Stromal and thylakoid-bound ascorbate peroxidases are produced by alternative splicing in pumpkin

Shoji Mano[a,b], Katsushi Yamaguchi[a], Makoto Hayashi[a,b], Mikio Nishimura[a,b,*]

[a]*Department of Cell Biology, National Institute for Basic Biology, Okazaki 444, Japan*
[b]*Department of Molecular Biomechanics, School of Life Science, the Graduate University for Advanced Studies, Okazaki 444, Japan*

Received 13 May 1997

Abstract A cDNA encoding stromal ascorbate peroxidase (sAPX) was isolated using poly(A)$^+$ RNA of pumpkin cotyledons by RT-PCR. The cDNA encodes a polypeptide with 372 amino acids and shares complete sequence identity with pumpkin thylakoid-bound ascorbate peroxidase (tAPX), except for the deletion of a putative membrane spanning region located in the carboxy domain of tAPX. Southern blot hybridization and analysis of intron structure indicated that mRNAs for sAPX and tAPX, whose suborganellar localizations in chloroplasts are different, are produced by alternative splicing. Immunoblot analysis showed that the accumulation of sAPX and tAPX was differently regulated during germination and subsequent greening of pumpkin cotyledons.

Key words: Alternative splicing; Chloroplast; Pumpkin (*Cucurbita* sp. cv. Kurokawa Amakuri Nankin); Stromal ascorbate peroxidase (EC 1.11.1.11); Thylakoid-bound ascorbate peroxidase

1. Introduction

Ascorbate peroxidase (APX), which uses ascorbate as the electron donor, has a role in the scavenging of H_2O_2. In higher plants, four isoenzymes of APX with different subcellular localizations have been reported, namely, microbody APX (mbAPX) [1,2], cytosolic APX (cAPX) [3,4], stromal APX (sAPX) [5,6] and thylakoid-bound APX (tAPX) [6,7]. cAPX is localized in the cytosol of both photosynthetic and non-photosynthetic tissues. The function is still obscure, although it has been reported that mRNA for cAPX was induced by environmental stimuli such as drought and heat stress [8,9]. mbAPX is localized on glyoxysomal and leaf-peroxisomal membranes and plays a role in the reduction of H_2O_2 which leaks from glyoxysomes and leaf peroxisomes [1]. Chloroplastic APXs (sAPX and tAPX) scavenge the H_2O_2 within chloroplasts. Chloroplastic APXs have been purified and determined their partial amino acid sequences [5,7,10]. Recently, cDNA for tAPX from pumpkin has been cloned and characterized in our laboratory [11] and Ishikawa et al. [6] also isolated cDNAs for tAPX and sAPX from spinach leaves, showing that amino acid sequences of tAPX and sAPX were identical except for the deletion of the C-terminal domain of tAPX, although both APXs had a putative transit peptide into chloroplasts.

In this paper, we report the characterization of pumpkin sAPX for the purpose of investigating whether sAPX and tAPX are produced by alternative splicing. We isolated cDNA for sAPX from greening pumpkin cotyledons. A Southern blot analysis and the characterization of genomic DNA revealed that the pumpkin gene encoding sAPX and tAPX is a single copy and both APXs were produced by alternative splicing. We also report here the developmental changes in the level of sAPX and tAPX proteins during germination and subsequent greening of pumpkin cotyledons.

2. Materials and methods

2.1. Plant materials

Pumpkin (*Cucurbita* sp. cv. Kurokawa Amakuri Nankin) seeds were soaked overnight and germinated in moist rock fiber (66R; Nitto Bouseki, Chiba, Japan) at 25°C in darkness. Some of these seedlings were transferred to continuous illumination at the 5-day stage.

2.2. Sequencing of cDNA for pumpkin sAPX

First-strand cDNA was generated using a Ready-To-Go T-primed first-strand kit (Pharmacia Biotech, Tokyo, Japan) from poly(A)$^+$ RNA template prepared from 5-day dark- and 4-day light-grown cotyledons using Oligotex-dT 30 <Super> (Roche Japan, Tokyo, Japan). PCR was used to isolate full-length cDNA for pumpkin sAPX. The reaction mixture contained 1 U of Ampli Taq DNA polymerase (Perkin Elmer Japan, Chiba, Japan), an upstream primer (ASA-S1; 5′-ATCGCGTTCATTGCCAGTTG-3′), a downstream primer (SAPX-II; 5′-GACACGGTCACAACAAAACATG-3′) which was synthesized corresponding to the genomic sequence obtained previously (Fig. 2) and an appropriate buffer in a total volume of 50 μl. Each cycle was at 94°C for 45 s, 55°C for 45 s and 72°C for 45 s. The DNA fragment was subcloned into a T-vector prepared using pBluescript KS+ as described previously [12]. The nucleotide sequence was determined with an automatic DNA sequencer (model 377; Perkin Elmer/Applied Biosystems) according to the manufacturer's instructions. The nucleotide and the deduced amino acid sequences were analyzed with DNA analytical software (GeneWorks; IntelliGenetics, Mountain View, CA).

2.3. Cloning of genomic DNA for pumpkin sAPX

An intron of the gene for pumpkin sAPX (tAPX) was amplified by PCR using total genomic DNA (5 ng) as a template. The genomic DNA was extracted from pumpkin leaves by the cetyltrimethylammonium bromide precipitation method [13]. The reaction mixture contained 1 U of Ampli Taq DNA polymerase (Perkin Elmer Japan, Chiba, Japan), an upstream primer (pumpchl A; 5′-GGTATTGTGATTGATGATGC-3′), a downstream primer (3′-non; 5′-GGATCGTGAAATCAGAAGACA-3′) and an appropriate buffer in a total volume of 50 μl. Each cycle was at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. Cloning and sequencing of the DNA were carried out as described previously (see Section 2.2).

*Correspondence author. Department of Cell Biology, National Institute for Basic Biology, Myodaiji, Okazaki 444, Japan. Fax: 81-564-55-7505.

Abbreviations: APX, ascorbate peroxidase; sAPX, stromal APX; tAPX, thylakoid-bound APX; mbAPX, microbody APX; cAPX, cytosolic APX; HPR, hydroxypyruvate reductase; RT-PCR, reverse transcription-polymerase chain reaction

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number D88420.

PII S0014-5793(97)00862-4

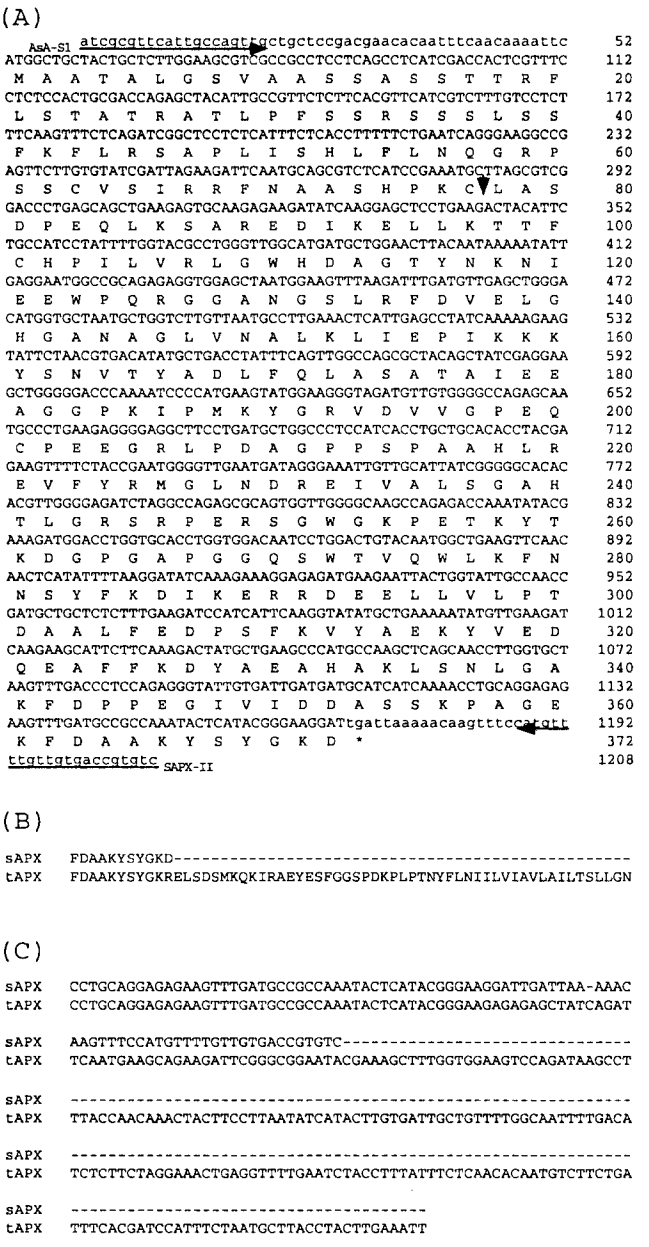

Fig. 1. Nucleotide and deduced amino acid sequences of pumpkin sAPX cDNA. A: The nucleotide sequence of the putative coding region is shown in uppercase letters and the 5′- and 3′-noncoding regions are shown in lowercase letters. The deduced amino acid sequence is presented in the single-letter code under the nucleotide sequence, starting at the first in-frame methionine residue and ending at the first stop codon, indicated by an asterisk. Arrowhead indicates the putative site of cleavage of the transit peptide, as estimated from the N-terminal sequence of tea sAPX and spinach tAPX. Primers for PCR (ASA-S1 and SAPX-II) are underlined with an arrowhead indicating the polarity. B: Comparison of the deduced amino acid sequences of the carboxy termini of sAPX and tAPX [11]. C: Comparison of the nucleotide sequences encoding the carboxy termini of sAPX and tAPX [11].

2.4. Southern blot analysis

Total DNA (10 μg) was digested with *Mlu*I, *Sal*I, *Xho*I and *Bam*HI. The products were fractionated on a 0.8% agarose gel, transferred to a Zeta-Probe blotting membrane (Bio-Rad, CA) by the capillary method and fixed by exposure to UV-light (Funa-UV-Linker, model FS-800; Funakoshi, Tokyo, Japan). The fragment corresponding to pumpkin sAPX was labeled with [^{32}P]dCTP (Amersham, Japan) using a Megaprime DNA labeling system (Amersham, Japan). The membrane was hybridized in 50% formamide, 0.12 M sodium phosphate (pH 7.2), 0.25 M sodium chloride, 7% SDS and 1 mM EDTA (pH 8.0) with 1.0×10^6 cpm·ml^{-1} of radiolabeled DNA probe for 18 h at 42°C. The membrane was washed at 42°C in 2×SSC plus 0.1% SDS for 15 min, in 0.2×SSC plus 0.1% SDS for 15 min, in 0.1×SSC plus 0.1% SDS for 15 min. The membrane was used to expose to the imaging plate of a Bio-Imaging analyzer (FUJIX BAS2000; FUJI Photo Film, Tokyo) with an exposure time of 18 h.

2.5. RT-PCR analysis

First-strand cDNA was synthesized as described previously (see Section 2.2) using poly(A)$^+$ RNA prepared from 5-day dark- and 2-day light-grown cotyledons. PCR was performed as described previously (see Section 2.2) except for EX Taq DNA polymerase (Takara Shuzo, Kyoto, Japan) and specific primers; the 5′-common sense primer of sAPX and tAPX (ASA3; 5′-CCTGGACTGTACAATGG-3′) and the 3′-specific antisense primers for tAPX (3′-non; see Section 2.3) and sAPX (SAPX-I; 5′-AGACGCGGACATCACAGATGTC-3′). The condition were 25 cycles of 45s at 94°C, 45s at 52°C and 45s at 72°C.

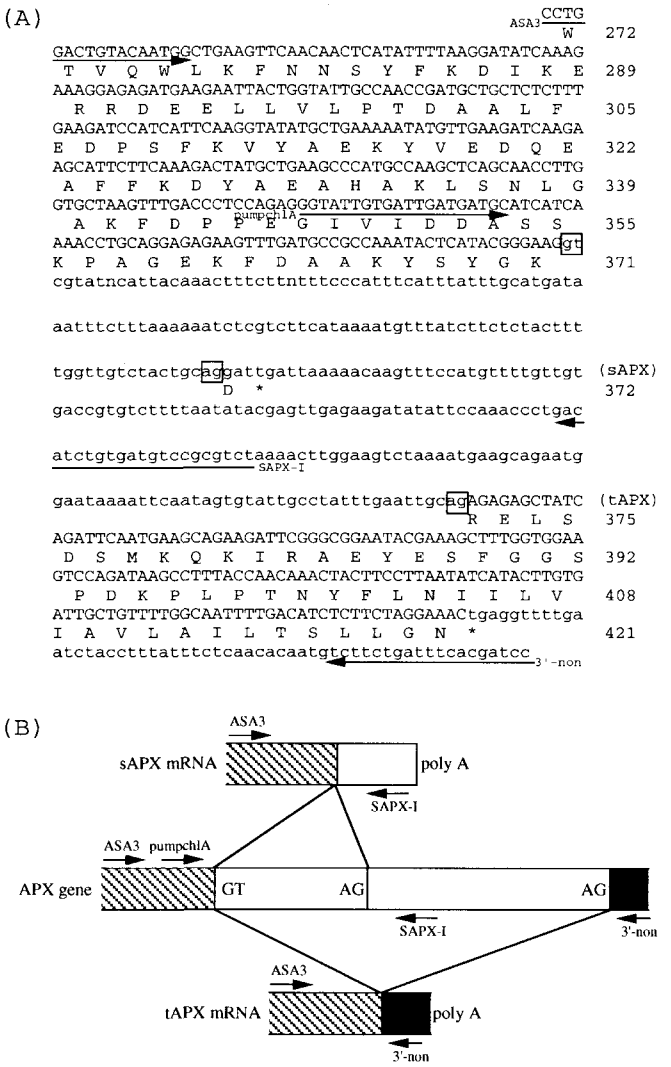

Fig. 2. A: The nucleotide sequence around the carboxy terminal of the pumpkin chloroplastic *APX* gene is shown. The nucleotide sequence of the exon is shown in uppercase letters and the intron and 3′-noncoding region are shown in lowercase letters. The deduced amino acid sequence is presented in the single-letter code under the nucleotide sequence with numberings on the right. Asterisks indicate the stop codons and boxes represent the consensus sequences found in the beginning (GT) and the end (AG) of an intron. PCR primers, ASA3, pumpchl A, SAPX-I and 3′-non, are underlined with an arrowhead indicating the polarity. B: Schematic representation of mRNAs and the gene. The hatched and filled boxes correspond to exons and the open boxes correspond to introns. Primers for PCR and the consensus sequences corresponding to the splice donor and acceptor sites are shown.

2.6. Immunoblot analysis

Pumpkin cotyledons grown under various conditions were homogenized with SDS-loading buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 0.2 mM NaCl and 10% SDS) and then the homogenates were centrifuged at 12 000× rpm for 10 min. Supernatants were subjected to SDS-PAGE on a 10% polyacrylamide gel as described by Laemmli [14] and transferred to a nylon membrane (Schleicher and Schuell, Dassel, Germany) in a semidry electroblotting system. Antibodies against pumpkin mbAPX prepared as described previously [1] were used and the polypeptides on the membrane were visualized with 1:5000 dilution of horseradish peroxidase antibodies against rabbit IgG (ECL System; Amersham, Japan).

3. Results and discussion

sAPX was cross-reacted with antibodies against the carboxy terminal 82-residue polypeptide of tAPX but cAPX and mbAPX were not [11]. The immunoreactivity suggests that sAPX contains homologous sequences to tAPX. Furthermore, we had determined the genomic sequence around the carboxy terminal region of tAPX (Fig. 2A). The genomic sequence showed the possibility that tAPX and sAPX are produced by alternative splicing. Therefore, RT-PCR was performed to amplify the coding region of pumpkin sAPX using primers of ASA-S1 and SAPX-II (Fig. 1). The 1.2-kb product was cloned into pBS vector and sequenced. The complete nucleotide and deduced amino acid sequences are shown in Fig. 1A. The cDNA consists of 1208 bp and contains a 1116-bp open reading frame that encodes a polypeptide with 372 amino acids with a total molecular mass of 40 661 Da. In the case of cleavage of the transit peptide on the C-terminal side of Cys-77, the calculated total molecular mass is 32 594 Da. This is in good agreement with the molecular mass of polypeptides recognized by antibodies against pumpkin mbAPX [11]. The nucleotide and deduced amino acid sequences of sAPX have been reported for spinach [6] and *Arabidopsis* (unpublished, accession no. X98925). The pumpkin sAPX was 76% identical with spinach sAPX. The sequence of the cDNA showed com-

plete identity with that of pumpkin tAPX cDNA [11] except for the carboxy terminal region, which contains a putative thylakoid-spanning domain (Fig. 1B,C). sAPX contained the transit peptide to chloroplasts but the lack of the thylakoid-spanning domain may make it remain in the stroma. The complete identity except for the carboxy terminal region between sAPX cDNA and tAPX cDNA suggests that alternative splicing causes a single gene to give rise to two mRNAs whose translated products are localized in different parts within cells. In higher plants, it has been reported that the synthesis of several enzymes are controlled by alternative splicing [6,15–20]. To investigate whether sAPX and tAPX in pumpkin are also produced by alternative splicing, we amplified pumpkin genomic DNA around the carboxy terminal region by PCR. A unique fragment of about 550 bp in length was amplified, subcloned into pBS vector and sequenced. The sequence showed the presence of a 291-bp intron that contains one donor site and two acceptor sites (Fig. 2A), suggesting that the synthesis of sAPX mRNA or tAPX mRNA depends on which acceptor site is used for the splicing (Fig. 2B), as occurs with the rat A3 adenosine receptor mRNA [21]. The nucleotide sequences of independently isolated products of PCR amplified from the genomic DNA had the same nucleotide sequence as shown in Fig. 2A.

To identify whether another *sAPX* gene exists, DNA from leaves of pumpkin was isolated, digested with *Mlu*I, *Sal*I and *Xba*I (none of which cuts pumpkin sAPX cDNA) and with *Bam*HI (which cuts the cDNA at one site) and then subjected to Southern blot hybridization using pumpkin sAPX cDNA as a probe. As shown in Fig. 3, the sAPX probe hybridized to a single band in the case of the digestion with *Mlu*I, *Sal*I and *Xba*I, while it gave two bands in the case of *Bam*HI. We have already obtained a cDNA clone for pumpkin mbAPX (Yamaguchi and Nishimura, unpublished data). The identity with amino acids between chloroplastic APXs and mbAPX is less than 30%. Although cDNA for pumpkin cAPX has not been cloned, comparison of cDNA sequences from another plants shows more than 80% identity between cAPXs and less than 30% identity between cAPX and chloroplastic APXs. These show that the probe used in Southern blotting (Fig. 3) does not hybridize to genes for mbAPX and cAPX under our experimental condition. These findings indicate that sAPX in pumpkin exists as a single-copy gene and alternative splicing produces two kinds of mRNAs for sAPX and tAPX.

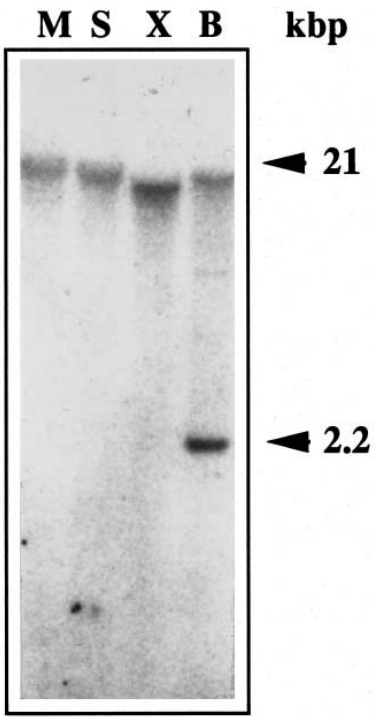

Fig. 3. Southern blot analysis of pumpkin genomic DNA. Genomic DNA (10 μg each) was digested with the restriction endonucleases indicated, separated on a 0.8% agarose gel, transferred to a Zeta-Probe blotting membrane (Bio-Rad) and probed with a ^{32}P-labeled pumpkin sAPX cDNA fragment. The size of DNA fragments are shown in bp on the right. M, *Mlu*I; S, *Sma*I; X, *Xho*I; B, *Bam*HI.

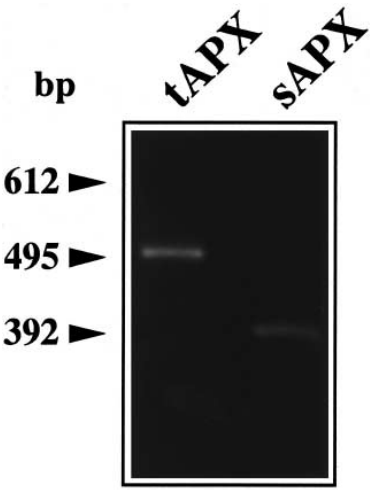

Fig. 4. PCR amplification of truncated sAPX- and tAPX-specific mRNA. RT-PCR was carried out using mRNAs prepared from 5-day dark- and 2-day light-grown cotyledons. The products were analyzed by electrophoresis on a 1% agarose gel and stained with ethidium bromide.

RT-PCR was performed to monitor the presence of two kinds of mRNA as templates of first-strand cDNAs synthesized from mRNAs of 5-day dark- and 4-day light-grown cotyledons. The 5′-common sense primer of sAPX and tAPX (AsA3), and the 3′-specific antisense primers for tAPX (3′-non) and sAPX (SAPX-I) were used. Judging from the nucleotide sequences of sAPX (Fig. 2A) and tAPX [11], the combination of the AsA3 and 3′-non primers should give a 508-bp fragment and that of the AsA3 and SAPX-I primers should give a 406-bp fragment. These bands can be seen in lanes tAPX and sAPX of Fig. 4, respectively. Fig. 4 shows that each band is amplified in accordance with specific primers, suggesting that two kinds of mRNA are expressed within pumpkin leaves.

How are the expressions of sAPX and tAPX regulated? To obtain a clue, we investigated developmental changes in the levels of the sAPX and tAPX proteins in pumpkin cotyledons during germination and subsequent greening. We previously detected four APX isoenzymes with molecular masses of 38, 34, 31 and 28 kDa by using antibodies against pumpkin mbAPX [11]. Furthermore, it was shown from subcellular fractionation experiments that APX isoenzymes with the same molecular masses are localized in the thylakoid, stroma, microbodies and cytosol, respectively [11]. The relative amounts of two kinds of chloroplastic APXs (tAPX and sAPX) in the extracts of pumpkin cotyledons grown in the

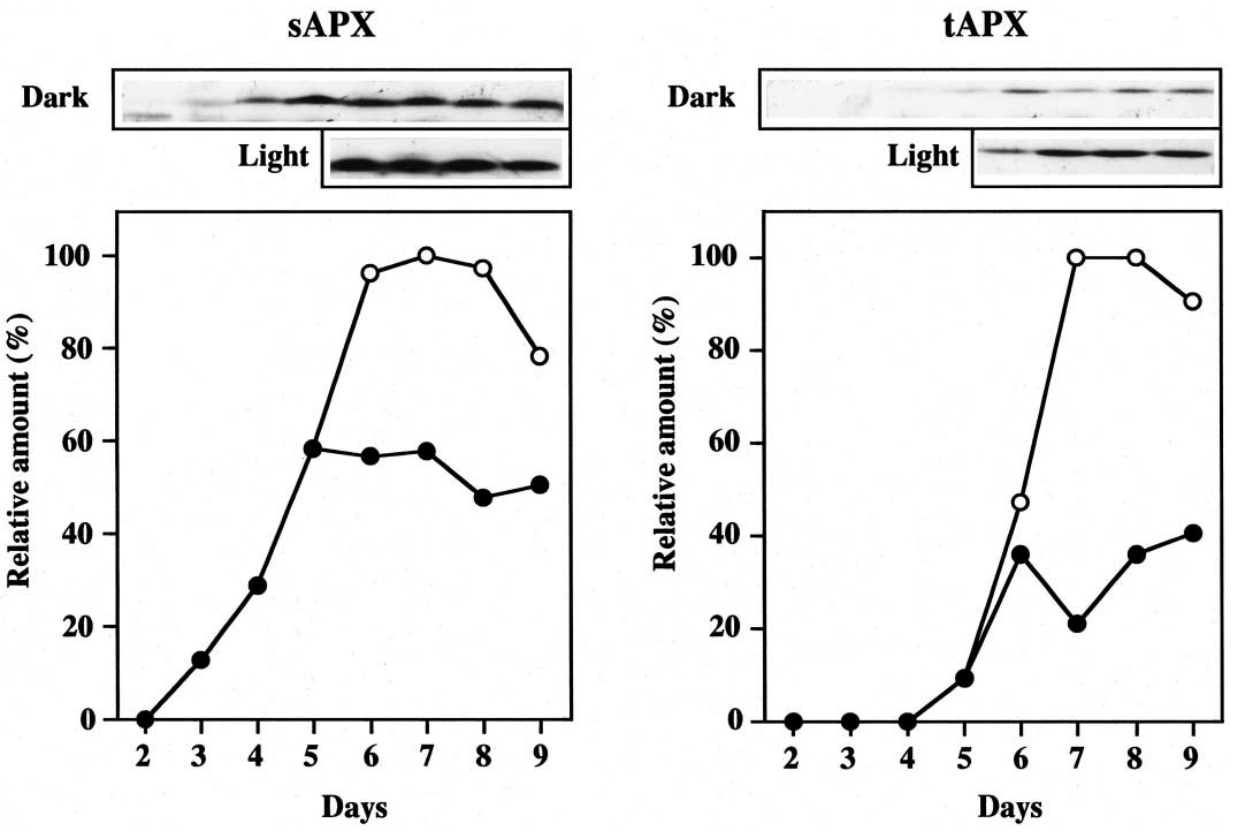

Fig. 5. Developmental changes in the relative amounts of sAPX and tAPX polypeptides in pumpkin cotyledons after germination. Total protein prepared from pumpkin cotyledons was subjected to SDS-PAGE, which was followed by immunodetection with antibodies against mbAPX (top panels). The relative amounts of the sAPX and tAPX polypeptides were determined densitometrically and plotted (bottom panels). (●) seedlings grown in darkness; (○) seedlings transferred to continuous illumination at the 5-day stage.

light and in the dark were determined by immunoblotting (Fig. 5, top panels). The immunoblot was quantified with a densitometer and plotted (Fig. 5, bottom panels). When seedlings were grown in darkness, the level of tAPX protein (right lower panel, closed circles) was hardly detected until the 5th day, whereas the level of sAPX protein (left lower panel, closed circles) gradually increased. The amounts of the two proteins increased rapidly when 5-day-old seedlings were transferred to an illuminated area (lower panels, open circles). Other chloroplastic enzymes also showed rapid increases as a result of illumination [22]. However, the different levels of induction of sAPX and tAPX in 7-day-old greening seedlings in the dark suggest that alternative splicing for sAPX and tAPX mRNAs in the cotyledons might be regulated by their developmental and environmental condition such as light. Although we performed this experiment several times with different extract preparations, the similar developmental patterns were obtained in all cases.

Alternative splicing is a well-known post-transcriptional regulatory mechanism in eukaryotic organisms. It has been reported that the synthesis of several enzymes in plants [15–20,23] and mammals [21,24,25] is controlled by alternative splicing. Previous work in our laboratory has shown that two kinds of cDNA clones for hydroxypyruvate reductase (HPR), HPR1 and HPR2, were produced by alternative splicing and their subcellular localizations were different because HPR1 protein contains the carboxy-terminal tripeptide Ser-Lys-Leu which is known as a targeting signal to microbodies [26,27] but the deduced HPR2 protein does not [17]. In this report, we provide another example that two proteins that are localized in different subcellular compartments and that are produced by alternative splicing. Further analysis at the mRNA level of sAPX and tAPX will provide new information on the regulatory mechanism of the expression of sAPX and tAPX by alternative splicing.

Acknowledgements: This work was supported by a grant for 'Research for the Future' Program (JSPS-RFTF 96L00407) from the Japan Society for the Promotion of Science and Grants-in-Aid for Scientific Research (No. 07456053, 04273101 and 04273102) from the Ministry of Education, Science and Culture, Japan, by a grant from the Nissan Science Foundation (Tokyo, Japan), by the NIBB program for Molecular Mechanisms of Stress Response.

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