Alternatively spliced mRNA variants of chloroplast ascorbate peroxidase isoenzymes in spinach leaves

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We have previously shown that stromal and thylakoid-bound ascorbate peroxidase (APX) isoenzymes of spinach chloroplasts arise from a common pre-mRNA by alternative splicing in the C-terminus of the isoenzymes [Ishikawa, Yoshimura, Tamoi, Takeda and Shigeoka (1997) Biochem. J. **328**, 795–800]. To explore the production of mature, functional mRNA encoding chloroplast APX isoenzymes, reverse transcriptase-mediated PCR and S1 nuclease protection analysis were performed with poly(A)$^+$ RNA or polysomal RNA from spinach leaves. As a result, four mRNA variants, one form of thylakoid-bound APX (tAPX-I) and three forms of stromal APX (sAPX-I, sAPX-II and sAPX-III), were identified. The sAPX-I and sAPX-III mRNA species were generated through the excision of intron 11; they encoded the previously identified sAPX protein. Interestingly, the sAPX-II mRNA was generated by the insertion of intron 11 between exons 11 and 12. The use of this insertional sequence was in frame with the coding sequence and would lead to the production of a novel isoenzyme containing a C-terminus in which a seven-residue sequence replaced the last residue of the previously identified sAPX. The recombinant novel enzyme expressed in *Escherichia coli* showed the same enzymic properties (except for molecular mass) as the recombinant sAPX from the previously identified sAPX-I mRNA, suggesting that the protein translated from the sAPX-II mRNA is functional as a soluble APX *in vivo*. The S1 nuclease protection analysis showed that the expression levels of mRNA variants for sAPX and tAPX isoenzymes are in nearly equal quantities throughout the spinach leaves grown under normal conditions. The present results demonstrate that the expression of chloroplast APX isoenzymes is regulated by a differential splicing efficiency that is dependent on the 3′-terminal processing of *ApxII*, the gene encoding the chloroplast APX isoenzymes.

Key words: mRNA processing, plant mRNA, polyadenylation, post-transcriptional regulation.

INTRODUCTION

Ascorbate peroxidase (APX: EC 1.11.1.11) has a crucial role in the detoxification of intracellular excess H_2O_2 in photosynthetic organisms. Over the past few years it has become apparent that APX isoenzymes are localized in three distinct cell compartments of higher plants: chloroplasts, cytosol and microbodies [1]. We have recently identified, in spinach leaves, full-length cDNA species encoding five types of APX isoenzyme: stromal (sAPX), thylakoid-bound (tAPX), microbody-membrane, cytosolic and unknown (SAP1) forms [2–4]. In our previous study, the first complete cloning of cDNA species and molecular characterization of sAPX and tAPX from spinach leaves have shown that the nucleotide sequence encoding the tAPX isoenzyme is identical with that of sAPX through the coding region up to residue 364, where the remainder of the C-terminal coding region is replaced by a different sequence that encodes 50 residues constituting the hydrophobic thylakoid membrane-binding domain [3]. On the basis of the isolation of the gene (*ApxII*) encoding the chloroplast APX isoenzymes, we have found that mRNA species for both isoenzymes are produced from one gene by alternative splicing of two 3′-terminal exons [5]. The same observation was also reported for pumpkin chloroplast APXs [6]. Although considerable information now exists on the molecular aspects of chloroplast APX isoenzymes, questions remain regarding the gene expression mechanism among the isoenzymes. Chloroplasts are potentially the most powerful source of oxidants and sites within the cell most at risk from photo-oxidative damage. To analyse the potential of the active oxygen-scavenging system of chloroplasts, we introduced *Escherichia coli* catalase, encoded by *katE*, into chloroplasts [7]. Interestingly, we found that chloroplast APX isoenzymes are completely inactivated by high irradiance under drought stress conditions both in the wild-type tobacco plants and in the transgenic plants. This fact suggests that the regulation of APX isoenzymes in chloroplasts might be one of the limiting factors in the mechanism of protection from photo-oxidative damage. Therefore it is important to elucidate how the expression of *ApxII* is regulated. As the first investigation of post-transcriptional regulation, reverse transcriptase-mediated PCR (RT–PCR) and S1 nuclease protection analysis were conducted with poly(A)$^+$ RNA or polysomal RNA from spinach leaves. Here we show that the expression of two chloroplast APX isoenzymes is regulated by pre-mRNA processing steps producing four mRNA variants, one form (tAPX-I) for tAPX and three forms (sAPX-I, sAPX-II and sAPX-III) for sAPX.

Abbreviations used: APX, ascorbate peroxidase; sAPX, stromal ascorbate peroxidase; tAPX, thylakoid-bound ascorbate peroxidase; RT–PCR, reverse transcriptase-mediated PCR.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers D77997 (tAPX), D83669 (sAPX), and AB002467 (*ApxII*).

MATERIALS AND METHODS

Materials

Spinach (*Spinacia oleracea*) seedlings, planted individually in pots, were grown in a climate chamber under the following conditions: an 8 h photoperiod, illumination 150 μE/s per m^2, temperature 15$\pm$2.5 °C and relative humidity 75$\pm$5%. In the middle of the photoperiod, 4-week-old plants were used for the present study. The cDNA species coding for spinach chloroplast APX isoenzymes were originally cloned into plasmid pBluescript SK(+) [3]. Commercial sources of materials were as follows: restriction enzymes and DNA-modifying enzymes from Takara (Kyoto, Japan), and the bacterial expression vector pET-3a and *E. coli* strain BL21(DE3)pLysS from Novagen (Madison, WI, U.S.A.). All other chemicals were of the highest grade of purity commercially available.

Preparation of poly(A)$^+$ RNA and polysomal RNA

Total RNA was prepared by the procedure of Shirzadegan et al. [8]. Poly(A)$^+$ RNA was purified by using the PolyATtract mRNA Isolation Systems (Promega, Madison, WI, U.S.A.). Polysomal RNA was prepared by the method of de Vries et al. [9]. Approx. 60 g of spinach leaves was frozen in liquid nitrogen and homogenized with a mortar and pestle. The leaf powder obtained was gently mixed with 180 ml of polysome buffer [50 mM Tris/HCl

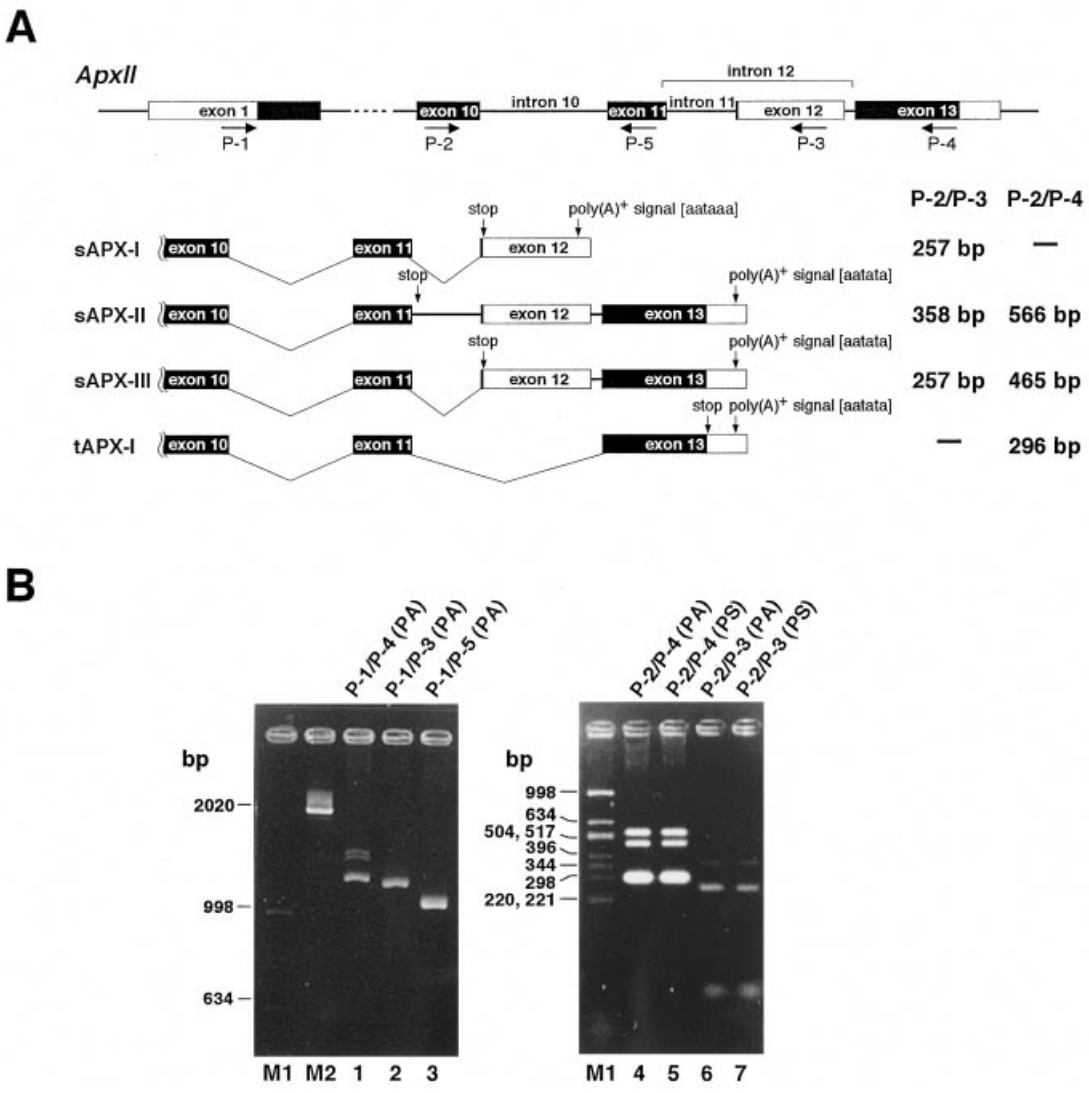


Figure 1 RT–PCR analysis of alternatively spliced mRNA variants of chloroplast APX isoenzymes

(**A**) Schematic representation of *ApxII* and 3′-terminal structures of alternatively spliced mRNA variants. Exon regions are shown as boxes and introns as lines. The open reading frame and untranslated regions are indicated by black boxes and white boxes respectively. Arrows (P-1 to P-5) indicate the locations of primers used in this study; their sequences are described in the Materials and methods section. The expected lengths of the RT–PCR products obtained by using sets of sense primer P-2 and anti-sense primers P-3 and P-4 are indicated. Functional stop codons and polyadenylation signals on each mRNA variant are indicated by stop and poly(A)$^+$ signal respectively. (**B**) RT–PCR analysis. Poly(A)$^+$ and polysomal RNA species from spinach leaves were reverse transcribed and used for RT–PCR analysis as described in the Materials and methods section. Amplified DNA was loaded on a 4% (w/v) agarose gel. The left panel shows results of the analysis with sets of sense primer P-1 and anti-sense primers P-3, P-4 and P-5. The right panel shows results of the analysis with sets of sense primer P-2 and anti-sense primers P-3 and P-4. Lane M1, *Eco*RI- and *Hinf*I-digested pBR322 DNA species used as size markers; lane M2, *Hin*dIII digested λ DNA species used as size markers. Abbreviations: PA, poly(A)$^+$ RNA; PS, polysomal RNA.

(pH 9.0)/50 mM $MgCl_2$/25 mM EGTA/250 mM NaCl/1 % (w/v) Nonidet P40] and ground gently until ice pieces completely disappeared. This suspension was centrifuged at 27000 ***g*** and 4 °C for 15 min, and the supernatant was filtered through a single layer of Whatman 3MM paper. The filtrate was overlaid on a 60 % (w/v) sucrose cushion and centrifuged at 180000 ***g*** and 4 °C for 3 h. The polysome/ribosome pellets obtained were suspended in 3 ml of gradient buffer [10 mM Tris/HCl (pH 8.5/10 mM $MgCl_2$/5 mM EGTA/50 mM NaCl], overlaid on a linear 10–40 % (w/v) sucrose gradient and centrifuged at 140000 ***g*** and 4 °C for 70 min. After fractionation, the polysomal fractions were collected and extracted with phenol/chloroform (1:1, v/v). The aqueous phase was precipitated with ethanol, and the resulting pellets containing polysomal RNA were dissolved in TE buffer [10 mM Tris/HCl (pH 7.0)/1 mM EDTA].

RT–PCR analysis

First-strand cDNA from poly(A)$^+$ RNA or from polysomal RNA obtained was synthesized by using SuperScript™ II RNase H$^-$ Reverse Transcriptase (Gibco BRL, Grand Island, NY, U.S.A.) with an oligo(dT) primer. The reaction was performed in a 40 μl mixture containing a standard enzyme buffer supplied by the manufacturer (Gibco BRL): 2 μg of poly(A)$^+$ RNA or polysomal RNA/1 μM oligo(dT) primer/10 mM dithiothreitol/500 μM dNTPs, containing 200 units of the enzyme. The reaction mixture was incubated at 42 °C for 50 min and then treated with RNase H at 37 °C for 20 min; 2 μl of the mixture was used for PCR analysis as a template cDNA.

To analyse the alternative splicing mechanism of chloroplast APX mRNA species, we used the following oligonucleotide primers: P-1, 5′-AAAACCACCCAATCTCACTCACTT-3′; P-2, 5′-TATGCAGCTGACCAAGAAGC-3′; P-3, 5′-CACAAGAAAATAGCTTCATCTTGC-3′; P-4, 5′-ACTGCCAAAACTCCAATCACAATC-3′; P-5, 5′-CTCCGGCAGGGGTTCCATTAA-3′. The locations of these primers on the chloroplast APX isoenzyme gene, *ApxII*, are shown in Figure 1(A). The PCR amplification was performed in 100 μl reaction mixtures containing 1 × PCR buffer supplied by the manufacturer (Takara), 2.5 mM $MgCl_2$, 200 μM dNTPs, 2.5 units of recombinant *Taq* DNA polymerase (Takara), each of the primers at 1.0 μM, and template cDNA. The PCR protocol consisted of 30 cycles of 94 °C for 60 s, 60 °C for 60 s and 72 °C for 90 s. RT–PCR products were purified by 4 % (w/v) agarose gel electrophoresis and subcloned into pT7Blue T-vector (Novagen). The resulting plasmids were sequenced by the dideoxy chain primer method with an automatic DNA sequencer (373A; Applied Biosystems, Foster City, CA, U.S.A.).

S1 nuclease protection analysis

The primers P-3 and P-4 were labelled at their 5′ ends with [γ-^{32}P]ATP (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.) by using T4 polynucleotide kinase. The probes were synthesized with *Tth* DNA polymerase (Toyobo, Osaka, Japan) and digested with restriction endonucleases. The DNA fragments were separated on 3.5 % (w/v) polyacrylamide/8 M urea gels and purified as single-strand DNA that was complementary to the mRNA. Spinach total RNA (50 μg) was hybridized with 2×10^4 c.p.m. of each probe in 50 μl of hybridization buffer containing 20 mM Hepes, pH 6.5, 50 % (v/v) deionized formamide and 400 mM NaCl. The hybridization mixture was incubated at 80 °C for 10 min, gradually cooled to 37 °C, then incubated at that temperature for 16 h. S1 nuclease (Toyobo) (50 units) was diluted with 300 μl of 30 mM sodium acetate, pH 4.5, containing 200 mM NaCl and 3 mM $ZnSO_4$, then added to each sample. The mixtures were incubated at 37 °C for 10 min. The reactions were terminated by the addition of 10 mM EDTA and the product was precipitated with ethanol. Dried pellets were dissolved in 80 % (v/v) formamide and subjected to electrophoresis on 3.5 % (w/v) polyacrylamide/8 M urea gels. The relative expression ratio of each protected APX transcript was calculated with a Mac BAS 2000 (Fuji Photofilm, Tokyo, Japan) and represent the means for three individual experiments.

Enzyme assays

The APX activity was assayed spectrophotometrically by the method of Shigeoka et al. [10]. Oxidation of alternative electron donors was measured in the same assay mixture as that used for ascorbate, but ascorbate was replaced by 20 mM pyrogallol (ϵ_{430} 2.47 $mM^{-1} \cdot cm^{-1}$), 10 mM guaiacol (ϵ_{470} 22.6 $mM^{-1} \cdot cm^{-1}$), 0.4 mM D-isoascorbate (ϵ_{290} 3.3 $mM^{-1} \cdot cm^{-1}$), 0.15 mM NAD(P)H (ϵ_{340} 6.22 $mM^{-1} \cdot cm^{-1}$) and 40 μM reduced cytochrome *c* (ϵ_{550} 19 $mM^{-1} \cdot cm^{-1}$) [11]. The activity of glutathione peroxidase was monitored spectrophotometrically by following the oxidation of NADPH in the presence of glutathione reductase [10].

Expression of the recombinant enzyme from sAPX-II mRNA in *E. coli*

To amplify the mature enzyme-coding region of the novel sAPX-II mRNA by PCR, we used an oligonucleotide (5′-TTTAGCACGCATATGTACGCTTCTGATCC-3′) containing an *Nde*I site as a sense primer and an oligonucleotide (5′-CTGTATGGATCCTAAGAATGATT-3′) containing a *Bam*HI site as an anti-sense primer. The PCR was performed under the same conditions as described above except that the temperature of the annealing step was 50 °C. The amplified DNA fragment was isolated and subcloned into a pT7Blue-T vector and then into the *Nde*I/*Bam*HI site of pET-3a. The sequence of the inserted region in the pT7Blue-T vector was verified by DNA sequencing. *E. coli* cells [strain BL21(DE3)pLysS] were transformed with the resulting plasmid, designated pET/sAPX-II, and grown in 50 ml of Luria–Bertani medium supplemented with 50 μg/ml ampicillin at 37 °C. When the culture reached a D_{600} of 0.6, isopropyl β-D-thiogalactoside was added to a concentration of 0.4 mM. The cells were grown further at 37 °C for 6 h, harvested by centrifugation, suspended in 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM ascorbate, and disrupted by sonication for preparation of the cell lysate. The resulting cell lysate was centrifuged at 15000 ***g*** and 4 °C for 20 min. The recombinant novel sAPX-II was partly purified by DEAE-Sephacel column chromatography from the cell lysate by the method of Yoshimura et al. [11]; the enzyme, with a specific activity of 168.8 μmol/min per mg of protein, was used for analysis of the enzymic properties.

Immunoblot analysis

Intact chloroplasts were isolated from spinach leaves by Percoll density centrifugation [12]. Proteins were separated by SDS/PAGE [12.5 % (w/v) gel] and transblotted to a nitrocellulose membrane with the transfer buffer of Towbin et al. [13]. The membrane was incubated with *Euglena* APX monoclonal antibody (EAP1), which reacted with chloroplast and cytosol APXs from higher plants [14]; proteins were then detected with horseradish peroxidase-conjugated goat anti-(mouse IgG) (Cappel, Organon Tekunika, Durham, NC, U.S.A.) and 4-methoxy-1-naphthol (Aldrich, Milwaukee, WI, U.S.A.).

RESULTS

Detection of alternatively spliced chloroplast APX mRNA species by RT–PCR analysis

The previous studies on cDNA and genomic cloning provided evidence that chloroplast sAPX and tAPX mRNA species are produced by alternative splicing of two 3′-terminal exons, the penultimate exon 12 for sAPX and the final exon 13 encoding the hydrophobic thylakoid-membrane-binding domain of tAPX [3,5]. To clarify the alternative splicing mechanism of chloroplast APX isoenzymes, we analysed the expression of chloroplast APX mRNA species in spinach leaves by RT–PCR analysis.

First we chose the 5′-common sense and 3′-common anti-sense primers, P-1 and P-5 for sAPX and tAPX, and the 3′-specific anti-sense primers, P-3 for sAPX and P-4 for tAPX respectively, as shown in Figure 1(A). The RT–PCR analysis with sets of primers (P-1 and P-3 for sAPX; P-1 and P-4 for tAPX) revealed the generation of several amplified products in addition to the expected product by agarose-gel electrophoresis (Figure 1B, lanes 1 and 2). When the RT–PCR analysis was also performed with a set of primers (P-1 and P-5) specific to the common region of both isoenzymes, only a single amplified product corresponding to the size predicted from the cDNA sequence was detected (Figure 1B, lane 3). The template RNA species used were treated with DNase I. Moreover, all of the RT–PCR products were detected by Southern blot analysis with both sAPX and tAPX cDNA species as probes (results not shown), indicating that they are not due to the amplification of unrelated mRNA species or the contamination of the genomic DNA. This suggested the possibility that the generation of several RT–PCR products results from alterations in the length of their 3′-terminal sequence region rather than alterations in the common coding sequence region consisting of exons 1–10.

Next we focused on the 3′-terminal sequence region of the APX isoenzyme mRNA species to clarify their structures. On the basis of the structure of the spinach chloroplast APX gene, *ApxII* [5], further RT–PCR analysis was performed with an upstream sense primer derived from exon 10 (P-2) and downstream anti-sense primers specific to the respective 3′-terminal region of each isoenzyme situated on positions in exons 12 (P-3) and 13 (P-4). The specific primer pair for sAPX (P-2 and P-3) produced two RT–PCR products: one fragment of 257 bp, corresponding to the expected size for the 3′-terminal sequence of the previously identified sAPX mRNA [3,5], and another fragment longer than 257 bp (Figure 1B, lane 6). The specific primer pair for tAPX (P-2 and P-4) produced three PCR products: one fragment of 296 bp, corresponding to the expected size for the 3′-terminal sequence of the previously identified tAPX mRNA [3,5], and two other marker fragments of more than 396 bp (Figure 1B, lane 4). The sequence analysis of the 257 and 296 bp products revealed that they were identical with the cDNA species derived from the respective mRNA spliced intron 11 or intron 12. Thus the mRNA variants containing the sequences of 257 and 296 bp products were designated sAPX-I and tAPX-I respectively (Fig-

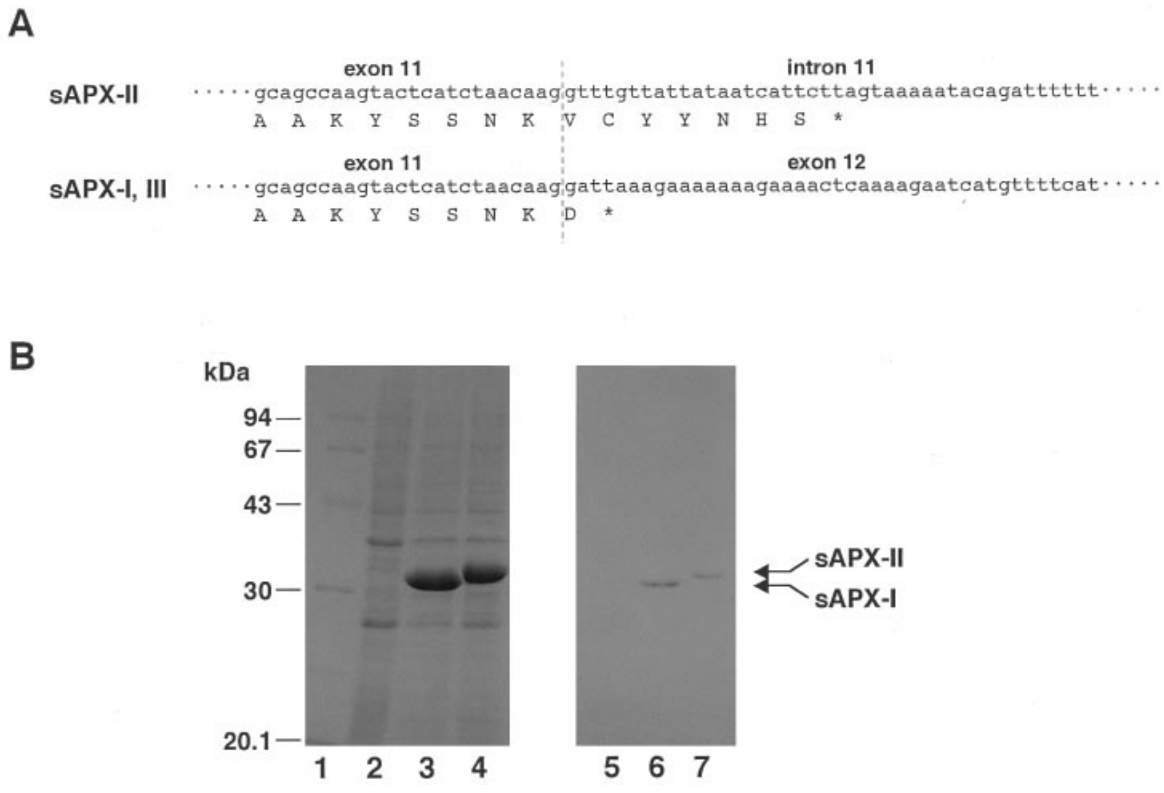


Figure 2 Expression of the previously identified sAPX (sAPX-I) and the novel sAPX (sAPX-II) in *E. coli*

(**A**) The 3′-terminal sequences of sAPX-I, sAPX-II and sAPX-III cDNA. The amino acid sequences deduced from each open reading frame are shown in single-letter code below the nucleotide sequences. (**B**) Protein samples (corresponding to 25 μg of total protein) prepared from cell lysates with induction by 0.4 mM isopropyl β-D-thiogalactoside for 6 h were analysed by SDS/PAGE [12.5% (w/v) gel] and immunoblotting. The left panel shows results of Coomassie Brilliant Blue staining; the right panel shows results of immunoblots with monoclonal antibody raised against *Euglena* APX [14]. Lane 1, molecular mass standards (Pharmacia); lanes 2 and 5, pET-3a-transformed *E. coli*; lanes 3 and 6, pET/sAPX-I-transformed *E. coli* [11]; lanes 4 and 7, pET/sAPX-II-transformed *E. coli*. The experimental conditions were as described in the Materials and methods section.

ure 1A). The sequence analysis also revealed that the remaining three RT–PCR products contained 358, 465 and 566 bp sequences. The nucleotide sequence of the 358 bp product was identical with that of the 5′ region of the 566 bp product, indicating that the 358 and 566 bp products were derived from the same mRNA. The sequence structures of the 566 and 465 bp products were characterized by alignment between these nucleotide sequences and *ApxII*. Interestingly, the 566 and 465 bp products were respectively 270 and 169 bp longer than that of the tAPX-I mRNA, and the respective insertional sequences were identical with the whole or partial sequence between exon 11 and 13 in *ApxII*. The 169 bp insert of the 465 bp product consisted of exon 12 and a 14 bp non-coding sequence (Figure 1A). Because exon 12 following exon 11 contained a stop codon (TAA), and exon 13 following the 14 bp non-coding sequence contained a polyadenylation signal (AATATA), the mRNA containing the sequence of 465 bp products would encode the previously identified sAPX protein and was therefore called sAPX-III (Figure 1A). The 270 bp insert in the 566 bp product consisted of intron 11, the penultimate exon 12 and the 14 bp non-coding sequence. In this case, it is worth noting that intron 11 directly following exon 11 is in frame with the coding sequence, and consequently the presence of intron 11 would lead to a product of the final seven residues (Val-Cys-Tyr-Tyr-Asn-His-Ser) of the deduced polypeptide sequence, leading to the synthesis of a novel sAPX protein as described below (see Figure 2A). The mRNA containing the sequence of the 566 bp product was therefore called sAPX-II.

To confirm the existence of four transcripts as functional mRNA species, we prepared polysomal fractions from spinach leaves and analysed the chloroplast APX mRNA species in the polysomal RNA pool by using RT–PCR (Figure 1B, lanes 5 and 7): all alternatively spliced mRNA species were found in the polysomal RNA pool. These results clearly indicate that the generation of the tAPX and three different sAPX mRNA species is apparently due to alterations in the length of the 3′ regions of the gene and that the alternatively spliced mRNA species are transported from the nucleus to the cytoplasm and incorporated into polysomes; therefore all of these mRNA variants participate in protein synthesis for chloroplast APX isoenzymes. The S1 nuclease protection analysis, discussed below, also supported this view.

Expression of recombinant novel sAPX derived from the APX-II mRNA in *E. coli*

The detection of sAPX-II in the polysomal fraction ruled out the possibility of its derivation from pre-mRNA, although the 3′-terminal sequence is identical with that of *ApxII*. The use of this insertional sequence led to the production of a novel protein that was identical with sAPX except that it had a C-terminus in which a seven-residue sequence replaced the last residue, Asp, of the previously identified sAPX (Figure 2A). The seven-residue sequence had no significant similarity to known organelle targeting signals and no hydrophobic domain available for binding to thylakoid membrane. As shown in Figure 2(B), the recombinant novel protein was expressed in *E. coli* by using the pET expression system. The product was detected in a soluble fraction as nearly 30% of the total protein in the *E. coli* cells by SDS/PAGE analysis (Figure 2B). The position of the protein band was consistent with the molecular mass of 34 kDa calculated from the deduced amino acid sequence of the novel protein, which was approx. 700 Da greater than that of the recombinant sAPX derived from the previously identified sAPX-I mRNA [11]. Immunoreactive bands obtained with the monoclonal antibody raised against *Euglena* APX were observed at the positions corresponding to the recombinant proteins derived from the previously identified sAPX-I and the novel sAPX-II mRNA species in gels stained with Coomassie Blue (Figure 2B). Table 1 shows a comparison of the enzymic properties of the recombinant enzymes derived from sAPX-II and sAPX-I mRNA species. The relative activities of electron donors with the novel recombinant sAPX were similar to those with the recombinant enzyme of the previously identified sAPX (Table 1). One of the specific properties of chloroplast APX isoenzymes is rapid inactivation in an ascorbate-depleted medium [1]. When the partly purified novel recombinant sAPX was diluted with ascorbate-depleted medium in accordance with the method described previously [4], the time to half inactivation of the enzyme was less than 1 min (results not shown), in agreement with that of the previously identified sAPX [11]. These results indicate that, in every respect, the novel sAPX has enzymic properties identical with those of the previously identified sAPX isoenzyme, except for its molecular mass; they also suggest that the sAPX-II mRNA containing the sequence of the 566 bp RT–PCR product encodes the novel sAPX isoenzyme and that the isoenzyme should be localized in the stromal fraction.

Table 1 Comparison of enzymic properties of recombinant enzymes derived from sAPX-II and sAPX-I mRNA species

Data for the recombinant sAPX-II are means for three individual experiments; data for the recombinant sAPX-I are taken from [11]. The peroxidase activity for ascorbate is shown as 100%. Data in parentheses are calculated from deduced amino acid sequences.

Property	Recombinant sAPX-II	Recombinant sAPX-I
Molecular mass (kDa)	34 (32.991)	33 (32.239)
Donor specificity (%)		
Ascorbate	100	100
Isoascorbate	53	58
GSH	0	0
Cytochrome *c*	0	0
NAD(P)H	0	0
Pyrogallol	14	19
Guaiacol	0.3	0.6
K_m (mM)		
Ascorbate	0.34	0.33
H_2O_2	0.04	0.04
Optimum pH	7.0	7.0

Determination of levels of the chloroplast APX mRNA variants by S1 nuclease protection analysis

As described above, the analysis of chloroplast APX mRNA species by RT–PCR showed that the use of the alternative 3′-terminal region of *ApxII* following exon 11 gives rise to four mRNA variants. To quantify the four types of chloroplast APX mRNA, S1 nuclease protection analysis was performed with the total RNA prepared from spinach leaves grown for 4 weeks under normal conditions. A diagram of the anti-sense probes designed and the detectable fragment sizes estimated is given in Figure 3(A). As shown in Figure 3(B), all of the mRNA variants, sAPX-I, sAPX-II, sAPX-III and tAPX-I, were also confirmed in this analysis. No additional band hybridizing with the probes used was detected at significant levels, supporting the belief that there is no extra form besides the four mRNA variants. The relative expression ratio calculated for the mRNA variants sAPX-I, sAPX-II, sAPX-III and tAPX-I was 21:5:32:42. A similar situation was also obtained with each mRNA variant in

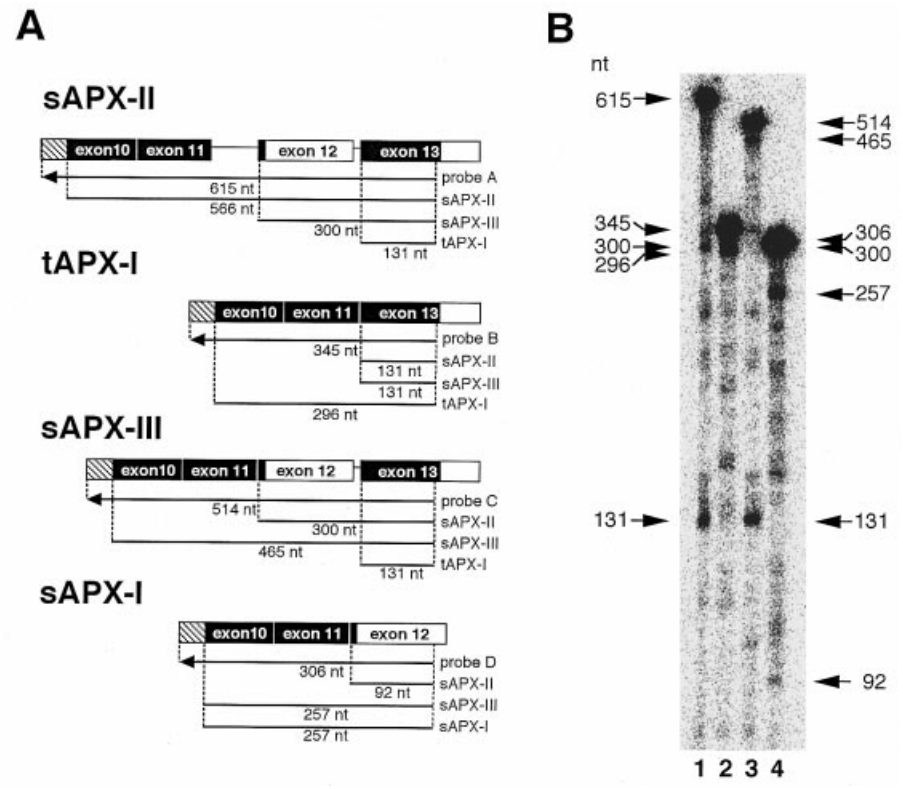


Figure 3 S1 nuclease protection analysis of alternatively spliced chloroplast APX mRNA species

(**A**) The experimental design of an anti-sense probe used for S1 nuclease analysis. Exon regions are shown as boxes and introns as lines. The open reading frame and untranslated regions are indicated by black boxes and white boxes respectively. Hatched boxes are residual regions of the plasmid vector after preparation of the probes. Arrows denote oligonucleotide probes (A–D) used for S1 nuclease protection analysis. Lines shown below are the sizes of the protected fragments. (**B**) Detection of mRNA variants by S1 nuclease analysis. Bands indicated by arrowheads with sizes in nt represent protected fragments from 50 μg of total RNA hybridized with single-stranded DNA probes A (lane 1), B (lane 2), C (lane 3) and D (lane 4). The experimental details were as described in the Materials and methods section.

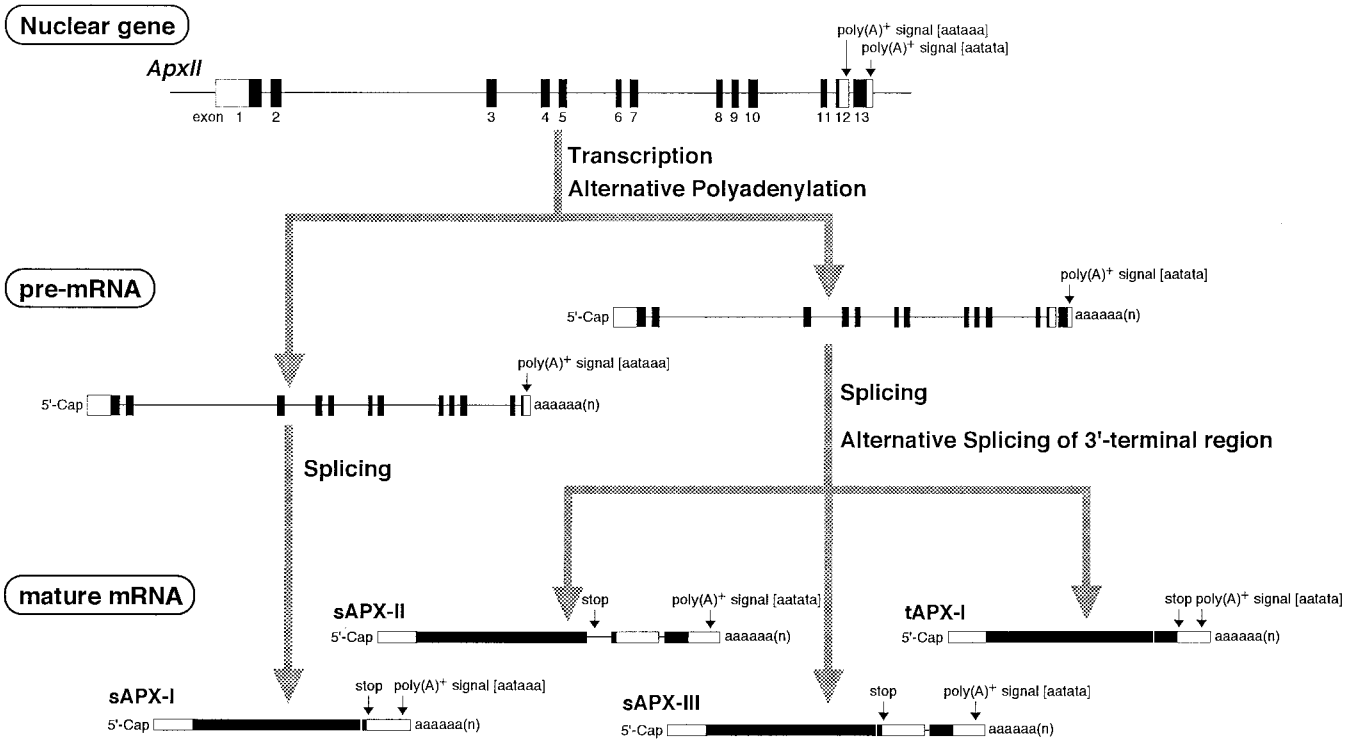
the polysomal RNA. Moreover almost the same results were obtained when the total RNA was prepared from spinach mature leaves grown for 12 weeks under normal conditions (results not shown).

The sAPX-II mRNA was expressed at a low level compared with other mRNA variants. This result is consistent with the fact that there was almost no detectable amount of its protein by immunoblot analysis with crude extracts from spinach leaves (results not shown). On the basis of the results reported here, the total mRNA expression ratio of sAPX to tAPX was 58:42, a value that was roughly in agreement with the fact that the enzyme activities for both chloroplast APX forms are in almost equal quantities throughout the spinach leaves under normal conditions [15].

DISCUSSION

The alternative splicing of pre-mRNA is a common mechanism for increasing the flexibility of eukaryotic gene expression by the generation of structurally distinct isoenzymes from a single gene. On the basis of the present results, a possible mechanism of generation for mature mRNA species encoding chloroplast APX isoenzymes is shown in Scheme 1. Four types of chloroplast APX mRNA variant are produced as a result of alternative polyadenylation sites and splicing in which three alternative 5′-splice sites compete for a common 3′-splice site of exon 11 of *ApxII*.

It is well established in animal cells that splicing and polyadenylation interact to influence the efficiency of both processes and that the polyadenylation signal is essential for 3′-end formation [16]. In contrast with the current state of knowledge in animal and yeast systems, little is known about the process of mRNA 3′-end processing or splicing in higher plants. However, recent studies suggest that 3′-end processing systems in higher plants are more complex and diffuse than those in animals. For 3′-end processing in animal systems, a highly conserved polyadenylation signal (AAUAAA) is essential and the spacing between the sequence and the downstream element, which is located downstream of the cleavage site and generally is GC- or U-rich, is also important [17]. In contrast, in higher plants the AAUAAA sequence is not necessarily required and many AAUAAA variants show almost the same 3′-end formation activity [18–20]. Rothnie et al. [20] have also reported that a repeated UUUGUA motif located upstream of the polyadenylation signal and a spacing between the motif and the signal are important for efficient 3′-end processing in cauliflower mosaic virus. Taken together, these results account for the production mechanism of chloroplast APX mRNA variants. In *ApxII*, two polyadenylation signals were located in exon 12 (AAUAAA) and exon 13 (AAUAUA) (Figure 1A), and significant repeated UUUGUA motifs were not identified upstream of both signals, suggesting that a 3′-end processing of the gene might show low efficiency and that the processing is



Scheme 1 Diagram of alternative splicing patterns producing spinach chloroplast APX mRNA species

The diagram is based on results from experiments described here. Functional stop codons and polyadenylation signals for each mRNA variant are indicated by stop and poly(A)$^+$ signal respectively.

dependent on the selection of either signal. If the AAUAAA signal in exon 12 is selected, the only resulting product followed by splicing of intron 11 should be sAPX-I mRNA encoding the previously identified sAPX isoenzyme (Scheme 1). In this case, the selective ratio of the AAUAAA signal in exon 12 was lower than that of the AAUAUA signal in exon 13 because the relative expression ratio of sAPX-I mRNA calculated by S1 nuclease protection analysis was 21 % (Figure 3B). In contrast, selection of the AAUAUA signal in exon 13 caused more complexity and, as a result, three mRNA variants (sAPX-II, sAPX-III and tAPX-I) were produced (Scheme 1). Intron 12 is composed of intron 11, exon 12 and the 14 bp encoding sequence, which is AU-rich (71.1 %) [5]. It has been shown that the splicing ability of an intron can depend on an AU-rich sequence [21,22]. For all of the coding exons, the intron–exon splice junctions of *ApxII* were readily identifiable, which conformed to the consensus sequences GT at the donor site and AG at the acceptor site [5]. After recognition of the polyadenylation site, mature sAPX-III and tAPX-I mRNA species encoding known chloroplast APX isoenzymes are generated by alternative excision of intron 11 or intron 12 as well as excisions of introns 1–10. Although the detectable level of the sAPX-II mRNA is very low (5 % of total) in comparison with that of other variants, the transcript was actually found in association with polysomes (Figures 1B and 3B), indicating that it probably gives rise to a novel sAPX isoenzyme that had seven additional residues at the C-terminus of the previously identified sAPX. The fact that the recombinant novel sAPX isoenzyme exhibited properties identical to those of the previously identified sAPX strongly supported the contention that the novel sAPX isoenzyme is functional *in vivo* as a stromal enzyme.

The relative expression ratio calculated for mRNA variants for sAPX and tAPX isoenzymes was nearly similar to that of the enzyme activities for both chloroplast APX forms in spinach leaves grown for 4 weeks under normal conditions. Similar results were also obtained with mature spinach leaves grown for 12 weeks (results not shown). Accordingly, it seems likely that the production of mature, functional mRNA species for sAPX and tAPX by alternative splicing, resulting in the expression of sAPX and tAPX isoenzymes in almost equal quantities, occurred constitutively in spinach leaves grown under normal conditions. Mano et al. [6] have reported that the accumulation of sAPX and tAPX isoenzymes in pumpkin cotyledons was regulated differently during germination and during subsequent greening. It is worth pointing out that some environmental stresses can lead to a splicing failure [23–25]. It might be that an unspliced mRNA such as sAPX-II accumulates in response to the presence or absence of environmental stresses. At the present time, we do not know clearly what *cis*-acting signals and *trans*-acting factors are involved in 3′-end processing including splicing to produce mature mRNA variants. These matters will be addressed in forthcoming papers.

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