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ScienceDirect
Plant Science 174 (2008) 420–431
PLANT SCIENCE
www.elsevier.com/locate/plantsci

Review

Abiotic stress response in plants: When post-transcriptional and post-translational regulations control transcription

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Received 12 December 2007; received in revised form 6 February 2008; accepted 6 February 2008
Available online 15 February 2008

Abstract

The molecular response of plants to abiotic stresses has been often considered as a complex process mainly based on the modulation of transcriptional activity of stress-related genes. Nevertheless, recent findings have suggested new layers of regulation and complexity. Upstream molecular mechanisms are involved in the plant response to abiotic stress, above all in the regulation of timings and amount of specific stress responses. Post-transcriptional mechanisms based on alternative splicing and RNA processing, as well as RNA silencing define the actual transcriptome supporting the stress response. Beyond protein phosphorylation, other post-translational modifications like ubiquitination and sumoylation regulate the activation of pre-existing molecules to ensure a prompt response to stress. In addition, cross-connections exist among these mechanisms, clearly demonstrating further and superimposed complexity levels in the response to environmental changes. Even if not widely identified, the targets of these mechanisms characterised so far are mainly regulatory elements of the stress response pathways. The network of post-transcriptional and post-translational modifications ensures temporally and spatially appropriate patterns of downstream stress-related gene expression. Future attempts of plant engineering could exploit insights from a deeper comprehension of these emerging sites of regulation of stress responses to develop stress resistant plants.

Keywords: Abiotic stress tolerance; Post-transcriptional regulation; Post-translational modification; Alternative splicing; Ubiquitination; Sumoylation

Contents

Abbreviations: ABF, ABRE-binding factor; Abh, ABA hypersensitive; ABI, ABA in sensitive; ASK1, *Arabidopsis* SKP1 (S-phase kinase associated protein 1); AtNCED3, *Arabidopsis thaliana* 9-*cis*-epoxycarotenoid dioxygenase 3; AtNUP160, *Arabidopsis thaliana* nucleoporin 160; AtTLP9, *Arabidopsis thaliana* TUBBY-like protein 9; AvrRxv, avirulence resistance gene from *Xanthomonas campestris* pv. Vesicatoria; CBF, C-repeat binding factor; COR, cold responsive; CSD, Cu/Zn superoxide dismutase; DCL1, Dicer like 1; DREB, drought responsive element binding; GRP, glycine rich protein; HOS1, high expression of osmotically responsive genes 1; HVD1, *Hordeum vulgare* DEAD box protein 1; ICE1, inducer of CBF expression 1; LEA, late embryogenesis abundant; LOS4, low expression of osmotically responsive genes 4; MAPK, mitogen-activated protein kinases; NUA, nuclear anchor; P5CDH, Δ^1-pyrroline-5 carboxylate dehydrogenase; PHR1, phosphate starvation response 1; PP2A, protein phosphatase 2A; RNP, ribonucleoprotein; SAD, supersensitive to ABA and drought; SCF, SKP1-CULLIN-Fbox; SDIR1, salt and drought-induced ring finger 1; SKP1, S-phase kinase associated protein 1; SIZ1, SAP (SAF-A/B; Acinus; PIAS motif) and Miz (Myc-interacting zinc finger protein) 1; STA1, STABILIZED1; STRS, stress response suppressor; TIR1, transport inhibitor response 1; UBC24, ubiquitin conjugating protein 24; YopJ, yersinia outer protein J.

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0168-9452/$ – see front matter
doi:10.1016/j.plantsci.2008.02.005

1. Introduction

Plants acquire resistance to stress environment by reprogramming metabolism and gene expression, gaining a new equilibrium between growth, development and survival. In the past two decades, important advances have been made in the understanding of transcriptional changes induced by environmental constraints and in the identification of signaling proteins and transcription factors which regulate the stress-induced gene expression. Outcomes depict a complex process constituted by several pathways starting from stress perception and ending with specific transcriptional changes [1]. The final scheme of the general response integrates both stress-specific requirement and cross-talking, ultimately resulting in specific and common outputs [2].

These findings supported the development of transgenic plants over-expressing regulators of the stress response in order to improve tolerance to single or multiple abiotic stresses. For example, the over-expression of the genes encoding the stress related DREB/CBF transcription factors improved cold and drought tolerance in *Arabidopsis* [3] and crops such as tomato [4,5] and rice [6]. Nevertheless, the over-accumulation of stress regulators *per se* is not always sufficient to improve stress tolerance because additional post-translational modifications may be required (i.e. *DREB2* [7]). Furthermore, when constitutive promoters are used, an enhanced level of stress tolerance is sometimes conferred at the expense of plant development and growth, due to metabolic costs of a misregulated stress response or to side effects of the transgene on the plant physiology [4,5,8–10]. Combined evidence suggests that more attention should be paid to the dynamic aspects of the activity of transcription factors and other regulatory proteins, often under the control of specific activation and modulation mechanisms.

Recent advances in proteomics and metabolic profiling have provided chances to integrate gene expression and protein activities. Outcomes indicate that further levels of regulation based on post-transcriptional and post-translational mechanisms are involved in the abiotic stress response. This review focuses on the most recent findings on differential regulations activated by plants after the perception of an abiotic stress and based on post-transcriptional and post-translational processes. They are raising as key mechanisms to finely modulate the amount and activity of pre-existing transcripts and proteins, respectively, with an ultimate effect on proteome and metabolome complexity [11,12].

Various steps of RNA processing affect quantitatively and qualitatively the mRNA population. Alternative splicing, which concerns up to two thirds of the genes, has important consequences on the availability of different kinds of transcripts, and ultimately of proteins [13]. RNA-mediated silencing is also emerging as an alternative mechanism to control the amount of specific transcripts by their degradation [14]. Subsequent steps of RNA processing, like the mRNAs transport through the nuclear envelope and the association of mRNAs to ribosomes, are other important check points of mRNA molecules and availability for protein synthesis [15,16]. After the translation, a plethora of molecules can constitutively or transiently interact with proteins modifying their activity, sub-cellular localization and half-life [17]. Phosphorylation is one of the best known post-translational protein modifications affecting conformation, activity, localization and stability of target proteins. It has a role in many biological processes, as phosphorylation cascades commonly translate extracellular stimuli into the activation of specific responses [18]. Since a number of recent reviews have been dedicated to kinases and stress response (see [19] for CDPKs and [20] for MAPKs), this topic will not be considered in this review. Among the polypeptides, ubiquitin and SUMO conjugations are emerging as major post-translational regulatory processes in all eukaryotes [21,22].

The emerging picture defines the post-transcriptional and post-translational mechanisms, as well as their interactions, as regulatory systems of the transcriptional changes related to the plant response to stresses. The network of such mechanisms is expected to effectively target transcription factors and other regulatory components of the stress signaling, resulting in either activation or repression of their activities. This ensures temporally and spatially appropriate patterns of downstream gene expression and ultimately the shaping of transcriptome and proteome of stress-exposed plants to switch on adaptive response. Although stress-responsive genes still represent an important aspect of stress adaptation, the simple observation of the transcriptome provides only a rough and largely incomplete picture of the response to stress. The characterization of post-transcriptional and post-translational regulatory systems is crucial for the deeper understanding of the molecular mechanisms governing plant adaptation to environment as well as for a practical purpose of plant improvement for stress tolerance.

2. Post-transcriptional processes affecting mRNA availability

The amount of mRNAs available for translation can be affected at different steps of the process of RNA maturation,

ranging RNA transcription to splicing, from transport to translation initiation, and degradation by RNAi.

Two main families of proteins, the RNA binding proteins and the RNA helicases, determine the fate of pre-mRNAs and mRNAs by regulating steps from transcription to protein translation. The RNA binding proteins bind RNA molecules immediately after the transcription, till the translation and constitute the mRNP complexes [23]. RNA helicases catalyse structural rearrangements acting as chaperone and allowing RNA molecules to fold properly [24]. Furthermore, RNA helicases promote the removal of RNA binding proteins from RNA-protein complexes before translation [25].

RNA binding proteins are characterised by conserved RNA-binding motifs, such as RNA recognition motif, glycine-rich motif, arginine-rich motif, zinc finger motif, and double-stranded RNA-binding motif [23]. The involvement of some glycine-rich RNA-binding proteins in response to a variety of environmental stresses, including cold, drought, UV radiation and heavy metals has been well established [26]. However, proof of their role in abiotic stress tolerance as RNA chaperones has been only recently shown for two *Arabidopsis* glycine-rich proteins, GRP2 and AtRZ-1a [27,28]. Both proteins accelerate seed germination and seedling growth under cold stress in an ABA-independent manner, and contribute to enhance cold and freezing tolerance. AtRZ-1a affects the translation of putatively target genes, including several proteins involved in stress resistance and in RNA and protein metabolism, while GRP2 modulates the expression and activity of various classes of mitochondrial-encoded genes by exhibiting transcription anti-termination activity. The transcripts coding for CCCH proteins, a family of RNA binding proteins with the CCCH zinc finger binding motif, also accumulate in response to cold and water stress in durum wheat [29].

The RNA helicases enzymes function as molecular motors that rearrange RNA secondary structure or act as mediators of RNA-protein association/disassociation. They are involved in many aspects of RNA metabolism including transcription, pre-mRNA splicing, mRNA export, RNA degradation, translation initiation and organellar gene expression [30]. Several RNA helicases are involved in the response to abiotic stress [24]. For example, the RNA helicase DCL1 [31] is responsible for the processing of the siRNA derived from *P5CDH* and *SRO5* transcripts during salt stress response [32]. The *Arabidopsis* gene *Los4* encodes a RNA-helicase constitutively expressed, but also involved in response to temperature stress [33]. The *los4-1* mutant has a reduced expression of *CBF3* and a delayed expression of *CBF1* and *CBF2* during cold acclimation resulting in chilling sensitivity, while *cryophyte/los4-2* mutant (allelic to *los4-1*) promotes a super-induction of *CBF2* under cold stress and an enhanced freezing tolerance. Both mutants are heat sensitive. This divergent response is mediated through a differential effect on nuclear mRNA export: inhibited by *los4-1* and enhanced by *los4-2* at low temperatures, while both mutations disrupt the mRNA export at high temperatures [34]. As the germination of *los4-2* seeds is more sensitive to ABA inhibition, this mutant provides further evidence for the connection between RNA metabolism and ABA signalling. This has been already suggested by the ABA-hypersensitive phenotype generated by mutations at the genes *Sad1* and *Abh1* encoding a Sm-like RNA splicing/export/degradation factor and a mRNA cap binding protein [35,36]. The finding of a stress-induced chloroplast-localized RNA helicase, HVD1, indicates the requirement of RNA helicase for stress response in organelles [37], suggesting a role in the regulation of photosynthesis-related RNAs. Two additional helicases, STRS1 and STRS2, play a negative regulatory role in stress response [38]. The *strs* mutants are more tolerant to salt, osmotic, and heat stresses due to an enhanced expression of *DREB/CBF* and heat shock transcription factor genes.

2.1. Stress-related transcripts from alternative splicing events

Alternative splicing is a mechanism by which multiple forms of mature mRNAs are produced from a single transcript, just after the transcript synthesis. The mRNAs of about 35–60% of human genes are considered to be alternatively spliced [39], while between 20 and 60% of plant genes, depending on the species considered, give rise to mRNA that are alternatively spliced [13]. Four main types of alternative splicing are known: exon skipping, alternative 5′ and 3′ splice sites and intron retention. Often, events of the first three alternative splicing types lead to functionally relevant changes in the protein products, such as replacement of the amino or carboxy terminus, or in-frame addition/removal of a functional unit. In this way, different polypeptides, with different functions or subcellular locations, are produced by a single gene. Exon skipping is the most frequent and intron retention the rarest alternative splicing form in animals [40], while intron retention is the most common alternative splicing in *Arabidopsis* and rice (>50% [40,41]). mRNAs with introns lead to truncated polypetides, or are subjected to nonsense-mediated mRNA decay, as retained introns often introduce in frame stop codons [42]. Nevertheless, the high rate of intron retention in plants and numerous studies available in literature suggest that it may represent the result of an active process inhibiting the splicing reaction rather than inaccurate intron recognition. The production of truncated, inactive transcripts at the expense of the corresponding full length mRNA, can work as a system regulating the amount of the active protein form finally produced, probably due to the lower level of correctly spliced transcript, or competition of different forms for association with ribosomes [42,43]. In addition, the shortened polypeptides formed following alternative splicing are not necessarily functionless forms of the full length protein, as shown for the N gene for resistance to tobacco mosaic virus where both full length and shortened alternative transcripts are needed for the complete resistance phenotype [44].

Alternative splicing events do not randomly affect mRNA of all genes, rather they seem to occur preferentially to mRNAs of certain classes of genes commonly involved in signal transduction, or encoding enzymes, receptors and transcription factors [45,46]. In plants some transcription factors undergo splicing in response to environmental constraints. The wheat

transcription factor gene *Wdreb2* generates three stress-regulated transcripts through exon skipping mechanism, in response to cold, drought, salt and exogenous ABA treatments. The three forms have different accumulation profiles and their expression is regulated through an ABA-dependent pathway during drought and salt stresses and an ABA-independent one under low temperature. Notably, the absence of second and third exons in one of the three transcripts do not impair its activity as transcriptional activator of downstream stress-related genes, like *cor* genes and LEA protein genes, in a yeast assay [47]. A subgroup of *MYB* transcription factor genes produces alternative transcripts whose accumulation is dependent on phytohormones and stress conditions in *Arabidopsis* and rice. Through alternative splicing, *AtMYB59* and *AtMYB48* genes are able to encode putative proteins differing for their MYB repeats and probably for their binding affinities to gene promoters [48]. Genes encoding proteins involved in post-translational modifications and signal transduction cascades may also be regulated by alternative splicing. The mRNA of a durum wheat gene encoding an ubiquitin ligase retains the 3′UTR-located intron in response to cold and dehydration stress [49]. The rice gene *OsBWMK1*, encoding a MAP kinase, has three transcript variants: *OsBWMK1L*, *OsBWMK1M* and *OsBWMK1S*. The second and third ones are induced by various stresses, while *OsBWMK1L* is constitutively expressed. Proteins deriving from the three transcripts are characterized by differential subcellular localizations: OsBWMK1S is primarily in the nucleus, while OsBWMK1L and OsBWMK1M are predominantly in the cytoplasm. Nevertheless, a treatment with defense signaling related molecules determines the translocation of OsBWMK1L from cytoplasm to nucleus [50].

The serine/arginine proteins are a class of RNA binding proteins with a role in splicing control. They are known to promote alternative splicing of their own transcripts as well as of other gene products [51,52]. Nineteen genes encoding serine/arginine proteins have been identified in *Arabidopsis*, and most of their mRNAs undergo alternative splicing following developmental and environmental stimuli producing 95 different transcripts [53,54]. The regulation by alternative splicing of genes whose products in turn alter the splicing of other genes may considerably enhance and amplify the signal transduction cascade in response to stress stimuli. The *Arabidopsis STA1* gene encodes a pre-mRNA splicing factor up-regulated by cold. The analysis of *sta1-1* mutant shows that STA1 protein can regulate the stability and splicing pattern of a number of endogenous gene transcripts related to abiotic stress response [55]. In particular, the *sta1-1* mutant is characterized by the non-correct splicing of *cor15a* mRNA, a messenger encoding a cold-induced chloroplast-targeted polypeptide. The finding that over-expression of *cor15a* enhances the *in vivo* freezing tolerance of chloroplasts in nonacclimated plants can therefore explain the chilling sensitivity of the *sta1-1* mutant [56].

Although only few alternative splicing events have been described so far in response to abiotic stresses, the recent findings indicate that a large proportion of the genes undergoes alternative splicing [13]. Therefore the effect of alternative splicing on the stress related transcriptome is probably still underestimated and a number of not yet known alternative splicing-based mechanisms are likely to play a role in the tolerance to abiotic stresses.

2.2. Nuclear trafficking affects response to stresses

The sole gateways of macromolecular trafficking between the cytoplasm and the nucleus are the nuclear pore complexes, large multiprotein complexes embedded in the nuclear envelope [15]. They consist of several copies of different proteins collectively called nucleoporins. The nuclear pore complex is a channel-like structure of eightfold symmetry divided into three elements: a nuclear basket, a central pore and cytoplasmic fibrils. Gene regulation in eukaryotes requires both the transduction of environmental signals into the nucleus, by means of specific regulatory proteins, and the export of mRNAs and non-coding RNAs from the nucleus to the cytoplasm. The mRNA export requires a RNA helicase, the DEAD-box protein 5, and several nucleoporins, besides the RNA binding proteins bound to mRNAs [57]. Conversely, karyopherin proteins mediate the transport of proteins and non-coding RNAs molecules through the nucleus envelope [58,59]. Karyopherins recognize the basic nuclear localization signal of proteins and nucleic acids and together with the protein or ribonucleic acid cargo form a heterotrimeric complex. This is targeted to the nuclear pore complex through the direct interaction of a karyopherin with specific nucleoporins and then translocated into the nucleus [60].

The nucleocytoplasmic trafficking is a regulated event. Recent works have suggested that nucleoporins and karyopherins are implicated in many aspects of plant life, including the abiotic stress response, by affecting the nuclear import and export [15]. The nucleoporin AtNUP160 is constitutively expressed at the nuclear rim, but it may become more important during cold stress in remodelling nuclear pore structures [61]. Indeed, even if the *atnup160-1* mutant is impaired in poly(A) mRNA export at both warm and cold temperatures, poly(A) mRNA accumulation in the nucleus is higher under cold stress. Moreover, the mutant has a reduced expression of the *CBFs* and other stress-related genes under cold stress, being therefore sensitive to chilling stress and defective in acquired freezing tolerance. A mutation in the *SAD2* gene encoding an importin β affects the expression of several ABA- and stress-responsive genes [62]. The *sad2-1* mutant is hypersensitive to ABA, salt and PEG treatment confirming the critical role of nucleocytoplasmic trafficking during stress response.

2.3. Degradation of stress related transcripts by nat-siRNAs and miRNAs

The recently discovered microRNAs (miRNAs) and endogenous small interfering RNAs (siRNAs) are emerging as important players in the regulatory network of the plant stress responses [14]. These small non-coding RNAs post-transcriptionally silence target genes either by guiding degradation or repressing translation of target mRNAs

[63,64]. Cold, dehydration, salt stress and nutrient starvation up-regulate and down-regulate the expression of different plant miRNAs, whose targets are supposed to be negative and positive regulators of stress tolerance, respectively [14,65,66]. The *Arabidopsis* miR393, up-regulated in response to most stress conditions, is putatively directed against several F-box E3 ubiquitin ligase transcripts, which are indeed down-regulated by abiotic stresses [67]. miR393 can probably act as positive regulator of stress tolerance by affecting the repressing activity of ubiquitination. The up-regulation of miR393 by low temperature might lead to a reduced proteolysis of ubiquitination targets through the cleavage of the mRNAs encoding ubiquitin ligases. The transcript of the conjugating enzyme UBC24, another component of the ubiquitination pathway, is the target of miR399 whose expression is up-regulated during exposure to low phosphate [68]. During phosphate starvation, the silencing of the conjugating enzyme may attenuate an ubiquitin pathway that negatively regulates the expression of phosphate transporters, thereby maximising phosphate uptake. Conversely, oxidative stress leads to the down-regulation of miR398, which, in turn, promotes the accumulation of transcripts coding for two Cu–Zn superoxide dismutase proteins (CSD1 and CSD2) involved in the detoxification from superoxide radicals [69].

Eukaryotic genomes contain many overlapping genes, approximately 10% of *Arabidopsis* genes are in convergent overlapping gene pairs, also known as natural *cis*-antisense gene pairs [70,71]. Although their functional significance is still unclear, one intriguing possibility is that overlapping transcripts in antisense orientation form double-stranded RNAs that may be processed into small RNAs. These nat-siRNAs (natural antisense transcripts-generated siRNAs) have recently emerged as important players in plant stress responses. For example an intriguing study in *Arabidopsis* explained the accumulation of proline during response to stress by means of the intervention of nat-siRNA. The gene *P5CDH*, involved in proline catabolism, is constitutively expressed, while salt stress induces the expression of *SRO5*, a gene of unknown function. During salt stress when transcripts of both gene, *P5CDH* and *SRO5,* are produced, the antisense overlapping gene pair between the two genes is also transcripted and generates two siRNAs of 24-nt and 21-nt. They lead to the salt-dependent down-regulation of *P5CDH* by mRNA cleavage and, as a consequence, to proline accumulation [32].

2.4. Differential association of stress-related transcripts to polysomes

The process of protein synthesis is regulated primarily during the initiation phase. As the mRNA-polysome level reflects the efficiency of initiation and re-initiation of translation, the analysis of the association of individual mRNAs with polysomes has revealed that the efficiency of translation significantly contributes to gene regulation under abiotic stresses. The amount of mRNAs in polysomes is generally reduced during exposure to dehydration or anoxia, while stress-induced mRNAs significantly increase in polysome association. Changes in polysome association can also happen without a concomitant change in steady-state accumulation of mRNAs [72,16]. The 5′UTR sequence [73] and the phosphorylation of the translational machinery [74,75] are critical factors for the efficiency of the initiation phase in response to stress, but RNA binding proteins could also be involved. In chloroplasts, RNA binding proteins and several nucleases have been described to adjust the relative half-life of their mRNAs in response to environmental cues, particularly light conditions [76]. A mechanism controlling the mRNA availability for ribosomes has been suggested to explain the significant imbalance between the mRNA expression levels and the amount of the corresponding proteins for tetrapyrrole pathway and photosystem complexes in response to variation in light intensity [77].

3. Protein degradation in response to stress: the role of ubiquitination

Ubiquitination is the covalent addition of the small protein ubiquitin to selected target proteins [78]. The attachment of ubiquitin is mediated by the sequential action of three enzymes: ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3). The addition of a multi-ubiquitin chain usually marks proteins for rapid intracellular degradation through the 26S proteasome, a multisubunit ATP-dependent protease whose main function is the degradation of proteins by proteolysis. Inactive proteins (i.e. incorrect folding) and proteins which are no longer required for cell are tagged by ubiquitin for proteolysis. Conversely, monoubiquitination regulate the location and activity of proteins, affecting various cellular processes from transcriptional regulation to membrane transport [79]. For example the activity of the PP2A phosphatase increases under low temperature or in dark after mono-ubiquitination [80]. The specificity of ubiquitination is ensured by the E3 enzymes which recognise target proteins. About 1400 *Arabidopsis* genes encode E3 enzymes, among them the RING-finger proteins and the SCF protein complexes are predominant [81].

A number of studies have pointed out the relevance of the ubiquitin-dependent protein degradation in stress-related signalling and response mechanisms [82–84]. Transcriptome and proteome analyses carried out in different plant species following exposure to abiotic stresses indicated that hundreds of ubiquitination-related transcripts/proteins are modified during stress responses suggesting a role for ubiquitination in determining the stress tolerance [81,85–87]. This is supported by the recent proteomic analysis of the flower buds of *Arabidopsis* plants carrying a mutation in the *ASK1* gene, a critical component of the SCF ubiquitin ligase complexes. In *ask1*, the impairment of the SCF ligase-mediated ubiquitination and the resulting accumulation of SCF targets allowed the identification of ubiquitination targets, among them a number of stress-related proteins [88].

Several RING-type E3 ligases are involved in the ABA-dependent molecular responses. The H2-type zinc-finger protein SDIR1 is a positive regulator of ABA signalling,

acting upstream of the main transcriptional regulators of the ABA molecular response [89]. Indeed, the up-regulation of *SDIR1* gene expression enhances ABA-induced stomatal closure resulting in increased drought tolerance. In cross-complementation experiments, the ABA-insensitive phenotype of the *sdir1-1* mutant can be rescued by several transcription factor genes acting in the ABA pathway (*ABI5*, *ABF3* and *ABF4*). Notwithstanding, the up-regulation of the *XERICO* gene, encoding a H2-type zinc-finger E3 ubiquitin ligase, results in increased drought tolerance due to an enhanced ABA-induced stomatal closure [90]. XERICO controls the level of ABA by enhancing the transcription of the key ABA-biosynthetic gene *AtNCED3*. XERICO also interacts with AtTLP9, an E3 TUBBY ligase acting as positive regulator of ABA signalling [91]. The findings indicate that the protein degradation mediated by the ubiquitin/proteasome pathway plays a fundamental role in ABA homeostasis and response.

Ubiquitination also plays a crucial role in responses to cold. *HOS1* encodes a RING-finger protein E3 ubiquitin ligase which exerts a negative control on cold response [92]. Indeed HOS1 mediates the ubiquitination of the master regulator for the response to cold, the transcription factor Inducer of CBF Expression 1, ICE1, leading to its proteasome-mediated degradation during exposure to cold. According to this function, *hos1* mutation enhances the induction of *CBFs* and of the downstream cold-regulated genes by low temperatures [93].

Variation in E3 ligase activities can be achieved through changes in the expression of the corresponding mRNAs [81], induction of multiple splice variants [49,94], miRNA-mediated gene silencing [14] and phosphorylation [95]. Phosphorylation in animals regulates the availability of many proteins as ubiquitination targets [96]. In addition, given that ubiquitination and sumoylation recognise the same lysine, sumoylation can prevent the protein degradation [97], as described in the following section. The E3 ligase activity can also be enhanced by conformational changes due to binding of specific ligands. The interaction of auxin, jasmonate or gibberellin molecules with the specific hormone receptor/F-box protein causes a conformational change in the corresponding E3 ligase complex resulting in activation of the enzyme and the subsequent degradation of target proteins [98–100].

4. Control of stress response by sumoylation

Sumoylation is a post-translational modification of protein substrates based on the covalent conjugation of the SUMO (Small Ubiquitin-like MOdifier) peptide [101]. The biochemical steps catalysing the conjugation are similar to those operating in the ubiquitination pathway, involving activating enzymes (E1), conjugating enzymes (E2) and E3 ligases. Sumoylation is a transient modification reversible by SUMO specific proteases which de-conjugate the substrates. In contrast to most of the ubiquitin conjugation systems that depend on E3 ligases for specific recognition of the target proteins, the E2 and E3 enzymes of the sumoylation machinery act on many different proteins. E2 can directly bind and sumoylate substrates *in vitro* by recognising the consensus motif CKxE/D (C: hydrophobic amino acid; K: SUMO target lysine; D/E acidic amino acids) [102,103]. Sumoylation is therefore expected to be specifically regulated at the target level, with phosphorylation accomplishing a critical role [96], while the dynamic aspects are regulated by the SUMO peptidase activity [104,105].

Sumoylation alters protein function by masking and/or adding interaction surfaces, or by inducing conformational changes. A wide variety of biological consequences of sumoylation have been observed, including sub-cellular re-localization, changes in enzymatic activity and protection from ubiquitin-mediated degradation. SUMO conjugation can promote transcription by enabling the nuclear import of transcription factors, but it can also impair the transcription by recruiting transcription factors in the repressive environment of particular sub-nuclear domains. SUMO can influence the assembly of transcription factors on promoters or the recruitment of chromatin-modifying enzymes, above all when associated to transcriptional repression [102].

Both loss and gain of function analyses, as well as the pattern of SUMO-conjugates revealed a key role of sumoylation in plants in response to environmental signals. A genome wide expression analysis in *Arabidopsis* identified 300 genes out of 1700 drought-induced sequences, whose up-regulation is mediated by the SIZ1 SUMO E3 ligase [106]. *Arabidopsis siz1* mutants are hypersensitive to phosphate deficiency [107], have reduced tolerance to high temperature, drought [106,108], chilling and freezing stresses [109]. Moreover, the phenotypic consequences of an increased SUMO content suggest a role for sumoylation in the control of the ABA signal transduction pathway with effects on the expression of stress-related ABA-responsive genes [110]. A general accumulation of SUMO conjugates is an early effect of the exposure to extreme temperatures, oxidative cues and dehydration stress [29,106,108,109,111,112]. Some stress-related transcription factors have been identified as SUMO conjugates in response to stress. Sumoylation activates the *Arabidopsis* MYB transcription factor PHR1, a determinant of the phosphate starvation response, resulting in the correct timely induction of some downstream genes related to phosphate starvation [107]. Sumoylation is essential for freezing tolerance through the stabilization of the transcription factor ICE1, inducer of *CBF* and repressor of *MYB15* expression [109]. This modification blocks the ubiquitin-mediated degradation of ICE1 allowing ICE1 to activate *CBF* transcription. The sumoylated isoform of ICE1 also has a negative effect on the transcription of *MYB15*, which functions as repressor of *CBF* genes. The final effect of the AtSIZ1-mediated sumoylation is therefore the attenuation of repressor systems that in normal growing conditions block part of the transcriptional response to cold.

5. A combinatorial network of post-transcriptional and post-translational regulations

Evidence is accumulating about reciprocal actions among different kinds of transcriptional, post-transcriptional and post-

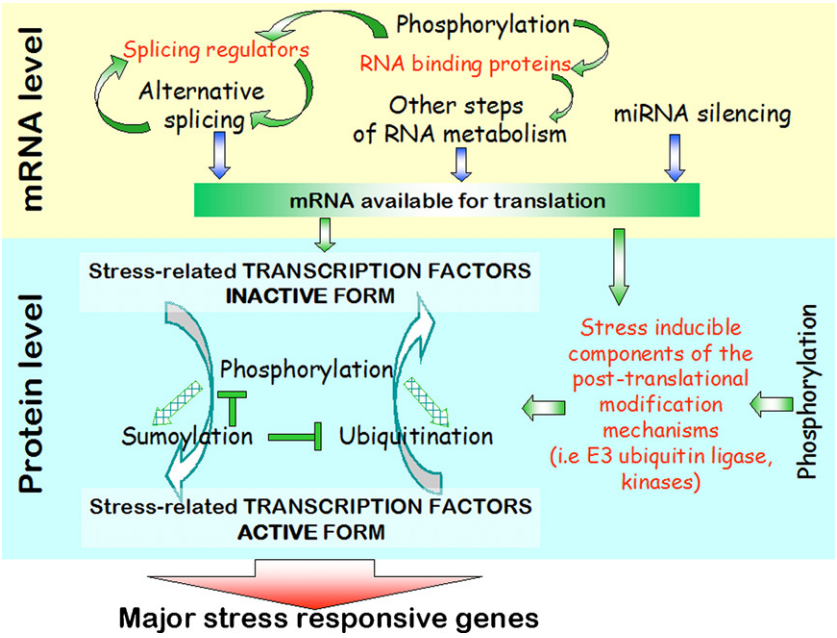

Fig. 1. Model describing the cross-talking among post-transcriptional (mRNA level) and post-translational (protein level) regulations involved in the control of the plant response to abiotic stress. See the text for details. Grating arrows indicate connections not yet reported in plants, but expected by evidence from animal studies.

translational regulations. The emerging picture is an increasing variety of interacting mechanisms shaping the transcriptome and proteome and contributing to the fine tuning of cell metabolism (Fig. 1).

The expression of genes encoding components of the post-translational control is often controlled at transcriptional level (i.e. many E3 ubiquitin ligases are stress induced), subjected to gene silencing by action of miRNA [67] or to alternative splicing events [49]. Furthermore the corresponding proteins might be phosphorylated [113]. Perusing lists of potential kinase substrates reveal intriguing connections between post-transcriptional mechanisms and phosphorylation. Splicing factors, RNA helicases as well as transcription factors were also among the targets of the stress-related MAP kinase3 and MAP kinase6 [114]. In *Arabidopsis* 79 unique phosphorylation sites were identified in 22 phosphoproteins having a role in RNA metabolism and mRNA splicing, including RNA helicases. As among them were some spliceosome SR proteins involved in hormone and abiotic stress response, the activation of specific splicing factors by phosphorylation during the exposure to abiotic stresses can be hypothesized [55,115]. Conversely, alternative splicing can also control protein phosphorylation. The rice gene *OsBWMK1*, encoding a MAP kinase, produces three protein variants based on alternative splicing events, two of them in response to various abiotic stresses [51]. A link between RNA processing and SUMO modification has been also recognized, in which SUMO pathway can be a possible mechanism to control nucleocytoplasmic transport of proteins [116]. Besides many hnRNPs, RNA helicases, and other proteins of RNA metabolism identified as substrates for SUMO modification in mammals [117], in plants a mutant in a nuclear pore protein, NUA, is affected both in SUMO homeostasis and nuclear RNA accumulation [118].

Multiple signaling pathways may converge on the same target protein by multisite modifications, resulting in complex combinatorial regulatory patterns that dynamically and reversibly affect the activity of a target protein. Different post-translational mechanisms may act together or have antagonistic effects. In animals, phosphorylation of a protein target is often essential to its ubiquitination [95]. For example, a whole class of F-box subunits of SCF ubiquitin–protein ligases binds to and thus recognizes phosphorylated epitopes on their substrates [119]. Sumoylation and phosphorylation reciprocally interact on the target proteins, with sumoylation only targeting phosphorylated proteins, or preventing phosphorylation [97]. In addition, ubiquitination and sumoylation often have antagonistic effects by acting on the same amino acid residues [120].

Understanding how different modifications act on the same target as well as the *in vivo* modalities and timings of these interactions, is a future challenge for the understanding of plant responses to abiotic stresses. Evidence about these networks in plants is still limited. However some recent insights on regulation of the activity of the transcription factor ICE1 offer a well characterized example of the complexity of these regulatory systems. *ICE1* is constitutively expressed, nevertheless it activates the expression of *CBF* genes only upon cold treatment [121]. Three different modifications are known, so far, to control the activity of ICE1 protein. At low temperature ICE1 can undergo sumoylation through the action of AtSIZ1 [109], resulting in a fully active transcription factor. Alternatively HOS1 can cause ubiquitination of ICE1 and consequently its proteosomal degradation [93]. ICE1 may be more or less available for ubiquitination and sumoylation depending on the protein phosphorylation status, which is most likely temperature dependent [84]. Similarly we can hypothesise a nuclear cold-induced localization of HOS1 by

phosphorylation. The balance between activation and degradation allows a perfect tuning of ICE1 activity which in turn leads to the activation of the cold-induced molecular response.

The signalling pathway controlling the phosphate homeostasis represents an example of how a cascade of different regulatory mechanisms can regulate the final expression of stress-related genes. The MYB transcription factor PHR1 is post-translationally regulated by the SUMO E3 ligase AtSIZ1 [107]. PHR1 is involved in the induction of miR399 in response to phosphate deprivation. The accumulation of miR399, in turn, represses the *PH2* gene encoding the ubiquitin conjugating enzyme UBC24 [68]. The final effect is, presumably, the attenuation of an ubiquitin pathway that negatively regulates the expression of phosphate transporters and root growth in normal conditions, maximising phosphate uptake during starvation.

Even if still speculative, interactions among post-transcriptional and post-translational regulations can be expected in the epigenetic component of the stress tolerance. Molecular mechanisms underpinning epigenetics include modification of histones and chromatin remodelling, besides DNA modification [122]. Many post-transcriptional and post-translational regulations are involved in epigenetic changes. The final effect of RNA-mediated gene silencing is often the methylation of the genomic region producing the target RNA. Phosphorylation, ubiquitination and sumoylation beyond acetylation and methylation, act on nucleosome core histones and sumoylation regulates the activity of the chromatin remodelling complexes [123]. All together these modifications constitute a histon code which activates or silences gene expression by modifying chromatin structure. Epigenetic changes have been implicated in the acclimation process, a phenomenon that allows a plant to become more resistant to future stress exposure after a previous stress sensing [124]. We believe that further progress on the understanding of the epigenetic contribute to stress tolerance will reveal new insights on the role of non-transcriptional regulations.

6. New targets for engineering stress tolerant plants?

A new generation of transgenic plants with improved performance under challenging environments could be developed using the increased knowledge on post-transcriptional and post-translational regulations. Regulators of post-transcriptional and post-translational mechanisms exert both positive and negative control activities of stress response. Therefore increasing the stress tolerance can be obviously obtained by enhancing activity of positive regulators or repressing activity of negative regulators. There is already some evidence of successful improvement in stress tolerance achieved through the positive or negative modification of regulators of post-transcriptional and post-translational mechanisms (Table 1), even though the exact functional mechanisms of stress tolerance are sometimes not completely defined. In *Arabidopsis* some successful examples of overexpression of positive regulators have been reported. Two genes encoding the serine/arginine proteins involved in alternative splicing were able to confer a higher tolerance to sodium and lithium chloride when expressed in plants as well as in yeast cells [125]. An improvement in freezing tolerance was observed over-expressing two RNA-binding proteins: GRP2, localized into the mitochondria, and AtRZ-1a [27,126]. Lastly, plants over-expressing the E3 ligase gene *XERICO* had increased ABA content and drought tolerance [90]. Two examples of mutation in a negative regulator have been reported so far. The mutation in the E3 ligase gene *HOS1*, which exerts a negative control on response to cold, enhanced cold tolerance promoting the induction of *CBFs* and downstream cold-regulated genes. A loss of function mutation in the two DEAD-box RNA helicases, STRS1 and STRS2, which have negative regulatory role in the stress response, increased tolerance to multiple abiotic stresses [38].

Despite the obvious advantage of using upstream general regulators, the identification of regulators that can increase stress tolerance without affecting plant growth and morphology can be actually problematic. Indeed as discussed in the previous paragraphs, post-translational and post-transcriptional regulations represent a complicated system based on a network of reciprocal interactions. In addition, such regulatory mechanisms control a broad array of basic cellular processes. For example the inhibition of enzymes common to the whole pathway of ubiquitination or sumoylation, like the proteasome or the SUMO E2 conjugating enzyme, may non-specifically affect many processes. The attack strategy of some plant pathogenic and symbiotic bacteria is an intriguing example of possible effects of the manipulation of the SUMO pathway. The

Table 1
Genes involved in post-transcriptional and post-translational regulations conferring increased abiotic stress tolerance

Gene name	Gene function	Modification	Transgenic phenotype	Reference
AtSRL1	Serine-arginine (SR) RNA binding protein	Up-regulation	Salt tolerance	[125]
GRP2	Glycine Rich RNA binding protein	Up-regulation	Cold and freezing tolerance	[27]
AtRZ-1a	Glycine Rich RNA binding protein	Up-regulation	Freezing tolerance	[126]
STRS1, STRS2	DEAD RNA helicase	Loss of function mutant	Tolerance to salt, osmotic, and heat stresses	[38]
CSD2	Cu/Zn Superoxide Dismutase	Mutagenesis of a miRNA recognition site	Tolerance to oxidative stress conditions (high light, heavy metal, and methyl viologen)	[69]
XERICO	E3 Ubiquitin ligase	Up-regulation	Drought tolerance by increased ABA level (up-regulation of *AtNCED3*)	[90]
HOS1	E3 Ubiquitin ligase	Loss of function mutant	Constitutively vernalized (enhanced cold-responsive gene expression)	[93]

avirulent factor YopJ/AvrRxv of *Xanthomonas campestris* strain XopD is a SUMO peptidase. This bacterial protein migrates to the nucleus of host cells and promotes the desumoylation of several nuclear proteins [127]. The impairment of the plant sumoylation system reprograms host cell functions allowing the bacteria to become pathogenic [128,129]. As evidenced by XERICO and HOS1 examples, in the case of ubiquitination the problem could be limited by specifically targeting E3 genes, the components of the ubiquitination pathway which ensure target specificity.

Alternatively, the engineering of specific stress-related targets of more general regulators of post-transcriptional and post-translational regulations could also assure a specific activity. For instance, the introduction of a *CSD2* gene with a defunct miR398 recognition site led to a substantial increase in oxidative stress tolerance [69]. The modification of specific targets can be achieved through the development of mutations (i.e. by TILLING [130]) in specific protein domains involved in the substrate recognition and modification.

Recent insights on post-transcriptional and post-translational mechanisms suggest that these mechanisms are exploited to strictly regulate and perfectly fine-tune the molecular responses to abiotic stresses. The final objective of plants is the achievement of the highest level of tolerance, by avoiding strongly physiological alteration and futile metabolic costs. Future attempts to minimise yield loss of plants exposed to environmental stresses should take into consideration such a requirement, and develop transgenic plants with physiological features closer to the wild type's ones. Current transgenic strategies based on a rough manipulation of regulatory factors produced plants with some increase of stress tolerance level at the expense of development and growth. Future aims will be the development of plants with a finer and more specific regulation of upstream general stress response regulators. With further improvement of knowledge on post-transcriptional and post-translational mechanisms, more promising scenarios in this direction can be hypothesised for plant engineering.

7. Post-transcriptional and post-translational regulations: future challenges for the understanding of the plant response to abiotic stresses

The recent progress of knowledge on plant abiotic stress response is depicting a frame where mechanisms controlling mRNA availability and protein activity act together to finely and timely adjust transcriptome and proteome to the continuous variations of environmental conditions. Future successful strategies to advance knowledge on plant responses to abiotic stresses will concern the functional characterization of key cellular regulators by genetic analyses of the corresponding mutants as well as by transcriptome and proteome surveys on transcriptome complexity, protein–protein interactions and post-translational modifications of proteins. These outcomes will lead to the identification of new environmental related pathways as well as of their target molecules. Though not yet documented in the context of the plant response to abiotic stress, knowledge from other organisms and experimental systems suggests that post-transcriptional and post-translational regulations are able to integrate external signals. For example, the activity of ubiquitination in the regulation of development processes is triggered by developmental hormones. Sensing of auxin is accounted directly by the F-box protein of an E3 ligase, TYR1 [99], while gibberellins bind to a protein that, in turn, associates with an E3 ligase [100]. Intriguing indications come also from plant defence responses to biotic stresses. The RNA mediated silencing is directly activated by virus nucleic acids [131] and alternative transcripts of some resistance genes are required for rapid and complete R gene-mediated resistance [44,132]. Moreover, ubiquitination in the defence response is directly triggered by jasmonic acid, the crucial plant hormone of host immunity [98]. These specific events may underline a more general situation where developmental- and environmental-related signals are integrated in the regulatory pathways controlling plant responses through post-transcriptional and post-translational regulation. For example, the post-translational regulation of ICE1 based on ubiquitination (HOS1)/sumoylation (SIZ1), could be functionally linked to cellular thermosensors and mediate the low temperature signal into the cell, in order to strictly modulate cold-responsive gene transcription by means of ICE1 activity.

Acknowledgements

This work was supported by Ministero delle Politiche Agricole Alimentari e Forestali (MiPAAF) of Italy, special grant PROTEOSTRESS, and by Ministero dell'Università e della Ricerca (MiUR) of Italy, special grant FISR ''Sistemi, metodologie e strategie per la caratterizzazione e valorizzazione della granella e degli alimenti derivati del frumento duro in ambienti marginali e/o vocazionali''.

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