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Gene Note

Identification of polyamine oxidase genes from apple and expression analysis during fruit development and cell growth

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Abstract Two cDNAs (*MdPAO1* and *MdPAO2a*) encoding polyamine oxidases (PAOs) were isolated from apple. Some notable features related to substrate specificity, catalytic activity and localization of MdPAO were predicted by comparison with those of other dicots- and monocots-PAOs. *MdPAO* was expressed in suspension cells at any time points tested during the culture period, but only in the young fruits among several organs analyzed.

Key words: *Malus sylvestris* var. domestica, polyamine oxidase.

Aliphatic polyamines, i.e. spermidine (Spd), spermine (Spm), and their obligate diamine precursor putrescine (Put), are implicated in a wide range of plant physiological processes such as morphogenesis, flower differentiation and initiation, pollen viability, root growth, somatic embryogenesis, anti-senescence, and biotic/abiotic stress responses (Galston and Sawhney 1990; Bouchereau et al. 1999). Put is synthesized from two pathways in plant, i.e. directly from ornithine by ornithine decarboxylase (ODC, EC 4.1.1.17) or indirectly from arginine by arginine decarboxylase (ADC, EC 4.1.1.19) via two intermediates, namely agmatine and *N*-carbamoylputrescine by respective agmatine iminohydrolase (EC 3.5.3.12) and *N*-carbamoylputrescine amidohydrolase (EC 3.5.1.53) (Malmberg et al. 1998). Put is then converted into Spd and Spm 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 SAM (*S*-adenosylmethionine) by SAM decarboxylase (SAMDC, EC 4.1.1.50). These biosynthetic genes have been isolated and relatively-well characterized in many plant species including fruit trees (e.g. reviewed by Kakkar and Sawhney 2002).

Compared with biosynthetic pathway, however, molecular cloning and characterization of the plant genes involved in the polyamine degradation are limited. One of those enzymes participating in polyamine degradation is polyamine oxidase (PAO, EC 1.5.3.11), which catalyze the conversion of Spd and Spm to 4-aminobutanal and *N*-(3-aminopropyl)-4-aminobutanal, respectively, along with 1,3-diamine propane in plants (Šebela et al. 2001). It is deemed that polyamine degradation through PAO is important for regulating intracellular polyamine titers, which then affect some physiological processes in plants (Bagni and Tassoni 2001). PAOs have been purified and partially characterized from a few plant species (Federico et al. 1989; Šebela et al. 2001). In particular, Gramineae *PAOs* such as maize (*MPAO*) and barley (*BPAO1* and *BPAO2*) are the most studied members of this class (Cervelli et al. 2000, 2001, 2004). In dicots, PAO activity in alfalfa (Bagga et al. 1991) and tobacco (Yoda et al. 2003) have been detected, and putative *PAOs* of *Arabidopsis thaliana* and *Nicotiana tabacum* have been registered at GenBank. However, their detailed properties remain unclear.

We have been characterizing most of genes involved in polyamine biosynthesis from apple, one of the most economically important fruit trees worldwide. To further understand the regulatory mechanism of polyamine metabolism in fruit development, ripening, and stress responses, the isolation of genes catalyzing polyamine degradation is required. Therefore, as the first step, *PAO* genes were isolated from apple, and its expression pattern was analyzed. To our knowledge, this is the first report on the characterization of dicots *PAO*.

Leaves, flowers at balloon stage, fruits at 19, 61, 103, 145, and 174 days after full bloom (DAF), and suspension cells of 'Orin' apple (*Malus sylvestris* var. *domestica*) were prepared as described by Hao et al. (2005). To isolate apple *PAO* homolog, total RNA from

Abbreviations: ADC, arginine decarboxylase; DAF, days after full bloom; ODC, ornithine decarboxylase; PAO, polyamine oxidase; Put, putrescine; SAM, *S*-adenosylmethionine; SAMDC, SAM decarboxylase; Spd, spermidine; SPDS spermidine synthase; Spm, spermine; SPMS, spermine synthase; UTRs, untranslated regions.

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apple fruits at 19 DAF, in which putative *PAO* was expressed in our preliminary experiment, was extracted as described by Hao et al. (2005). First-strand cDNA was synthesized using 1 μg of total RNA with a SMART™ RACE cDNA amplification kit (Clontech). To amplify the partial DNA, three degenerate primers [5′-ATS-TCYGGKATHTCGGCR-3′ (pao-f) for first RT-PCR and nested-PCR, 5′-TGTGYTCBCCKGTRAAGWA-3′ (pao-r2) for first RT-PCR and 5′-AYVWDRATRTCVGTN-GCKT-3′ (pao-r1) for nested-PCR] were designed based on the conserved regions of *PAO* homologous sequences from maize, barley, *Amaranthus hypochondriacus* and *Arabidopsis thaliana* (Figure 1). RT-PCR was conducted in a 50 μl reaction mixture containing 2.5 μl of first-strand cDNA, 1 μM of each primer, 200 μM dNTPs, and 0.5 U Ex *taq* polymerase (TaKaRa) in 1×Ex *taq* polymerase buffer using a DNA thermal cycler (Perkin Elmer). RT-PCR conditions were 95°C for 15 s, 45°C for 30 s, and 72°C for 2 min (35 cycles). The reaction was diluted 100-fold, and 1 μl was removed as a template DNA for nested PCR, which was carried out under the same conditions for RT-PCR. First and nested RT-PCR yielded a fragment in size of ca. 930 bp from fruits at 19 DAF using the degenerated primers. Subsequently, the sequences of 5′- and 3′-untranslated regions (UTRs) corresponding to this fragment were determined by 5′- and 3′-RACEs. Two full-length cDNAs, designated as *MdPAO1* (DDBJ accession no. AB250234) and *MdPAO2a* (AB250235), were re-amplified with specific primers designed based on the 5′- and 3′-UTR sequences. Sequences of *MdPAO1* and *MdPAO2a* showed the same length, i.e. each 1872 bp nucleotides with 497 amino acids. However, three nucleotides were different between their ORFs, which caused two synonymous-substitutions and one nonsynonymous-substitution, i.e. Val 451 in MdPAO1 to Ala 451 in MdPAO2a (Figure 1). MdPAOs shared approximate 50% identity to monocots PAOs such as in maize (MPAO) (Tavladoraki et al. 1998), barley (BPAO1 and BPAO2) (Cervelli et al. 2001) and rice (*RPAO*, GenBank accession number AC105731). In contrast, it shared a high identity (72 %) to dicots, *Arabidopsis* PAO (*APAO*; AK118627).

N-terminal regions of MdPAO and APAO had shorter respective twenty-six and twenty-nine residues than that of MPAO in which included signal peptides (Figure 1). On the other hand, the region from Met 1 to Ala 21 in MdPAO (Figure 1) was also predicted as signal peptides by algorithm of SingalP3.0 Server (Technical Univ. of Denmark; http://www.cbs.dtu.dk/services/SignalP/index.php), although the value of signal peptide probability was 0.79 which was lower than 1.0 in MPAO. In addition, a potential consensus sequence of N-glycosylation at Asn 137 in MdPAOs was predicted (Figure 1) by algorithm of Prosite (Swiss Institute of Bioinformatics; http://kr.expasy.org/prosite) like MPAO (Figure 1). According to three-dimensional structural modeling analysis (Cervelli et al. 2001), Phe 431/Tyr 431 (MPAO and BPAO1/ BPAO2 and APAO; numbered based on MPAO and shown by box in Figure 1), Glu 90/Ala 90 (MPAO, BPAO1 and BPAO2/APAO; shown by box in Figure 1) and Glu 198 (conserved in all; shown by box in Figure 1) are key amino-residues for PAO activity (Figure 1). In MdPAO, Phe 431 in MPAO was substituted by Tyr and Glu 198 in MPAO was conserved (Figure 1). These sequences in MdPAO were the same as those in BPAO2 and APAO. Furthermore, Glu 90 in MPAO was substituted by a neutral residue Val in MdPAO, as the same case of a neutral substitution by Ala in APAO (Figure 1). As reviewed by Cerevelli et al. (2001), the substitution of Glu 90 by Val might lead to a different substrate specificity and/or catalytic activity of MdPAO. In C-terminal region, both MdPAO and APAO as well as BPAO2 were longer in size than MPAO and BPAO1 (Figure 1). The secondary structure analysis suggested the highly polar of eight residues at C-terminal extension in BPAO2 resulting in forming α-helix (Cervelli et al. 2001) and Cervelli et al. (2004) confirmed that the region conferred the symplast localization. In MdPAO, the C-terminal region between 427th - 459th amino acid was highly polar and predicted to form α-helix, as in BPAO2 did so (Cervelli et al. 2001). This implies a possibility that MdPAO might not localize in apoplast like MPAO, but in symplast like BPAO2. However, further protein analysis is needed to clarify its localization caused by the feature of C-terminus, together with functions of deduced signal peptide and key amino acids for enzyme activity.

Interestingly, the phylogenetic analysis of plant PAOs roughly classified them into two groups, i.e. monocots- and dicots-groups (Figure 2). Their low similarity and classification between monocots- and dicots-group are supported by DNA blot analysis by Cervelli et al. (2000), in which they reported that MPAO could not hybridize to diocots genome such as *Medicago sativa*, pea and *Nicotiana benthamiana*.

Apart from *MdPAO2a*, another clone (*MdPAO2b*) was found to possess a 186-nucleotide insertion between 348th- and 349th-nucleotide of *MdPAO2a*. Resultantly, *MdPAO2b* was predicted to encode a truncated product by frame-shift. The insertion site in MdPAO2b was located between Ser 101 and Gly 102 in MdPAO1 and 2a, corresponding to the site between Asp 128 and Gly129 in MPAO, Asn 124 and Gly 125 in BPAO1, Ser 124 and Gly 125 in BPAO2 as well as Ser 98 and Gly 99 in APAO (shown by black triangle in Figure 1), into which the second intron was inserted. These predictions suggested that the *MdPAO2b* was a defective derivative from the same genomic DNA as *MdPAO2a* possibly due to the failure to splice the second intron, although analysis of *MdPAO2* genomic sequence is still required.

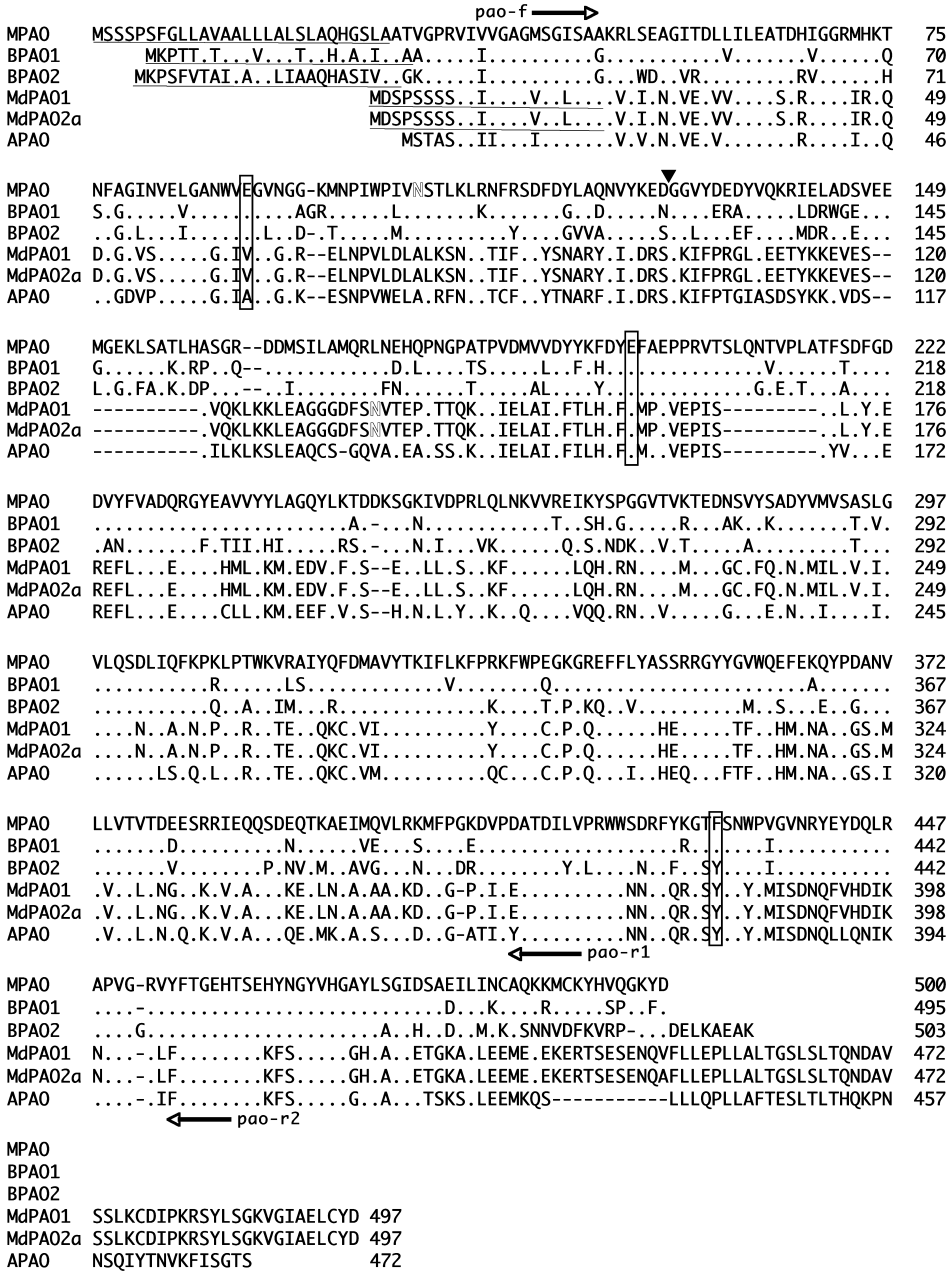

Figure 1. Comparison of amino acid sequences of plant PAOs. Underline and box indicate the putative signal peptides and residues putatively involved in the catalytic activity, respectively. Potential glycosylation sites are indicated by white-letter. Dot and hyphen indicate identical-sequence and gaps, respectively. Black triangle and arrows represent the insertion site of second intron and the degenerated primer sets used for RT-PCR, respectively. MPAO; maize, BPAO; barley, APAO; *Arabidopsis thaliana*, MdPAO; *Malus domestica*.

Subsequently, DNA gel blot analysis was carried out. Genomic DNA was extracted from apple leaves as described by Porebski et al. (1997) and digested with *Eco*RI, *Eco*RV, *Hin*dIII, *Spe*I and *Xba*I, respectively. Since specific probe could not be designed due to the high similarity between *MdPAO1* and *MdPAO2a*, a 930-bp (nucleotides 203rd–1132nd) *MdPAO1* fragment was used as probes in this study. No cut site for used restriction enzymes was found in probe region. One or two major bands were detected for each digestion (Figure 3A), suggesting the presence of two *MdPAO*-related sequences in apple genome, implying *MdPAO1* and *MdPAO2*.

Expression analysis of *MdPAO* in different apple tissues was carried out using the same probe as in DNA gel blot analysis. Total RNA was extracted from suspension cells, leaf, and fruit flesh tissues according to Wan and Wilkins (1994). To investigate the importance of *MdPAO* in cell division/proliferation, its expression was analyzed with apple suspension cells over the culture period (Kitashiba et al. 2005). The growth curve of these cells showed a typical sigmoid shape. During the initial

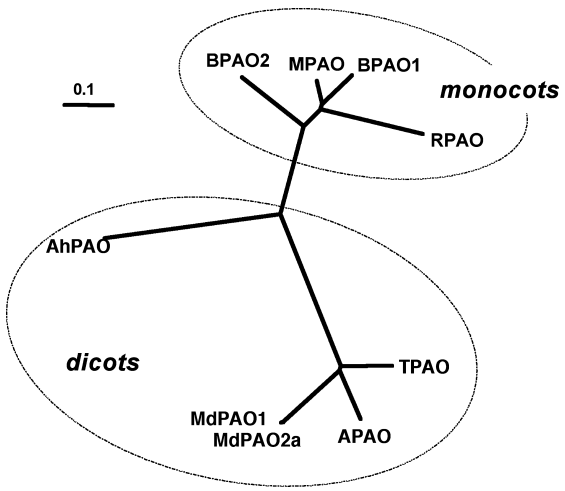

Figure 2. Phylogenetic tree of proteins related to polyamine oxidase, which was created with TreeView program (Page 1996). The distance scale represents evolutionary distance expressed as the number of substitution per residue. RPAO; *rice*, AhPAO; *Amaranthus hypochondriacus*, TPAO; tobacco.

stationary stage from day 0 to 4, the growth rate was quite low. Following the stationary stage, cell growth entered into logarithmic stage which lasted approximately 1st week from day 6 to 14. Subsequently, the cells gradually slowed their growth rate, and eventually entered into the second stationary stage from day 16 to 20. Two bands were detected; lower one possibly corresponding to *MdPAO1* and/or *MdPAO2a* and upper one to *MdPAO2b*. Both signals were low on day 2 but increased gradually and reached a peak on day 20 under this condition (Figure 3B). Since the dramatic increase of *MdPAO* expression at the senescence stage from day 16 to 20 was observed, *MdPAO* might be implicated in maturation and/or senescence of living cells.

In reproductive tissues, the accumulation of *MdPAO* transcript was detected only in fruitlets at 19 DAF with a very low intensity. In fruits at other stages, signal was undetectable (Figure 3C). Similarly, the expression of *MdPAO* in vegetative tissues of apple was barely detected either (Figure 3C). In contrast, *PAO* signals were detected in the vegetative tissues of monocots such as maize and barley (Cervelli et al. 2000, 2001).

Since the expression of *MdPA*O was highly induced at the senescence stage of apple suspension cells (Figure 3B), we expected high expression of *MdPAO* at matured organs of both reproductive and vegetative tissues such as 174 DAF fruits and mature leaf. Its expression, however, was unexpectedly under detectable level, indicating that *MdPAO* may not have positive implication in growth of fruit and leaves.

To our knowledge, this is the first report to isolate and characterize PAO genes from dicots. In this report, different primary features of both amino acid sequences and tissue specificity of gene expression were revealed

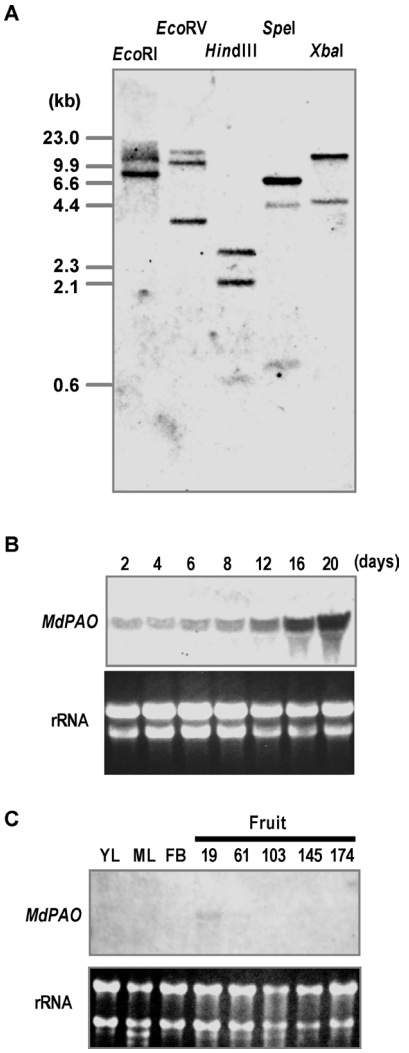

Figure 3. (A) DNA gel blot analysis of *MdPAO* genes in 'Orin' apple. Ten μg of apple genomic DNA was digested with different restriction enzymes. The digested DNAs were fractioned on a 1.0% agarose gel and transferred onto a Hybond N membrane (Amersham Biosciences). Hybridization, washing, and detection were performed as described by Kitashiba et al. (2005). (B) RNA gel blot analysis of *MdPAO* in suspension cells induced from fruitlets of 'Orin' apple (Hao et al. 2005). (C) RNA gel blot analysis of *MdPAO* in various organs of 'Orin'. YL; young leaf, ML; mature leaf, FB; flower bud, 19, 61, 103, 145, and 174; fruits at 19, 61, 103, 145, and 174 days after full bloom, respectively.
In both RNA gel blot analyses, ten micrograms of total RNA were electrophoresed on a 1% agarose gel and then transferred to a Hybond N membrane (Amersham Biosciences) by blotting with 20×SSC. Hybridization, washing, and detection were performed as described by Kitashiba et al. (2005).

between monocots- and dicots-PAOs. Although more detailed analysis such as PAO activity, localization in cells and genomic structure in dicots such as apple and *Arabidopsis* is needed, comparison of PAOs between monocots and dicots is helpful for evolutional analysis as well as to understand their physiological roles and polyamine metabolic profiles.

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