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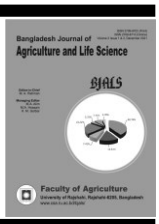

Alternative splicing of AT1G29800 and AT4G06634 genes and its effect on protein structure and functionality in *Arabidopsis thaliana*

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ABSTRACT

Alternative splicing is a post and co-transcriptional regulation of gene expression. Recently it has been revealed that not only PPR (Pentatricopeptide repeat) family genes but also other additional family genes MORF/RIP, ORRM and Zinc finger are involved in RNA editing. The aim of this study is to find out the tissue-specific expression and alternative splicing of Zinc finger family genes AT1G29800 and AT4G06634 and their effect on protein structure and functionality. AT1G29800 and AT4G06634 genes are functionally very importance which are probably alternatively spliced and are located in the protein coding region which is tentatively determined using the Arabidopsis database. Tissue-specific alternative splicing in AT1G29800 with intron retention and AT4G06634 with 5' alternative splice site genes of zinc finger family was confirmed by PCR and sanger sequencing. This study suggests that tissue-specific expression of different alternatively spliced transcript happen even in different developmental stages.

Introduction

Alternative splicing is a bimolecular process where multiple mRNAs are generated from the same gene through the different selection of splice site. This process is one of the key element in living organism during development that is regulated in response to environmental factors (Graveley 2001; Lareau et al. 2004; Kelemen et al. 2013). Such kind of splicing phenomenon affect the biological process like development, progeny fate selection even gametocytes differentiation in *Plasmodium Spp*. (Gomes et all, 2022). Alternative splicing is one of the important phenomena in plants biology. In *Arabidopsis thaliana* approximately 15 years ago

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it seems that 1.2% genes undergo alternative splicing but in the era of the next generation sequencing technology it is surprising that, multiexon containing genes undergo up to 60% alternatively spliced (Syed et al. 2012). Core circadian clock genes are also controlled by alternative splicing in *Arabidopsis* during different environmental conditions (James et al. 2012) (Syed et al. 2012). In previous studies alternative splicing studied most of the cases by whole plants however in this study we went for tissue-specific even at the different developmental stage. However, there is few report on pollen specific alternative study in case of *Arabidopsis spp*. (Estrada et al. 2015). The functional consequences and effect of alternative splicing on phenotype is very important and still under study (Syed et al. 2012). In case of human transcriptome >95% of the genes go through alternative splicing thus the huge diversity of protein occurs due to alternative splicing (Pan et al. 2008).

In plants, the conversion of C-to-U as affected by cytidine deaminase has been shown to be dependent on zinc ions. This is possibly due to the requirement for Zn^{2+} in the active center of cytidine deaminase (Vasudevan et al. 2013). In numerous plant ancestries, the ubiquitous zinc finger family may be discerned, including in *Selaginella*, which may suggest that it plays an evolutionarily conserved role in the plant editosome (Sun et al. 2015; Sun et al. 2016). Recent advancements made in the field of plant RNA editing have begun to focus on the non-PPR editing factors, which highlight an unpredicted diversity in the plant editosome (Sun et al. 2016). Amongst the most predominant motifs in eukaryotic proteins may be found the Zinc Finger. They are characterized following the number as well as order of the histidine and cysteine residues present in the amino acid chain, as these interact with zinc ions to maintain the tertiary structure of the proteins. Analogous to the function at the molecular level, it was discovered that Zinc Finger Proteins play a role in a wide variety of different biological processes including the regulation of transcription, modification of chromatin and degradation of proteins by ubiquitin (Ingo Appelhagen, Gunnar Huep et al. 2010). The RanBP2 Zinc Finger family protein is a potential chloroplast RNA editing factor (Sun et al. 2015). The efficiency of editing RNAs has been seen to vary in different tissues and at different stages of development e.g. in non-green tissues and seedlings while a majority of editing sites were found in green seedlings and green tissues (Tseng et al. 2013). The greatest number of proteins in the Organelle zinc finger family are essential in RNA editing and also play a role in other aspects of RNA metabolism in plant organelles (Sun et al. 2015). Correspondence has been identified amongst the Zinc Finger proteins based on their zinc finger motifs. The structures of the Zinc Finger proteins are defined by their evolutionarily conserved zinc finger motifs. A number of Zinc Finger proteins are used in multiplexed autoantibody assays for the minimally invasive detection of colorectal cancer (O'Reilly J-A et al. 2015).

Tissue-specific alternative splicing may alter the stability of the protein, activity of the enzyme, localization at the sub-cellular level as well as other features in mammalian tissues (Merkin et al. 2012). Tissue-specific alternative splicing in plants, however, is as yet not well understood.

The aim of this study is to identify and extends of alternative splicing of nominated accession no. AT1G29800 and accession no. AT4G06634 genes sequences in various plant tissues including the leaf, stem, stipe and root as well as the whole seedling. The expression patterns of the alternatively spliced mRNAs have been compared and investigated using different Arabidopsis tissues at varying developmental stages i.e. at 4, 8, 12, 16, 21, 27 and 32 days. Using web tools, the way in which the alternative splicing influences the resulting protein diversity in Arabidopsis has also been investigated.

Materials and methods

Arabidopsis thaliana sample collection and growth conditions

Arabidopsis thaliana ecotype Colombia (Col-0) seeds were sown in paper pots were gently filled with a mixture of horticultural perlite, peat moss and vermiculite in a compositional ratio of 1:2:1. The Arabidopsis seeds were then sowed in the paper pots, and the pots covered using cling film, before being kept under dark conditions for three to four days. Afterward, they were transferred to the Green Farm U.ING (made in Japan) kept in a growth room, under the following conditions: fixed growth room temperature 22°C, relative humidity 45%, 16 hours' exposure to light and 8 hours in darkness. After germination, watering was carried out twice daily (every morning and evening), fertilization two times per week, and different kinds of samples collected. The following samples were

collected: seedling (whole plants at 4, 8 and 12 days) and 16, 21, 27, 32 days old leaf, stipe, stem and root.

Extraction of Plant RNA and cDNA synthesis

We have extracted RNA from those sample by using Qiagen plant mini kit (Germany, catalog no. 74904) according to the manufacturer's instructions. The extracted RNA was then treated with DNase (RQ1 RNase free DNase; Promega, Madison, WI, USA) to digest the contaminating genomic DNA. The samples were then purified by phenol-chloroform and ethanol precipitation. The NanoDrop™ (Thermo Scientific) was finally used to quantify the RNA obtained. Using this purified RNA, cDNA was then synthesized by reverse transcriptase (Superscript III, Invitrogen) with oligo dT primer and Random hexamer primers. cDNA synthesis was confirmed using a housekeeping primer for *Arabidopsis thaliana*; GAPDH forward primer: GTTGTCATCTCTGCCCCAAG, reverse primer: TGCAACTAGCGTTGGAA ACA.

Selection of probably alternatively spliced AT1G29800 and AT4G06634 genes sequences from the Arabidopsis database

Candidate genes for probable alternative splicing were identified by combing through the NCBI gene database (https://www.ncbi.nlm.nih.gov/gene/). The accession numbers pertaining to each zinc finger gene sequence identified were sourced from the NCBI database and input into the *Arabidopsis thaliana* Plant Genome Database (http://www.plantgdb. org/ AtGDB/) with the aim of establishing the genomic map of each gene. Using this information, probable alternatively spliced genes were selected (whole genome model) by At- TAIR10. Information on the full-length genomic DNA, mRNA and cDNA was obtained as well. Ultimately, the genomic sequence of each gene, as well as information relating to intron and exon sequences, coding sequences, transcript and resulting amino acid sequence were obtained from The Arabidopsis Genome Integrative Explorer (http://atgenie.org/) and cross-checked. From these databases we selected AT1G29800 and AT4G06634 functionally important genes for further detailed study.

Design of PCR Primers

Primers were designed using the Primer3 primer design software （*primer3 software bioinfo.ut.ee/ primer3-0.4.0/primer3/）and primer blast was performed by NCBI primer blast.* In case of failure or inappropriate outcome with the first designed primer set, another set of primers were designed. Primers were purchased from (Eurofins, Japan); stored in TE buffer at a concentration of 50 pmol/µl in salt-free condition. The primer set was diluted to 10 pmol/µl with TE buffer as working concentration.

Polymerase Chain Reaction and analysis of polyacrylamide gel image

Accession no. AT1G29800, Zinc finger C-x8-C-x5-C-x3-H type family protein; annealing temperature 57°C 30cycles, Forward Primer TCGCTTGAAGGA AGCATTTT Reverse primer CAACGAGA TGA A GCGAATC. Accession no. AT4G06634, Zinc finger (C2H2 type) family protein; annealing temperature 55°C 35 cycles, Forward Primer CTTTCGAGAGAC GACCCATC Reverse primer TTCCCGCAACCAT CATAACT. The denaturation temperature was set at 94°C and elongation temperature of 72°C. The annealing temperatures were set according to each primer as suggested by the primer 3 software after primer design. A 6% polyacrylamide gel was used for electrophoresis which was stained with the SYBR® Green dye (Lonza, Rockland, ME, USA). Equal amounts of PCR product were loaded onto the polyacrylamide gel and electrophoresis carried out. Each gel image was photographed using different exposure times in order to obtain high-quality images for analysis. Gel images were visualized and photograph were taken using the LAS3000 (Fujifilm, Tokyo, Japan).

Sequencing of the transcript

PCR products were sequenced in Applied Biosystems (Applied Biosystems 3130xl Genetic Analyzer, Foster City, CA, USA). The desired bands from PAGE were excised from the gel and kept in disposable pellet pestle/Tissue grinder tubes (Kimble®, Capitol Scientific, Inc. TX 78758, USA, Catalogue no. 749520-0090) before being frozen at -80°C for 1 hour. After that, the frozen gel pieces were well crushed using the pestle. About 10 µl 0.1X TE was added to the crushed powder before being subjected to further grinding. The pestle was then discarded and the tubes vortexed for 10 minutes. Following this, the tubes were centrifuged

at full speed at 4°C for 20 minutes in a tabletop centrifuge. The supernatant was transferred into another tube and 3 µl was used as a sequencing sample. The Big dye Terminator V3.1 sequencing standrd kit (Austin, TX, USA catalog no. 4336935) was used for sequencing. Sequence results with the reverse primer were reverse complemented using the Sequence Manipulation Suite online software, http://www.bioinformatics.org/sms2/reference.html (Stothard 2000). The entire set of sequencing results were aligned with those of the *Arabidopsis thaliana* genome by way of BLAST (Kent 2002).

Determining of tissue-specific expression of the isoforms of AT1G29800 and AT4G06634 genes

The products obtained from PCR were loaded onto a 6% polyacrylamide gel and run in the 1XTBE buffer at 200 volts for 20 minutes. SYBR® Green dye (Lonza, Rockland, ME, USA) was then used to stain the gel in the presence of 1XTBE buffer and placed on a rocker for 20 minutes. After this, the gel was placed in the LAS3000 (Fujifilm, Tokyo, Japan) and its image captured. Finally, the image obtained was analyzed using the Image J (NIH, Maryland, USA) software whereby densitometry analysis was done on each of the resulting bands in order to compare the expression levels of the transcripts that exhibit alternative splicing. Sanger sequencing technique was applied to confirm the PCR products.

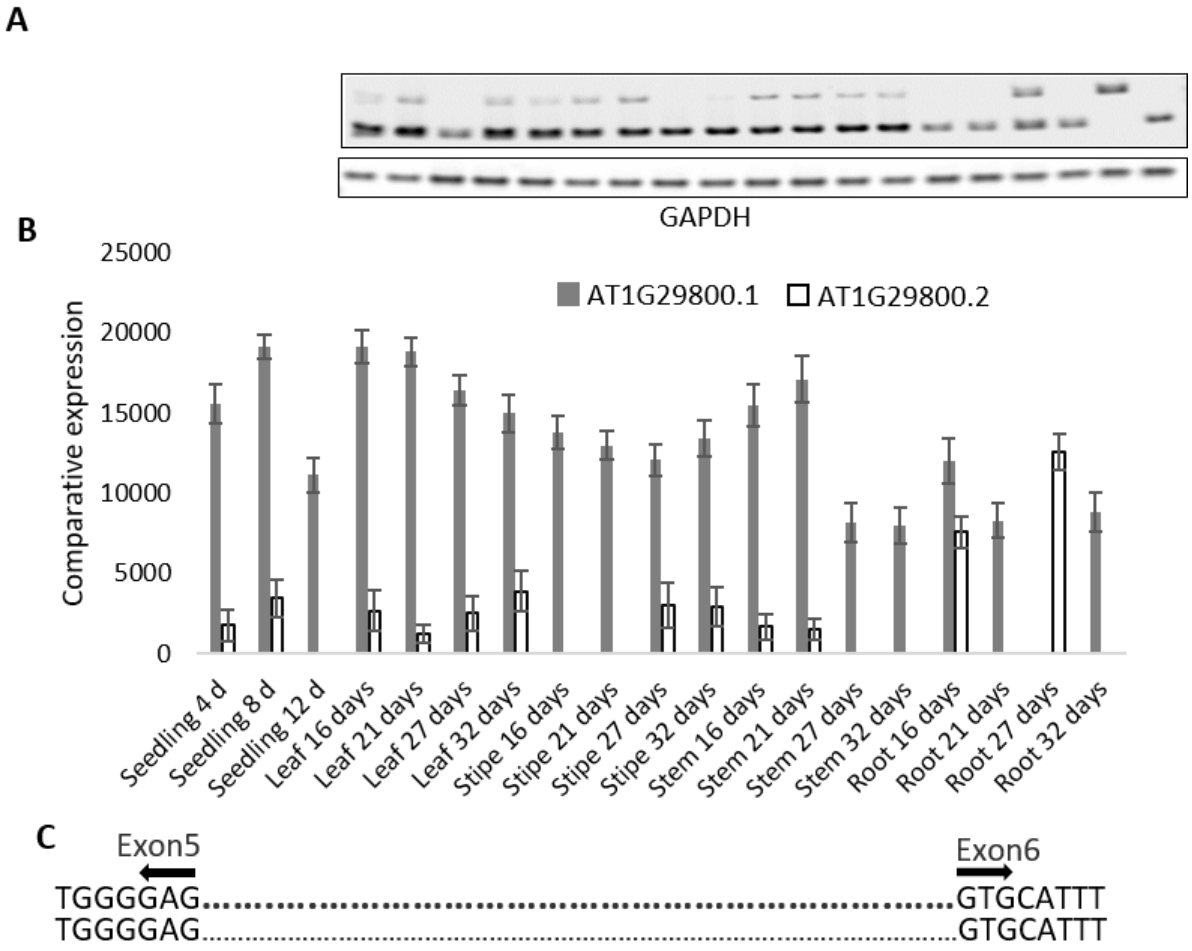

Fig. 1. Accession no. AT1G29800. A. Polymerase Chain Reaction (PCR) amplification and expression of seedling, leaf, stipe, stem and root. Estimated fragment size from gel in base pairs (bp). B. Comparative expression of two spliced isoforms among different tissues. C. The genomic sequence of AT1G29800 from exon 5 to exon 6. Arrow indicates exon 5 and exon 6 boundaries, dot indicates intron sequences. Bold dot indicate intron retained.

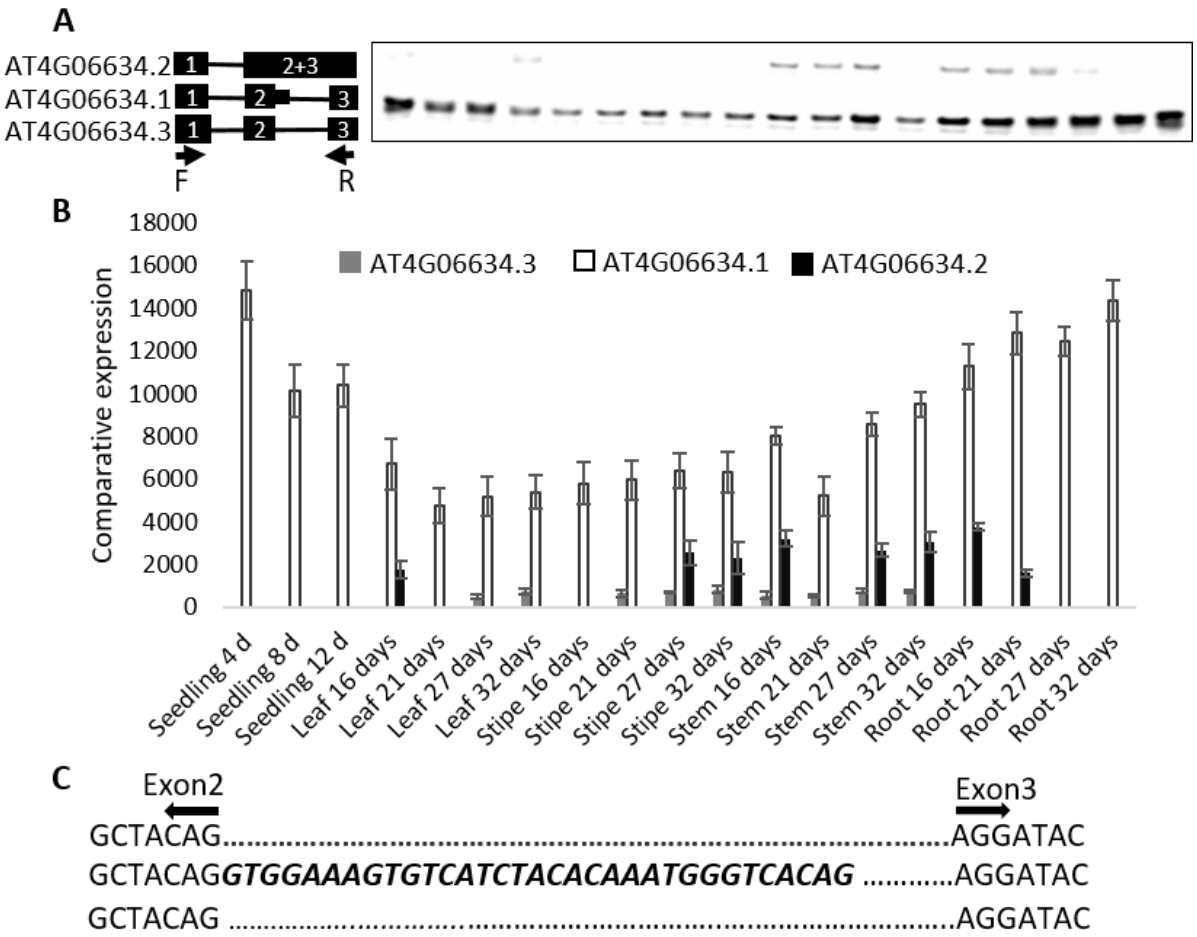

Fig. 2. Accession no. AT4G06634. A. PCR amplification of seedling, leaf, stipe, stem and root. Estimated fragment size from gel in base pairs (bp). B. Comparative expression of different tissues in two spliced variants. C. The genomic sequence of AT4G06634 from exon 1 to exon 3. Arrow indicates exon 1 and exon 3 boundaries, dot indicates intron sequences. Bold dot indicate intron retained. Italic sequences and dash indicate exon 2. Bold sequences and dot indicate 5' alternate splice site

Determining of protein diversity

The effects of alternative splicing on the resulting protein isoforms were studied using the I-TASSER (Iterative Threading Assembly Refinement) bioinformatics tool (Roy et al. 2010; Yang et al. 2014). Ligand binding sites and the distances between residues were also studied using I-TASSER. The full-length amino acid sequences were submitted to the I-TASSER server. Initially, an institutional email address is required for user registration in the I-TASSER server. 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. Then the amino acid was submitted to the server with the desired output parameters. 1-2 weeks after submission, different data sets were provided in a link to the submitting authority. Different parameters between the alternatively spliced isoforms were analyzed comparatively.

Results

In this study, we identified AT1G29800 and AT4G06634 alternatively spliced genes of Zinc finger family proteins from Arabidopsis database TAIR (https://www.arabidopsis.org/) that were differentially expressed in tissues and development specific aspect.

Determining of tissue-specific expression of the isoforms of AT1G29800 and AT4G06634 genes

In AT1G29800, 89 nt intron retained between exon 5 and 6, creating Intron Retention (AT1G29800.2) Fig. (1A and C). Intron retention was activated in root 27 days Fig. (1A). In leaf AT1G29800.1 (Shorter isoform) 16, 21, 27 and 32 days expression gradually decreased Fig. (1B). In AT4G06634, 110 nt intron retained between exon 2 and 3, creating AT4G06634.2 (Intron Retention) Fig. (2A and C). 5' alternative spliced site, 33 nt added with exon 2 creating AT4G06634.1. This 5' alternative spliced site was selected in all tissues Fig. (2A).

Determining of protein structure affected by alternative splicing of AT1G29800 and AT4G06634 genes

Alternative splicing events located in the coding region of AT1G29800 and AT4G06634 genes. Next, we investigated whether alternative splicing affects protein structure. In AT1G29800, 89(GTGATGTTAACACAGTTTAAGGGAATTTGTAA TTCTGA CTTTAATGATGGAAAA GTTCCTGACC TGG AAAACTTCTTGTTGGTGTGCAG) nt addition with shorter isoform resulted in frameshift Table (1).

In case of AT4G06634, we identified three references isoform; AT4G06634.3(shorter isoform), AT4G06634.1 (5' alternative spliced site) and AT4G06634.2 (Intron Retention), 33 nucleotides added (GTGGAAAGTGTCATCTACACAAATGGGTCACAG) with AT4G06634.3 then creating AT4G06634.1 Fig. (2A and C) that consist of 11 inframe added amino acids GGKCHLHKWVT (40-50) Table (1). At the same time, in the reference isoform AT4G06634.2, 110 nucleotides were added by intron retention between the exon 2 and 3 Table (1). In AT4G06634.2, 110 nucleotide addition resulted in frameshift Table (1). At the same time, in the reference isoform AT4G06634.1, 5' alternative spliced site added 33 nucleotides that make an 11 amino acid. Due to the addition of these 11 amino acids with AT4G06634.3, beta sheet disappeared in the middle but alpha helix appeared in the C-terminal of protein Fig. (3A). Ligand binding site may change in AT4G06634.1 and AT4G06634.3 but in case of zinc and nucleic acid, ligand binding residues were different between two isoforms Fig. (3C and D).

Discussion

The alternative splicing process is evidently greatly varied in plants and heavily influences transcript diversity. In the plant *Arabidopsis thaliana*, instances of splicing that involve only single-nucleotide exons have been reported (Guo and Liu 2015). In this study, alternative splicing occurring amongst the Zinc Finger gene family is the main focus. Information obtained from the Uniprot database **(**http://www.uniprot.org**)** describes the functions. CELL DEATH RELATED ENDOSOMAL FYVE/SYLF PROTEIN 1 (CFS1) is responsible for autophagy regulator and repressor of cell death (Sutipatanasomboon et al. 2017). CFS1 and AT1G29800 have similar FYVE and actin binding SH3YL1 Ysc84/Lsb4p Lsb3p plant FYVE (SYLF) domain. CFS1 and AT1G29800 display a high degree of amino acid sequence conservation with 75% similarity in the FYVE domain and 71% similarity in the SYLF domain (Sutipatanasomboon et al. 2017). The alternate splicing portion of our interest is located at downstream of SYLF domain. In this study, shorter isoform (AT1G29800.1) expression was higher in almost in all tissues except root 27 days Fig. (1A and B). On the other hand, intron retention (AT1G29800.2) between exon 5 and 6 expressed comparatively lower level in seedling 4, 8 days; in leaves; stipe 27and 32 days; stem 16, 21 days; root 16, 27 days Fig. (1A and B). It may be stated that the intron retention (AT1G29800.2) play a vital role in the normal physiology of the plants.

Accession no. AT4G06634 consist of mainly five C2-H2 zinc-finger motifs that can be classified as the N-terminal four fingers tandem arrays, another one fifth zinc finger is 114 nucleotides downstream from the fourth zinc finger. It is reported that AT4G06634 is a negative regulator of abscisic acid (ABA) pathway (Li et al. 2016). ABA is an important hormone for plant development including seedling growth, seed maturation, and response to various environmental stresses. AT4G06634 has both transcription activation and repression domain. In previous finding expression of longer isoform AT4G06634.2 found moderate in seedling, higher in flower and absent in root (Li et al. 2016). This finding is similar with shorter isoform (AT4G06634.3) that expressed in leaves, stipe, stem, and in 16 days old root. However, we found the longer isoform AT4G06634.1 expressed in almost all tissues even higher in seedling and root Fig. (3A and B). This discrepancy may be due to our isoform level expression study and larger

number sample size with age variation. The important thing that the splicing portion is located in the N-terminal region, therefore according to the finding it can be stated that this splicing activity may affect the repression activity of this gene.

The alternative splicing of different Zinc Finger genes results in varied expression levels in different tissues. In this study, it was identified that instances of intron retention were better expressed than alternative 5' splice sites. Previously reported that, in the case of Arabidopsis, intron retention is more common types than other types of alternative splicing (Li et al. 2016). RNA-editing in *ndhD-1* site of Arabidopsis chloroplasts has also been conducted by the DYW1 domain of PPR which exhibits the zinc-binding active site signature motif HxE(x)nCxxC. This motif is essential for RNA editing (Boussardon et al. 2014). Taking into account the results of this study and the aforementioned findings, it may be said that the alternative splicing of the Zinc Finger motif may affect RNA editing at the tissue-specific level.

Table 1. Summary of alternative splicing affecting protein structure of studied genes

Accession number of gene	Alternative - splicing events	Effect on		No. of alpha helices	No. of beta sheets	No. of random coils
		Nucleotide	Amino acids			
AT1G29800.1	Reference isoform Shorter	-	-	14	13	48
AT1G29800.2	Reference isoform Intron retention	89 nucleotide addition (GTGATGTTAACACAG TTTAAGGGAATTTGTA ATTCTGA CTTTAATGATGGAAAA GTTCCTGACCTGGAA AACTTCTTGTTGGTGT GCAG)	Frameshift	17	15	43
AT4G06634.3	Reference isoform Shorter	-	-	7	13	46
AT4G06634.1	Reference isoform 5' alternative spliced site	33 nucleotide addition (GTGGAAAGTGTCATC TACACAAATGGGTCA CAG)	11 amino acid addition (GGKCHLH KWVT)	7	12	42
AT4G06634.2	Reference isoform Intron retention	110 nucleotide addition (GTGGAAAGTGTCATC TACACAAATGGGTCA CAGGTGAAATTGTGA TGTTTTAATCTAATTT TCATGTTTGTGAATGT TAAATTGTATCATTGT GTTGTTGTTATTACCA G)	Frameshift	6	11	38

The table represents that alternative splicing affected protein our studied genes of Accession no. AT1G29800 and Accession no. AT4G06634.

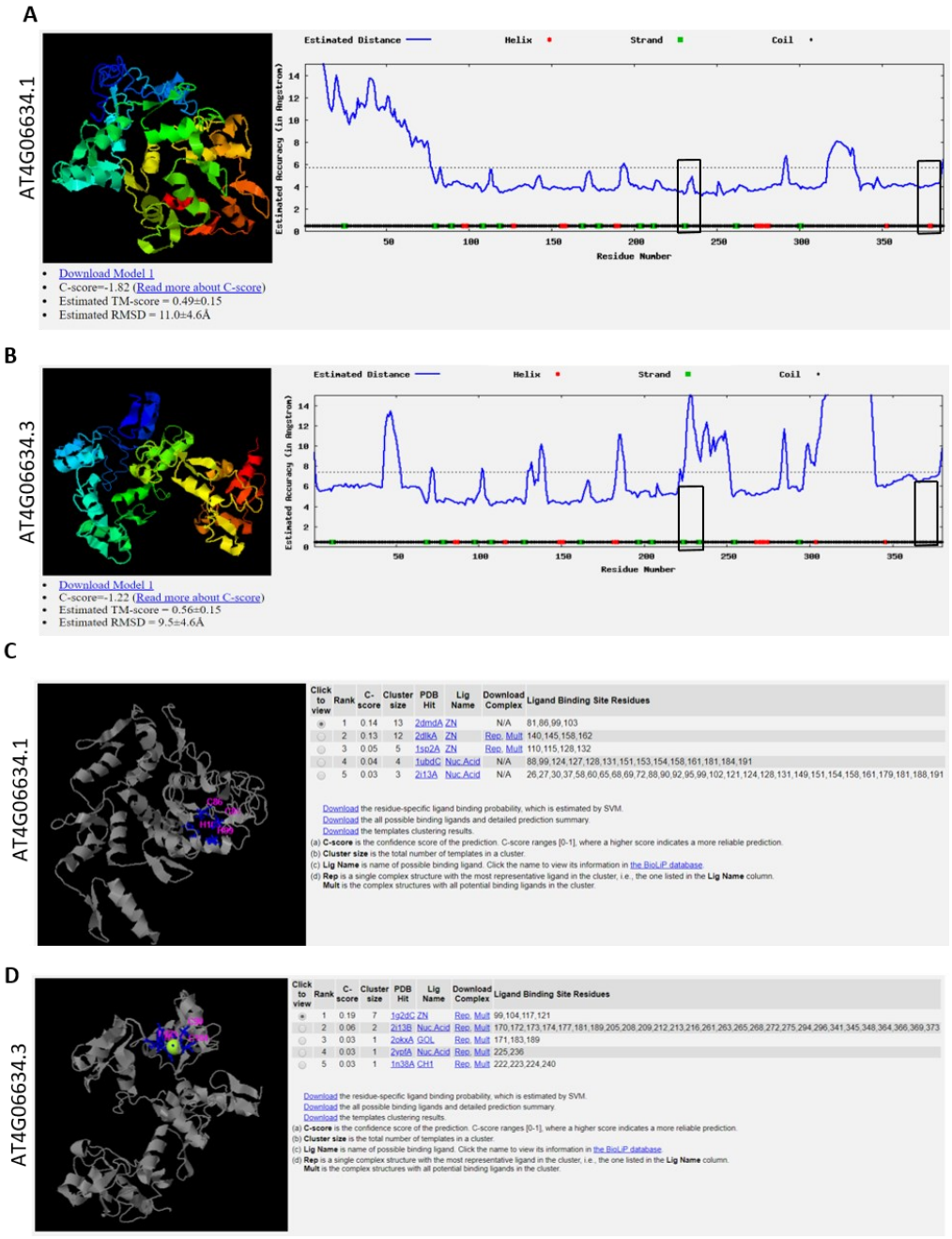

Fig. 3. Due to 11 amino acid addition and deletion affecting the structure and alteration of ligand binding site residues by I-TASSER bioinformatics tool in accession no. AT4G06634 A. Beta sheet disappeared in the middle due to addition. B. Beta sheet appeared in the middle due to deletion. C-D. Ligand binding site may change in two isoforms but in case of Zinc and Nucleic acid, ligand binding residues different between two isoforms

This study revealed that zinc finger proteins are greatly diversified as a result of alternative splicing. In another study, it was also discovered that the protein-protein interacting pathway was also altered as a result of tissue-specific alternative splicing in Arabidopsis? (Ellis et al. 2012). Alternative splicing producing two different proteins. We tested and found that number of alpha helix and beta sheet altered as a result the ligand binding activity on the Zinc finger protein altered due to alternative splicing. Accordng to the result the longer isoforms binding to the ligands Zinc and Nuclic acid. And the shorter isoform binds to the ligands Zinc, Nuclic acid, Gol and CH1. Due

to alternative splicing ligand binding site also changed between two isoforms.

Different transcription factors have several zinc finger motifs which are involved in DNA binding and protein-protein interactions. Following the research on plants, several zinc finger motifs were identified which play a key role. These proteins actually have a key role in different biological processes such as pathogenic immunity, floral development and light mediated morphogenesis (Noman et al. 2019). This proves that alternative splicing events actually alter binding locations as well as change the binding affinity.

conclusion

It can be stated that alternative splicing events in the zinc finger transcript probably largely affecting Arabidopsis plant physiology. More investigation regarding the localization of the alternatively spliced transcript and protein, site-specific and tissue-specific Alternative splicing need to be investigated for understanding their effect on growth and development. More experimental results are needed to understand the precise effect of alternative splicing isoforms of these genes.

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Author's Contributions

TT and UQ designed research: UQ performed the experiment; UQ and MTAA analyzed the data. UQ wrote the paper. UQ and MTAA revised the manuscript.

Conflict Interest: The authors declare no conflicts of interests.

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