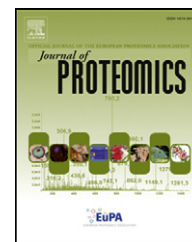


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## Review

# Coping with abiotic stress: Proteome changes for crop improvement☆

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## ABSTRACT

Plant breeders need new and more precise tools to accelerate breeding programs that address the increasing needs for food, feed, energy and raw materials, while facing a changing environment in which high salinity and drought have major impacts on crop losses worldwide. This review covers the achievements and bottlenecks in the identification and validation of proteins with relevance in abiotic stress tolerance, also mentioning the unexpected consequences of the stress in allergen expression. While addressing the key pathways regulating abiotic stress plant adaptation, comprehensive data is presented on the proteins confirmed as relevant to confer tolerance. Promising candidates still to be confirmed are also highlighted, as well as the specific protein families and protein modifications for which detection and characterization is still a challenge.

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## 1. Introduction

The global financial crisis (rising inflation and unemployment) together with the increases in food prices and world population are serious threats to the Millennium Development Goals that aimed to eradicate extreme poverty and famine. As wisely warned by Albert Sasson [1] a key cause to the global crisis has been the reduced investment in agriculture (only 4% of public investments and 4% of development aid) and the present worldwide insufficient food supply.

Having reached 7 billion people in October 2011, the world population may still increase by 34% until 2050 (70% being located in cities). With the strong competition for land use, feeding the expected 9 billion people will necessarily depend on increasing yields per unit area, rather than increasing cropping area [2]. This may be especially difficult under the changing environmental conditions. To meet the sustainability requirements of “producing more with less” (water, nutritional inputs, pests and disease treatments), most challenges thus fall on agricultural practices (agronomy) and genotype improvement (breeding). As warned by FAO, cereal crops will need to rise to about 3 billion tons (from the present 2.1 billion) [3]. Moreover, the fact that several food crops are currently being used for the production of biofuels puts an extra pressure on world food supplies [4].

In spite of the minor benefits to agriculture from the global environmental changes (e.g.: alleviation of low-temperature growth inhibition, or increased CO<sub>2</sub> fertilizing effect on C3 crops), several major negative effects challenge human capacity to mitigate shortage in food and energy supplies [4].

Among these, the capacity to tolerate drought and salinity is of particular relevance in a global scale for the impact that these stresses have in numerous agricultural regions where subsistence farming depends on improved food crops (mainly Africa and Asia) [1].

To meet these challenges, the plant breeding programs must be increasingly grounded on scientific knowledge and supported by tools that permit to trace, along breeding, markers for relevant traits. The ability to survive abiotic stresses and also to maximize performance under less favoring conditions are thus major topics for research investment (although the mechanisms involved are often opposite). For its global relevance and impact on food security, in this review on translational proteomics we address the influence of abiotic stress, mainly salinity and drought, on plant growth and survival. The review focuses on the proteins (and their modifications) that have been identified and characterized as relevant for the plant tolerance strategy (spotting signaling, oxidative stress and photosynthesis and metabolism), aiming to identify the major breakthroughs and bottlenecks in the use of proteomics to improve crop adaptation to abiotic stress.

## 2. Crop proteomics for abiotic stress tolerance and the contribution from xerophytes and halophytes

Among crops, rice, wheat and maize provide approximately half of the calories consumed worldwide.

Despite the potential of proteomics to advance stress tolerance in crops, this is still an emerging field of research.

In recent past, numerous studies in crop proteomics were conducted using different genotypes and tissues, submitted to different stresses, thus complicating comparative analyses. To overcome this problem, several repositories where researchers can find proteomics information for functional analysis were built, such as the International Proteomics Research in Arabidopsis ([www.masc-Proteomics.org/](http://www.masc-Proteomics.org/)), the Maize Proteome DB ([ppdb.tc.cornell.edu/dbsearch/searchcomp.aspx](http://ppdb.tc.cornell.edu/dbsearch/searchcomp.aspx)), the Soybean Proteome DB ([proteome.dc.affrc.go.jp/Soybean/](http://proteome.dc.affrc.go.jp/Soybean/)) or the Organellome ([podb.nibb.ac.jp/Organellome](http://podb.nibb.ac.jp/Organellome)) among many other databases (reviewed in [5]). Recently, a global plant proteomics organization was developed to establish, preserve and disseminate collected information on plant proteomics, the 'International Plant Proteomics Organization (INPPO; [www.inppo.com](http://www.inppo.com)) [6], and hopefully the increasing INPPO community will boost plant proteomics research.

In plant abiotic stress studies, it is common to analyze proteomes by contrasting stressed plants against control ones, attempting to correlate changes in protein accumulation with the plant phenotypic response. Additionally, comparisons between genotypes with different sensitivity towards stress (sensitive vs. tolerant) are crucial to understand the putative influence of differentially abundant proteins in tolerant genotypes. In Fig. 1 we present a general workflow of the process of protein discovery and functional validation. Positive leads are selected for crop improvement and the plants assessed for safety and quality in field trials. As a general tendency, it is observed that tolerant genotypes not only increase oxidative stress metabolism and the scavenging capacity of reactive oxygen species (ROS), but also show enhanced carbohydrate metabolism and more efficient photosynthesis [7–10].

A protein change in response to abiotic stress is a complex mechanism, in most cases species- and genotype-dependent. For instance, in wheat, the tolerant genotype 'Khazar-1' showed an increase in accumulation of thioredoxin (Trx h) under drought field conditions while, in contrast, the sensitive genotypes 'Afghani' and 'Arvand' showed a decrease [7]. However, under salt-stress, an increase in Trx was observed in both tolerant and sensitive barley genotypes [11]. Moreover, Hajheidari et al. [7] observed the existence of three isoforms of Trx h that showed different response patterns in the contrasting genotypes; two of the isoforms increased in the tolerant genotypes and decreased in the sensitive accession. Another example of the different mechanisms of protein regulation during stress response comes from drought stressed maize, where two isoforms of HSP26 responded differently in two contrasting genotypes [12]. Furthermore, the embryo proteome of six different rice genotypes (with contrasting responses to drought, salt and cold) showed differences in the post-translational status of a LEA rice Rab21 [13]. This stress protein was found more strongly phosphorylated in the embryos of sensitive genotypes than in tolerant ones. These examples highlight the diversity of regulation mechanisms controlling plant proteome and response to stress, and alert for the relevance of transient modifications controlling protein function, such as post-translational modifications (PTMs).

Introgression lines sharing the same genome and only differing in stress tolerance capacity are another interesting resource to search for putative markers of stress tolerance. The

effect of drought and salinity was evaluated by 2-DE/MS in the seedlings of wheat introgression line 'Shanrong No. 3' (tolerant) which derives from an alien chromatin introgression from tall wheatgrass and its parent line 'Jinan 177' (sensitive). The results showed that the tolerant genotype (Shanrong No. 3) had a constant expression of two ATPases, although that was not the case in the roots of the sensitive genotype [14]. Interestingly, a parallel transcriptomic study revealed identical expression levels of both mRNAs in the roots of the two genotypes suggesting that the specific detection of these proteins in the tolerant genotype resulted from either translational regulation or post-translational modification [14]. The study of a complex trait such as stress response is necessarily enriched by integrating data obtained through different approaches, including physiology and phenotypic data. By correlating maize proteomic and physiological results, Benešová et al. [12] postulated that the hypersensitive early stomatal closure in the sensitive genotype caused the inhibition of photosynthesis, thus leading to a less efficient synthesis of the protective/detoxification proteins associated with drought tolerance. Peng et al. [14] suggested that stress tolerant wheat genotypes are more capable of coping with salt due to the combined effect of ion homeostasis and a more effective capacity to scavenge ROS and other toxic by-products.

Important contributions to abiotic stress proteomics have also been obtained from comparisons with plants adapted to extreme salt or drought stress environments, namely halophytes and xerophytes, respectively.

A leaf proteomic comparison of *Arabidopsis* and its close halophyte relative *Thellungiella*, revealed significantly changed proteins under salt-stress [15]. The authors found more changes in protein abundance in *Arabidopsis* (88 spots against 37 in *Thellungiella*), with most of the identified proteins being involved in photosynthesis, energy metabolism, and stress response [15]. Another proteome comparison between rice (two contrasting genotypes) and *Porteresia coarctata* (a halophytic type of wild rice), allowed identifying in *P. coarctata* 16 proteins involved in osmolyte synthesis, photosystem functioning, RuBisCO activation, cell wall synthesis and chaperone activity [16]. It is interesting to note that two *Porteresia* genes (*PcINO1* and *PcIMT1*, absent in *Oryza sativa*) conferred salt-tolerance when over-expressed in tobacco [17,18]. However, the products of these genes could not be detected in *Porteresia* proteome in the study conducted by Sengupta and Majumder, using two-dimensional gel electrophoresis (2-DE) followed by MALDI-TOF [16]. This fact highlights the need for development of new methodologies for proteomic studies, for instance targeting specific organelles to improve the detection of low abundance proteins.

As for the xerophytes, it was found that, under drought, the proteome of the model resurrection plant *Sporobolus stapfianus* revealed changes in proteins associated with chromatin structure and function, namely an increased abundance of the SNF2P protein (an ATP-dependent chromatin-remodeling enzyme) [19]. Such modifications may be implicated in expression changes in genes important for drought adaptation. Although largely studied at the transcription level, resurrection plants have not been much studied by proteomics. Still, there are several examples of post-translational modifications of dehydration-induced proteins in these plants, namely evidence for phosphorylation of LEA proteins [20].

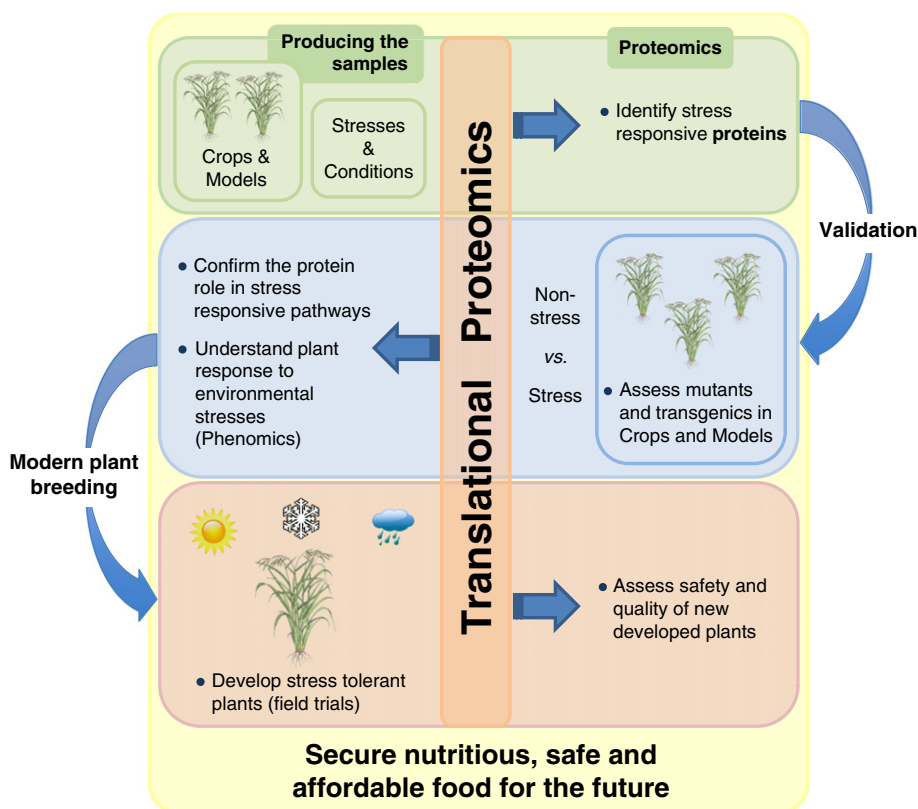


**Fig. 1 – Workflow for abiotic stress tolerance improvement in crops, identifying the three main stages: first the experiments leading to protein discovery, then the validation of protein function, and finally the application in modern plant breeding.**

There is still much to investigate regarding the proteome of halophytes and xerophytes as putative donors of stress tolerance, namely regarding post-translational modifications.

To better understand how crops cope with abiotic stress several authors selected for analyses the different plant tissues, organelles and developmental stages, depending on the targeted stress (reviewed by [21]). For instance, aiming to understand salt stress impact on rice fertility, Dooki and co-workers [22] targeted the proteome of young panicles of salt-stressed plants and identified 13 proteins significantly changed. The high sensitivity of rice to cold at the reproductive stage was also expressed in the differential abundance of 37 proteins [23].

Due to the importance of membranes, proteome studies specifically targeting the plasma-membrane (PM) have also proved crucial to identify some membrane-located low-abundance proteins. By targeting the plasma-membrane proteome in salt-stressed versus control rice, Cheng et al. [24] were able to reveal 18 proteins that changed their abundance, six of them for the first time reported as salt-responsive. Additionally, the work of these authors led to the identification of the novel leucine-rich-repeat type receptor-like protein kinase, OsRPK1, as a salt-responding protein. In soybean, through a combined gel-based approach and LC-MS/MS-based proteomics, it was possible to detect calnexin accumulation in the PM under osmotic stress [25]. The authors also suggested that osmotic stress led to the increase of ion efflux by the up regulation of a PM H1-ATPase protein [25].

Besides the need to develop specific methodologies to identify low-abundant proteins or especially relevant protein modifications (Section 3), or to specifically target organs or cell compartments to better understand the tolerance mechanisms, it is also crucial to give support to the proteomics information with phenotypic data, in order to extrapolate biological meaning (Section 4). In Fig. 2 we highlight some of the relevant issues in the process of protein discovery for abiotic stress tolerance.

### 3. Methods in proteomics to address abiotic stress

In response to stress, plants activate specific cellular programs that result in the accumulation of protective proteins and in modifications in metabolism and development. To achieve this, plants experience dramatic changes in their gene expression programs. The modulation of gene expression is operated by several families of TFs and by signal-sensing networks of phosphorylation/dephosphorylation events and other PTMs that can stabilize TFs or target them for degradation. The usual low abundance of TFs and the rapid and transient nature of protein PTMs (e.g., phosphorylation, cysteine oxidation/reduction) complicate their detection and quantification through proteomics [26]. So far the proteomic methodologies targeting the identification of low abundant proteins and PTMs rely mostly on the use of the model plant *Arabidopsis*, due to the

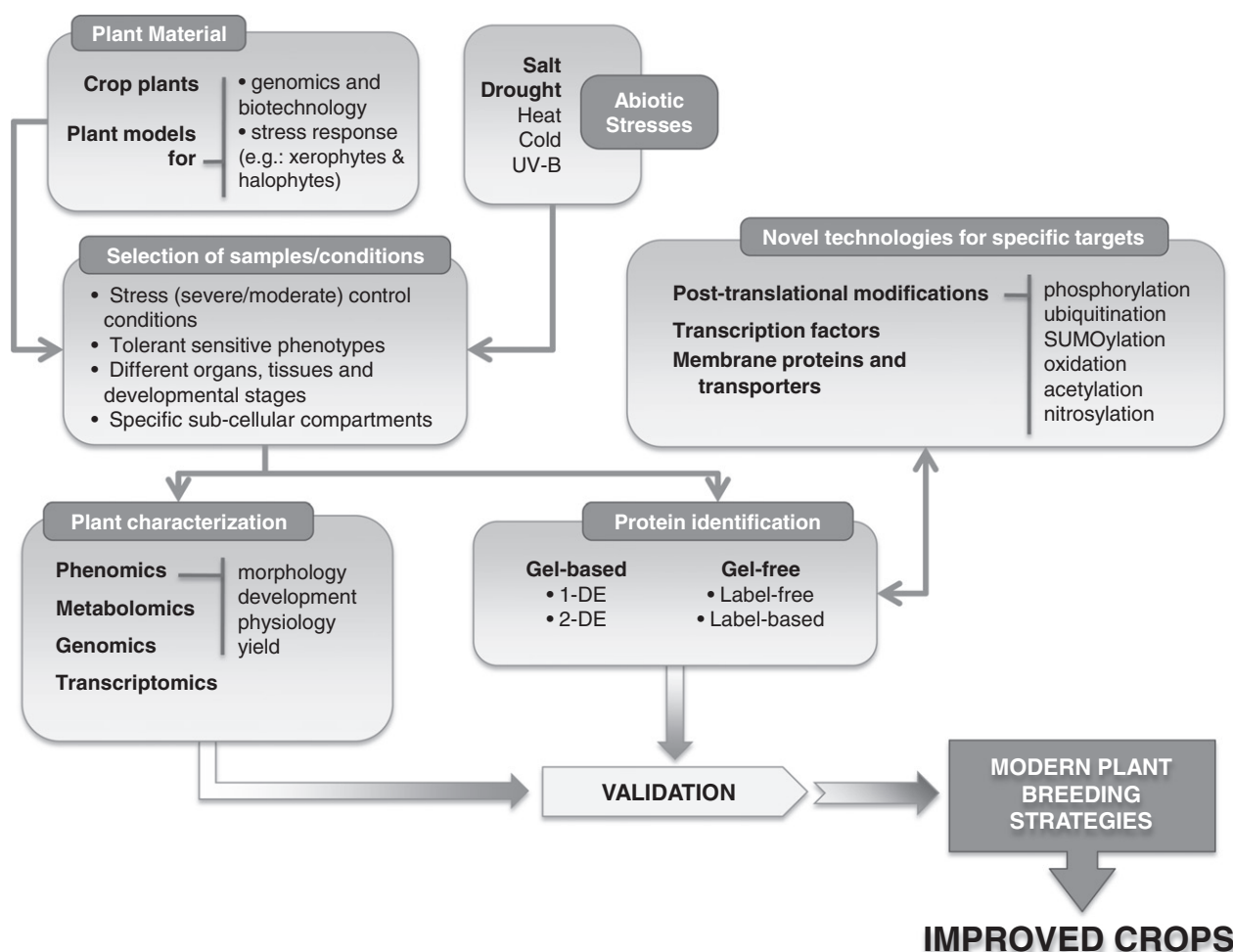


**Fig. 2 – Detail of Fig. 1 workflow, highlighting the importance of the selection of samples and conditions, of precise plant characterization and of novel technologies to identify proteins and their modifications that may be useful for crop improvement.**

numerous tools and methodologies already available or that can rapidly be produced (e.g.: establishment of auxotrophic mutant cell lines; production of transgenic lines overexpressing tagged proteins; available cellular fractionation methodologies). But, although most of these findings can be translated into crops, it is likely that specific protein regulation may differ among species. For instance it is known that in *Arabidopsis*, the NAC transcription factors (TFs) mediate gene expression in response to dehydration, whereas in rice they show additional functions [27]. Thus it is crucial to complement *Arabidopsis* proteomic studies with studies done in other plant models, such as rice as the main cereal model crop. This chapter covers the most relevant proteomic methodologies available and emerging techniques that may prove instrumental to advance translational plant proteomics.

### 3.1. Gel-based and gel-free quantitative proteomics approaches

The techniques employed in crop proteomics are still largely dependent on gel-based approaches rather than gel-free based methodologies involving liquid chromatography coupled to

mass spectrometry (LC-MS). It is not surprising that two-dimensional gel electrophoresis is still the preferred method for protein separation in crop proteomic studies [28] considering that nanoscale LC chromatographic separation techniques are technically demanding, requiring highly purified samples. Considering iTRAQ (isobaric tags for relative and absolute quantification) [29,30], a gel-free quantitative proteomics strategy involving protein chemical labeling, the sample preparation is complex, time-consuming, and specific software is needed to analyze the MS-derived data [31]. Nevertheless, there is an increasing number of studies addressing abiotic stress responses through iTRAQ approaches [15,32–34].

Since its implementation in the middle 70s [35,36], 2-DE application expanded considerably [37] and has evolved to more sophisticated methodologies e.g., two-dimensional difference-in gel electrophoresis (2-D DIGE) [38–40], to overcome problems such as gel-to-gel variations. 2-D DIGE is one of the most popular non-MS based quantitative methods for detecting differences in protein abundance arising with abiotic stress [41–45]. It allows running multiple protein samples in the same gel and identifying protein provenience, because each sample is labeled with a different fluorescent dye. Other similar methodologies based on

multiplex-fluorescence 2D gel electrophoresis are also available, like Refraction-2D™ (NH DyeAGNOSTICS) [46]. Nevertheless, the tendency is that gel-free based techniques will be increasingly used in plant proteomics research as the complexity of samples to be analyzed is also increasing e.g., in studies of proteome-dynamics to obtain information about protein synthesis and degradation; and to fully characterize PTMs as essential players of functional proteomics.

One of the major drawbacks of 2-DE is its limitation for detection of low-abundance proteins such as those involved in signal transduction pathways (e.g., transmembrane receptor-like kinases, intracellular receptors and kinases) or regulatory proteins (e.g., transcription factors) which have a crucial role in abiotic stress response. These signaling/effector proteins can be separated using gel-based approaches (either 1-DE or 2-DE) but often require pre-fractionation methods to enrich in specific sub-cellular compartments followed by isolation of specific proteins or protein complexes by immuno-precipitation or affinity purification of (usually tagged) proteins [47]. Still, a 2-D gel provides a visual representation of the proteome in which distinct protein isoforms resulting from changes in Mr and/or pI can be observed, something that LC-MS-based methods do not allow [31]. 2-DE is thus expected to work as a complementary tool to novel gel-free based approaches for quantitative proteomics [31,48].

### 3.2. Gel-free quantitative proteomics approaches involving metabolic labeling

MS-based methodologies may be either label-based or label-free. Label-free methodologies rely on the relative comparison of MS ion signals e.g., LC/MS<sup>E</sup> that averages the signal intensity of the three most intense tryptic peptides [49], whereas label-based methods involve the chemical or metabolic labeling of proteins or peptides. Bindschedler and Cramer [50] and Abdallah et al. [51] recently reviewed the techniques that are being used in plant quantitative proteomics (MS-based techniques involving both label-based and label-free strategies, and non-MS based), considering the advantages and drawbacks of each technique (see [31] for quantitative methods applied to crop proteomics). Also recently, Matros et al. [48] focused on the progress in LC-based protein separation and label-free quantitative methodologies employed in functional proteomic analyses in plants. These reviews provide extensive information about the use of quantitative proteomics approaches in plant biology. This review mainly focuses on gel-free quantitative approaches using metabolic labeling due to their relevance for abiotic stress studies.

#### 3.2.1. Stable isotope labeling by amino acids in cell cultures (SILAC)

Metabolic labeling approaches are most powerful tools to address dynamic quantitative proteome changes occurring during the activation of plant signaling events in response to stress [52–55]. This method however, still relies on the *in vivo* labeling of cultured cells, that limits its application to the study of fast proteomic responses (e.g.: cell signaling) and to those species for which cell cultures are available. Moreover, undifferentiated cell cultures may be unable to mimic cell behavior in fully differentiated plants.

Metabolic labeling is achieved either by the incorporation of stable isotopes at single amino acid level, as in the case of SILAC [56], or through the enrichment of growth media with <sup>15</sup>N-labeled inorganic salts [57], therefore allowing protein labeling at the time of protein synthesis. By contrast, chemical labeling (e.g., isotope-coded affinity tag/iCAT [58] and iTRAQ [29,30]) occurs only after protein extraction or even after proteolytic digestion, thus reflecting a more “static” view of the proteome as compared to the “dynamics” of metabolic labeling. Nevertheless, chemical-labeling is essential to analyze quantitative changes in the proteome of stressed plants as it may target downstream stress-responsive proteins with a direct role in cellular protection, such as LEAs or those involved in oxidative stress protection, as further discussed below.

In a typical SILAC experiment, two cell cultures should fully incorporate certain essential amino acids (i.e., Arg or Lys), one culture being fed the naturally occurring amino acid (the “light” cell culture) and the other the amino acid with its isotope counterpart (the “heavy” cell culture), to enable the relative quantification of protein abundance between the two cultures [56,59]. Moreover, the cell lines to use must be auxotrophic for the amino acid to be labeled. So, it can be argued that SILAC seems to be less appropriate for quantitative proteomics in plants due to their autotrophic nature, thus resulting in low efficiency labeling with exogenously supplied labeled amino acids [51,60,61]. But still it can be most useful and applied to characterize plant protein expression in cell cultures in response to stress treatments, with a special focus on the signaling events triggered by PTMs on specific proteins [52,53,59]. SILAC in plants was first described in *Arabidopsis* suspension cells to accurately quantify the changes in abundance of several glutathione S-transferase isoforms in response to salicylic acid and to analyze the signal-dependent phosphorylation events during that treatment [52]. One of the major drawbacks was that the maximal incorporation of labeled amino acids was only about 80% after 5 days in culture. To overcome the bias introduced by incomplete labeling in plants, a simple modification of SILAC was achieved enabling similar quantitation accuracy and reproducibility as conventional SILAC in animal cells, by comparing *Arabidopsis* cells cultures labeled with two versions of heavy Lys (Lys-4 and Lys-8) [59]. The strategy still suffers notable limitations, like being restricted to dark-grown cells, but the authors argue that it can be applicable to the analysis of short-term processes like signal transduction. However, for the photosynthetic green alga *Chlamydomonas reinhardtii*, an emerging model system for plant biology, SILAC has been successfully employed to study the dynamic changes of metabolism and proteome turnover under salt stress conditions [53]. In this study, *de novo* protein synthesis under different salt concentrations could be analyzed using an arginine auxotrophic strain, cultivated for 24 h in the presence of a stable isotope-labeled arginine.

Although the full implementation of SILAC technique in higher plants could be extremely complex, requiring for instance the establishment of auxotrophic mutant cell lines for Arg and Lys it could offer unique insights into the early events of signal transduction and protein turnover in response to environmental changes.

### 3.2.2. Metabolic labeling for quantitative proteomics

Metabolic labeling with  $^{15}\text{N}$  has been successfully applied for quantitative proteomics in several eukaryotic model organisms, including plants and green algae [54,55,57,62–65]. The growth of cells in medium containing  $^{15}\text{NH}_4\text{Cl}$  as nitrogen source is notably cheaper than the  $^{13}\text{C}$ -Arg used in SILAC. However, the quantification of  $^{15}\text{N}$ -labeled tryptic digests can be quite challenging due to the limitations in the current available tools that do not support either  $^{15}\text{N}$ -based quantification or large amounts of data, thus requiring new software solutions. Mühlhaus and co-workers [54] developed a software framework, IOMIQS, to overcome these problems, and were able to employ successfully  $^{15}\text{N}$  metabolic labeling and MS quantitative shotgun proteomics to monitor the time-dependent proteome dynamics in response to heat stress, in the green alga *C. reinhardtii*. More recently, Martin et al. [64] developed a method to quantify protein turnover and calculate synthesis and degradation rate constants of individual proteins in autotrophic organisms like algae and plants. The automated analysis of partial stable isotope incorporation with  $^{15}\text{N}$  was conducted over a 144-hour time frame, and this was only possible due to the development of a novel suite of Perl scripts named “Protein TurnStILE (turnover by stable isotope labeling experiments). This new and exciting approach offers new opportunities to determine the changes in protein turnover rates when a plant has to adapt to new environmental conditions. The general strengths and weaknesses of using  $^{15}\text{N}$  labeling were recently reviewed by Arsova et al. [66].

Like SILAC, the above mentioned methodologies rely essentially on the use of plant cell cultures or unicellular organisms e.g., *C. reinhardtii* and *Ostreococcus tauri*, raising again the question of what happens at the whole organism/plant level. In contrast, the hydroponic isotope labeling of entire plants (HILEP) enables the metabolic labeling of the whole plant, by growing plants in hydroponic culture containing  $^{15}\text{N}$  inorganic salts as the sole nitrogen source [67,68]. HILEP was most suitable for relative protein quantification in *Arabidopsis* plants submitted to oxidative stress, with close to 100% of  $^{15}\text{N}$ -protein labeling success [68]. Alternative methods employing in vivo metabolic labeling at the whole plant level make use of solid medium to grow plants, instead of hydroponics, thus allowing a better simulation of the natural environment. This is the case of SILIA (stable isotope labeling in *Arabidopsis*), which uses a modified agar-based medium [69] and SILIP (stable isotope labeling in *planta*) which uses a soil-based medium [70]. Nevertheless, hydroponics overcomes the need of having plants in aseptic conditions (as required for SILIA). Hydroponics is not only extensively used in plant research, but also as a modern farming technique for intensive and high-yield crop production. Regarding response to abiotic stress, hydroponics has been largely and successfully used to assess differences in transcript and protein abundance, in response to drought [71,72], salt [73,74], oxidative [75] or even heat stress [76]. The addition of polyethylene glycol (PEG) 6000 in hydroponic cultures to induce water stress has been employed to disclose the mechanisms of stress tolerance among wheat genotypes with different levels of drought tolerance [77,78], as a screening system for drought tolerance in sunflower [79], to test the level of water stress tolerance of maize transgenic

plants overexpressing the *LEA Rab28* gene [71], to induce water stress in maize plants analyzing inhibition of leaf elongation [80], and in *Medicago* seeds to study DNA repair responses activated during seed imbibition [81]. Also, the application of NaCl in hydroponic culture has been widely used to screen for plant salt tolerance [82,83]. The technical simplicity associated to the establishment of hydroponic cultures, together with their cost effectiveness, is making HILEP or similar  $^{15}\text{N}$ -labeling methods increasingly popular for in vivo labeling of plant proteins in quantitative proteomic approaches [67]. The big challenge for the implementation of such techniques is data analysis, due to the presence of peptide doublets with variable mass difference and incomplete labeling. Specific software is therefore needed to allow automation of  $^{14}\text{N}/^{15}\text{N}$  peak picking for peptide identification and measurement of ion intensities in extracted ion chromatograms (as reviewed by Bindschedler et al. [67]).

To sum up, the application of stress treatments either in hydroponic or solid media combined with metabolic labeling either at cell culture level or whole plant level will definitely contribute to deepen our knowledge on the cellular dynamics involving proteome changes during plant stress adaptation. The relevance of these techniques should be highest when addressing the rapid and transient protein PTMs regulating the signaling pathways in response to abiotic stress.

An emerging technology in plant proteomics with enormous potential to study crop responses to abiotic stress is MALDI-imaging mass spectrometry. This MS-based technique identifies which molecules and to which extent they are expressed in a certain cell or tissue; however, it is still very far from routine application, requiring many improvements in sample preparation and data handling (recently reviewed in [84,85]). In most cases, this technique has been applied to metabolite rather than peptide identification and the technique has not yet been extended to detect changes occurring in plant tissues subjected to abiotic stress.

The implementation of quantitative proteomics in plants can be most difficult due to the differences in genome, physiology, and culture, as highlighted by Bindschedler and Cramer [50]. In fact, the high dynamic range of protein abundances in plant proteomes, the high level of proteases, the presence of substances that can interfere with reproducible separation and/or MS detection (cell wall polyphenols, polysaccharides, starch and lipids) can turn the routine implementation of protein quantitative analyses quite challenging [48,50].

## 4. Phenomics to interpret proteomics data

Precise phenotypic evaluation of plants grown in highly standardized environments is essential to allow comparisons of data sets [86], namely those derived from proteomic studies in order to fulfill the goals of translational proteomics for abiotic stress tolerance in the most important crops.

The high standardization of plant growth and stress imposed conditions, in which the physiological status is strictly monitored along time (through, for instance, non-destructive imaging techniques) is now emerging under the name of phenomics. To achieve the required level of standardization, high-throughput, controlled-environmental facilities are required not only to

improve precision, but also to reduce the need for replication in the field [86]. Although high-throughput phenotyping is still not widely available due to the high costs associated, several initiatives to implement appropriate phenotypic assessment have been undertaken, either improving imaging (Lemnatec GmbH, Aachen, Germany, [www.lemnatec.de](http://www.lemnatec.de)), or other quantification strategies for non-visible traits (e.g.: nutrient content). Recently, the European Plant Phenotyping Network (EPPN, [www.plant-phenotyping-network.eu](http://www.plant-phenotyping-network.eu)) started offering access to 23 different phenotyping facilities (Mar. 2012–Dec. 2015) distributed in Germany, France, Hungary and UK (UK National Plant Phenomics Centre, [www.phenomics.org.uk](http://www.phenomics.org.uk)). Also in Australia, plant phenotyping was recognized as a priority for crop improvement, and the Australian Plant Phenomics Facility (APPF, [www.plantphenomics.org.au](http://www.plantphenomics.org.au)) was created, joining facilities in Adelaide and Canberra. Moreover, Australia, Canada, France and Germany integrate the International Plant Phenotyping Network (IPPN, [www.plantphenomics.com](http://www.plantphenomics.com)).

One of the first biotech companies to recognize the importance of plant phenotyping was CropDesign (Ghent, Belgium, [www.cropdesign.com](http://www.cropdesign.com)) that dedicates most efforts to improve maize and rice.

Phenomics is now applicable to a wide range of plants and research areas, making high-throughput screens possible by reducing the time and labor needed for plant characterization [87]. When using high-throughput phenotyping to compare response curves of leaf development changes to soil water deficit, Granier and collaborators [60] were able to identify one drought-tolerant accession (An1) among 10 *Arabidopsis* genotypes. The potential of phenomics to generate reliable phenotypic data that can be correlated with protein function is particularly important to improve crop stress response.

## 5. Key metabolic pathways in abiotic stress adaptation

### 5.1. Signal transduction

#### 5.1.1. Calcium-sensing proteins

The signal transduction pathways of all organisms are complex systems of metabolic control and/or adaptation to the environment. In these mechanisms,  $\text{Ca}^{2+}$  plays an important role as a universal secondary messenger [88]. Transient changes in  $\text{Ca}^{2+}$  concentration in the cytoplasm are sensed and decoded by several  $\text{Ca}^{2+}$  sensors. Such sensors are often proteins that bind to  $\text{Ca}^{2+}$  and change their conformation in a  $\text{Ca}^{2+}$ -dependent manner [88]. Several families of  $\text{Ca}^{2+}$  sensors have been identified in plants. Among them, calmodulins (CaM), calmodulin-like proteins (CML) and also CaM-binding proteins (CaMBP) are known to be up-regulated by stress-induced  $\text{Ca}^{2+}$  signaling [89].

In *Arabidopsis*, the calmodulin AtCaM3 is involved in the activation of HSP by specific TFs, thus establishing the link between calmodulins and thermo-tolerance [90,91]. Other calmodulin-binding transcription factors (CAMTA) such as AtCAMTA1 and AtCAMTA3 are positive regulators of the CBF family, thus being fundamental in low temperature acclimation through the CBF regulon [92]. Moreover, in *Arabidopsis*,

several reports linked overexpression of endogenous CaMPBs and CLMs to an increased cold, salt, drought-tolerance and also ABA sensitivity [93–95]. Using *Arabidopsis* as a heterologous system, it was found that a soybean CaMBP receptor-like kinase (GsCBRLK) enhanced salt-tolerance [96]. Also, transgenic *Arabidopsis* expressing rice CML (OsMSR2) showed an increased drought- and salt-tolerance [97]. Altogether, these studies demonstrate the importance of calmodulins as key players in the transduction of stress-response signals.

Another  $\text{Ca}^{2+}$ -binding protein, calreticulin, which is a known chaperone responsible for protein folding in the endoplasmic reticulum [98], is differentially accumulated in response to salt stress [99–103]. Transgenic *Arabidopsis* plants overexpressing calreticulin CRT2, displayed reduced resistance to virulent *Pseudomonas syringae* pv. tomato, thus demonstrating the CRT2 response to pathogen defense and regulation role in the plant innate immunity [104]. The importance of calreticulin in response to abiotic stress was highlighted by the overexpression of wheat TaCRT in tobacco plants that showed an overall enhanced drought tolerance [105].

#### 5.1.2. 14-3-3 proteins

14-3-3 proteins are phosphoserine-binding proteins that are modulators of a wide array of targets through direct protein–protein interactions [106]. In plants, an increasing number of 14-3-3 proteins were found, suggesting their importance in plant development [107], and affecting the activity of various enzymes and ion channels of central importance in plant biochemistry [106]. 14-3-3 proteins were already linked to nutrient deficiency responses in tomato and *Arabidopsis* [108–110]. Numerous proteomic studies have shown a differential accumulation of 14-3-3 proteins in response to abiotic stresses (namely drought and salt) in crops such as rice, wheat and maize [19,24,25,102,111–116]. One of the ways 14-3-3 proteins appear to influence abiotic stress responses is through the stress-induced ABA pathway [107]. This mutual regulation is supported by transactivation studies [117] and by its interaction with AREB/ABF/ABI5-like transcription factors which are direct regulators of ABA-responsive genes [118]. A major effect of ABA response is stomatal closure to prevent water loss, which is partially due to the reduction of an  $\text{H}^{+}$ -ATPase that is a direct interactor of 14-3-3 proteins [119]. Cotton transgenic plants overexpressing an *Arabidopsis* 14-3-3 protein (GF14 $\lambda$ ) were found to be drought tolerant due to a higher photosynthetic efficiency when compared with control plants, related to an increased stomatal aperture [120]. Altogether, these studies reinforce the role of 14-3-3 proteins in stress response through the regulation of ABA pathway.

#### 5.1.3. G-proteins

G-proteins (either heteromeric G-proteins or small monomeric G-proteins) are GTP-binding proteins that, together with G-protein coupled receptors, represent an essential component of signal transduction pathways in all eukaryotes [121,122]. In plants, small GTPase Rab proteins are the largest monomeric family of G-proteins, and in the past decade, they were linked to abiotic stress responses [123]. In *Arabidopsis*, small GTPase Rab7 was implied in an enhanced salt and osmotic stress tolerance as well as a reduced ROS accumulation [124]. Interestingly, the overexpression in transgenic tobacco plants of the small

GTPase Rab7 from two stress-tolerant species (*Proposis juliflora*, a high-temperature, drought and heavy metal tolerance, and pearl millet, a drought-tolerant grain crop), resulted in a similar increase in salt-tolerance [125,126]. In rice, the overexpression of the small GTPase Rab7B3 was shown to be salt- and ABA-responsive [127], further reinforcing the key role of small GTPase Rab proteins in stress signaling.

#### 5.1.4. Kinases

A great number of serine/threonine kinases, including mitogen-activated protein kinases (MAPKs) have emerged from proteomic studies as being differentially accumulated under stress [15,111]. Since the role of MAPKs in stress signaling is vast and overlapping [128], it is difficult to imply the role of these kinases in a specific stress response. For instance, although an *Arabidopsis* MAPK (AtMPK4) was identified as salt-responsive [15] it has also been characterized as differentially regulated in every major abiotic stress condition [129].

A receptor-like protein kinase (OsRMC — rice root meander curling) was first identified by 2-DE, and its role was subsequently validated by RNA interference in transgenic rice. The results showed that transgenic knock-out lines of OsRMC have improved salt stress tolerance when compared to wild type rice plants [130].

### 5.2. Photosynthesis and metabolism

#### 5.2.1. Photosynthesis-related proteins

Photosynthesis plays a central role as energy source for plant metabolism and it is therefore highly correlated with plant productivity and consequently with crop yields. Among the different biological processes influenced by stress, photosynthesis is one of the most affected. Its efficiency is highly reduced by adverse environmental conditions, such as high salinity and drought, which induce stomatal closure, slowing CO<sub>2</sub> assimilation and consequently reducing photosynthetic rate [131,132]. Abiotic stresses negatively affect photosynthesis at different levels: CO<sub>2</sub> diffusion, photosystem II (PSII) efficiency, electron transport, ROS formation, RuBP content (dependent on ATP and NADPH supply), RuBisCO activity, and photorespiration [133]. Nevertheless, plants have developed many strategies to acclimate to adverse conditions [132,134]. The main goal is to maintain the photosynthetic efficiency as high as possible, but avoid the energy imbalance that results from the stress, leading to over-excitation of the photosynthetic apparatus and consequently to photo-oxidative damage [131]. The ability of plants to adapt and/or acclimate to adverse environments is related to the plasticity and resilience of photosynthesis, which, in combination with other processes, determines plant growth and development. An important aspect of the plant response to salt and drought stress is the recovery of photosynthesis following stress relief. Photosynthesis recovery is dependent on the intensity of photosynthesis decline under stress and the way plants recover largely determines plant tolerance to water deficits and salinity [135,136].

Identification of photosynthesis-related proteins that are differentially abundant under water deficits and high salinity is essential to better understand the mechanisms underlying plant response to these stresses and will definitely be useful to improve (through breeding) agricultural crops. So far, many

photosynthesis-related proteins have been shown to increase or decrease in response to high salinity and drought [19,137–139]; however, only a few of those have been functionally characterized and shown as having potential for crop breeding.

Regarding the light reactions of photosynthesis, a number of proteins have been shown to be highly responsive to both salt and drought stresses. However, among these, only the light-harvesting chlorophyll a/b binding proteins (LHCB) and the chloroplastic ferredoxin-NADP(H) reductase have been functionally characterized, using transgenic plants, in order to better understand their role in stress response. Xu and co-workers [140] showed that down-regulation or disruption of any member of the LHCB family, LHCB1, LHCB2, LHCB3, LHCB4, LHCB5, or LHCB6, reduces responsiveness of stomatal movement to ABA, and therefore results in a decrease in plant tolerance to drought stress in *Arabidopsis thaliana*. They showed that LHCBs play a role in guard-cell signaling in response to ABA and suggested that they may be involved in ABA signaling partially by modulating ROS homeostasis. These data indicate that LHCBs are interesting targets for crop breeding, but further studies are needed. When the chloroplastic ferredoxin-NADP(H) reductase was overexpressed in tobacco, transgenic plants displayed normal rates of photosynthesis and an increased tolerance to oxidative stress [141]. Given its role in oxidative stress protection, it is likely that overexpression also confers tolerance to salt and/or drought stress, but this is still to be investigated. Overexpression of the LHCBs and chloroplastic ferredoxin-NADP(H) reductase in crop plants followed by abiotic stress tolerance assessment is a priority.

Carbonic anhydrase (CA) has also been shown to be differentially regulated under drought and salt stresses [15,142]. CA has a relevant role in CO<sub>2</sub> exchange by influencing the internal conductance [143], especially under limiting conditions of CO<sub>2</sub> supply, as it happens under severe drought and salinity stresses. It was observed that CA silencing in tobacco inhibited up to 99% of CA activity but had no significant impact on net CO<sub>2</sub> assimilation [144]. However, susceptibility to water stress appeared to increase in response to the decline in CA activity. In addition, transgenic *Arabidopsis* overexpressing OsCA1 had a greater salt tolerance at the seedling stage than wild-type plants [145]. These studies show that CA has an important role in abiotic stress responses and that it may have an enormous potential in crop improvement. It is however necessary to investigate how the main crops respond to high salinity and/or drought when expressing enhanced levels of CA.

RuBisCO activase (RCA) is another photosynthesis-related protein shown to be differentially regulated under abiotic stress. In most cases, the level of this protein is reduced under stress; however, there are a number of salt tolerant plant species that showed an accumulation of the protein in response to salinity [15,16,146]. This indicates that enhanced levels of RCA may be somehow related to stress tolerance. Although no functional studies were performed to investigate RCA role in salinity, the overexpression of a thermostable RCA1 variant in *Arabidopsis* conferred moderate tolerance to heat stress [147]. It is known that plant photosynthesis declines when the temperature exceeds its optimum range and this reduction is linked to the inhibition of RCA. These results provide clear evidence that RCA is a major limiting factor in plant photosynthesis under moderately elevated

temperatures, but also suggest that it has potential to improve crop plants productivity not only under heat stress conditions, but also under drought or salinity stresses. Again, it is urgent to investigate how crops overexpressing RCA (or a more stable isoform) behave under drought or salt stress.

Sedoheptulose-1,7-bisphosphatase (SBPase) has a key role in regulating the photosynthetic Calvin cycle and is normally down regulated under abiotic stress. However, transgenic rice plants accumulating SBPase in chloroplasts resulted in an enhanced tolerance to salt stress at the young seedlings stage [148]. The analysis of chlorophyll fluorescence and the activity of SBPase indicated that the enhancement of photosynthesis in plants subjected to salt stress was not related to the function of PSII but to the activity of SBPase. In addition, transgenic rice plants accumulating SBPase were shown to be more tolerant at high temperature and again it was demonstrated that the enhancement of photosynthesis to high temperature was not related to the function of photosystem II but to the content and activation of SBPase [149]. These results suggest that overexpression of SBPase may be an effective method to improve abiotic stress tolerance, although to better understand SBPase role, it is essential to test how its overexpression in rice or other crops modifies plant behavior under moderate and severe drought stress.

There are still other salt and drought regulated proteins, such as PsbP and chloroplastic ATP synthase [15,19], that would be interesting to investigate as putative targets to improve abiotic stress tolerance in crops. PsbP protein has been shown to be essential for the regulation and stabilization of photosystem II in higher plants [150]. The chloroplastic ATP synthase coupling factor is a key element in the drought stress responses. Water stress was shown to inhibit plant photosynthesis by decreasing coupling factor and ATP [151].

#### 5.2.2. Other carbohydrate metabolism-related proteins

Besides the photosynthesis-related proteins, many other proteins related to the carbohydrate metabolism are differentially abundant under drought and salt stresses [19,137,138]. The functional characterization of these proteins is however scarce, only few of them have been overexpressed and/or silenced in order to evaluate their role in stress response. For instance, the level of fructose 1,6-bisphosphate aldolase (FBA) was found to decline under salt stress in most of the plants studied [75,113,152]. In addition, it was found that mutant plants of an *Arabidopsis* FBA (AtFBA6, cytoplasmic isoform) showed salt and mannitol tolerance and an ABA sensitive phenotype at the germination stage [153]. Although these results indicate that lower levels of cytoplasmic FBAs increase salt stress tolerance, they must be taken with caution, as AtFBA6 is only one among the five cytoplasmic isoforms. Interestingly, in *Salicornia europaea*, a halophytic perennial dicot plant, it was reported that FBA (cytoplasmic isoform) accumulated under salt stress [146]. Altogether, these results suggest that different cytoplasmic isoforms may have different functions and behaviors under salinity stress. More studies are needed, particularly in crop plants, to clarify the role of the FBAs in salt and drought stress tolerance and their potential use in plant breeding.

The cytosolic glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein, involved in glycolysis, has been shown to

accumulate under drought and salt stresses [15,138]. Moreover, when a stress-inducible GAPDH gene from *Pleurotus sajor-caju* (oyster mushroom) was overexpressed in potato plants, these showed an enhanced tolerance to salt stress [154]. Although this is a good indication that cytosolic GAPDH plays an important role in salt stress tolerance, further studies are required. For instance, it is relevant to analyze the effect of its overexpression in other crops, especially regarding tolerance to drought or other abiotic stresses.

Another glycolytic protein that is highly responsive to salt and drought stresses and may be a potential target for crop breeding is phosphoglycerate mutase [137,155]. Zhao and co-workers showed that phosphoglycerate mutases and glycolytic activity are critical for guard-cell function in *Arabidopsis* [156]. Given that stomatal closure is one of the primary plant responses to drought and high salinity, it is likely that these proteins play some role in these stresses. In order to investigate whether this protein has potential to improve crop plants, it is essential that transgenic crop plants (overexpressing and silencing phosphoglycerate mutase) are produced and assessed for abiotic stress tolerance.

Proteins involved in the tricarboxylic acid (TCA) cycle are also differentially regulated by abiotic stresses [137,155]. For instance, different studies have shown that the cytosolic NADP-dependent isocitrate dehydrogenase (ICDH) protein level is induced in response to either salt or drought stress [137,146]. The cytosolic NADP-dependent ICDH catalyzes the reversible conversion of isocitrate to 2-oxoglutarate (2-OG) and links C and N metabolism [157]. When the maize ZmICDH was overexpressed in *Arabidopsis* [158], it enhanced salt stress tolerance, thus showing the capability of this protein to be used in plant breeding. Again, it is essential to characterize the function of this protein in important food crops, such as rice, wheat or maize.

### 5.3. Oxidative stress

#### 5.3.1. Superoxide elimination

Superoxide ( $O_2^-$ ) is formed from the one-electron reduction of oxygen ( $O_2$ ) and is the first species of ROS to be formed. In eukaryotic aerobes, the major site for  $O_2^-$  formation is the mitochondrial respiratory chain, where 1–2% of transferred electrons end up reducing  $O_2$  [159]. In plants, chloroplasts add up as the major  $O_2^-$ /ROS cellular site [160], followed by peroxisomes [161]. The  $O_2^-$  radical is rapidly eliminated by resident superoxide dismutases (SOD). There are 4 known types of SOD, the Cu,Zn-containing SOD (Cu,Zn-SOD) [162], the Fe-containing SOD (Fe-SOD) [163], the Mn-containing SOD (Mn-SOD) [164], and Ni-containing SOD (Ni-SOD) [165]. In plants, typically Mn-SOD is mitochondrial, Fe-SOD is plastidic, and Cu,Zn-SOD can be plastidic, peroxisomal or cytosolic.

Field trials with alfalfa overexpressing tobacco Mn-SOD in mitochondria or chloroplasts, show that transgenics perform better than control plants, with a higher survival and yield after winter and water-deficit, although in greenhouse trials no significant differences could be observed [166–168]. This indicates not only a potential role for Mn-SOD in crop improvement, but also the importance of testing plants in field conditions, or simulating multiple stresses in fully controlled environments (see Section 4). Nevertheless, much care is needed when

overexpressing Mn-SOD in mitochondria, since the levels of Mn-SOD overproduction play a critical role in determining the effects of oxidative stress originated within this organelle [169]. Additionally, tobacco plants overexpressing a chloroplast-localized Cu,Zn-SOD showed an enhanced tolerance to high light intensity and low temperature stress [170]. Extensive proteomic studies showing an increased accumulation of SOD proteins in response to several abiotic stresses reinforce the functional evidence for potential use of SODs in crop plant improvement. Cu,Zn-SOD is the most commonly found, possibly because some of the isoforms are usually abundant in plants. In crops, Cu,Zn-SOD increases in response to drought and salinity [152,171–179] and decreases in response to heat stress [180]. Mn-SOD was also found to increase in response to salt and water stresses in alfalfa, wheat, and pine [14,138,181,182], while Fe-SOD increased in citrus, in response to salinity [75]. In salt-stressed barley, a genotype-dependent response was observed for Cu,Zn-SOD, with the salt-tolerant genotype ‘Afzal’ showing a higher protein accumulation than observed in the salt-sensitive ‘L-527’ [183].

### 5.3.2. Enzymes of the glutathione-ascorbate cycle

Monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) are enzymes involved in the maintenance of the pools of reduced ascorbate and glutathione in cells, in the glutathione-ascorbate cycle. Both ascorbate and glutathione are important antioxidants involved in enzymatic and non-enzymatic cellular reactions (for important reviews in the plant biology field see [184,185]). During the glutathione-ascorbate cycle, which is one of the most important cellular mechanisms for hydrogen peroxide ( $H_2O_2$ ) detoxification, ascorbate is oxidized to monodehydroascorbate by ascorbate peroxidase (APX) with the concomitant reduction of  $H_2O_2$  to water. Monodehydroascorbate can be re-reduced to ascorbate by MDHAR at the expense of NAD(P)H or spontaneously dismutate to ascorbate and dehydroascorbate. Dehydroascorbate is then reduced to ascorbate by DHAR, using reduced glutathione, which becomes oxidized. The cycle is completed with the re-reduction of oxidized glutathione by GR, with oxidation of NADPH to NADP<sup>+</sup>.

DHAR was found to increase in several proteomic studies in crops, such as rice, wheat, barley, alfalfa, citrus, and watermelon, in response to several abiotic stresses (water-, heat-, salt-, cold-, and oxidative-stresses) [22,114,138,75,178,180,186–190]. It has also been identified as the protein with a higher increase (12 fold) during dehydration followed by rehydration in *Physcomitrella patens*, a highly drought-resistant moss [142] and severe water stress (4.8 fold) of *Elymus elongatum*, a drought tolerant tall wheatgrass [191]. In a wheat proteomics study comparing drought response in a tolerant versus sensitive genotype, DHAR was found to increase in the tolerant genotype, and decrease in the sensitive one [7]. These results, together with the fact that DHAR is a key enzyme in the glutathione-ascorbate cycle, highlight the potential of its use in translational proteomics. A few functional studies further indicate such potential. Tobacco plants expressing a human DHAR in chloroplasts are more resistant to oxidative stress caused by methyl viologen or  $H_2O_2$  treatments. Young seedlings also performed better under cold and salt stresses [192]. Interestingly, these plants also show a slight increase in the levels of glutathione reductase, underlining

the tight regulation within the glutathione-ascorbate cycle. Transgenic tobacco overexpressing wheat DHAR showed an increased tolerance to ozone exposure (leading to oxidative stress) [193]. Arabidopsis DHAR overexpression in tobacco also resulted in an increased tolerance to ozone exposure, drought, salt and PEG stresses [194]. Oddly, tobacco plants overexpressing DHAR showed a higher susceptibility to drought than wild-type plants, due to a higher number of open stomata, pointing out the importance of further research on this topic [195]. A moderate expression of rice DHAR in Arabidopsis resulted in an increased tolerance to salt stress, during germination, but had no effect during chilling or heat stresses [196]. In the above cases, overexpression of DHAR led to increased ascorbate and reduced glutathione.

MDHAR was found to decline in response to salt stress in barley, Arabidopsis, and the halophytic perennial dicot *S. europaea* [10,99,146]. So far, its increase was only found in citrus in response to salinity [75]. Moreover, functional studies showed that its overexpression in tomato decreased the ascorbate levels [197]. This variability makes MDHAR not a very promising target to improve crop abiotic stress tolerance.

Proteomic studies have shown slight increases of GR in rice, after ABA/salt and salt stresses [100] and in banana, after osmotic stress [137]. Ectopic expression of *Escherichia coli* GR in tobacco chloroplasts led to an increase in the damage caused by cadmium and UV-B stresses [198]. The same laboratory has combined the overexpression of GR with rice DHAR and with *Escherichia coli* glutathione S-transferase (GST) in tobacco chloroplasts. These double-transgenics showed an enhanced tolerance to the oxidative stress inducing agent methyl viologen, a feature not observed for plants expressing DHAR, GR or GST alone [190,198]. Individual expression of GST resulted in an increased tolerance to salt and cold stresses [190].

### 5.3.3. Other glutathione dependent enzymes

Glutathione S-transferase (GST) is another promising target to improve crop abiotic stress tolerance. GST is a part of the plant protection mechanisms against toxic  $O_2$  intermediates, together with superoxide dismutases, catalases, ascorbate peroxidases and glutathione peroxidases (GPX). GST limits oxidative damage by removing both ROS during stress, or detoxifying xenobiotics under normal conditions. GST and GPX activities depend on the pool of reduced glutathione, which is maintained by the GR at the expense of NAD(P)H (see above). Transgenic tobacco seedlings co-overexpressing GST and GPX are more tolerant to thermal and salt stresses than non-transgenics. They show attenuated growth arrest and steady levels of lipid peroxidation at 20 °C, 30 °C, and 100 mM NaCl in the dark [199]. In Arabidopsis expressing tobacco GST, membrane lipid peroxidation in roots was reduced to half the amount observed in non-transformed plants, but in this case no root growth arrest rescue was observed [200]. The choice of the target GST has to consider the type of response desired. Plants usually have big families of these proteins, but although many are functionally redundant, they may still have differential responses to different abiotic stresses [201]. Some proteomic studies in Arabidopsis [99], citrus [75], and barley [183] additionally show a number of GST isoforms responding differently (some levels increasing and others decreasing) to the same stress. Also, co-overexpression of GST with other enzymes involved in the

maintenance of the pools of reduced ascorbate and glutathione may also be necessary to balance the remaining antioxidant system, during stress. As an example, the overexpression of tobacco GST in cotton seedlings failed to increase resistance to abiotic stress and instead lower levels of DHAR and more oxidized glutathione were observed in salt-stressed plants [202]. Still, the importance of GST induction in crop stress response is well visible in numerous proteomic studies [7,14,75,101,111,114,137,180,183,187,188,203–206]. An interesting example of genotype-dependent variation comes from the proteomics study on wheat drought response, where GST is increased 2-fold in the drought tolerant genotype ‘Khazar-1’, while being decreased by a similar factor in the sensitive genotypes ‘Arvand’ and ‘Afghani’ [7].

#### 5.3.4. Ascorbate peroxidase (APX)

Hydrogen peroxide is the product of the one-electron reduction of  $O_2^-$ . Excess accumulation of  $H_2O_2$  is one of the mechanisms by which plants are damaged, during several abiotic stresses, particularly, salinity and drought [207,208]. Hydrogen peroxide can cross biological membranes and exert its effect (deleterious or as a signaling agent) far away from its origin, hence the importance of its regulation. APX can reduce  $H_2O_2$  to water, but it depends on the availability of the reduced ascorbate pool maintained through the glutathione-ascorbate cycle. Thus, its overexpression in crop stress-tolerance must be carefully designed, as discussed for GST (see above). Co-expression of cassava Cu,Zn-SOD and pea APX was done in tall fescue, a cool-season grass, targeting both proteins to the chloroplast driven by a stress-inducible promoter. When tested with methyl viologen,  $H_2O_2$  and heavy metal exposures, the transgenic plants showed lower accumulation of  $H_2O_2$ , lower lipid peroxidation and higher maintenance of chlorophyll content, than control plants [209], indicating APX- and SOD-mediated protection against oxidative stress. In Arabidopsis, a combination of drought and heat stresses induced APX1 expression and accumulation at the protein level [210]. The selectivity for a specific APX isoform was also shown in the same work, since only knock-out mutants for Apx1 showed  $H_2O_2$  accumulation and increased sensitivity to the combination of drought and heat stress, while no changes were observed for mutants of the thylakoid or stomatal/mitochondrial APXs, when compared to wild-type plants [210]. In several proteomic studies of crops responding to abiotic stresses, APXs were found to vary increasing or decreasing their abundance, depending on the conditions and the identified isoform [9,22,75,114,173,175,181,211–213].

#### 5.3.5. Other hydrogen peroxide eliminating enzymes

Catalase is an enzyme that eliminates  $H_2O_2$  producing  $O_2$  and water. Catalase isozymes are located in peroxisomes and mitochondria, where they exert their function in  $H_2O_2$  detoxification [214]. In crop proteomic studies, catalase has been shown to increase in wheat responding to drought [181], and in rice roots and leaves challenged with salt stress [74,100]. In opposition, catalase levels declined after salt stress in cucumber, citrus, and barley [10,75,175]. In wheat, salt stress increased the amount of catalase in a tolerant genotype, while in a sensitive one, catalase was found to decrease [14]. To our knowledge, there are no functional studies supporting the use

of catalase in the improvement of crop performance under stress and, at this point, this protein does not look like a promising target.

### 5.4. Stress-responsive proteins

#### 5.4.1. HSP70 (protein synthesis/assembly and stability)

Heat Shock Proteins (HSP) are molecular chaperones whose gene expression is regulated by heat shock factors (HSFs) that bind to heat shock elements in HSP gene promoters [215]. HSPs are not only involved in plant heat stress response, but also in the response to other abiotic stresses. In fact, HSF involvement in several abiotic stresses has been described [216–220]. In crops, HSP level variations have been reported in several proteomic studies, as a response to drought, salt-, oxidative-, and heat-stresses [10,74,75,100,101,112,114,175,180,188,189,204,212,213,221–227]. Most of the time, an increase in protein level is observed, making the members of this protein family potential good targets for plant stress tolerance improvement. HSP70 is the most important HSP known as being involved in various plant abiotic stress responses [228]. An interesting example is that HSP70 levels were found to increase in a tolerant genotype of barley, while decreasing in a sensitive one [204]. Functional studies have also proved the relevance of HSP70 in abiotic stress response. NtHsp70-1 was constitutively overexpressed in tobacco and shown to help to prevent DNA fragmentation and degradation and chlorophyll breakdown during heat stress. Additionally, NtHsp70-1 overexpression increased the tolerance to heat stress and to drought [229]. Also, a fungal heat shock protein (HSP7 from *Trichoderma harzianum*) led to identical responses when overexpressed in Arabidopsis. It induced an increased tolerance to heat, osmotic, salt, and oxidative stresses and prevented growth inhibition by heat stress [230].

Additional strategies relevant to improve crop tolerance to abiotic stress may include the overexpression of proteins that stabilize HSP70, such as the HSP70-binding protein AtFes1A (containing the armadillo repeat motif, which is a characteristic repetitive amino acid sequence) [231]. Considering the successful increase in abiotic stress tolerance achieved by the ectopic expression of OsHsfA2e in Arabidopsis [232], also other selected HSFs will likely prove effective to improve crop stress tolerance.

#### 5.4.2. Late embryogenesis abundant (LEA) proteins

Late embryogenesis abundant (LEA) proteins were first identified in cotton embryos [233]. LEAs accumulate extensively in the later stages of seed maturation during the desiccation process, disappearing after germination [234]. Initial evidence showed that, in the embryo, LEAs are induced by ABA [235,236] and salt stress [236], while in vegetative tissues, LEAs are induced by water and cold stresses, by exogenous ABA, and wounding [235,237]. LEAs are organized in several families [238], with group 2 LEAs or dehydrins, which include rice Rab21 [236] and some of cold-regulated (COR) proteins [237] as the best characterized. Besides plants, LEAs can also be found in other organisms, but their presence in desiccation-tolerant organisms, such as nematodes [239], together with their importance along seed maturation supports their role in desiccation tolerance, and their potential to increase crop drought tolerance. LEAs were reported to protect specific cellular structures

or mitigate the effect of drought stress by sequestering ions and maintaining minimum cellular-water requirements [240,241]. But still, their precise role remains unclear. Heterologous expression of the salt-tolerant rice 'Pokkali' Rab16A (coding for Rab21) in tobacco, under the control of its own (stress-inducible) promoter, led to an increase in salt tolerance [242]. Moreover, overproduction of this gene in a rice transgenic sensitive background resulted in an improved salt-tolerance and related physiological traits [243]. In addition, expression of the rape LEA ME-LeaN4 in lettuce led to an enhanced growth ability under unfavorable conditions, such as high salinity and water-deficit [244]. In rice, overexpression of OsLEA3, transcriptionally induced by water deficit and salt stress, renders transgenic seedlings an increased tolerance to such stresses [245]. As expected, proteomic studies performed at the grain level in rice, wheat, and maize revealed the accumulation of several LEA proteins [13,181,223,246,247]. In vegetative tissues, proteomic studies have revealed LEAs accumulation in rice seedlings after drought [176], and after salt stress when applied after ABA treatment, but not when salt stress was applied alone [100]. LEAs also accumulated in roots and hypocotyls of soybean after salt stress [221] and in wheat leaves after salinity [173]. Interestingly, proteomics of unstressed leafy-shoots of the desiccation-tolerant moss *P. patens* revealed 13 protein spots, corresponding to 7 LEA genes [142]. So far, a few hundred of LEA proteins have been identified (see LEAPdb [238]), but in most cases, LEA transcripts are only detected under stress conditions, or during the late stages of plant seed development. This "constitutive" expression of *Physcomitrella* LEAs can constitute important evidence that artificially forcing LEA expression in vegetative plant tissues has a great potential to increase stress tolerance. Despite the constitutive expression of 7 LEA genes, dehydration of *Physcomitrella* induced the increase of 27 spots corresponding to LEAs, with fold increases up to 13.7, while rehydration induced 15 LEA spots [139]. Altogether, products of 11 LEA genes were identified in response to stress, 9 belonging to group 3 LEAs and 2 dehydrins [142]. Group 3 LEA proteins displayed multiple isoforms that responded dynamically to dehydration and rehydration [142], which can indicate a modulation of LEA function by PTMs. Rice dehydrin Rab21 (encoded by Rab16A) was shown to be differentially phosphorylated depending on the studied genotype [13]. The most basic isoform (less phosphorylated) was found predominantly in the grain of drought-tolerant rice genotypes [13].

#### 5.4.3. PR (Pathogenesis-related proteins)

Pathogenesis-related (PR) proteins are a group of plant proteins induced in response to pathogen infection. PR proteins were classified into 17 different families (PR-1 to PR-17) depending on their biological function or according to primary structure similarity [248–250]. Despite the numerous studies presently available, the function of many PR proteins is still unknown. Nevertheless, in over-expression studies, PR-10 proteins were shown as particularly promising to improve abiotic stress tolerance.

PR-10 proteins respond not only to biotic, but also to abiotic stresses, such as drought, salt-, cold-, and oxidative-stresses, and UV-irradiation [251–264]. As examples, rice RSOsPR10 and Osdrr genes were found to be upregulated in rice roots when subjected to drought and salt stresses [253,257]. Additionally,

PR-10 protein levels were highly induced in rice seedlings and leaves under high ozone and UV-mediated stresses, respectively [254,265]. In other proteomic studies, PR-10 proteins were shown to accumulate in response to salt-stress in grass pea, in a barley salt-tolerant variety, and in the wine grape cultivar Cabernet Sauvignon [111,187,227]. Interestingly, a comparison of the proteomes of a salinity-tolerant peanut callus cell line against its sensitive counterpart revealed that most of the differently abundant low molecular proteins were PR-10 [258]. Taken together, this evidence suggests that PR-10 protein abundance may be manipulated to improve abiotic stress tolerance in crops. In fact, the constitutive expression of a pea PR-10 gene in *Brassica napus*, enhanced germination and growth rates under salinity [266]. Moreover, the expression of potato PR-10a in faba bean improved tolerance to drought and salt stresses [267].

The over-expression of PR proteins, however, may also have some drawbacks, not only because of their involvement in plant growth and development, but also because the group includes known allergens such as Bet v 1 and Ara h 8 proteins.

## 6. Abiotic stress impact on food quality and safety

Improving plant survival or performance under adverse conditions will certainly be based on proteins known to have defensive or protection functions. Since, it is already known that proteins belonging to the plant defense system are often also allergens [268], one may speculate that alterations in their expression may increase plants potential allergenicity. Moreover, since abiotic and biotic stimuli can alter global protein expression, and differences in the environmental growth conditions of a given food plant may also have consequences on its protein composition. These hypotheses guide us to a broader discussion regarding the impacts that climate change may have on the safety [269] and quality [270] of plant food products.

Proteins are involved in virtually all plant cell functions, such as defense, catalyzing biochemical reactions, in signaling, structural support, and amino acid storage. Plant proteins and peptides may also possess specific functional properties that facilitate processing and serve as the basis of product performance [271], or may eventually be bioactive, having antihypertensive [272,273], antilipemic [274–276], and antioxidant [277,278] actions (among others).

There is a great lack of studies correlating the effects of altered environmental conditions on plant food protein composition and the quality and safety of derived products.

Regarding plant environmental growth conditions and their relation to plant food quality, there are some studies demonstrating that elevated CO<sub>2</sub> leads to a reduction in grain nitrogen and protein concentration [279]. This may be a problem in a world with a rising population and constant demand of protein-rich diets. Also, in some cases a reduction in protein concentration may also affect food products performance. For example for adequate bread-making quality, wheat grain protein concentrations higher than 11.5% are required. Under CO<sub>2</sub> enrichment, protein concentrations in wheat grains may decrease to values below the minimum

quality standard for bread-making [280]. Although this effect may be minimized under abundant nitrogen fertilization [281], it may be economically impracticable and environmentally unacceptable. Furthermore, overcoming the CO<sub>2</sub>-induced reduction in grain protein by increases in N fertilization may conduct to undesired alterations of other quality characteristics [282] or may be translated into higher biomass rather than an enhanced redistribution of N from vegetative pools to the grains, thus leading to a decreased grain protein concentration [283]. Nitrogen increases in the soil may also have a negative impact on the environment.

Some studies have also demonstrated that elevated CO<sub>2</sub> may, for instance, also impact protein composition, affecting grain traits relevant to eating quality [284], and amino acids concentrations [280].

The number of scientific studies relating environmental growth conditions with plant food safety is also very scarce. Still, there are some recent studies that clearly relate increases in atmospheric temperatures and carbon dioxide concentrations, with the rise of pollen concentration and consequent contribution to an increased incidence and prevalence of allergic diseases [269,285,286].

Additionally to the increase of pollen concentration, Singer and co-workers [287] published a study concerning ragweed pollen, where the increase of Amb a1 content (a common aero-allergen) was related to the higher concentration in atmospheric CO<sub>2</sub>. Other authors used SDS-PAGE and IgE immunoblotting to study the influence of temperature on the expression of the major birch pollen allergen Bet v1. The results presented suggest that Bet v1 amount was higher in trees grown at higher daily mean temperatures [288].

Regarding food allergens, Hänninen and co-workers [289] used whole and wounded turnips (treated with salicylic acid or ethephon) protein extracts and performed IgE immunoblotting with sera from patients allergic to natural rubber latex (cross-allergies are common among these patients) to demonstrate that activation of certain defense mechanisms could increase turnip allergen content. Also, Pühringer and co-workers [290] used *Nicotiana tabacum* plants transformed with the GUS reporter gene driven by the promoter of apple Ypr10 (encoding the major allergen Mald1) to correlate plants' growth conditions with a potential increase in allergenicity. The group demonstrated that apple Ypr10 promoter may be induced by both abiotic stress (salicylic acid and reduced glutathione) and pathogens (virus infection and fungal elicitors). They have also confirmed an increase in transcriptional and translational expression of Mald1 in young apple leaves after the same abiotic stimuli and after treatment with fungal elicitors.

More recently, Armentia et al. [291] verified that the wheals obtained in skin prick tests of tomato allergic patients were significantly higher with extracts of tomato treated with ethylene and salicylic acid as compared with extracts from non-treated tomatoes.

These studies clearly demonstrate that environmental stimuli may have a crucial role in plant allergen expression. In order to prevent and control potential allergy-related health problems associated with the urgent need of getting higher yields of more nutritious food in less friendly lands and

meteorological conditions, it will be crucial to develop plants able to resist these new challenging stimuli, while maintaining or increasing their nutritional quality, and maintaining or reducing their allergenic potential.

Proteomics has been proven to be a fundamental tool in allergen identification. Usually, the experimental approach starts with protein separation by 2-DE followed by protein electrotransfer to a membrane. Identification of immunoreactive spots is performed by IgE immunoblot with sera from allergic patients or monoclonal antibodies and protein identification of the selected spots is usually accomplished by mass spectrometry.

Several different studies have been conducted using this methodology [292–296] and there are already some online databases integrating information about identified potential allergens ([www.allergome.org](http://www.allergome.org); [www.allergenonline.org](http://www.allergenonline.org)). However, there is still much information about these proteins that must be gathered, for instance regarding the physiological functions, the expression patterns along development and under variable environmental conditions, and the potential post-translational processing.

Proteomics approaches to dissect stress responsive pathways have been crucial to understand plant responses to environmental stresses. This knowledge, together with effective approaches for simultaneous quantification of major allergens, as well as non-allergenic proteins potentially affecting traits of nutritional or agronomic value, are critical to foster plant breeding strategies.

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## 7. Conclusions and future perspectives

Although proteome analysis to advance crop breeding for stress-tolerance has been the focus of numerous research and revision papers, it is still on a discovery mode. In the same way that human proteomics is expected to reveal diagnostic and prognostic biomarkers of human diseases, also quantitative proteomic tools for plants may help identifying stress-tolerance “biomarkers” for crop plant breeding. Comparative analyses are still difficult due to the different plants and stress conditions analyzed, but efforts to standardize experiments and gather the information in comprehensive databases are arising. The study of different plant models, including those adapted to extreme conditions, has unveiled a number of proteins potentially relevant for crop breeding. However, the fact that in many cases such proteins induce different phenotypic responses, depending on the host genome, justifies the need to further investigate crops, not only for protein functional validation, but also for identification of the most relevant proteins associated with the desired phenotypes.

From the search of the available literature, it is evident that the methodologies applied in plants for protein identification (and quantification) are insufficient. Probably due to their reduced amounts in the cell, it is very rare that transcription factors or proteins involved in PTMs are identified using the available techniques. Eventually, the selection of specific developmental stages, organs/tissues or organelles may allow for protein enrichment and successful analyses. So far, however, we have important gaps in knowledge regarding the regulation of abiotic stress plant response. Also membrane

proteins and transporters are still poorly described due to the difficulties in protein purification.

The description of the protein effect on plant development, productivity and physiology, at whole plant level and in field conditions, is imperative for application in crop breeding programs. Numerous tools have recently become available for plant phenomics, although access to them is still not as widespread as needed.

As summarized in Fig. 3, up to date, the proteins that have effectively been confirmed as conferring abiotic stress tolerance are involved in signal transduction (Rab7 and GF14 $\lambda$ ), in protection from oxidative stress (DHAR, GST), related to protein protection (HSP70, Rab21, ME-LeaN4, LEA3), and in biotic/abiotic stress crosstalk (PR-10a). Some proteins as it is the case of the glutathione–ascorbate cycle, seem to be more effective when co-expressed (GST with GPX, GR with DHAR, or GR with GST). Among the photosynthesis-related proteins regulated by the adverse environmental conditions, some of them have already shown potential to improve abiotic stress tolerance in crops, such as CA1 and SBPase, while for other proteins further studies are needed. With less supporting data, but also appearing promising for crop improvement, there are proteins such as HSP70-stabilizing and HSFs for protein protection, OsRMC in stress signaling, Mn-SOD and

specific APX isoforms in oxidative stress protection and PsbP, chloroplastic ATP synthase, GAPDH, and ZmICDH for improvement of photosynthesis/metabolism. Confirmation of their relevance must be checked in homologous or heterologous systems prior to use in target crops.

To address the challenge of securing nutritious, safe and affordable food for the future, coordinated international research is essential to contribute for improved knowledge on the physiological functions of plant proteins, as well as their expression and post-translational modifications, under diverse environmental conditions.

The improvement of crop production and yields to feed the world's growing population dependent on an ever-smaller number of farmers, will likely be achieved through the increasing knowledge of plant systems biology and production of plants more resistant to stressful environments.

So far, breeding for abiotic stress tolerance has been achieved through crosses with tolerant donors, identification of key QTLs and introduction of responsive genes such as ion transporters, and transcription factors as well as LEAs for salt and drought-tolerance, respectively. Due to their low concentration, ion transporters and transcription factors are typically not detected in two-dimensional gels. In the near future, we expect a major breakthrough in the discovery of these proteins

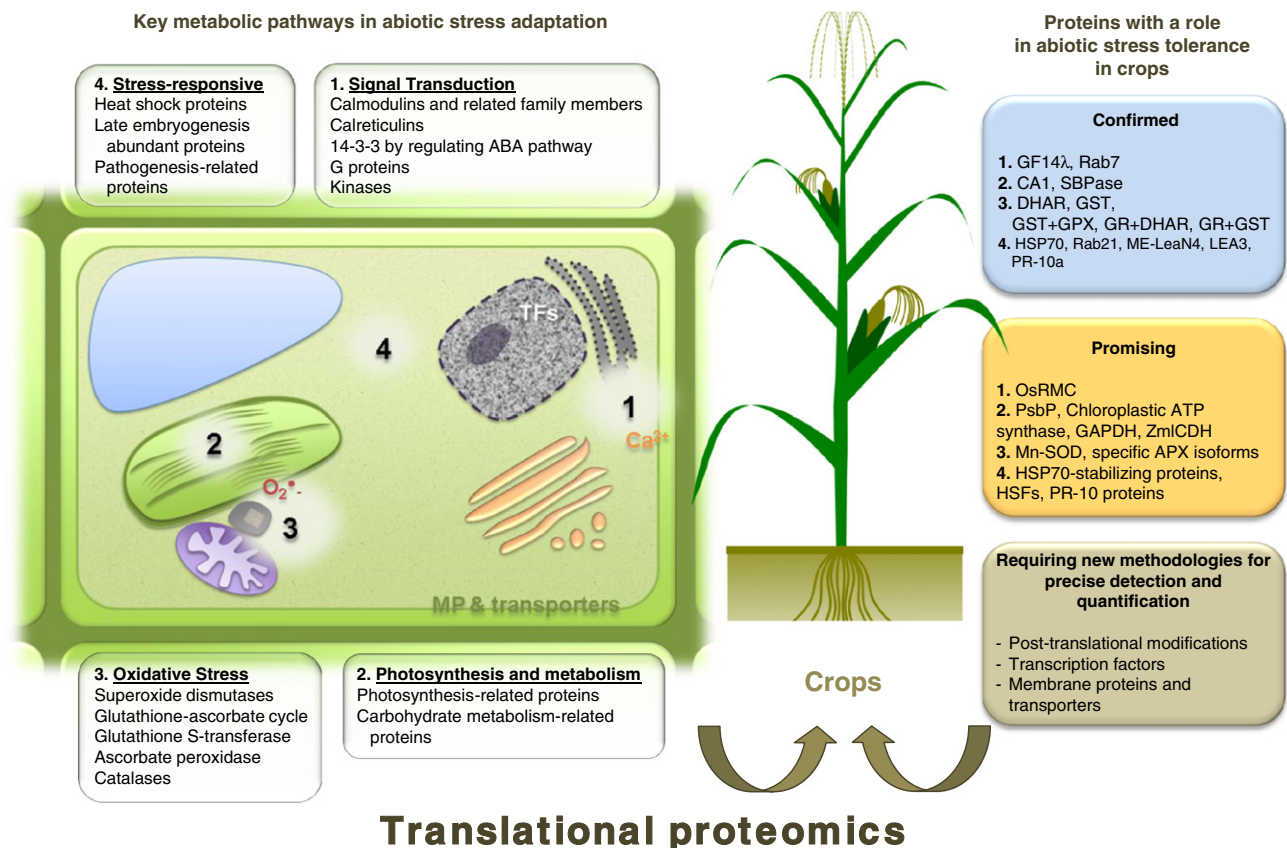


**Fig. 3 – Translational proteomics for abiotic stress tolerance improvement in crops.** Key metabolic pathways (1., 2., 3. and 4.) were identified in proteomic studies of crops and model plants. Confirmed proteins with a role in abiotic stress tolerance in crops were identified in functional studies using mutant and transgenic plants. Promising proteins must still be validated in functional studies, and new methodologies are required to address still unveiled issues (PTMs — post-translational modifications) or protein families (TFs — transcription factors, and membrane proteins — MP and transporters).

by gel-free techniques. As it happened with quantitative trait loci (QTLs) in general, that proved to be extremely valuable to find candidate genes effective in stress tolerance [297], maybe also the protein quantity loci (PQL) can help finding good markers. After identification of the differentially abundant proteins, the locus controlling the variations can be mapped on the genome. A map coincidence of a PQL peak with its protein locus could indicate the effect of different alleles on protein amount, while a map coincidence of a PQL and a QTL for a different trait could implicate a role of the candidate protein concentration on expression variations of the trait. In maize, it was shown that PQLs co-localizing with QTLs for drought could be candidate proteins, since a more abundant protein with high PQLs (P71) was associated with growth reduction during drought-stress [298]. Other PQLs were also mapped and co-localized with a frost-damage-resistance QTL in pea [299]. Altogether, the use of PQLs is still limited although they are an interesting strategy for crop improvement.

As happened in recent years, technological advances will determine future progress in biological sciences, in particular plant sciences [300,301]. Expectedly, new technologies will help answering major questions to keep pace with future demands for food (and feed), energy and environment [300]. The integration of all the information coming out from high-throughput OMICS approaches is a challenging task, but it is crucial for making the step forward from data collection to modeling and prediction of how biological systems function in myriad situations [301]. Despite its current limitations, proteomics will continue to provide important contributions that focus exclusively on proteins — the cellular effectors. Translational proteomics will pave the way to apply this knowledge in crop abiotic stress improvement.

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