

An oligonucleotide/oligosaccharide-binding-fold protein enhances the alternative splicing event producing thylakoid membrane-bound ascorbate peroxidase in *Nicotiana tabacum*

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Abstract

The stromal and thylakoid membrane-bound ascorbate peroxidase isoforms are produced by the alternative splicing event of the 3'-terminal region of the *APXII* gene in spinach (*Spinacia oleracea*) and tobacco (*Nicotiana tabacum*), but not in Arabidopsis (*Arabidopsis thaliana*). However, all alternative splicing variants were detected in *APXII* gene-transformed Arabidopsis, indicating the occurrence of its regulatory mechanisms in Arabidopsis. The efficiency of this alternative splicing event in producing thylakoid membrane-bound ascorbate peroxidase mRNA is regulated by a splicing regulatory *cis* element, but *trans* splicing regulatory factor(s) for alternative splicing remain unclear. To identify this factor, we conducted a forward genetic screen using Arabidopsis in combination with a luciferase reporter system to evaluate the alternative splicing efficiency of thylakoid membrane-bound ascorbate peroxidase mRNA production. We isolated 9 mutant lines that showed low efficiency of the AS in producing thylakoid membrane-bound ascorbate peroxidase mRNA compared with that in the control plants. From one mutant [*APXII alternative splicing inhibition (apsi1)*], the causal gene responsible for the phenotype, AT5G38890 (oligonucleotide/oligosaccharide-binding-fold protein, *APSI1*), was identified. The levels of thylakoid membrane-bound ascorbate peroxidase mRNA from the transformed *APXII* gene decreased and increased in *APSI1* knockout and *APSI1*-overexpressing plants, respectively. *APSI1* was localized to the nucleus and specifically bound to the splicing regulatory *cis* element sequence. Tobacco plants that disrupted the closest homologs of *APSI1* showed low levels of endogenous thylakoid membrane-bound ascorbate peroxidase mRNA. These results indicate that *APSI1* is an enhancing component of the alternative splicing event of *APXII*.

Keywords: ascorbate; ascorbate peroxidase; alternative splicing

Introduction

Photosynthesis in the chloroplasts of plant leaves is a source of reactive oxygen species (ROS) (Asada 1999). ROS, such as O₂⁻, hydrogen peroxide (H₂O₂), singlet oxygen, and hydroxyl radicals are cytotoxic molecules because they can potentially oxidize and destroy various cellular components (e.g. proteins, nucleic acids, and lipids), which ultimately leads to cell death (Apel and Hirt 2004; Noctor et al. 2016). However, ROS are also key regulators of many biological processes, such as growth, cell cycle, programmed cell death, hormone signaling, development, and activation of defense pathways to environmental stresses (Mittler et al. 2004; Foyer and Noctor 2005; Fujita et al. 2006; Mignolet-Spruyt et al. 2016; Mittler 2017; He et al. 2018; Mhamdi

and Van Breusegem 2018). Therefore, plants must tightly regulate the balance between the production and scavenging of ROS in the processes of acclimation against fluctuating environmental conditions.

The O₂⁻ produced from the photosynthetic electron transport chain in chloroplasts is disproportionated into O₂ and H₂O₂ either spontaneously or enzymatically by the reaction of superoxide dismutases, which are localized to both the stroma and the surface of the thylakoid membrane (Asada 1999). H₂O₂ is then reduced to water by the reaction of 2 chloroplastic isoforms of ascorbate peroxidase (APX), stromal (sAPX) and thylakoid membrane-bound (tAPX), using ascorbate as an electron donor (Asada 1999; Shigeoka et al. 2002; Yoshimura et al. 2002; Maruta

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et al. 2016; Kameoka et al. 2021). That is, the reaction pathway on the surface of the thylakoid membrane acts first on the scavenging of O_2^- produced from the photosynthetic electron transport chain, and the pathway in the stroma acts on the O_2^- escaped from there. These reactions in the chloroplasts begin with water production and end with water formation without gain or loss of any molecule; therefore, the overall electron transfer reaction is referred to as the water–water cycle (Asada 1999). The water–water cycle acts not only as an antioxidant system, but also as a system for dissipating excess electrons from photosynthetic electron transport, that is, an electron sink (Asada 1999). Therefore, appropriate regulation of the expression levels of both sAPX and tAPX is important for maintaining such multifunctional ROS metabolic processes in chloroplasts.

We have previously demonstrated that chloroplastic APX isoforms are encoded by a single gene (APXII) and are produced by alternative splicing (AS) event in the 3'-terminal region of its pre-mRNA in spinach (*Spinacia oleracea*) (LOC110797270) and tobacco (*Nicotiana tabacum*) (LOC107792162) plants (Ishikawa et al. 1997; Yoshimura et al. 1999, 2002). APXII contains 13 exons split into 12 introns, and the transit peptide sequence and catalytic domain for sAPX and tAPX are encoded by exons 1–11 (Fig. 1a). The penultimate exon 12 encodes the terminal amino acid residue (Asp) and the stop codon for sAPX mRNA before the polyadenylation signal. The final exon 13 encodes the thylakoid membrane-spanning domain and the stop codon for tAPX mRNA before the polyadenylation signal. As a result of both alternative 3' splice site selection and alternative polyadenylation site selection, the AS event in the 3'-terminal region of APXII produces 4 types of mRNA variants, 1 form (tAPX-I) encoding tAPX and 3 forms (sAPX-I, -II, and -III) encoding sAPX (Yoshimura et al. 1999, 2002). Among them, sAPX-II mRNA contains an insertional sequence (intron 11) that causes the addition of 7 amino acid residues to replace the last residue of the C-terminus in the sAPX protein (Yoshimura et al. 1999), although the mRNA containing such a structure is likely to be a target of nonsense mediated mRNA decay (Kwon et al. 2014). These findings indicate that the AS event in the APXII gene is a mechanism reasonable for the production of the sAPX and tAPX isoforms.

AS produces different RNA isoforms from one gene, enhancing the complexity of genic output, and it seems likely that plants have evolved AS to increase the repertoire of responses to different environmental conditions (Laloum et al. 2018; Chaudhary et al. 2019; Punzo et al. 2020). RNA splicing is catalyzed by spliceosomes, large complexes composed of 5 small nuclear ribonucleoproteins (U1, U2, U4, U5, and U6 snRNPs) and numerous proteins (Wilkinson et al. 2020). AS involves decisions regarding which introns are to be removed and which exons are to be included in mature mRNA. These decisions are achieved by the interaction between cis regulatory element(s) on premature RNA, such as exon splicing enhancer (Chen and Manley 2009), and trans splicing regulatory factor(s), such as serine/arginine-rich (SR) proteins (Reddy and Shad Ali 2011; Li et al. 2021).

Information on the regulatory mechanisms of AS events as well as spliceosome composition and splicing mechanisms has mostly been derived from studies on yeasts and metazoan cells (Papasaiakas and Valcárcel 2016). Although the basic mechanisms of AS in higher eukaryotes are similar, some differences exist between plants and animals in gene architecture and splicing machinery, suggesting plant-specific regulation of AS (Kornblihtt et al. 2013; Irimia and Roy 2014; Matera and Wang 2014). However, information on plants is largely unclear because of a delay in the development of an efficient *in vitro* splicing system

(Albaqami and Reddy 2018). Forward genetic approaches have been useful for identifying novel splicing-related factors in plants (Hugouvieux et al. 2001; Marquardt et al. 2014; Zhan et al. 2015). However, adopting this approach to identify the factors involved in the regulation of well-defined AS events in plants is difficult because of the difficulty in evaluating AS efficiency in a large number of mutants. Recently, Kanno et al. (2020) developed a novel green fluorescent protein (GFP) reporter system to carry out a forward genetic screen. The researchers identified some genes encoding previously unpublished splicing factors, clearly indicating that a forward genetic screen in combination with a reporter gene is appropriate for identifying novel splicing factors involved in a well-defined AS event.

In the case of APXII, the splicing regulatory cis element (SRE), which is highly conserved among APXII genes in plants, is located upstream of the acceptor site in intron 12 and acts as a splicing enhancer of the intron, resulting in an increase in the tAPX-I mRNA variant (Yoshimura et al. 2002). We also previously confirmed that the efficiency of alternative 3' splice site selection regulated by SRE modulates the production rate between sAPX and tAPX proteins in a tissue-specific manner, in contrast to the constant selection rate of alternative polyadenylation sites. Although the cis element has been cleared, trans splicing regulatory factor(s) of the AS of the APXII gene remain unclear. Here, we aimed to use a forward genetic screen to identify the factors affecting the AS event that produces the tAPX-I-type splicing variant using transgenic Arabidopsis expressing a reporter gene. We identified a causal gene, APSI1 [oligonucleotide/oligosaccharide-binding (OB)-fold protein], in one of the isolated mutants (*apsi1*). Further functional analyses of the APSI1 gene demonstrated that APSI1 binds to SRE in the nucleus and acts as an enhancer of the AS event producing tAPX-I-type mRNA in Arabidopsis and tobacco.

Materials and methods

Plant materials and growth conditions

Arabidopsis ecotype Col-0 was used as the wild-type (WT) plant. The T-DNA insertion line *apsi1* knockout (KO-*apsi1*; SALK_004562) was obtained from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA).

Arabidopsis seeds were sown on half-strength Murashige and Skoog's (MS) medium (0.8% agar) containing 2% sucrose and stratified in the dark for 2–3 days at 4°C. Plants were grown under normal conditions (16 h of light at 80 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ and 8 h of darkness, 23°C) for 2–4 weeks in a growth chamber (LPH-241, NK System, Tokyo, Japan). Spinach (*S. oleracea*) was grown on soil in a growth chamber for 4 weeks under the following conditions: 8 h of light at 200 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ and 16 h of darkness at 15°C. Tobacco plants (*N. tabacum* L. cv. SR1) were grown on MS medium (0.8% agar) containing 3% sucrose in a growth chamber for 4 weeks under the following conditions: 12 h of light at 300 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ and 12 h of darkness at 28°C. Plants were collected, frozen immediately in liquid N_2 , and stored at -80°C until used in the analyses described in the text. All other chemicals were of the highest commercially available grade.

Plasmid construction to generate transgenic plants

The plasmid for the expression of the 3' region of the APXII gene (exons 11–13) from spinach, driven by the cauliflower mosaic virus (CaMV) 35S promoter, was constructed using GATEWAY cloning technology (Thermo Fisher Scientific, Waltham, MA, USA).

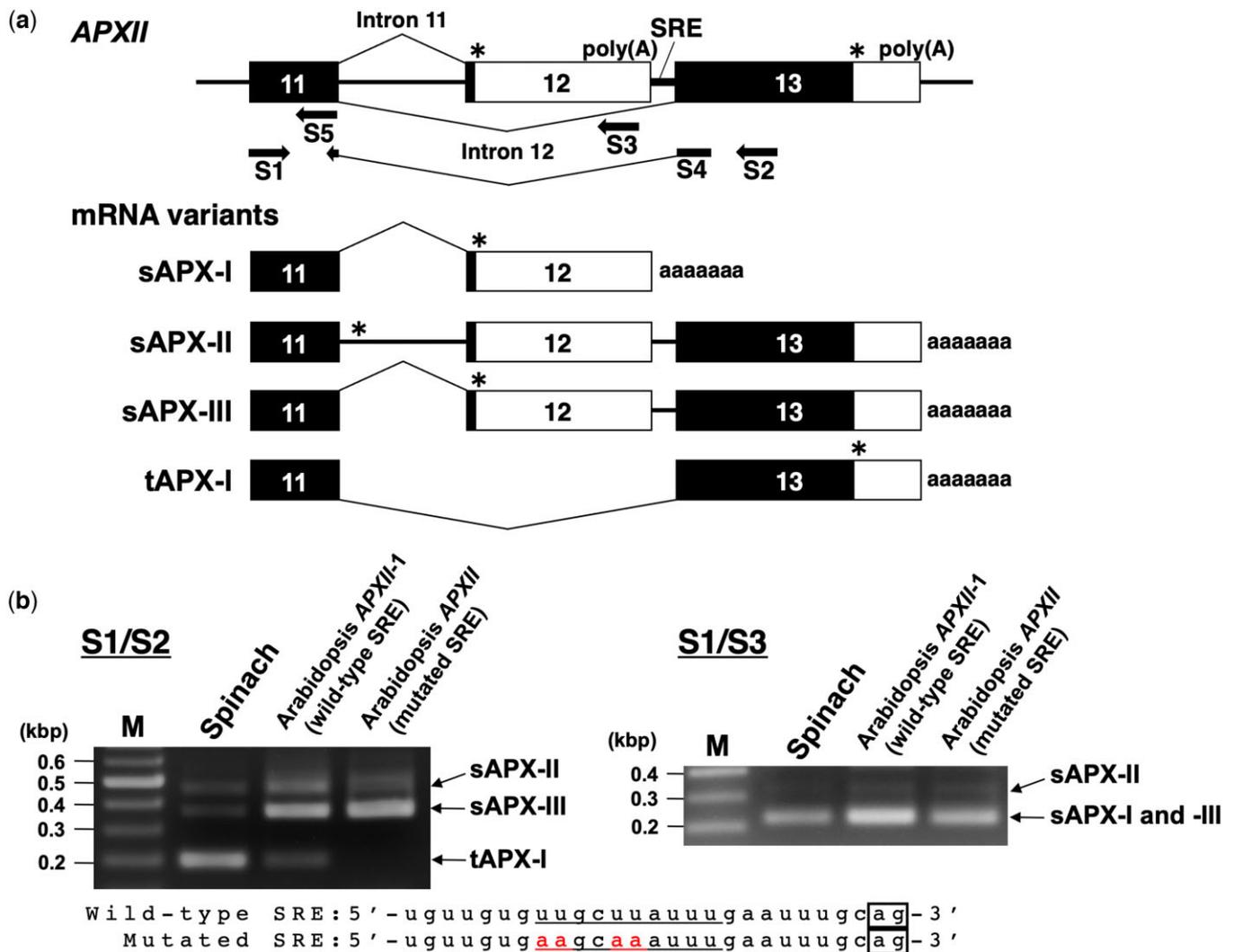


Fig. 1. The AS event of APXII in Arabidopsis. a) Schematic representation of the AS pattern producing spinach chloroplastic APX mRNAs. Partial genomic DNA and mRNA sequences corresponding with the 3'-terminal regions of chloroplastic APXs (exons 11–13) are shown based on data from experiments reported previously (Yoshimura et al. 1999). Exon regions are shown as boxes and introns as lines. The open reading frame and untranslated regions are indicated by black and white boxes, respectively. Functional stop codons and polyadenylation signals for each mRNA variant are indicated by asterisks and poly(A), respectively. The position of the SRE sequence is indicated by a bold line. Arrows (S1–S5) indicate the location of primers used in this study (Supplementary Table 1). b) Semiquantitative RT-PCR analysis to detect the chloroplastic APX mRNA variants (sAPX-I, -II, -III, and tAPX-I) in spinach and Arabidopsis. The 3' region of the APXII gene (exons 11–13) with either WT or mutated SRE sequences from spinach driven by the CaMV 35S promoter was transformed into Arabidopsis using *A. tumefaciens*. The sequences of WT and mutated SRE are shown. The line indicates the SRE region (Yoshimura et al. 2002). The terminal dinucleotides of acceptor site of intron 12 (ag) are indicated by boxes. Semiquantitative RT-PCR analysis (26 cycles) was carried out using sets of primers to detect the mRNA variants produced from the endogenous chloroplastic APX gene and the transgene in extracts from the leaves of 4-week-old spinach and 2-week-old transgenic Arabidopsis [Arabidopsis APXII-1 (wild-type SRE) and Arabidopsis APXII (mutated SRE); T3 generation], respectively. Amplified products were electrophoresed on a 2% (w/v) agarose gel. M, molecular marker. The experiments were repeated at least 3 times using plants grown independently, and the representative results are shown.

The 3' region of APXII was amplified from the spinach genome using specific primers with the attB1 and attB2 sequences, as shown in Supplementary Table 1. The amplified gene was cloned into the donor vector pDONR201 and then recloned downstream of the CaMV35S promoter in the binary vector pFAST-G02 (Inplanta Innovations, Yokohama, Japan). Mutation of the SRE sequence was achieved by site-directed mutagenesis using the pDONR201/APXII gene (exons 11–13) and the primer set shown in Supplementary Table 1.

The plasmid expressing the chimeric gene, in which the 3' region of the spinach APXII gene was fused with firefly luciferase (*Fluc*) cDNA driven by the CaMV35S promoter, was constructed using In-Fusion cloning technology (TaKaRa, Shiga, Japan). The region from the donor site of intron 11 to the acceptor site of

intron 12 including SRE of the spinach APXII gene was amplified from the spinach genome using specific primers with a portion of the neighboring sequence at the final construction, as shown in Supplementary Table 1. The anterior region corresponding to exons 1–3 and the posterior region corresponding to exons 4–7 of *Fluc* cDNA were amplified from the cDNA using specific primers with a portion of the neighboring sequence at the final construction. The binary vector pRI101-AN (TaKaRa) was also amplified using specific primers with a portion of the neighboring sequence at the final construction. All obtained fragments were ligated to construct the pRI101-AN/APXII-*Fluc* gene using the In-Fusion HD Cloning Kit (TaKaRa).

The plasmid expressing APS11 (AT5G38890) under the control of its own promoter (1,500bp) was constructed using a

conventional method with restriction enzymes. The region from 1,500 bp upstream of exon 1 to the putative termination sequence of *APSI1* was amplified from the Arabidopsis genome using specific primers with the sequences of either HindIII or KpnI as shown in [Supplementary Table 1](#). Fragments containing the *apsi1* mutation were amplified from the genome of the *apsi1* mutants. The amplified gene was cloned into a cloning vector, pUC118 (TaKaRa), and then recloned into the region between the HindIII and KpnI sites of the binary vector pRI201 (TaKaRa).

The plasmid expressing *APSI1* cDNA driven by the CaMV35S promoter was constructed by a conventional method using restriction enzymes. The WT and mutated *APSI1* cDNA were amplified from Arabidopsis using specific primers with the sequences of either NdeI or EcoRI, as shown in [Supplementary Table 1](#). The amplified cDNA was cloned into a cloning vector, pUC118 (TaKaRa), and then recloned into the region between the NdeI and EcoRI sites of the binary vector pRI101 (TaKaRa).

The plasmid expressing *APSI1* cDNA fused in-frame with GFP at the N-terminus was constructed using GATEWAY cloning technology (Thermo Fisher Scientific). *APSI1* cDNA was amplified from Arabidopsis using specific primers with the attB1 and attB2 sequences, as shown in [Supplementary Table 1](#). The amplified fragment was cloned into the donor vector, pDONR201, and then recloned downstream of the CaMV35S promoter in the binary vector pGWB506 ([Nakagawa et al. 2007](#)).

PCR amplifications were performed using PrimeSTAR HS DNA Polymerase or PrimeSTAR GXL DNA Polymerase (TaKaRa). Recombination was performed according to the manufacturer's instructions. The sequence of each construct was verified by DNA sequencing.

Transformation of Arabidopsis

Agrobacterium tumefaciens strain C58C1, which was transformed with the constructs described above by electroporation, was used to infect Arabidopsis (Col-0) using the floral dip method ([Clough and Bent 1998](#)). The transformed plants were screened for 2 weeks on half-strength MS medium (0.8% agar) containing 2% sucrose and appropriate antibiotics depending on the selection marker gene in the construct and then transferred to soil. Among the T3 seeds obtained by self-fertilization, lines in which the transgene was introduced homozygously were selected based on tolerance against antibiotics and PCR analysis results.

Mutagenesis and screening of Arabidopsis

A transgenic line (T3) harboring one copy of the *APXII-Fluc* gene was used for mutagenesis with ethyl methanesulfonate (EMS). Mutagenized M2 seeds were sterilized and sown on a half-strength MS medium (0.8% agar) containing 2% sucrose. Seedlings grown for 2 weeks in a growth chamber (LPH-241, NK system) were sprayed with 2.5 mM luciferin (potassium salt, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and grown for 2 h. Luminescence derived from Fluc was detected using an EM-CCD camera (C9100-13, Hamamatsu Photonics, Hamamatsu City, Japan).

Semiquantitative RT-PCR

Total RNA was extracted from plant materials using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan). After DNase treatment, total RNA was used to synthesize first-strand cDNA using reverse transcriptase (ReverTra Ace, Toyobo, Osaka, Japan) with an oligo (dT)₂₀ primer. Assays were performed according to the manufacturer's instructions. PCR amplification was performed with 24–28 cycles of 95°C for 10 s, 55–65°C for 10 s, and 72°C for 60 s, followed

by 72°C for 10 min, using PrimeSTAR HS DNA Polymerase (TaKaRa). The primers used are listed in [Supplementary Table 1](#). The amplified products were electrophoresed on a 1.5–2% (w/v) agarose gel.

Immunoblotting

Immunoblot analysis was performed using polyclonal mouse antibodies raised against the Fluc protein (Thermo Fisher Scientific). Soluble protein samples (30 µg) extracted from Arabidopsis leaves were used for analysis. Protein concentration was determined using the method described by [Bradford \(1976\)](#). Fluc protein was detected using the antibody as the primary antibody and antimouse IgG-horseradish peroxidase conjugate as the secondary antibody. Protein bands were detected using an ECL Western Blotting Analysis System (GE Healthcare, Milwaukee, WI, USA).

Analysis of luciferase activity

Luciferase activity in the soluble protein extract was measured using a Luciferase Assay System (Promega, Madison, WI, USA) and luminometer (Gene Light, GL-200, Microtec, Chiba, Japan) according to the manufacturer's instructions.

Identification of the causal gene for the mutants

Genomic DNA was extracted from the leaves of >20 seedlings of either the control plants or the F2 progenies of mutants, which were obtained by backcrossing with the control plants, showing the same phenotype as that of the mutant line using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The extracted DNA was fragmented by sonication (S220, Covaris, M&S Instruments, Osaka, Japan) and then converted to a library using TruSeq DNA Sample Prep Kits (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. The libraries were sequenced using NextSeq500 (Illumina). The obtained data were analyzed using Mitsucal computer system ([Suzuki et al. 2018](#)) to identify the mutation(s) responsible for the phenotype.

Subcellular localization of GFP–*APSI1* fusion protein

Transgenic plants expressing the GFP–*APSI1* fusion protein were grown on half-strength MS medium (0.8% agar) containing 2% sucrose for 1 week. The fluorescence of the GFP fusion proteins in the roots was monitored using a fluorescence microscope (BZ-X800, Keyence, Osaka, Japan) and laser scanning confocal fluorescence microscope (LSM700, Carl Zeiss, Jena, Germany). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific).

Gel shift assay

The plasmid expressing *APSI1* and *NtAPSI1-1* cDNAs fused with the hexa-histidine tag at the N-terminus was constructed using In-Fusion cloning technology (TaKaRa). The cDNAs of *APSI1*, *NtAPSI1-1*, and an expression vector, pE SUMOstar (LifeSensors, Malvern, PA, USA), were amplified using specific primers with a portion of the neighboring sequence at the final construction, as shown in [Supplementary Table 1](#). The obtained fragments were ligated to construct pE SUMOstar/*APSI1* and pE SUMOstar/*NtAPSI1-1* using an In-Fusion HD Cloning Kit (TaKaRa). PCR amplifications were performed using PrimeSTAR HS DNA Polymerase or PrimeSTAR GXL DNA Polymerase (TaKaRa). Recombination was performed according to the manufacturer's instructions. The sequence of each construct was verified by DNA sequencing. *APSI1* and *NtAPSI1-1* proteins were expressed

in *Escherichia coli* and purified by nickel affinity chromatography (HiTrap Chelating HP, GE Healthcare) followed by dialysis. Biotin-labeled and unlabeled RNA probes were synthesized by Hokkaido System Science (Hokkaido, Japan).

Gel shift assay was performed using a LightShift Chemiluminescent RNA Electrophoretic Mobility Shift Assay (EMSA) Kit (ThermoScientific) according to the manufacturer's instructions. In brief, 50 fmol of protein was incubated with 20 fmol RNA probe for 25 min at 25°C in a 20 µl mixture containing 1× binding buffer, 5% glycerol, and 10 ng tRNA. The mixtures were then loaded onto a 4% acrylamide gel and run in 0.5× Tris/borate/EDTA buffer at 4°C. The gel was transferred to a nylon membrane (Hybond-N⁺, GE Healthcare) using a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. After treatment with the solutions in the kit, the RNAs in the gels were visualized using an LAS3000 Mini imaging system (Fujifilm, Minato, Japan).

Generation of NtAPSI1 genes-disrupted tobacco plants by the CRISPR/Cas9 system

NtAPSI1 genes-disrupted tobacco plants were generated using the clustered, regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated 9 (Cas9) genome editing system. Specific guide sequences targeting the 5' of NtAPSI1-1 and NtAPSI1-2 were selected using CRISPR-P (<http://cbi.hzau.edu.cn/cgi-bin/CRISPR>) (Lei et al. 2014), taking into account the predicted single-guide RNA (sgRNA) efficiencies using sgRNAscorer (<https://crispr.med.harvard.edu/sgRNAScorer/>) (Chari et al. 2015). For each guide sequence, 2 complementary 24-bp oligos with 4-bp overhangs (5'-attgACTCAAGAGGTGGTGACGCC-3' and 5'-aaacGGCGTACCACCTCTTGAGT-3' for sgRNA1 and 5'-attgGGGCTGAGGAACAGTCCAT-3' and 5'-aaacATGGAGCTGTCTCAGCCC-3' for sgRNA2; overhang sequences are indicated by lowercase letters) were annealed and inserted via a cut-ligation reaction with BbsI (Thermo Scientific) and T4 DNA ligase (Thermo Scientific) in either pMR217 for sgRNA1 or pMR218 for sgRNA2 (Ritter et al. 2017). Using a MultiSite Gateway LR reaction, both sgRNA modules were combined with pDE-Cas9 (Km) to yield the final expression plasmid (Ritter et al. 2017). The expression plasmid was transformed in *A. tumefaciens* C58C1 and used to transform tobacco (cv. SR1) according to Yoshimura et al. (2002). Twenty-four primary transformants (T0) were selected using kanamycin, and genomic DNA from each seedling leaf was extracted. For each gene, an ~900-bp genomic region spanning the predicted Cas9 cut sites was amplified using specific primers, as shown in Supplementary Table 1. Deletion of the amplicon sequence was verified by cleaved amplified polymorphic sequence analysis using the NcoI restriction enzyme. The selected T0 plants were self-fertilized to obtain T1 seeds. The amplified PCR products of the T1 lines, #13-1 and #17-3, were cloned into a TA vector, and each plasmid was sequenced to identify mutations in the vicinity of the target sequences. After selfing, T2 plants were checked for segregation of the transgene locus using kanamycin resistance and DNA sequencing.

Data analyses

Statistical analyses of data were performed using Student's t-test. Calculations using Microsoft Excel were performed on at least 3 independent biological replicates (see figure legends). In all experiments, fully expanded leaves from more than 3 plants were pooled and used as one biological replicate.

Results

Distribution of the AS event of APXII in the plant kingdom

The N-terminal 364 amino acid sequence of tAPX in spinach and tobacco is completely identical to that of sAPX, whereas the remainder of the C-terminal region (terminal one amino acid) of sAPX is substituted by 50 different amino acids, which constitute a hydrophobic thylakoid-membrane-binding domain (Ishikawa et al. 1996; Yoshimura et al. 2002). Similar structures of sAPX and tAPX have also been reported in pumpkin (Mano et al. 1997). We searched for nucleotide sequences encoding APX cDNA in NCBI database and found both sAPX and tAPX in charophytes, pteridophytes, and various terrestrial plants (pteridophytes, bryophytes, monocots, and eudicots) (Maruta et al. 2016). Among these sequences, we found that the sAPX and tAPX sequences from many eudicots (e.g. *Beta vulgaris*, *Chenopodium quinoa*, *Cucurbita lanatus*, *Glycine max*, *Ipomoea nil*, *Mesembryanthemum crystallinum*, *Nicotiana attenuata*, and *Populus trichocarpa*) have similar structures to those from spinach and tobacco (Supplementary Table 2), suggesting that sAPX and tAPX are generated by AS in these plants. However, the sequences of sAPX and tAPX from charophytes (*Klebsormidium nitens*), pteridophytes (*Selaginella moellendorffii*), monocots (*Musa x paradisiaca*, *Oryza sativa*, *Zea mays*), and some eudicots, including *Arabidopsis lyrata*, *A. thaliana*, *Brassica napus*, *Solanum lycopersicum*, and *Solanum tuberosum*, were found to be considerably different, suggesting that these organisms produce chloroplastic APX isoforms individually from different genes.

Genetic screening of a regulatory factor for the AS event of APXII from Arabidopsis

To examine whether the AS event of APXII is conserved in Arabidopsis, the 3' region of APXII from spinach (exons 11–13), where the AS event for production of sAPX and tAPX mRNAs takes place (Fig. 1a), driven by the CaMV35S promoter was transformed into Arabidopsis (Col-0) using *A. tumefaciens*. All mRNA variants (sAPX-I, -II, -III, and tAPX-I) were detected in the leaves of transgenic Arabidopsis (*Arabidopsis* APXII-1; T3 generation) using semiquantitative RT-PCR and sequence analysis and were also observed in spinach leaves (Fig. 1b). Compared with the AS efficiencies of tAPX-I and sAPX-III mRNAs in spinach, those in transgenic Arabidopsis were decreased and increased, respectively. In other transgenic Arabidopsis lines (*Arabidopsis* APXII-2 and -3) with low expression levels (a total of the AS variants) of the spinach APXII gene detected by a set of primers positioned in exon 11 of APXII, the levels of tAPX-I mRNA as well as sAPX-III mRNA were decreased compared with those in the Arabidopsis APXII-1 line (Supplementary Fig. 1). The expression levels of endogenous sAPX (AT4G08390) and tAPX (AT1G77490) genes remained unchanged in these transgenic lines. The partial nucleotide substitutions in the SRE of APXII, in which the 4 uridine nucleotides were substituted by adenines, resulted in a decrease in tAPX-I mRNA (Fig. 1b), as reported previously for tobacco (Yoshimura et al. 2002). These results indicate that the mechanism for the AS event of APXII is conserved in Arabidopsis, implying its role in AS events of other endogenous genes.

To incorporate a reporter system for genetic screening, we constructed a Fluc cDNA interrupted by intron 12 of the spinach APXII gene under the control of the CaMV35S promoter (Fig. 2a). The type of AS event for APXII is the alternative 3' splice site selection between the acceptor sites of introns 11 and 12; thus, the region from the donor site of intron 11 to the acceptor site of intron 12, including SRE, which acts as the splicing enhancer of

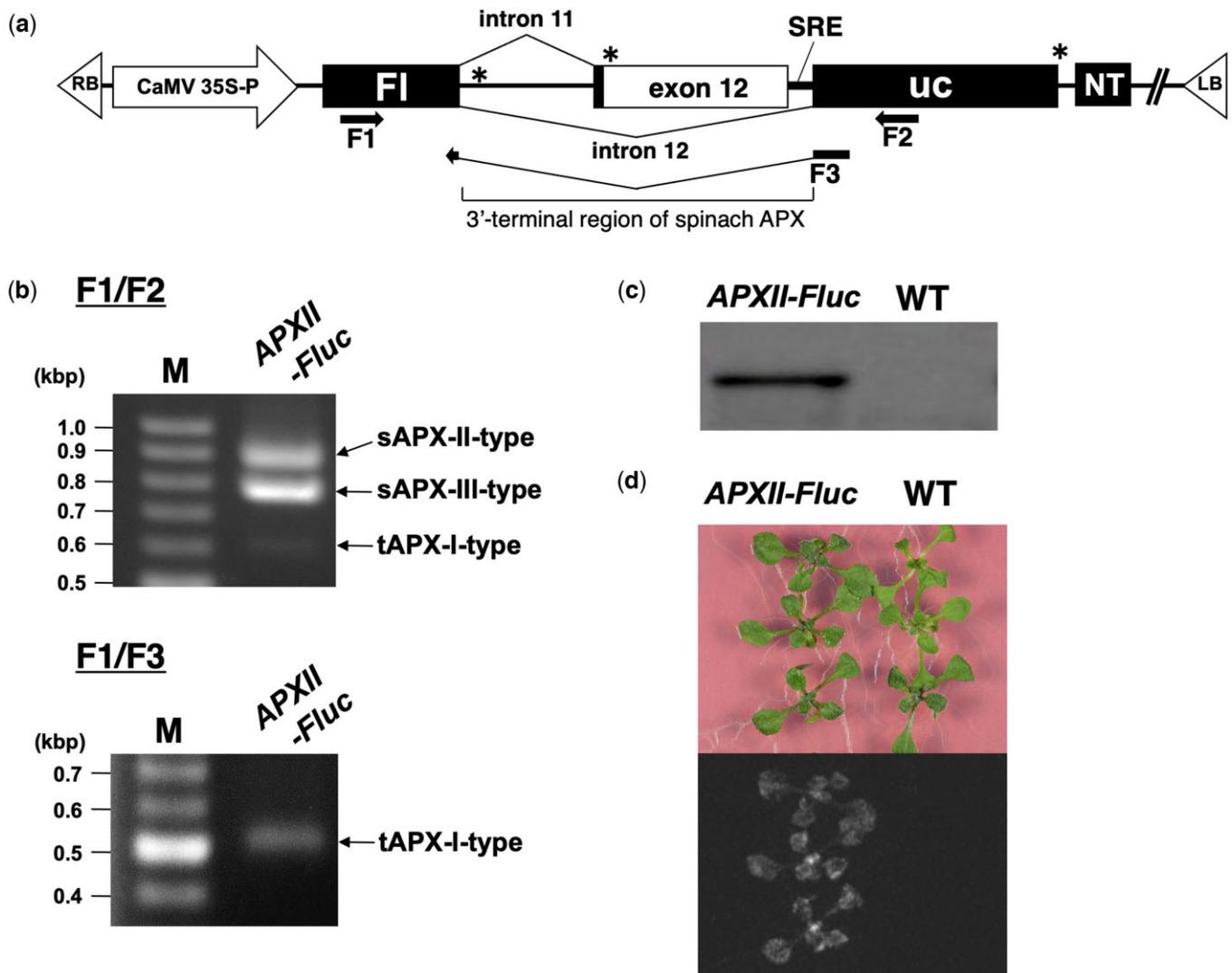


Fig. 2. Transgenic Arabidopsis expressing the APXII-Fluc chimera gene. a) Schematic representation of the APXII-Fluc chimera gene. The chimera gene, in which the donor site of intron 11 to the acceptor site of intron 12 including SRE of the spinach APXII gene is fused with the Fluc cDNA at its intron 3-spliced site, driven by the CaMV 35S promoter was transformed into Arabidopsis using *A. tumefaciens*. Exon regions are shown as boxes and introns as lines. The open reading frame and untranslated regions are indicated by black and white boxes, respectively. Functional and premature stop codons are indicated by asterisks. The position of the SRE sequence is indicated by a bold line. Arrows (F1-F3) indicate the location of primers used in this study (Supplementary Table 1). LB, left tDNA border; RB, right tDNA border. b) Results of semi-quantitative RT-PCR analysis to detect the AS variants (sAPX-II-, -III, and tAPX-I types) in the APXII-Fluc chimera gene-transformed Arabidopsis (APXII-Fluc). Semi-quantitative RT-PCR analysis (26 cycles) was carried out using sets of primers in the extract from the rosette leaves of 2 weeks old APXII-Fluc plants (T3 generation). Amplified products were electrophoresed on a 2% (w/v) agarose gel. M, molecular marker. c) Results of immunoblot analysis to detect the Fluc protein in the APXII-Fluc and WT Arabidopsis. Immunoblot analysis was performed using the antibody raised against the Fluc protein produced from the tAPX-I-type mRNA in extracts from the rosette leaves of 2-week-old APXII-Fluc and WT Arabidopsis. d) Luminescence image of APXII-Fluc and WT Arabidopsis. Fluc-dependent luminescence was imaged in 2-week-old plants by EM-CCD camera. The experiments were repeated at least 3 times using plants grown independently, and the representative results are shown.

intron 12, was inserted into the intron 3-spliced site of the Fluc cDNA. This chimeric gene (APXII-Fluc) produced 3 types of mRNA variants: tAPX-I-, sAPX-II-, and sAPX-III-type. Among them, only tAPX-I-type mRNA, resulting from the splicing of intron 12, produced a functional Fluc protein (Fig. 2a). In contrast, both sAPX-II- and sAPX-III-type mRNAs encoded truncated Fluc proteins because of the appearance of a premature stop codon in either intron 11 or exon 12 derived from APXII. The AS efficiency of the APXII-Fluc gene in the transgenic Arabidopsis line (APXII-Fluc) was analyzed using semi-quantitative RT-PCR. tAPX-I-type mRNA was slightly detected in the transgenic plants, and sAPX-II- and -III-type mRNAs were detected at almost equal levels (Fig. 2b). To detect tAPX-I-type mRNA clearly, a primer (F3) designed to match

the exon junction was used for semi-quantitative RT-PCR analysis (Fig. 2a). As expected, a single band was amplified in APXII-Fluc plants, and the sequence was confirmed to encode tAPX-I-type mRNA (Fig. 2b). Fluc protein was also detected by immunoblot analysis (Fig. 2c), and clear luminescence was detected (Fig. 2d). These results clearly indicated that pre-mRNA from the APXII-Fluc gene was alternatively spliced in Arabidopsis.

For the forward genetic screen, seeds of APXII-Fluc Arabidopsis were treated with EMS, and ~2,000 M1 plants were grown and self-fertilized to produce M2 seeds. Approximately 20,000 M2 plants were screened based on the Fluc luminescence intensity relative to that of the APXII-Fluc plants. For each putative mutant line, M3 populations were rescreened using Fluc luminescence

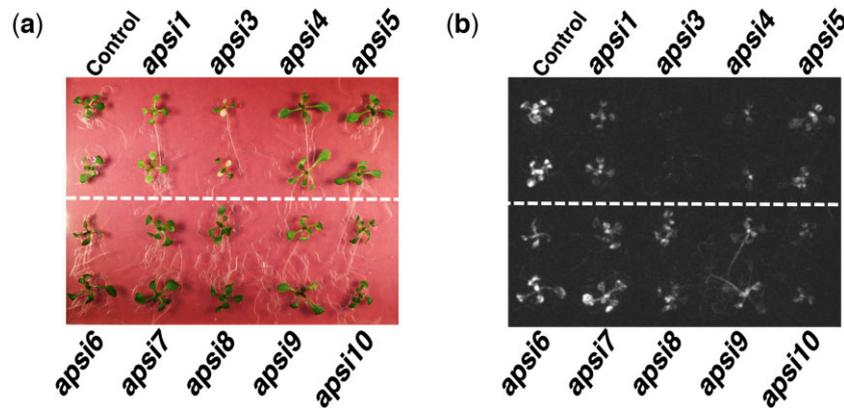


Fig. 3. Mutants showing low intensities of Fluc luminescence. Bright (a) and luminescence (b) images of the mutants. Fluc-dependent luminescence was imaged in the 2-week-old M3 generation of EMS-mutated *APXII-Fluc* plants (*apsi*) using EM-CCD camera. Control, *APXII-Fluc* plants.

analysis. As a result, 9 *APXII* alternative splicing inhibition (*apsi*) mutant lines were isolated and showed lower intensities of Fluc luminescence (Fig. 3, a and b). Under normal conditions, these mutants, except for *apsi3* and *apsi10*, showed no obvious phenotypes in growth or morphology (Fig. 3a); the *apsi3* and *apsi10* mutants showed slight growth inhibition, but normal reproductive development. The intensity of Fluc luminescence in each mutant was consistent with the level of Fluc activity detected in the soluble extract (Supplementary Fig. 2).

Semiquantitative RT-PCR analysis revealed that all *apsi* mutants showed lower levels of tAPX-I-type mRNA compared with those in the *APXII-Fluc* plants (Fig. 4), indicating that the changes in Fluc luminescence of these mutants resulted from changes in the efficiency of AS in producing tAPX-I-type splicing variants. The levels of sAPX-II- and -III-type mRNAs in all *apsi* mutants were similar to those in the *APXII-Fluc* plants (Fig. 4).

Identification of the causal gene for *apsi1* mutants

To identify the corresponding mutation responsible for the phenotypes of the mutants, the *apsi1* mutants were backcrossed to the parental control (*APXII-Fluc*) plants, and F2 progenies were obtained. Among the 135 F2 progenies, 35 siblings showed low Fluc luminescence, suggesting that the phenotypes of *apsi1* mutants resulted from a single recessive mutation occurring in the nuclear genome. DNA was extracted from the bulked seedlings of the F2 progenies showing low Fluc intensities and sequenced using next-generation sequencing. A total of 1.19 Gbp, ~10-fold the size of the Arabidopsis genome, was sequenced, and the obtained short reads were analyzed using the Mitsucal computer system (Suzuki et al. 2018). Around the 17-Mbp region of chromosome 5, numerous nearly homozygous mutations were detected (Supplementary Fig. 3a). Among them, 1 nucleotide substitution, from C to T, was located at the 3' end of the first exon of the gene labeled AT5G38890. This substitution caused a nonsense mutation in the codon for Gln (Supplementary Fig. 3b), which was confirmed by conventional sequencing and was unique to *apsi1* mutants (M3 generation). Therefore, we named the gene labeled AT5G38890 as *APSI1*.

The *APSI1* gene placed under the control of its 1.5-kbp promoter was introduced into the *apsi1* mutants. The obtained transgenic plants (*apsi1/APSI1*, T3 generation) showed similar intensities of Fluc luminescence, as well as levels of tAPX-I-type mRNA, to those in the *APXII-Fluc* plants (Supplementary Fig. 4). However, the *apsi1* mutation (*apsi1/apsi1*, T3 generation) failed to complement the low intensity of Fluc luminescence in the *apsi1* mutants. We obtained

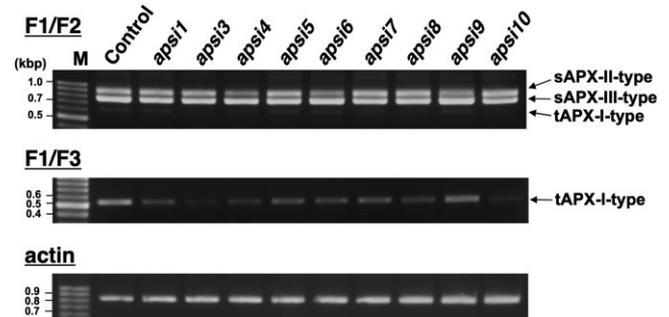


Fig. 4. AS efficiency of *APXII-Fluc* gene in the *apsi* mutants. Semi-quantitative RT-PCR analysis detected AS variants (sAPX-II, -III, and tAPX-I types) produced from the *APXII-Fluc* gene in *apsi* mutants. Semi-quantitative RT-PCR analysis (24–28 cycles) was carried out using sets of primers (Supplementary Table 1) in extracts from the rosette leaves of 2-week-old *apsi* mutants (M3 generation). The primer positions are shown in Fig. 2a. Amplified products were electrophoresed on a 2% (w/v) agarose gel. Equal loading of each amplified cDNA was determined using the control actin2 PCR product. M, molecular marker. Control, *APXII-Fluc* plants. The experiments were repeated at least 3 times using plants grown independently, and the representative results are shown.

KO-*apsi1* Arabidopsis plants (SALK_004562) from the ABRC (<https://abrc.osu.edu>). The T-DNA insert was located in the final exon (exon 6) of the gene (Supplementary Fig. 5a). Under normal conditions, these plants showed no obvious growth or morphological phenotypes. Then, to confirm the role of *APSI1* in the AS event of *APXII*, but not the chimeric gene, transgenic plants expressing the 3' region of spinach *APXII* (exons 11–13) driven by the CaMV35S promoter were fertilized by KO-*apsi1* and WT plants. The plants were then self-fertilized to obtain seeds in which both transgenes were introduced homozygously and named KO-*apsi1/APXII* and WT/*APXII* plants. The expression of *APSI1* was completely defective in KO-*apsi1/APXII* plants (Supplementary Fig. 5b). All 3 types of mRNA variants were detected in KO-*apsi1/APXII* and WT/*APXII* plants, but the levels of tAPX-I mRNA were decreased in KO-*apsi1/APXII* plants (Fig. 5a). These results indicate that the AT5G38890 (*APSI1*) mutation is responsible for the phenotypes of the *apsi1* mutants.

Effect of *APSI1* overexpression on the AS event of *APXII* in Arabidopsis

We generated *APSI1*-overexpressing Arabidopsis plants (*APSI1ox*) in which *APSI1* cDNA driven by the CaMV35S promoter was introduced homozygously. Under normal conditions, these plants

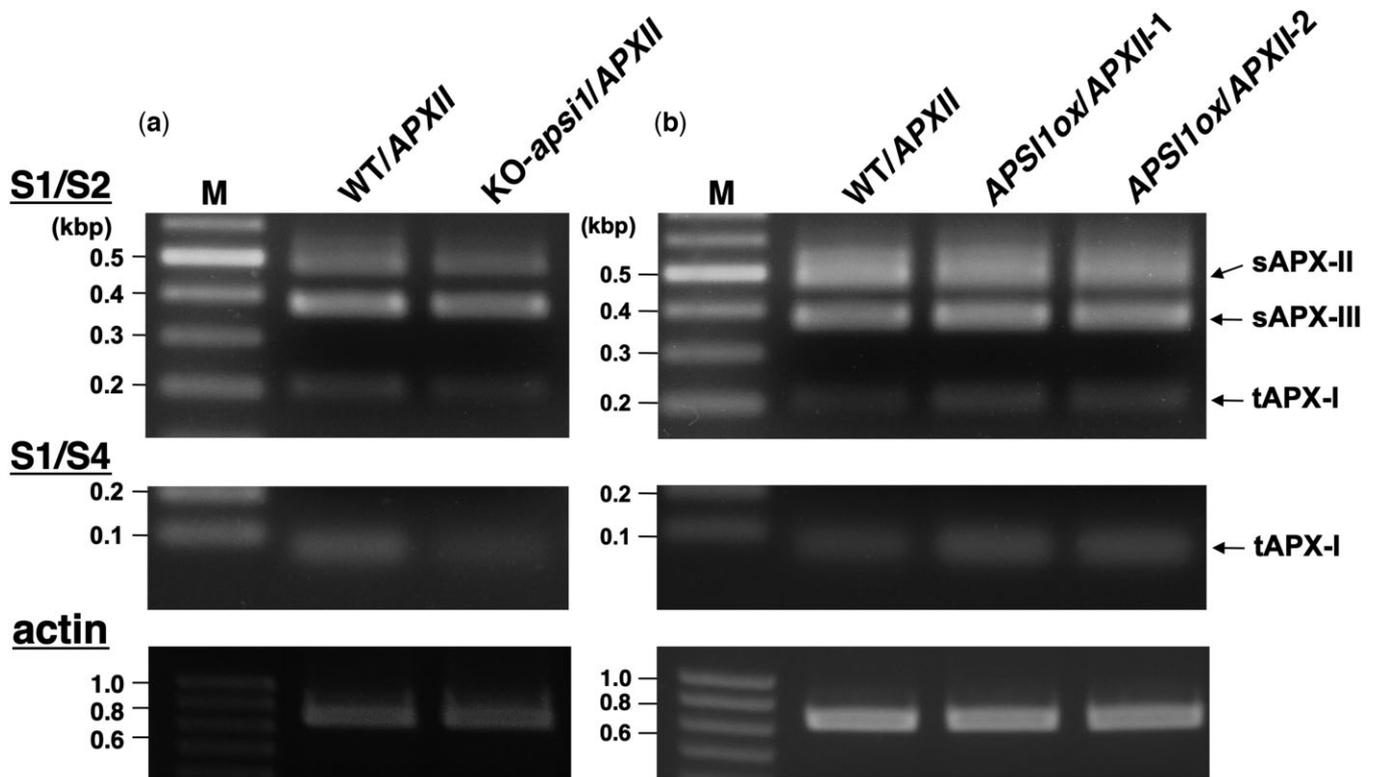


Fig. 5. Effect of the disruption and overexpression of *APSI1* on the AS event of *APXII* in Arabidopsis. Semi-quantitative RT-PCR analysis detected AS variants (sAPX-II, -III, and tAPX-I mRNAs) produced from the spinach *APXII* gene in KO-*apsi1/APXII* (a) and *APSI1ox/APXII* (b) plants. Semiquantitative RT-PCR analysis (24–28 cycles) was carried out using sets of primers (Supplementary Table 1) in extracts from the rosette leaves of 4-week-old plants (T3 generation). The primer positions are shown in Fig. 1a. Amplified products were electrophoresed on a 2% (w/v) agarose gel. Equal loading of each amplified cDNA was determined using the control actin2 PCR product. M, molecular marker. The experiments were repeated at least 3 times using plants grown independently, and the representative results are shown.

showed no obvious growth or morphological phenotypes. Then, transgenic plants expressing the 3' region of spinach *APXII* (exons 11–13) driven by the CaMV35S promoter were fertilized with *APSI1ox* plants. The transformants were self-fertilized to obtain seeds in which both transgenes were introduced homozygously, and named *APSI1ox/APXII* plants. Semi-quantitative RT-PCR analysis revealed that 2 lines of the *APSI1ox/APXII* plants (*APSI1ox/APXII*-1 and -2) showed enhanced expression levels of *APSI1* compared with those in the *WT/APXII* plants (Supplementary Fig. 5b). All 3 types of mRNA variants were detected in the *APSI1ox/APXII* plants as well as in the *WT/APXII* plants, but the levels of tAPX-I mRNA were increased in the *APSI1ox/APXII* plants (Fig. 5b). These results clearly indicate that *APSI1* positively impacts the AS efficiency of not only the chimeric *APXII* gene but also the native *APXII* gene; that is, *APSI1* acts as an enhancer of the AS event producing tAPX-I mRNA.

APSI1 localization in the nuclei of Arabidopsis cells

Based on the UniProt database (<https://www.uniprot.org>), *APSI1* was predicted to be localized in the cytosol, although factors involved in the regulation of AS events should be localized in the nucleus, and the subcellular localization of the protein was determined. The *APSI1* cDNA was fused in-frame with GFP at the N-terminus and then introduced into Arabidopsis plants (*GFP-APSI1*). In the root cells from *GFP-APSI1* plants, the fluorescence was predominantly enriched in the nucleus, whereas the fluorescence in control plants (*GFP*) was spread throughout the cytosol (Supplementary Fig. 6). Further observation using a confocal laser

scanning microscope (LSM700, Carl Zeiss) showed fluorescence in *GFP-APSI1* plants distributed in nucleus and enriched in nucleolus-like regions (Fig. 6). These results suggest that *APSI1* plays a role in nucleolus function, including RNA metabolism.

APSI1 binding to the RNA with SRE

APSI1 encodes OB-fold protein, which are demonstrated to play essential roles in a number of cellular processes, including DNA replication, DNA recombination, DNA repair, transcription, translation, cold shock response, and telomere maintenance (Theobald et al. 2003; Amir et al. 2020). According to the Pfam domain database (<http://pfam.xfam.org>), the OB-fold domain is located at 66–186 of the *APSI1* amino acid sequence, which overlaps with the ribosomal protein S1-like RNA-binding domain (70–149 a.a.) (Fig. 7a). The N-terminus of *APSI1* (5–42 a.a.) was similar to the exosome complex exonuclease RRP4. The OB-fold domain has been reported to bind to single-stranded DNA or RNA (ssDNA/ssRNA) (Theobald et al. 2003; Amir et al. 2020), implying that the *APSI1* protein can bind to the SRE sequence as a *trans* splicing regulatory factor. We performed an *in vitro* gel shift assay using recombinant *APSI1* proteins to determine whether the OB-fold domain binds to ssRNA. The recombinant *APSI1* protein fused with a hexa-histidine tag was expressed in *E. coli* and purified by nickel affinity chromatography (Supplementary Fig. 7a). When the recombinant *APSI1* protein was mixed with the ssRNA probe containing the SRE sequence, slowly migrating bands were detected, indicating complex formation between SRE and *APSI1* (Fig. 7b). There were 2 shifted bands, suggesting oligomer formation of *APSI1*. The nonlabeled competitor diminished the shifted

species ($2n = 4x = 48$) derived from a natural hybrid between *Nicotiana sylvestris* ($2n = 2x = 24$) and *Nicotiana tomentosiformis* ($2n = 2x = 24$) (Shibata et al. 2013), and these 2 genes are speculated to be homeologs.

Next, we performed a gel shift assay using purified recombinant NtAPSI1-1 proteins fused with a hexa-histidine tag (Supplementary Fig. 7b). The recombinant NtAPSI1-1 protein also efficiently bound to the RNA with the SRE sequence, but not to the mutated SRE, and the shifted band abolished with adding a high amount of unlabeled RNA with the SRE sequence as a competitor (Fig. 7c). These results suggest that APSI1 and its tobacco ortholog specifically bind to SRE in the pre-mRNA produced from APXII in cells.

Effect of the disruption of NtAPSI1 on the AS event of endogenous APXII in tobacco

To test whether NtAPSI1 is involved in the AS event of APXII, we used CRISPR-Cas9 genome editing to generate tobacco plants (cv. SR1) in which both NtAPSI1 genes were disrupted (Fauser et al. 2014; Ritter et al. 2017). Two identical nucleotide sequences between the NtAPSI1-1 and NtAPSI1-2 genes following the protospacer-adjacent motif sequence were used to design sgRNAs (Supplementary Fig. 8a). Two sgRNAs were simultaneously introduced into tobacco plants, and 2 independent transgenic lines (#13-1 and #17-3) were obtained. The genes NtAPSI1-1 and NtAPSI1-2 from the T2 generation of transgenic tobacco plants were amplified by PCR using specific primers (Supplementary Table 1). The amplified PCR products were cloned into a TA vector, and each plasmid was sequenced to identify deletions in the vicinity of the target sequences. In 18 clones from the NtAPSI1-1 gene of #13-1, 2 types of mutant alleles were identified, but no WT allele was found (Supplementary Fig. 9). One allele had a 47-base deletion downstream of sgRNA1 and a 1-base deletion upstream of sgRNA2, and the other had a combination of a 7-base deletion and 1-base insertion. In contrast, all 10 clones from the NtAPSI1-2 gene of #13-1 had a complete deletion between sgRNA1 and sgRNA2, but no WT allele was found. Similarly, we confirmed deletions in the NtAPSI1-1 and NtAPSI1-2 genes of #17-3 (Supplementary Fig. 9). In 12 clones from the NtAPSI1-1 gene of #17-3, the sequence from downstream of sgRNA1 to 157-base downstream of sgRNA2 was deleted. In 9 clones from the NtAPSI1-2 gene of #17-3, a 198-base deletion in the sequences downstream of sgRNA1 and a 1-base substitution upstream of sgRNA2 were found. No WT allele was found in clones from either gene of #17-3. In the mRNA extracted from #13-1 lines, only truncated transcripts for NtAPSI1-1 and NtAPSI1-2 were detected (Supplementary Fig. 8b). In the #17-3 lines, the expression of NtAPSI1-1 and NtAPSI1-2 mRNAs was completely lost. These results indicated that the genome edited tobacco lines, #13-1 and #17-3, could not express normal NtAPSI1-1 and NtAPSI1-2 proteins.

All 3 types of mRNA variants produced from the endogenous APXII gene were detected in the #13-1 and #17-3 lines as well as in the WT (cv. SR1) tobacco plants (Fig. 8). The levels of tAPX-I mRNA were considerably decreased in the #13-1 and #17-3 lines. These results clearly indicate that NtAPSI1-1 and NtAPSI1-2 positively affect the AS efficiency of APXII in tobacco cells.

Effect of APSI1 on the tissue-specific AS event of APXII in Arabidopsis and tobacco

The AS event of APXII in spinach and tobacco was previously demonstrated to be regulated in a tissue-specific manner (Yoshimura et al. 2002). The ratio of the levels of sAPX mRNAs (a

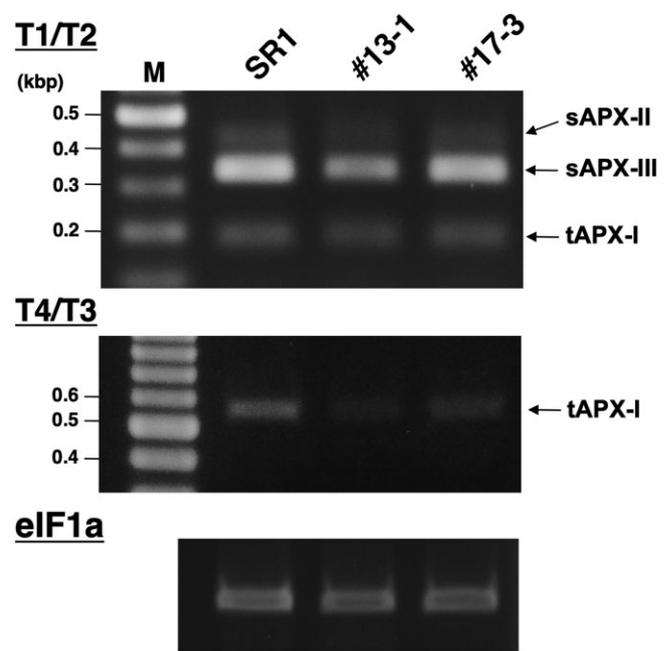


Fig. 8. AS efficiency of the endogenous APXII gene in NtAPSI1-disrupted tobacco plants. Semiquantitative RT-PCR analysis detected AS variants (sAPX-II, -III, and tAPX-I mRNAs) produced from the endogenous APXII gene in genome-edited tobacco lines #13-1 and #17-3, and WT tobacco plants (SR1). Semiquantitative RT-PCR analysis (24–28 cycles) was carried out using sets of primers (Supplementary Table 1) in extracts from the leaves of 4-week-old plants (T2 generation). The positions of primers, except for T4, are similar to those of the spinach APXII gene in Fig. 1a. The T4 primer was designed based on the middle region of exon 5. Amplified products were electrophoresed on a 2% (w/v) agarose gel. Equal loading of each amplified cDNA was determined using the control eukaryotic translation initiation factor 1A (eIF1a) PCR product. M, molecular marker. The experiments were repeated at least 3 times using plants grown independently, and the representative results are shown.

total of sAPX-I, -II, and -III) to tAPX-I mRNA was close to 1 in leaves; in contrast, the ratio in roots was greatly elevated due to an increase in sAPX-III and a decrease in tAPX-I resulting from changes in AS efficiency of intron 11 and intron 12. In spinach and tobacco, gel shift analysis demonstrated that SRE interacts strongly with nuclear proteins from leaves but not from roots (Yoshimura et al. 2002). Using mRNA extracted from various tissues, including rosette leaves, stems, and roots from APXII-Fluc plants grown for 4 weeks, the relationship between the frequency of AS event of APXII and the expression level of APSI1 was tested. While the levels of APSI1 expression were highest in the roots and lowest in the leaves of APXII-Fluc plants, the efficiency of AS in producing tAPX-I-type mRNA was almost equal in all tissues (Fig. 9). In the *apsi1* mutants, an additional transcript with unspliced intron 1 was detected, as was the fully spliced transcript. The levels of fully spliced APSI1 mRNA in the *apsi1* mutants were markedly lower than those in APXII-Fluc plants. The *apsi1* mutation close to the 5' acceptor site of intron 1 may have reduced its splicing of intron 1. Since *apsi1* mutants had a nonsense mutation that resulted in the production of considerably short proteins, the gene products from APSI1 were clearly not functional in any tissues of the mutants. The AS efficiency in producing tAPX-I-type mRNA in all tissues of the *apsi1* mutants was considerably lower than that in APXII-Fluc plants (Fig. 9). These results indicate that the expression of APSI1 and the AS event of APXII in Arabidopsis are not regulated in a tissue-specific manner.

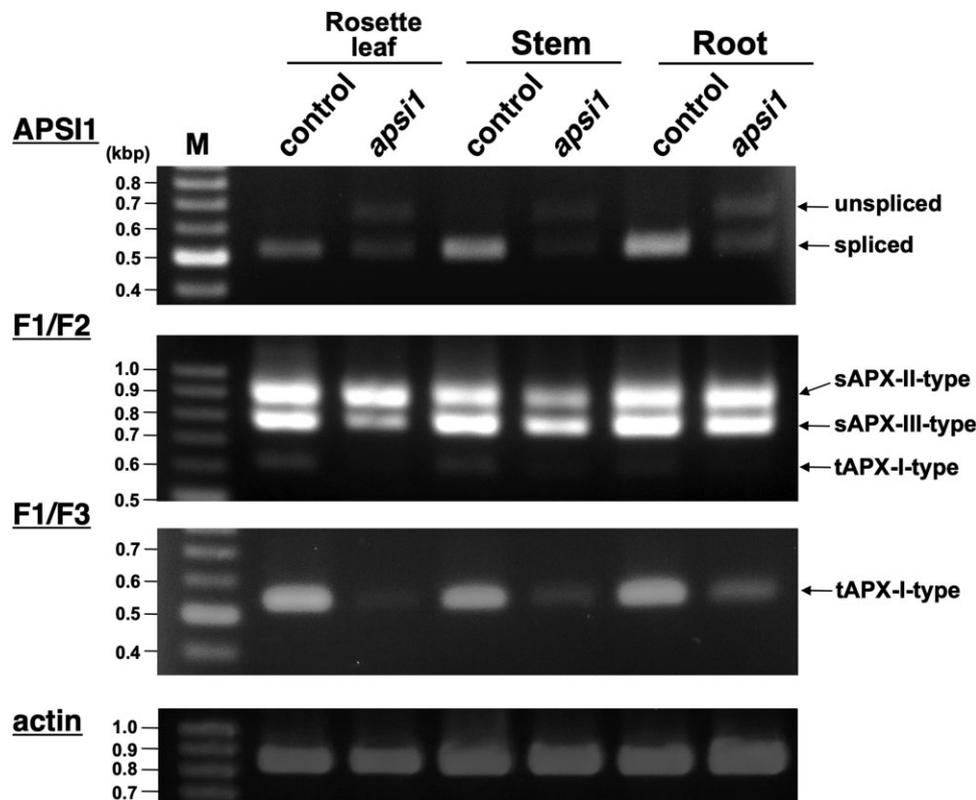


Fig. 9. AS efficiency of *APXII-Fluc* in the tissues of *APXII-Fluc* plants and *apsi1* mutants. Semiquantitative RT-PCR analysis detected *APS11* mRNA and AS variants (sAPX-II, -III, and tAPX-I types) produced from the *APXII-Fluc* gene in *apsi1* mutants. Semiquantitative RT-PCR analysis (24–28 cycles) was carried out using sets of primers (Supplementary Table 1) in extracts from the rosette leaves, stems, and roots of 4-week-old *APXII-Fluc* plants and *apsi1* mutants (M3 generation). The primer positions are shown in Fig. 2a. Amplified products were electrophoresed on a 2% (w/v) agarose gel. Equal loading of each amplified cDNA was determined using the control actin2 PCR product. M, molecular marker. Control, the *APXII-Fluc* plants. The experiments were repeated at least 3 times using plants grown independently, and the representative results are shown.

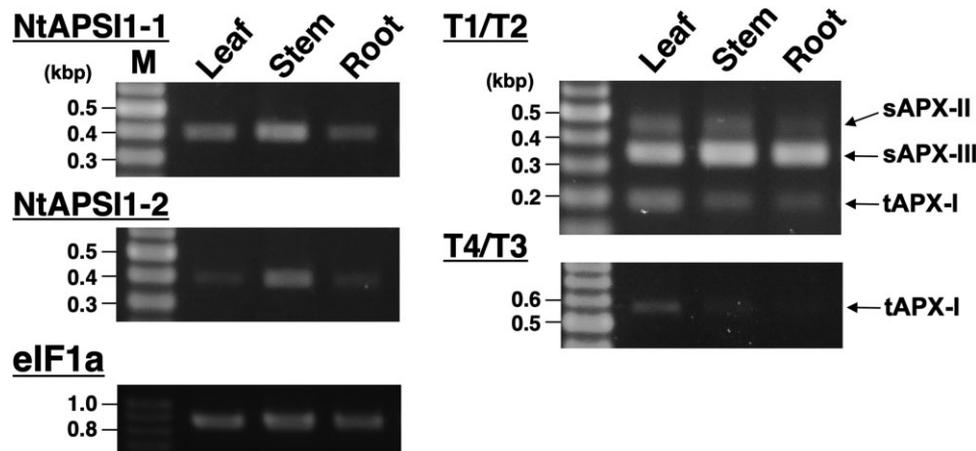


Fig. 10. Expression of *APS11* orthologs and AS efficiency of *APXII* in tissues of tobacco plants. Semiquantitative RT-PCR analysis detected *NtAPS11-1* and *NtAPS11-2* mRNAs, and the AS variants (sAPX-II, -III, and tAPX-I mRNAs) produced from the endogenous *APXII* gene in *N. tabacum* L. cv. SR1. Semiquantitative RT-PCR analysis (24–28 cycles) was carried out using sets of primers (Supplementary Table 1) in extracts from the leaves, stems, and roots of 4-week-old plants. The positions of primers, except for T4, are similar to those of the spinach *APXII* gene in Fig. 1a. The T4 primer was designed based on the middle region of exon 5. Amplified products were electrophoresed on a 2% (w/v) agarose gel. Equal loading of each amplified cDNA was determined using the control eIF1a PCR product. M, molecular marker. The experiments were repeated at least 3 times using plants grown independently, and the representative results are shown.

Next, we analyzed the expression levels of *NtAPS11-1* and *NtAPS11-2* in the leaves, stems, and roots of tobacco plants (cv. SR1). The expression levels of *NtAPS11-1* and *NtAPS11-2* were highest in the stems of these tissues (Fig. 10). In contrast, in

agreement with previous report (Yoshimura et al. 2002), the efficiency of AS in producing tAPX-I mRNA from endogenous *APXII* in tobacco leaves was higher than that in the stems and roots (Fig. 10). These results indicate that, like *Arabidopsis*, the

expression of *NtAPSI1* in tobacco is not regulated in a tissue-specific manner and that the *NtAPSI1-1* and *NtAPSI1-2* proteins are not involved in the tissue-specific regulation of AS event.

Discussion

Chloroplastic APXs are believed to be a bottleneck in the reaction pathways of ROS metabolism in chloroplasts because of their extremely fragile nature; these enzymes are highly sensitive to H_2O_2 at low ascorbate levels (Chen and Asada 1989; Miyake and Asada 1996; Ishikawa et al. 1998; Yoshimura et al. 2000). In contrast, *Arabidopsis* and rice mutants that either lack or suppress the expression of chloroplastic APXs have been found not to exhibit any stress-sensitive phenotypes, at least, under laboratory conditions (Giacomelli et al. 2007; Kangasjärvi et al. 2008; Maruta et al. 2010; Caverzan et al. 2014), indicating that the actions of chloroplastic APXs could be complemented by other antioxidative systems. However, Kameoka et al. (2021) demonstrated that the proton gradient regulation 5-dependent mechanism, which is a major factor for cyclic electron flow around photosystem I and acts on the generation of a *trans*-thylakoid proton gradient that controls H_2O_2 production from photosynthesis, compensates for chloroplastic APXs in *Arabidopsis*, and vice versa. Therefore, chloroplastic APXs are reasonably assumed to be significant for the maintenance of normal photosynthesis and for plant tolerance to environmental stresses.

Both sAPX and tAPX are highly conserved in terrestrial plants, although unicellular algae have only sAPX as the chloroplastic isoform (Maruta et al. 2016). Among terrestrial plants, pteridophytes, monocots, and some eudicots, including *Arabidopsis*, seem to produce chloroplastic APX isoforms from individual genes, although many eudicots, such as spinach and tobacco, appear to produce sAPX and tAPX by the AS event from a single gene. These findings suggest that the AS event of the chloroplastic APX gene was distributed in eudicots after the divergence of monocots and eudicots in angiosperms. However, *Arabidopsis* could correctly produce sAPX and tAPX mRNAs by AS from the spinach *APXII* gene (Fig. 1), suggesting that mechanisms involved in the AS event are at least partially conserved in eudicots and that the regulatory factors for the AS event play a role in other AS events of endogenous genes. More information on the sequences of chloroplastic APX isoforms and evidence for the conservation of AS events from other plant species are required for accurate discussion of the evolution of the AS mechanisms of chloroplastic APX.

In *Arabidopsis*, more than 61% of all transcripts from intron-containing genes are alternatively spliced, resulting in transcriptome and subsequent proteome diversities for cellular processes, although some of these proteins are degraded by nonsense-mediated decay (Marquez et al. 2012). Moreover, it has been estimated that more AS events induced by various stressful conditions can confer stress tolerance. AS can be classified into 4 main types: (1) intron retention (IR), (2) exon skipping (ES), (3) alternative 5'-splice sites (Alt 5'ss), and (4) alternative 3'-splice sites (Alt 3'ss). In humans, ES accounts for 38.4%, followed by Alt 3'ss (18.4%), Alt 5'ss (7.9%), and IR (2.8%). In contrast, the most frequent AS event in *Arabidopsis* is IR (39.6%), followed by Alt 3'ss (15.5%) and Alt 5'ss (7.5%), with ES being the rarest (2.7%) (Carvalho et al. 2013). These facts imply that the regulatory mechanisms of AS events differ considerably between plants and animals. In fact, there is increasing evidence for novel splicing factors in plants (see Review, e.g. Liu et al. 2022). In the case of *APXII*, Alt 3'ss selection, the efficiency of which is regulated by

SRE in a tissue-specific manner, occurs in the 3'-terminal region, but *trans* splicing regulatory factor(s) remain unclear.

Here, to identify a regulatory factor for the AS event of *APXII*, we employed the following strategy: first, we conducted genetic screening in combination with a reporter system in *Arabidopsis* and then carried out functional analyses of its ortholog using tobacco plants, which are experimentally easier to treat than spinach. We then successfully developed a reporter system that could easily monitor AS efficiency to produce a tAPX-I-type splicing variant in *Arabidopsis* (Fig. 2). In both spinach *APXII*-expressing *Arabidopsis* plants and *APXII-Fluc* plants, the AS efficiency producing tAPX-I (-type) mRNA was decreased compared with that in spinach, resulting in an increase in the efficiency in producing sAPX-III-type mRNA (Figs. 1, 2, and 5). In addition, the AS efficiency in producing tAPX-I-type mRNA was almost equal in the rosette leaves, stems, and roots of *Arabidopsis* but not in those of tobacco (Figs. 9 and 10). These findings suggest that the AS efficiency of ectopically expressed genes is somewhat unclear. The levels of tAPX-I mRNA and sAPX-III mRNAs were decreased depending on the expression levels of *APXII* in *Arabidopsis* leaves (Supplementary Fig. 1). The expression of *APXII* did not affect the expression levels of endogenous sAPX and tAPX genes. These findings suggest that the AS efficiency of *APXII* is constant in *Arabidopsis* and the AS event does not impact on the splicing events of other genes.

The 9 mutants screened using the reporter system showed changes in AS efficiency in producing a tAPX-I-type splicing variant, consistent with changes in the intensity of *Fluc* luminescence (Figs. 3 and 4). These results clearly indicate that this reporter system can successfully monitor the efficiency of AS production of tAPX-I mRNA in the *APXII* gene by the intensity of *Fluc* luminescence and can screen mutants with abnormalities in the AS event. We identified the causal gene, *APSI1*, responsible for the phenotypes of *apsi1* mutants (Supplementary Figs. 3 and 4). The *APSI1* gene encodes an OB-fold protein, which has an OB-fold domain, a small structural motif originally named for its oligonucleotide/oligosaccharide binding properties (Theobald et al. 2003; Amir et al. 2020). This domain also acts on protein-protein interactions. OB-fold proteins comprise a superfamily, and the nucleic acid-binding family is known to be the largest among the OB-fold proteins. Proteins containing this domain are involved almost any time an ssDNA or ssRNA is present or requires manipulation, such as during DNA replication, DNA repair, transcription, translation, cold shock response, or maintenance of telomeres (Theobald et al. 2003; Amir et al. 2020). In plants, distinct roles of some proteins with an OB-fold domain have been demonstrated. *Arabidopsis* POT1 was found to be an accessory factor for telomerase, which is required for positive regulation of telomere length (Surovtseva et al. 2007). In contrast, *Arabidopsis* mutants of the *RMI1* gene were found to be sterile because of extensive chromosome breakage during meiosis I, the first division phase of meiosis, indicating that the protein is essential for meiotic recombination in plants (Bonnet et al. 2013). Recently, the OB-fold domain in RNA helicase A—employed by a number of viruses including HIV-1 to facilitate their replication—was found to be involved in modulating a subset of viral RNA splicing events (Xing et al. 2014; Shi et al. 2021), implying the role of *APSI1* in the AS event of *APXII*. Although *Arabidopsis* has a large number of genes encoding OB-fold proteins, there is no close homolog of *APSI1* in the plants. Therefore, plants are thought to have independently evolved specific OB-fold proteins to regulate various cellular responses.

Analysis using GFP fusion proteins demonstrated that APS11 was distributed in the nucleoplasm and was enriched in a nucleolus-like region (Fig. 6). Eukaryotic cells are known to regulate gene expression by spatially organizing the molecular machinery involved in many nuclear events, including mRNA splicing. Several factors involved in RNA and DNA metabolism, including DNA repair and rRNA synthesis, are localized in the nucleolus (Vascotto et al. 2009; Jobert et al. 2013; Iarovaia et al. 2019). The nucleolus has also been reported to operate as a site for the storage and preservation of enzymes until they are needed in the nucleoplasm (Lirussi et al. 2012). In addition, the nucleolus can transiently hold epigenetic regulators during heat shock, allowing restoration of the epigenomic landscape after stress conditions (Azkanaz et al. 2019). Furthermore, small nucleolar RNAs—which act as components of ribonucleoproteins responsible for post-transcriptional modification of RNA targets, ribosomal and small nuclear RNAs—are known to be enriched in the nucleolus (Bratkovič and Rogelj 2014). These facts imply that the subnuclear localization of APS11 may be involved in its regulatory function in AS events.

Various factors involved in the splicing machinery are localized primarily in irregularly shaped domains called nuclear speckles (Corkery et al. 2015; Gordon et al. 2021). Nuclear speckles are highly enriched in dozens of RNA binding proteins that include not only spliceosomal snRNPs, but also other splicing factors such as SR proteins and SR-like proteins, transcription factors, cleavage and polyadenylation proteins, and mRNA export proteins (Corkery et al. 2015; Gordon et al. 2021). Nuclear speckles act as storage, assembly, and/or modification sites from which splicing factors are recruited to perform mRNA splicing at the transcription sites. SR proteins are known to play roles in the determination of alternative splice sites and trafficking of mRNAs between the nucleus and cytosol (Ali et al. 2003; Mori et al. 2012). This discrepancy in subnuclear localization between APS11 and other splicing factors will be elucidated by further studies of the temporal changes in the spatial distribution of the protein in the nucleus.

Disruption and overexpression of APS11 resulted in a decrease and increase, respectively, in the efficiency of AS production of tAPX-I mRNA from APXII in *Arabidopsis* (Fig. 5). Disruption of NtAPS11 also resulted in a decrease in the efficiency of AS in tobacco (Fig. 8). In addition, both APS11 and NtAPS11-1 proteins bound to RNA with the SRE sequence (Fig. 7). These findings suggest that APS11 activates efficiency of AS in the production of tAPX-I mRNA from the APXII gene via binding to SRE. However, the expression of APS11 is not regulated in a tissue-specific manner in either *Arabidopsis* or tobacco, whereas the AS event of APXII in tobacco is (Figs. 9 and 10). These results suggest that the expression levels of APS11 and its tobacco orthologs in leaves are sufficient to maintain the AS event that produces tAPX-I mRNA and there is an additional factor that competitively inhibits the action of APS11 in root and stem tissues. In addition, the fact that disruption of the APS11 gene in both *Arabidopsis* and tobacco did not cause complete suppression of tAPX-I mRNA production suggests that APS11 is not a key component for the AS event that produces tAPX-I mRNA but acts to maintain the high efficiency of tAPX-I mRNA production, implying the complexity of the regulatory mechanism. We are progressing in the elucidation of the role of APS11 in other AS events in tobacco and *Arabidopsis* to uncover whether APS11 acts generally on various AS events. In addition, we aim to identify the causal gene for the phenotypes of other *apsi* mutants. Studies on the causal genes responsible for other

mutants will shed light on the whole regulatory mechanism for the AS event of APXII.

Data availability

Seeds of all mutant and WT *Arabidopsis* lines are available at the ABRC, and all DNA sequence data are available at NCBI under the following accession numbers: APXII, NCBI accession numbers: LOC110797270 (spinach), LOC107792162 (tobacco); APS11, AT5G38890, KO-*apsi1*, ABRC stock number: SALK_004562.

Supplemental material is available at G3 online.

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Conflicts of interest

The authors of this manuscript declare no conflicts of interest.

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