
Abiotic Stress Responses in Plants: Unraveling the Complexity of Genes and Networks to Survive

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1. Introduction

Plants are often subjected to unfavorable environmental conditions – abiotic factors, causing abiotic stresses - that play a major role in determining productivity of crop yields [1] but also the differential distribution of the plants species across different types of environment [2]. Some examples of abiotic stresses that a plant may face include decreased water availability, extreme temperatures (heating or freezing), decreased availability of soil nutrients and/or excess of toxic ions, excess of light and increased hardness of drying soil that hamper roots growth [3]. The ability of plants to adapt and/or acclimate to different environments is directly or indirectly related with the plasticity and resilience of photosynthesis, in combination with other processes, determining plant growth and development, namely reproduction [4]. A remarkable feature of plant adaptation to abiotic stresses is the activation of multiple responses involving complex gene interactions and crosstalk with many molecular pathways [5, 6].

Abiotic stresses elicit complex cellular responses that have been elucidated by progresses made in exploring and understanding plant abiotic responses at the whole-plant, physiological, biochemical, cellular and molecular levels [7]. One of the biggest challenges to modern sustainable agriculture development is to obtain new knowledge that should allow breeding and engineering plants with new and desired agronomical traits [8]. The creation of stress-tolerant crop either by genetic engineering or through conventional breeding covered almost all aspects of plant science, and is pursued by both public and private sector researchers [9].

During the last decade, our research groups have focused their research on elucidating the different components and molecular players underlying abiotic stress responses of a broad range of species both model and crops plant. Several attempts to engineer those species with improved abiotic stress traits (drought and salinity) were made and the response of genetically engineered plants was deeply studied after establishment of adequate physiological methods. Now, we are moving efforts to expand our knowledge on plants response to abiotic stresses using holistic System Biology approaches, taking advantage of available high throughput tools such as transcriptomics, proteomics and metabolomics.

The aim of this chapter is to provide a general overview of the main studies made and how the different expertises of our team were pooled to improve our understanding of the biology of abiotic stress responses in plants. We present some details about the main results and perspectives regarding other possible approaches to develop plants better adapted to face the environmental constraints.

2. Physiological mechanisms underlying abiotic stress responses

Stress is a concept imported from physics. It was introduced in the theory of elasticity as the amount of force for a given unit area [10]. In a biological context, stress is usually defined as an external factor that exerts a disadvantageous influence on the plant [11]. Alternatively, stress could be defined as a significant deviation of the optimal condition of life [12].

2.1. Physiological responses to early abiotic stress: Functional decline in the alarm phase — The stress reaction

Three main phases may be considered on plant stress events and responses: i) the phase of alarm; ii) the phase of resistance; and iii) the phase of exhaustion [12]. Lichtenthaler [13] added a fourth phase, the regeneration phase, which occurs only when the stressor is removed before damage being too severe, allowing partial or full regeneration of the physiological functions. The alarm phase starts with the so-called stress reaction, characterized by functional declines due to the stressor factor, offset by restitution counter reactions, in the transition to the phase of resistance. Stressors rarely act separately and individually on a plant. Generally, several stress factors act simultaneously, such as the frequently combined, at sunny, warm and dry summer periods, heat, water and high-light stress [14].

Sensing is the very first event experienced by a plant when one or more environmental factors (biotic or abiotic) depart from their optimum. Stress sensing is a complex issue and there is not a single sensing mechanism common to all stresses. For instance, some stresses directly affect the underground parts of plant bodies (e.g. drought, flooding) whereas other stresses (e.g., photoinhibition) affect directly the aboveground structures of plant bodies. It is, thereby, expected that different sensing mechanisms will be involved. The most common model of sensing external stimuli is that of a chemical ligand binding to a specific receptor [15]. This model, however, is suitable only for chemical stresses (e.g., heavy metal stress, nutrient depletion stress), not for physical stresses: primary sensing of temperature stress (heat stress

or chilling / freezing) do not involve any chemical ligand. The same applies to radiation stress, although in this case an analogy between “ligand – receptor” and “photon – receptor” could be made. Even when molecules are involved, the universal character of the ligand - receptor model is debatable. In fact, in what concerns the rooting system, it is unclear if cells can sense the water concentration in the soil [16]. In contrast, experimental evidences point to the possibility of sensing cell water homeostasis. The isolation of a transmembrane hybride-type histidine kinase from *Arabidopsis thaliana* provides experimental evidence for osmosensors in higher plants [17]. Also sugars generated by photosynthesis and carbon metabolism in source and sink tissues play an important role in sensing and signaling, modulating growth, development, and stress responses [18].

Following sensing, one or more signaling and signaling transduction cascades are activated, preparing restitution counter reactions which will lead to the phase of resistance to stress. Meanwhile, functional declines are generally observed, including the photosynthetic performance, transport or accumulation of metabolites and/or uptake and translocation of ions, as described later in section 2.3. If these declines are not counteracted, acute damage and death may occur. The importance of restitution counter reactions is highlighted in experiments where different rates of stress imposition are compared: a more pronounced decline of physiological functions (photosynthesis, photosynthetic capacity and electron transport rate) was observed when higher plants were rapidly dehydrated than when the rate of water loss was slower [19]. In desiccation resistant bryophytes there is a threshold of water loss rate behind which no physiological restoration is observed [20]. Increased damage with more rapidly imposed stress is due, at least in part, to increased production of active oxygen species (AOS) [21]. Significant differences in the physiological behavior between the phase of alarm and the phase of resistance were highlighted by Marques da Silva and Arrabaça in [22], who found in the C4 grass *Setaria sphacelata* a decrease on the activity of the enzyme phosphoenolpyruvate carboxylase after several days of water stress, in sharp contrast with the several-fold increase of its activity observed after a short period of acute stress.

2.2. Common and distinctive features of salinity, cold and drought stress

Salinity, cold and drought stress are all osmotic stresses: they cause a primary loss of cell water, and, therefore, a decrease of cell osmotic potential. However, the elicitor of cell water loss differs between stresses: i) salinity stress decreases cell water content due to the decrease of external water potential, caused by the increased ion concentration (mainly Na⁺ and Cl⁻), turning more difficult water uptake by roots and water translocation to metabolically active cells; ii) cold stress decreases cell water content due to the so-called physiological drought, i.e., the inability to transport the water available at the soil to the living cells, mainly the ones of the leaf mesophyll; iii) the decrease of the cell water content under drought stress is due to water shortage in soil or/and in the atmosphere. Anyway, dehydration triggers the biosynthesis of the phytohormone abscisic acid (ABA) and it has been known for a long time that a significant set of genes, induced by drought, salt, and cold stresses, are also activated by ABA [23].

As a consequence of water loss and decreased cell volume, cell sap solute concentrations increase and thereby cell osmotic potential decreases. As cell turgor also decreases, an early effect common to these stresses is a sharp decrease in leaf expansion rate and overall plant growth rate. Furthermore, an additional active decrease of the cell sap osmotic potential is observed, as an attempt to keep cell hydration. In fact, at the metabolic level, a common feature to these three stresses is the osmotic adjustment by synthesis of low-molecular weight osmolytes (carbohydrates [24], betain [25] and proline [26]) that can counteract cellular dehydration and turgor loss [27]. On the other hand, differences between these stresses do also exist. While drought stress is mainly osmotic, ion toxicity, namely Na^+ , is a distinctive feature of salinity stress. Cold stress, behinds physiological drought, has an impact on the rate of most biochemical reactions, including photosynthetic carbon metabolism reactions, as enzyme activities are extremely temperature-dependent. Also water stress and salinity stress decrease photosynthesis, which create conditions to increased photoinhibition, particularly under high irradiances.

2.3. Plant bioenergetics as a core to stress sensor

Despite the different physiological responses to early abiotic stress discussed previously, a common point observed is the changes in the plant bioenergetic status. Such changes may involve a decrease in the energy production and/or an increase in energy demand to overcome the stress. The bioenergetics status is often considered as the chemical energy provided by adenylate energy charge (AEC), as defined in [28], for which plants are mainly dependent on photosynthesis.

The effect of abiotic stresses on photosynthesis can be perceptible: i) within the photochemical reactions in the tylakoid membrane; ii) in the carbon reduction cycle in the stroma; iii) in the carbohydrate use in the cytosol and; iv) on the CO_2 supply to the chloroplast dependent of stomata, mesophyll and chloroplast conductance (reviewed by [29,30]). ATP and NADPH resulting from photochemical reactions are used in all others processes except CO_2 supply to the chloroplast in C3 plants, so any limitation in photosynthesis such as those imposed by drought, can alter the plant bioenergetics status [31].

When the ATP and NADPH production by photochemical processes exceed the capacity for utilization in CO_2 fixation, plants can use several processes to dissipate energy and avoid or minimised photoinhibition (see 2.4). These processes include alternative electron sinks dependent of O_2 such as the oxygenase reaction catalised by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, E. C. 4.1.1.39) which initiates photorespiration [32]. The light-dependent O_2 uptake by photorespiration not only use ATP and reducing power from photosynthetic electron transport system but also cause a loss of the CO_2 fixed by Calvin cycle. Even in plants under no photoinhibitory conditions, photorespiration occur due to the capacity of Rubisco to catalise the carboxylation and oxygenation of ribulose-1,5-bisphosphate, depending on the CO_2/O_2 ratio. At 25 °C, photorespiration increases the cost of carbon (C) fixation to 4.75 ATP and 3.5 NADPH per C fixed under atmospheric CO_2 and O_2 Concentrations, which compares to 3 ATP and 2 NADPH per C fixed under no photorespiration conditions, e. g. only 2% O_2 instead of atmospheric 21% O_2 [33]. In plants submitted to drought, a reduction

of photosynthesis and photorespiration is observed as a result of the lower CO₂ and O₂ availability in the chloroplast. However, in this situation, the photorespiratory pathway is less decreased than photosynthesis, as firstly suggested Lawlor and co-workers [34, 35]. In fact, despite the much higher affinity of Rubisco for CO₂ than O₂, the CO₂ concentration is almost at the sub saturating level in C3 plants. Thus any decrease in stomatal conductance or in the gases solubility limits the carboxylase activity while the oxygenase activity is unaffected or less affected [36, 37]. In C4 plants, the higher CO₂ concentration at the Rubisco level allows a lower decrease in the photosynthesis / photorespiration ratio under water deficit [38] than the one observed in C3 plants, despite the C4 pathway having per se specific energy costs. The less efficient light use for CO₂ fixation caused by photorespiration lowers the quantum yields of photosynthesis in C3 plants under drought [39] or high temperature but this was not observed in C4 plants [40]. Since photorespiration is the major cause of a lower bioenergetic balance in photosynthetic tissues of C3 plants, increasing plant growth by overcoming the limitation of photosynthesis imposed by Rubisco is still an important target of research and plant improvement [41-46].

In C3 and C4 plants under water deficit, the photosynthetic rate decreases with the leaf relative water content and water potential [47-52]. This decrease is frequently correlated to the impairment of photochemical processes in C3 plants [53, 54], including inhibition of ATP synthesis [55-56]. It is still unclear if photosynthesis is primarily limited by water deficit through the restriction of CO₂ supply to metabolism (stomatal limitation) [47] or by the impairment of other processes which decrease the potential rate of photosynthesis (non-stomatal limitation). Nevertheless research efforts on these subjects are relevant to improve plants responses to stress [56].

Biochemical modeling of leaf photosynthesis in C3 and C4 plants [57-61] can provide useful insights into the evaluation of stomatal and non-stomatal limitations of photosynthesis, as previously shown in drought stressed *Medicago truncatula* plants [52] and *Paspalum dilatatum* plants under water deficit [38], elevated CO₂ [62] and dark chilling [63]. Photosynthesis light curves allow the determination of the relative contribution of respiration, photosynthesis and photorespiration to the light energy dissipation [64]. Additionally, they are an expeditious method to screen plants with improved resistance to water deficit, as also shown with *M.truncatula* transgenic lines [39].

The role of plant mitochondria in the bioenergetic balance is complex and involves cytochrome *c* oxidase but also several other processes such as alternative dehydrogenases and alternative oxidase that are independent of the adenylate control [65]. An increase in leaf respiratory energy demand to overcome the drought stress *via* respiration was referred in leaves in few studies [66-69]. More often, in drought plants, no change or a decrease in respiration is observed in leaves but the variations were always minor comparing to photosynthesis, despite the interdependence of the two processes through photorespiration [70]. However, at the whole-plant level, the contribution of respiration to the plant bioenergetics status is relevant because respiration can account for a release of 30-70% of the C fixed daily in well-watered plants, whereas in drought plants the proportion of C lost increases, mainly due to the decrease observed on photosynthesis [69-73].

2.4. Stress interaction: Photoinhibition as a case study

Photoinhibition, the decrease of photosynthesis and/or photosynthetic capacity due to exposure to excess photosynthetically active radiation, is dependent not only on the radiation level but also on the level of metabolic activity.

Thereby, all stresses that decreased energy demand increased photoinhibition. In fact, photoinhibition occurs when the demand from the carbon reduction cycle for ATP and, mainly, reductive power is decreased and, thereby, not enough NADP⁺ is available to act as the terminal electron acceptor of the linear photosynthetic electron transport chain. In these circumstances, the photosynthetic electron transport chain becomes over-reduced and AOS such as hydroxyl radicals, the superoxide anion and hydrogen peroxide are formed [74], causing oxidative damage to the components of the photochemical apparatus. It is well established that the main target of oxidative damage is the D1 protein and that photoinhibition occurs when the accumulation of photooxidized D1 surpasses its *de novo* synthesis [75]. Plants developed several mechanisms to cope with high irradiance and avoid photoinhibition. These range from the anatomical to the molecular level. Paraheliotopic leaf movements [76] or leaf nastic growth [77], allowing the vertical orientation of leaves, optimizes the leaf to irradiation angle in order to decrease energy load and prevent photoinhibition. Leaf chloroplast movements, to minimize exposition to high irradiation [78] or to fulfill auto-shading, represents another example of strategies to avoid or minimize photoinhibition.

At the molecular level, non-photochemical quenching of chlorophyll fluorescence regulates energy dissipation at the primary photosynthetic reactions and therefore constitutes the first protection line against photodamage. This dissipative pathway is controlled by the thylakoid lumen pH and the xanthophyll cycle [79] which increases the dissipation of excitation energy by inducing an enzymatic conversion of the carotenoid violaxanthin into antheraxanthin and zeaxanthin. Additionally, a second line of defense is provided by alternative electron cycling such as photorespiration. When photooxidation cannot be avoided, damage in the photosynthetic apparatus occurs, especially in PSII, where the reaction center D1/D2 heterodimer is the main site to be affected, mainly D1 while D2 is affected in a lesser extent [80]. The repair of damaged components is then activated, as D1 has a high turnover rate. However, if the rate of repair fails to keep pace with the rate of damage, photosynthesis is decreased and photoinhibition occurs [75]. Nuclear-encoded early-light inducible proteins (ELIPs) may play a relevant role in the protection mechanism discussed above [81] and it will be addressed in a subsequent 4.3 section of this chapter.

2.5. Stress and plant life-cycle: The case of drought stress

It is well known that drought stress at the early stages of plant life, shortly after germination, may have devastating impacts as both the root system is not yet fully established, in one hand, and stomatal control is not yet fine tuned. However, drought stress at this early life stage did not attract much research attention, because it is easily overcome by farmers through an accurate choice of seedling dates. Drought stress at later phenological stages received most attention, particularly the comparison between drought effects on the vegetative phases and in the reproductive phases over grain production. It is now well established that the effects of

stress may vary significantly with the phenological stage of plants. Reproductive stages are generally more sensible to stress than vegetative ones, but differences can also be made between different phases of the reproductive stage. Mouhouche *et al.* [82] found in *Phaseolus vulgaris* that periods of flowering were more sensitive than pod elongation and grain filling phases. Casanovas *et al.* [83] reported a decrease of both leaf physiology and grain yield in maize subjected to drought during flowering. Boonjung and Fukai [84] reported that when drought occurred during vegetative stages, it had only a small effect on subsequent development and grain yield. The effect of water stress on yield was most severe when drought occurred during panicle development.

Grapevine provides an interesting example of the complexity of the relationships between drought stress and plant phenology. Traditionally, grapevine is a non-irrigated crop that occupies extensive areas in dry lands and semi-arid regions [85]. Recently, in the Mediterranean region, irrigation was introduced to increase the low land yield. However, wine quality is strongly dependent on the organoleptic characteristics of grapes which, in turn, particularly in what concerns soluble sugar contents, are dependent on moderate drought stress during berry expansion (i.e. in the phases from fruit set to *veraison*). The irrigation strategy must therefore maximize the vineyard production without decreasing berry quality, an objective suitable for deficit irrigation programs (DRI).

Furthermore, a deep understanding of plant carbon assimilation and partitioning mechanisms under different water regimes will be required in the frame of precision agriculture, as, in fact, these mechanisms play a key role in the fine tuning of the balance between berry yield and quality. Hopefully, this will lead to the adoption of criteria for irrigation scheduling based on vine physiology [85].

3. Gene expression and regulation under abiotic stress

3.1. Complexity of gene expression and regulation

Plants have evolved intricate mechanisms at multiple levels that increase tolerance in order to adapt to adverse conditions and to an ever sessile living. Plant growth and productivity are affected to a great extent by environmental stresses such as drought, high salinity, and low temperature. Expression of a variety of genes is induced by these stresses in various plants. The products of these genes impact not only stress tolerance but also in stress response.

Genes induced during stress conditions function not only in protecting cells from stress by producing important metabolic proteins, but also in regulating genes for signal transduction in the stress response. The first group includes proteins that probably function in stress tolerance, such as chaperones or late embryogenesis abundant (LEA) proteins. The second group contains protein factors involved in further regulation of signal transduction and gene expression that probably function in stress response [86]. In some cases networks and cascades of expression are activated in response to a stress condition. The regulation of the expression of these networks is being studied during the last decades.

The use of microarray approaches, and more recently, of the Next Generation Sequencing (NGS) methodologies have unveiled new regulatory mechanisms that complicate the understanding and most of all the possibilities to modulate and control these processes in view of improving plant responses and productivity.

The regulation of plant genes can be observed at three levels: transcriptional; post-transcriptional and post-translational. In each level, actions depend on specific molecular elements as well as molecular networks and cascades.

The transcriptional regulation involves the interplay of three major elements: chromatin and its modification and remodeling; cis-regulatory elements which are often binding sites, such as enhancers and promoters, located upstream and downstream the coding region; and trans-regulatory elements, usually transcription factors. Chromatin modification and remodeling involved in plant abiotic stress response have been observed in numerous situations [87]. The sensitization of stress responsiveness is called priming [88, 89]. Priming boosts the plant's defensive capacity and brings it into an alarmed state of defense. Recently, priming was correlated with chromatin modification of promoter region of WRKY transcription factors [90]. The involvement of epigenetic mechanisms in the response to environmental cues and to different types of abiotic stresses has been documented [91,92]. Recent reports have shown that different environmental stresses lead to altered methylation status of DNA as well as modifications of nucleosomal histones.

Promoters are regulatory regions of DNA located upstream of genes that bind transcription factor IID (TFIID) and allow the subsequent coordination of components of the transcription initiation complex, facilitating recruitment of RNA polymerase II and initiation of transcription [93].

Members of dehydration-responsive element-binding (DREB) or C-repeat binding factor (CBF), MYB, basic-leucine zipper (bZIP), and zinc-finger families have been well characterized with roles in the regulation of plant defense and stress responses. Most of these transcription factors (TFs) regulate their target gene expression through binding to the cognate cis-elements in the promoters of the stress-related genes [94]. More recently the WRKY transcription factors are becoming one of the best-characterized classes of plant transcription factors [95]. Several WRKY proteins were shown to be involved in plant drought and salinity stress responses [96]. For example, overexpression of the *Oryza sativa* WRKY11 under the control of Heat Shock Protein 101 (HSP101) promoter led to enhanced drought tolerance [97]. Similarly, the altered salt and drought tolerance of 35S:OsWRK45 and 35S:OsWRK72 Arabidopsis plants may be attributed to induction of ABA/stress-related genes [98,99].

NAC (N-acetylcysteine) proteins are plant-specific TFs which have been shown to function in relation to plant development and also for abiotic and/or biotic stress responses. The cDNA encoding a NAC protein was first reported as the RESPONSIVE TO DEHYDRATION 26 (RD26) gene in Arabidopsis [100]. For example OsNAC6 expression is induced by cold, drought, high salinity, and ABA [101]. OsNAC6 showed high sequence similarity to the Arabidopsis stress-responsive NAC proteins ANAC019, ANAC055, and ANAC072 (RD26). It seems that abiotic stress-responsive NAC-type transcription factors, especially the SNAC

group genes, have important roles for the control of tolerance against environmental stresses such as drought [102].

Post-transcriptional regulation is a second level of gene expression modulation which is represented by four groups of processes: pre-messenger (mRNA) processing (capping, splicing, and polyadenylation), mRNA nucleocytoplasmic trafficking, mRNA turn-over and stability, and mRNA translation [103].

Alternative splicing is widely known to regulate gene expression in plants subjected to low and high temperatures [104]. For example, it was shown that *STABILIZED1 (STA1)*, a gene coding for a nuclear pre-mRNA splicing factor is important under cold stress conditions in *A. thaliana* [105]. Alternative splicing has been reported upon water deficit as well [106].

Since the early 2000's, several reports have associated small RNAs to abiotic stress responses, showing that post-transcriptional regulation of gene expression plays an important role in these phenomena [107]. Small RNAs (20 to 25 nt) are processed from non-coding double-stranded RNA precursors by RNases of the DICER-LIKE (DCL) family and mediate a series of gene silencing mechanisms. One of these mechanisms cleaves mRNAs or prevents their translation through the mediation of 21 nt microRNAs. The discovery that stress can regulate microRNA (miRNA) levels, coupled with the identification of stress-associated genes as miRNA targets provided clues about the role of miRNAs in stress responses. Functional analyses have demonstrated that several plant miRNAs play vital roles in plant resistance to abiotic stresses [108-110]. Their role in abiotic stress responses will be further addressed in section 3.2.

Messenger RNA translation is dependent on mRNA cytoplasmic cycling [111] namely compartmentalization in P bodies and association to ribosomes. The amount of mRNAs in polysomes is generally reduced during exposure to dehydration or anoxia, while stress-induced mRNAs significantly increase in polysome association [112]. 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 [113].

At the post-translational level phosphorylation, sumoylation and ubiquitination of proteins are processes that play major roles in the modulation of plant response to abiotic stress. Phosphorylation and de-phosphorylation play major roles in the responses to abiotic stress. Several signal transduction cascades formed by mitogen-activated protein kinases (MAPKs) and SNF-1-related protein kinases (SnRKs) are activated upon water deprivation and osmotic stress through the phosphorylation of specific residues [114]. Among these, SnRK2 proteins have been shown to be involved in ABA-dependent responses to water deficit, like stomata closure [115].

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 [116]. *XERICO* controls the level of ABA by enhancing the transcription of the key ABA biosynthetic gene *AtNCED3*. The findings indicate that the protein degradation mediated by the ubiquitin/proteasome pathway plays a fundamental role in ABA homeostasis and response [112].

Sumoylation was also reported to participate in responses to phosphate starvation, and to the tolerance to low and high temperatures [117]. An increase in the levels of SUMO-protein conjugates was also detected in water-deprived plants [118].

The concerted actions of the transcriptional, post-transcriptional and post-translational mechanisms 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. The complete understanding of the interplay of these three regulatory systems is crucial for the understanding of the molecular mechanisms governing plant adaptation to environment as well as for plant improvement for stress tolerance.

3.2. miRNAs in plant responses to abiotic stress – An additional post-transcriptional regulation layer may apply

Plant responses to abiotic stress such as water deficit involve an intricate regulation of gene expression at the transcriptional and post-transcriptional levels. MicroRNAs (miRNAs) are a class of small non-coding RNAs molecules (21-24 nt) involved in post-transcriptional regulation of gene expression. miRNAs were shown to be involved in plant development [119-124], biotic [125, 126] and abiotic stress responses [108, 110, 127-130].

In plants, microRNAs repress gene expression by directing mRNA degradation or translational arrest: miRNAs guide Argonaute (AGO) proteins to bind to matching target mRNAs in a RNA-induced silencing complex (RISC), promoting cleavage of mRNAs with near perfect base complementarity and/or inhibiting translation of those with lower complementarity [131-133].

The first reports assigning miRNAs to have a role in shaping plant responses to abiotic stresses were based on small RNA cloning and sequencing [134], complemented with analyses of miRNA expression profiles and miRNA target prediction [108]. Since then, the application of high-throughput sequencing technology and genomic approaches like microarray analyses to evaluate the profile of miRNA expression in various tissues and conditions, associated to improved bioinformatic tools to identify miRNAs and their targets, have allowed an extensive recognition of stress-responsive small RNAs and their targets in various plant species (reviewed in [107]).

Sequencing of miRNAs in Legumes was first reported in *Medicago truncatula* [135] and *Glycine max* [136] but there are references to small RNAs in other Legumes back to 2004, with a size population of small RNA molecules being identified in the phloem sap of *Lupinus albus* [137]. These findings were the basis of a systemic signalling mechanism in which small RNAs movement is facilitated by chaperone proteins to exert their action at a distance.

One of the most extensively studied miRNAs in the context of abiotic stresses have been the miRNAs involved in nutrient deprivation miR395, miR398 and miR399, all identified in the phloem sap of nutrient deprived plants. In fact, studies in *Arabidopsis* have established that miR395 (sulphate), miR399 (phosphate) and miR398 (copper) regulate these nutrients homeostasis by moving along the phloem to inform the roots of the nutrient status of the shoot [138-139].

The miR395 gene-family targets genes involved in sulphate translocation (the low-affinity transporter *SULTR2;1*) and assimilation (the ATP sulphurylases, *APS*) [134, 140,141]. Importantly, miR395 itself is regulated by a transcription factor, the SULFUR LIMITATION 1 (*SLIM1*) [141]. The miR395/*APS-SULTR2;1/SLIM1* regulatory module is involved in root-to-shoot sulphate translocation as a strategy to improve sulphate assimilation in the leaves during sulphate starvation [142].

The miR399 gene-family is strongly and specifically induced by inorganic phosphate limitation in the shoot and targets *PHO2*, an E2 ubiquitin-conjugating enzyme that represses Pi uptake [109, 140,143-144]. As for miR395, also the expression of miR399 is regulated by a transcription factor, the MYB TF PHOSPHATE STARVATION RESPONSIVE1 (*PHR1*; [109]). The miR399/*PHO2/PHR1* regulatory module operates under Pi deprivation: miR399 is induced by *PHR1* in the leaves, travels along the phloem to repress *PHO2* expression in the roots thereby releasing several protein targets from ubiquitinylation-dependent degradation, including transporters involved in Pi allocation inside the plants and increasing Pi content in the shoot. A worth mentioning aspect of the miR399 regulatory module is the extra layer of miR399 activity regulation exerted by *IPS1* (induced by phosphate starvation1) [145]. *IPS1* is a non-protein coding transcript with sequence complementarity to miR399 that sequesters miR399 thus inhibiting its repressing activity over its target. This mechanism designated as target mimicry was first described in plants [145] and more recently discovered in animals [146] and expands the regulatory post-transcriptional gene expression network in which miRNAs are involved.

The miR398 (and miR408) are induced by copper limitation and target genes encoding copper proteins like Copper/Zinc superoxide dismutases, cytochrome *c* oxidase and plantacyanin [147, 148]. Similar to miR395 and miR399, also miR398 and miR408 are regulated by a transcription factor, the *SQUAMOSA* promoter binding protein-like7 (*SPL7*) that regulates the expression of several copper-responsive genes [149]. Copper in contrast to sulphate and phosphate is a micronutrient but still the regulation of this nutrient homeostasis is basically similar, as it involves systemic signalling, a well established regulatory module involving a transcription factor, the miRNA and its target.

The miR395, miR399 miR398 and miR408 were identified in *M. truncatula* by sequencing libraries of small RNAs from the aerial part [135]. Homologs of known miRNA target genes were identified, such as low affinity sulphur transporter for miR395, *COX5b* (subunit 5b of mitochondrial cytochrome *c* oxidase) for miR398, *PHO2* for miR399 or plantacyanin for miR408. However, our computational prediction identified many hypothetical genes for miRNA targeting ([135] - Additional File 1), rendering experimental confirmation a laborious and unsuccessful task (Trindade, unpublished data).

Some miR398 and miR408 predicted targets were validated by 5'RACE and miR398 and miR408 expression was further investigated in different plant parts and in specific water deficit conditions, showing up-regulation in water deprivation and concomitant down-regulation of their validated targets [129]. These targets were further confirmed by deep sequencing of cleaved miRNA targets (Parallel Analysis of RNA Ends - PARE) [150-151] in *M. truncatula* in

collaboration with the Tamas Dalmay laboratory (School of Biological Sciences, UEA, Norwich, UK) (unpublished data).

Still, the bioinformatic prediction of many hypothetical genes for miRNA targeting raises the question whether we are dealing with true or instead pseudo targets and can have a strong implication on our assumptions about the mechanisms of miRNA functioning as they impose an additional layer of post-transcriptional regulation.

Seitz [152] proposed that many computational identified miRNA targets are indeed pseudotargets that prevent miRNAs from binding their true targets by sequestering them. They would have the basic features of miRNA targets identified by the target prediction algorithms: complementarity to miRNAs and phylogenetic conservation but are instead modulators of miRNA expression.

These pseudotargets occur naturally in plants [145] and animals [146] and were firstly associated to miRNA regulation of nutrient deprivation but their involvement in other abiotic stress conditions like water deprivation may also be envisaged.

A 5-year EU FP7 project designated “ABStress - Improving the resistance of legume crops to combined abiotic and biotic stress” was recently started [153]. This project will study the small RNAs and epigenetic regulation involved in abiotic and biotic stresses in Legumes using *Medicago truncatula* as a model and it is certainly expected to bring new information about the complex network of regulatory circuitries in which miRNAs participate.

4. Transgenic approaches to improve abiotic stress resistance

The advance in genetic engineering offers new ways to understand the genetic mechanisms of stress-related genes and their contribution to the plant performance under stress [154]. However, while a great degree of success has been obtained in the production of herbicide-, virus- and fungal-resistant plants and plants with fortified nutritional values using transgenic tools, the same has not been the case in production of abiotic stress-tolerant crops [155]. This is largely due to the complex genetic mechanisms that govern abiotic stress tolerance. Additionally, as previously referred, in natural conditions, crops can suffer from different stress combinations, at different development stages and during different time periods.

Recently, several reviews were published concerning genetic engineering for abiotic stress tolerance, most focused in model but also in crop plants (e.g. [156 -161]). Possible targets for genetic engineering towards abiotic stress in plants are genes belonging to structural and regulatory categories. They can be modified (for example truncated) and fused to other genetic components such as signal peptides that direct their expression to specific organelles and/or reporter genes for early detection in transgenic plants. After the proper cloning of the desired genes, they are engineered for their expression to be regulated in a time and space context, using specific promoters. The approach can take into account if it is desirable to have the gene expression upregulated, by sense overexpression of the transgene, or downregulated, by the antisense or RNA interference (RNAi) techniques.

Presently, numerous genes associated to plant responses to abiotic stress have been identified and characterized in laboratory studies (reviewed in [157, 162-163]). Engineered overexpression of biosynthetic enzymes for osmoprotectants such as glycine betaine [164,165]; stress induced proteins such as LEA proteins [166-167]; scavengers of reactive oxygen species [168,169]; transcription factors [170, 171] or signal transduction components [172-173] were reported. Since stress resistance is a complex trait regulated by several genes acting in a concerted way during the process, it is not surprising that transgenic approaches using a single stress-related gene will only lead to marginal stress improvement [174]. One of the major challenges is the introduction of multiple genes by pyramiding strategies or co-transformation [175-176].

It is also expected that several areas, such as post-transcriptional regulation involving protein modification, protein degradation and RNA metabolism will emerge [163]. An example is the application of miRNAs in the improvement of stress resistance. The discovery of miRNAs involved in the regulation of stress responses and discovering the potential use of these miRNAs to modulate or even increase stress resistance in plants is an open field of research as previously discussed in section 3.2 of this chapter. As an example, Sunkar and co-workers [110] have generated transgenic *Arabidopsis thaliana* plants overexpressing a miR398-resistant form of a plastidic Cu/Zn Super Oxide Dismutase (Cu/Zn-SOD;CSD2) and confirmed that transgenic plants accumulate more CSD2 mRNA than plants overexpressing a regular CSD2 and are consequently much more tolerant to high light, heavy metals, and other oxidative stresses. These results suggest that understanding posttranscriptional gene regulation is important to widen our ability to manipulate stress tolerance in plants and offer an improved strategy to engineer crop plants with enhanced stress tolerance.

The process of generating transgenic lines requires success in the transformation method and proper incorporation of stress resistance genes into plants. The most used method to transfer foreign genes into plant cells and the subsequent regeneration of transgenic plants is based on the natural system, the *Agrobacterium*-mediated plant transformation [177]. Particle bombardment has also been exploited extensively for plant transformation especially in species recalcitrant to *Agrobacterium* infection such as maize. The development of new plant transformation vectors namely using new-plant associated bacteria (such as from the *Rhizobiacea* family) has also proved to be an effective approach to generate transgenic plants from explants/genotypes unsuitable for *Agrobacterium*-mediated transformation methodology [178].

The promoters that have been most commonly employed in the production of abiotic stress-tolerant plants include the cauliflower mosaic virus (CaMV) 35S promoter (mostly used for dicot crops) and the actin 1 promoter (Act-1) (used for expression of transgenes in monocot crops) [155]. As these promoters are constitutive, the downstream transgenes are expressed in all organs and at all stages which is unnecessary as well as demanding on the energy reserves of the cell [170]. In some cases, constitutive expression of a gene normally only induced by stress can have negative effects on growth and development when stress is not present (pleiotropic effects). The use of inducible promoters that allow the expression of a transgene only when it is required could therefore be the ideal solution [179, 180]. There is a strong need to obtain an increased array of inducible promoters, which are expressed only when exposed

to stress situations, and to pair such promoters with the stress tolerance-related genes in the adequate cloning vectors [181]. Additional tests need to be performed to guarantee that obtained stress-inducible promoters work in heterologous plant systems.

Concerning the improvement of stress resistance, the past decade has witnessed the utilization of transgenic approaches for experimental purposes, mainly in model plant systems but not in important agricultural species or crops. Nevertheless, the creation of stress-tolerant crops either by genetic engineering or through conventional breeding has covered almost all aspects of plant science, and is pursued by both public and private sector researchers [161]. One of the major goals of transgenic technology is to produce plants not only able to survive stress, but also capable to grow under adverse conditions with substantial biomass production, thus overcoming the negative correlation between drought resistance traits and productivity, which was often present in past breeding programs [155, 182]. In the case of crop plants, it is ultimately the yield of genetically altered plants under specific field conditions that will determine whether or not a specific gene, or metabolic or signaling pathway, is of technologic importance [3]. One successful case in releasing tolerant plants to abiotic stresses is the transgenic maize line resistant to drought developed by the Monsanto company. This maize line (MON87460) was recently approved in the USA and is able to grow in soils with reduced water content due to the presence of a cold shock protein –CSPB- from *Bacillus subtilis* [183].

During the last decade, our group has engineer model species like tobacco and *Medicago truncatula* with improved abiotic stress traits (drought and salinity), using different stress related genes.

4.1. Engineering trehalose accumulation

Trehalose is a disaccharide, containing two glucose molecules. Trehalose was first discovered in 1832 from the Ergot of rye [184-186] and since then isolated from numerous organisms, including algae, fungi, bacteria, insects and crustaceans. Trehalose is nevertheless considered non-occurring in measurable amounts in plants, with the exception of a few species [184], notably the so called “resurrection plants”, able of surviving the loss of most of their water content until a quiescent stage is achieved and upon watering rapidly revive and restored to their former state [187].

Trehalose can be synthesized by three different pathways [188] and the most frequent in nature involves the enzyme trehalose-6-phosphate synthase (TPS; EC 2.4.1.15) that catalyzes the transfer of glucose from UDP-glucose to glucose-6-phosphate to produce trehalose-6-phosphate plus UDP. Another enzyme, trehalose-6-phosphate phosphatase (TPP; EC 3.1.3.12) converts trehalose-6-phosphate to free trehalose [184, 186, 189, 190]. Genes codifying both enzymes have been isolated in several species including *Sacharomyces cerevisiae* and *Escherichia coli* and several plant species such as *Arabidopsis* and rice [191]. Trehalose may be degraded by the enzyme trehalase (EC 3.2.1.28) [186, 191].

In living organisms, several functional properties have been proposed for trehalose: energy and carbon reserve, protection from dehydration, protection against heat, protection from damage by oxygen radicals and protection from cold [186]. As trehalose, sucrose is one of the

few free disaccharides in nature. Both are non-reducing sugars and synthesized by similar pathways. Contrary to trehalose, sucrose synthesis is mainly limited to photosynthetic organisms [192], where it holds a central position as the major product of photosynthesis and as a transport molecule involved in growth, development, storage, signal transduction and acclimation to environmental stress. Sucrose transport is finally energetically superior to trehalose transport making it more “preferred” to plants metabolism. It is hence often suggested that trehalose is evolutionary more ancient than sucrose [192].

As trehalose is present in so low or in undetectable amounts in most plants, it is unlikely that under natural conditions and with the exception of desiccation tolerant plants, this sugar might play a role in stress protection in plants [193]. Nevertheless, other roles have been proposed for trehalose and trehalose-6-phosphate synthase: regulation of plant growth and development; broad spectrum agent preventing symbiosis between susceptible plants and trehalose producing microorganisms [193-194]; the regulation of carbohydrate metabolism or the perception of carbohydrate availability [190,194-197]; the regulation of embryo maturation [197-199]; implication on vegetative growth and transition to flowering [200]; implication on seedling development [201-202]; and regulation of glucose, abscisic acid and stress signaling [203-205]. According to [190], trehalose plays several roles in carbohydrate metabolism, with a number of processes and pathways being affected.

For all that was stated above, trehalose is one of the most studied osmoprotectants and in recent years there has been a growing interest in trehalose metabolism as a means of engineering stress tolerance in crop plants [191]. Several experiments have been conducted to obtain transgenic plants over-expressing genes codifying enzymes of the trehalose biosynthetic pathway of *E. coli* and *S. cerevisiae*, using both model plants like tobacco (*Nicotiana tabacum*) and crop plants such as potato (*Solanum tuberosum*), rice (*Oryza sativa*) and more recently tomato (*Lycopersum esculentum*). Additional, attempts have been made using an alternative approach: the inhibition of the expression of trehalase gene. Those experiments and their main results are summarized in Table 1.

The previously mentioned genetic engineering obtained a variable degree of success. Generally speaking, transgenic plants were found to have higher tolerance than controls to some form of water stress imposed, following in most cases, confirmed trehalose accumulation. Albeit such fact, trehalose engineered plants frequently had altered phenotypes, particularly dwarfism and leaf abnormalities. Such fact was particularly true for the first transformation events in which genes of microbial origin were used. Later events, in which endogenous or plant origin genes were used seem to counter that tendency [217, 218]. Genetic engineering of plants with trehalose biosynthesis genes seems therefore to be of extreme pertinence to the increase of abiotic stress tolerance in plants, particularly plants of agricultural importance such as cereals and legumes.

4.2. Engineering polyamine accumulation

Polyamines (PAs) are small (low-molecular-weight), positively charged, aliphatic amines that are found in all living organisms. The major forms of PAs are putrescine (Put), spermidine (Spd) and spermine (Spm), although plants also synthesized a variety of other related com-

pounds. Arginine (Arg) and ornithine (Orn) are the precursors of plant PAs. Ornithine decarboxylase (ODC; EC 4.1.1.17) converts Orn directly into Put. The other biosynthetic route to Put, via arginine decarboxylase (ADC; EC 4.1.1.19), involves the production of the intermediate agmatine (Agm) followed by two successive steps catalysed by agmatine iminohydrolase (AIH, EC 3.5.3.12) and *N*-carbamoylputrescine amidohydrolase (CPA, EC 3.5.1.53). In animals and fungi Put is synthesized primarily through the activity of ODC while in plants and bacteria the main pathway involves ADC. Aminopropyl groups, donated by decarboxylated *S*-adenosyl methionine (dcSAM), must be added to convert Put into Spd and Spm in a reaction catalysed by spermidine synthase (SPDS; EC 2.5.1.16) and spermine synthase (SPMS; EC 2.5.1.22), respectively (reviewed in [220]). Polyamines levels in plants increase under a number of environmental stress conditions, including drought and salinity [221-223]. Several biological roles were proposed for polyamines action in stress situations; PAs could act as osmoprotectants, as scavengers of active oxygen species (AOS) or by stabilizing cellular structures, such as thylakoid membranes [222, 224, 225]. The first reports of transgenic approaches using genes responsible for PA biosynthesis were conducted in two species, tobacco and rice [226-230]. Recently, new insights into the role and regulatory function of polyamines in plant abiotic stress tolerance have been achieved, with several abiotic (salt, drought, freezing, heat) stress tolerant transgenic plants overproducing polyamines being described in the following reviews [220, 231-233].

Among abiotic stresses drought is the main abiotic factor as it affects 26% of arable area [229]. Plants respond to changes in water status by accumulating low molecular-weight osmolytes including PAs. Polyamines may have a primary role of turgor maintenance but they may also be involved in stabilizing proteins and cell structures. The polycationic nature of PAs at physiological pH is believed to mediate their biological activity, since they are able to bind to several negatively charged molecules, such as DNA, membrane phospholipids, pectic polysaccharides and proteins [225].

In respect to the antioxidant activity of PAs, the research data is contradictory; on the one hand, PAs have been suggested to protect cells against AOS and on the other hand, their catabolism generates AOS [232]. PA catabolism produces H_2O_2 , a signaling molecule that can act promoting activation of antioxidative defense response upon stress, but can also act as a peroxidation agent. In a recent study, the effect of increased putrescine (Put) accumulation was found to negatively impact the oxidative state of poplar cells in culture due to the enhanced turnover of Put [233]. Gill and Tuteja [234] stated that, while increase Put accumulation may have a protective role against AOS in plants, enhanced Put turnover can actually make them more vulnerable to increased oxidative damage. The higher polyamines, Spd and Spm are believed to be most efficient antioxidants and are considered scavengers of oxyradicals [235].

As plants with elevated putrescine contents are able to tolerate drought stress because Put has a direct protective role in preventing the symptoms of dehydration, higher PAs (Spd and Spm) appear to play an important role in stress recovery [236]. Recently, transgenic rice plants overexpressing *samdc* (*S*-Adenosyl methionine decarboxylase gene), with increased Spd and Spm levels, were considered to be non drought tolerant, but showed a more robust recovery

Gene/Promoter	Origin	Plant	Main Effects	Ref.
<i>tps</i> ; Rsu- rubisco small unit promoter	Yeast	Tobacco	Increased trehalose levels; Transgenic plants showed less water loss upon leaf detaching.	[206]
<i>otsA</i> ; <i>otsB</i> ; CaMV 35S	<i>E. coli</i>	Tobacco	Low levels of trehalose in leaves.	[207]
<i>otsA</i> ; <i>otsB</i> ; CaMV 35S	<i>E. coli</i>	Potato	Absence of trehalose detection.	[207]
<i>tps1</i> ; CaMV 35S	Yeast	Tobacco	Higher levels of trehalose; Phenotypic alterations (stunted growth; lancet shaped leaves); Improved drought tolerance.	[208]
<i>otsA</i> ; <i>otsB</i> ; CaMV 35S	<i>E. coli</i>	Tobacco	Phenotypic alterations (larger leaves and altered stem growth); Higher growth under drought stress.	[209]
<i>otsA</i> ; CaMV 35S	<i>E. coli</i>	Tobacco	Altered phenotypes; Transgenic plants showed less water loss upon leaf detaching.	[210]
<i>otsA</i> ; <i>otsB</i> ; Rsu and ABA-inducible promoter	<i>E. coli</i>	Rice	Higher trehalose levels; Sustained plant growth; Less photo-oxidative damage Favorable mineral balance under abiotic stress; Stress tolerance.	[211]
<i>otsA</i> ; <i>otsB</i> ; Ubi-1 promoter	<i>E. coli</i>	Rice	Increased trehalose levels; Absence of phenotypic alterations and altered growth. Tolerance to drought, salt and cold.	[212]
<i>otsA</i> ; <i>otsB</i> ; CaMV 35S	<i>E. coli</i>	Tobacco	Altered photosynthesis in transgenic plants.	[213]
<i>tps1</i> ; CaMV 35S	Yeast	Tomato	Higher trehalose content; Altered phenotypes; Tolerance to drought, salt and oxidative stress.	[214]
<i>tp</i> ; CaMV 35S	Pletorus sajor-caju	Tobacco	Higher trehalose content; Unaltered phenotypes; Tolerance to water deficit.	[215]
<i>tre</i> (Antisense); CaMV 35S; Rd29A- osmotic stress inducible	<i>Medicago sativa</i>	Tobacco	Reduced trehalase activity in transgenic plants.	[216]
<i>tps</i> ; CaMV 35S		Tobacco	Transgenic plants with higher tolerance to several osmotic stresses.	[217]
	<i>A. thaliana</i>		Transgenic lines with higher tolerance to moderate water deficit or ability to recovery from severe water deficit.	[218]
		<i>M. truncatula</i>		
<i>tps</i> ; Act-1 promoter	<i>O. sativa</i>	Rice	Improved the tolerance of rice seedling to cold, high salinity and drought.	[219]

Table 1. Genetic Engineering of plants towards trehalose accumulation

from drought compared to wild type [236]. The *de novo* synthesis of Spd and Spm in transgenic plants under drought stress, at the expenses of Put, was responsible for the stress tolerance observed in these plants.

The covalent linkage of PAs to proteins appeared to be of extreme importance in plant light-induced stabilization of the photosynthetic complexes and Rubisco therefore exerting a positive effect on photosynthesis and photo-protection. Also in the cytosol, they are involved, mediated by transglutaminase (TGase) activity, in the modification of cytoskeletal proteins and in the cell wall construction/organization [237]. In a recent study, the characterization at the proteomic level of the TGase interaction with thylakoid proteins, demonstrated its association with photosystem II (PSII) protein complexes using maize thylakoid protein extracts [238]. Binding of Put to thylakoid membranes has been proposed to be a photoadaptation response under controlled stress conditions. Campos and collaborators [238] results reinforce the importance of the TGase in photo-protection by polyamine conjugation to light-harvesting complex II (LHCII) proteins.

Recently, PAs were proposed to be components of signaling pathways and fulfill the role of second messengers [220, 231]. Studies with ABA-deficient and ABA-insensitive Arabidopsis mutants with differential abiotic stress adaptations [239] support the conclusion that the up-regulation of PA biosynthetic genes and Put accumulation under water stress are mainly ABA-dependent responses. To reinforce the fact that PAs biosynthesis may be regulated by ABA, several stress-responsive elements, like drought responsive (DRE), low temperature-responsive (LTR) and ABA-responsive elements (ABRE and/or ABRE-related motifs) are present in the promoters of the polyamine biosynthetic genes [239]. Liu *et al.* [240] also found that inward potassium channels were targets for PA regulation of stomatal movements. Since ABA signaling pathway in stomata regulation involves many different components including signaling molecules like AOS, IP_3 , Ca^{2+} and nitric oxide (NO), evidences point to an interplay between ABA, polyamines, H_2O_2 and NO in stomata regulation [220].

In our experiments, we transformed the model legume *Medicago truncatula* cv. Jemalong with the arginine decarboxylase gene (*adc*) from *Avena sativa* to overexpress the heterologous ADC enzyme aiming to increase the levels of polyamines in transgenic plants [241, 242]. Several transgenic lines overexpressing This oat *adc* construct were obtained. The oat *adc* cDNA under the control of a CaMV 35S constitutive promoter was previously transferred into rice plants [228] and those authors found increased Put levels in regenerated plants and observed minimized chlorophyll loss during drought stress. However, constitutive over-expression of this gene severely affected developmental patterns of those plants. Afterwards, the same group used the monocot maize's ubiquitin-1 (Ubi-1) promoter to overexpress the *Datura adc* gene and found that transgenic plants, with increased Put levels, were tolerant to drought stress [230]. The Ubi-1 promoter is known to contain a number of stress-responsive elements that enhance transgene expression under drought stress [230] and hence function as a stress-inducible promoter. Roy and Wu [229] also found that the expression of the *adc* transgene under the control of an ABA-inducible promoter led to stress-induced upregulation of ADC activity and polyamine accumulation in transgenic rice plants. Second-generation transgenic rice plants showed an increase in biomass under salinity-stress conditions.

In our *M. truncatula* system, no altered external morphology was observed in *adc* transgenic plants, that were successfully developed without phenotypic visible alterations and produced seeds (T₂ generation) [241, 242]. One specific transgenic line (L108) expressing the heterologous *adc* transgene had a very high accumulation of Agmatine (22-fold) (the direct product of the ADC enzyme and intermediate in the Put biosynthesis) and moderately related increase of Put (1.7-fold) and Spd (1.9-fold) levels, compared to control plants [242]. These results are consistent with several reports that suggest PAs levels are under strict homeostatic regulation [227, 243].

Nevertheless, several recent studies have concluded on the feasibility of PA biosynthesis engineered for the production of stress-tolerant plants. Accumulating experiments and their main results are summarized in Table 2. The constitutive expression of homologous *adc1* and *adc2* in *Arabidopsis* resulted in freezing and drought tolerance, respectively [244-245]; with a patent application for “Plant resistance to low-temperature stress and method of production thereof” by [244]. In another work, transgenic tomato lines transformed with the yeast *samdc* fused with a ripening-specific promoter E8, over-accumulate Spd and Spm and, interestingly, showed phenotypes of agronomical importance such as enhanced phytonutrient content and fruit quality [246-247]. Polyamine-accumulating transgenic eggplants exhibited increased tolerance to multiple abiotic stresses (salinity, drought, low and high temperature and heavy-metal) and also biotic resistance against fungal disease caused by *Fusarium oxysporium*. These authors used a construct similar to ours, with the *adc* gene from oat under the control of the constitutive CaMV 35S promoter and found that some transgenic eggplants lines showed an enhanced level of Put, Spd and in some cases also Spd. These lines also showed increase in ADC and also on the activity of the PA catabolic enzyme, diamine oxidase (DAO) [248].

There are several reports in which the plant response to diverse abiotic stress is associated to the stimulation of polyamine oxidation [249]. However, the precise role of polyamine catabolism in the plant response to environmental stress remains elusive [249-250]. Considering these results, further research concerning the PAs changes and the global response of our *M. truncatula* diverse germplasm with altered PA content to multiple stresses should be developed in the near future.

4.3. Engineering accumulation of photo-protective proteins — ELIPs

To cope with environmental stresses, plants activate a large set of genes, which lead to the accumulation of specific stress-associated proteins (reviewed in [253]). The stomatal limitation on photosynthesis imposed by the earlier stages of water deficit (WD) result in a decrease of primary electron acceptors available for photochemistry [47]. If protection mechanisms are not activated, the excess of absorbed energy may induce photo-oxidative damage in chloroplast structures. The nuclear-encoded early-light inducible proteins (ELIPs) may play a relevant role in the protection mechanisms discussed above.

ELIPs and ELIP-like proteins are pigment-binding components of the thylakoid membrane widely distributed among plant species and belong to the chlorophyll a/b-binding protein (cab) family (reviewed in [254, 255]). ELIPs are widely present among different plant species like pea [256], barley [257], *Craterostigma plantagineum* [258], *Dunaliella bardawil* [259], *Sporobolus*

Gene/Promoter	Origin	Plant	Main Effects	Ref.
<i>odc</i> ; CaMV 35S	<i>S. cerevisiae</i>	Tobacco	Increased ODC activity; Increased Put and Nicotine	[251]
<i>samdc</i> ; CaMV 35S	Human	Tobacco	Increased SAMDC activity; Spd and Spm levels. Lower Put levels. Thick leaves, stems and stunting.	[252]
<i>adc</i> ; Tet- inducible promoter	Oat	Tobacco	Increased ADC activity; Phenotypic alterations pp to Put levels (thin stems and leaves, leaf necrosis, chlorosis, short internodes and growth inhibition)	[226]
<i>adc</i> ; CaMV 35S	Oat	Tobacco	Increased ADC activity; ODC and SAMDC normal; Increased Agm; Put, Spd and Spm normal.	[227]
<i>adc</i> ; CaMV 35S	Oat	Rice	Increased Put and less chlorophyll loss during drought. Severe altered phenotypes.	[228]
<i>adc</i> ; ABA- inducible promoter	Oat	Rice	Increased Put, ADC activity and biomass under salt stress.	[229]
<i>samdc</i> ; E8 promoter	Yeast	Tomato	Increased Spd and Spm. Enhanced phytonutrient content and fruit quality	[246, 247]
<i>adc</i> ; Ubi-1 promoter	<i>D. stramonium</i>	Rice	Higher Put, Spd and Spm levels and drought tolerance	[230]
<i>adc</i> ; CaMV 35S	Oat	<i>M. truncatula</i>	Increased Agm, Put and Spd levels. Absence of phenotypic alterations and altered growth (second generation homozygous plants).	[241, 242]
<i>adc</i> ; CaMV 35S	Oat	Eggplant	Increased Put, Spd and Spm levels; multiple abiotic stress resistance and fungal resistance.	[248]
<i>adc1</i> ; <i>adc2</i> ; CaMV 35S	Arabidopsis	Arabidopsis	Increased Put; freezing and drought tolerance.	[244, 245]

Table 2. Genetic Engineering of plants towards polyamine accumulation

stapfianus [260], *Arabidopsis thaliana* [261], *Tortura ruralis* [262], *Nicotiana tabacum* [263] and recently found in *Coffea canephora* [264].

Contrary to the other members of the *cab* family that are expressed constitutively, ELIPs accumulate transiently during the greening of etiolated plants [265] and in developing plastid membranes [266]. In mature plants, ELIPs also accumulate in response to various stress conditions including ABA or desiccation [258], nutrient starvation [259], high light [267, 268], UV-B [269], cold [270], methyl jasmonate [271], salinity [262] and senescence [263]. ELIPs and

ELIP-like proteins are thought to protect the chloroplast apparatus from photooxidation by: a) acting as transient pigment-binding proteins during biogenesis or turnover of chlorophyll binding proteins [262, 266, 268, 272]; b) binding or stabilising carotenoids like zeaxanthin and lutein [266, 268, 273, 274]; c) stabilising the pigment-protein complexes and/or favouring their appropriate assembly [268, 272, 274, 275]; d) dissipating the excessive absorbed light energy at the reaction center of the PSII, in the form of heat or fluorescence [276].

We decided to express the *dsp22* gene from *Craterostigma plantagineum* [258] in *M. truncatula*, aiming to investigate the protective role of this ELIP-like protein in the photosynthetic apparatus, during the dehydration and rehydration [81, 241]. We assessed the photochemical performance of in *dsp22* transgenic (A.27) and wild type (M9-10a) plants together with leaf pigment contents and biomass accumulation during dehydration and subsequent recovery. Transgenic *M. truncatula* plants overexpressing the ELIP-like DSP22 protein display higher amount of chlorophyll (Chl), lower Chl *a*/Chl *b* ratio and higher actual efficiency of energy conversion in PSII after dehydration and rehydration, also suggesting a role in pigments stabilization during WD stress [81]. Our results are in agreement with the transient photosynthetic pigment binding function postulated for ELIPs and ELIP-like proteins under disturbing environmental conditions [266, 268]. Additionally, the results indicate that DSP22 may contribute to reduce the impact of photooxidative damage on the PSII complex of *M. truncatula* resulting from WD and recovery treatments. Despite of this assumption, the mechanisms by which DSP22 leads to enhanced photooxidative protection in this model legume are yet not clear and further studies are necessary to support these hypothesis. Nevertheless, the results supports that the expression of photoprotective proteins, such as ELIPs, can be considered a valuable approach to improve abiotic stress resistance in crops.

5. Omics and system biology approaches to understand abiotic stress responses

During the last decade, the “reductionistic” molecular biology and functional biology approaches are being progressively replaced by the “holistic” approach of systems biology. However, molecular biology and systems biology are actually interdependent and complementary ways in which to study and make sense of complex phenomena [277]. Presently, the use and development of post-genome methodologies, such as global analysis of transcriptomes, proteomes and metabolomes integrated in solid bioinformatics platforms, has noticeably changed our knowledge and holistic understanding various plants function, including the response to abiotic stresses [278]. System-based analysis can involve multiple levels of complexity, ranging from single organelles or cells, tissues, organs to whole organisms. These variables can be still combined with multiple developmental stages and environmental interactions suggesting an infinite number of permutations to this complexity [279].

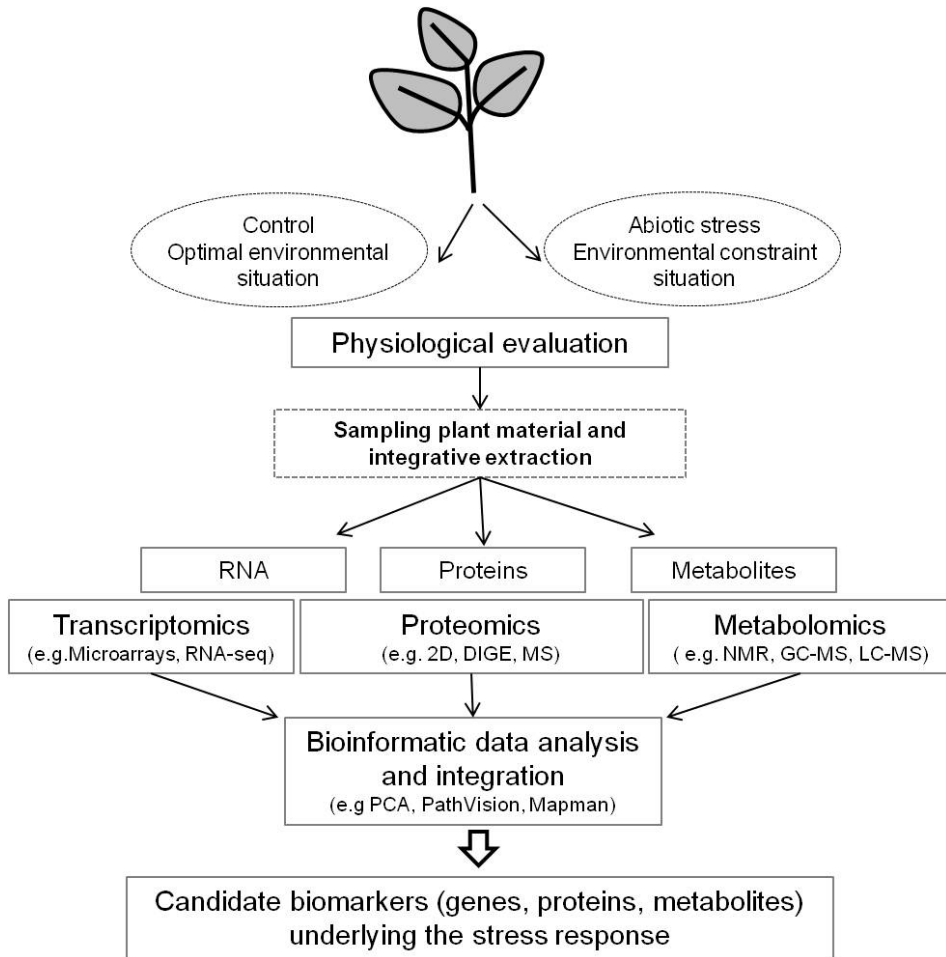


Figure 1. Schematic overview of a common System Biology approach to study abiotic stress responses in plants.

The breakthrough in Omics technologies has led to designing better experiments which provide deep insight into the function of genes and also their effects on phenotypic change in a specific biological context [280]. System biology approaches can circumvent some barriers that had previously blocked the translation of knowledge gained from model plants, like *Arabidopsis thaliana* and *Medicago truncatula*, to other economically important plant species in light of current progress in generating new crop genome sequences and functional resources [279, 281]. It is anticipated that this trend will continue into the next decade in light of current developments in crop functional resources [281] and in view of the exponential number of papers published on abiotic stress studies in plants using a systems biology approaches during the last decade [279].

Most of the plant system biology approaches relied on three main axes: transcriptomics, proteomics and metabolomics (see Figure 1).

In addition to these previous studies, interaction between DNA-proteins and Proteins-proteins – interactomes - are being also used with success to identify regulatory proteins involved in complex whole plant responses [282]. Bioinformatics has been crucial in every aspect of Omics-based research to manage various types of genome-scale data sets effectively and extract valuable information and facilitate knowledge exchange with other model organisms [278, 283]. A comprehensive list of the analytical bioinformatics platforms available constituting an essential infrastructure for systems analysis can be found in [278].

5.1. Transcriptomics

Transcriptomics, also referred as expression profiling, captures spatial and temporal gene expression within plant tissues or cell populations on a specific biological context (e.g. genotype, growth or environmental condition). In many instances transcriptomic analysis is used to screen for candidate genes for abiotic stress improvement programs [280] or to predict the tentative gene function by the association of differently expressed or co-expressed genes with the plant phenotype alteration [284]. Transcriptomic approaches should incorporate highly specific, sensitive and quantitative measurements over a large dynamic range with a flexibility to identify unanticipated novelties in transcript structures and sequences [285].

Determination of large scale transcript profiles or identification of differentially regulated genes in plants can be performed by various techniques, such as DNA microarrays, serial analysis of gene expression (SAGE) or more recently Digital Gene Expression (DGE) profiling taking advantage of next-generation sequencing (NGS) based tools such as RNA sequencing (RNA-seq) [279, 280, 285]. The hybridization-based method, such as that used in microarray analyses, together with the availability of completed genomes sequences and increasing public repositories of available microarray data and data analysis tools have opened new avenues to genome-wide analysis of plant stress responses [278, 280].

Cassava (*Manihot esculenta* Crantz) is an important tropical root crop adapted to a wide range of environmental stimuli, such as drought and acid soils, but it is an extremely cold-sensitive species [286]. A transcriptome profiling of cassava apical shoots, that were submitted to a progressive cold stress, was conducted using a dedicated 60-mer oligonucleotide microarray representing 20,840 cassava genes has identified a total of 508 transcripts [287]. Those differentially expressed transcripts were identified as early cold-responsive genes in which 319 sequences had functional descriptions when aligned with Arabidopsis proteins. Various stress-associated genes with a wide range of biological functions were found, such as signal transduction components (e.g., MAP kinase 4), transcription factors (TFs, e.g., RAP2.11 and AP2-EREBP), and active oxygen species scavenging enzymes (e.g., catalase 2), as well as photosynthesis-related genes (e.g., PsaL). This work provided useful candidate genes for genetic improvement in this species and suggested that the dynamic expression changes observed reflect the integrative controlling and transcriptome regulation of the networks in the cold stress response of this important tropical root crop.

Drought is the major constraint to increase yield in chickpea (*Cicer arietinum*) [288]. SuperSAGE, an improved version of the serial analysis of gene expression (SAGE) technique, has been employed in the analysis of gene expression in chickpea roots in response to drought [289]. To achieve this goal 80,238 26 bp tags were sequenced representing 17,493 unique transcripts (UniTags) from drought-stressed and non-stressed control roots. A total of 7,532 (43%) UniTags were more than 2.7-fold differentially expressed, and 880 (5.0%) were regulated more than 8-fold upon stress. Their large size enabled the unambiguous annotation of 3,858 (22%) UniTags when searched against public databases. This comprehensive study demonstrated that signal transduction, transcription regulation, osmolyte accumulation, and AOS scavenging undergo a strong transcriptional remodeling in chickpea roots in early drought stress responses, suggesting potential targets for breeding for drought tolerance.

High-throughput transcriptome sequencing and digital gene expression (DGE) profiling are cost-efficient platforms that are predicted to change transcriptomic analysis, eliminating the need for restriction enzyme digestion of DNA samples, PCR-based genomic amplification and ligation of sequence tags; they are additionally a suitable choice for characterizing non-model organisms without a reference genome [290-291]. Furthermore, RNA-seq can produce a complete coverage of transcripts, providing information about the sequence, structure and genomic origins of the entire transcript [285]. The dynamic transcriptome expression profiles of poplar (*Populus simonii* × *Populus nigra*) under salt stress were investigated using Solexa/Illumina digital gene expression technique [292]. A total of 5453, 2372, and 1770 genes were shown to be differentially expressed after exposure to NaCl for 3 days, 6 days and 9 days, respectively. Differential expression patterns throughout salt stress identified 572 genes, most of them mapped to the Gene Ontology term “receptor activity”, “transporter activity” and “response to stress”. Importantly this study showed that the greatest upregulation was observed for the POPTR_0018s02240.1 transcript encoding a serine/threonine protein kinase. Serine/threonine protein kinases have been reported to confer enhanced multi-stress tolerance in many plants [293], suggesting that this gene can be a suitable target for biotechnological manipulation with the aim of improving poplar salt tolerance.

The recent rapid accumulation of dataset containing large-scale gene expression profiles has supported the development of dedicated web databases acting as large public repositories, where data and underlying experimental conditions are widely described. A very complete and comprehensive list of searching database may be found in [294]. With the completion of the genome sequencing of several model and crop plants, these repositories can constitute important functional resources to be explored to decipher the molecular mechanisms underlying abiotic stress responses.

5.2. Proteomics

Proteomics may be defined as the science that studies the proteome, i.e. the number of proteins expressed in a given cell, tissue, organ, organism or populations. Proteomics is normally associated to two types of studies: 1) the characterization of a proteome in which all the proteins expressed in a given cell, tissue, organ, organism or populations are identified; and 2) differential proteomics in which a proteome of for instance a plant under control conditions is

compared to the proteome of the same plant under study conditions such as the exposure to a heavy metal or water deficit, or in another example the comparison of protein expression profiles between different varieties of wheat.

Proteomics is heavily dependent on two laboratory techniques, protein electrophoresis (particularly two-dimensional electrophoresis and DIGE – Difference In Gel Electrophoresis) and protein identification using mass spectrometry. For further information on these approaches, kindly refer to the reviews by Minden [295] and Soares *et al.* [296] on respectively DIGE and mass spectrometry based protein identification strategies. Proteomics, particularly differential proteomics, has been widely applied to the study of the effects of several abiotic stresses on plant organs and tissues. The subject has been the object of a recent and extensive review [297]. For this reason, in this section we will provide examples on the use of proteomics to study the effects of abiotic stress in plants.

Evers *et al.* [298] have used both transcriptomics and proteomics to study the effects of cold and salt stresses on the leaf transcriptome and proteome of potato (*Solanum tuberosum*). Results pointed out to a number differentially regulated genes and proteins at the level of both stresses. Interestingly, salt exposure results displayed a strong down-regulation of genes implicated in primary metabolism, detoxication apparatus and signal transduction, whereas upon cold exposure, up and down-regulated genes were similar in number. On the contrary, proteome analysis seems to point out to an increase in protein expression of almost every protein with the exception of those with a role in photosynthesis. The results from this study highlight not only the differences between transcriptome and proteome expression as a consequence of cold and salt stresses but it particularly shows how the proteome analysis tends to be much more thorough and complete than transcriptome analysis.

In another example, DIGE has been used to study the effects of high level of UV radiation on the leaf proteome of artichoke, particularly targeting the levels of inducible antioxidants present in this species [299]. Authors observed a total of 145 spots showing differential expression and were able to identify 111 of them. Most of the proteins differentially modulated were chloroplast located, involved in photosynthesis, sugar metabolisms, protein folding and stress responsive, shedding a new understanding on the physiological and metabolic alternations induced by UV radiation exposure.

The embryo proteome of six rice varieties subjected to water deficit stress has been compared in order to further understand the mechanisms leading to water-stress tolerance in this crop [300]. A total of 28 proteins were identified involved in stress tolerance (LEA proteins), nutrient reservoir activity, among other proteins implicated in diverse cellular processes potentially related to the stress response (e.g., mitochondrial import translocase) in this cereal. Authors were also able to identify several differences and the post-translational level, particularly in the late embryogenesis abundant Rab21 that was more strongly phosphorylated in the embryos of the sensitive varieties than in the embryos of the tolerant ones. Similarly to the example by Evers previously mentioned, this study clearly demonstrates the broadness and completeness of proteome studies, particularly at the level of Post Translational Modifications (PTMs).

These three simple examples illustrate the advantages of the use of (differential) proteomics to study the effects of different abiotic stresses such as water deficit, temperature or UV exposure. Results show a large number of proteins being affected by abiotic stresses and the metabolic pathways that are subsequently affected and at what levels they are affected. The advantages of proteomics are further highlighted by the possibility to study PTMs of key importance in plant's physiological and biochemical responses to stress.

5.3. Metabolomics

Higher plants have the remarkable ability to synthesize a vast array of compounds that differ in chemical complexity and biological activity, playing indispensable roles in chemical defenses against biotic and abiotic stresses [301, 302]. In such context, it is obvious that Metabolomics (i.e. the study of the metabolome, or the set of metabolites found in a given plant tissue or organ) plays a significant role in bridging the phenotype-genotype gap [303]. The increasing number of publications in this subject also supports that metabolomics is not just a new Omics but a valuable tool to study phenotypes and changes in phenotypes induced by biotic and abiotic stresses (reviewed in [303]).

Metabolomics experiments start with the acquisition of metabolic fingerprints or metabolite profiles using various analytical instruments and separation technologies based in the physico-chemical properties of each metabolite [280]. Since there is no single technology currently available (or likely in the near future) to detect all compounds found in plants or any other organism, a combination of multiple analytical techniques, such as gas chromatography (GC), liquid chromatography (LC), capillary electrophoresis (CE) coupled to Mass Spectrometry (MS), and Nuclear Magnetic Resonance (NMR) are generally performed following established protocols (reviewed in [280, 301]).

Metabolomic profiling of plants under stress is an important approach to study stress induced change in metabolites pools. In most of these studies, metabolite profiles are analyzed in combination with transcriptomic analysis: a strong correlation between metabolite levels is often correlated to a specific gene underlying a specific response or phenotype observed [280, 304]. In the recent past, the majority of the metabolic works have occurred in model species such as *Arabidopsis* [305] but nowadays, such metabolomic technologies are being used with success in forages [306], cereals [307] and other food crops [308].

Common bean (*Phaseolus vulgaris* L.) is one of the most important legume crops for human consumption but its productivity is often limited by low Phosphorus (P) levels in the soil [309]. Coupled to a transcriptomic approach, a non-biased metabolite profiling of bean roots using GC-MS was done to assess the degree to which changes in gene expression in P-deficient roots affect overall metabolism [308]. A total of 81 metabolites were detected and 42 were differentially expressed between -P to +P response ratios. Stress related metabolites identified such as polyols accumulated in P-deficient roots as well as sugars, providing additional support for the role of these compounds for P stress. The metabolomic data supported the identification of candidate genes involved in common bean root adaptation to P deficiency to be used in improvement programs.

A recent study in maize was conducted to understand the combined effects of enhanced atmospheric CO₂ and drought on the stress responses by monitoring foliar metabolites (LC and GC-MS) and transcripts [307]. The concentrations of 28 out of 33 leaf metabolites were altered by drought. Soluble carbohydrates, aconitate, shikimate, serine, glycine, proline and eight other amino acids increased, and leaf starch, malate, fumarate, 2-oxoglutarate and seven amino acids decreased with drought. Overall analysis of both transcriptomic and metabolomic data supported that water stress inhibited C₄ photosynthesis and induced photorespiration in this species.

In plants, isoprene is a dual purpose metabolite that can act as thermo-protective agent proposed to prevent degradation of photosynthetic enzymes/membrane structures [310] and/or as reactive molecule reducing abiotic oxidative stress [311]. Gene expression and metabolite profiles of isoprene emitting wild type plants and RNAi-mediated non-isoprene emitting grey poplars (*Populus x canescens*) were compared by using poplar Affymetrix microarrays and non-targeted FT-ICR-MS (Fourier Transform Ion Cyclotron Resonance Mass Spectrometry) [312]. A transcriptional down-regulation of genes encoding enzymes of phenylpropanoid biosynthetic and regulatory pathways, as well as distinct metabolic down-regulation of condensed tannins and anthocyanins, in non-isoprene emitting genotypes was seen, when high temperature and light intensities possibly caused a transient drought stress. The results suggested that non-isoprene emitting poplars are more susceptible to environmental stress and provided new evidences about the physiological and ecological roles of isoprene in the protection of plants from environmental stresses.

6. Conclusions and final remarks

The Intergovernmental Panel on Climate Change 2012 (IPCC, 2012) indicated that temperature rising, drought, floods, desertification and deterioration of arable land and weather extremes will severely affect agriculture, especially in drought-prone regions of the developing world [313]. Regarding food security, this threatening scenario highlights the need for a globally concerted research approach to address crop improvement to mitigate crop failure under marginal environments. One of the major goals of plant improvement is to develop crops fit to cope with environmental injuries but still capable to achieve substantial yield under abiotic stress.

Data from traditional breeding, plant molecular breeding based in the development of molecular markers, candidate gene identification or gene expression profiles and from the use of transgenic approaches are becoming more and more frequent. Resulting plants are being evaluated in controlled conditions (greenhouse and growth chambers) but also, importantly, in the field to confirm the generation of improved cultivars. Despite the difficulty to establish reliable methods to assess new breed or engineered plant phenotypes as result of those approaches, some efforts are anticipated to fulfill the gap between plant molecular biology and plant physiology.

Several stress-resistant genes encoding for functional proteins were identified and introduced via genetic engineering into model species such as *Medicago truncatula*, *Nicotiana tabacum* or *Arabidopsis thaliana*, producing plants with improved abiotic stress tolerance. These results support the future use of this technology into economically important plants species namely crops and trees. As a consequence of the novel findings on the mechanisms underlying the regulation of gene expression under abiotic stress, we could speculate that future genetic engineering approaches might be targeted to these regulatory pathways. Emerging reports where the expression of regulatory molecules such as transcription factors (e.g. NAC proteins) or components of the small RNA pathway (e.g. miR398) are described to successfully produce abiotic stress resistant plants, supporting our hypothesis. Nevertheless, it should be kept in mind that the success of this approach relies on the development of efficient regeneration and transformation methods adequate to the target species or genotype. Future research efforts should be directed to overcome this significant limitation. Although the use of a constitutive promoter (e.g. CaMV 35S) ensured the expression of the target coding sequence, it presents some disadvantages as discussed previously. The use of inducible promoters (e.g. rd29A) that allow the expression of a transgene only when it is required could therefore be the ideal solution.

As stated previously across this manuscript, the nature and complexity of abiotic stress responses supports the use of global, integrative and multidisciplinary approaches to understand the different levels of regulation of stress responses. The emerging holistic System Biology approaches still enclose a myriad of unexploited resources for Plant and Agricultural Sciences. Given the increasing development of high throughput genomic tools and concomitant release and progress on plants genome sequencing, it is now possible to gain information in a global scale, providing an overall comprehensive and quantitative overview on the gene-to-metabolite network associated to a particular plant response. The use of such cutting-edge methodologies to a specific plant species requires a previous study of the availability of reference genomes (e.g. Phytozome [314]), metabolite (e.g. Plant Metabolic Network [315]) or proteomic databases (e.g. UniProtKB [316]). Additionally, it requires appropriate laboratory, equipment and bioinformatics facilities and know-how that can be accessed using own institutional infrastructures or taking advantage of established collaborations with renowned research institutional research platforms and /or commercial service providers.

Presently, we are exploring the molecular mechanisms underlying *Medicago truncatula* and *Phaseolus vulgaris* adaptation to water deprivation using a System Biology approach that combines whole plant physiology data with transcriptomics, proteomics and metabolomics. We aim to identify candidate genes to be used in legume improvement programs and also fundamental knowledge on points of transcriptional, post-transcriptional and post-translational regulation of the gene expression under stress in these species. This highlights the efforts that we are currently doing to transfer the developed tools and information gained with the model *Medicago truncatula* to an important grain legume crop. A robust identification of the molecular targets to be used in biotechnological applications will be elucidated. Additionally, some clues about the signaling, regulation and interaction between the different cellular players involved are also expected.

In due time, it is expected that Omics and System Biology approaches provides a comprehensive knowledge of the plant responses to abiotic stresses making a significant progress in developing crops and trees with desirable traits as increasing yield and quality under abiotic stress and contribute to sustainable agriculture development.

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