used a Ds insertion mutant of the AtADC2 gene (adc2-1) to prove that the Arabidopsis ADC gene (AtADC2) plays an important role in salt tolerance. Similarly, Arabidopsis mutants, spe1-1 and spe2-1 showed reduced ADC activity and decreased salt tolerance compared with the wild types (Kasinathan and Wingler, 2004).

Compared with ADC, there have only been a few reports on the isolation of complete ODC genes from plants (Michael et al., 1996; Kwak and Lee, 2001). Meanwhile, heterologous ODCs, isolated from yeast and mouse, were introduced into different plants to demonstrate their impact on ODC activity and polyamine titre. Polyamine biosynthesis of transgenic carrot cells and poplar with mouse ODC was investigated in detail (Andersen et al., 1998; Bhatnagar et al., 2001, 2002). The increased putrescine biosynthesis in transgenic carrot cells and rice callus with mouse ODC enhanced the somatic embryogenesis and regeneration potential, respectively (Bastola and Minocha, 1995; Kumira and Rajam, 2002). Furthermore, genetic manipulation of heterologous ODC can even affect another secondary metabolism pathway, in which transgenic tobacco over-expressing yeast ODC showed a 2-fold higher nicotine level than the control (Hamill et al., 1990).

Fruit trees once planted are exposed to various environmental stresses for a longer time than annual crops. However, molecular functions of polyamines in cell growth and stress response have not been fully studied. To gain the first insights into the molecular role of polyamines in cell growth and stress response in fruit trees, a full-length ADC gene (MdADC) was isolated from apple, and its expression patterns in different tissues at different growth stages and under different stress conditions were analysed. Although many attempts were made to isolate the ODC gene from apple using different tissues and organs, it was difficult to get a full-length ODC gene. Instead, a partial ODC homologue (pMdODC) was obtained with a second PCR from the first RT-PCR product. When polyamine biosynthesis inhibitors were added to the medium, the growth of callus was arrested by d-arginine, an ADC inhibitor, but to a lesser degree by N-difluoromethylornithine (DFMO), an ODC inhibitor. Based on these results, the involvement of MdADC in cell growth and stress responses to chilling, salt, and dehydration, as well as the primary biosynthetic pathway for putrescine biosynthesis in apple, were discussed.

Materials and methods

Plant materials

Leaves, shoots, and flowers (balloon stage) of the apple cultivar ‘Orin’ (Malus sylvestris var. domestica) were collected from the experimental farm of the National Institute of Fruit Tree Science (Tsukuba, Japan). Fruits were collected at 19, 61, 103, 145, and 174 d after full bloom (DAF) from the Apple Research Center, National Institute of Fruit Tree Science (Morioka, Japan).

Apple callus was induced from young fruits and maintained at 1 month intervals on callus subculture medium containing MS-salts (Murashige and Skoog, 1962), Nitsch organic components (Nitsch and Nitsch, 1969), 3% sucrose, 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 1 μM N⁶-benzylaminopurine (BA), and 0.8% agar in the dark at 25 °C. For suspension culture, the callus was transferred into the liquid subculture medium and placed on a rotary shaker at c. 120 rpm in the dark at 25 °C. The suspension cultures were subcultured three times at 2 week intervals before being used in the growth experiment. In order to make a growth curve, aliquots from the 12-d-old cultures were transferred to new medium, and were collected and weighed from day 0 to day 20 at 2 d intervals. In this case, the cultures at day 0 are the same as those in 12-d-old cultures. Meanwhile, the cultures collected were also used for RNA gel blot analysis and measurement of polyamine contents.

In vitro apple shoots were maintained on the shoot subculture medium containing MS-salt (Murashige and Skoog, 1962), B5-organic (Gamborg et al., 1968), 3% sucrose, 4.5 μM BA, 0.5 μM 3-indolebutyric acid (IBA), and 0.8% agar under a 16 h photoperiod at 25 °C.

Isolation of ADC from apple

Total RNA was isolated from apple shoots as described by Wan and Wilkins (1994). First-strand cDNA was synthesized using a First-Strand cDNA Synthesis Kit (Amersham Pharmacia) following the manufacturer’s instructions. Degenerate primers 5’TACCCARGGDDHTHTAYCCDGAGGA-3’ (sense) and 5’-CTKG-GYCCRCRRAAYAGRTTGGAGA-3’ (antisense) were designed based on the conserved regions of ADC amino acid sequences of other plants. RT-PCR conditions were 95 °C for 12 min followed by 35 cycles of 94 °C for 45 s, 52 °C for 60 s, and 72 °C for 45 s. The PCR product was designated as pMdADC and subcloned into pCR®2.1 vector (Invitrogen) for sequencing as described by Zhang et al. (2003). After confirming it as the homologue fragment of the ADC gene, the fragment was labelled with digoxigenin-dUTP (Roche) for DNA and RNA gel blot hybridization. 5’-RACE and 3’-RACE (Rapid Amplification of cDNA Ends) were used to isolate the full-length apple ADC by amplifying the upstream and downstream regions of pMdADC. One microgram of shoot RNA was used to prepare RACE-Ready cDNAs with a SMART™ RACE cDNA Amplification Kit (Clontech Laboratories, Inc.) following the manual. The 5’-RACE and 3’-RACE were carried out in a Perkin-Elmer DNA Thermal Cycler 480 with 5’ and 3’
gene specific primers (GSP) which were designed based on the sequence of pMdADC. The amplification programme contained one cycle of 2 min at 95 °C; five cycles of 30 s at 94 °C, 3 min at 72 °C; five cycles of 30 s at 94 °C, 30 s at 72 °C, 3 min at 72 °C; 25 cycles of 30 s at 94 °C, 30 s at 68 °C, 3 min at 72 °C; and one cycle of 7 min at 72 °C. The amplification products were subcloned into pCR®2.1 vector (Invitrogen) for sequencing as described by Zhang et al. (2003). The resultant sequences were aligned with pMdADC to get a full-length ADC gene. The full-length gene was designated as MdADC.

Isolation of ODC from apple

The first-strand cDNA, the same as that in ADC isolation, was used for RT-PCR to isolate the ODC fragment. Degenerate primers 5'-TTTAYGCYTTAATGYAAACC-3' (sense) and 5'-CRRCCRGAYRTCKCADC-3' (antisense) were designed based on the conserved regions of ODC amino acid sequences in other plants (Michael et al., 1996). RT-PCR conditions were 95 °C for 12 min followed by 35 cycles of 94 °C for 60 s, 52 °C for 60 s and 72 °C for 110 s. Second PCR conditions were 94 °C for 10 min followed by 35 cycles of 94 °C for 60 s, 54 °C for 60 s, and 72 °C for 110 s. The PCR product was subcloned into the pCR®2.1 vector (Invitrogen). After confirming it as a homologue of the ODC gene, the apple ODC homologous fragment, designated as pMdODC, was labelled with digoxigenin-dUTP by PCR (Roche) for DNA and RNA gel blot analysis or cDNA library screening.

DNA gel blot analysis

Genomic DNA was isolated from apple leaves according to Porebski et al. (1997). Ten micrograms of DNA samples were digested with Dral, XhoI, or XhoI for ADC and DraI or SpeI for ODC. Digested DNA samples were fractioned on 0.8% agarose gel and transferred onto a Hybond N membrane (Amersham Pharmacia). Hybridization was performed using digoxigenin-labelled pMdADC and pMdODC, respectively, at 43 °C in a solution containing 7% SDS, 0.05 M phosphate (pH 7.0), 5× SSC, 1% blocking reagent, and 0.1% N-lauroylsarcosine. The membranes were washed twice in a solution containing 0.2× SSC and 0.1% SDS for 5 min at room temperature, followed by washing twice in a solution containing 0.1× SSC and 0.1% SDS for 15 min at 68 °C.

Polyamine and inhibitor treatments

To investigate the effects of exogenous polyamines and/or inhibitors of polyamine biosynthesis on the growth of apple callus, \( \alpha \)-arginine (5, 8, or 10 mM) or DFMO (15 mM) was added to the medium. Proteins were quantified by the method of Bradford (1976) using bovine serum albumin as a standard.

Polymine analysis

To extract free polyamines, 0.1 g of callus and suspension cultures were homogenized in 1 ml of 5% perchloric acid and extracted on ice for 30 min. After being centrifuged at 2000 g and 4 °C for 5 min, the supernatant was transferred to another tube and kept on ice. The pellet was extracted again with 1 ml of 5% perchloric acid on ice for 30 min and then centrifuged at 2000 g and 4 °C for 5 min. The supernatants derived from the two centrifugations were combined. One hundred microlitres of supernatant were moved to a 0.5 ml tube and adjusted to pH 7.0 with saturated Na₂CO₃. Eighty microlitres of neutralized extract were then transferred into a glass V-vial followed by the addition of 10 μl of 1 M sodium borate buffer (pH 6.3) and 100 μl of 5 mM 9-fluorenylmethyl chloroformate (FMOC) (Sigma) in acetone. After being vortexed for 1 min at room temperature, 200 μl of 1-adamantanamine (ADAM) (ICN) in acetone was added and vortexed for another 30 s. The mixture was centrifuged at 2000 g at 4 °C for 5 min. Ten microlitres of supernatant was loaded to analyse the polyamine–FMOC derivatives with high-performance liquid chromatography (HPLC; Shimadzu LC10–VP). Reversed-phase HPLC of derived standards and extracts was carried out according to Yokota et al. (1994).

Enzyme analysis

Polyamine biosynthetic enzymes, ADC and ODC, in 2-week-old callus on MS medium with or without \( \alpha \)-arginine (8 mM) and DFMO (15 mM) were extracted in 100 mM potassium phosphate (pH 8.0) containing 20 mM sodium ascorbate, 1 mM pyridoxal-5’-phosphate, 10 mM dithiothreitol, 0.1 mM EDTA, and 0.1 mM phenylmethylsulphonyl fluoride. The homogenate was centrifuged at 25 000 g for 20 min, and the supernatant was used to measure enzyme activity. Enzyme activity was determined at 30 °C as described by Song et al. (2001). The substrates used for ADC and ODC activities were 9 mM \( L \)-arginine labelled with 370 kBq ml⁻¹ \( L \)-[^14C]arginine and 63 mM \( L \)-ornithine labelled with 370 kBq ml⁻¹ \( L \)-[^14C]ornithine, respectively. Proteins were quantified by the method of Bradford (1976) using bovine serum albumin as a standard.

Stress treatments

Two-day-old in vitro shoots were exposed to different stresses including cold, high temperature, and salt stress. For cold and high temperature treatments, in vitro shoots were treated at 4 °C or 35 °C, respectively. The samples were collected at 6, 24, 72, and 120 h, respectively. For the salinity stress treatment, in vitro shoots were transferred to subculture medium containing either 0 or 300 mM NaCl, under a 16 h photoperiod at 25 °C. The samples were collected at 6, 12, 24, 48, 72, 96, and 120 h. Meanwhile, fresh young shoot samples from the experimental farm were given the dehydration treatment. These young shoots were incubated at 25 °C in the dark with or without a water supply. The samples were collected at 1, 2, 3, 5, 7, 10, 24, 36, and 48 h.

Expression analysis of MdADC or pMdODC in apple tissues and under stress conditions

Total RNAs were isolated according to Wan and Wilkins (1994). Ten micrograms of total RNA were used for RNA gel blot analysis. After electrophoresis in a 1.2% formaldehyde-denatured agarose gel, RNA patterns were blotted onto a Hybond N membrane (Amersham Pharmacia) and hybridized with either digoxigenin-labelled pMdADC or pMdODC probes under the same conditions as described in DNA gel blot analysis, with the exception of setting the hybridization temperature to 48 °C. Loading equality and transferring efficiency of RNA into membranes were confirmed by staining with ethidium bromide and methylene blue, respectively.

Results

Isolation of ADC and ODC genes from apple

A fragment of c. 1400 bp in size was amplified from shoot cDNA with a ADC-specific degenerate primer set and identified as an apple ADC homologue (pMdADC) after sequencing and sequence alignment. In order to perform 5’- and 3’-RACEs for isolation of full-length ADC, 5’-GSP (ADC-R5) and 3’-GSP (ADC-R3) were designed as 5’-TTGCAACAAGCAGCCTCATTGGC-3’ (1248–1229th) and 5’-TGCTAGAGCTTGAAGCGGAT-3’ (2361–2380th), respectively, based on the sequence (Fig. 1). Subsequently, 5’- and 3’-RACEs obtained two fragments of 1300 bp and
Fig. 1. Nucleotide sequence and deduced amino acid sequence of *MdADC*. The primer positions are underlined. 5'9-GSP (ADC-R5), gene specific primer for 5'9-RACE; 3'9-GSP (ADC-R3), gene specific primer for 3'9-RACE; P1-S and P1-A, sense and antisense primers, respectively, for the amplification of full length *MdADC*; P2-S and P2-A, sense and antisense primers, respectively, for the amplification of conserved regions of *MdADC*. 

Hao et al.
sequence, ODC-specific primers 5′-TGCCGTCAAATG-TAACCCCTC-3′ (sense) and 5′-GAGGAGCATG-CATTTTGGG-3′ (antisense) were designed for RT-PCR. A series of RT-PCRs using cDNAs of young leaves, mature leaves, flower buds, young fruits, and callus did not amplify any fragments (data not shown). Alternatively, in order to get full-length ODC cDNA, a sense ODC-specific primer (3′-GSP) was used for 3′-RACE and an antisense ODC-specific primer (5′-GSP) for 5′-RACE. But neither RACE produced any fragments from the cDNAs of young leaves, mature leaves, flower buds, young fruits, and callus of apple. Since hybridization should be more sensitive for the detection of homologous sequences with a specific probe than PCR, both young apple leaf and flower cDNA libraries, 600 000 plaques for each, were also used for screening ODC with digoxigenin-labelled pMdODC. However, no positive clone could be obtained from the screening.

DNA gel blot analysis

Digoxigenin-labelled pMdADC fragment was used for DNA gel blot analysis (Fig. 3A). This probe detected one strong band with additional faint bands in Drl digestion and two bands in XbaI or XhoI digestions, respectively. Since there are no XbaI sites in MdADC and also no XhoI sites in the probe region, the two bands detected by XbaI or XhoI were possibly alleles of the same locus or originated from different loci in the genome. These results indicate that a single or two copies of the MdADC gene might be present in the apple genome.

At the same time, digoxigenin-labelled pMdODC was used for DNA gel blot analysis of ODC (Fig. 3B). The result showed that two bands were detected in all digestions by restriction enzymes Drl and SpeI, suggesting that pMdODC might be a single or two copy genes in the apple genome.

Effects of the inhibitors of putrescine biosynthetic enzymes

Two weeks after the callus was transferred to subculture medium, the fresh weight increased about 20 times relative to the initial weight. In the preliminary experiment, 5 mM D-arginine, an ADC inhibitor, caused partial suppression of apple callus growth, but 10 mM D-arginine repressed the

Fig. 2. Alignment of amino acid sequence of Malus domestica (pMdODC) and corresponding parts in ODCs of Datura stramonium (DDBJ accession number X87847), Glycine max (AJ563382), and Nicotiana tabacum (AF127247). Asterisks and dots indicate the identical and similar amino acid residues, respectively.

900 bp in size, respectively. Both fragments were sequenced. The alignment of these two fragments with the pMdADC showed that they could be combined into one contig, which encoded a putative full-length ADC gene designated as pMdADC.

Only one ADC gene was isolated here. Due to the high heterozygosity of fruit trees, possibly more than one ADC homologous gene is present in apple. In order to confirm or eliminate this kind of possibility, six other clones were sequenced. Three of these six clones were amplified by RT-PCRs from shoot cDNAs with primer sets 5′-AA-CAACCCCAAAAGCCATTC-3′ (sense, P1-S) and 5′-ACTCTCTCTATCACGTCCCTTGCT-3′ (antisense, P1-A) that were designed based on the 5′ upstream and 3′ downstream regions (Fig. 1) and could, theoretically, cover about 2700 bp containing the full-length ADC cDNA. Meanwhile, another three clones were produced by RT-PCRs from shoot cDNAs with primer sets 5′-TAC-CAGGGCGTGTACCCGGTGAAAT-3′ (sense, P2-S) and 5′-GAAGGAGGTGTTGGACTCCT-3′ (antisense, P2-A) that were designed based on highly conserved amino acid regions in predicted ADC protein compared with other plant ADC proteins and could amplify a fragment of about 1300 bp. Sequence comparisons showed that all DNA sequences were completely identical to pMdADC (data not shown).

The full-length ADC gene pMdADC (3344 bp) consisted of a 2184 bp ORF encoding 728 amino acids with 78 kDa. pMdADC had a long 5′ region that contained an additional short upstream open reading frame (uORF) encoding 11 amino acids (Fig. 1). The full-length cDNA sequence of pMdADC was deposited in the DDBJ database under the accession number AB181854.

By contrast, the RT-PCRs did not produce any fragments from shoot cDNA with different combinations of the ODC-specific degenerate primer set. After a 100-fold dilution of the first PCR product, the RT-PCR product was used for DNA gel blot analysis of MdADC (data not shown).

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callus growth completely and addition of putrescine did not recover the growth during the course of the experiment. Therefore, 8 mM D-arginine was used in this experiment. When either 8 mM D-arginine or 15 mM DFMO was added to the subculture medium, the callus growth was inhibited slightly by DFMO, regardless of the high concentration, and almost completely inhibited by D-arginine (Fig. 4A). Two weeks later, the callus growth was conspicuous on medium containing DFMO, but it had ceased completely on medium containing D-arginine. Correspondingly, the fresh weight of DFMO-treated and D-arginine-treated calluses were only 43% and 4%, respectively, of that of the control. However, the application of 1 mM putrescine partially resumed the callus growth inhibited by 8 mM D-arginine (Fig. 4A).

Free polyamine contents were measured using 2-week-old callus (Fig. 4B). In the control, the contents of free putrescine, spermidine, and spermine were 610.0 ± 67.0, 89.3 ± 7.9, and 10.8 ± 0.8 nmol g⁻¹ fresh weight (FW), respectively. In DFMO-treated callus, however, the free putrescine and spermidine contents decreased to about half that of the control, while free spermine content increased to 14.8 ± 0.5 nmol g⁻¹ FW. Similarly, in D-arginine-treated callus, the free putrescine and spermidine contents decreased to 70.3 ± 11.8 and 57.2 ± 10.4 nmol g⁻¹ FW, respectively, while free spermine content sharply increased to 83.3 ± 10.1 nmol g⁻¹ FW, which is much higher than those in the control and DFMO-treated calluses.

The ADC and ODC activities were also measured (Table 1). In the control, ADC activity was slightly higher than ODC activity. When exposed to D-arginine, an ADC inhibitor, ADC activity was completely inhibited, but ODC activity was positively induced two times higher than the control. Surprisingly, when ADC and ODC activities were measured in the callus treated by DFMO, an ODC inhibitor, ODC activity was not affected but ADC activity was repressed to a large extent.

Expression of MdADC and pMdODC in suspension cultures and different apple tissues

The expression of MdADC gene in apple suspension cultures was also analysed during the culture period. The growth curve of apple suspension cultures was typically S-shaped (Fig. 5A) and can be divided into three stages, I, II, and III. The growth rate was quite slow during the initial stage (stage I) from day 0 to day 6 and reached its peak during the logarithmic stage (stage II) from day 8 to day 16. Subsequently, growth slowed down and the culture eventually entered the stationary phase (stage III) from day 16 to day 20. Correspondingly, the MdADC expression level varied with the changes in the cell growth rate (Fig. 5B). At stage I, the expression level of the MdADC gene increased gradually from day 2 to day 4. Six days after subculture, the highest expression level of MdADC was detected. The
MdADC transcripts decreased to a very low level on day 20. In order to confirm the relationship between the increase in MdADC gene expression and cell growth, the polyamine contents in suspension cultures were measured (Fig. 5C). The result showed that the polyamine contents, especially putrescine, were nearly in agreement with the MdADC expression levels and the growth curve, suggesting that MdADC is surely involved in cell growth of apple suspension cultures.

Both vegetative tissues, including young and mature leaves, and reproductive tissues such as flower and fruits at...
different stages were used for \( MdADC \) expression analysis (Fig. 6). The RNA gel blot showed that young leaves expressed a high level of \( MdADC \) transcripts relative to the mature leaf. In the reproductive tissues, flowers showed the highest level of \( MdADC \) expression. Young fruit showed a lower level of \( MdADC \) transcript at 19 DAF, i.e. the cell division stage in fruit, than flowers. With the fruit development and ripening, the expression level of the \( MdADC \) gene decreased gradually from 61 to 103 DAF, and was hardly detectable in fruit during ripening stages from 145 to 174 DAF.

By contrast, the \( pMdODC \) probe did not detect any signals in suspension cultures at any stage or in any tissues tested, even when the amount of total RNA was increased to 30 \( \mu g \) (data not shown).

Expression of \( MdADC \) or \( pMdODC \) under environmental stresses

The morphology of in vitro shoots remained normal throughout the treatment at 4 °C but in those at 35 °C the leaf edges started to turn brown at 120 h (data not shown). The responses of \( MdADC \) expression to low and high temperatures were different (Fig. 7A). At 4 °C, \( MdADC \) transcripts increased at 6 h and maintained this level until 120 h. At 35 °C, however, \( MdADC \) transcripts sharply decreased to a trace level at all stages of the treatments.

\( MdADC \) expression of in vitro shoots remained almost at the same level from 6 to 120 h in the subculture medium without NaCl (0 mM NaCl). When they were exposed to 300 mM NaCl stress, their appearance remained normal from 6 to 72 h, but they started to turn brown along the veins of leaves at 96 h (data not shown). Correspondingly, the level of \( MdADC \) transcripts varied with treatment time from 6 to 120 h (Fig. 7B). In general, \( MdADC \) expression levels in the treated samples were much higher than those in the non-treated samples.

Upon exposure to dehydration treatments, the young fresh shoots cut directly from the orchard showed different changes in weight under two conditions, i.e. with and without a water supply. Their weight remained stable if water was supplied, while it gradually decreased from 1 to 48 h in the ambient conditions without water supply (data not shown). The \( MdADC \) expression level of the control shoots increased at 1 h and then gradually decreased to the level at 0 h from 2 to 5 h. Thereafter, the expression level was kept constant until 48 h. As for the dehydrated shoots, the \( MdADC \)-expressing level increased to a relatively higher level than the initial level at 0 h from 7 to 48 h (Fig. 7C).

By contrast, no \( pMdODC \) signals were detected in the samples exposed to the above-mentioned stresses by RNA gel blot analysis (data not shown).

Discussion

In this study, a full-length \( ADC \) gene (\( MdADC \)) was successfully isolated from apple. In addition to the main ORF, \( MdADC \) possessed a uORF in its 5' upstream region, in agreement with the structure of \( ADC \) genes from other plant species. The uORF of carnation \( ADC \) encodes seven amino acids, MQKSLHI (Chang et al., 2000), while those of both tobacco and pea \( ADC \) genes encode eight amino acids, i.e. MVLCCFDS (accession number AB012873) and MLCFPLRA (Pérez-Amador et al., 1995), respectively. By
contrast, the uORF of MdADC encoded 11 amino acids, MHCMFGSDFGA, that possessed two methionine (M) residues. The starting site for uORF transcription remains to be clarified. Although no consensus exists for the length and amino acid sequences of these uORFs, it has been documented that uORF inhibits the translation of downstream ORF in the ADC gene (Chang et al., 2000), which is pertinent to maintaining polyamine homeostasis. A similar mechanism is also reported in other polyamine biosynthetic genes such as ODC (Kwak and Lee, 2001) and SAMDC (Hanfrey et al., 2002). The abrogation of SAMDC uORF leads to abnormal morphogenesis in tobacco (Hanfrey et al., 2002), and polyamine levels (homeostasis) are regulated through the uORF-mediated translation.

Analysis of genomic MdADC sequences indicated the presence of a single gene or two loci in the apple genome. In the case of a single gene, the faint band that appeared in the DNA gel blot analysis may be ascribed to the allelic gene in the same locus because apple is genetically a heterozygous plant. However, the possibility of the presence of a second locus still remains because it has been reported that apple (n=17) is derived from an ancient hybridization of a single progenitor (n=9) (Evans and Campbell, 2002). Even if there are two ADC loci in the apple genome, MdADC may be preferably transcribed from a single sequence (gene), because the sequences of all isolated ADCs were completely identical to each other.

Many plants also produce putrescine by the ADC pathway in addition to the ADC pathway. In this study, a partial ODC homologue (pMdODC) was obtained from the RT-PCR product with a second PCR and used as a probe for DNA and RNA gel blot analysis. It was difficult, however, to isolate ODC genes from tissues including leaf, flower, shoot, callus, suspension cultures, and flesh by RT-PCR. Using pMdODC as a probe for cDNA library screening, no positive clones could be revealed from several cDNA libraries. In addition, no signals could be detected in a series of RNA gel blot analyses in various tissues at different developmental stages or exposed to different environmental stresses. These results indicated that its expression level is very low in the apple tissues tested, although the ODC gene exists in the apple genome. It has been reported that there are no ODC homologous sequences in the Arabidopsis genome (Hanfrey et al., 2001). Recently, however, putative ODC activity was found in Arabidopsis (Tassoni et al., 2003), indicating that Arabidopsis may encode an ODC gene sequence with low homology to the known ODC genes in other higher plants. Although no signal was detected with the probe pMdODC in apple tissues in this study, ODC activity was also found in apple fruit (Biasi et al., 1991) and callus in this study. These results may suggest, therefore, the presence of another type of ODC gene with low homology to the known ODC genes in other plant species.

As is well known in plants, ADC- and ODC-mediated putrescine biosynthesis pathways play different roles in plant development and growth (Tabor and Tabor, 1984). It seems that ADC is related to tissue maturation and response to environmental stresses, while ODC plays the main role in cell division (Slocum et al., 1984; Tabor and Tabor, 1984; Evans and Malmberg, 1989). Furthermore, it has been reported that the ADC pathway for polyamine biosynthesis is predominant in higher plants (Bierecka et al., 1985; Rajam, 1993; Minocha and Minocha, 1995). In this study, 8 mM D-arginine was applied to inhibit ADC activity. As expected, ADC activity was completely inhibited, while ODC activity was positively induced. In addition, D-arginine treatment seriously inhibited cell growth and caused a dramatic decrease in putrescine content, suggesting that ADC is the major enzyme in the production of putrescine in apple callus, while ODC yields putrescine inefficiently. The inhibition of apple cell growth could be recovered by adding putrescine exogenously, indicating that D-arginine inhibits cell growth by changing the putrescine content. Meanwhile, 15 mM DFMO was used to inhibit ODC activity in this study. Compared with the DFMO concentrations applied in other studies, generally from 5 mM to 2 mM (Lee et al., 1996; Aziz et al., 1998; Fos et al., 2003), the concentration in the present study should be high enough to inhibit ODC activity. However, it only caused a decrease in ADC activity and an incomplete inhibition of cell growth without affecting ODC activity. It has been known that α-difluoromethylarginine (DFMA), an inhibitor for ADC, is converted to urea and DFMO by arginase (Slocum and Galston, 1985). At present, however, it is not possible to explain why DFMO inhibited the activity of ADC, but not that of ODC. Collectively, D-arginine, an ADC inhibitor, induced much more serious inhibition in cell growth and putrescine content than DFMO, an ODC inhibitor. In other words, the growth of apple callus, to a large degree, depends on the putrescine produced by the ADC pathway.

In suspension cultures, the accumulation patterns of MdADC transcripts were in accordance with the cell growth patterns and putrescine titre. MdADC also showed a higher expression level in the young tissues, including young leaf, flower, and young fruit, where cells are dividing and growing more actively than old tissues such as the mature leaf and ripening fruit. Thus, there was also a positive correlation between cell growth and MdADC expression in apple. Furthermore, free putrescine content in young fruits was about 6–10 times that in ripening fruits as previously described by Zhang et al. (2003), indicating that MdADC expression during apple fruit development was positively related with the putrescine titre. Therefore, it can be inferred that ADC is a major pathway for putrescine biosynthesis in apple, although the ODC pathway is reported to be responsible for cell division in actively growing tissues in other plant species (Slocum et al., 1984; Tabor and Tabor, 1984; Evans and Malmberg, 1989).
Induced expressions of ADC upon stresses were reported in potassium-deficient Arabidopsis (Watson and Malmberg, 1996), acid-stressed soybean hypocotyls (Nam et al., 1997), salt-stressed rice (Chattopadhyay et al., 1997) and Arabidopsis (Urano et al., 2004), wounded Arabidopsis (Pérez-Amador et al., 2002), and chilling-, salt-, and osmotically stressed mustard (Mo and Pua, 2002), which demonstrated that ADC genes play important roles in plant responses to stress in the environment. Since the expression levels of MdADC were positively or negatively induced by environmental stresses, including extreme temperatures, high salinity, and dehydration, MdADC might also be involved in the response to stress in addition to cell growth. However, physiological reason(s) for the repression of MdADC under high temperature in the present research has not yet been ascertained.

Based on the results, the following conclusions can be drawn: (i) the ODC pathway is active in apple, but the expression level of the ODC gene homologous with its counterparts found in other plant species is quite low in the apple tissue tested; (ii) MdADC expression correlates with cell growth and stress responses to chilling, salt, and dehydration, suggesting that ADC is a primary biosynthetic pathway for putrescine biosynthesis in apple. Besides the possibility of the presence of an ODC which is quite different in sequence from the known ODCs and pMdODC, the localization of ODC should also be considered because most of the full-length ODC cDNAs in the DNA database were isolated from root-related tissue, i.e. root cultures of Datura stramonium (Michael et al., 1996), roots of Lycopeicum esculentum (Kwak and Lee, 2000) and Nicotiana tabacum (accession number AF127242), nodules of Lotus japonicus (AJ575746), and seedlings of Glycine max (AJ563382, AJ563383). The highest ODC activity was found in the root tip of Helianthus tuberosus (Bagni et al., 1983). In apple, Wang and Faust (1986) suggested that ODC plays a more important role than ADC in root initiation and growth through experiments using ADC and ODC inhibitors. The tissues used in this study did not include root-related tissues, which may explain the negative results in the isolation of full-length ODC cDNA. Further studies are needed to address these issues. Nevertheless, it can be inferred that MdADC is a major gene in putrescine biosynthesis, at least in the conditions employed in this study.

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