

A conserved alternative splicing event in plants reveals an ancient exonization of 5S rRNA that regulates TFIIIA

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Abbreviations: 5S rRNA, 5S ribosomal RNA; AS, alternative splicing; *At*TFIIIA, transcription factor IIIA from *Arabidopsis thaliana*; EI, exon3 including isoform of TFIIIA mRNA, with premature stop codon; ES, exon3 skipped isoform of TFIIIA mRNA encoding the functional protein; PTC, premature termination codon; NMD, nonsense-mediated mRNA decay; RUST, regulated unproductive splicing and translation; Mya, million years ago

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Uncovering conserved alternative splicing (AS) events can identify AS events that perform important functions. This is especially useful for identifying premature stop codon containing (PTC) AS isoforms that may regulate protein expression by being targets for nonsense mediated decay. This report discusses the identification of a PTC containing splice isoform of the *TFIIIA* gene that is highly conserved in land plants. *TFIIIA* is essential for RNA Polymerase III-based transcription of 5S rRNA in eukaryotes. Two independent groups have determined that the PTC containing alternative exon is ultraconserved and is coupled with nonsense-mediated mRNA decay. The alternative exon appears to have been derived by the exonization of 5S ribosomal RNA (5S rRNA) within the gene of its own transcription regulator, *TFIIIA*. This provides the first evidence of ancient exaptation of 5S rRNA in plants, suggesting a novel gene regulation model mediated by the AS of an anciently exonized non-coding element.

Alternative splicing (AS) creates multiple mRNA transcripts or isoforms, from a single gene. Alternative splicing is common in metazoans and, to a lesser extent, in plants.¹ Upwards of 80% of human genes have been demonstrated to undergo AS,^{2,3} while 20–40% of plant genes produce two or more transcript isoforms.^{1,4} While the proportion of plant genes that generate alternative transcripts is far less than that of animal genes, another intriguing difference is the predominance of certain alternative splicing mechanisms. The basic classes of alternative transcripts

(exon-skipping, alternative acceptor/donor sites, etc.) are present in both plants and animals; however, the most abundant human AS event is exon skipping and the most abundant plant AS event is intron retention. Cases of exon skipping and intron retention are relatively rare in plants and animals, respectively. This bias may reflect differences in the molecular mechanism of splice site recognition between plants and animals.¹

Approximately 60–75% of all AS events occur within the translated regions of mRNAs,^{5,6} which can effect protein function (reviewed in ref. 6) and influence protein diversity. It is also quite common for alternative splicing to result in isoforms that contain a premature termination codon (PTC) which, subsequently, become targets for nonsense mediated decay (NMD).⁷ NMD is an RNA surveillance system that recognizes mRNAs containing premature termination codons (PTCs) and targets them for degradation.⁸ Lewis and Brenner⁹ proposed that a process of regulated unproductive splicing and translation (RUST) may function to regulate protein expression by generating NMD-targeted isoforms. Premature termination codon containing isoforms that result from alternative splicing are common in plants^{1,4,10} and animals.⁹ However, it is not clear to what extent PTC containing isoforms are indeed coupled to NMD as a mode of post-transcriptional gene regulation versus simply being the by-products of noisy splicing. Recent studies suggest that a large fraction of AS events are a result of noise in the splicing machinery and lead to unstable protein products if translated^{11,12} rather than being

an effector of an NMD mediated expression regulation process.¹³

While many PTC containing transcript isoforms may result from imprecise splicing, there remain several examples of genes that are truly regulated by coupling PTC containing alternatively spliced transcript isoforms with NMD. The majority of these encode components of the ribosome^{14,15} and splicing machinery.¹⁴⁻¹⁸ One interesting feature associated with some genes regulated by AS-coupled NMD is the presence of ultraconserved sequence elements. Lareau et al.¹⁸ identified ultraconserved elements associated with cassette exons within some mammalian serine/arginine (SR) rich proteins that result in PTC containing transcript isoforms when spliced in. Evidence suggests that the PTC containing isoforms are subject to NMD and that when abundant, these SR proteins affect the splicing of their own mRNA to increase the proportion of NMD target isoforms, which are degraded by the NMD pathway. Thus, the regulation of unproductive splicing of SR protein transcripts is proposed to play a role in the auto-regulation or homeostatic control of these splicing regulators.¹⁸ Consistent with this, Zhang et al.¹³ examined human and mouse orthologues of well annotated protein coding genes for PTC containing transcript isoforms and determined that only the small fraction (2%) that exhibited strong evolutionary conservation of the PTC containing transcript isoforms were likely to represent bona fide examples of regulated unproductive splicing coupled to NMD. These observations suggest that the portions of the gene directly influencing production of the PTC containing isoform are under purifying selection and will remain conserved between organisms with conserved PTC containing splice isoforms, assuming that the mechanisms of alternative splice determination and PTC recognition were also conserved. Therefore, most genes that truly utilize AS-coupled NMD to regulate protein expression post-transcriptionally should have evolutionarily conserved PTC containing isoforms that can be identified during comparative genomics searches.

Recently, two independently conducted comparative genomic analysis identified

a highly conserved sequence element associated with an alternatively spliced cassette exon in the gene encoding the TFIIIA transcription factor in rice (*Oryza sativa*) and *Arabidopsis thaliana*^{19,20} that diverged 140–150 Mya.²¹ Hammond et al.²⁰ searched for structured cis-regulatory RNA elements based on nucleotide and secondary structure conservation between noncoding regions in rice and *Arabidopsis* orthologous genes. Fu et al.¹⁹ identified the same sequence element while performing a comparative genome-wide search for AS events conserved between *A. thaliana* and *O. sativa*. TFIIIA (Transcription Factor for polymerase III A) was the first zinc finger transcription factor characterized, and is essential for the Pol III-based transcription of 5S ribosomal RNA (5S RNA) that is required for the assembly of the large ribosomal subunit.²² TFIIIA can bind to both 5S rRNA and 5S rDNA,^{23,24} and these binding properties have led to the proposal of a negative feedback auto-regulation model for 5S rRNA transcription in metazoans.²⁵ In contrast to the regulation of 5S RNA by TFIIIA, and its interaction with 5S rRNA, the transcriptional regulation of TFIIIA is not well understood; however, post-transcriptional regulation of TFIIIA had never been reported previously in any species.

The *Arabidopsis* TFIIIA (*AtTFIIIA*) gene consists of seven exons, the third of which is alternatively skipped (ES: Exon-Skipped isoform) or included (EI: Exon-Inclusive isoform) to produce either of two transcript isoforms. The *AtTFIIIA* ES isoform encodes the fully functional peptide containing all nine zinc fingers, while the EI isoform contains a PTC that resides within the cassette exon retained in this isoform. Translation of the EI isoform results in a truncated protein that contains only the first two zinc-fingers and is unable to bind 5S rRNA or initiate transcription of 5S rDNA.¹⁹ A dramatic accumulation of the PTC containing transcript in *Arabidopsis* seedlings following cycloheximide treatment¹⁹ or in the presence of a UPF1 mutant background²⁶ demonstrates that the *AtTFIIIA* EI transcript is a target of NMD.

Fu et al.¹⁹ provide further support that the *AtTFIIIA* EI isoform is a target of NMD by evaluating phenotype

and transcript abundance in transgenic *Arabidopsis* overexpressed in either of the two isoforms. The hypothesis that expression of TFIIIA is regulated by RUST predicts that the EI isoform serves no purpose other than as a ‘dead-end’ to translation. Therefore, exogenous overexpression of the EI transcript isoform should not affect the expression level of the endogenous ES isoform or plant fitness. Indeed, the phenotype of transgenic *Arabidopsis* plants that overexpress the EI isoform were indistinguishable from non-transgenic (wild-type) *Arabidopsis* plants. On the other hand, exogenous overexpression of the functional transcript would produce an “overexpression” phenotype that may affect morphology or plant fitness. In addition, if the cell regulates *AtTFIIIA* expression by RUST, then the cell should respond to rising TFIIIA protein and/or 5S rRNA levels by influencing the alternative splicing decision during processing of the endogenous *AtTFIIIA* pre-mRNA to increase the ratio of PTC containing EI to ES isoform. Three independent transgenic ES overexpression events were assessed for EI and ES isoform expression levels and their affect on *Arabidopsis* phenotype. ES isoform overexpression lines exhibit abiotic stress-related phenotypes, specifically sensitivity to salt and osmotic shock, with severities that correlate to the relative level of accumulation of the transgenic *AtTFIIIA* ES isoform. Additionally, the endogenous *AtTFIIIA* EI/ES ratios were strongly positively correlated with the levels of ES isoform accumulation in the transgenic lines. These results demonstrated that increasing the expression of *AtTFIIIA* (and presumably the amount of TFIIIA protein and 5S rRNA) with *AtTFIIIA* overexpression constructs results in a corresponding increase in the amount of EI isoform produced during processing of the endogenous *AtTFIIIA* mRNA. This result demonstrated that high levels of *AtTFIIIA* protein and/or 5S rRNA cause a greater fraction of the *AtTFIIIA* transcripts to be spliced into the unproductive isoform and then degraded through NMD, thereby downregulating *AtTFIIIA* production.

Because genes whose protein expression levels are truly modulated by regulated unproductive splicing coupled to

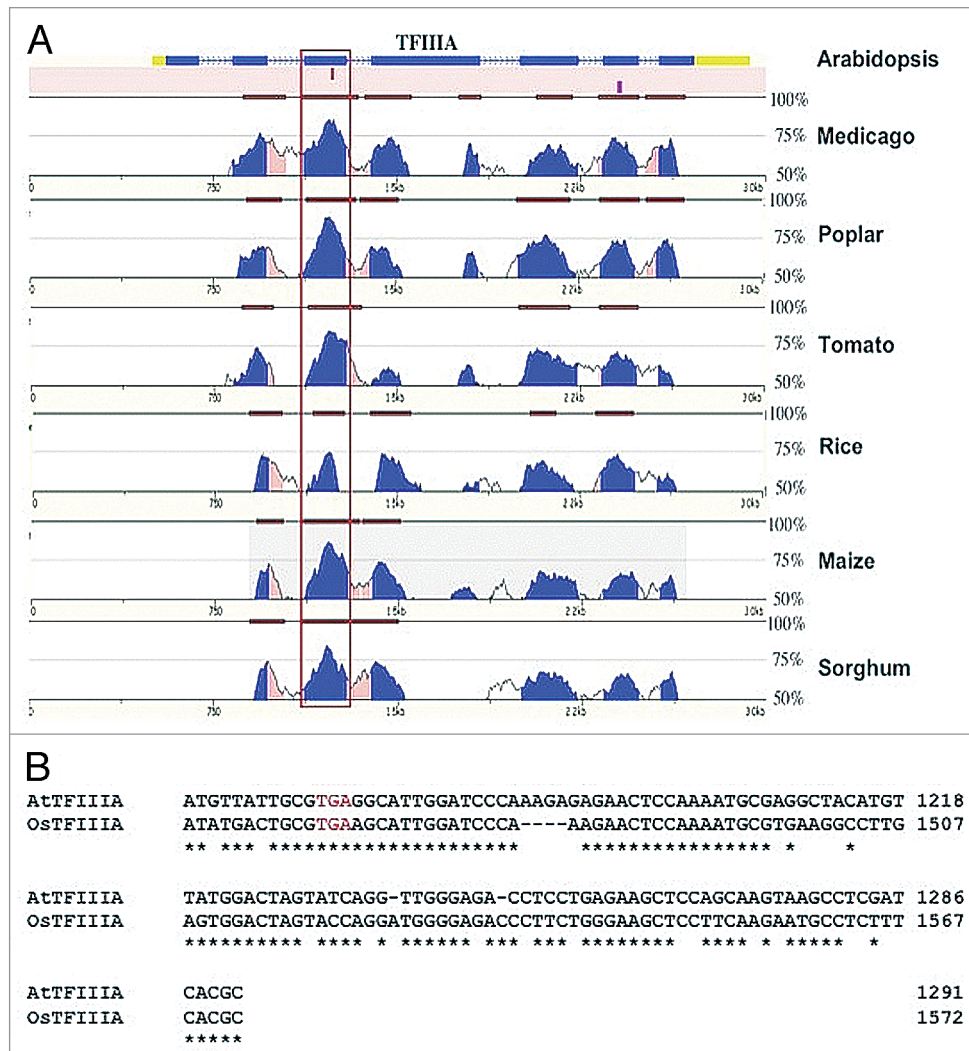


Figure 1. An example of sequence conservation between the alternative exons in a selection of plant *TFIIIA* genes. (A) Vista plots of pair-wise alignments between *TFIIIA* from Arabidopsis and its orthologue in medicago, poplar, tomato, rice maize and sorghum. The alternative exon (boxed) is the most conserved feature between Arabidopsis *TFIIIA* and its orthologues in other plant species. Levels of sequence identity between Arabidopsis *TFIIIA* and its orthologues in the six other plants species displayed are depicted as blue peaks. Brown bars signify segments that pass the alignment criteria of 70% identity over a window of 100 bp. (B) Detailed sequence alignment between the PTC containing alternative exon of Arabidopsis *TFIIIA* and one of the PTC containing alternative exons of rice *TFIIIA*—rice contains a duplication of the alternatively spliced exon (see text). Sequence identity across the alternatively splice exon is 74%. The in-frame stop codon introduced when this exon is included in the transcript is represented by red text.

NMD are expected to exhibit strong evolutionary sequence conservation of the PTC containing transcript isoforms, and given the presence of PTC-containing AS isoforms conserved between *A. thaliana* and *O. sativa*, it is not unexpected that this regulatory mechanism is associated with *TFIIIA*. What was surprising was the degree of sequence conservation observed across a very wide evolutionary time scale. Available plant EST and genomic sequences provide the means to examine the extent of conservation of the alternative exon within the *TFIIIA* gene of many plant species. Analysis of available

and experimentally isolated sequences identified 52 *TFIIIA* orthologues from a diverse group of land plants that include many flowering plants, the bryophyte moss (*Physcomitrella patens*), and an early vascular plant (*Selaginella moellendorffii*) that last shared a common ancestor with angiosperms over 400 Mya. No evidence for the alternative exon was found in the green alga *Chlamydomonas* or other non plant genomes.¹⁹ The pervasiveness of the alternative exon throughout a large and evolutionary diverse collection of land plants suggests it performs an important functional role, which is underscored by

the finding that the alternative exon was the most conserved feature of the *TFIIIA* orthologues (Fig. 1). Most significant was the obvious sequence similarity between the alternative exon and 5S rRNA. Multiple sequence analysis identifies highly conserved sequence blocks between all 5S-like alternative exons and 5S rRNA sequences, suggesting that the 5S rRNA-like element may adopt a similar secondary structure to that of 5S rRNA.^{19,20} Computational^{19,20} and experimental²⁰ evidence suggests that the 5S rRNA-like element associated with the alternative exon adopts many 5S rRNA secondary

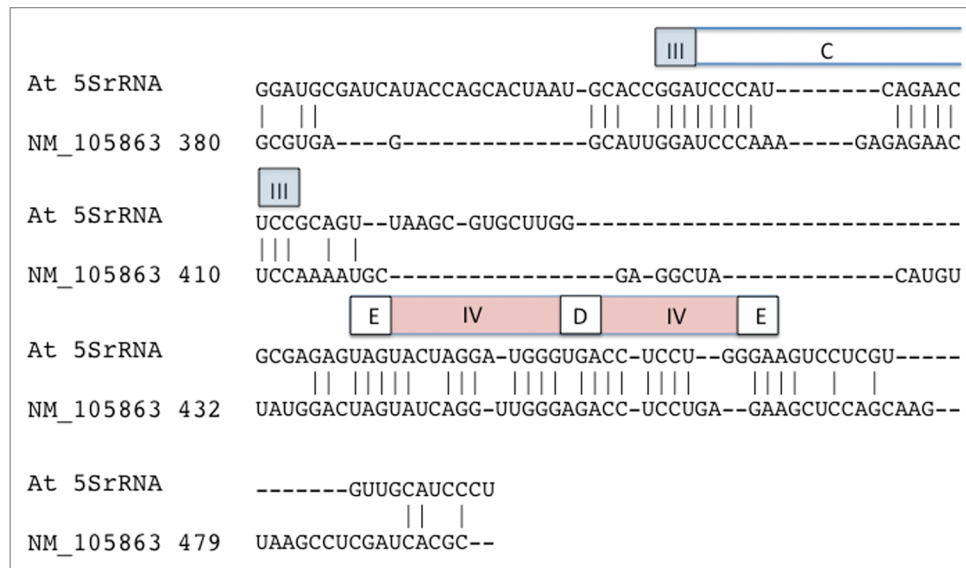


Figure 2. Alignment of the alternative exon of Arabidopsis TFIIIA to Arabidopsis 5S rRNA. The alternative exon sequence is represented within GenBank accession NM_105863 from nucleotide position 380 to 479. Regions corresponding to 5S rRNA secondary structure features are depicted above the sequence segment in which they are found. Elements are named according to the standard 5S rRNA nomenclature: helices are designated by roman numerals (III–V) in colored boxes; loop regions are designated by letters (C–E) in open boxes.

structure elements including Helix III and loop D that are required for ribosomal protein L5 binding, and helix IV and loop E that can interact with TFIIIA protein^{19,22} (Fig. 2). The invariance throughout all land plants examined for nucleotides that may maintain these specific 5S rRNA-like secondary structural elements within the 5S rRNA-like alternative exon suggests that this element is under strong purifying selection to maintain these structural elements and that regulation of *TFIIIA* alternative splicing may occur via protein factors that normally interact with 5S rRNA.

What is the molecular mechanism responsible for regulating the splicing decision? The SC35 SR protein in mammals is thought to regulate its own expression through a negative feedback loop mediated by alternative splicing and NMD.¹⁸ Likewise, since TFIIIA is known to bind 5S rRNA, Fu et al.¹⁹ proposed a simple model by which TFIIIA protein binds the 5S rRNA-like element associated with the alternative exon and affects splicing by either acting as a splicing factor or competing with another splice factor by occupying splice sites or cis-splicing regulatory regions such as exon splice enhancers or suppressors. Under this model, and consistent with experimental observations, TFIIIA

would interact with its own pre-mRNA via the 5S rRNA-like structural elements helix IV and loop E. This would influence inclusion of the PTC containing exon and trigger NMD. Conversely, when TFIIIA levels are low, the PTC containing exon is skipped during mRNA processing so that the ES isoform is produced, which leads to an increase in TFIIIA protein levels. This model suggests that TFIIIA interacts directly with *TFIIIA* pre-mRNA; however, Fu et al. did not rule out the possibility that other factors in addition to or in place of, TFIIIA are involved in the regulation model.¹⁹ Since TFIIIA is essential for the transcription of 5S rRNA, it is reasonable to expect that the mechanism regulating expression of *TFIIIA* would be sensitive to 5S rRNA levels. One glaring deficiency of the *TFIIIA* auto-regulation model is that it is not clear how regulation of *TFIIIA* is influenced by the level of 5S rRNA.

Elegant biochemical analysis by Hammond et al.²⁰ demonstrated that ribosomal protein L5 directly binds the conserved helix III-like structure of the 5S rRNA like element associated with the alternative exon, which parallels a known binding interaction between L5 and 5S rRNA.²² Hammond et al. further determined that the binding of L5 to the 5S rRNA like element is responsible for

splicing control. Binding of L5 protein to the 5S rRNA element associated with the alternative exon in the *AtTFIIIA* pre-mRNA results in skipping of this exon during mRNA production, while absence of L5 binding results in production of the EI isoform that is subject to NMD. When free levels of L5 are low due to low production or to complex formation with 5S rRNA, L5 is less available to bind to the 5S rRNA-like element within the *AtTFIIIA* pre-mRNA and splicing results in the PTC containing EI form. Conversely, the binding of L5 to *AtTFIIIA* pre-mRNA influences the splicing machinery to favor production of the ES isoform. This regulatory logic coordinates the levels of 5S rRNA with L5 ribosomal protein and makes sense in light of the observation that 5S rRNA must be bound with L5 prior to its incorporation into the ribosome.²² Hammond et al.²⁰ suggest that L5 binding may displace an exonic splice factor from the pre-mRNA, thus effecting the splicing decision. However, it may be the secondary structure of the 5S rRNA-like element that actually influences the splicing decision. There is evidence that secondary structure can influence alternative splicing^{27,28} potentially by masking splice sites.²⁸ Schwartz et al.²⁷ determined that secondary structure plays a role in

exon recognition and that increased stability of secondary structure is inversely correlated to exon inclusion levels. Perhaps L5 binding further stabilizes the secondary structure of the 5S rRNA-like element containing alternate exon leading to its exclusion during *AtTFIIIA* pre-mRNA processing. Whether it is secondary structure or presence/absence of protein factors that further influence the splicing decision remains to be determined.

The summation of the experimental analysis by Fu et al.¹⁹ and Hammond et al.²⁰ indicates that TFIIIA protein level in *Arabidopsis* is regulated post-transcriptionally by coupling unproductive splicing with NMD; and, this depends on direct binding of ribosomal protein L5 to an ultra-conserved sequence element with striking similarity to 5S rRNA. Evidence for this 5S rRNA-like alternate exon has been found in all land plants examined (>50), but is absent in metazoans and green algae. Furthermore, phylogenetic analyses suggests that the 5S rRNA element originated from insertion of 5S rRNA into an intron of *TFIIIA* after the separation of land plants and unicellular green alga, but before the divergence of nonvascular and vascular plants, suggesting that this event occurred over 400 Mya.¹⁹ A single 5S rRNA-like element is associated with an alternate exon in most plant *TFIIIA* genes examined. However, the 5S rRNA like element has been duplicated in at least six plant species: 4 monocots from the family Poaceae and 2 Eudicots from the family Asteraceae. Although the origin of the 5S rRNA element is not known, some alternate exons have been shown to originate from retrotransposon insertions such as *Alu* elements in mammals,^{29,30} and, the *Cassandra* retrotransposon carries a 5S rRNA sequence within each of its two long terminal repeats and is present in many plant genomes.³¹ If the 5S rRNA-like element was derived from an ancient insertion of a *Cassandra* retrotransposon into an ancestor of land plants it is likely that one copy was lost shortly after insertion, and those species that have two copies of the 5S rRNA-like element acquired this through subsequent duplication.

The discovery of a 5S rRNA-like element acting to regulate TFIIIA protein

expression represents the first example of 5S rRNA, a non-coding gene, being exonized into genic sequence in plant genomes. Furthermore, this exonization event is an example of molecular exaptation, where a previously adapted sequence is co-opted to serve a different function. In this instance, the 5S rRNA like element within the plant *TFIIIA* gene is probably evolved from a 5S rRNA carrying transposon or pseudogene that inserted into the *TFIIIA* locus. Once present in the *TFIIIA* locus, the 5S rRNA-like structural features of the 5S rRNA-like element have been co-opted to function in a regulatory role that controls *TFIIIA* expression post transcriptionally in land plants. This represents a particularly intriguing event because 5S rRNA has thus been co-opted to play a role in regulating the expression of TFIIIA, which is the very transcription factor responsible for activating 5S rRNA transcription. The observation that regulation of *TFIIIA* by unproductive splicing with NMD is limited to land plants, and overexpression of the *AtTFIIIA* ES isoform is associated with osmotic stress phenotypes, allows that this regulatory circuit may have contributed to the adaptation of plants to stress when their ancestors invaded land.

While the prevalence of AS in plants is still being determined, and most known plant AS events have not been functionally characterized, evidence suggests that AS participates in important plant functions including development and stress response.¹ Using comparative genomics to identify conserved alternative splicing events among evolutionarily distant species can prioritize AS events for functional characterization, help uncover relevant cis- and trans-regulatory factors, and distinguish regulated AS events from noise in the transcription machinery. With more available plant genomes, deeper transcriptome sequencing and the power of comparative genomics, we expect to uncover more conserved AS events and perhaps more cases of coexonization-exaptation in plants.

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