Alternative splicing: Enhancing ability to cope with stress via transcriptome plasticity

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1. Introduction

Alternative splicing is involved in many physiological processes, including the response to biotic and abiotic stresses [1]. The sessile growth habit of plants prompted the evolution of unique adaptive developmental and physiological strategies that cope with environmental stress. The plant response to any given stress is a complex phenomenon that is based on the interactions and cross-talk between a number of mechanisms that are involved in the regulation of gene expression at different stages, from transcriptional to post-translational levels. In the light of findings over the last decade, alternative splicing has been proposed as one of these
Fig. 1. Main types of alternative splicing events: A, fully spliced transcript; B, exon skipping; C, alternative 5′ splice site; D, alternative 3′ splice site; E, intron retention.

regulatory mechanisms that is active in the promotion of genome plasticity and versatility, through amplification of the number of proteins and quantitative regulation systems from a single coding unit [2].

This review is focused on the extent and the role of alternative splicing in plant responses to environmental stresses (both abiotic and pathogenic) in the era of the “-omic” technologies. Particular attention is devoted to the following aspects: (i) the description of stress-related genes and gene networks where alternative splicing has been clearly demonstrated to have a functional role, with particular attention on families of regulatory genes; (ii) the conservation of alternative splicing events across species and its evolutionary implications; (iii) the study of the genetic basis of alternative splicing as a suitable tool to generate more resistant crop varieties; (iv) the contribution of genome-wide approaches, with the more recent technologies based on next-generation sequencing to achieve an understanding of the role of alternative splicing in the plant stress response.

2. Molecular mechanisms of pre-mRNA alternative splicing

Pre-mRNA splicing consists of the removal of intron sequences from pre-mRNAs by a large RNA-protein complex, the spliceosome, which recognizes specific sequences at the splice sites. These splice sites can be recognized in pairs either across the intron (intron definition model) or across the exon (exon definition model) [3,4]. Alternative splicing is a process that generates two or more different transcripts from the same pre-mRNA molecule by using different splice sites. Four main types of such splicing are known: exon skipping, alternative 5′ and alternative 3′ splice sites, and intron retention [3]. The resulting splice variants are, respectively: cassette exons, competing 5′ splice sites, competing 3′ splice sites, and retained introns (Fig. 1). These do not occur with the same frequencies in the different kingdoms, as they are affected by the mechanisms used to identify and process splice sites in any given organism. Thus, in a comprehensive survey of alternative splicing across 42 eukaryote species belonging to different kingdoms, they were all found to use these four main kinds of alternative splicing in different proportions. In particular, the ratio of competing 3′ acceptor and competing 5′ donor sites was nearly constant across the organisms considered, while the ratio of retained introns to cassette exons varied substantially among the kingdoms [4]. In more detail, this ratio was very high in all 14 fungi, low in 13 multicellular animals, and intermediate in 6 plant species.

In plants, the prevalence of retained introns with respect to cassette exons has been confirmed by numerous studies [4–13]. The differences in these splice-variant ratios across the kingdoms can be related to the most prevalent splice site recognition mechanism, given that splicing variations are more likely to result in retained introns with the intron definition model (fungi and plants), and in cassette exons with the exon definition model (animals). However, it is likely that almost all eukaryotes can recognize splice sites via both of these mechanisms [3,4].

These different proportions of alternative splicing types have clear implications for the functional meaning of alternative splicing events. Indeed, events based on exon skipping and on alternative 5′ or alternative 3′ splice sites more easily lead to functionally relevant changes in the protein products. These will include changes in the amino- or carboxy-terminus, and in-frame addition/removal of a functional unit, which can have consequences on the subcellular localization, binding properties, and activity or stability of the resulting protein. In this last case, alternative splicing provides regulation that is not only qualitative, but also quantitative [1].

Most intron retention events result in the insertion of an in-frame premature termination codon within the transcript, which can lead an mRNA to different fates. If this codon is located more than 50 nucleotides upstream of an exon–exon junction, the transcript will be targeted for degradation by nonsense-mediated decay, an mRNA surveillance mechanism that is believed to prevent accumulation of truncated, and potentially harmful, proteins.
Alternative splicing coupled with nonsense-mediated decay represents a mechanism for the fine tuning of the amount of a functional transcript in the cell. Otherwise, mRNAs with premature termination codons can be translated into truncated proteins, which will often lack some active domains that are present in the full-length protein product. In this way, these truncated proteins can contribute to the control of the amount of functional protein that is produced. Nevertheless, truncated proteins might themselves have functional roles in processes in which the gene is involved, as has been demonstrated for many resistance genes in plants [16–18] (Fig. 2).

3. Is alternative splicing a matter of balance between stress protection and detrimental effects of stress resistance proteins on plant growth?

Plants can cope with environmental stress by reorganizing their metabolism and gene expression, reaching a new balance between growth, development and survival. The transcriptional changes induced by environmental constraints have been investigated over the past two decades, and many genes and proteins involved in stress signaling and in the regulation of stress-induced gene expression have been identified and characterized. The outcome of these studies consists of a complex network in which several pathways act through their cross-talk, starting from stress perception and ending with specific transcriptional changes [2,19]. The whole process is thus hypothesized to be finely tuned at different levels, ensuring the best response possible. Different mechanisms have been described that regulate transcript synthesis, maturation, stability, nuclear trafficking and association to polysomes. Furthermore, post-translational modifications that can modulate protein activity or control protein degradation, such as sumoylation and ubiquitination, have been well characterized in response to abiotic stress [2]. In such a complex framework, alternative splicing contributed an additional control level for stress-responsive genes, both in qualitative and quantitative regulation of gene transcripts.

3.1. The role of alternative splicing in the response to abiotic stresses

Interestingly, most of the alternative splicing events that have been described in response to abiotic stress concern genes with regulatory roles, covering all levels of regulation of gene expression, as shown in Table 1. These include genes involved in post-translational regulation mechanisms that are initially activated in the stress-signal cascade, such as ubiquitin-mediated protein degradation and protein phosphorylation [20–23]. The E3 ubiquitin ligases are a very large gene family that acts in proteasome-mediated protein degradation and is involved in many biological processes, including stress responses [20,21]. Splicing events were described for a number of genes encoding E3 ligases following exposure to dehydration and cold stress [22,23] (Table 1).

Mitogen-activated protein kinases (MAPKs) have roles in phosphorylation/dephosphorylation cascades, and regulate
stress-signal transduction in both animals and plants [24,25]. The expression of 20 Arabidopsis MAPK genes was analyzed by RT-PCR, which revealed the production of splice variants for five of these genes [26]. The regulation of protein kinase activity is exerted through alternative splicing in different manners. In some cases, alternative splicing altered the domain architecture of the kinases, influencing their subcellular localizations but not their activities. This mechanism was described for two out of three alternative proteins coded by the rice gene OsBWMK1, which were induced by various stress conditions and had different subcellular localizations. In particular, treatment with defense-signaling-related molecules promoted the translocation of one of these forms from the cytoplasm to the nucleus [27]. In other cases, the alternative splicing events can affect protein kinase activities, although the resulting non-active proteins are not necessarily without function. The MPK13 gene, with six exons and five introns, generated at least three splice variants: a full-length transcript (MPK13 Full), and two mRNAs that retained the 4th or 5th introns (MPK13 I4 and I5). Although the MPK13 I4 protein had no protein kinase activity, it enhanced the MKK6-dependent activation of the MPK13 Full protein [26].

Transcription factors are also modulated by alternative splicing under stress conditions. Two members of the MYB gene family of transcription factors (AtMYB59 and AtMYB48) encode alternative proteins that differ in their MYB repeats, and also probably in their binding affinities to gene promoters [28]. The CBF/DREBs are a well-characterized gene family of transcription factors that control resistance to abiotic stress in many plant species, such as for low temperature and dehydration [29,30]. Two splice variants of the OsDREB2B gene are differentially expressed in response to drought and heat stress in rice [31]. In particular, the OsDREB2B1 transcript is more abundant in non-stress conditions and contains a shorter open-reading frame because of a frame shift caused by insertion of the second exon into the mature mRNA. This exon was spliced out in the full-length OsDREB2B2 transcript that accumulated following stress exposure. A similar alternative splicing mechanism was described for the WDREB2 gene in wheat, and for the barley HvDRF1 and maize ZmDREB2A orthologs [32–34]. The meaning of the differential expression of the two OsDREB2B splice variants in rice might relate to fine quantitative regulation of the functional transcript [31]. In the absence of stress, the cells are provided with the non-functional transcript, which can be rapidly converted to the full-length, fully functional transcript by changing the splicing pattern of the gene, and therefore avoiding the time necessary for transcriptional activation and pre-mRNA accumulation. Indeed, a rapid change of the gene splicing pattern can

Table 1
Some examples of plant genes that undergo alternative splicing in response to biotic and abiotic stresses.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene product</th>
<th>Gene function</th>
<th>Species</th>
<th>Stress</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZmCOI6.1</td>
<td>Unknown</td>
<td>–</td>
<td>Zea mays</td>
<td>Cold, drought</td>
<td>[85]</td>
</tr>
<tr>
<td>19KDa complex I subunit</td>
<td>Hydrolase</td>
<td>Metabolism</td>
<td>Dunaliella salina</td>
<td>Anoxia, salt, rotenone</td>
<td>[86]</td>
</tr>
<tr>
<td>S21C</td>
<td>Superoxide dismutase</td>
<td>ROS control</td>
<td>Populus trichocarpa</td>
<td>Abiotic and biotic stress</td>
<td>[87]</td>
</tr>
<tr>
<td>hips-SOD1</td>
<td>Respiratory burst oxidase homolog</td>
<td></td>
<td>Zea mays</td>
<td>Cold, heat, salt, ultraviolet light</td>
<td>[37]</td>
</tr>
<tr>
<td>ZmbohB</td>
<td>MYB transcription factor</td>
<td>Arabidopsis thaliana</td>
<td>Arabidopsis thaliana</td>
<td>Jasmonic acid, salicylic acid, auxin, gibberellin, salt</td>
<td>[28]</td>
</tr>
<tr>
<td>AtMYB59</td>
<td>transcription factor</td>
<td>Arabidopsis thaliana</td>
<td>Arabidopsis thaliana</td>
<td>Jasmonic acid, salicylic acid, auxin, gibberellin, salt</td>
<td>[28]</td>
</tr>
<tr>
<td>OsDREB2B</td>
<td>DREB/CBF transcription factor</td>
<td>Transcriptional regulation</td>
<td>Oryza sativa</td>
<td>Drought</td>
<td>[31]</td>
</tr>
<tr>
<td>WDREB2</td>
<td>DREB/CBF transcription factor</td>
<td>Transcriptional regulation</td>
<td>Triticum aestivum</td>
<td>Drought</td>
<td>[32]</td>
</tr>
<tr>
<td>HvDRF1</td>
<td>DREB/CBF transcription factor</td>
<td>Transcriptional regulation</td>
<td>Hordeum vulgare</td>
<td>Drought</td>
<td>[33]</td>
</tr>
<tr>
<td>ZmDREB2A</td>
<td>DREB/CBF transcription factor</td>
<td>Transcriptional regulation</td>
<td>OsBWMK1</td>
<td>Drought</td>
<td>[34]</td>
</tr>
<tr>
<td>TFIIIA</td>
<td>Transcription factor for polymerase</td>
<td>Oryza sativa</td>
<td>Arabidopsis thaliana</td>
<td>Osmotic stress, salt</td>
<td>[70]</td>
</tr>
<tr>
<td>III A</td>
<td>Splicing regulators</td>
<td>Post-transcriptional regulation</td>
<td>various species</td>
<td>Abscisic acid, indoleacetic acid, 6-benzyl aminopurine, salt, heat, cold</td>
<td>[38–41]</td>
</tr>
<tr>
<td>A1GRP7</td>
<td>Splicing regulators</td>
<td>Post-transcriptional regulation</td>
<td>Arabidopsis thaliana</td>
<td>Cold</td>
<td>[46]</td>
</tr>
<tr>
<td>A1GRP8</td>
<td>Splicing regulators</td>
<td>Post-transcriptional regulation</td>
<td>Arabidopsis thaliana</td>
<td>Cold</td>
<td>[46]</td>
</tr>
<tr>
<td>6G2</td>
<td>E3 ubiquitin ligase</td>
<td>Triticum durum</td>
<td>Arabidopsis thaliana</td>
<td>Cold</td>
<td>[22]</td>
</tr>
<tr>
<td>At4g39140</td>
<td>E3 ubiquitin ligase</td>
<td>Arabidopsis thaliana</td>
<td>Arabidopsis thaliana</td>
<td>Cold</td>
<td>[22]</td>
</tr>
<tr>
<td>At2g32190</td>
<td>E3 ubiquitin ligase</td>
<td>Arabidopsis thaliana</td>
<td>Arabidopsis thaliana</td>
<td>Cold</td>
<td>[22]</td>
</tr>
<tr>
<td>ASK 20</td>
<td>E3 ubiquitin ligase</td>
<td>Post-translational regulation</td>
<td>Oryza sativa</td>
<td>Cold, salt, wounding</td>
<td>[26]</td>
</tr>
<tr>
<td>MPK13</td>
<td>Protein kinase</td>
<td>Arabidopsis thaliana</td>
<td>Arabidopsis thaliana</td>
<td>Cold</td>
<td>[23]</td>
</tr>
<tr>
<td>OsBWMK1</td>
<td>Protein kinase</td>
<td>Arabidopsis thaliana</td>
<td>Arabidopsis thaliana</td>
<td>Cold, salt, wounding</td>
<td>[26]</td>
</tr>
<tr>
<td>OsBIP2C2</td>
<td>Protein phosphatase</td>
<td>Arabidopsis thaliana</td>
<td>Arabidopsis thaliana</td>
<td>Fungal elicitors, jasmonic acid, salicylic acid, salt</td>
<td>[27]</td>
</tr>
<tr>
<td>VbCP15-7</td>
<td>Chitinase</td>
<td>Arabidopsis thaliana</td>
<td>Arabidopsis thaliana</td>
<td>Cold</td>
<td>[26]</td>
</tr>
<tr>
<td>ICS</td>
<td>Isocitriose synthase</td>
<td>Defense</td>
<td>Citrus clementina</td>
<td>Tetranychus urticae infection, methyl jasmonate</td>
<td>[49]</td>
</tr>
<tr>
<td>Rpg1</td>
<td>Protein kinase</td>
<td>Metabolism</td>
<td>Hordeum vulgare</td>
<td>Puccinia graminis sp. tritici infection</td>
<td>[58]</td>
</tr>
<tr>
<td>RPS6</td>
<td>NBS-LRR protein</td>
<td>Arabidopsis thaliana</td>
<td>Arabidopsis thaliana</td>
<td>Pseudomonas syringae infection</td>
<td>[61]</td>
</tr>
<tr>
<td>Pr-ta</td>
<td>NBS-LRR protein</td>
<td>Arabidopsis thaliana</td>
<td>Arabidopsis thaliana</td>
<td>Pseudomonas syringae infection</td>
<td>[61]</td>
</tr>
<tr>
<td>RLM3</td>
<td>NBS-LRR protein</td>
<td>Arabidopsis thaliana</td>
<td>Arabidopsis thaliana</td>
<td>Leptosphaeria maculans infection</td>
<td>[16]</td>
</tr>
<tr>
<td>N gene</td>
<td>NBS-LRR protein</td>
<td>Arabidopsis thaliana</td>
<td>Arabidopsis thaliana</td>
<td>Tobacco mosaic virus infection</td>
<td>[17]</td>
</tr>
<tr>
<td>RPS4</td>
<td>NBS-LRR protein</td>
<td>Arabidopsis thaliana</td>
<td>Arabidopsis thaliana</td>
<td>Bacterial pathogens expressing avrRps4</td>
<td>[18]</td>
</tr>
<tr>
<td>BPP1</td>
<td>NBS-LRR protein</td>
<td>Arabidopsis thaliana</td>
<td>Arabidopsis thaliana</td>
<td>AvrRps4</td>
<td>[63]</td>
</tr>
<tr>
<td>BPP5</td>
<td>NBS-LRR protein</td>
<td>Arabidopsis thaliana</td>
<td>Arabidopsis thaliana</td>
<td>Pseudomonas syringae infection</td>
<td>[64]</td>
</tr>
</tbody>
</table>

ROS, reactive oxygen species; NBS-LRR, nucleotide binding site-leucine repeat start.
be obtained through the post-transcriptional or post-translational activation of splicing regulators. Furthermore, this mechanism can keep the transcription of OsDREB2B constitutively active without affecting plant growth. Many proteins that are responsible for resistance to both biotic and abiotic stress can negatively affect plant growth and development when they are not needed, or can have a high metabolic cost, and therefore their levels need to be precisely regulated [20].

Another example is given by a maize gene that belongs to the class of respiratory-burst-oxidase homolog (RBOH) genes, which are involved in reactive oxygen species (ROS) production in response to avirulent pathogens and in many abiotic stress conditions [35,36]. ROS levels must be finely tuned to prevent their toxicity, even controlling their activity as signal molecules. Analysis of the maize ZmrbohB gene revealed the generation of two transcript isoforms, ZmrbohB-α and ZmrbohB-β, that arised from the retention of intron 11, which carried a premature termination codon and probably led to nonsense-mediated decay in response to several abiotic stimuli such as cold, heat, UV and salinity stress [37].

An even more interesting case was observed for the stress-related alternative splicing of genes coding for splicing regulators. The serine/arginine (ser/arg) proteins are a highly conserved family of RNA-binding proteins that have key roles in the execution and regulation of pre-mRNA splicing specifically in different plant tissues, at different developmental stages, and in response to abiotic stress [38]. Twenty genes encoding ser/arg proteins have been identified in Arabidopsis, and most of their mRNAs (16) undergo alternative splicing following developmental and environmental stimuli; this can produce nearly 100 different transcripts [39,40]. Intriguingly, these can promote alternative splicing of their own transcripts, as well as of other gene products. Overexpression of the SR3055 and RSZ2356 genes in Arabidopsis, as well as the OsSRZ236 and OsSRY33 genes in transgenic rice, alters the splicing patterns of their own pre-mRNAs and those of several other ser/arg genes [38]. The functional significance of most of the reported splice variants from ser/arg genes remains unknown; nevertheless, many alternatively spliced transcripts contain a premature termination codon. Thirteen ser/arg genes that were alternatively spliced generated 75 transcripts [41]. Out of these, 53 contained a premature termination codon, and half were shown to be targets of degradation by nonsense-mediated decay by using an Arabidopsis mutant that lacked UPF3, one of the core components of the nonsense-mediated decay machinery. More details are found in numerous reviews on this gene family [38,42–45].

Arabidopsis RNA-binding protein AtGRP8 has a similar auto-regulation and cross-regulation system that promotes the use of a cryptic 5′ splice site and generates an alternatively spliced transcript, as AtGRP8. This contains a premature termination codon and accumulates in particular in response to cold stress [46]. This transcript is a direct nonsense-mediated decay target, thus limiting the production of the functional AtGRP8 protein when its levels pass a threshold. In addition to its own pre-mRNA, AtGRP8 negatively regulates the AtGRP7 transcript via formation of the equivalent alternatively spliced as AtGRP7 transcript, which leads to a decrease in AtGRP7 abundance. Furthermore, AtGRP7 itself negatively auto-regulates its own transcript and the AtGRP8 transcript through alternative splicing linked to nonsense-mediated decay [46]. The feedback loops identified for these genes are thought to operate as slave oscillators downstream of the circadian clock [46]. In particular, the negative auto-regulation and cross-regulation may fine tune and balance the expression of both proteins. Furthermore, as the two genes show differences in their expression in response to external stimuli [46], the connection between the two regulatory circuits may serve to integrate input by diverse environmental stimuli. Finally, three Arabidopsis polypyrimidine-tract-binding protein genes have been shown to undergo similar auto-regulation and cross-regulation pathways [47]. These proteins belong to the heterogeneous nuclear ribonucleoprotein (hnRNP) family, which, antagonistically to ser/arg protein action, commonly bind to splicing silencers, and therefore repress the use of splice sites [48]. Even if independent of stress stimuli, this mechanism underlines the importance of the coupling of alternative splicing and nonsense-mediated decay as a regulatory mechanism in cell metabolism.

In summary, these genes are part of a network of interlocking feed-back loops whereby under abiotic stress conditions these RNA-binding proteins regulate themselves and are reciprocally cross-regulated through coupling unproductive splicing to nonsense-mediated decay. Alternative splicing regulation of genes that have products that alter the splicing of other genes in turn might considerably enhance and amplify the signal-transduction cascade in response to stress stimuli. Further studies are needed to clarify the effects of such regulatory loops on the downstream genes that have their splicing patterns influenced by these important regulators. It would be interesting for example altering the expression level of one of the genes acting in a regulatory loop or both of them and studying the effect on the plant transcriptome in different stress conditions, together with the consequences on the phenotype of the plant. Of great importance will be also the identification of RNA targets of the splicing regulators, in order to construct networks of cross-talk among different pathways of stress signal transduction. The action of splicing regulation might be exerted in particular on other regulatory genes, so further amplifying the signal cascade, or directly on downstream genes.

3.2. The role of alternative splicing in biotic stresses

Plants can be attacked by a wide set of pathogens, including viruses, bacteria, and fungi, as well as by nematodes and insects. Thus, plants have developed a number of defense mechanisms at both local and systemic levels. Chemical and physical barriers are constitutive and/or inducible elements of plant-cell responses that are aimed at physically counteracting the penetration of a pathogen into plant tissues and cells. Chitinases are pathogenesis-related (PR) proteins, and their activities can be induced by viral infections and fungal and bacterial cell-wall components, as well as more general sources of stress, such as wounding. A defense-related acidic chitinase II gene is alternatively spliced in Tetrazylluchus urticae-infested Citrus clementina plants, as well as in response to pathogen infection and to methyl jasmonate treatment. The plants produced an additional transcript containing a premature termination codon after the first 135 amino acids [49].

Cyclotides are a novel family of plant-derived defense peptides characterized by insecticidal [50–53] and antimicrobial activities [54]. Different genes coding for cyclotides were identified in Viola baoshanensis, and alternative splicing increased the diversity of cyclotide expression via the recombination of N-terminal repeat regions and cyclotide domains [55].

A key aspect of biotic stress resistance is the ability of plants to detect the presence of attacking agents, so as to promptly activate mechanisms that are aimed against the invaders. Two relevant groups of host receptors are recognized as having key roles in this recognition process. The first comprises protein recognition receptors, which are usually membrane-bound receptor-like proteins or receptor-like kinases that can recognize pathogen-associated molecular patterns [56]. Arabidopsis has a large superfamily of at least 610 such genes, including both transmembrane and cytoplasmic receptor-like kinases, and leucine-reach repeat receptor-like kinases. This is the largest subfamily in the Arabidopsis genome, with at least 223 members [57]. These receptor-like kinase proteins are regulated by alternative splicing. Indeed, several alternative splicing forms have been identified, such as for the barley stem rust
resistance gene Rpg1, which can be translated into a predicted full-length protein product that has a unique structure of a receptor-like kinase with dual kinase domains. In this case, alternative splicing contributed enhanced resistance to the stem rust pathogen Puccinia graminis f. sp. tritici [58]. A subset of 33 genes encoding leucine-reach repeat receptor-like kinases was analyzed [59], and alternative transcripts were found for most of them.

The second group is composed of the resistance proteins involved in the intracellular detection of pathogen effectors when these are introduced into the host cells [56]. Nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins are divided into two classes according to the presence at their C-terminal of a Toll/interleukin-1 receptor (the TIR-NBS-LRRs, which appear to be absent in monocotyledons [60]) or a coiled-coil domain (the CC-NBS-LRRs). The majority of plant-disease resistance genes that have been reported to undergo alternative splicing belong to the TIR-NBS-LRRs, even if some examples have been reported for CC-NBS-LRRs in cereals.

Alternative splicing affects the expression of resistance genes mainly through induction of the synthesis of protein forms that contain different combinations of functional domains. Three alternative transcripts derived from the Arabidopsis RPS6 (resistance to Pseudomonas syringae) gene. The full length protein contained three functional domains (TIR, NBS and LRR), while the two alternative transcripts were characterized by a premature termination codon and encoded truncated proteins in which only one or two domains were present [61]. Other examples are the rice Pi-ta gene that confers resistance to races of Magnaporthe oryzae [62] and the RLM3 (Resistance to Leptosphaeria maculans 3) gene in Arabidopsis [16] (Table 1). Interestingly, the shorter peptide produced by the RLM3 gene had a positive effect on resistance.

The positive effects of truncated proteins produced via alternative splicing of resistance genes is a common defense strategy, which has been demonstrated also for the Nicotiana glutinosa N gene [17], and for the RPS4 gene, an Arabidopsis TIR-NBS-LRR gene that is involved in resistance to bacterial pathogens that express avrRps4 in a specific manner. In particular, alternative transcripts containing a shorter open-reading frame were shown to be translated into truncated proteins that are characterized by different stabilities and that have clear roles in inducing hypersensitive response-like cell death in the absence of avrRps4 when transiently expressed in Nicotiana benthamiana [18]. A similar approach has demonstrated that the truncated RPP1 and RPS5 proteins also have roles in the induction of cell death [63,64]. Such positive roles in resistance for truncated proteins might be obtained by alleviating self-inhibition of the full-length proteins [18] or by functioning as adaptors for downstream signaling events [38,60]. Further studies based on biochemical assays for protein–protein interactions are required to clarify the molecular details of this mechanism.

In the gene-for-gene model [65], plant disease resistance genes devoted to pathogen recognition must be expressed at a basal level in plant cells so as to recognize the avirulence factors that are produced by a pathogen during an infection, and thus to promptly induce the defense response. However, once the presence of a pathogen is recognized, the resistance genes can also be up-regulated to promote these resistance responses. As seen for some key regulators of the plant response to abiotic stresses, alternative splicing participates at this point as one of the multiplicity of mechanisms that co-operate in the fine-tuning of the activities of proteins, the amounts of which need to be balanced to enhance disease resistance and to limit cellular damage and metabolic cost [66].

Alternative splicing coupled to nonsense-mediated decay, and the production of proteins with different domain rearrangements (which might even be truncated), both contribute to this balance of genes involved in resistance to abiotic stress, such as non-optimal temperatures and dehydration, as described in the previous section. Nevertheless, to our knowledge, nonsense-mediated decay has not been clearly implicated in the regulation of pathogen-related and resistance genes. Even if studies of the role in resistance to various biotic agents of alternative splicing coupled to nonsense-mediated decay will be available in the near future, it appears that the production of alternative polypeptides that are characterized by different combinations of protein sub-modules, as seen for the cyclotides and for the domains of the resistance proteins, represents the main alternative splicing-based regulation mechanism in plant responses to biotic attacks. Resistance genes are very numerous in the plant genome, and they often occur in clusters at specific loci following gene duplication and amplification events [67]. In this manner diverse protein products are generated that facilitate the genetic evolution of resistance to newly evolved pathogen races that express new avirulence molecules that overcome plant resistance. In light of this, alternative splicing can participate together with gene duplication in the amplification of resistance gene variation and complexity, that help plants to cope with biotic stress in plant–pathogen co-evolution.

4. Conservation of alternative splicing regulation, and the evolutionary implications related to stress response

The conservation of splicing variants among plant species can provide insights on evolution of alternative splicing. First of all, the extent of alternative splicing conservation appears to be related to the phylogenetic distance among the species. Genome-wide studies have identified many alternative splicing events that are well conserved between dicotyledons and monocotyledons, and also only within one of these two classes; these have therefore occurred before or after, respectively, the dicotyledon–monocotyledon division. The ratio of homologous pairs undergoing alternative splicing is higher between Medicago and Arabidopsis (10–15%) than between Medicago and rice (0.8%), and rice and Arabidopsis (0.9%) [5]. Furthermore, about 15% of the alternative splicing events observed in Medicago were conserved among the legume species, but only half of them were also in common with Arabidopsis [7]. Interestingly, the number of conserved alternative splicing events appears to significantly increase when gene classes that are specifically involved in stress response are considered. As an example, the analysis of a subset of about 150 ESTs that are members of the ROS gene network, as selected from the Populus EST database, has revealed that more than 50% of alternative splicing events in Populus correspond to Arabidopsis homologs [68]. The stress-dependent intron–retention event in the 3′–UTR of the durum wheat 6G2 gene, encoding an E3 ubiquitin ligase, is structurally conserved in bread wheat, barley and Arabidopsis [22]. The intron retention pattern of the AtGRP7 gene was one of the six clusters of (a total of 1,360) genes with similar alternative splicing events across Medicago, Arabidopsis and rice [5]. Furthermore, the pattern of auto-regulation and cross-regulation coupled to nonsense-mediated decay for the ser/arg and poly pyrimidine tract-binding protein genes has been widely described not only in different plant species, but also in humans [69], which indicates the importance of this mechanism of regulation across the kingdoms.

Such conserved alternative splicing regulatory systems occurred very early during the evolution of multicellular living organisms, and provide advantages in terms of the ability to cope under different kinds of adverse conditions. The same conclusions can be drawn for splicing events that occur in plant-specific genes. An interesting example is seen by the exonization of 5S ribosomal RNA (5S rRNA) within the gene of its own transcriptional regulator, TFIIB (transcription factor for polymerase III A), which is essential for RNA-polymerase–III-based transcription of 5S rRNA.
in eukaryotes. Under osmotic and salt stress, the Arabidopsis gene underwent quantitative auto-regulation based on alternative splicing of the exonized 5S RNA, coupled with nonsense-mediated decay, resembling that described for the ser/arg genes [70]. The 5S-RNA-derived exon in the TFIIIA gene is present in all land plant species tested but not in green algae and non-plant species. Thus it was proposed that the conservation of 5S-RNA-like elements, as well as the alternative splicing pattern of the TFIIIA genes, might have had a role in the successful colonization of land by these organisms, due to its role in the regulation of homeostasis under water and osmotic stress conditions [70].

This conservation of alternative splicing events may provide a strong indication of functional products, and distinguish splicing errors from functional alternative splicing [71]. Nevertheless, the identification of a high number of non-conserved alternative splicing events might be due to a rapid evolution of splicing diversity, which can contribute to an explanation of some of the morphophysiological differences among species. As an example, the ICS gene that codes for isochorismate synthase converts chorismate to isochorismate in the biosynthesis of phylloquinone, an essential cofactor for photosynthetic electron transport [72]. The Arabidopsis ICS gene is also involved in salicylic acid synthesis during plant defense, while in Populus and other species, salicylic acid is mainly produced via the phenylpropanoid pathway. In these two species, evolution has led to the uncoupling or differential regulation of the biosynthesis of phylloquinone and salicylic acid as defense responses by means of two different strategies: gene duplication has led to the evolution of two distinct paralogs in Arabidopsis, AtICS1 and AtICS2, which are differentially regulated by environmental stress, and which under certain conditions promote the synthesis of salicylic acid (present at a very low levels in this species). Salicylic acid is normally present in Populus at much higher levels in its role as a defense against herbivores (1–3 orders of magnitude higher than those following pathogen attack in Arabidopsis), and it is produced via the phenylpropanoid pathway, with a single copy of the ICS gene present. This single ICS Populus gene is subject to alternative splicing, with the production of several transcript forms, which is not seen for the Arabidopsis ICS genes [72]. According to this study, alternative splicing and gene duplication can act as two contrasting mechanisms for the modulation of ICS function in higher plants.

Ploidy and interspecific hybridization directly act in plant adaptive evolution and speciation, and they often determine phenotypic differences, and affect the evolution of new alternative splicing events in response to stress. The ploidy levels in both natural and synthetic wheat lines change the stress-dependent alternative splicing patterns of WDREB2 transcripts, probably due to new interactions among the splicing regulators and the splice sites coded by the slightly different A, B and D genomes [73]. The alternative splicing patterns of 40 genes were analyzed in an interspecific Populus hybrid and in its parents [74]. New alternative splicing variants were observed in the hybrid, with respect to the parents. Some of these concerned two ser/arg genes, which indicated that even if variations in alternative splicing in plant stress responses are numerically limited in hybrids, their impact can be considerable on overall gene expression [74]. The alternative splicing can be determined by interactions between cis-acting sequences that are recognized by the spliceosomal machinery and trans-acting splicing factors that can be regulated by stress conditions. Thus suboptimal interactions might take place in hybrids between species with divergent orthologously interacting proteins [75]. Therefore alternative splicing might function as one of the factors that form the basis of the dynamic nature of genomes and heterotic gene expression patterns in hybrids, which includes up-regulation and down-regulation of gene expression, gene silencing, chromosomal rearrangements, and cytosine methylation changes [74].

In conclusion, miss-splicing events that arise from different kinds of DNA mutations, chromosomal rearrangements or epigenetic effects, can occur at low levels, and a “basal level” of splicing errors might be maintained as a source of new genetic variability, which will thus promote evolution.

5. Studying the genetic basis of alternative splicing: a suitable tool for improvement of stress resistance in crops?

Despite the large number of alternative splicing studies in the literature, the extent of intra-species variations of alternative splicing has been poorly investigated. The Columbia and Wassilewskija ecotypes of Arabidopsis differ in alternative splicing in the RPT5b gene, which encodes a proteasome subunit that is essential for gametophyte development. The RPT5a and RPT5b genes are fully redundant in Columbia but not in Wassilewskija, in which the RPT5b gene was partially mis-spliced in the transcript, with its function abolished. This mis-splicing event was due to a SNP in the seventh intron of the RPT5b gene, with a natural variation in a collection of 487 Arabidopsis accessions [76]. The gametophyte development was not affected in accessions carrying the mis-spliced RPT5b transcript due to the presence of a fully functional RPT5a allele.

The rate of alternative splicing among different genotypes of the same species was similar within the majority of dicotyledon species [77]. This was probably due to the very recent origin of most of the cultivars. The greatest differences in the alternative splicing rates were found between the Columbia and Wassilewskija ecotypes of Arabidopsis (30%). Lower differences were observed in cereals, and in particular, in maize and barley (20%), probably due to the intense selection pressures exerted by the earlier domestication process and by the later plant breeding.

The natural genetic and phenotypic variations that occur in crop plants are the main resources for contemporary breeding strategies. Linkage and association mapping are useful tools for the identification of loci that underlie phenotypic variations. Ongoing efforts to improve important agronomic traits have resulted in the mapping of many quantitative trait loci (QTLs) using traditional genetic marker technologies. In contrast, the identification of the gene(s) responsible of phenotypic variation and an understanding of their allelic variations and modes of action have proven to be difficult, especially in species that are characterized by large and complex genomes.

Expression genetics, which combines gene-expression studies with genetic linkage and association mapping analysis, is now considered a fundamental tool for the discovery of expression variants of genes involved in the control of quantitative phenotypes [78]. The use of microarray technology allowed the scoring of differential gene expression in large sets of samples, which can be considered as quantitative traits and be mapped as gene expression QTLs. Similarly, metabolite and protein levels, as well as alternative splicing variations, can potentially be mapped as quantitative traits. Many studies have been carried out in animals and humans, but very little evidence is available for plants. The first genome-wide analysis of genetic variations of alternative splicing was in the nematode Caenorhabditis elegans, using tiling microarray (microarray platforms that are sensitive to splicing) data with multiple probes that targeted every exon of each gene [79]. A genome-wide association mapping approach was proposed to identify QTLs that can explain the regulatory variations in Arabidopsis [80]. A collection of F1 hybrids obtained by randomly crossing 111 accessions was used to test the associations of various expression traits with nearly 142,000 SNPs [80]. An expression analysis of single exons and introns was performed to assess the genetic regulation of splicing variation. Both local and distant associations were detected, which confirmed that both cis-acting and trans-acting factors can regulate alternative splicing [80].
Overall, the number of distant associations was higher than local associations for both introns and exons, even if these local associations explained a larger proportion of the observed variation for the splicing traits. Furthermore, a refined expression analysis was carried out of introns and exons belonging to the same gene in which the SNP was located. This analysis revealed that the 3’-end of introns was the most abundant site for splicing QTLs.

Similar approaches can be applied to crop species exposed to different abiotic or biotic stress treatments, and these should reveal the stress-related alternative splicing events that are important for resistance. In particular, co-mapping of splicing QTLs with QTLs for stress resistance traits might indicate candidate genes for stress resistance and provide insights into the molecular basis of these resistance mechanisms. The evaluation of genetic variability in large collections of commercial cultivars and wild accessions might help to select genotypes for alternative splicing events with a clear effect on resistance in pre-breeding and breeding marker-assisted programs. As a complementary approach, the splicing pattern of identified genes might be regulated in transgenic plants by means of the ectopic expression of genes carrying modified splice junctions or genes encoding particular allelic forms of splicing regulators involved in the plant stress resistance. The transgenic approach could be of great importance in demonstrating the effective role of splice variants of particular genes in contributing to resistance. Furthermore, transgenic plants thus improved for stress resistance could be used to transfer the resistance determinants to elite cultivars in pre-breeding marker-assisted programs.

### 6. Genomic approaches reveal new insight on the extent of alternative splicing in plant stress responses

Sequence information has been used for computational studies that are aimed at describing the extent of alternative splicing in a given species, and to compare its characteristics in different species and taxa. The results are strongly dependent on the experimental or computational approaches used and the species considered, as summarized in Table 2. In all cases, intron retention was the most represented type of alternative splicing, representing more than 50% of the events. For the distribution along the gene, about 80% of the events occurred in the coding region in Arabidopsis [71,81] and Brachypodium distachyon [11], with a small proportion in untranscribed regions. Similar results were obtained for Medicago truncatula [5].

There are several technical issues that still need to be solved to properly not only detect but also to quantify the abundance of the alternative transcripts generated from the same pre-mRNA. Besides tiling microarrays, new bioinformatics tools have been recently developed and made publicly available to the scientific community that can be applied to study the plant transcriptome at the isoforms level also via RNA-seq [82].

Genomic studies on alternative splicing indicate that the appearance of many splice variants is induced by environmental stresses. As an example, the measured number of alternative splicing events was significantly higher for cDNA libraries developed from Arabidopsis plants exposed to different kinds of stress, in particular to low temperatures, compared to cDNA libraries derived from control plants [83]. This might indicate a role for alternative splicing in the regulation of gene expression in plants under stress conditions, or it might suggest that most splice variants detected under stress can be ascribed to stress-damage-driven errors or inaccuracies of the splicing machinery. In support of the second hypothesis, only a few cases of functional regulation by alternative splicing have been described in the last few years with respect to the high number of stress-related alternative splicing events that have been described in genome-wide studies. Furthermore, only a small fraction of splicing variants that have been identified in these studies can be translated into functional proteins in Arabidopsis, Medicago truncatula and Chlamydomonas [5,9,10].

Nevertheless, if alternative splicing is a non-specific consequence of stress damage, a random distribution would be expected among genes. On the contrary, the functional distribution of the transcripts containing retained introns was skewed towards stress and external/internal stimuli-related genes [84]. Eighteen percent of alternative splicing events identified in Arabidopsis by an approach based on tiling arrays were differentially included in transcripts between various stress treatments and controls [13]. Furthermore, splicing and intron retention in particular might have roles in plant stress responses, as supported by the observation that they occur preferentially in genes with regulatory roles. The transcript groups, based on gene ontology, that were enriched in the retained introns included mostly RNA processing and signal transduction in Arabidopsis [77]. The Arabidopsis intron retention was frequently associated with specific types of abiotic stress, and most of the highly conserved intron retention events have been detected in essential regulatory genes [8].

Taken together, results from genome-wide studies indicate that the extent of alternative splicing in plant responses to stress has been significantly underestimated. Continuous advances in technology and computational methods, and exploitation of a larger variety of abiotic and biotic stress treatments will allow to discover ever more alternative splicing events that are potentially involved in the regulation of the stress response.

### Table 2: Rate of alternative splicing in different plant species, evaluated with different methods.

<table>
<thead>
<tr>
<th>Species</th>
<th>Method</th>
<th>Rate of alternative splicing (% of expressed genes)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis thaliana</td>
<td>EST/genomic sequence comparison</td>
<td>3–35</td>
<td>[7,77]</td>
</tr>
<tr>
<td>Oryza sativa</td>
<td>Analysis of next generation sequencing data</td>
<td>42a</td>
<td>[8]</td>
</tr>
<tr>
<td>Hordeum vulgare</td>
<td></td>
<td>26–31</td>
<td>[77]</td>
</tr>
<tr>
<td>Sorghum bicolor</td>
<td></td>
<td>52–60</td>
<td>[77]</td>
</tr>
<tr>
<td>Medicago truncatula</td>
<td></td>
<td>46</td>
<td>[77]</td>
</tr>
<tr>
<td>Malus domestica</td>
<td></td>
<td>69</td>
<td>[77]</td>
</tr>
<tr>
<td>Lotus japonicus</td>
<td></td>
<td>72</td>
<td>[77]</td>
</tr>
<tr>
<td>Solanum lycopersicum</td>
<td></td>
<td>18</td>
<td>[5]</td>
</tr>
<tr>
<td>Glycine max</td>
<td></td>
<td>4</td>
<td>[10]</td>
</tr>
<tr>
<td>Lactuca sativa</td>
<td></td>
<td>3</td>
<td>[5]</td>
</tr>
<tr>
<td>Brachypodium distachyon</td>
<td></td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Chlamydomonas reinhardtii</td>
<td></td>
<td>5–46</td>
<td>[5,7,77]</td>
</tr>
<tr>
<td>Populus trichocarpa</td>
<td></td>
<td>69</td>
<td>[77]</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td></td>
<td>46</td>
<td>[5,7,77]</td>
</tr>
<tr>
<td>Oryza sativa</td>
<td></td>
<td>72</td>
<td>[77]</td>
</tr>
</tbody>
</table>

* a % of intron containing gene.

Overall, the number of distant associations was higher than local associations for both introns and exons, even if these local associations explained a larger proportion of the observed variation for the splicing traits. Furthermore, a refined expression analysis was carried out of introns and exons belonging to the same gene in which the SNP was located. This analysis revealed that the 3’-end of introns was the most abundant site for splicing QTLs.
7. Conclusions

Only few years ago alternative splicing was considered a marginal genetic phenomenon with little effect on plant homeostasis under stress conditions. Alternative splicing has emerged as a powerful mechanism for the regulation of plant stress response. In particular, it has evolved along with the other regulatory levels, from transcriptional to post-translational, to contribute to the creation and maintenance of regulatory networks that allow plants to adapt to their changing environmental conditions. In light of these considerations, alternative splicing events that increase stress resistance should be associated to specific genes or genomic regions in crops, and selected to obtain more resistant varieties. This might represent a good strategy for obtaining good yield performance in the framework of the scenario of climate change, in which water will become less available and new and more virulent pathogen strains will spread.

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