

Regulation of tissue-specific alternative splicing of chloroplastic ascorbate peroxidase isoenzymes in spinach

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Introduction

Even under optimal conditions, the process of chloroplastic electron transport produces active oxygen species such as superoxide radical, H₂O₂, and hydroxyl radical. APX (EC 1.11.1.11), which has an important role in scavenging excess H₂O₂, exists as stromal soluble (sAPX) and thylakoid membrane-bound (tAPX) forms in chloroplasts of higher plants (Asada 1999). We have previously shown that, in spinach, chloroplastic APX (chlAPX) isoenzymes are encoded by a single gene (*ApxII*) (Ishikawa et al. 1997) and four types of chlAPX mRNA variants, one form encoding tAPX (tAPX-I) and three forms (sAPX-I, sAPX-II, sAPX-III) encoding sAPX, are produced by the alternative splicing event of the 3'-terminal region (Yoshimura et al. 1999). In this study, we analyzed the expression ratio of chlAPX isoenzymes in several tissues of mature spinach plant and the existence of the possible regulation factor as a *cis*-element involved in alternative splicing.

Materials and methods

Spinach seedlings (*Spinacia oleracea*) were grown in a climate chamber under the following normal conditions: an 8-h photoperiod, illumination of 300 $\mu\text{E m}^{-2} \text{s}^{-1}$, 15 \pm 2.5°C, and a relative humidity of 75 \pm 5% for 4 weeks to obtain mature plants. Preparation of total RNA from leaves, stems, and roots of spinach plants and Northern blot analysis were carried out as described in Yoshimura et al. (2000). The RNase protection analysis was carried out according to the instruction manual of the RPAII kit (Ambion, TX, USA). The extraction of protein from nuclei was carried out according to Mikami et al. (1994) with some modifications. The RNA mobility-shift analysis was performed essentially according to Guo et al. (1991) with some modifications.

Results

The steady-state transcript levels of chlAPX isoenzymes in leaf, stem, and root--- To determine whether the transcription level of *ApxII* was altered in several tissues, we analyzed the steady-state transcript levels of total chlAPX in several tissues of mature spinach plants by Northern blotting using tAPX cDNA as a probe. Interestingly, even in the non-photosynthetic tissue such as root, the chlAPX gene was expressed and almost equal quantities of chlAPX mRNA variants were detected in the leaf, stem, and root of mature plants (data not shown).

The expression ratios of alternatively spliced chlAPX mRNA variants in each tissue--- We analyzed the expression ratio of four types of mRNA variants in several tissues of mature spinach plants by RNase protection analysis (Fig. 1). The expression ratio of those mRNA variants was extremely different in each tissue. In leaf, the relative expression ratio of three types of sAPX mRNAs to tAPX mRNA was in almost equal quantities. In contrast, the ratios of sAPX mRNAs to tAPX mRNA in stem and root strongly increased. The differences of those ratios in both tissues were caused by the increase in sAPX-III mRNA and the decrease in tAPX-I mRNA. The ratio of sAPX-I and sAPX-II was not altered. This result clearly indicates that the expression ratio of chlAPX isoenzymes is regulated by tissue-specific alternative splicing.

The putative cis-element to regulate tissue-specific alternative splicing--- Significant structural difference among four spliced mRNA variants of chlAPX is within the 3'-terminal region consisting of intron 11 to exon 13 of the gene (*ApxII*). This fact indicates that an essential regulation factor as a *cis*-element for alternative splicing would be appeared within this 3'-terminal region. Thus, we carried out the computer-assisted analysis using chlAPX genes reported previously (Yoshimura et al. 1999). Interestingly, high homology was observed in introns 11 and 12 in these regions of three plant species and the immediately upstream region of exon 13 in these sequences was highly conserved (data not shown). These facts suggest that this specific region may be a *cis*-element to regulate tissue-specific alternative splicing. We designated this region as a putative splicing regulatory element (SRE; Fig. 2).

Identification of a trans-acting factor that binds to SRE by gel-shift analysis--- We carried out gel-shift analysis to detect the RNA-protein interaction between the SRE and the nuclear proteins extracted from the leaf, stem, and root of spinach. When the 32 P-labeled RNA probe encoding the 3'-terminal sequence of the spinach chlAPX gene including the SRE region (T1; line in Fig. 3) was expressed *in vitro* and then incubated with nuclear proteins prepared from each tissue, a RNA-protein complex with different mobility from that of probe RNA was

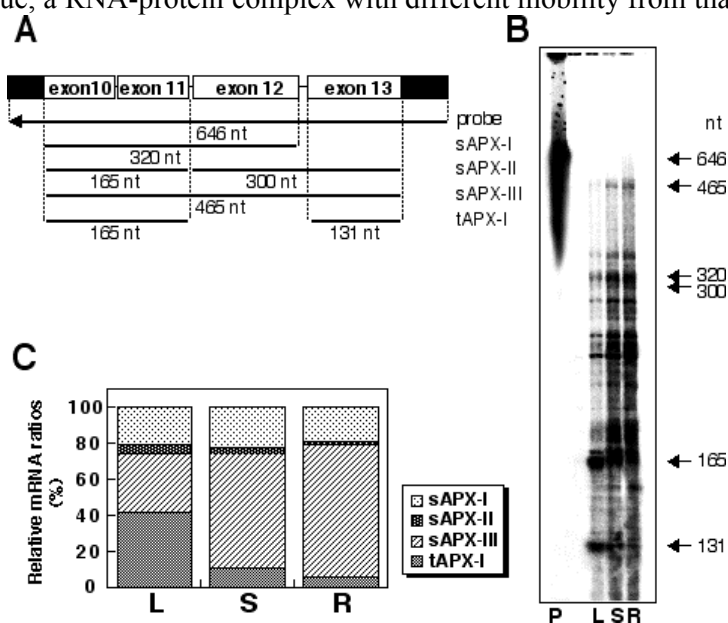


Fig. 1 Expression ratios of alternatively spliced chlAPX mRNA variants in each tissue of spinach. (A) The experimental design of an antisense probe was used for the RNase protection analysis. Arrows represent the antisense RNA probe corresponding to a portion of the plasmid vector (black box) and a 3' portion of sAPX-III. Exons and introns are shown by boxes and lines, respectively. (B) RNase protection analysis. Bands indicated by arrowheads with size in nt represent protected fragments hybridized with the antisense RNA probe. P, RNA probe; L, leaf RNA; S, stem RNA; R, root RNA. (C) Relative expression ratios of chlAPX mRNAs (sAPX-I, sAPX-II, sAPX-III, and

detected (Fig. 3). Interestingly, the highest degree of intensity of the mobility band was detected in the leaf nuclear proteins. Furthermore, when an unlabeled RNA probe (T1) was added in the reaction as a competitor, the mobility band disappeared (data not shown). No significant mobility band was observed in root nuclear proteins. When the 32 P-labeled RNA probe encoding the acceptor site of intron 11 in the spinach chlAPX gene, which did not include the SRE region (T2; dotted line in Fig. 3), was used, two RNA-protein complexes with different mobility from that of the probe RNA were detected. However, the intensities of these mobility bands in each tissue were almost of equal degree. These results suggest that

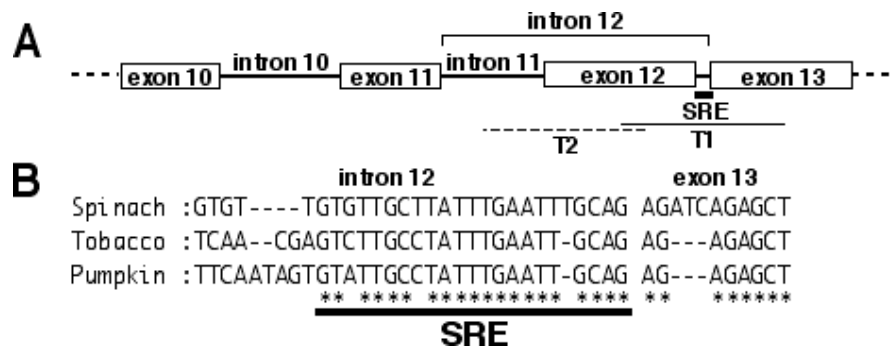


Fig. 2 Comparison of the nucleotide sequences of 3'-terminal regions of chlAPX gene in spinach, tobacco, and pumpkin. (A) A schematic representation of the 3'-terminal region of the chlAPX gene. The bold line indicates the SRE region. The solid line and the dotted line indicate the sequences of T1 and T2 probes, respectively, used in the gel mobility shift analysis. (B) The sequence alignment is shown for the nucleotide sequences of the chlAPX genes from higher plants. The bold line indicates the SRE region.

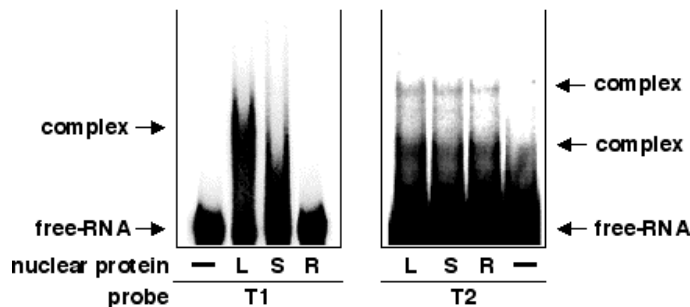


Fig. 3 The complex formation of SRE in chlAPX mRNA and a nuclear protein. (A) Detection of the RNA-protein interaction by the gel mobility shift analysis. The positions of free RNA and complex are indicated by arrows. The nuclear proteins prepared from leaf, stem, and root are represented by L, S, and R, respectively.

nuclear extracts from leaf contain a *trans*-acting factor that binds to the SRE region, which is clearly distinct from factors for the constitutive splicing.

Discussion

When the polyadenylation signal in exon 12 was selected, the only resulting product followed by the splicing of introns 1-11 was sAPX-I mRNA (Yoshimura et al. 1999). Accordingly, the lack of change in the ratio of sAPX-I mRNA to total chlAPX mRNAs indicates that the ratio of two polyadenylation site selection is not regulated in each tissue (Fig. 1). The selection of the polyadenylation signal in exon 13 leads to the generation of three mRNA variants (sAPX-II, -III, and tAPX-I) followed by the alternative excision of intron 11 or intron 12. Therefore, it is likely that the alternative splice-site selection in the alternative splicing event of chlAPX pre-mRNA is a principal regulation mechanism to change the expression ratio of chlAPX isoenzymes in each tissue. We found that the SRE sequence, which might be involved in the

alternative splicing event, in the immediately upstream region of exon 13 of *ApxII* (Fig. 2). Based on the possible mechanism of the generation of mature mRNA variants (sAPX-I, sAPX-II, sAPX-III, and tAPX-I) (Yoshimura et al. 1999), the relative expression ratio of each mRNA (Fig. 1), and the relative amounts of a *trans*-acting factor binds to the SRE region in several tissues (Fig. 3), a possible model of the regulation mechanism of the alternative splicing of chlAPX was proposed. When a *trans*-acting factor binds to the SRE region in chlAPX pre-mRNA, the splicing efficiency of intron 12 may increase, leading to the enhancement of production of the tAPX-I mRNA. Accordingly, a *trans*-acting factor may function as an enhancer of the intron-12 splicing. We are progressing in the identification of a *trans*-acting factor and the explanation of the regulation mechanism of alternative splicing.

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