Charles University

Faculty of Science

Study programme: Special Chemical and Biological Programmes

Branch of study: Molecular Biology and Biochemistry of Organisms

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The Role of Alternative Splicing in Plants
Úloha alternativního sestřihu u rostlin

Bachelor´s thesis

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Prague, 2023

Acknowledgments

I would like to express my sincere gratitude to my supervisor, Mgr. Božena Klodová, for her invaluable guidance and support throughout the writing of my thesis. Her expertise, knowledge, and patience were essential for the completion of this bachelor's thesis. To my family and friends, thank you for always being there for me. Your love and support have helped me during my studies.

Declaration

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Abstract

Alternative splicing is a mechanism of gene expression regulation that maintains, regulates, and creates genomic diversity and tissue specificity in plants. It involves the differential joining of exons in precursor mRNAs, leading to multiple mRNA isoforms from a single gene. The formation of these isoform variants and their subsequent translation leads to subfunctionalization of proteins, generating diversity in structure and function. Therefore, alternative splicing is often important in various biological processes in plants, such as development, stress response, immunity, and reproduction. Key types of alternative splicing events include intron retention, exon skipping, alternative 5'/3' splice sites, and mutually exclusive exons. Regulation of alternative splicing involves cis-regulatory elements and trans-acting protein factors such as serine/arginine-rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs). This thesis aims to summarise the mechanisms and consequences of alternative splicing in plant development, including maturation of male and female gametophytes, meiosis, stress, and cell differentiation. It also describes methodological approaches that allow for a genome-wide study of alternative splicing, including microarrays, RNA-seq, and PCR. A better understanding of alternative splicing will provide insights into plant biology and may facilitate agricultural and biotechnological applications.

Keywords: alternative splicing, stress response, RNA sequencing, plants, proteome diversity

Abstrakt

Alternativní sestřih představuje mechanismus regulace genové exprese, který udržuje, reguluje a vytváří genomovou diverzitu a pletivovou specificitu u rostlin. Zahrnuje odlišné spojování exonů v prekurzorových mRNA, což vede k více mRNA isoformám z jednoho genu. Vznik těchto isoformních variant a jejich následný překlad vede k subfunkcionalizaci proteinů a vytváří diverzitu ve struktuře a funkci. Alternativní sestřih je proto často důležitý v různých biologických procesech u rostlin, jako je vývoj, stresová odpověď, imunita a reprodukce. Klíčovými typy událostí alternativního sestřihu jsou retence intronů, vynechání exonů, alternativní 5‘/3‘ sestřihová místa a vzájemně se vylučující exony. Regulace alternativního sestřihu zahrnuje cis-regulační elementy a trans-působící proteinové faktory, jako jsou serin/arginin bohaté (SR) proteiny a heterogenní jaderné ribonukleoproteiny (hnRNP). Tato práce shrnuje mechanismy a důsledky alternativního sestřihu ve vývoji rostlin, včetně zrání samčích a samičích gametofytů, meiózy a diferenciace buněk. Popisuje také metodologické přístupy, které umožňují studium alternativního sestřihu v celém genomu, včetně microarrays, RNA-seq a PCR. Lepší porozumění alternativnímu sestřihu poskytne pohledy do biologie rostlin a může usnadnit zemědělské a biotechnologické aplikace.

Klíčová slova: alternativní sestřih, reakce na stres, sekvenování RNA, rostliny, diverzita proteomu

List of frequently used abbreviations

3'SS	3' splice site
5'SS	5' splice site
ABA	abscisic acid
Alt3'SS	alternative 3' splice sites
Alt5'SS	alternative 5' splice sites
AltA	alternative acceptor
AltD	alternative donor
AltLE	alternative last exon
AltP	alternative position
AltTSS	alternative transcription start site
APA	alternative cleavage and polyadenylation
AS	alternative splicing
ATP	adenosine triphosphate
Avr	avirulence
BCP	bicellular pollen
BPS	branch point sequence
CBC	cap-binding complex
CPKs	calcium-dependent protein kinases
DEU	differential exon usage
DIU	differential isoform usage
EJC	exon-junction complex
ESE	exonic splicing enhancers
ESS	exonic splicing silencers
ETI	effector-triggered immunity
ExS	exon skipping
FER	feronia receptor
FER	female fertile line
GRP7	glycine-rich RNA-binding protein 7
hnRNP	heterogeneous nuclear ribonucleoproteins
HS	heat stress
IL-1	mammalian Interleukin
IR	intron retention
ISE	intronic splicing enhancers
ISS	intronic splicing silencers
LRR	leucine-rich repeat
MAMPs	microbe-associated molecular patterns
MAPKs	mitogen-activated protein kinases
ME	mutually exclusive exons
MOS	proto-oncogene serine/threonine kinase
mRNA	
NBS	nucleotide binding site
NMD	nonsense-mediated decay
OsGS	rice glutamine syntetaze
PAMPs	pathogen-associated molecular patterns
PCR	polymerase chain reaction
PIRL	plant intercellular Ras-group leucine-rich repeat protein
Pol II	RNA polymerase II
pre-mRNA	precursor mRNA
PRR9	pseudo-response regulator 9
PTC	premature termination codon
PTI	primal plant defence system PAMP-triggered immunity
qPCR	quantitative PCR
R	resistance
RALF1	rapid alkalinisation factor 1
RBP	RNA-binding protein
rca	rubisco activase mutant
RNA-seq	RNA sequencing
RNP	ribonucleoprotein
ROS	reactive oxygen species
RRM	RNA recognition motif
RT-PCR	real-time PCR
SAD1	supersensitive to aba and drought 1
SF1/BBP	splicing factor 1/branch point binding protein
siPEPs	small interfering peptides
snRNPs	small nuclear RNPs
SR	serine/arginine-rich
STE	sterile line
TCP	tricellular pollen
TF	transcription factor
TFIIB	transcription factor IIB
UNM	uninucleate microspores
uORF	open reading frame
UTR	untranslated region

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1 Introduction

Alternative splicing (AS) is a fundamental cellular mechanism that enhances the regulatory potential of the genome and contributes to the remarkable diversity of transcripts and proteins observed in living organisms. Unlike constitutive splicing, where a single set of splice sites is consistently used to generate a pre-mRNA transcript from a given gene, AS involves the selection of alternative splice sites, leading to the production of multiple mRNA isoforms from a single gene. This process plays a crucial role in modulating gene expression and protein function, enabling organisms to adapt to diverse environmental conditions and developmental stages (Reddy 2001).

In plants, AS has emerged as a key regulatory mechanism that governs numerous biological processes, including development, stress response, and environmental adaptation. Studies have shown that a significant proportion of plant genes undergo AS. Nine taxa of angiosperms across the angiosperm phylogenetic tree were analysed, seven eudicots, one monocot and *Amborella trichopoda*, which is considered as sister species of all other angiosperms. In this analysis, it was found that 70.4% of multi-exon genes in *Amborella* undergo AS, 64.4% in grape (*Vitis vinifera*), 53.2% in poplar (*Populus trichocarpa*), 52.9% in *Arabidopsis thaliana*, 50.2% in soybean (*Glycine max*), 46.6% in rice (*Oryza sativa*), 44.9% in common bean (*Phaseolus vulgaris*), 44.7% in *Medicago* and 39.1% in tomato (*Solanum lycopersicum*) (Chamala et al. 2015). These estimates can be lower compared to other studies, as only four AS event types (AltA, AltD, ExS, and IR) were analysed (Chamala et al. 2015). A previous study of AS in *Arabidopsis* reported that 61.2% of the expressed multi-exon genes undergo AS, as in this study the 10 most frequent types of AS were used to estimate the frequency of AS (Marquez et al. 2012). Furthermore, alternative splicing has been found to be tissue-specific in some cases, suggesting that it plays a role in tissue differentiation and specialisation (Qulsum and Tsukahara 2018). This widespread prevalence of AS highlights its importance in generating a diverse repertoire of transcripts and proteins that contribute to plant growth, development, and resilience.

The regulation of AS in plants is complex and involves a network of interacting factors, including cis-regulatory elements within the pre-mRNA, trans-acting RNA binding proteins, and the spliceosome machinery. These factors work together to influence the selection of splice sites, ultimately determining which exons are included or excluded from the mature mRNA transcript (Staley and Guthrie 1998).

AS contributes to plant stress responses by generating protein isoforms that are better suited to withstand environmental challenges. For example, under salt, drought, or heat stress conditions, AS can lead to the production of protein isoforms with improved stress tolerance, for example in maize (*Zea mays*), wheat (*Triticum aestivum*), *Arabidospis*, and rice (*Oryza sativa*) (Thatcher et al. 2014; Z. Liu et al. 2018; H. Yang et al. 2022; X.-X. Liu et al. 2022). Furthermore, AS plays a role in plant-pathogen interactions by regulating the expression of defence genes and signalling pathways, influencing the plant's ability to

resist pathogen resistance, examples were observed in wheat (*Triticum aestivum*), tabaco (*Nicotiana*), *Medicago,* flax (*Linum usitatissimum*), and *Arabidopsis* (S. Yang, Tang, and Zhu 2014; Kim et al. 2009; Andersen et al. 2020; Ling et al. 2015).

In summary, AS has emerged as a critical regulatory mechanism in plants, shaping the transcriptome and proteome to facilitate development, stress response, and environmental adaptation. Understanding the mechanism of AS regulation and its impact on plant biology could have significant implications for agricultural biotechnology and crop improvement strategies.

2 Molecular mechanism of splicing

Shortly after discovery of the intron-exon architecture of eukaryotic genes, Gilbert asked, „Why genes in pieces?" (Gilbert 1978). First, it was proposed that exons should reassemble to form some functional unit of proteins, which would allow the easy evolution of new proteins and functions through exon shuffling. The study carried out with large-scale genomic data found that protein domain boundaries, which can be encoded by multiple exons, have been shown to align with exon borders (M. Liu and Grigoriev 2004). Later, in a revision study, it was proven that a significant number of domain borders correspond to exon borders throughout all chordates, plants, nematodes, arthropods, and some fungi and protists (Smithers, Oates, and Gough 2019). This clarifies that alignment of exon and domain boundaries is a general property of the eukaryotic genome. However, not all exon boundaries align with domain borders, indicating that some disordered regions of exons can be shared across multiple structural domains. Interestingly, more recently evolved proteins exhibit a higher proportion of clear exon boundaries aligned with structural domain boundaries compared to older proteins. These newer proteins, having undergone domain rearrangements since their last common ancestor, often possess unique domain architectures. This unique domain architecture can enhance functional diversification and reflects the evolutionary plasticity of the gene structure. Exon shuffling, the recombination of exons from different genes, can contribute to functional diversity in multicellular organisms. László Patthy proposed that exon shuffling probably played a role in the evolution of multicellularity, as most exon-shuffled proteins have extracellular functions, such as components of the extracellular matrix, cell adhesion proteins, and receptor proteins (Patthy 1999). This theory is supported by evidence suggesting that AS may have driven protein evolution in long-lived multicellular organisms. Notably, distorted regions, thought to result from exon shuffling or AS, are frequently found in proteins involved in signalling and homeostasis (P. R. Romero et al. 2006).

Splicing is carried out in cooperation with ribonucleoproteins. The final mRNA, which acts as a template for protein synthesis, is achieved from pre-mRNA, obtained by transcription in the nucleus. The pre-mRNA undergoes a maturation process, which consists of mRNA capping of the 5' end, splicing of noncoding introns, and polyadenylation. Successful completion of maturation steps allows multiple proteins to bound the mRNA to a ribonucleoprotein complex (mRNP), which is then selectively exported

to the cytoplasm for translation (Vorländer, Pacheco-Fiallos, and Plaschka 2022), overview of this process can be seen in Figure 1.

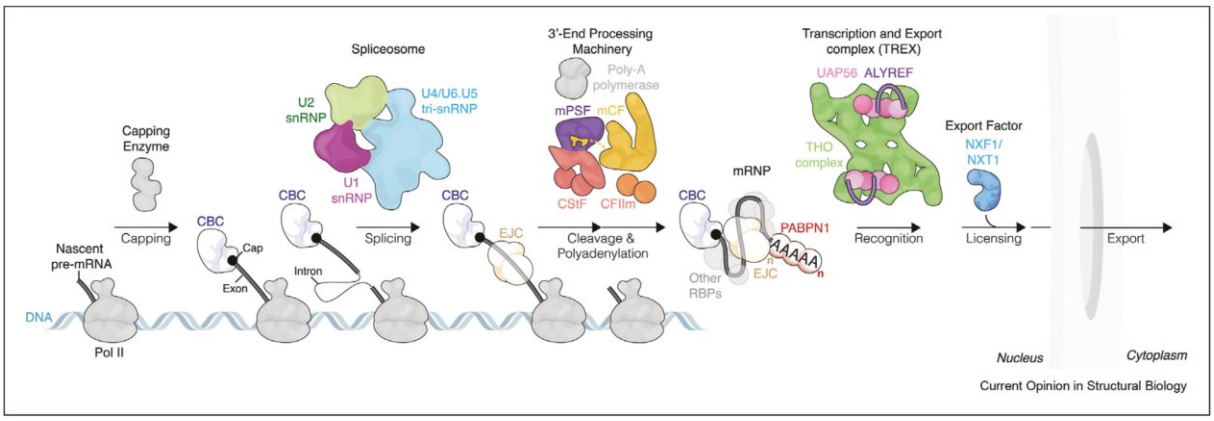

Figure 1 Overview of nuclear mRNA synthesis and maturation. Pre-mRNA is synthesised by RNA polymerase II (Pol II), capped by a cap-binding complex (CBC) at its 5'end. Introns are spliced mainly during transcription, and the resulting splice junctions are marked by an exon-junction complex (EJC). The mRNA is then cleaved of any polyadenylated polymer by the 3'end processing machinery and this poly-A tail is fused by the nuclear poly-A binding protein (PABPN1) to the 3'end of the mRNA. The alternative 3'end processing machines are not shown in the image, as they are used in specific circumstances. The mature mRNA is then recognised by the transcription and export complex (TREX), which loads the nuclear export factor, NXF1 / NXT1, onto the mRNA. Modified from Vorländer, Pacheco-Fiallos, and Plaschka 2022.

The alternative splicing is carried out by a dynamic ribonucleoprotein (RNP) machine known as the spliceosome. This multicomponent complex comprises of several small nuclear RNPs (snRNPs), each playing a crucial role in navigating the pre-mRNA and selecting the appropriate splice sites. The U1, U2, U4/U6, and U5 snRNPs, along with numerous auxiliary proteins, assemble in a precisely orchestrated sequence to form the spliceosomal complex, as depicted in Figure 2.

In some metazoans and plants, a second smaller spliceosome was built from structurally distinctive and functionally analogous U11/U12 and U4atac/U6atac snRNPs. U5 is conserved in both types of spliceosomes (reviewed by Patel and Steitz 2003). The smaller spliceosome, sometimes referred to as U12, was not observed in some metazoans, *Saccharomyces cerevisiae,* and *Caenorhabditis elegans* (Burge, Padgett, and Sharp 1998). Consensual, stepwise, and simple assembly of major (U2-type) spliceosome, acquired by *in vitro* studies using native gel electrophoresis, affinity selection, and glycerol gradient centrifugation, is described as the flow of these steps, reviewed in Wahl, Will, and Lührmann 2009: as first E complex is built from ATP-independent U1 snRNP, which is bound to the 5 'splice site (SS) of the intron by basepairing with the 5' end of U1, this weak RNA-RNA interaction is in higher eukaryotes supported by serine and arginine rich proteins (SR) and U1 snRNP. This initial interaction is accompanied by binding of the proteins of the splicing factor 1/branch point binding protein (SF1 / BBP) and the auxiliary factor (U2AF). U2 binds to the branch-point sequence (BPS) and the polypyrimidine tract located downstream of BPS. U2AF is a protein that binds to pre-mRNA and helps to splice out introns. It is made up of two subunits, U2AF65 and U2AF35. The first step in binding to U2AF is carried out by U2AF65, which binds to the RNA recognition motif (RRM) of pre-mRNA. The second step is carried out by U2AF35, which binds to the AG dinucleotide of the 3' splice site (3'SS). These two steps are

important for the recognition of the 5' splice site (5'SS) and 3'SS, which are the signals that tell the splicing machinery where to start and stop splicing (Wahl, Will, and Lührmann 2009).

After the composition of the E complex, the formation of the A complex occurs. This process is initiated by the interaction of the ATP-dependent base pairing of U2 with the pre-mRNA. The base-pairing interaction involves stabilisation by heteromeric protein complexes redated to the U2 snRNP, SF3a, SF3b, and SR domain of U2FA65. The association of the U2 complex leads to the displacement of the SF1/BBP proteins from BPS. The final steps to form the A complex are the interaction of SF3b14a/p14 with BPS adenosine and the interaction of SF3b155 with the C-terminal RNA recognition motif of U2AF65.

The assembly of the B complex follows next. This step requires the U5 and pre-assembled U4/U6 subunits. First, the catalytically inactive form is constructed from all needed snRNPs. Then, the catalytically active B* complex emerges. During the transition to the catalytically active state of the spliceosome, RNP rearrangements occur, involving the handover of one or more binding partners from one RNP to another. In this specific transition, the U1 and U4 RNPs are destabilised or released. Following the first catalytic step, the C complex is formed. The second catalytic step involves rearrangements of the spliceosomal RNP network (Konarska, Vilardell, and Query 2006). Subsequently, the spliceosome is dismantled, releasing spliced mRNA in association with RNPs. U2, U5, and U6 snRNPs are recycled for the next splicing event.

The spliceosome is assembled on a pre-mRNA substrate (reviewed in Hastings and Krainer 2001), although Nilsen suggested that there is no requirement of a pre-mRNA substrate for assembly of the complex (Nilsen 2002). Although there is less evidence for this opinion, the spliceosome is still a dynamic molecular machine, and both options could be possible. The possibility of obtaining purified multi-snRNP complex suggests that the maturation process can involve pre-assemblance, hence RNA-dependent ATPases were missing in this purified multi-snRNP complex (Stevens et al. 2002). The idea of a preassembled spliceosome is also supported by the existence of long introns in metazoans and the need for cross-exon assembly of the first complex (U1 recognising the downstream 5’SS and U2 and U2AF binding the upstream polypyrimidine tract and BPS), suggesting the existence of cross-exon interaction and different assembly pathways at least in early stages of spliceosome assembly (reviewed in Wahl, Will, and Lührmann 2009).

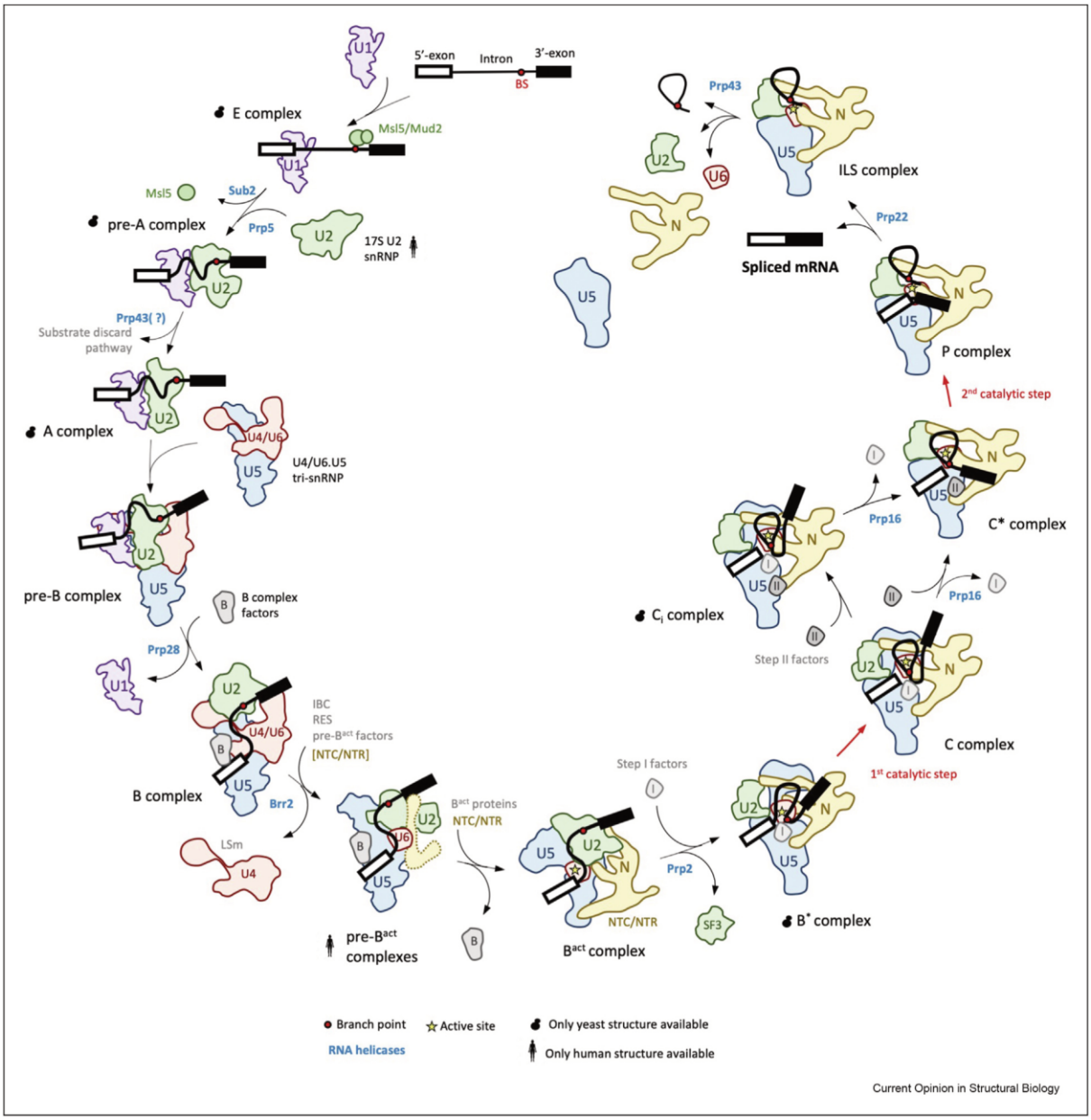

Figure 2 Stepwise assembly of yeast and human spliceosome, cartoon shapes of splicing complexes are based on yeast structures, except the 17S U2 snRNP and preB^{act} complex, where only summary structure is available. Modified from Tholen and Galej 2022.

2.1 Mechanism of alternative splicing

Alternative splicing is performed by the same spliceosome as constitutional splicing. In case of AS events nonconstitutional slice sites, alternative intron-exon junctions are recognised by the spliceosome; this selection is influenced by position and function of the *cis*-regulatory elements, which have protein-protein interaction domains and one or more RNA-binding domains. These elements are categorised into four groups: exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs), and intronic splicing silencers (ISSs). ISSs and ESSs are generally recognised by heterogeneous nuclear RNPs (hnRNPs), (Smith and Valcárcel 2000). ESEs are usually bound by proteins from the SR protein family (Long and Caceres 2009). Mechanisms of selection of ISE site are not clearly specified, but it is known that they are recognised by hnRNP F, hnRNP H, neurooncological

ventral antigen 1 (NOVA 1), NOVA 2, FOX1 and FOX2 (also known as RBM9) (Ule et al. 2006; Mauger, Lin, and Garcia-Blanco 2008). The choice of an AS site frequently occurs during the early assembly of the spliceosome, during splice site recognition. Further studies also observed that a decision can occur during a later stage of splicing and even during conformational changes of the spliceosome (Lallena et al. 2002; House and Lynch 2006).

AS encompasses a diverse array of mechanisms that alter the final mRNA transcript. These mechanisms can be broadly classified into several categories based on the specific exons or splicing sites that are affected: intron retention (IR), where the intron is not removed from the pre-mRNA during splicing; cassette exons (ExS, in Figure 3) are exons that can be included or excluded from the mature mRNA transcript; alternative transcription start site (AltTSS), involving the selection of an alternative transcription start site, leading to the production of an mRNA transcript with a different 5' untranslated region (UTR); alternative polyadenylation (APA), the polyadenylation site, which marks the end of the mRNA transcript, is selected from multiple alternative sites; alternative 5' splice sites (Alt5'SS) as an alternative donor (AltD) in Figure 3, alternative 5’ slice site within the intron is utilised by the spliceosome, resulting in the inclusion of a different portion of the intronic sequence in the mature mRNA; alternative 3' splice sites (Alt3'SS) as alternative acceptor (AltA) in Figure 3, this mechanism is similar to Alt5'SS, but different alternative 3' splice site is selected.

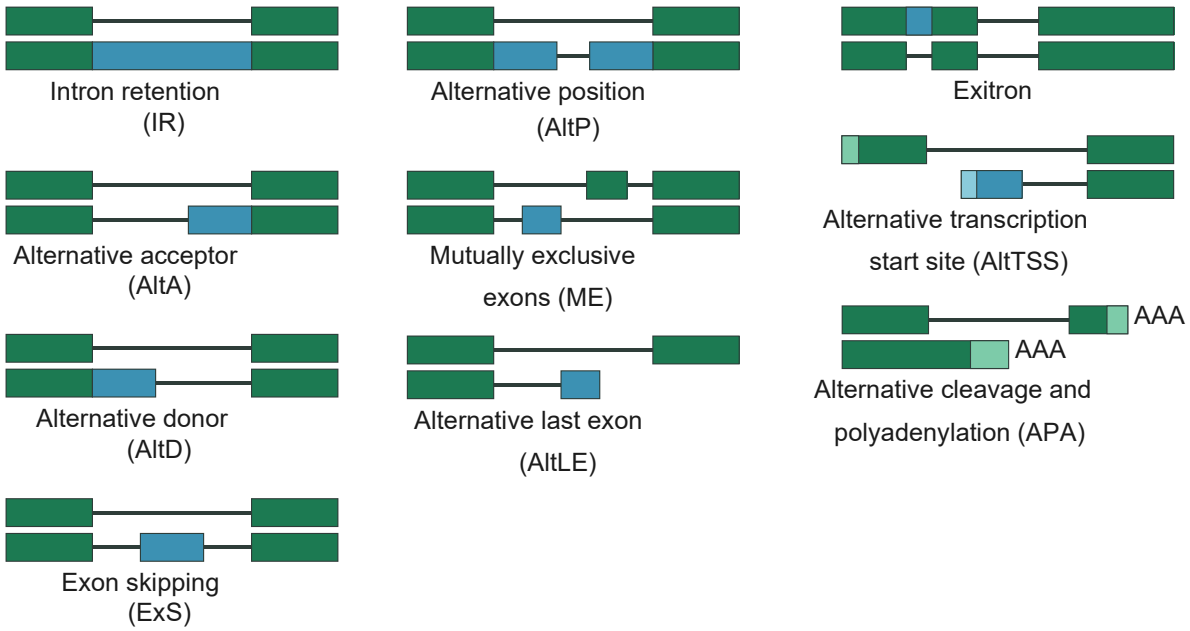

Figure 3 Types of alternative splicing. Dark green and dark blue represent constitutive and alternative coding regions of the coding, respectively; light green and light blue represent constitutive and alternative regions, respectively, modified from Timofeyenko et al. 2023.

The choice of AS categorisation can vary depending on the specific research context. For instance, the terms "exon skipping (ExS)," "alternative donor (AltD) or acceptor (AltA) site," and "intron retention (IR)" are sometimes used instead of "cassette exon," "Alt5'SS," and "Alt3'SS," respectively (B.-B. Wang and Brendel 2006). Additionally, the grouping used in a recent study describing a new machine learning algorithm to determine the conservation of protein variants included additional types such as AltTSS and APA (Timofeyenko et al. 2023).

2.2 Nonsense-mediated decay

Nonsense-mediated decay (NMD) is a conserved cellular mechanism that targets mRNA transcripts containing premature termination codons (PTCs) for degradation. PTCs can arise from genetic mutations, alternative splicing events, or errors in transcription and translation (Kurosaki, Popp, and Maquat 2019). NMD acts as a quality control mechanism that prevents the translation of truncated proteins that can be harmful or dysfunctional. NMD has been shown to play important roles in various biological processes, such as development, cellular differentiation, and disease (reviewed in Isken and Maquat 2007).

NMD is a complex process that involves multiple protein factors and RNA binding complexes. These factors recognise the presence of a PTC within an mRNA transcript and trigger the degradation of the transcript. The NMD process first involves the recruitment of exon junction complex (EJC)-associating NMD factors upstream of each exon-exon junction. After this, the mRNA is exported to the cytoplasm. In the cytoplasm, three events can occur: EJC-dependent NMD, which results in exonucleolytic mRNA decay, EJC-independent NMD, resulting in endonucleolytic mRNA decay or normal translation without NMD. These processes can be seen in Figure 4.

Not all transcripts possessing PTC undergo NMD, initiation of NMD depends on more factors. For example, the distance between the 3 ‘UTR PTC’ and the 3’end of the transcript is suggested to activate the NMD pathway. In plants, it was observed that the typical length of 3’-UTR is 241 nt (Pesole et al. 1997). In other analysis of length between a PTC and constitutional stop codon in typical isoform, which triggers NMD, was estimated with length about 300 nt (Schwartz et al. 2006). In a later study, it was observed that constructs with a >350nt long 3’-UTR have a higher ratio of exceptions, which can escape NMD (Kalyna et al. 2012). In plants, NMD has been shown to be regulated by various environmental and developmental cues. For example, NMD activity has been found to be upregulated in response to abiotic stress conditions such as salt, drought, and heat in *Arabidopsis* (Kalyna et al. 2012).

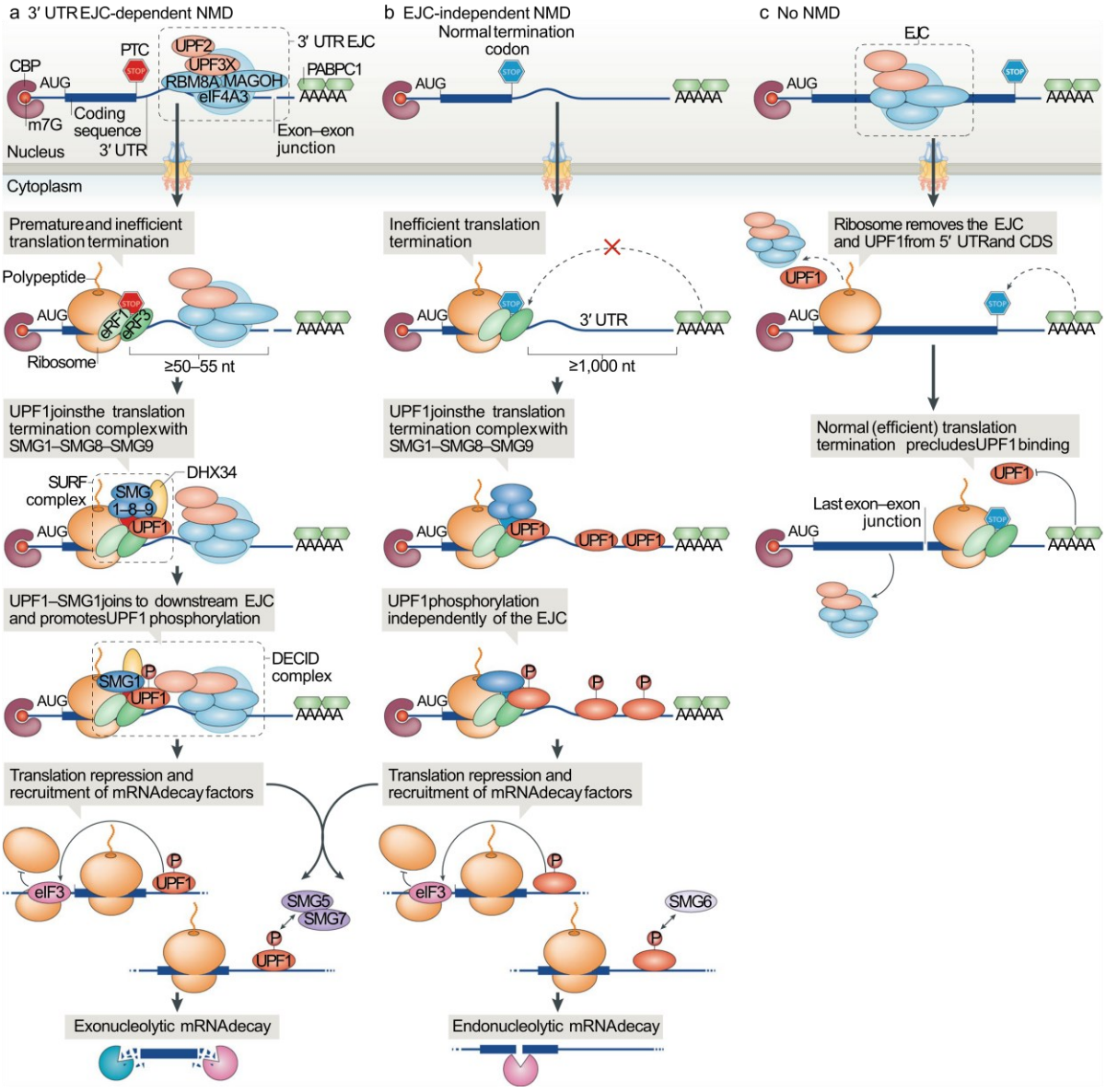

Figure 4 The discrimination between targets and nontargets of nonsense-mediated mRNA decay is shown in part **a.** Translation is terminated using a 3' untranslated region, with PTC located ≥50–55 nt upstream of an exon–exon junction, resulting in exonucleolytic mRNA decay. In part **b,** is shown EJC-independent NMD, where polyadenylate-binding protein 1 (PABPC1) is too distant to efficiently recruit eukaryotic release factors (eRF1–eRF3) to initiate translation termination, resulting in phosphorylation and an increasing probability of NMD. In part **c,** no NMD occurs and translating ribosome removes the EJCs. Modified form Kurosaki, Popp, and Maquat 2019.

2.3 *Trans*-splicing

AS events can result in fusion of pre-mRNA parts from different genes resulting in chimeric transcript. This is quite common in humans (Akiva et al. 2006). *Trans-splicing*, a distinct splicing process, involves the fusion of a 5' splice donor from one pre-mRNA with the 3' splice acceptor of another pre-mRNA. This phenomenon was observed and confirmed in rice through a real-time polymerase chain reaction (PCR) by (G. Zhang et al. 2010). They identified 234 trans-splicing fusion events, with 173 occurring intrachromosomally (precursor pre-mRNAs from different chromosomes) and 61 occurring intrachromosomally (precursor pre-mRNAs from the same chromosome). Interestingly, 25 of these events involved neighbouring genes, while 36 involved distant genes. Notably, many fused transcripts generated

through *trans*-splicing possess open reading frames (ORFs) that combine specific protein domains from different genes, resulting in proteins with novel functional interactions (Akiva et al. 2006). Although the precise evolutionary origins of *trans-splicing* remain unclear, it is hypothesized that it has emerged either as a byproduct of *cis*-splicing or as a form of 'splicing noise' (G. Zhang et al. 2010). Regardless of its origins, *trans*-splicing potentially represents a novel form of AS with a broader functional impact (Gabr, Stephens, and Bhattacharya 2022). Recent studies have delved further into the role of *trans*-splicing in plants, demonstrating its involvement in various biological processes. For instance, a study in *Arabidopsis thaliana* revealed that trans-splicing is essential for embryo patterning (Tadini et al. 2018).

2.4 Regulation of alternative splicing

Regulation of AS is strict, as both transcriptional and post-transcriptional processes can be involved. The spliceosome can be regulated during the formation of each complex by the spliceosomal proteins. Pre-mRNA itself can act as an AS regulator by cis-acting sequences; they can be distinguished into categories such as splice sites, motifs identified by enhancers and repressors, and other sequences that influence the formation of secondary structures (Dvinge 2018). Splice sites usually have conserved boundaries at the 5' and 3' ends of the junction sites, mostly represented by dinucleotides GU and AG, respectively, but other neighbouring nucleotides can also be involved during the splicing process (Yeo and Burge 2004). Furthermore, the splice sites can be categorised into strong and weak, corresponding to constitutively and alternatively spliced exons (M. Chen and Manley 2009). Other mechanisms that regulate AS are bulged pre-mRNA/snRNA duplexes that can emerge during binding of the U1 snRNP to the non-canonical 5'splice site (Roca et al. 2012). Short conserved sequences contained in pre-mRNA can influence assembly or modify accessibility of pre-mRNA because they can act as binding sites for RNA-binding proteins (RBPs) (Warf and Berglund 2010). Most sequences and motifs regulating splicing were discovered by transcriptome-wide determination of human RBP binding sites. These binding sites were also observed in plants, for example, rice, obtained by microarray data (Morris et al. 2011) or in *Arabidopsis* where they are connected to the stress response (Lorković 2009). The next mechanism of regulating AS are secondary structures formed within the pre-mRNA. They may regulate accessibility to splice sites or to RBP's binding sites. One of the simpler ways is the formation of an RNA harpin that bury the 3' or 5' splice site and excludes the exon. The formation of RNA duplexes can loop out whole exons, and RNA secondary structures can also enhance AS by bringing other splicing elements into the neighbourhood (Warf and Berglund 2010; Estes, Cooke, and Liebhaber 1992).

The AS in plants can also be regulated by environments. Specifically, it can be influenced by abiotic and biotic stresses, developmental ageing, circadian rhythms, and microbiome interactions (reviewed in Staiger and Brown 2013). For example, the AS response to temperature stress typically occurs in one of the three ways (J. Jiang et al. 2017). One of them is intron retention, leading to the insertion of premature termination codons in the spliced transcript. Intron retention limits the appearance of the functional

transcript of an actively transcribed gene. It was suggested that this can be a faster molecular response than regulating its transcription, making it convenient for a fast stress response (Sureshkumar et al. 2016). The second mode is the peptide interference caused by small interfering peptides (siPEPs) that affect the translation of alternative spliced mRNA into shortened proteins, forming non-functional heterodimers, lacking transcription regulation or DNA-binding domains. This makes small interfering peptides competitive inhibitors of targeted transcript factors (Seo et al. 2011). Activation of a transcription factor (TF) is the third distinguished mode described in the AS of HSFA2 in *A. thaliana*. The AS transcript is truncated and *C*-terminal modified with an extra leucine-rich motif, which can activate its own transcription by binding to its own promoter, resulting in its positive autoregulation (Z. Liu et al. 2018). Another example of regulating AS can be observed in relation to circadian system, rhythmically oscillating splice forms of pseudo-response regulator 9 (PRR9) in *Arabidopis* (Sanchez et al. 2010).

The regulation of AS in plants is a complex process that involves both transcriptional and post-transcriptional mechanisms. Splice sites, pre-mRNA secondary structures, and splicing-regulating sequences and motifs are among the key factors that regulate AS. Additionally, environmental factors such as temperature variation, light, and symbiotic interactions with arbuscular mycorrhizal fungi can also influence AS in plants. For example, temperature stress can lead to intron retention, peptide interference, and activation of transcription factors, affecting the splicing patterns of key stress-response components (reviewed in Staiger and Brown 2013). Understanding the mechanisms underlying the regulation of AS in plants is essential for both fundamental science and practical applications, as it can provide insights into how plants cope with environmental stresses and potentially lead to the development of stress-resistant crops.

3 Differences in the alternative splicing mechanism in eukaryotes

Genes in plant cells are less likely to undergo alternative splicing compared to mammalian cells. Only about 33% of genes are alternatively spliced in rice (G. Zhang et al. 2010), which is more than previously reported 21.2% for rice and 21.8% for *Arabidopis* (B.-B. Wang and Brendel 2006), compared to 50%-75% of multiexon genes of humans (Pan et al. 2008; Kwan et al. 2007).

The structure of exons and introns varies between eukaryotes; generally, vertebrates have large introns and small exons; in lower eukaryotes, exons are larger than introns (Sterner, Carlo, and Berget 1996). In *Arabidopsis*, the average length of genes is about 5 kb, with average exons and introns lengths about 250 and 167 bases (The Arabidopsis Genome Initiative 2000), which is five to six times smaller than the average length of exons in animals. Furthermore, the average number of introns per gene is smaller than in humans, 5 compared to 8.8 in humans with an average gene size of approximately 27 kb, 1340 bp of exons, and a length about ~3.4 kb in humans (Lander et al. 2001). The 5' and 3' splice sites of U2-type introns share similar characteristics with non-plant models, since conserved GU and AG dinucleotides of the splice ends were also found in plants (Brown, Smith, and Simpson 1996). This was verified

by experiment, where *Arabidopsis rubisco activase mutant* (*rca)* with the mutated 5' splice site of intron 3 in rubisco activase mutated from GU to GA resulted in accumulation of partially processed introns (H. X. Liu and Filipowicz 1996). The branch site of plant introns is less conserved than in mammalian models, where the polyprimide tract precedes the 3' splice site of the intron, the corresponding region in plant introns is instead rich in uridines (Domon et al. 1998). However, it is important to note that the 3' splice site seems to be more flexible than the 5' splice site, which was observed in all three kingdoms: animals, fungi, and plants (Frey and Pucker 2020).

The spliceosomal machinery seems to be conserved between plants and animals, the discovery of U12-type intron orthologs in *Arabidospis thaliana* suggests the existence of a minor U12-type spliceosome in plants (Marquez et al. 2012). The representation of these two types can be seen in Figure 5. An earlier study of U12-type spliceosome conservation suggests the existence of this machinery before the divergence of plants and metazoans (Lorković et al. 2005). The U12-type intron was not found in genomes of *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (Burge, Padgett, and Sharp 1998).

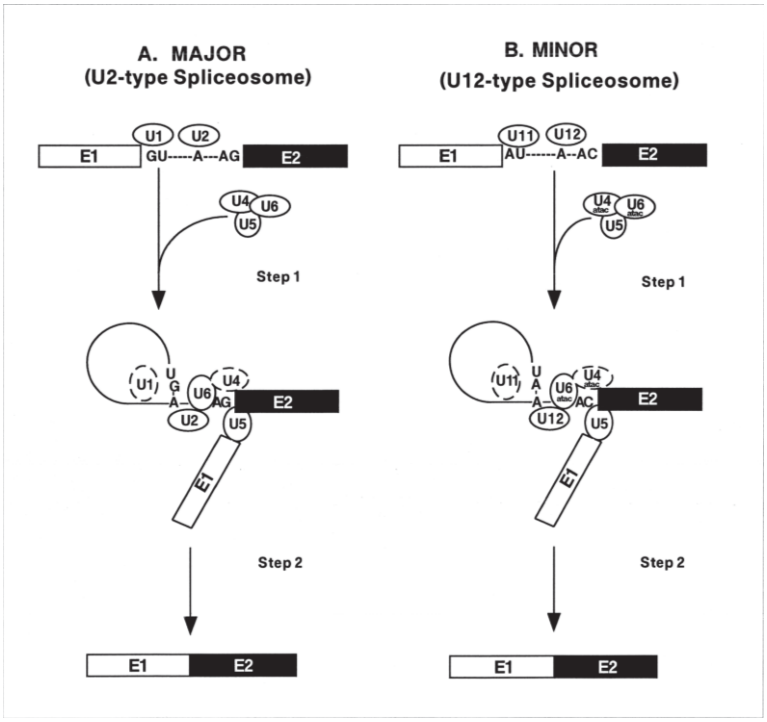

Figure 5 Simplified diagram of major and minor splicing, modified from Reddy 2001

AU rich sites are needed for efficient intron splicing in plants as demonstrated in (Goodall and Filipowicz 1989), this discovery is further developed into an idea of lowering the recognition role of 3'SS and 5'SS itself as an AU rich signal is probably more important. AU rich sites are defined as islands of 4-7 nt. The experiment observing the inefficient splicing of AU-poor wheat amylase intron in tobacco, the amylase intron contains approximately 55% of the AU content, showed an accurately spliced product, but its efficiency was about 2%. In comparison, a pea legumin intron congaing 73% of AU content was spliced with an efficiency about 82% (Simpson et al. 1996). When hybridised introns of amylase

and legumin were imported into cells, the splicing efficiency was higher, the 5’ end of the amylase intron fused with the 3’ end legumin intron showed approximately 64% efficiency. Similarly, it was found that the 5’ end of the legumin intron fused to the 3’end of the amylase intron was spliced with a 92% efficiency. In the fourth experiment, it was observed that point mutations in the branch points of the amylase and legumin intron, resulted generally in a lower efficiency of splicing or total abolishment of spliced transcripts. These results implies that the high ratio of AU content in intron branchpoint serves as an efficient recognition motif in dicots.

In conclusion, the alternative splicing mechanism in plant species exhibits significant differences compared to other organisms. While about 33% of rice genes undergo alternative splicing, recent studies suggest that 50%–75% of multiexon genes in humans undergo this process (Pan et al. 2008; Kwan et al. 2007; G. Zhang et al. 2010). The structure of exons and introns varies across eukaryotes, with the average length of introns in humans being ~3.4 kb (Lander et al. 2001). The 5’ and 3’ splice sites of U2-type introns in plants share similar characteristics with non-plant models, with conserved GU and AG dinucleotides. However, the 3' splice site appears to be more flexible than the 5' splice site across all kingdoms. Point mutations in the amylase and legumin introns can reduce splicing efficiency, highlighting the importance of these sequences in the splicing process.

4 Role of alternative splicing in plants

4.1 Stress response

Plants, as well as other sessile organisms, deal with stress through a complex reaction based on interactions and crosstalk between many mechanisms that regulate gene expression. AS being one of them, as it takes place in the promotion of genome plasticity and versatility, by increasing the number of isoforms and proteins with potentially different functions from a single coding unit (reviewed in Mastrangelo et al. 2012).

Stresses caused by soil salinity, the presence of heavy metals, or other changes in soil composition can trigger the AS event of various genes. For example, a higher nitrogen content may generate the second functional transcript of *OsGS1:1*, a glutamine synthase. It was observed that precursor mRNA can be spliced into two mature transcripts, longer transcript *OsGS1:1a* and truncated one *OsGS1:1b*. It was observed that plants overexpressing *OsGS1:1a* produced seeds with a higher amylose content, while *OsGS1:1b* produced seeds with higher content of other proteins than amylose (X. Liu et al. 2022). Salt stress has a wide AS effect on more than 6000 genes, which was proposed by Feng et al. 2015 in their study they observed the germination rate of *Arabidopsis* seedlings on agar plates with 100 mM, 120 mM and 150 mM concertation of NaCl. In the following RNA-seq analysis, they observed 12 218 novel splicing events, indicating that salt stress increases genome-wide AS.

Drought is an important stress factor in plant life, affecting its development and influencing their yield. The rapid alkalinisation factor 1 (RALF1) complex participates in the drought response through its interaction with the feronia receptor (FER). FER phosphorylates the glycine-rich RNA-binding protein 7 (GRP7), which influences the spliceosome and acts as hnRNP. The resulting hnRNP affects the splicing of genes participating in ABA signaling, by interaction with U1 snRNP. The observed changes were in corelation with other RNA binding proteins, eg GRP8 (paralog of GRP7). Suggest that RALF1 and its involvement through FER in the stress response pathway (L. Wang et al. 2020). Another example of a drought response may be supersensitivity to abscisic acid (ABA) and silencing of drought 1 (SAD1) encoding Sm-like protein 5, part of U6 snRNP. U6 participates in the catalytic activity of pre-mRNA intron excision, *sad1* mutants have defective recognition of 3' and 5' splicing sites that manifest in hypersensitivity to ABA, drought, and salt stress during germination and root growth (Cui et al. 2014).

4.2 Immunity

Biotic stresses are mainly caused by herbivory or insects oviposition. The plant defence mechanism consists of rapid changes in gene expression. Although recent studies suggest the involvement of AS in defence, knowledge of AS mechanism in defence against biotic stress is still limited. A study of AS in biotic stress was performed as a genome-wide analysis on *Nicotiana attenuate*. The AS splicing rates between *N. attenuate* leaves and roots were compared after infecting the plants with tobacco hornwood (*Manduca sexta*) and letting it feed on the leaves for 5 hours. In leaves, reduction of AS events by 7.3% compared to uninfected plants was observed, whereas in roots the AS events increased by 8.0% (Ling et al. 2015). By further analysis, it was found that supresses AS in genes in leaves were involved in primary metabolism. Suppression of primary metabolism is a common reaction of plants after herbivore attack (Bilgin et al. 2010). Whereas AS enriched genes in roots are related with RNA modification and protein glycosylation, these genes might help with herbivory tolerance and defence (Erb et al. 2009).

Several other studies suggest the regulatory potency of plant-microbe interactions (S. Yang et al. 2008; Xue-Cheng Zhang, Zhang, and Gassmann 2007). Most pathogens produce elicitors, molecules such as bacterial flagellin peptides and fungal chitin, and heptaglucosides, alternatively called pathogen-associated molecular patterns (PAMPs). PAMPs trigger PAMP-triggered immunity and regulate mitogen-activated protein kinases (MAPKs) and other signalling kinases that act as phosphoregulators of splicing proteins (Tsuda and Katagiri 2010). Such an example can be SC35-like splicing factor, an SR-rich protein that controls AS events, which can have several sites phosphorylated by Mitogen-activated protein kinase 4 (MPK4) (Yan et al. 2017). The study of *Brachypodium distachyon* revealed a small but significant change in the ratio of alternatively spliced and constitutive isoforms between infected and control plants. This finding suggests that a range of immune-related genes, including those coding for receptor-like kinases, transcription factors, RNA silencing, resistance proteins, and splicing-associated proteins, undergo AS events during viral infection (Ling et al. 2015; Mandadi and Scholthof 2015). Understanding the specific AS events in immune-related genes can provide insight into how plants modulate their

immune responses at the post-transcriptional level. For example, truncated isoforms generated by AS may participate in resistance to plant diseases by suppressing the negative regulation of immunity initiation or directly engaging in effector-triggered signalling (S. Yang, Tang, and Zhu 2014).

The vast majority of resistance (R) genes undergo AS, and some resistance genes need to produce alternative spliced transcripts to correctly recognise pathogen signals, as constitutional isoforms are autoinhibited (Takken and Goverse 2012). Furthermore, alternative spliced isoforms are induced by the presence of a pathogenic signal (Takken and Goverse 2012). Primal plant defence system PAMP-triggered immunity (PTI), induced by conserved pathogen (or microbe)-associated molecular patterns (PAMPs or MAMPs), can be suppressed by pathogens. In PTI, pathogen molecules that are conserved across pathogenic species are recognised by the corresponding receptor on the plasma membrane. Recognition of the microbial elicitor induces several pathways that lead to the defence of plants. For example, it induces rapid influx of Ca^{2+} ions, production of reactive oxygen species (ROS) such as $O_2^{\cdot-}$ and H_2O_2. Additionally, it leads to activation of four different mitogen-activated protein kinases (MAPKs) as calcium-dependent protein kinases (CPKs). PTI can then be inhibited by effector-triggered immunity (ETI) (J. Zhang and Zhou 2010). As a second defence, plant cells start to produce R proteins, which recognise Avirulence (Avr) proteins. R proteins interact with pathogen proteins directly, by directly bounding with effectors (Dodds et al. 2006), or indirectly by detecting effects caused by Avr proteins on other cellular proteins; this mechanism was proposed as guard hypothesis by (van der Biezen and Jones 1998).

The R genes usually consist of a central nucleotide binding site (NBS) and a C-terminal leucine-rich repeat (LRR) region. The NBS region is usually built from three subdomains, the NBS subdomain, ARC1 and ACR2, containing aspartate kinase, chorismate mutase, and TyrA. NBS includes ATP or GTP binding sites required for the initiation of signalling cascades that result in resistance response. The ACR subdomains, which occur in Apaf-1, R proteins, and CED-4, are highly conserved and crucial for intramolecular interactions of R proteins. On the other hand, the LRR region provides recognition specificity to the plant defence (Staskawicz et al. 1995; Albrecht and Takken 2006).

By the N-terminal structure of NSB-LRR, R proteins can be divided into two subgroups, Drosophila Toll and mammalian Interleukin (IL)-1 receptor domain homologous (TIR-NBS-LRR), which is exclusive for dicots (S. Yang, Tang, and Zhu 2014). The second subgroup is typical for its putative coiled-coil domain (CC-NBS-LRR), which is found in both dicots and monocots. Most of the TIR- and CC-NBS-LRRs do not contain transmembrane domains or organelle-targeting signals; in such a manner they are predicted to be cytosolic, some of them showing dynamic changes in subcellular location. Alternative isoforms have been discovered for many TIR-NBS-LRR genes, including flax *L* and *M* loci (Ayliffe et al. 1999), *RPP1* (AT3G44480), *SNC1* (AT4G16890), *RPS4* (AT5G45250), *RPS6* (AT5G46470), *RPP5* (AT4G16950) (Parker et al. 1997; Kim et al. 2009; W. Gassmann, Hinsch, and Staskawicz 1999; Li et al. 2010), *Medicago truncatula RCT1* (S. Yang et al. 2008) or *Nicotiana glutinosa* virus

resistance (N) gene (Whitham et al. 1994). AS events were also observed in the CC-NBS-LRRs sub-family, but only AS events of rice *RCA5* have been studied more (Cesari et al. 2013). This may be due to unknown regulatory roles in disease resistance of these genes. AS does not seem to have a great impact on the regulation of the R genes itself, although response to the pathogen and suppression of autoimmunity symptoms can be regulated by NMD activity. NMD also suppresses the formation of working pathogenic transcripts (Gloggnitzer et al. 2014).

Truncated proteins generated by AS may have a different subcellular localisation than their full transcript counterparts, an example of this behaviour can be the truncated *RPS4* and the tobacco TMV resistance protein N. The study carried out by Wirthmueller et al. 2007 observed that alternative spliced RPS4^{nls}-HA-StrepII with missing RPS4 nuclear pool resulted in losing its location and function. Mutation of *SNCL1* led to the identification of MOS genes, which are assumed to play a regulatory role at AS of R genes in various stages of protein maturation. Only *SNC1* and *RPS4* genes have well studied regulation by proto-oncogene, serine/threonine kinase (*MOS)* genes (Palma et al. 2007; F. Xu et al. 2012; S. Xu et al. 2011). The loss-of-function mutation for *MOS4*, *MOS12*, or *MOS14* was observed to show an altered splicing pattern for *SNC1* and *RPS4,* suggesting their contribution to the splicing of target *R* genes (F. Xu et al. 2012). From homologous studies on human and yeast it is believed that MOS14 plays a key role in the spliceosomal complex named MOS4-associated complex (MAC). From planta assays the interaction between MOS14 and *Arabidospis* homologs of cell division cycle 5 such as protein (CDC5L) and the pleiotropic regulator 1 (PRLG1) was established (Palma et al. 2007). MAC3A, MAC3B, MAC5A and MAC5B are redundant homologs of precursor RNA processing 19 (Prp19), pre-mRNA-splicing factor RBM22, respectively, influencing AS of *R* genes possibly during participation in pre-mRNA splicing (Monaghan et al. 2009; 2010). Co-immunoprecipitation of MOS12, a homologous SR protein to human cyclin L, with MOS4 proposes association with the MAC complex. Mutants *mos12* show altered splicing patterns of *RPS4* thus non-fully functional RPS4-mediated resistance, nevertheless, *RSP6* splicing pattern seems to be unchanged as RPS6-mediated resistance. This indicates the possible existence of more spliceosomal complexes in plants (F. Xu et al. 2012). Loss-of-function mutation od *MOS14*, which encodes the transportin-SR homologous nuclear protein, results in reduction in *SNC1* and *RPS4* expressions. This may be due to the mislocalization of MOS14 cargos. In these mutants, the localisation of four different SR proteins was disrupted because the *C*-terminus interaction with MOS14 was lacking (S. Xu et al. 2011).

Studies of AS of *R* genes are limited due to the required presence of the pathogen to conduct phenotypic changes. Most AS transcripts may be potential NMD targes, which serves as quality control in many eukaryotes. Why and how some of the AS transcripts escape NMD is unknown, as some NMD mutants show the same phenotypes as their wild-type counterparts upon defence response (Jeong et al. 2011). As the analysis in *P. syringae*-infected *Arabidopsis* in the study carried by (Howard et al. 2013) shows low NMD activity, during infection, NMD of *R* genes is repressed resulting in the accumulation of

alternative *R* gene transcripts (Rayson et al. 2012). The low abundance of *R* genes such as *N* and *RPS4* can also be caused by the absence of their corresponding effector which corresponds to low NMD activity. AS transcripts targeted for NMD usually have an open reading frame (uORF) or a larger 3'UTR region. Some intron retention shows these characteristics, but they are not affected by NMD (Kalyna et al. 2012). Studies of *MOS* genes brought some insight into AS of *R* genes; however, other splicing factors and RNA-binding proteins of *R* genes need to be studied. Most *R* genes also required the presence of a pathogen effector to produce noticeable phenotypes.

The AS in the plant serves as another regulatory layer in response to various stresses that plants have to deal with in the rapidly changing environment. AS is a fast and efficient way of reacting in comparison with another mechanisms. Alternative spliced transcripts participated in different pathways, truncated isoforms of the same gene often interact with the same substrate, and the ratio between alternative spliced isoforms and constitutional ones can serve as regulation of the stress-response pathway. AS is a complex mechanism in plant cells influenced by other regulatory events, such as reversible phosphorylation, epigenetics, and histone markers (X.-X. Liu et al. 2022).

4.3 Alternative splicing in male gametophyte

The male gametophyte, haploid male sexual stage of life plants, which is reduced into two or three cellular structure in angiosperm plants. During maturation, it goes through huge developmental changes within an anther, and mature pollen grain is released. Each mother pollen mother cell undergoes miosis forming four recombinant microspores (MS), each maturing into a single pollen grain (reviewed in Sorojsrisom et al. 2022; Hafidh and Honys 2021). During pollen maturation in *Arabidopsis*, AS increases substantially, allowing a single gene to produce multiple mRNA transcripts through intron retention, exon skipping, alternative splice sites, and other mechanisms. These splicing events alter hundreds of genes involved in meiosis, cell division, vesicle transport, and other processes (Misra et al. 2023).

In a study conducted by (Estrada et al. 2015), which compares AS events between pollen and leaf tissue using semiquantitative PCR. It was observed that the SR genes At-SR30 (AT1G09140) and At-RS41 (AT5G52040), involved in splicing regulation (Barta, Kalyna, and Reddy 2010), have different spliced transcripts than leaves. The leaf preferential isoform of At-SR30 contains introduced PTC, which removes 23 amino acids from the C-terminal of the protein, including five serine-rich (SR) repeats, which are suggested to interact with other proteins by phosphorylation. The preferred pollen isoform includes an alternative exon that opens a new translation start leading to the loss of 35 N-terminal amino acids, resulting in the loss of part of RNA recognition motif. The starch synthase-like protein has been found to have multiple alternative spliced 5 'regions, some of them indicated alternative donor sites and some unannotated variants with retained introns. The conserved protein of unknown function AT4G21720 was occasionally seen to retain an alternative spliced intron in both leaves and pollen, but its abundance

was low. Another gene of unknown function (AT3G17120) suggests the use of an alternative transcription start site in pollen, suggesting that different isoforms emerging in pollen use an alternative transcription start site and the requirement of a different 5' donor site in splicing (Estrada et al. 2015). Putative RING-type ubiquitin ligase (AT3G06330), together with ULS2 that regulates leaf senescence and stomatal closure of the ABA transporter (Wei et al. 2022), appeared to have an alternative transcription start site in a pollen product, resulting in 455 bp in both leaves and pollen, and a smaller product, about 395 bp product, in pollen only. Galactose oxidase/kelch repeat superfamily protein (AT1G67480) has shown coexpression of multiple variants in pollen and leaves, these variants indicated the use of alternative transcription start sites, a novel splice form or retention of intron, the abundance of these variants was different between pollen and leaves. Some genes were not observed to be alternative splices using this method, for example RNA-seq data for the histone chaperone (AT2G19480) suggest a preference of exon-skipped form in pollen and the non-skipped form in leaves, but PCR assessment confirmed the existence of the exon-skipped form in both pollen and leaves (Estrada et al. 2015).

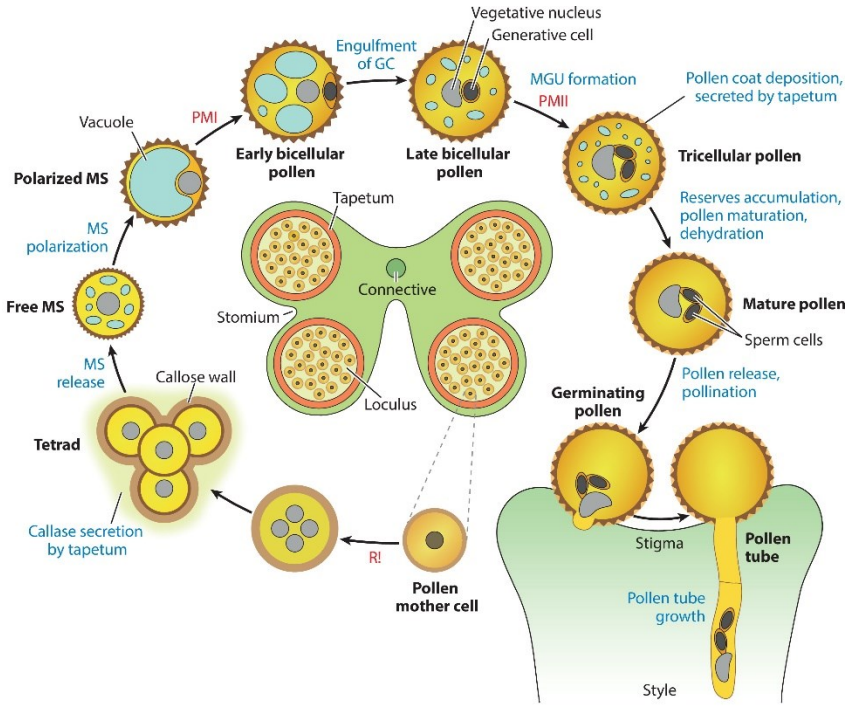

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Figure 6 Overview of pollen development and maturation within an anther. Diploid pollen mother cells undergo two meiotic divisions, tetrad of four haploid microspores (MS) is produced. The released MS from the tetrad is referred to as uninucleate MS (UNM) in the text. UNM is then polarised and undergo asymmetrical division, forming bicellular pollen (BCP). The genetic cell then divides once more, forming tricellular pollen (TCP), this can happen before or upon pollen maturation. Figure retrieved from Hafidh and Honys 2021.

Analysis of AS of *Arabidopsis* sperm cells (SC), vegetative nucleus, and egg cells (EG) showed that in all three cell types, the most prevalent AS type was alternative acceptor (AltA), in vegetative nucleus and egg cells it was seen that intron retention (IR) was second most predominant, although alternative donor (AltD) was observed as second prevalent in sperm cells (Misra et al. 2023). In all three cells, exon skipping (ExS) was found to be the least used type of AS. Among these three cell types, 884 common

AS events that corresponded to 634 genes were found (Misra et al. 2023). The proportion of AS events in maize and rice gametes was found to differ from those in *Arabidopsis*. In maize ECs, ExS was found to be the most widely used type of AS and is second most prevalent in SCs. In the rice germline, IR was observed to be the most predominant form of AS, with AltA being the second, as observed in *Arabidopsis*. The data collected by this analysis highlight the tissue-specific complexity of AS in plants male gametophyte (Anderson et al. 2013; J. Chen et al. 2017).

Analysis of the transcriptome in the development of *Arabidopsis* pollen, using RNA-seq data, showed a high similarity of the transcripts between the early stages of pollen development, uninucleate microspores (UNM) and bicellular pollen (BCP). The transcriptome changes observed during the later stages of pollen development, specifically between tricellular pollen (TCP) and mature pollen grain (MPG), showed a high degree of similarity (Honys and Twell 2004). During the transition between each development stage, changes of differential exon usage (DEU) and differential isoform usage (DIU) were observed. DEU refers to changes in the relative usage of exons within a gene, whereas DIU refers to changes in the dominant expressed isoform. During the transition from UNM to BCP, 1 769 exons used exons in 1 132 genes were observed. During the BCP to TCP transition, 1 769 exons belonging to 1 132 genes underwent DEU and 1 037 exons in 588 genes changed during the TCP to MPG transition. DIU events were much less common than DEU; in the transition from UNM to BCP 336 isoform switches were identified in 204 genes, during BCP-TCP there were 837 isoform switches in 458 genes and 129 isoform switches in 78 genes during the TCP-MPG transition. Only four isoforms were switched between all stages; most of the isoform switches were stage-unique. Not all isoform switches resulted in functional consequences in the resulting peptides. However, some isoform switches resulted in loss or gain of the signal peptide, domain presence, or changes in NMD sensitivity. Observation of GO enrichment for the DEU and DIU for UNM-BCP transition resulted in enrichment of genes involved in meiosis cytokinesis and chromosome organisation. During the BCP-TCP transition, GO analysis showed enrichment in GTPase activity, docking, mRNA splicing, protein transport, and vesicle trafficking. In the transition from TCP to MPG enrichment of genes associated with transporters was observed. Regulation of AS expression represents an important mechanism during pollen development. These data suggest the potential of AS in pollen development regulation (Klodová et al. 2022).

The study that observed the maturation of pollen grains under heat stress (HS) of tomatoes conducted by (Peet, Sato, and Gardner 1998), points out that the maturation stage is the most sensitive to heat stress. Both heat-stressed and non-stressed female receptor plants did not produce any fruits when crossed with heat-stressed male donors. The heat stress of the donor plant before pollen release showed a decrease in the number of pollen grains than in plants where stress was applied during ovule development; this suggests that other stages of pollen development, such as pollen germination, tube growth, and fertilisation, of pollen development, are less sensitive to heat stress than pollen maturation. Other studies used *Brassica napus* (canola) as a model organism to observe the crossing of male donors and

female receptors. Stressed emasculated receptor plants crossed with control pollen had about 37% reduction in seeds, and even greater loss of seeds showed plants that were crossed with heat-stressed pollen. This was also proven by an *in vitro* assay, in which inversible defects were observed in pollen germination (Young, Wilen, and Bonham-Smith 2004).

Heat stressed pollen from the Moneymaker tomato cultivar (with 39° C for approximately one hour) did not show differences in pollen vitality, compared to a 21% reduction in the pollen grains in Red Setter cultivar (Keller et al. 2016). Genome-wide transcriptome analysis showed that IR, ExS, AltA, and AltD were observed in both control flowers, kept at 25° C, and in heat-stressed ones. However, different numbers of genes underwent AS in each condition and cultivar. In Moneymaker, 8 415 genes exhibited IR under heat stress and 4 578 genes underwent IR in control. In Red Star a slight increase in the number of genes undergoing IR was observed, 8 795 genes in heat stressed samples, and 4 578 in control. An increase of other alternative splicing events was also observed in both cultivars, 684 and 612 undergo ExS in the Moneymaker and Red Star under control conditions, respectively, to 1 225 and 901 in the heat stressed ones. AltA and AltD did not show an increase under heat stress in either cultivar. More than half of the exon skipped genes were shared in both cultivars, respectively 54% in Money Maker and 61% in Red Setter, 81/89% genes underwent intron retention in both cultivars and under both conditions. The ratio of alternative spliced transcripts after heat stress is comparable between both cultivars; however, the amount of IR and ER events between cultivars in non-stressed pollen was different. The number of AS events is higher in Moneymaker. Most of the AS events targeted genes are involved in protein folding and gene expression. A further study of two genes coding the ubiquitin ligase complex subunit Cullin 1B (Solyc09g074680) and the regulatory serine / threonine protein phosphatase 6 subunit 3 (PP6R3, Solyc11g069490), which have relatively constant expression of constitutively spliced isoforms in control, shows that upon heat shock, intron retention leads to PTC and C-terminal truncation of the resulting protein. From these findings, it is implied that AS can serve as an alternative layer of heat shock regulation (Keller et al. 2016).

Furthermore, the accumulation and reduction of heat shock-induced AS events were studied. From comparison of tomato cultivars, it was found that heatshock-dependent accumulation of IR and ExS appears to be more global and reduction on the other hand, is more cultivar specific. Subsequent analysis of gene expression in both cultivars under both conditions did not show the dependence of the IR on the number of introns in the genes or any relation to the underlaying genes. However, the comparison showed that alternative splicing events such as ExS and IR are generally more abundant during recovery from heat stress than reduction of them. Analysis of the 451 genes with HS-dependent IR or ER reduction did not show any enrichment in the Gene ontology (GO) term database, although the HS-dependent accumulation of 2 343 genes was found to be enriched in 33 GO terms, which could be described with four general functional categories, including gene expression, RNA modification, translation, and protein folding. The domain architecture of several genes was alternated, and five proteins have gained functional

domain. For example, the F-box / LRR repeat protein (Solyc10g080020) was found to gain a new F-box domain along with the loss of the Fist_C domain. Other proteins showed an AS-induced domain split, resulting in the detection of two subdomains or an AS-induced domain transition. One example is Calpain-2 catalytic subunit (Solyc11g068460) which gained IR induced switch from EF_hand_5 to left-hand domain. Several proteins were observed to have lost at least one domain, such as transcription initiation factor IIB (Solyc10g079360.5) without the two TFIIB repeats (TFIIB). The transcript of pyruvate dehydrogenase kinase (Solyc12g098930.1) did not encode the ATPase domain (HATPase_c) in HS. Some other proteins have lost repeated domains or motifs, for example, Solyc01g065490.1, Solyc05g053190.1, or Solyc11g069490.1. The loss of functional domains or motifs raises the question of whether these transcripts are aimed at NMD or remain partially functional with reduced or alternative activity (Keller et al. 2016).

AS adds an extra layer of control over constitutively expressed genes in the male gametophyte, likely fine-tuning pollen development. Splicing patterns also change in response to stresses like heat, regulating the activity of heat shock proteins. Differential splicing between species and cultivars demonstrates evolutionary conservation, but also flexibility.

4.4 Alternative splicing in female gametophyte

Similarly in pollen, alternative splicing seems to have a regulating role in ovules as well. Study of plant intercellular Ras-group leucine-rich repeat protein (PIRLs) expose its essential function in female and male gametophytes. Comparison of PIRL6 expression in vegetative parts and generative parts revealed that vegetative organs lack fully spliced PIRL6 transcripts. In the PIRL6 locus, overlapping genes were found, PIRL6 overlaps with AT2G19340, however, their expression is not linked together. Seven PIRL6 transcripts were observed, one fully functional and six with residual introns containing PTCs. This could explain why PIRL6 is selectively downregulated in vegetative organs by AS, while AT2G19340 can remain transcriptionally active. PIRL6 is proposed to play a key role during differentiation of male and female gametophytes during early stages and may thus be tightly restricted in non-generative parts of plant. No parallels for PIRL6 were found in animals, which proposes its novel cellular function, which is critical for plant and other organisms, such as mitosis, its initiation or regulation, cell positioning, differentiation, and gene regulation required for proper timing and spatial coordination (Forsthoefel et al. 2018).

Analysis of the *Pinus tabuliformis* transcriptome revealed the mechanism of ovule abortion. Comparison of gene expression between the fertile and sterile line revealed that genes related to carbohydrate metabolism and signal transduction, including sucrose synthase, phosphofructokinase, chorismate synthase, clavata-1-like protein, tetraspanin, reticulons, and plasma membrane ATPase-related genes, were in the the lower abundance in the sterile line (STE) than in female fertile line (FRL). Whereas genes associated with mitosis and apoptosis were more expressed in the FRL line. Expression levels of some

stress response genes also differed between lines. Furthermore, genes related to auxin signaling components were observed to be expressed differently in both lines, in FRL line were upregulated. Four genes encoding auxin-responsive protein were upregulated, four encoding auxin response factor 2, and two transport inhibitor response 1.

In the FRL line, it was observed that 42.9% of the genes had only one isoform, whereas in the STE line 39.91% of the genes of one isoform were present. In the entire transcriptome genes with more than 15 isoforms were represented by 1.28% in the STE and 1.33% in the FRL line. In the FRL and STE lines, there were found 1830 and 1243 AS events, respectively. The most prevalent AS was IR, then the 5' and 3' alternative splice sites with mutually exclusive exons being the least observed one. Genes that were considered to affect female sterility were further investigated. The most diverse in FRL and STE were *AGPL1* (associated with starch metabolism), *bHLH66* (associated with plant growth and development), and *TUBA* (associated with mitosis), in the first three genes, IR was the most used AS mode, and *TUBA* used AltD. The number isoforms of AGPL*1* and *TUBA* differed in FRL and STE, *bHLH* had only one isoform in both lines, but with differently spliced form in each line (Gong et al. 2021). These differences indicate that the development of ovules is a complex process, and metabolism genes, alterative splicing of genes, auxin-related genes, and genes involved in cell division play a key role in this process.

5 Research methods of alternative splicing

5.1 Microarrays

The microarrays technology for studying alternative splicing uses oligonucleotides fused into glass slides, produced by inkjet printing or photolithography. Inkjet method, as an advantage of creating 'in-house' microarrays in the laboratory, has limitations in the maximum spots that can be printed, around 150 000, while the limit for photolithography is around 5500 00 spots (Johnson et al. 2003; Gardina et al. 2006; Krawetz 2009). Two main ways of using microarrays is targeting exon-bodies or exon-junctions, use of exon-junction probes allows direct detection of exon-junctions. Tiling arrays, on the other hand, allow one to observe a relative change in exon expression. The exon-junction way uses junction probes that hybridise against half to the end of one exon and half the beginning of the next exon. Observation of signals from constitute exons, showing the general transcript level, allows distinguishing between alternative splicing and simple change in transcript abundance (Krawetz 2009; Srinivasan et al. 2005). Flexibility of design for exon-body analysis can be achieved by changing of the hybridisation temperature (Relógio et al. 2002), but there is no flexibility in design of exon-junction analysis, which can be accomplished by offsetting exon-junction probes by one or two nucleotides. This could be an issue of detecting small exons, as their probes are too short, and these exons are often observed as 3-nt long variants of the alternative 3 'splice site' (Sinha et al. 2010). Tiling arrays could be used to observe AS in unannotated genomes; they use 25-mer oligonucleotides that are spaced 5-35 bp apart, which

allows one to cover complete genome. For each oligonucleotide, a mismatch nucleotide serves as control (Krawetz 2009).

There are several problems when using microarrays, one of them is low reproducibility with other methods such as RT-qPCR, which are used as validation of findings. The validation rate could be as low as 35% but mainly reaches 50-70%. Furthermore, this number shows only false positive cases, and the real number including false negatives could be even higher (Krawetz 2009). One reason for this error rate can be the large number of unknown RNAs that overlap with the known transcript, where the unknown RNA contains an alternative exon, and this newly seen transcript can somehow stimulate alternative splicing events. It cannot be distinguished which isoform is upregulated (Cheng et al. 2005).

Another complication of using microarrays is the lack of connectivity of the information between distant exons, when two or more exons are detected in one transcript, and it is not clear if the sample contained two generations of mRNA containing one alternative exon in each generation or appearance of mRNAs that contain both exons (Krawetz 2009). As next-generation sequencing became more and more affordable, RNA-seq started to be the main competitor of microarrays. The comparison of microarrays and RNA sequencing shows that both technologies provide accurate identification of splice events and prediction of splice sites (P. R. Romero et al. 2006). RNA-seq leads to better coverage of genome as it is not limited by physical limitations of spots on slide, allowing unlimited detection of novel events, and shows better flexibility. Another advantage of RNA-seq is higher validation rates using other methods. On the other hand, microarrays tend to be more sensitive in the detection of some weakly expressed events, which are often missed by RNA-seq. Microarrays also showed less resource consumption during the treatment of the obtained data, which shows that microarrays are a considerable alternative to RNA-seq (J. P. Romero et al. 2018).

5.2 High-throughput RNA sequencing (RNA-seq)

RNA sequencing is a powerful and widely used method to profile gene expression and identifying alternative splicing events in plants or any other organism. The discovery of parallel RNA sequencing could reduce the cost and time needed to acquire gene expression data from whole transcriptomics data from a species; these datasets are usually large and require high technical skills and high computational resources. The major caveat of RNA-seq is its acquisition of sequence data, which are obtained using short reads; typical length of reads is 50 bp to 150 bp. This is seen as a greater problem with dealing with *de novo* assembled full-length transcriptomes (Conesa et al. 2016), short reads of input sequences are also problematic when transcript isoforms of the same gene are studied. The complications of short reads could be overcome by using techniques with longer sequence reads, which are starting to be used in recent days. The main drawback of these techniques is the higher error rate of these reads, which could disrupt *de novo* transcript identification. One solution of this problem is the matching of these long reads with a short one (Au et al. 2012). The general pipeline for AS identification using RNA-seq

can be seen in Figure 7. As sequencing started to be more affordable, the depth of sequencing had increased. This in recent days leads to overwhelm of hardware resources and sometimes causes troubles when using some heuristic algorithms, which are not scalable to the quantity of acquired data (Roberts 2013).

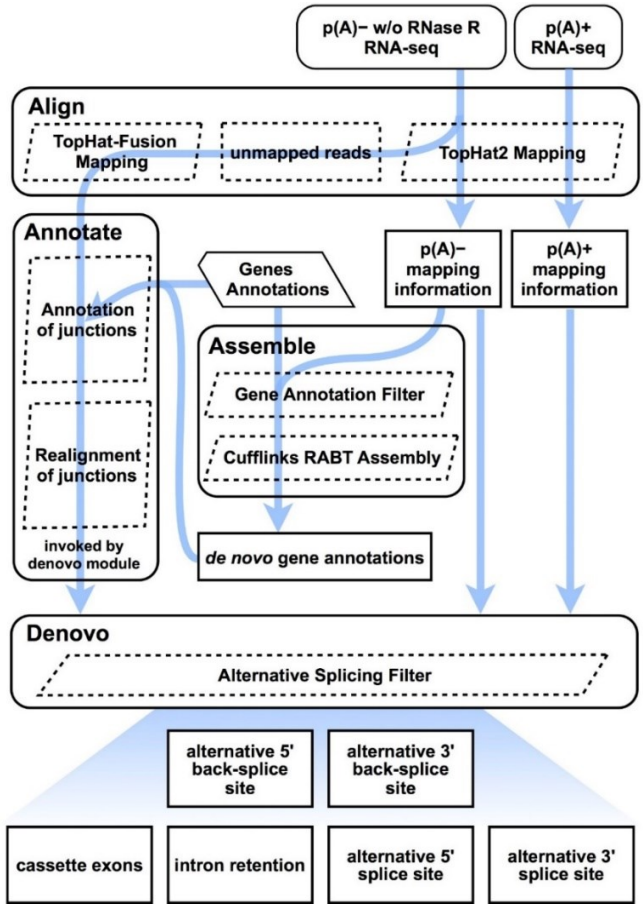

Figure 7 General pipeline of alternative splicing analysis using RNA-seq, retrieved from X.-O. Zhang et al. 2016.

In AS analysis, two approaches commonly used, intron-centric and exon-centric. The intron-centric way quantifies detection of differential expression of each isoform within the total gene expression; then the AS ratio can be estimated based on the ratio isoforms including or excluding exons of interest. The second approach is exon-centric skip estimation of isoform expression and detects alternative spliced isoforms using comparison of reads on exons and junctions of the genes between compared samples. This approach is based on the idea that isoform expression can be observed using the signal of exons and their junctions (Conesa et al. 2016; W. Jiang and Chen 2021).

In recent years, the emerge of machine learning has attracted attention to predict AS sites and branchpoints. These neural networks generally use *ab initio* learning, without any knowledge hard coded into the code, to form positive splice site examples from available genome annotations and large-scale RNA-seq datasets. For reduction of false positive identifications, negative examples from random genomic background are used. Tools such as COSSMO (Bretschneider et al. 2018) and LaBranchoR

(Paggi and Bejerano 2018) or other neural networks usually show a high percentage of correctly predicted branchpoints; these tools can also have better performance using the right conditions than methods using secondary structures, phylogenetic conservations, binding sites for splicing regulators, and splice-site strength, which are estimated using MaxEntScan (Yeo and Burge 2004). The use of neural networks also brings some scepticism, as they are often seen as 'black boxes' becuase their exact mechanism is not always clear (Bretschneider et al. 2018; Louadi et al. 2019; Oubounyt et al. 2018; Paggi and Bejerano, n.d.).

5.3 PCR

Reverse transcriptase PCR (RT-PCR) and quantitative PCR (qPCR) are commonly used as secondary assets in AS analysis. However, the usage of these methods by high-throughput is limited due to their tedious laboratory work. Furthermore, the purity of RNA sample plays a key role, as contaminating DNA can produce indistinguishable product from unspliced pre-mRNA. Contaminating DNA can be removed using a second DNase treatment in some cases (Simpson et al. 2019). Some protocols aimed to reduce the lab work needed to use these methods with a large number of samples, such as using *in vivo* luciferase and fluorescent proteins to identify modulators of alternative splicing (Stoilov et al. 2008; Younis et al. 2010). However, these attempts often suffer from a high error rate, and thus require secondary assays to confirm their findings. RT-PCR is often considered as the golden standard for these secondary assays, as it is relatively easy to perform, has high resolution, offers high flexibility, and in some cases requires a smaller input sample (Freeman, Walker, and Vrana 1999; Simpson et al. 2019). The high resolution of this method may also be its drawback, as it is sensitive to biotic and abiotic stresses, and circadian rhythm; therefore, several repetitions are required on multiple samples (Simpson et al. 2019).

Basic RT-PCR starts from RNA isolation, either total RNA or polyadenylated RNA, obtaining a single-strand complementary DNA copy (cDNA), amplification of target cDNA using PCR and quantification of amplification products by end-point product, using fluorescent intercalating dyes, measuring of incorporated radioactivity by autoradiography or phosphor imaging or real-time monitoring (Freeman, Walker, and Vrana 1999).

6 Conclusion

Alternative splicing in plants proves to be an important regulatory mechanism during mRNA maturation. It offers easy and flexible mechanisms to cope with various stresses and adaptations to them. The role in protein diversity has yet to be discussed, as many alterative transcripts are present in particular tissues, under the activity of some stresses, and many of them undergo nonsense-mediated decay (Göhring, Jacak, and Barta 2014). Alternative splicing has been studied more in animal models, mainly in humans, as it is often pathogenic and leads to various diseases (Poulos et al. 2011). Although many features of transcription are conserved among plants and humans, there are some differences such as the

different ratio of AU rich sites, as they are needed for efficient intron splicing in plants (Goodall and Filipowicz 1989). Plants have a higher ratio of IR, observed, for example, in rice, *Arabidopsis,* and maize (Marquez et al. 2012; Thatcher et al. 2014; B.-B. Wang and Brendel 2006) than in animal models where ExS is the predominant type. Interesting is the observation of group II introns in host organelles, mitochondria, and plastids, despite their bacterial origin. These introns are believed to have emerged as result of genomic transition between host and endosymbiont (Schmitz-Linneweber et al. 2015). It was also observed that splicing is largely co-transcriptional; this behaviour means that the chromatin environment, such as histone modification, methylation status, nucleosome occupancy, and RNA polymerase II processivity, has a strong influence on splicing outcomes (Jabre et al. 2019). This can also make it difficult to study alternative splices as they are influenced by more processes in the cell.

AS has been shown to play an important role in a variety of biological processes, including plant development, as it allows the production of different proteins that are required for different stages of growth (Muhammad et al. 2022). AS allows rapid modulation of gene function in response to stresses (Young, Wilen, and Bonham-Smith 2004; Staiger and Brown 2013). Key stress response pathways are regulated by AS, including heat shock proteins, receptor-like kinases, and disease resistance genes attack (Z. Liu et al. 2018; Walter Gassmann 2008). AS also shapes the development of male and female gametophytes, fine-tuning the expression of genes involved in meiosis, cell division, and differentiation.

Various experimental techniques have been utilised to study genome-wide AS patterns in plants. Microarray analysis allows interrogation of known exon-exon junctions. They can use exon-body or exon-junction probes to assay splicing patterns (J. P. Romero et al. 2018; Krawetz 2009). This method is limited by the number of probes and detection of novel events. However, RNA sequencing (RNA-seq) has become the dominant approach due to its unbiased detection of novel splice events. Short-read and emerging long-read sequencing strategies are applied. Computational methods like machine learning predict splice sites and model AS regulation mechanisms (X.-O. Zhang et al. 2016; W. Jiang and Chen 2021). Secondary techniques like RT-PCR confirm AS events and patterns. Using quantitative PCR, the isoform abundances can be measured.

In general, alternative splicing is an widely used and important mechanism of gene regulation in plants that contributes to the diversity of gene expression and protein isoforms in these organisms. More research is needed to fully understand the roles of alternative splicing in plant biology and to develop tools for manipulating alternative splicing for agricultural and biotechnological applications.

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