Elucidation of salt stress defense and tolerance mechanisms of crop plants using proteomics—Current achievements and perspectives

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Salinity is a major threat limiting the productivity of crop plants. A clear demand for improving the salinity tolerance of the major crop plants is imposed by the rapidly growing world population. This review summarizes the achievements of proteomic studies to elucidate the response mechanisms of selected model and crop plants to cope with salinity stress. We also aim at identifying research areas, which deserve increased attention in future proteome studies, as a prerequisite to identify novel targets for breeding strategies. Such areas include the impact of plant-microbial communities on the salinity tolerance of crops under field conditions, the importance of hormone signaling in abiotic stress tolerance, and the significance of control mechanisms underlying the observed changes in the proteome patterns. We briefly highlight the impact of novel tools for future proteome studies and argue for the use of integrated approaches. The evaluation of genetic resources by means of novel automated phenotyping facilities will have a large impact on the application of proteomics especially in combination with metabolomics or transcriptomics.

Keywords:
Crop productivity / Microbial communities / omics / Phenotyping / Plant proteomics / Salinity

1 Introduction

1.1 Soil salinity represents a serious restraint to plant productivity

Crop plant productivity can be heavily curtailed by abiotic stress, one of the most serious of which is soil salinity. Soil salinity depresses plant growth initially in the form of osmotic stress, and later causes ion-specific toxicity [1, 2]. During the initial phase, the plant's ability to absorb water...
is increasingly compromised, resulting in the triggering of a range of cellular and metabolic processes also observed during drought stress. Cell expansion, cell wall synthesis, protein synthesis, stomatal conductance, and photosynthetic activity are all inhibited by osmotic stress, while compatible solutes and abscisic acid (ABA) are accumulated. A series of analyses have identified both enzymatic and nonenzymatic components of this response, many of which are concerned with the elimination or neutralization of ROS and low molecular weight antioxidants [3, 4]. During the second phase, the salt that has entered the root is transported via the xylem to the shoot, where it contributes to growth inhibition and finally accelerates senescence of mature leaves. The basis of salinity tolerance is largely multigenic, and mechanisms conferring tolerance during the second phase include the inhibition of sodium ion (Na\(^+\)) ingress into the shoot, the limitation of Na\(^+\) transport to the shoot meristem, and a reduction in the Na\(^+\) concentration in the cytosol [5].

1.2 Plants differ in their salt tolerance

Among the cereals, barley (Hordeum vulgare), which is grown both for feed and malting purposes, is considered to be inherently more tolerant than either rice (Oryza sativa) or bread wheat (Triticum aestivum). In dicotyledonous species the variation is even greater, ranging from very sensitive species such as Arabidopsis thaliana to halophytes such as Mesembryanthemum crystallinum, saltbush (Atriplex spp.), and Thellungiella salsuginea (previously T. halophila) [6]. Unfortunately, the domestication-driven selection for yield has reduced genetic variation for this trait in most of our crop species [7]. Since the 1970s, attempts have been made to identify genes or gene products, which are responsive to the presence of salinity stress, based on comparisons made between related species or accessions within a species. The earliest example of this approach dates back to the study of Rush and Epstein [8] working with tomato. More recently, the salinity response of the glycophyte model plant A. thaliana has been compared with that of the halophyte T. salsuginea [9–11], and similar studies have been made between contrasting cultivars of tomato, rice, wheat, and barley [12–14].

Numerous physiological, molecular, and functional studies have provided a wealth of information on salt-responsive genes and gene products, and shed light on species-related differences in mechanisms of salinity tolerance. The bulk of this work has been carried out using the three model plant species, namely A. thaliana, O. sativa, and M. crystallinum. Apart from these plants, others like T. salsuginea, Xerophyta humilis, Populus euphratica, Setaria italica, Glycine soja, H. vulgare, Sorghum bicolor, and T. aestivum have been exploited to understand the differences in gene/protein expression between salt-sensitive and tolerant species. Excellent reviews have been published previously, and the interested reader is referred to them for complementary information [6, 15, 16].

The aim of the review is to summarize the insights into salt stress responses of plants achieved by proteome studies. Many research groups interested in salt stress defense mechanisms have integrated proteome techniques into their repertoire and this is reflected by the rapid increase in related publications. We do not aspire to comprehensively include all these citations into our review. Instead, we will focus on work performed with cereals as important crop plants. Work with model plants Arabidopsis and Mesembryanthemum will be summarized. We will also highlight selected areas of subproteome analysis, namely the analysis of plasma membranes, tonoplast, and apoplastic, which are known to be major sites harboring salt stress defense components due to their role in ion transport, receptor signaling, and ion sequestration. We will also summarize recent progress in chloroplast and mitochondria salt stress proteomics, which underscores the role of these organelles in ROS generation and scavenging. We will address signaling components and summarize current knowledge on hormonal control of salt stress defenses. We then emphasize the potential impact of plant–microbe interaction on the capacity for salt tolerance, and important aspect often ignored under lab conditions. Finally, we will derive conclusions for future research directions in salt stress proteomics.

2 Plant proteomics to unravel mechanisms of salinity tolerance

Salinity stress is known to cause a multigene response and therefore a precise analysis of the proteome is essential for understanding the underlying stress physiology. During the last decade proteome analyses have gained popularity in plant science, and have mostly relied on 2DE for whole tissue protein separation. However, recent years have seen the development of a number of novel approaches to access individual classes of proteins, which enable deeper analysis of the proteome from particular tissues, cells, or subcellular fractions; these generally complement rather than replace the well-established 2DE approach. It has become common practice to apply MS-based proteomic approaches to complex mixtures as well as to prefraccionated extracts in order to identify post-translationally modified peptides [17]. The complete description of a proteome remains challenging, given the level of complexity introduced by the presence of mRNA splicing, various PTMs, particular isoforms, and variable degradation products. However, an adjustment in the expression of a certain isoform or of a particular protein modification could well be an important prerequisite of a resistant genotype to perform better under stress such as salinity, and thus represent a particular target to monitor.

To date, plant proteome analysis allowed for the identification of more than 560 unique salt-responsive proteins in shoots, leaves, roots, seedlings, radicles, hypocotyls, grains, gametophytes, and unicells from 34 plant species [16]. A summary of salinity tolerance mechanisms reflected by these protein candidates has been ascribed to functional categories such as photosynthesis, ROS scavenging system, osmotic homeostasis, signal transduction, ion homeostasis and...
cross-membrane transport, protein fate, cytoskeleton dynamics, and cross-tolerance to multiple stresses [16]. Equivalent investigations of salinity response and tolerance, especially in the context of transmembrane ion transport, plasma membrane properties and cellular compartmentalization, signal perception and transduction, as well as long-distance signaling and energy metabolite distribution are rare or even lacking.

In the model plant *A. thaliana*, the effects of salinity and hyperosmotic stress on the proteome of cell suspension cultures have been investigated by means of 2D DIGE technology [18]. Among the proteins responsive to 200 mM NaCl and/or 400 mM sorbitol treatment were H^-ATPases, signal transduction related proteins, transcription/translation-related proteins, detoxifying enzymes, amino acid and purine biosynthesis-related proteins, proteolytic enzymes, HSPs, carbohydrate metabolism associated proteins, and proteins of unknown function. Another study, employing 2DE to investigate salt-responsive proteins in *Arabidopsis* roots after treatment with 150 mM NaCl for either 6 or 48 h identified proteins related to oxidative stress as well as ATPases [19]. More recently, the combination of 2DE and an LC-MS-based (iTRAQ) approach was utilized to compare the salinity responses of *A. thaliana* and *T. salsuginea* [10]. Overall, there were more changes in protein abundance upon salinity detected in *Arabidopsis* when compared to *Thellungiella* and the responsive proteins were different between both species. A more efficient way of stress perception was suggested for *T. salsuginea*, thereby leading to faster achievement of homeostasis under salt stress conditions. In contrast, a strong hyperosmotic reaction has been detected for *Arabidopsis*.

Salinity-induced tissue-specific and subcellular-specific alterations to the proteome, as well as a number of post-transcriptional modifications, have been widely explored in both *A. thaliana* [10,19–21] and rice [22–28]. However, results mostly pointed to responses of previously characterized salt-induced proteins rather than providing new mechanistic insights on salinity tolerance. Moreover, extrapolation of results on salinity tolerance in *Arabidopsis* to cereal species turned out to be difficult due to species-specific differences in salt tolerance mechanisms [29]. The analysis of the response to salinity stress has tended to be focused on whole organs, with little emphasis being given to the transcriptome, proteome, or metabolome at the specific cell-type or subcellular level. In the *A. thaliana* root, the salinity stress response appears to be regulated in a cell- and tissue-specific manner [30,31]. Transcriptional changes induced by severe salinity stress are largely constrained by developmental parameters, reflected by differential regulation of specific biological functions in subsets of cell layers. The physiological response to salinity stress differs along both the longitudinal and the radial axis of the root. Accordingly, the subcellular distribution of regulatory proteins appears to be cell-type dependent. An example of this behavior is given by members of the chromatin-associated high mobility group A. *thaliana* proteins [32]. While HMGB2 and HMGB4 localize preferentially to the nucleus in root meristem cells, the distribution of HMGB1 is less well defined [33]. A recent study elegantly showed that the expression of the cereal Na^- transporter HKT1;1 in the mature root stele of *A. thaliana* decreases Na^- accumulation in the shoot by 37 to 64%, leading to an increased salinity tolerance of respective lines [34]. Such observations serve to emphasize the need for the spatial analysis of proteomic and metabolomic data.

Mesembryanthemum crystallinum (ice plant) is a member of the *Aizoaceae* family in the order Caryophyllales of which sugar beet is the most economically valuable crop. Plants in this order are often found in marginal, stressful habitats and show a remarkable ability to tolerate diverse abiotic stress; particularly cold and salt stress [35]. The ice plant is a highly salt tolerant species native to South Africa but now naturalized worldwide, predominantly in coastal areas, which show a Mediterranean climate [36]. For most common crop plants, the presence of salt restricts the uptake of water and necessary nutrients, and once accumulated within the plant sodium can quickly reach toxic levels [6]. The ice plant has developed adaptive mechanisms to enable the uptake of water under high salinity conditions and to actively accumulate the salt into the aerial parts of the plant [36,37]. These plants show very high water use efficiency as a result of facultative Crassulacean acid metabolism [38]. The unique properties of the ice plant have attracted many labs to exploit this plant as a model system to study salt tolerance, despite lacking reliable tools required for molecular genetic analysis. Studies focusing on particular cellular adaptive mechanisms have helped to gain an understanding of specific processes involved in salt tolerance of this plant, however, few proteomics studies have been carried out. The specialized epidermal bladder cells of *M. crystallinum*, which swell up into balloon-like sacs when plants are treated with Na^+, have recently been subjected to proteomic analysis [39,40]. Identified proteins showed diverse biological function and confirmed a role for these cells in ion and water homeostasis, as well as in plant defense, carbohydrate metabolism, and photosynthesis. Proteomic studies such as these in *M. crystallinum*, combined with large-scale mRNA expression profiling [41] will help to identify proteins and pathways that are essential for plant salt tolerance and can be exploited for improving crop plant responses to salinity. Subcellular proteome analysis, focusing on salt-responsive proteins at the tonoplast of *M. crystallinum* will be discussed below in the appropriate section.

Barley (*H. vulgare*) is known to be one of the most salinity tolerant of the cereals and has become a model in elucidating, for example, developmental processes or biotic interactions [42,43]. Cultivated and wild barley have been exploited to generate a substantial body of physiological [25,44–47], genetic [48–51], and molecular [52–55] data relating to their response to salinity stress. Despite significant technological advances, little use has been made of proteomics-based approaches [56–59]. Barley shows remarkable genotypic diversity in tolerance to unfavorable environmental conditions, including salinity tolerance as observed in the parents of the

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Oregon Wolfe Barley and Steptoe-Morex mapping populations. Proteomic profiling of mature grains of Oregon Wolfe Barley lines with differing salinity responses has revealed that the level of 6-phosphogluconate dehydrogenase and glucose/ribitol dehydrogenase are positively correlated with salinity tolerance during germination. In addition, heterologous expression of glucose/ribitol dehydrogenase increased the tolerance of a salt-sensitive yeast strain [60]. A comparison of the root proteomes of cvs. Steptoe (salinity sensitive) and Morex (tolerant) successfully identified a number of cultivar-specific and salinity stress responsive proteins. Among those, proteins involved in ROS detoxification were more abundant in the tolerant genotype, while proteins involved in iron uptake were more pronounced in the sensitive one [60]. Accordingly, differences in the expression of ROS scavenging proteins have been detected in two contrasting barley genotypes during long-term salinity exposure [58]. On the other hand, results obtained from two contrasting rice genotypes indicated a higher capability of Na$^+$ exclusion for the tolerant genotype rather than other detoxifying responses [61].

The level of growth reduction during salinity stress appears to be directly influenced by the sensitivity of the crop species and by the genotype. Maize (*Zea mays* L.) is considered as a salt-sensitive plant [62], showing a strong and rapid growth reduction in response to salt stress, which is reflected by many changes in the proteome [63]. However, a salt-resistant maize hybrid showed an apoplastic acidification in the leaves under salt stress and was able to maintain leaf growth [64]. The function of apoplastic proteins in the process of mitigation of salt stress in maize remains uncertain but could benefit from a subcellular proteomic approach to elucidate candidate proteins for possible stress; please refer to Section 3.

Rice (*O. sativa* L.) is the primary staple food for more than 2 billion people and supplies 23% of the total calories consumed globally [65]. Rice is also a model for functional genomics studies of graminaceous crops due to its small genome, high degree of synteny with other cereal crops, availability of a fully sequenced genome, and ease of genetic manipulation, providing a major advantage for functional studies [66]. Salinity is a major factor that limits rice production worldwide. While rice is relatively tolerant to salt stress during germination and active tillering, it is considered to be sensitive to salinity during the early seedling and reproductive stages [67]. Even varieties such as Pokkali, which can withstand moderate salt stress in mid-to-late vegetative stage, succumb if exposed to moderate salt during seedling establishment or reproduction [67].

Investigation of salt stress responsive proteins in various rice tissues including roots [23, 24, 26, 68–72], shoots [27, 73, 74], young panicles [75], and anthers [76] have been the focus of several studies. These studies focus on ubiquitin-related proteins [68], apoplastic proteins [69], and membrane-associated proteins [26, 77], with the majority showing the consistent regulation of proteins involved in carbohydrate, nitrogen, and energy metabolisms, ROS scavenging, cell signaling, mRNA and protein processing, and cytoskeleton stability [78].

Like most plants rice presents distinguishable responses to salt stress [79]. While most of the studies on rice response to salinity applied a short-term salt stress [23, 24, 27, 68–71, 73, 80], a few groups investigated the effects of long-term exposure [72, 74–77]. Together these studies show that the early responses to water and salt stress are essentially identical and salt-specific effects develop with time [44]. Parker et al. demonstrated specific differences in the proteins expressed during the first and second phases of response to salt by comparing 24 h with 7 days salt stress [74]. A study by Song et al. [69] showed that protein abundance changed in the rice root apoplast changed significantly following 1 h of salt stress treatment, underscoring the rapidness of the response. It is important to be aware that some changes associated to plant stress tolerance involve post-translational regulation such as protein phosphorylation, and this modification may take place within the short term. This is highlighted by a study in rice, which demonstrates that following 4 and 10 h of salt stress, 10 of the 17 responsive proteins identified were likely to be regulated at the post-translational level instead of protein abundance [24]. Although rice is much more sensitive to salt stress at the reproductive than at the vegetative stage, little is known about the physiological or proteomic response of the plant to salinity during reproduction. A proteome analysis of rice panicle responses to salinity at the early reproductive stage revealed a role for several salt-responsive mechanisms [75]. Proteome analysis of anthers in two contrasting rice genotypes IR64 (salt-sensitive) and Cheriviruppu (salt-tolerant) under salt stress showed the possible involvement of carbohydrate/energy metabolism and anther and pollen wall remodeling/metabolism in the adaptation of rice to salt stress at the reproductive stage [76].

Wheat is the most widely cultivated crop in the world, and is clearly a major component of the human diet. Because of the size and complexity of the wheat genome, wheat proteome analysis has lagged behind other plant species, particularly compared to the model plants such as rice and *Arabidopsis*.

While early proteome studies of wheat response to salt stress showed a few differentially expressed proteins [81–83], more recent attempts to analyze the wheat leaf proteome response to salinity resulted in identification of a long list of salt-responsive proteins [84], including carbon, amino acid, and nitrogen metabolism proteins, signal transduction related proteins, and detoxification and defense-associated proteins.

These studies focused on the effects of short-term salt stress in wheat. By comparing the proteome of salt-resistant and salt-sensitive wheat genotypes under 125 mM NaCl, Saqib et al. [81] showed that the initial reaction to salinity at the protein level is an unspecific response and the genetic variation in salt resistance of the tested wheat genotypes becomes more clear in the second phase of salt stress, which takes a few weeks to develop [81]. It was suggested that the initial response may be a secondary effect rather than a specific adaptation. This observation is in line with the findings made by Peng et al. [13]. By analyzing the proteome of roots...
and leaves under salinity and drought conditions, they observed that the number of salinity-responsive proteins was greater than the number of drought-responsive proteins and most of the drought-responsive proteins also responded to salinity [13].

3 Subcellular proteomics

The complex response to salt stress at the cellular level is reflected by adjustments in the transcriptome [85], the proteome [4,86], and the metabolome [87,88], which lead to physiological and developmental alterations depending on stress intensity and stress duration. While available tools for expression analysis of transcripts allowed for the identification and quantification of thousands of RNA molecules from single cells and tissues [30,89], proteome studies are more challenging due to the lack of amplification technology and the biochemical heterogeneity of polypeptides. A means to dig deeper into the proteome is to fractionate the samples and analyze the subcellular proteome responses to salinity stress by focusing on specific organelles or membranes. To date, plant salinity responsive subcellular proteomics has been performed on microsomal membranes (A. thaliana and T. halophila), plasma membranes (A. thaliana, O. sativa, Beta vulgaris, and Dunaliella salina), mitochondria (T. aestivum), chloroplast (Z. mays, T. aestivum, Salicornia europaea, and A. thaliana), apoplast (O. sativa, Nicotiana tabacum, and Z. mays), and tonoplast (M. crystallinum); the reader is also referred to [16]. Membrane proteins are known to act as facilitators and regulators of ion transport and as such have an important role in plant stress tolerance, in particular salt stress. Most proteomic studies on membrane proteins concentrated on the construction of protein catalogues of plasma membranes, thylakoid membrane, tonoplast, ER, and nuclear matrix, among others [90,91]. Here we describe in more detail the contribution of specific subcellular compartment/membrane proteomes to salinity stress tolerance.

3.1 Plasma membrane proteomics

At high ambient salinity, proteins embedded in the lipid bilayer of the plant plasma membrane fulfill key functions in signal transduction, and regulate active and passive ion fluxes, thus restricting increases in intracellular sodium and chloride concentrations.

Despite the great biological significance of plasma membrane proteins and innovations in analytical methodology [17], comprehensive analysis still remains a challenging task. Quality and quantity of gene products are affected by the high level of dynamic processes in plasma membranes, including exocytosis, endocytosis, lateral movement, protein degradation, and PTMs. Furthermore, their investigation is hampered by their low abundance and hydrophobic biochemical properties, necessitating the development of methods for plasma membrane enrichment, removal of nonspecifically associated soluble proteins, and peptide and protein analyses [92].

Current methodologies for identifying plasma membrane proteins that can be used as targets to increase salt tolerance have been applied to crops such as rice and barley. Salinity stress induced changes in the proteome of the rice seedling plasma membranes treated with 100 mM NaCl for 2 wk included 24 proteins, which were present at different abundances on 2DE gels [77]. Identification was successful for eight of those, and included remorin, putative 1,4-benzoquinone reductase, two 14-3-3 proteins and a hypersensitive-induced response protein. In another study, 2DE separation of root tip plasma membrane proteins of rice seedlings challenged with 150 mM NaCl for 48 h revealed expression changes of 34 proteins with roles in membrane stabilization (peroxidase, remorin), ion homeostasis (14-3-3 protein), signal transduction (calmodulin, DREPP proteins, putative receptor protein kinase), and defense-related proteins (osr40c1, hypersensitive-induced response protein, 1,4-benzoquinone reductase) [26].

3.2 Apoplast proteomics

Within a plant, the apoplast is the free diffusional region outside the plasma membrane, consisting mainly of a continuum of cell wall as well as the extracellular spaces [93,94]. It can serve as a storage space for solutes and is involved in physiological reactions such as intercellular signaling, defense against biotic and abiotic stresses, and is important for the acquisition of water and nutrients. Sodium accumulation in the leaf apoplast has been suggested to lead to dehydration, wilting and finally, the death of the affected leaves. The soluble proteins within the apoplastic space can be considered as the apoplastic proteome.

One of the first reports of salt stress response in the apoplastic proteome was from tobacco [95]. In this study, the expression of two chitinases and a germin-like protein increased significantly under salt stress, while two lipid transfer proteins were expressed de novo.

Specific effects of salinity on the apoplastic β-expansin proteins, which are known to be involved in loosening cell walls and promoting cell growth, were recently investigated in maize cultivars differing in salt sensitivity, following short-term exposure to 100 mM NaCl [96]. According to the results the authors [96] suggested that the upregulation of the expansins, ZmExpB2, ZmExpB6, and ZmExpB8, may sustain the stable expression of these proteins under conditions of salt stress. By means of 2DE and western blot analysis, the β-expansin isofrom 6 (ZmEXPB6) was established to be decreased in growth-inhibited leaves of the salt-sensitive maize hybrid Lector. In contrast, in the salt-resistant hybrid SR03 the stable abundance of β-expansin proteins maintains shoot growth [64].
In rice, a study investigated the apoplastic proteome in 10-day-old shoots by means of 2D DIGE following exposure to 200 mM NaCl for 1, 6, or 12 h [69]. This study identified 69 salt-responsive proteins of which 37 are known apoplastic proteins [69]. A similar study, applying 200 mM NaCl for 1, 3, or 10 h to 10-day-old rice seedlings, revealed changes in ten proteins, one of which corresponded to a strongly upregulated apoplastic protein, *O. sativa* root meander curling, with extracellular domain-like cysteine-rich motifs (DUF26) [97]. Interestingly, *O. sativa* root meander curling RNA, transgenic rice showed improved salt stress tolerance.

### 3.3 Tonoplast

The vacuole is known to play a key role in plant salt tolerance, sequestering Na\(^+\) away from metabolic enzymes and helping to maintain cytoplasmic Na\(^+\)/K\(^+\) ratios low. Quantitative proteomics of salt-responsive proteins identified from highly pure tonoplast fractions of *M. crystallinum* leaf tissue identified a small number of proteins whose abundance changed when plants were grown in the presence of NaCl [98]. These included expected proteins such as subunits of the V-ATPase, the vacuolar proton pump, which plays a key role in the sequestration of Na\(^+\) into the vacuole; but also unexpected proteins, the glycolytic enzymes, aldolase and enolase. Commonly presumed to be cytosolic, the membrane association of these proteins suggested an alternative function for these enzymes, and evidence was presented that showed aldolase could interact with the V-ATPase B subunit and regulate V-ATPase activity [98]. The lack of identification of salt-responsive integral membrane proteins may reflect the use of in-gel proteomic approaches and future work combining gel and gel-free quantitative approaches may serve to increase the number of proteins identified as salt-responsive.

### 3.4 Chloroplast proteomics

Studies on chloroplast metabolism have shown that photosynthesis is directly affected by salinity [99]. The chloroplast response is complex as the salt stress reduces stomatal opening, which decreases CO\(_2\) availability, limiting photosynthesis; but also salinity causes structural changes in the chloroplast and results in the overproduction of ROS causing oxidative stress. While total protein proteomic studies have identified chloroplast proteins responsive to salinity, studies in which chloroplasts are first isolated and purified provide a more detailed picture of the organelle specific effects of the stress.

In *S. europaea*, subcellular proteomic investigation of chloroplast proteins by the application of 2DE and blue-native PAGE in combination with MS/MS analysis, identified 90 proteins whose expressions were altered under long-term, high salt treatment [100]. Analysis of gene expression patterns of 12 selected proteins, including RbcL, seduheptulose, bisphosphatase, RuBisCO activase, and phosphoglycerate kinase, provided evidence for correlations between transcription and proteomics data. The authors concluded that chloroplast proteins that participate in Calvin cycle, light reaction, and nitrogen metabolism are critical in governing salt tolerance of *S. europaea*.

In salt-stressed wheat chloroplasts, 21 protein spots showed differential abundance during salt stress, which was monitored daily for a period of 3 days. From these spots 65 different proteins were identified, not all corresponding to chloroplast proteins. Those that showed consistent up-regulation over the 3-day stress period included cytochrome b6–f complex, glutamine synthetase, fructose-bisphosphate aldolase, and S-adenosylmethionine synthase [101].

A proteomic study of maize chloroplasts aimed to investigate the effects of a short-term salt exposure (up to 4 h), as sodium ions were shown to accumulate rapidly in maize chloroplasts in the initial phase of moderate salt stress [102]. 2DE revealed 12 salt-responsive chloroplast proteins increased 2- to 6.7-fold while eight chloroplast proteins decreased. It was suggested that the enhanced abundance of the ferredoxin NADPH reductase, the 23 kDa polypeptide of the photosystem II, and the FtsH-like protein might reflect a mechanism to attenuate the detrimental effects of Na\(^+\) on the photosynthetic machinery.

### 3.5 Mitochondrial proteomics

The response of mitochondria to salinity stress appears to be complex; while mitochondrial respiration has been shown to be differentially regulated by salinity stress depending on species, tissue, or cell type, in a manner unrelated to tolerance levels, there are convincing links between increases in mitochondrial antioxidant defense proteins and salinity tolerance [103]. Furthermore, whole cell proteomic studies have identified mitochondrial proteins as being particularly stress-responsive [104]. However, these proteins are all predominantly highly abundant relative to other cellular proteins. Few studies have employed isolated mitochondria to study the effects of salinity on these organelles.

Employing two economically important wheat cultivars, Jacoby et al. [105] quantitatively compared variations in shoot mitochondrial protein abundance between the salt-tolerant cultivar Wyalkatchem, and the salt-sensitive cultivar Janz, in the presence and absence of salt stress. Differentially expressed proteins included Mn-superoxide dismutase, cytochrome synthase, nucleotide diphosphate kinase, and the voltage-dependent anion channel. The large amount of differences in proteins involved in ROS scavenging suggested that alterations in ROS defense pathways between the mitochondrial proteomes of the two wheat varieties may correlate with whole plant salt tolerance.

Another study, using 2DE of purified mitochondria proteins isolated from rice root tip cells exposed to high salt stress, shown to induce responses associated with
programmed cell death (PCD), identified alterations in nine PCD-related proteins [106]. These included three mitochondrial proteins, which were upregulated in the presence of salt: glycoside hydrolase, mitochondrial HSP 70, and Cu/Zn-superoxide dismutase; and two mitochondrial proteins, which were downregulated, namely ATP synthase beta subunit and cytochrome c oxidase subunit 6b. Three other nonmitochondrial proteins were also identified as salt-responsive, including S-adenosylmethionine synthetase 2, transcription initiation factor eIF-3 epsilon, and 20S proteasome subunit. Although these proteins were assigned as contaminants, it was suggested that they do have roles in PCD.

3.6 Conclusive remarks on subcellular proteomics

Innovations in gel-free and gel-based proteomic concepts have facilitated the investigation of subcellular protein distributions, as well as PTMs. However, analyzing subproteomes such as the complex and highly dynamic plasma membrane still represents a challenging task. Nevertheless, in-depth knowledge and characterization of subcellular proteomes will be essential to the understanding of salt stress responses in plants and to the development of salt-tolerant crops. In addition to the enrichment techniques for the isolation of particular organelles or subcellular structures, complementary analysis by directed approaches will become more widespread. This will be discussed further below.

4 The impact of microbial communities on salinity tolerance

The rhizosphere and endosphere of plants is colonized by a range of microorganisms, of which some are able to enter and colonize the plant interior, usually exhibiting growth promoting activity in the host [107]. The beneficial effects provoked by endophytes result from nitrogen fixation, increased phytohormone production, and nutrient supply, as well as pathogen suppression [108, 109]. These mechanisms help to alleviate detrimental effects of unfavorable environmental conditions on the host plant.

Though proteomic studies on plant/microbe interactions are rare, ample evidence has been presented to show that plant growth promoting rhizobacteria (PGPR) mitigate salt stress in crop plants, as shown when wheat seedlings were inoculated with PGPR Azospirillum lipoferum [110]. Nowadays, it is generally accepted that PGPR can stimulate not only plant growth and yield, but also ameliorate the effect of biotic and abiotic stresses [111, 112]. For instance, it has been noted that PGPR that contain the enzyme 1-aminocyclopropane-1-carboxylate deaminase facilitate plant growth and development by decreasing plant ethylene levels, especially following a variety of environmental stresses [111, 113].

Also, arbuscular mycorrhizal fungi mitigate the effects of salt stress in plants by several different physiological mechanisms, for review [114–116]. More recently, ultrastructural evidence of the salt stress mitigation has been shown with arbuscular mycorrhizal fungi Glomus intraradices in Trigonella foenum [117].

Other studies using endophytic fungi showed an improvement of growth and a significant mitigation on the impact of salinity and drought for cucumber, via gibberellins [118] and indol acetic acid effects [119] as well as for soybean associated with Metarhizium anisopliae through isoflavonoids influence [120]. Also, it has been reported that exopolysaccharides from cyanobacteria showed a remarkable ability to improve seed germination and vigor index in wheat, maize, and rice under different salt concentrations [121]. Co-inoculation of Bacillus subtilius SU47 and Arthrobacter sp. SU18 on wheat cultivated under different salinity regimes showed that the activity of antioxidant enzymes in wheat leaves decreased under salinity stress suggesting alleviation of the effect of saline stress on wheat growth [122].

Though there is a vast amount of genomic information on bacteria and plant interactions, the analysis of the proteome still represents a key approach to curate or complement genomic-based predictions or information [123–125]. Using a metaproteogenomic approach, the phyllosphere and rhizosphere microbiota of rice cultivars has been characterized [126].

Also, it has been shown that Pseudomonas fluorescens MSP-393, a proven biocontrol agent for many of the crops grown in saline soils of coastal ecosystem, was not hampered with higher salinity in soil. Peptide mass fingerprinting revealed the upregulation of many of salt-regulated proteins and de novo—synthesis of osmolytes in the cytosol [127].

5 Hormones and other signaling mechanisms

The central role(s) of hormones in the plants response to salt stress has not been a topic adequately reflected in proteome studies. While the majority of proteome studies have monitored the impact of salinity stress on protein patterns after a mid- or long-term exposure, short-term studies focusing on characterizing proteins involved in signaling cascades in response to the stress and/or hormone application are rare. We will summarize current knowledge, which will drive future integrated studies toward early salinity response of crop plants.

Long-distance hormonal signaling between roots and shoots under salt stress has received special attention during the last few years [128, 129]. The basis for this lies in the knowledge that xylem hormonal status plays a role in mediating shoot responses not only in terms of growth but also for stress adaptation and, therefore can directly affect crop salt tolerance. Manipulation of xylem sap cytokinin (CK) composition (e.g. by grafting plants on selected rootstocks) has produced positive effects on tomato growth and yield under salinity [130, 131]. Although root-sourced Cks, ABA, and the ethylene precursor aminocyclopropane-1-carboxylic
acid seem key in controlling shoot performance under abiotic stress, further insights about their physiological, biochemical, and genetic determinants and regulation at their sites of production and action will certainly facilitate genetic improvement of crop salt tolerance. However, other hormones with a nonspecific root origin such as auxins, gibberellins (GAs), salicylic acid, and brassinosteroids (BRs) also seem to have an important direct or indirect role in salt tolerance [6, 116, 128, 132–134].

Recently, it has been reported that 6-benzylaminopurine differently affects the proteome in shoots and roots in Arabidopsis, including hormonal homeostasis [135]. While 6-benzylaminopurine upregulated proteins involved in carbohydrate/energy metabolism and ABA biosynthesis/response in the shoots, the CKs upregulated most of proteins involved in ethylene biosynthesis in the roots. In turn, ethylene and the auxin, indole-3-acetic acid (IAA) seem to regulate similar physiological processes in Arabidopsis roots but through different proteins [136]. Hence, it is important to elucidate how CKs and signaling components modulate not only stress tolerance, especially those associated with ABA, but also development, in particular with auxins (i.e. root growth) and ethylene, and how these responses can be translated from Arabidopsis to crop species.

A comprehensive study in A. thaliana attempted to analyze the signaling events occurring during salt stress by studying salt-induced changes in the root microsomal proteome, using 2DE combined with protein gel blotting [137]. Ca\(^{2+}\)-dependent membrane-binding proteins, designated annexins, were identified as the major signaling components, a result validated by the observation that T-DNA insertion mutants of two isoforms of annexin displayed hypersensitivity to osmotic stress and ABA treatment during germination and early seedling growth. Mutants with ABA deficiency showed reduced tolerance toward abiotic stresses, including salt stress [138]. Since ABA regulates nearly 10% of the protein-coding genes, a much higher percentage than other plant hormones [139], the dissection and manipulation of its signaling pathways offers potential opportunities for improving crop stress tolerance, especially via manipulation of transcription factors (TFs) and hormone receptors. Among the TFs, the SnRK2-AREB/ABF pathway governs the majority of ABA-mediated, ABRE-dependent, gene expression in response to osmotic stress during the vegetative stage. The ABL1 protein (ABILike1) of rice is a key component of ABA signaling codifying a basic region/leucine zipper motif TF that is expressed in various tissues and is induced by the hormones ABA and IAA and water stress conditions including salinity [140]. The ABA receptor PYR/PYL/RCAR family is well conserved in crop species and their exogenous modulation by pyrabactin, a selective ABA agonist, will enable new chemical strategies for modulating ABA receptor activity and crop stress responses on a commercial scale [see [141] for a review].

Protein phosphorylation/dephosphorylation is a central PTM that affects protein structure and may play a key role in phytohormone-mediated physiological responses affecting plant development and adaptation to environmental stresses. Many differentially phosphorylated proteins are involved in this process, including various families of kinases, receptor-like kinases (RLKs), and protein phosphatases. Indeed, the ABA-signaling model is expanding since the analysis of the phosphoproteome using \(^{15}N/^{15}N\) metabolic labeling in Arabidopsis following ABA application for a short period (5–30 min) revealed changes in 50 polypeptides, including more than 20 unknown proteins [142]. Increases in phosphorylation were observed in a conserved N-terminus of sucrose non-fermenting related kinases and basic leucine zipper TFs that putatively regulate AREB activity or affinity [142, 143]. However, four aquaporin members were dephosphorylated in a conserved C-terminal serine residue, supporting the role of ABA in the reduction of water flow and adaptation to drought and salinity. The authors suggest that the model invoking phosphatase inhibition by the ABA receptor seems to be insufficient to explain the decreased phosphorylation level of many other unknown proteins [142].

RLKs are considered as key regulators of plant architecture and growth behavior, with also a function during abiotic stress [see [144] for a review]. A single amino acid replacement in the RLK BRI1 brassinolide receptor, that negatively regulates its activity, strongly promotes shoot growth, together with increased proline production that is normally associated with water and salt stresses [145]. Recently, it was also shown that overexpression of the RLK, Os-SIK1 affects stomata density in the leaf epidermis of rice and leads to higher tolerance to salt and drought stresses [146]. This modulation of stomata density was recently supported by the uncovering of molecular interactions between BR and stomatal signaling pathways [133]. Another possible molecular mechanism that links BRs with growth acclimation to abiotic stress tolerance involves regulated intramembrane proteolysis triggered by endoplasmic reticulum stress signaling [147, 148].

As in the case of ABA, it can be expected that synthetic molecules that activate or repress regulatory proteins such as RLKs could provide powerful chemical tools to interfere with the corresponding signaling pathways [144]. However, only a few small molecules have been reported that efficiently and specifically modulate plant-signaling cascades. This may in part be due to the fact that many plant-signaling pathways are initiated by protein–protein interactions, as recently defined for auxin, GA, ABA, and BR hormone sensing [see [144] for a review]. The target proteins of such modifications are largely unknown and need to be identified for a better understanding of phytohormone effects [143].

Since the hormonal responses to salt stress are complex, the effects of the qualitative and quantitative hormonal changes on the proteome and especially on post-translational signaling modifications should be initially approached by studying individual hormones to allow for dissection (i) the specific mechanisms of response to each hormone, (ii) the interactions among hormones, (iii) the relationship between hormones and specific physiological effects, and (iv) to identify potential target proteins for improving salt.
tolerance. Comparative phytohormone-responsive phosphoprotein analysis by using TiO2-phosphopeptide enrichment mass accuracy precursor alignment has been performed in Arabidopsis following ABA, auxin, GA, jasmonic acid (JA), and CK treatments [143]. This study revealed that different hormones differentially regulate phosphorylation sites in distinct amino acid residues in members of the same protein families, as well as the existence of hormone-specific responsive phosphopeptides, demonstrating hormone-specific phosphorylation sites and independent post-translational programs. It was also reported that other sites are common for multiple hormones, confirming the global crosstalk among hormone signaling pathways and the complexity of establishing predictive models on the hormone-mediated plant-proteome responses to salinity due to the differential spatial and temporal hormonal changes and tissue specificity.

The regulation of ion transporters by hormones is of special relevance in the context of salinity in order to maintain ionic and nutritional homeostasis. For example, the K+ transporter (KUP5) and the Na+/H+ exchanger, SOS1/NHX7 were regulated by ABA, while other members of these gene families, KUP7 and NHX2 were regulated by IAA, and two peptides for Cl− transporters were regulated by IAA and kinetin [143]. An Mg2+ transporter was regulated by JA alone, while phosphorylation of a high-affinity NO3− transporter was found to be downregulated exclusively by ABA. The phosphorylation of the NH4+ transporter AMT1 was highly upregulated by GA and JA [16]. The enhancer of PINOID (ENP) was also activated by phosphorylation following IAA treatment, which in turn phosphorylated PIN auxin transporter’s cell polarity of auxin signaling pathways [143], and could be involved in root growth maintenance under salinity [149]. Tyrosine phosphatase 1, required for ABA signaling, and protein phosphatase 2C were phosphorylated by ABA and GA, while the F-box protein was differentially phosphorylated by IAA and GA. However, GA seems to be more exclusive in activating efficient trafficking in the ER through calnexin phosphorylation [143].

The interesting finding that hormones target various members of protein families involved in growth, together with the identification of a small number of proteins that were co-regulated by multiple hormones, indicate synergistic action of pathways. Such pathway interactions probably occur through combinatorial regulation of common target proteins by various hormone-controlled kinases [143], with the common objective of adapting growth to the environmental conditions in order to improve plant survival. However, the biotechnological manipulation of the key proteins or hormones should focus on not only the ecological adaptation in terms of survival but also the agronomical stability for securing food production. For example, ABA induces the phosphorylation and activation of the AREBs TFs and the overexpression of the phosphorylated AREB1 induces the expression of many ABA-inducible genes [150, 151]. Indeed, the overexpression of the SlAREB1 gene in tomato improved plant survival under drought and salinity stress through senescence-delaying and water-conservative mechanisms [152]. Those ABA-mediated mechanisms are probably incompatible with growth maintenance, for which the coordination with other growth-promoting hormones/proteins is required. One core regulatory element for plant growth seems to be the protein G α-subunit, whose phosphorylation site Tyr166 is sensitive to several hormones, thus adapting cellular sensitivity to environmental cues in different ways [143].

The identification of 84 methyl-jasmonate (MeJA) responsive proteins in Brassica napus guard cells has also revealed interesting molecular mechanisms underlying MeJA function in stomatal movement, which include homeostasis of H2O2 production and scavenging, signaling through calcium oscillation and protein (de)phosphorylation, gene transcription, protein modification, energy balance, osmoregulation, and cell shape modulation [153]. Although some of these proteins (i.e. phosphatase 2A, OST1, myrosinase, CPDKs, and MAPKs) are shared with ABA-signaling pathway, other MeJA-specific proteins could not be directly involved in stomata closure [153]. Those results are consistent with the down-regulation of photosynthesis and carbohydrate anabolism related proteins in response to MeJA in A. thaliana, and the upregulation of stress-related proteins, thus redirecting resources from growth to defense mechanisms [154]. MYC2 protein seems to have a key role in the JA post-translational regulation [155]. However, salicylic acid application improved both growth and drought stress tolerance in wheat seedling through upregulating 35 critical proteins involved in those physiological processes, essentially activating Rubisco, glycolysis, tricarboxylic acid cycle, and antioxidant enzymes [156], some of which could be used as targets or markers in breeding programs.

The manifold physiological and biochemical salinity adaptation responses are mirrored to a large extent at the level of transcriptional control. Regulatory networks of cis-acting elements and TFs underlying salt stress responses have been elucidated [157]. Interaction of the salt-responsive network with hormonal control as well as overlap of regulator circuits of biotic as well as abiotic stress have been defined based on work with Arabidopsis and rice (see [7] for a discussion).

In conclusion, unraveling hormone and other signaling components in Arabidopsis and other species will contribute to create new strategies for enhancing crop stress tolerance through chemical and conventional or biotechnological genetic methods. Future proteome studies will have to integrate these aspects into their experimental design (see below).

6 Challenges and future integrative research strategies

In addition to the power of existing proteomics tools and approaches, several important challenges are emerging for future research in the area of stress proteomics. The fraction of the proteome reached in current studies is far from giving a comprehensive view. Analysis of subcellular fractions clearly
extends the depth of the proteome to be investigated. In the majority of the cited papers, 2DE is still the basic technique for separating proteins. However, LC-based proteome analysis is now established in many labs. Both separation approaches offer specific advantages. With the classical 2DE approach, protein variants and degradation processes can be rapidly visualized. Two Arabidopsis germin-like proteins differing in only one amino acid could be distinguished on a 2DE gel [158]. Also, the occurrence of protein degradation at certain developmental stages becomes immediately apparent on 2DE gel images. A major advantage of the LC-based proteomics is in most cases the low quantity of protein needed for an analysis. This aspect favors the analysis of samples only available in minute amounts, such as pure plasma membrane protein fractions or cell-type specific fractions. LC-MS also complements the poor capacity of 2DE gels for analyzing hydrophobic proteins such as those from membrane preparations. MS techniques for the targeted analysis of select proteins such as selected reaction monitoring/MRM will complement the biochemical approaches and allow to quantify a desired subset of the proteome relevant in a specific biological context as demonstrated for yeast and human cell culture [159, 160].

Another important lesson is the prime importance of good phenotyping to complement the massive amounts of proteomics data being obtained [78, 161]. The combination of physiological measurements with proteomics analyses should provide important information for the development of new strategies for molecular plant breeding under stress conditions [161]. High-throughput noninvasive phenotyping facilities for plants have been installed or are currently extended; several of them will serve as core facilities offering services to external research groups (e.g. the European Plant Phenotyping Network (http://www.plant-phenotyping-network.eu/) and the German DPPN (http://www.dppn.de)). Another major facility is the Australian site established at the University of Adelaide (http://www.plantaccelerator.org.au/). Advanced phenotyping of plant collections will identify novel genotypes with specific stress defense responses. In particular, improved phenotyping of offspring lines from mapping populations or introgression lines will facilitate the identification of useful stress defense related candidates by “genetical proteomics” [162]. The combination of proteomics with other omics techniques such as metabolomics, transcriptomics, and fluxomics is also still in its infancy [163, 164] but a promising strategy toward systems biology analysis of salt stress defense responses to support breeding of tolerant crop lines. Using complementary genomics and metabolomics approaches and bioinformatic tools enabled the prediction of phenotypic performance in Arabidopsis [165, 166], tomato [167], and maize [168]. Similar value will be added with information from proteomic approaches.

The analysis of PTMs and of protein complexes is of particular priority in future studies [169]. Although powerful bioinformatic tools exist, experimental confirmation is mandatory. Given the large number of already described modifications of proteins, it is apparent that large efforts of the proteomics community are required to develop reliable tools for analysis. Phosphoproteome analysis has been a focus of intensive PTM research and improved lab protocols for analysis have become available [170]. Furthermore, databases with experimentally determined phosphopeptides as well as predictive tools constitute important resources [21, 171–174] for the proteomics community. Shoot microsomal fractions of rice plants that were subjected to 200 mM NaCl for 2 days were analyzed for alterations in the post-translational phosphorylation patterns of proteins [21]. Multiple phosphorylated peptides were identified in aquaporin isoforms OsPIP2;1, OsPIP2;6, and OsPIP2;7, reflecting the importance of water flux control in response to salinity. Accordingly, regulation of water transport activity of barley aquaporin isoforms HvPIP1 and HvPIP2 was suggested to require phosphorylation. Moreover, heteromerization of both isoforms resulted in significantly increased water transport activity [175]. Phosphorylation of aquaporins isoforms AtPIP2;1 and AtPIP2;4 has also been demonstrated in membrane preparations from 200 mM salt-treated Arabidopsis plants [20].

Information on the spatial distribution of proteins and metabolites will be a prerequisite to improve current models of stress defense responses and to elaborate the specific functions of individual tissues. MS-based imaging techniques have been recently introduced into plant research [176], but are focused on the analysis of metabolites and peptides. Imaging of larger proteins and the identification of proteins with specific spatial patterns is still a challenge.

As outlined above, hormonal control is an important aspect of stress defense; due to the low abundance, MS-based imaging of phytohormones has not yet been achieved. Laser microdissection of tissues followed by sensitive LC-MS analysis provides an alternative to the imaging strategy [177]. Spatial resolution of metabolite and protein imaging is currently restricted to a dimension of about 10–20 µm. Live cell imaging in combination with suitable molecular tools can also provide spatial information at the subcellular level. Recently, the lateral distribution of 279 plasma membrane proteins was investigated in yeast using green fluorescent protein labeling in combination with total internal reflection microscopy [178]. Results were consistent with a patch-wise organization of the proteins into numerous domains. It was suggested that the domain organization of proteins had an impact on their function [178]. At this point one can only infer on the significance of topological rearrangement of plasma membrane or tonoplast proteins as part of plant salt defense responses.

B.J.B. wishes to thank UNAM-DGAPA-PAPIIT (IN203913) for funding proteomics studies in the lab. H.P.M. wishes to thank BMBF for funding proteome-based studies and the EU for funding of the COST action FA 603 on plant proteomics. B.J.B. and H.P.M. appreciate funding for an exchange program co-funded by the German DAAD (PPP Mexico, ID 56269749) and CONACYT (#189170). A.M. gratefully acknowledges the financial support from German Academic Exchange Service (ID 50755371).
and the German Federal Ministry of Education and Research (BMBF, 0313821A). J.L.D. and T.C. wish to express their gratitude for mobility grants of the CONACyT and the BMBF. F.P.A. thanks the European Commission (ROOTPOWER Contract # 289365), the EU COST office (action FA1204 on vegetable grafting), and the Spanish MEC-FEDER (AGL2011-27996) for support of hormonal signaling research.

The authors have declared no conflict of interest.

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