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Identification of Zinc Finger (ZnF) Genes that Undergo Tissue-specific Alternative Splicing in *Arabidopsis thaliana*

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RNA editing involves the post-transcriptional modification of genes, whereas alternative splicing involves both post- and co-transcriptional regulation of gene expression. Our previous findings reported that tissue and different developmental stage-specific alternative splicing and RNA editing occur in pentatricopeptide repeat (PPR) family genes. In this study, we found that important Zinc Finger (ZnF) genes undergo tissue-specific alternative splicing, which affects protein structure and function. Five ZnF genes that were likely to undergo alternative splicing were selected based on their functional importance. Most of these genes are located in protein-coding regions, which were predicted using the Arabidopsis database. Tissue-specific alternative splicing in all five ZnF genes was confirmed by quantitative PCR and sequencing. AT1G75340/ZnF2c, AT1G75340/ZnF2d, AT3G61850/ZnF3b, AT1G06040/ZnF5b show intron retention, AT2G47850/ZnF1b contains a 3' alternative splice site, and exon skipping is detected in AT1G75340/ ZnF2a. Alternative splicing events occurred in the coding regions of four genes and in the 3' UTR of AT3G51950/ ZnF4. We identified three novel and previously unannotated alternatively spliced isoforms by quantitative PCR analysis and sequencing; ZnF4b arose from an alternative 3' splice site and ZnF2c, ZnF2d from intron retention. AT2G47850/ ZnF1 and AT3G61850/ZnF3 isoforms affect protein structures, ligand binding sites and ultimately functions. There were previously unannotated isoforms AT1G75340/ZnF2c and AT1G75340/ ZnF2d that resulted in frameshift mutations. The functionality of alternatively spliced isoforms need to be elicited in plant physiology.

Keywords: Alternative splicing, Arabidopsis, RNA editing, Zinc finger motif

Introduction

Alternative splicing is a bimolecular process that involves the generation of multiple mRNAs from a single gene sequence (Tognacca et al., 2022). Alternative splicing is also a vital process in the development of living organisms and is influenced by environmental factors (Kelemen et al., 2013). The application of next-generation sequencing technologies has shown that up to 60% of genes containing multiple exons exhibit alternative splicing (Syed et al., 2012; Huang et al., 2022; Qulsum & Tsukahara, 2018); for example, the primary genes involved in the circadian clock of Arabidopsis are heavily influenced by alternative splicing under different environmental conditions (James et al., 2012; Syed et al., 2012; Huang et al., 2022). As well as having a considerable

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impact on phenotype, alternative splicing has major ramifications for functionality, and study into these effects is ongoing (Syed et al., 2012). Earlier studies on alternative splicing used whole plant samples; however, we used a tissue-specific approach to observe different stages of plant development.

Recent studies of plant RNA editing have focused on non-PPR (pentatricopeptide repeat) editing factors, and have highlighted an unpredicted diversity in the plant editosome (Sun et al., 2016; Qulsum et al., 2019). In plants, the conversion of cytidine to uridine (C-to-U) by cytidine deaminase is dependent on zinc ions. This may result from the requirement for Zn^{2+} in the active center of cytidine deaminase (Vasudevan et al., 2013). The zinc finger protein family is found in numerous plant families, including the spikemoss Selaginella, which suggests that it plays an evolutionarily conserved role in the plant editosome (Sun et al., 2015, 2016).

The zinc finger (ZnF) is one of the predominant motifs found in eukaryotic proteins. ZnF proteins are characterized according to the number and order of histidine and cysteine residues present in the polypeptide chain. These amino acids interact with zinc ions to maintain the tertiary structure of the proteins. Analogous to their function at a molecular level, ZnF proteins are involved in a wide variety of different biological processes, including the regulation of transcription, modification of chromatin, and degradation of proteins by ubiquitin (Laity et al., 2001; Appelhagen et al., 2010; Diaz-Ramirez et al., 2022). The zinc finger protein RanBP2 is a potential chloroplast RNA editing factor (Sun et al., 2015). The efficiency of RNA editing varies across different tissues and at different stages of development; for example, a higher number of editing sites are found in green seedlings and green tissues than in non-green tissues and comparatively mature seedlings (Tseng et al., 2013; Qulsum et al., 2019). Most proteins in the organelle zinc finger family are essential for RNA editing and are also involved in other aspects of RNA metabolism in plant organelles (Sun et al., 2015).

The evolutionarily conserved zinc finger motif defines the structure of ZnF proteins, and thus the different members of this protein family are grouped according to the level of correspondence between their ZnF motifs. A number of ZnF proteins are used in multiplexed autoantibody assays that detect colorectal cancer in a minimally invasive manner (O'Reilly et al., 2015). In mammalian species, tissue-specific alternative splicing may alter protein stability, enzyme activity, and the sub-cellular level of localization, as well as other features (Merkin et al., 2012). Tissue-specific alternative splicing in plants, however, is not yet well understood.

This study was designed to identify instances of alternative splicing of specific ZnF genes in various plant tissues, including the leaf, stem, stipe, and root, as well as in whole seedlings. The expression patterns of alternatively spliced mRNAs were compared in different Arabidopsis tissues across a variety of developmental stages such as days 4, 8, 12, 16, 21, 27 and 32. A range of web-based tools were used to investigate the manner in which alternative splicing influenced protein diversity in Arabidopsis.

Materials and Methods

Plant growth conditions and sample collection

Paper pots were gently filled with a mixture of horticultural perlite, peat moss, and vermiculite in a ratio of 1:2:1. Arabidopsis (*Arabidopsis thaliana*) Col-0 accession seeds, were sowed onto the surface of the mixture. The pots were covered with cling film, kept under dark conditions for 3 to 4 days, and then transferred to a growth room at the Green Farm U.ING (Osaka, Japan). Plants were grown at 22°C constant temperature and 45% relative humidity under a photoperiod of 16 hours of light and 8 hours of darkness. After germination, plants were watered twice daily (every morning and evening); fertilizer was applied twice each week. Samples of seedlings (whole plants) were collected when the plants were 4, 8, and 12 days old; samples of leaves, stipes, stems, and roots were collected from plants that were 16, 21, 27, and 32 days old.

Plant RNA extraction and cDNA synthesis

Total RNA was extracted from each sample using the Qiagen plant mini kit (Qiagen, Germany, catalog no. 74904), according to the manufacturer's instructions. Samples were treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) to remove all contaminating genomic DNA and then purified by phenol-chloroform and ethanol precipitation. The RNA content of each sample was quantified using a

NanoDrop™ spectrophotometer (Thermo Fisher Scientific, USA). cDNA was synthesized from purified RNA by reverse transcription using Superscript III reverse transcriptase (Invitrogen, USA) and oligo dT primers. cDNA synthesis was confirmed by polymerase chain reaction (PCR) amplification of GAPDH, a housekeeping gene of Arabidopsis, using *GAPDH* forward (GTTGTCATCTCTGCCCCAAG) and reverse (TGCAACTAGCGTTGGAAACA) primers.

Selection of alternatively spliced Arabidopsis ZnF gene sequences

Arabidopsis ZnF genes likely to undergo alternative splicing were identified using the NCBI gene database (https://www.ncbi.nlm.nih.gov/gene/). The accession numbers of the ZnF gene sequences identified in the NCBI database were input into the *Arabidopsis thaliana* Plant Genome Database (http://www.plantgdb.org/AtGDB/) to establish the genomic map position of each gene. Using this methodology, probable candidates for alternatively spliced genes (whole genome model) were selected using the TAIR10 annotation of the Arabidopsis genome. Information on the expression of sequences in different tissues, obtained from The Arabidopsis Information Resource (TAIR; https://www.arabidopsis.org/), enabled the identification of 25 alternatively spliced ZnF genes. Finally, the genomic sequence of each gene, as well as information relating to its intron and exon sequences, coding sequences, transcript, and resulting amino acid sequence, was obtained from The Arabidopsis Genome Integrative Explorer (http://atgenie.org/) and cross-checked against the other sources. Of the 25 genes identified, five functionally important genes were selected for further detailed study.

Primer design

Primers were designed using the Primer3 primer design software (bioinfo.ut.ee/primer3-0.4.0/primer3/). The specificity of primer sets to the intended PCR target was checked using NCBI primer-blast. Another set of primers was designed if the first set failed or gave an inappropriate outcome. Primers were purchased from Eurofins (Japan) and stored in TE buffer at a concentration of 50pmol/µL in salt-free conditions. Primers were diluted to a working concentration of 10pmol/µL with TE buffer.

Analysis by PCR and polyacrylamide gel electrophoresis (PAGE)

The numbers of thermal cycles used for the PCR analysis of expression of each gene are shown in Table 1. In each case, the denaturation temperature was set at 94°C and the elongation temperature at 72°C. The annealing temperatures were set individually for each primer set, as suggested by the Primer3 software used for primer design (Table 1).

TABLE 1. Identification of the ZnF family genes used in this study. Each gene is listed according to the tentative gene name assigned to it in this study, together with its TAIR gene ID/accession number. Genes are annotated according to the description in the database. PCR analysis and sequencing were used to confirm each gene. The annealing temperature, number of thermal cycles, and gene-specific primer sequences used in the PCR analyses are listed in the table

Tentative gene name	Gene ID/ Accession no.	Annotation	AT°C	Cycle	Forward	Reverse
ZnF1	AT2G47850	Zinc finger C-x8-C-x5-C-x3-H-type family protein	58	30	CATGAGCCA-CGTTCCACTAA	GGCGCCTTG-CATATAAGAAC
ZnF2	AT1G75340	Zinc finger C-x8-C-x5-C-x3-H-type family protein	55	35	CCCTTCTCC-AGCATTCTCTG	GTTGACGCT-GAAAGCATTTG
ZnF3	AT3G61850, DAG1	Dof-type zinc finger DNA-binding family protein	57	30	CCAGTCGG-AGGTAGCTCAAG	GGCATTGTT-GGAAACCCTAA
ZnF4	AT3G51950	Zinc finger CCCH-type family protein	55	35	CAACCTGTC-GTGTTGGGATA	TGTGTGGTC-CATCATCACAT
ZnF5	AT1G06040, STO	B-box zinc finger family protein	58	35	CAGCAGCAA-CAACAACCTTC	CACCGAAGA-ATCCCATGTCT

A 6% polyacrylamide gel was used for electrophoresis of PCR products. Equal amounts of PCR products were loaded onto the polyacrylamide gel, and electrophoresis was carried out. The gels were stained with SYBR® Green dye (Lonza, Rockland, ME, USA). Each gel image was photographed using different exposure times to obtain high-quality images for analysis. Gel images were analyzed using a LAS3000 Luminescent Image Analyzer (Fujifilm, Tokyo, Japan).

Transcript sequencing

The desired bands were excised from the gel following electrophoresis and transferred to disposable pellet pestle/tissue grinder tubes (Kimble®, Capitol Scientific, Inc., TX, USA, catalog no. 749520-0090) before being frozen at -80 °C for 1 hour. The frozen gel pieces were crushed in the grinder tubes using the pestle, and approximately 10 µl 0.1× TE was added to the crushed powder prior to further grinding. The tubes were vortexed for 10 minutes and then centrifuged at full speed at 4°C for 20min in a tabletop centrifuge. The supernatant was transferred into another tube, and a 3µL sample was extracted for sequencing.

PCR products were sequenced using a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing was performed using the Big dye Terminator V3.1 sequencing standard kit (Austin, TX, USA, catalog no. 4336935). Sequencing results obtained using the reverse primer were reverse complemented using the Sequence Manipulation Suite online software (http://www.bioinformatics.org/sms2/reference.html (Stothard, 2000)). The complete set of sequencing results were aligned with the *Arabidopsis thaliana* genome using BLAST (Kent, 2002).

Determination of tissue-specific expression of different ZnF isoforms

PCR products were loaded onto a 6% polyacrylamide gel and run in 1× TBE buffer at 200 volts for 20 minutes. The gel was stained with SYBR® Green dye (Lonza, Rockland, ME, USA) in the presence of 1× TBE buffer and placed on a rocker for 20min. The image of the stained gel was captured using a LAS3000 (Fujifilm, Tokyo, Japan) and subjected to densitometry analysis using Image J software (NIH, MD, USA). Densitometry analysis of the bands enabled a comparison of the expression levels of the transcripts that exhibited alternative splicing. These results were confirmed by sequencing.

Determination of protein diversity resulting from alternative splicing

The effects of alternative splicing on the resulting protein isoforms were determined using the I-TASSER (Iterative Threading Assembly Refinement; https://zhanggroup.org/I-TASSER/) bioinformatics tool (Roy et al., 2010; Yang et al., 2014). The full-length amino acid sequences were submitted to the I-TASSER server. I-TASSER was also used to determine the ligand-binding sites and the distances between residues. The protein sequences from each of the spliced variants were established using AtGenIE (http://atgenie.org); however, the novel variant that was discovered in the course of this study was transcribed using the Expasy translator tools (https://web.expasy.org/translate/) before the amino acid sequence was submitted to the I-TASSER server, together with the desired output parameters. Different parameters between the alternatively spliced isoforms were analyzed comparatively.

Results

We used the TAIR (https://www.arabidopsis.org/) database to identify 25 alternatively spliced genes encoding ZnF family proteins that were differentially expressed across tissues or showed developmentally specific expression. For a given gene, alternatively spliced isoforms are represented as a, b, c, and d.

Determination of tissue-specific expression of different ZnF isoforms

A 3’ alternative splice site prior to exon 5 added 78 nucleotides to ZnF1 to create ZnF1b (Fig. 1A and C). Transcript containing this 3’ alternative splice site was observed in 4-day-old seedlings, in leaves from 16-, 21-, and 27-day-old plants, and in the stipes of 32-day-old plants (Fig. 1A). Expression of ZnF1a gradually increased in roots over time, but expression of ZnF1b gradually decreased; however, expression of ZnF1b increased again in the roots of 32-day-old plants (Fig. 1B).

Exons 7 and 8 of ZnF2, which contained 78 and 93 nucleotides, respectively, were skipped to create ZnF2a (Fig. 2A and C). We observed two novel and unannotated spliced isoforms of ZnF2: ZnF2c, which retained a 100 nucleotide intron between exons 7 and 8, and ZnF2d, which retained a 106

nucleotide intron between exons 8 and 9 (Fig. 2A and C); both isoforms were confirmed by Sanger sequencing. ZnF2c was expressed in 32-day-old leaves and 16-day-old stipes, and ZnF2d was expressed in stems of 16- and 27-day-old plants and in roots of 16-day-old plants (Fig. 2A). ZnF2b was strongly expressed in all tissues (Fig. 2B). Expression of ZnF2d was low in stem tissue from 16- and 27-day-old plants and was not detected in the stems of 21- and 32-day-old plants (Fig. 2B).

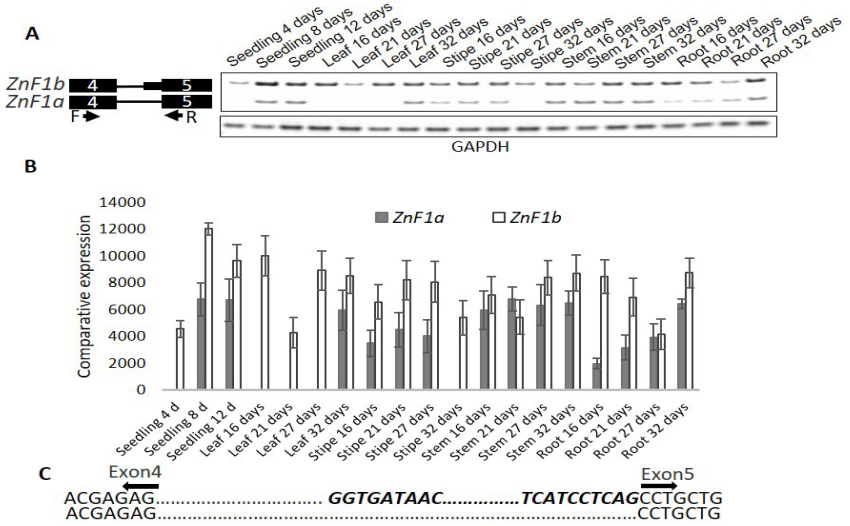

Fig.1. Alternative splicing in ZnF1 (accession no. AT2G47850) transcripts. a PCR analysis of ZnF1 expression in seedlings and in leaf, stipe, stem, and tissue. The estimated sizes of the fragments on the gel are shown in base pairs (bp). b Comparative expression of two differently spliced isoforms of ZnF1 in different tissues. c The genomic sequence of ZnF1 between exon 4 and exon 5. The arrows indicate the exon boundaries, and the dots indicate the intron sequences. Bolded italic sequences and dots indicate the 3' alternative splice site

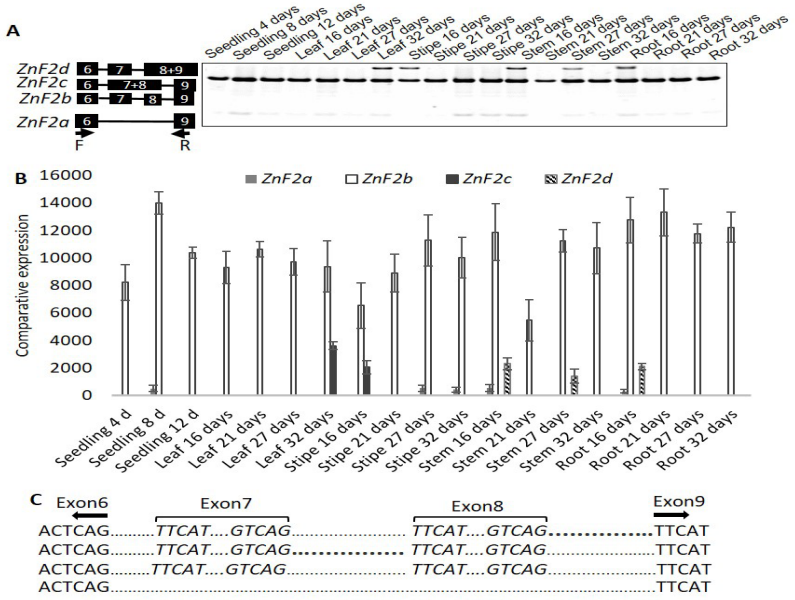

Fig. 2. Alternative splicing in ZnF2 (accession no. AT1G75340) transcripts. a PCR analysis of ZnF2 expression in seedlings, and in leaf, stipe, stem, and root tissue. The estimated sizes of the fragments on the gel are shown in base pairs (bp). b Comparative expression of four alternatively spliced isoforms in different tissues. b The genomic sequence of ZnF2 between exon 6 and exon 9. The arrows indicate the boundaries of exons 6 and 9, and the dots indicate the intron sequences. Exons 7 and 8 are indicated by italic sequences and dots; bold dots indicate retained introns

An 84-nucleotide intron between exons 2 and 3 of ZnF3 was either retained to create ZnF3b or spliced out to produce ZnF2a (Fig. 3A and C). Intron retention was not detected in transcripts from 4-day-old seedlings, in transcripts from the leaves of 16-, 21-, 27-, and 32-day-old plants, or in transcripts from the stipes and stems of 21-day-old plants (Fig. 3A). ZnF3a expression in leaves gradually decreased between day 16 and day 32 (Fig. 3B). Although ZnF3b was not expressed in 4-day-old seedlings, its transcript was detected in 8- and 12-day-old seedlings. ZnF3a expression gradually increased in seedlings between days 4 and 12 (Fig. 3B).

An 88-nucleotide intron was retained between exons 9 and 10 of ZnF4 to produce ZnF4c (Fig. 4A and C). A 3' alternative splice site in ZnF4 added 32 nucleotides to exon 10 to create ZnF4b, a new and previously unannotated isoform (Fig. 4A and C). The PCR analysis detected ZnF4b transcript only in 4-day-old seedlings (Fig. 4A). ZnF4c transcript was not detected in 12-day-old seedlings or in the roots of 16-day-old plants (Fig. 4A); however, ZnF4c was expressed in the roots of older plants, and its levels rose gradually between days 21 and 32 (Fig. 4B).

PCR analysis with a ZnF5-specific primer set revealed that an 82-nucleotide intron was retained between exons 2 and 3, creating ZnF5b (Fig. 5A and C). Transcripts retaining the intron were not detected in 4-day-old seedlings, in stipes from 16-, 27-, or 32-day-old plants, in the stems of 21-, 27-, or 32-day-old-plants, or in the roots of 27- and 32-day-old plants (Fig. 5A).

Determination of protein structure affected by alternative splicing

As alternative splicing sites were detected in the coding regions of ZnF1, ZnF2, ZnF3 and ZnF5, we investigated whether alternative splicing affected protein structure. Alternative splicing of ZnF1 produced a transcript encoding a protein, ZnF1b, which contained an additional 26 amino acids in comparison with the reference isoform (Table 2); the presence of the additional amino acids was confirmed by PCR and sequencing. The protein secondary structure differed between the two isoforms of ZnF1 (Table 2). The presence of the additional 26 amino acids (GDNECSYYLKTGQCKFGITCKFHHPQ; amino acids 138–163) resulted in a change in the number of α-helices, β-strands, and random coils (Fig. 6A and B). In ZnF1b, an upstream β- strand and α-helix disappeared but a novel α-helix formed in the middle of the protein (Fig. 6A). In addition, the presence or absence of these 26 amino acids may have altered the ligand-binding ability of the isoforms, ligand binding residues thus changing the types of ligands associating with the protein (Fig. 6C and D).

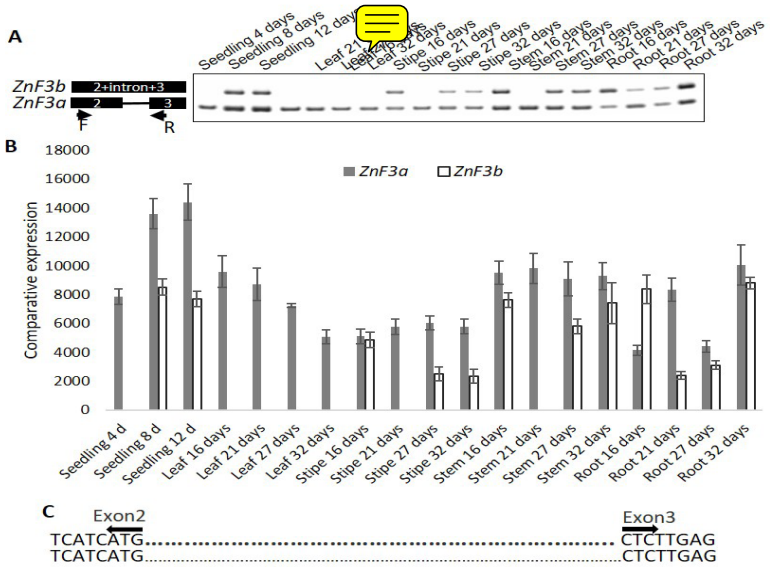

Fig. 3. Alternative splicing of ZnF3 (accession no. AT3G61850; DAG1) transcripts. a PCR analysis of ZnF3 expression in seedlings, and in leaf, stipe, stem, and root tissue. The estimated sizes of the fragments on the gel are shown in base pairs (bp). b Comparative expression of two alternatively spliced isoforms of ZnF3 in different tissues. c The genomic sequence of ZnF3 between exon 2 and exon 3. The arrows indicate the boundaries of exons 2 and 3, and the dots indicate the intron sequences; bold dots indicate a retained intron

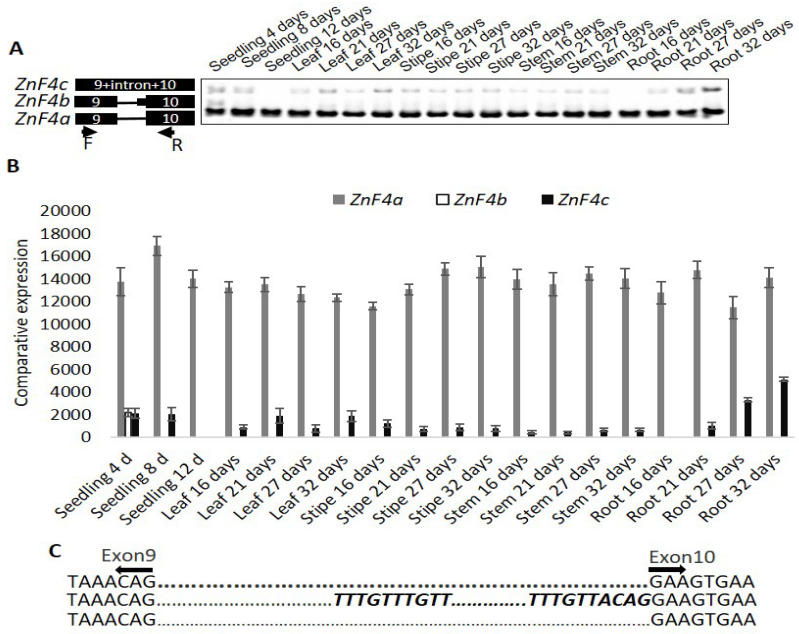

Fig. 4. Alternative splicing of ZnF4 (accession no. AT3G51950) transcripts. a PCR analysis of ZnF4 expression in seedlings, and in leaf, stipe, stem, and root tissue. The estimated sizes of the fragments on the gel are shown in base pairs (bp). b Comparative expression of three alternatively spliced isoforms of ZnF4 in different tissues. c The genomic sequence of ZnF4 between exon 9 and exon 10. The arrows indicate the boundaries of exons 9 and 10, and the dots indicate intron sequences; italic sequences and dots indicate the 3' alternative splice site; bold dots indicate a retained intron

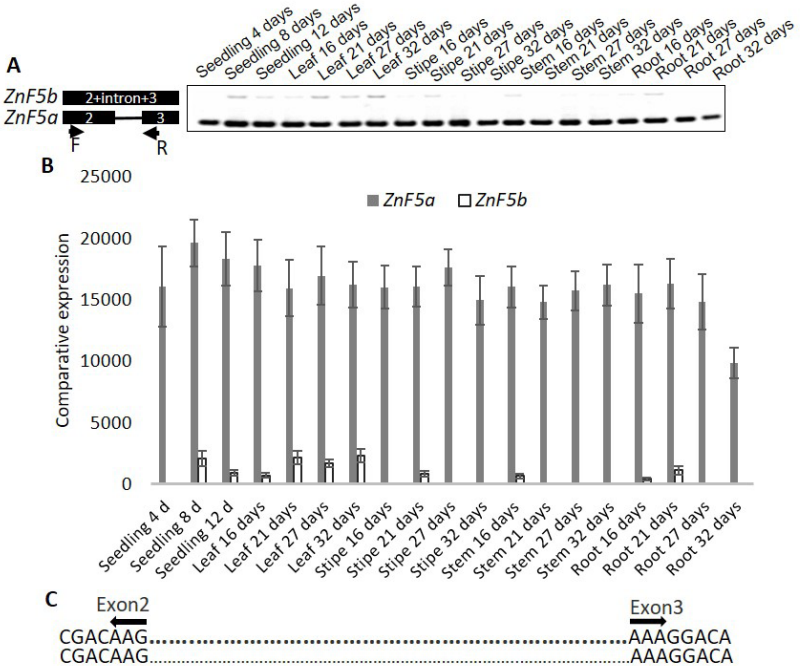

Fig. 5. Alternative splicing of ZnF5 (AT1G06040; STO) transcripts. a PCR analysis of ZnF5 expression in seedlings, and in leaf, stipe, stem, and root tissue. The estimated sizes of the fragments on the gel are shown in base pairs (bp). b Comparative expression of two alternatively spliced isoforms of ZnF5 in different tissues. c The genomic sequence of ZnF5 between exon 2 and exon 3. The arrows indicate the boundaries of exons 2 and 3, and the dots indicate intron sequences; bold dots indicate a retained intron

TABLE 2. Summary of alternative splicing affecting protein of studied genes

Tentative gene name	Alternative splicing events	Effect on	
		Nucleotide	Amino acids
ZnF1a	Reference isoform Shorter	-	-
ZnF1b	Reference isoform 3' alternative spliced site	78 nucleotide addition	26 amino acid addition
ZnF2a	Reference isoform exon skipping	-	-
ZnF2b	Reference isoform longer	171 nucleotide addition	57 amino acid addition
ZnF2c	New unannotated Intron retention	100 nucleotide addition	Frameshift
ZnF2d	New unannotated Intron retention	106 nucleotide addition	Frameshift
ZnF3a	Reference isoform Shorter	-	-
ZnF3b	Reference isoform Intron retention	84 nucleotide addition	28 amino acid addition
ZnF5a	Reference isoform Shorter	-	-
ZnF5b	Reference isoform Intron retention	82 nucleotide addition	Frameshift

The table represents that alternative splicing affected protein our selected genes of ZnF1, ZnF2, ZnF3 and ZnF5. The alternative splicing sites in case of ZnF4 were selected in UTR regions.

The reference isoform for ZnF2 is ZnF2a. Transcripts encoding ZnF2b and two other novel and previously unannotated isoforms, ZnF2c and ZnF2d, were identified by a PCR analysis that determined whether two introns, one containing 100 and the other 106 nucleotides, had been retained (Table 2; Fig. 2A and C). Although the addition of 57 amino acids (QQTTFPNTNAGGVSSSGPPNPFASFTQQSNNQQTAFSNTNAGGLSSSGPPNAFASFN; amino acids 253–309) to ZnF2 greatly affected the protein structure (β-strand, α-helices and random coils) between the residues. ZnF2c and ZnF2d, the novel isoforms of ZnF2, contained an additional 100 and 106 nucleotides, respectively, which resulted in frameshifts during translation (Table 2).

Retention of an intron between exons 2 and 3 of ZnF3 added an extra 84 nucleotides to ZnF3a to produce the longer ZnF3b transcript (Table 2; Fig. 3A and C). This encoded an ZnF3 isoform incorporating an additional 28 amino acids (GMSHFFHMPKIENNNTSSSIYASSSPVS; Table 2), whose presence resulted in the loss of a β-strand from the C-terminal region and of an α-helix from the middle of the ZnF3b protein (Fig. 7A). In addition, the presence or absence of these 28 amino acids resulted in changes to the ligand-binding ability of the ZnF3 isoforms, thus altering the types of ligands associating with the protein (Fig. 7C and D). The 28-amino acid peptide present in ZnF3b was highly conserved between ZnF family proteins, both within a species and across plant species (Fig. 7E). The addition of 82 nucleotides to ZnF5 resulted in frameshifts during translation (Table 2).

Discussion

The alternative splicing process varies greatly between plants and heavily influences the extent of transcript diversity. Instances of splicing that involve only single-nucleotide exons have been reported in *Arabidopsis thaliana* (Guo & Liu, 2015; Qulsum & Tsukahara, 2018) although sequences involved in intron recognition and splicing are mainly located in introns, exonic sequences also contribute to intron splicing. The smallest constitutively spliced exon known so far has 6 nucleotides, and the smallest alternatively spliced exon has 3 nucleotides. Here we report that the Anaphase Promoting Complex subunit 11 (APC11. The focus of the current study was alternative splicing of genes in the zinc inger family. Functional data deposited in the Uniprot database s(http://www.uniprot.org) suggest that ZnF1 and ZnF5 are involved in binding zinc ions, although the exact biological mechanism has not yet been fully elucidated. Exons 8 and 9 of ZnF2 generate circular RNA, which may bind stably to cognate DNA and form a DNA-RNA hybrid (Conn et al., 2017) although sequences involved in intron recognition and splicing are mainly located in introns, exonic sequences also contribute to intron splicing. The smallest constitutively spliced exon known so far has 6 nucleotides, and the smallest alternatively spliced exon has 3 nucleotides. Here we report that the Anaphase Promoting Complex subunit 11 (APC11. The formation of such DNA-RNA hybrids may result in lower transcription efficiency. We found that ZnF2b, which contained exons 7 and 8, was highly expressed in all tissues whereas ZnF2a, which skipped both these exon was expressed at very low levels in 8-day-old seedlings, the stipes of 27- and 32-day-old plants, and the stems and roots of 16-day-old plants. Exon 9, however, was present in both these isoforms.

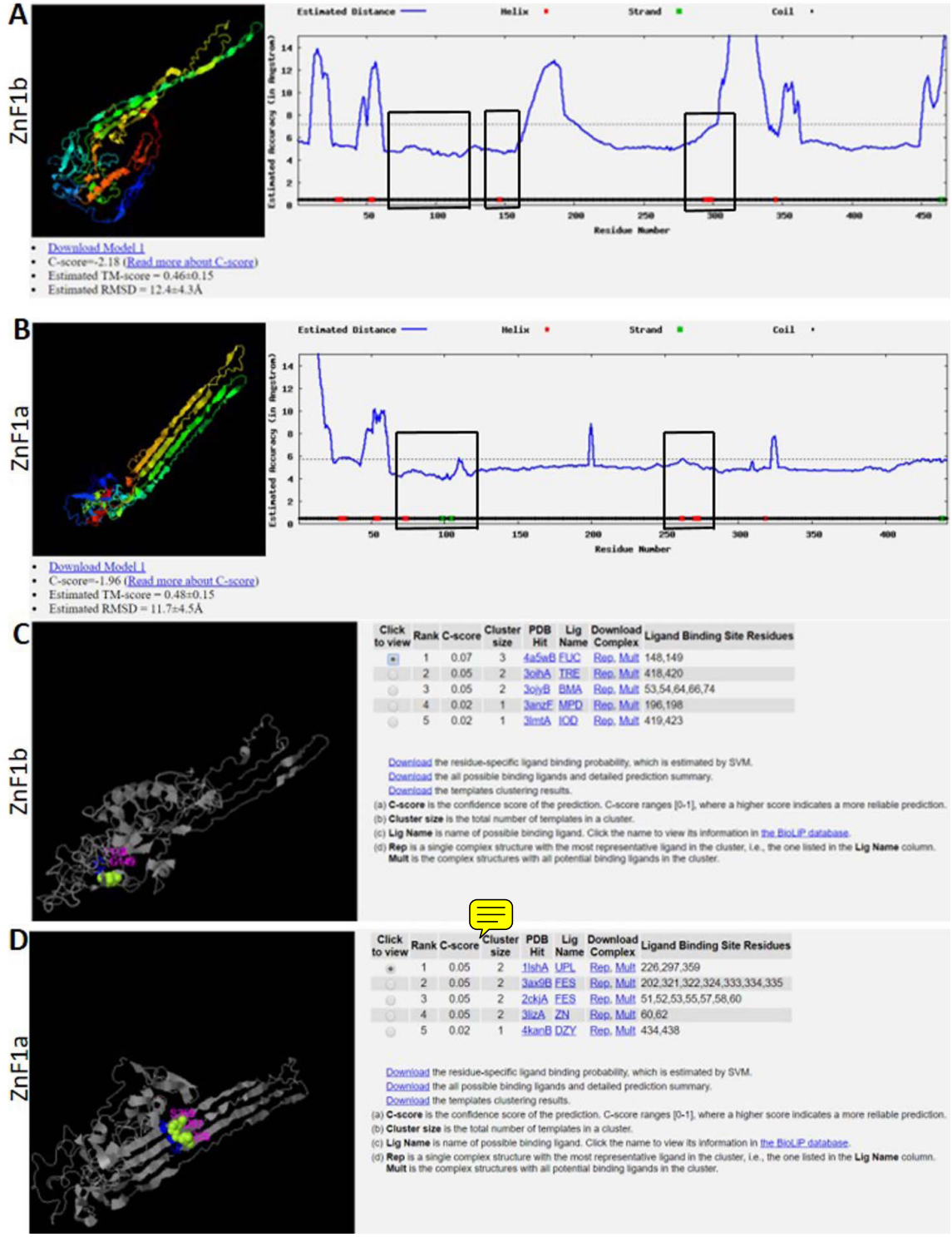

Fig. 6. Effect of a 26-amino acid addition in ZnF1 (accession no. AT2G47850) on the structure of ligand-binding site residues, as determined by the I-TASSER bioinformatics tool. A, B. Addition of 26 amino acids to ZnF1b result in a loss of β- strand and an α-helix from the N-terminal region and the gain of an α-helix in the middle region of the protein. Absence of these 26 amino acids result a β- strand and an α-helix in the C-terminal region of ZnF1a and another α-helix in the middle of the protein (marked by rectangular boxes). C, D. Changes in the ligand-binding site between the two isoforms of ZnF1

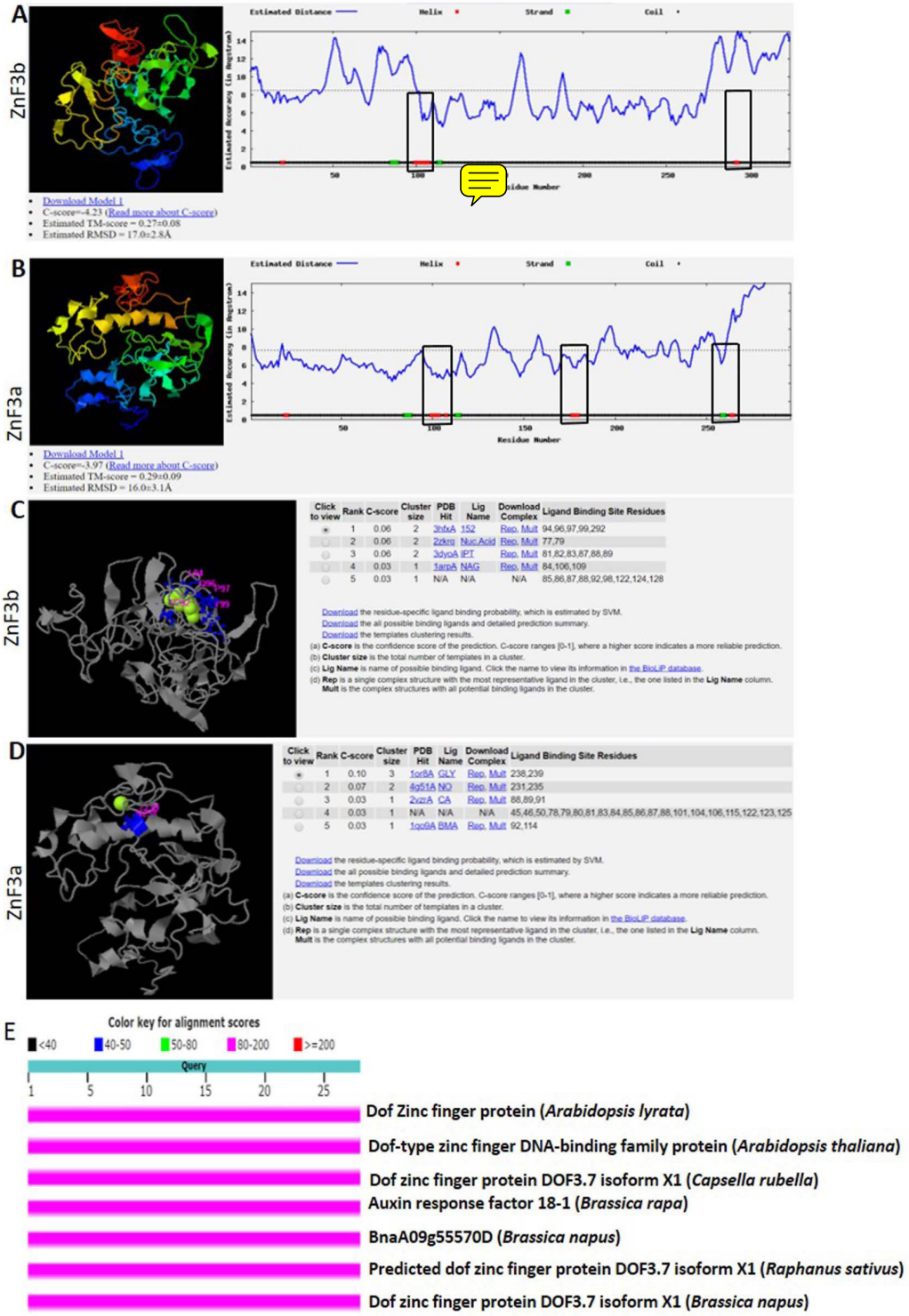

Fig .7. Effect of the presence and absence of a 28-amino acid sequence on the structure and ligand-binding sites of ZnF3 (accession no. AT3G61850; DAG1). A, B. The addition of 28 amino acids caused the loss of a β-strand from the C-terminal region and the loss of an α-helix from the middle of the protein (marked by rectangular boxes). Absence of these additional 28 amino result no β-strand in its C-terminal region; loss of a α-helix in the middle of the protein (marked by rectangular boxes). C, D. Changes in the ligand-binding site between the two isoforms of ZnF3. E. Homology of the 28-amino acid peptide found in alternatively spliced isoforms of ZnF3 (accession no. AT2G47850) to peptides in ZnF proteins from other plant species

ZnF3 is a member of the DNA-binding One Zinc Finger (DOF) family of proteins. DOF proteins contain a highly conserved DNA-binding domain and a C2-C2 zinc finger motif (Yanagisawa & Sheen, 1998; Chen et al., 2020). A nonconserved transcriptional factor in the C-terminal region of Dof proteins may interact with various partners (Yanagisawa & Sheen, 1998; Wang et al., 2018) particularly in the Euphorbiaceae family. DNA binding with one finger (Dof. Intron retention in ZnF3 was observed mainly in stipes, stems, and roots (Figure 3A), and the shorter isoform was expressed in all tissues (Fig. 3A). In maize, DOF1 is a transcriptional activator whereas DOF2 is a transcriptional repressor; light induces binding of DOF1 to its target site (AAAAGG) in green leaves, which activates transcription (Yanagisawa & Sheen, 1998; Chen et al., 2020). In non-green leaves, the transcriptional activity of DOF1 was inhibited. We did not detect the ZnF3b isoform in 4-day-old non-green seedlings (Fig. 3A). Intron retention in ZnF3b may cause the protein to lose its transcriptional activity.

In addition to binding Zn ions, ZnF4 binds DNA, catalyzes hydrolysis reactions, exhibits nuclease activity, and binds to RNA (Zhen & James, 2018).

The alternative splicing of different ZnF genes resulted in different levels of expression in different tissues. Transcripts with retained introns were more often expressed than those with alternative 5' and 3' splice sites. Previous reports suggest that intron retention is more common than other types of alternative splicing in Arabidopsis (Li et al., 2016). Higher expression of ZnF3 in specific tissues was observed in this study. RNA editing at the ndhD-1 site in the Arabidopsis chloroplast involves the DYW1 domain of PPR, which contains the zinc-binding active site signature motif HxE(x)nCxxC that is essential for RNA editing (Boussardon et al., 2014). This observation, together with our results, suggests that alternative splicing of the zinc finger motif may affect RNA editing at the tissue-specific level.

When we considered the effects of alternative splicing of transcripts on the proteins, we found that ZnF3b contained a 28-amino acid peptide that was highly conserved in ZnF family proteins, both within and between plant species. This implied that this peptide has an important conserved function, and protein structure and function may be changed by alternative splicing.

Alternative splicing produced a wide range of diversity within ZnF proteins. A previous study showed that the protein-protein interacting pathway may be altered by tissue-specific alternative splicing (Ellis et al., 2012) yet the functions of the vast majority of differentially spliced exons are not known. In this study, we observe that brain and other tissue-regulated exons are significantly enriched in flexible regions of proteins that likely form conserved interaction surfaces. These proteins participate in significantly more interactions in protein-protein interaction (PPI. Different transcription factors contain several ZnF motifs that are involved in DNA binding and protein-protein interactions. Research on plants has identified several ZnF motifs that play key roles in these processes and also in other biological processes such as pathogenic immunity, floral development, and light-mediated morphogenesis. Alternative splicing events in such proteins alter binding locations and also change the binding affinity. Zinc-finger proteins contain several types zinc fingers motifs- CCCH type, Dof type and B-box type. These proteins play important roles in different environmental stress like drought, cold, temperature in plants (Chen et al., 2020; Bu et al., 2021). In Plants, different types of alternative splicing events are regulated in stress conditions and tissue specific levels (Punzo et al., 2020; Martin et al., 2021). *Arabidopsis thaliana* utilize alternative splicing mechanism for stress responses and thus alter tissue-specific transcriptomic and proteomic (Martin et al., 2021; Steward et al., 2022; Tognacca et al., 2022).

Conclusion

It is clear that alternative splicing events in ZnF transcripts have a major effect on Arabidopsis physiology. Further investigations to determine the localization of the alternatively spliced transcripts and proteins, as well as site-specific and tissue-specific RNA editing, are required for a full understanding of their effects on growth and development. These will provide a picture of the precise effects of alternative splicing and editing events. Such studies should address natural editing events during gene transcription and determine how the resultant changes to the proteins affect substrate recognition and plant physiology.

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