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Review Article

UPDATE IN ROOT PROTEOMICS WITH SPECIAL REFERENCE TO ABIOTIC STRESSES: ACHIEVEMENTS AND CHALLENGES

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Abstract: Abiotic stresses, such as drought, high salt, waterlogging, extreme temperatures, ultraviolet-B radiation, CO_2 and mineral deficiencies or toxicities severely reduce crop/plant productivity. The plants have evolved highly complex but well coordinated adaptive responses operating at the transcriptional, post-transcriptional, translational and post-translational levels, to alleviate the cellular damage caused by different stress factors. A comprehensive understanding of regulation either at all levels or one of these will provide better tools to improve plant's performance under stress. Roots are primary organs in plants exhibiting cell defenses to several abiotic stresses and are highly sensitive; compared to proteins in leaves and stems, the root proteins have shown rapid responses. This review focuses on root proteomic studies conducted on different crop/plant species after subjecting to different abiotic stress factors, such as drought, salinity, waterlogging, heavy metal and temperature stress. In addition, this review discussed the protein level changes in roots and their possible role to cope with each of the stress factors. Such knowledge on root response characteristics are ultimately helpful in understanding abiotic stress effects in plant roots at molecular level, and in improving crops for tolerance against abiotic stress through gene manipulation, either through conventional breeding or advance biotechnological approaches.

Keywords: Abiotic stress; root; plant proteomics; salt stress; drought; waterlogging.

1. Introduction

Plant growth is greatly diminished by environmental stress factors such as drought, high salinity, extreme temperatures, ultraviolet-B radiation, CO_2 and mineral deficiency and toxicity. These abiotic stresses are severe limiting factors of plant growth and crop production. Abiotic stresses are projected to reduce yields to less than half of that possible under ideal growing conditions, across a range of cropping systems around the globe. The assessment of potential yield losses by individual abiotic stresses is estimated at 17% (drought), 20% (salinity), 40% (high temperature), 15% (low temperature) and 8% by other factors (Shafiq-ur-Rehman *et al*., 2005). It has been estimated that 90% of arable land experience, different abiotc stresses, singly or in combination (Leopold, 1990). Plants respond and adapt to these stresses in order to survive. For example, drought and high temperatures can be exacerbated by mineral toxicities that limit root growth. Higher plants have evolved multiple, interconnected strategies that enable them to survive under abiotic stress (Surabhi *et al*., 2003; Kumari *et al*., 2007; Surabhi *et al*., 2008; Veeranagamallaiah *et al*., 2008; Singh *et al*., 2010; Witzel *et al*., 2009; 2010). However, these strategies are not well developed in most agricultural crops. Although, different plant species have variable thresholds for stress tolerance, and some of them can successfully tolerate severe stresses until completion of their life cycles, after long periods of stress exposure most cultivated crop plant species

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are highly sensitive and either they die or suffer from productivity loss. Thus, understanding and improving of stress tolerance in crop plants not only presents a challenging basic research problem, but could also have significant impact on agricultural productivity.

Roots play a vital role in uptake of water and nutrients, and for anchorage in the soil. They are highly plastic, able to adjust physiologically and developmentally to changing environmental conditions (Iyer-Pascuzzi *et al*., 2009). The importance of root growth for maintaining a crop yield is becoming recognized and of increasing interest to plant breeders (Gewin, 2010). With the growing demand for food globally, it is particularly important that we have a good understanding of the processes affecting root growth under variable soil water regimes associated with changing weather patterns. In terms of physical limitations to root growth, the major causes of poor root system and development are water stress (too little water for root growth), hypoxia or anoxia (too little oxygen) and mechanical impedance (soil that is too hard for roots to penetrate rapidly) (Gewin, 2010).

The main hitch of transcript-based approaches is the lack of information on the state of the proteome. Profiling proteins instead of mRNAs is more informative and since proteins are regarded as the 'effectors' of cellular functions (Bachi and Bonaldi, 2008). Thus the most suitable approach for gene phenotyping and characterization of functional states consists of a global and unbiased analysis of the proteome or of specific proteome subsets (Vij and Tyagi, 2007). Analysis of the proteome provides a direct link between genome sequence and biological activity. Additionally, expression levels and post-translational modifications may help to understand the complex cellular processes at the protein level in roots.

2. Root proteome analysis in response to abiotic stresses

Within a plant the specifics of proteome response to abiotic stress vary from tissue to tissue and organ. Roots are highly sensitive and have been shown to be the primary organs to exhibit specific cell defenses to different biotic and abiotic stresses (Nozu *et al*., 2006). The MS-based proteomics allow isoform specific protein identification and hence are able to differentiate specific and shared functions within a protein family (Ghosh and Xu, 2014). This level of detection is often not possible in transcriptomic studies. Importantly, this review is focused on root proteomics, because huge amount of information exists on the effects of abiotic stressors on the plant shoot parts. Further, reviewing root responses can complement the mechanistic paradigms of stress tolerance proposed from such studies on the plant shoot parts (Trinidad *et al*., 2016). The proteome-wide identification and functional analysis of proteins provide further insights in addition to the findings obtained from transcriptional level and thereby allow a holistic view of abiotic stress response pathways in plant roots. The root proteomic response for each of the abiotic stress factors are emphasized in detail in the following sub-headings.

2.1. Salt stress

Salt tolerance is a complex phenotype which is controlled by multiple genes. Identifying novel genes, determining their expression patterns in response to salt stress, and exploring their functions in stress adaptation are the basis for implementing effective engineering strategies to improve salt tolerance in plants (Cushman and Bohnert, 2000). It is estimated that salt stress may affect half of all arable lands by 2050, and will be a major factor responsible for the loss of arable land for the coming decades (Wang *et al*., 2003). Proteomic studies on the analysis of plant salt stress response on root tissue are rather scanty (Table 1). It was revealed in one of the previous proteomic studies that salt stress caused the accumulation of a lectin-like protein (SalT) in rice roots (Claes *et al*., 1990). The influence of plant hormone, abscisic acid (ABA) in regulating salt stress induced protein synthesis was investigated in roots of *Solanum lycopersicon* Mill cv. Alisa Craig (AC) and the near isogenic ABA-deficient mutant, *flacca* (*flc*), using 2-DE analysis (Chen and Plant, 1999). The polypeptide profiles of salt-treated AC and *flc* roots were similar, suggesting that the synthesis of most novel polypeptides in salt-treated roots is not dependent on an elevated level of endogenous ABA (Chen and Plant, 1999). The effect of salt stress on the polypeptide levels was examined using 2-DE in roots of two contrasting wheat (*Triticum durum*) cultivars, i.e. sensitive cv. Ben Bachir and tolerant cv. Chilli. The net synthesis of a 26 kDa polypeptide was significantly changed in the tolerant cultivar (Majoul *et al*., 2000).

Table 1
A summary of root proteome analysis under different abiotic stresses.

Plant species	*Stress condition*	*Duration and treatment*	*Methods*	*Key findings*	*Reference*
Rice (*Oryza sativa* L.)	Salt stress	4-days; 1% NaCl	2-DE, N-terminal sequencing	• The organ-specific response of *sa/T* in response to salt stress.	Claes *et al*. (1990)
Rice (*Oryza sativa* L.)	Salt stress	14-days; 0 (control) and 100 mM NaCl	2-DE, MALDI-TOF/MS, ESI-Q-TOF-MS/MS	• Constitutive high expression of APX and Up-regulation ofASR1 and CCOMT in sensitive and tolerant genotypes.	Salekdeh *et al*. (2002)
Rice (*Oryza sativa* L.)	Salt stress	24, 48, and 72 h; 0 (control) and 150 mM NaCl	2-DE, MALDI-TOF/MS	• Six-novel salt-responsive proteins were identified, i.e. UDP-glucose pyrophosphorylase, cytochrome*c* oxidase subunit 6b-1, glutamine synthetase root isozyme, putative nascent polypeptide associated complex alpha chain, putative splicing factor-like protein and putative actin-binding protein.	Yan *et al*. (2005)
Rice (*Oryza sativa* L)	Salt stress	0, 10 and 24 h; 0 (control), 150 mM NaCl	2-DE, MALDI-TOF/TOF MS	• Most proteins with quantity change during salinity stress are not phosphoproteins or have no change in phosphorylation level.	Chitteti and Peng (2007)
Rice (*Oryza sativa* L)	Salt stress	48 hrs; 0 (control) and 150mM NaCl	2-DE, MALDI-TOF/TOF MS	• A new leucine-rich-repeat type receptor-like protein kinase (OsRPK1) was identified as a salt-responsive protein. • Immuno-blots indicated that OsRPK1 is also induced by cold, drought, and abscisic acid. • The immuno-histochemical techniques determined the expression of OsRPK1 in the plasma membrane of cortex cells of roots.	Cheng *et al*. (2009)
Rice (*Oryza sativa* L.)	Salt stress	1, 3, or 6h; 200 mM NaCl,	MALDI-TOF/MS 2-DE	• The study indicates that knocking down the expression level of OsRMC in transgenic rice led to insensitive seed germination, enhanced growth inhibition, and improved salt stress tolerance. • These results indicate that plant apoplastic proteins may have important roles in the plant salt stress response.	Zhang *et al*. (2009)
Wheat (*Triticum durum* L.)	Salt stress	4-days; 0 (control) 200 mM NaCl	2-DE, N-terminal sequencing	• Up-regulation of anti-oxidative defense protein such as peroxiredoxin.	Majoul *et al*. (2000)
Wheat (*Triticum aestivum* L.)	Salt stress	10-days; 0 (control) and 125 mM NaCl	2-DE	• The salt-resistant and sensitive genotypes showed a similar biochemical reaction at the level of proteins after 10-day exposure to 125mM NaCl.	

contd. table 1

Plant species	*Stress condition*	*Duration and treatment*	*Methods*	*Key findings*	*Reference*
				• The difference between the genotypes in various categories of protein change (up-regulated, down-regulated, disappeared, and new-appeared) was only 1 - 8%.	Saqib *et al.* (2006)
Barley (*Hordeum vulgare* L.)	Salt stress	5-days; 0 (control) and 0.2 M NaCl	2-DE, LC-MS/MS	• This study revealed that salt tolerant barley develops additive and synergistic biochemical strategies, such as selective accumulation of ions, control of ion uptake by roots and transport in to the leaves, and compartmentalization of ions at the cellular and whole plant levels, in addition to induction of ROS- scavenging enzymes to counteract the salt stress.	Sugimoto and Takeda (2009)
Barley, (*Hordeum vulgare* L.)	Salt stress	13-days; 0 (control), 100, 150 mM NaCl.	2-DE, MALDI-TOF/MS nano LC-ESI-Q-TOF MS/MS	• Two proteins involved in the glutathione-based detoxification of reactive oxygen species (ROS) were more abundant in the tolerant genotype, while, proteins involved in iron uptake were expressed at a higher level in the sensitive one. • This study emphasizes the role of proteins involved in ROS detoxification during salinity stress, and identified potential candidates for increasing salt tolerance in barley.	Witzel *et al.* (2009)
Barley, (*Hordeum vulgare* L.)	Salt stress	1, 4, 7 and 10-days; 0 (control), 100 and 150mM NaCl.	2-DE nano LC-ESI-Q-TOF MS/MS	• Proteins involved in detoxification pathways and terpenoid biosynthesis were detected as early responsive to salinity.	Witzel *et al.* (2014)
Barley, (*Hordeum vulgare* L.)	Salt stress	6-days; 0 (control) and 100 mM NaCl,	2-DE and MALDI-TOF/TOF MS	• Forty-seven spots exhibiting abundance changes under salt stress were successfully identified. The most significant differences concerned the proteins that are involved in signal transduction, detoxification, protein folding processes and cell wall metabolism.	Mostek *et al.* (2015)
Maize (*Zea mays* L.)	Salt stress	1h; 0 (control) and 25 mM NaCl	2-DE, MALDI-TOF/MS	• This report provides new insights for the initial reaction of the proteome and phosphoproteome of maize roots after adjustment to saline conditions and reveals early members of sugar signaling and cell signaling path-ways such as calmodulin.	Zorb *et al.* (2010)

contd. table 1

Plant species	*Stress condition*	*Duration and treatment*	*Methods*	*Key findings*	*Reference*
Pea (*Pisum sativum* L.)	Salt stress	1-week (short-term stress) and 6-week (long-term stress); 0 (control), 75 mM and 150mM NaCl.	2-DE, ESI-Q-TOF-MS/MS	• Study suggested the possible existence of a novel signal transduction pathway involving SOD, H_2O_2, NDPK and PR-10 proteins with a potentially crucial role in abiotic stress responses.	Kav *et al*. (2004)
Tomato (*Solanum lycopersicum* L.)	Salt stress	14-days; 0 (control) and 100 mM NaCl	2-DE, LC-MS/MS	• The carbon metabolism and energy-related proteins were mainly up-regulated by salt stress and exhibited most-tolerant versus most-sensitive abundance variations. • Unexpectedly, some antioxidant and defence proteins were also down- regulated, while some proteins putatively involved in osmoprotectant synthesis and cell wall re-inforcement were up-regulated by salt stress mainly in tolerant genotypes.	Manaa *et al*. (2011)
Tomato (*Lycopersicon esculentum* Mill.)	Salt stress	30min, 8h, 24h, 48h, 96 h (time course); 24h salt stress and 48h recovery; 170 mM NaCl	2-DE	• Study confirms that the elevated level of endogenous ABA may not play a major role in regulating the expression of many salt-induced proteins in tomato root.	Chen and Plant (1999)
Cotton (*Gossypium hirsutum* L.)	Salt stress	24h; 200mM NaCl,	iTRAQ, LC-MS/MS	• Some novel salt-responsive proteins were identified, which are involved in cell cytoskeleton metabolism, membrane transport, signal transduction and stress responses.	Li *et al*. (2015)
Arabidopsis	Salt stress	6 and 48 h; 0 (control) or 150mM NaCl	2-DE, LC-MS/MS	• Poor statistical correlation (6 h, r= –0.13; 48 h, r=0.11) of the protein expression data with previous microarray results was detected. • The study supports the concept that post-transcriptional regulation plays an important role in stress-responsive gene expression and highlighting the need for combined transcriptomic and proteomic analyses.	Jiang *et al*. (2007)
Rice (*Oryza sativa* L.)	Drought	21-days; 100% reposition (control) and 50% reposition (stress) of the water lost daily.	2-DE, MALDI-TOF/TOF	• Twenty-two proteins putatively associated to drought tolerance were identified by mass spectrometry. • Several protein families identified in the proteomic analysis were not detected in the cDNA analysis.	Rabello *et al*. (2008)

contd. table 1

Plant species	*Stress condition*	*Duration and treatment*	*Methods*	*Key findings*	*Reference*
Rice (*Oryza sativa* L.)	Drought	7-days (water stress) and 24h re-watering	2-DE; MALDI-TOF-MS/MS	• A novel protein, R40C1 was reported to be up-accumulated in roots of transgenic plants which may play an important role in generation of drought tolerant plants.	Paul *et al.* (2015)
Maize (*Zea mays* L.)	Drought	24 h and 48h; Water potential -0.03 MPa for (control) and -0.16MPa stressed.	2-DE, ESI-Q-TOF/MS	• This large-scale proteomic analysis provides novel insights into the complexity of mechanisms that regulate root growth under water deficit conditions and highlights the spatial differences in CWP composition in the root elongation zone.	Zhu *et al.* (2007)
Soybean (*Glycine max* L.)	Drought	4-days of drought stress and 4-days recovery.	nano- LC-MS; nanospray LTQ Orbitrap mass spectrometer	• Post-drought recovery response mechanisms in soybean root including hypocotyl were analyzed using gel-free proteomic technique. • The investigation revealed that peroxidase and aldehyde dehydrogenase were increased in protein abundance under drought stress.	Khan and Komatsu (2016)
Sunflower (*Helianthus annuus* L.)	Drought	6-days; Relative water content 87-78% for control and diminution by 10-15% for stress.	2-DE, nano LC-MS/MS	• The study indicate that changes in energy usage, water transport and ROS scavenging are important mechanisms for maintaining root growth as the soil dries.	Ghaffari *et al.* (2013)
Wild Watermelon (*Citrullus lanatus* sp.)	Drought	1 and 3-days; Soil water content for unstressed, stressed for 2 and 4-days were 41.7, 34.2 and 27.5% (w/w), respectively.	2-DE, LC–MS/MS	• The investigation suggests that the xerophyte switches survival strategies from drought avoidance to drought tolerance during the progression of drought stress, by regulating its root proteome in a temporally programmed manner. • This study provides new insights into the complex molecular networks within plant roots involved in the adaptation to adverse environments.	Yoshimura *et al.* (2008)
Alfalfa (*Medicago sativa* L.)	Drought	12-and 15days of water stress and re-watering for 3-days	2-DE; Voyager-DE STR MALDI-TOF/MS	• Two proteins, namely cytosolic ascorbate peroxidase (APX2) and putative F-box protein were newly detected on 2-DE maps of WD-treated plants.	Atikur *et al.* (2015)

contd. table 1

Plant species	*Stress condition*	*Duration and treatment*	*Methods*	*Key findings*	*Reference*
Holm oak (*Quercus ilex subsp. Ballota* [Desf.] *Samp.*)	Drought	10 and 20-days followed by 10-days of recovery; Relative water content 80 and 58% for control and stress, respectively.	2-DE, MALDI-TOF/TOF Mass Spectrometry	• The holm oak roots subjected to water limitation treatment point at early activation of the proteins involved in secondary metabolism, down-regulation of glycolysis and stimulation of ATP synthesis, accumulation of some enzymes related to amino acid and one-carbon metabolism, and complex changes in protein synthesis, folding/processing and degradation processes.	Simova-Stoilova *et al.* (2015)
Rice (*Oryza sativa* L.)	Heavy metal stress (Cu)	3-days; 8 and 0.32 µM Cu^{2+}	2-DE, MALDI-TOF/TOF-MS.	• In total, twenty-sevendifferentially expressed novel Cu-binding proteins were identified., of this 16-proteins were not previously identified asCu-IMAC-binding proteins from plants or animals.	Chen *et al.* (2015)
Rice (*Oryza sativa* L.)	Heavy metal stress (Cd)	15-days; 10 µM (control) and 100 µM Cd	2-DE, MALDI-TOF/MS	• 100µM Cd concentration strongly induced the expression of regulatory proteins and some metabolic enzymes in root tissues.	
				• The treatment with 10 µM of Cd induced changes in metabolic enzymes, but it mainly activated defense mechanisms by the induction of transporters and proteins involved in the degradation of oxidatively modified proteins.	Aina *et al.* (2007)
Soybean (*Glycine max* L.) Merr	Heavy metal stress (Al)	24, 48 and 72 h; 0 (control) and 50 µM Al^{3+}	2-DE, MALDI-TOF/MS	• The heat shock protein, glutathione S-transferase, chalcone-related synthetase, GTP-binding protein and ABC transporter ATP-binding protein were previously detected at the transcriptional or translational level in other plants. Other proteins, identified in this study, are new Al-induced proteins.	Zhen *et al.* (2007)
Indian mustard (*Brassica juncea* L.)	Heavy metal stress (Cd)	3-days; 0 (control) and 250 mM $CdCl_2$	2-D DIGE, iTRAQ	• This study determined the involvement of enzymes such as peptide methionine sulfoxide reductase and 2-nitropropane dioxygenase in alternatives redoxregulation mechanisms, as well as O-acetylserine sulfhydrylase, glutathione-S-transferase and glutathione-conjugate membrane transporter, as essential players in the Cd hyper- accumulation and tolerance of *B. juncea*.	Alvarez *et al.* (2009)
Chinese silver grass (*Miscanthus sinensis* L.)	Heavy metal stress (Sb)	3-days; 0,50,100, 200, 300, 500, 750 and 1000µm of $KSb(OH)_6$	2-DE, MALDI-TOF/MS and MALDI-TOF/TOF-MS	• The candidates conferring Sb tolerance include Dnaj-like protein, CBS domain-containing protein, HSP90, PR, ZFP, GR1, ACC oxidase, ATP synthase, APX, POD, Prx and GST.	Xue *et al.* (2015)

contd. table 1

Plant species	*Stress condition*	*Duration and treatment*	*Methods*	*Key findings*	*Reference*
Ceylon spinach (*Talinum triangulare* Jacq. [Willd.])	Heavy metal stress (Pb)	7-days; 0(control) and 1.25 mM Pb $(NO_3)_2$	2-DE, MALDI-TOF/TOF-MS	• The abundant proteins during 1.25 mM Pb stress conditions revealed indication about their involvement in root architecture, energy metabolism, reactive oxygen species (ROS) detoxification, cell signaling, primary and secondary metabolisms, and molecular transport systems.	Kumar and Majeti (2014)
Sour pummel (*Citrus sinensis*) and Xuegan (*Citrus grandis*)	Heavy metal stress ($MnSO_4$)	17-weeks; 2 µM (control) and 600 µM $MnSO_4$	2-DE,MALDI-TOF/MS	• Fifty-three and thirty-nine differentially expressed protein spots were isolated from Mn-toxic *Citrus sinensis* and *Citrus grandis* roots, respectively. • Mn-toxicity-induced changes in protein profiles greatly differed between the two species.	You *et al.* (2014)
Poplar *(Populus spp.)*	Heavy metal stress (Zn)	28 days; 1mM of Zinc and 1µM as a control (0.065 ppm).	2-DE, Nano LC-ESI-LIT–MS/MS	• Zn was accumulated preferentially in roots, that the antioxidant system, the carbohydrate/energy and amino acid metabolisms were the main pathways modulated by Zn excess. • The mitochondria and vacuoles were the cellular organelles predominately affected by Zn stress.	Romeo *et al.* (2014)
Elsholtzia (*Elsholtzia splendens*)	Heavy metal stress (Cu)	48h; 25, 50, 75 and 100 µM of Cu^{2+}.	SDS-PAGE, LC-ESI-MS/MS	• The majority of the twenty-two up-regulated proteins were involved in the antioxidant defense pathway, cell wall polysaccharide remodeling, and cell metabolism process. • The thirty-three down-regulated proteins were involved in the signal pathway, energy, and protein synthesis.	Liu *et al.* (2014)
Arabidopsis (*Arabidopsis thaliana* L.)	Heavy metal stress (Cd)	24 h; 0 (control) and 10µM Cd^{2+}	2-DE, MALDI-TOF/MS	• Most of the identified proteins belong to four different classes: metabolic enzymes such as ATP sulphurylase, glycine hydroxymethyltransferase, and trehalose-6-phosphate phosphatase; glutathione S -transferases; latex allergen- like proteins; and unknown proteins.	Roth *et al.* (2006)
Soybean (*Glycine max* [L.] Merr)	Waterlogging	3 and 7-days	2-DE, MALDI-TOF/MS,ESI–MS/MS	• The identified proteins include well-known classical anaerobically induced proteins as well as novel waterlogging-responsive proteins, that were not known previously as being waterlogging responsive candidates.	Alam *et al.* (2010)
Soybean (*Glycine max* [L.] Merr)	Waterlogging	2-days	Nano LC-MS	• Functional categorization of identified glycoproteins indicated that the majority were related to glycolysis and protein degradation.	Mustafa and Komatsu (2014)

contd. table 1

Plant species	*Stress condition*	*Duration and treatment*	*Methods*	*Key findings*	*Reference*
Tomato (*Lycopersicon esculentum* L.)	Waterlogging	24 and 72 h	2-DE, MALDI-TOF/MS	• Identified several novel proteins, including 3-beta-hydroxylase, phenylalanine ammonia-lyase, glutamyl-tRNA reductase, flavanone 3-hydroxylase, mitochondrial ATPase alpha subunit, cysteineprotease, DWARF1, and NIM1-like protein 2.	Ahsan *et al.* (2007)
Rice (*Oryza sativa* L.)	Cold stress	24 and 72 h; 28°C (control) and 10°C (chilling treatment)	2-DE, MALDI-TOF/MS, ESI-MS/MS	• A group of novel proteins were identified including acetyltransferase, phosphogluconate dehydrogenase, NADP- specific isocitrate dehydrogenase, fructokinase, PrMC3, putativealpha-soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein, and glyoxalase1. • Gene expression at the mRNA level of some selected proteins revealed that transcription levels are not always concomitant to the translational level.	Lee *et al.* (2009)
Rice (*Oryza sativa* L.)	Cold stress	48h; 5°C	SDS-PAGE, 2-DELC-MS/MS	· The identified proteins were involved in different metabolic pathways such as receptor –type protein kinase, GPI-anchored protein, leucine-rich repeat transmembrane protein kinase, water channel protein, plasma membrane integral protein, lipid transfer protein, phosphate transporter, MAP3K like protein kinase.	Hashimoto *et al.* (2009)
Pea(*Pisum sativum* L.)	Cold stress	Chilling (11-days), frost (4-days), recovery (8-days)	2-DELC-MS/MS	· Investigation revealed no clear specific organ response to chilling exposure could be distinguished.	Dumont *et al.* (2011)
Bentgrass (*Agrostis stolonifera* L. and *Agrostis scabra* L.)	High temperature stress	2- and 10-day; 20 °C (control), 30 °C (moderate heatstress), or 40 °C (severe heat stress)	2-DE, MALDI-TOF/TOF	· The study suggested that the up-regulation of sucrose synthase, GST, SOD, and HSP Sti (stress-inducible protein) may contribute to the superior root thermo tolerance of *A. scabra*.	Xu and Huang (2008)

Using a proteomic approach Salekdeh *et al*., (2002) have studied salt-responsive proteins in roots of the salt-tolerant and -sensitive rice varieties, Pokkali and IR-29, respectively. In their study, an ABA and stress responsive protein (ASR1), ascorbate peroxidase and caffeoyl-CoA *O*-methyltransferase (CCOMT) were identified among salt responsive proteins. Further, CCOMT was markedly up-regulated in Pokkali, but slightly changed in IR-29. The CCOMT plays a key role in biosynthesis of suberin and lignin, and increased lignification may help to reduce the bypass water flow that allows Na^+ ions to enter rice roots via an apoplastic route (Salekdeh *et al*., 2002).

A systematic proteomic analysis was carried out on rice root proteins under high salinity (Yan *et al*., 2005). In their study, ten different salt stress-responsive proteins were identified, which are involved in a wide range of cellular processes, *e.g.* carbohydrate, nitrogen and energy metabolisms, ROS scavenging, mRNA and protein processing and cytoskeleton stability. Four of them i.e. triosephosphate isomerase, enolase, SAMS2 and peroxidase were previously shown to respond to salt stress either at the RNA level or at the protein level. Further, six novel salt stress-responsive proteins were identified, i.e. UGPase, COX6b-1, GS root isozyme, putative a-NAC, putative splicing factor-like protein and putative ABP. These results necessitate further investigation to disclose the functional role of identified individual proteins towards salt stress response, using biotechnological and genetic approaches (Yan *et al*., 2005). It is anticipated that the rice genome contains 32000 to 50000 genes and each gene may give rise to multiple proteins by means of alternative splicing or PTM. The proteins analyzed by Yan *et al*., (2005) represent only a small part of the rice proteome and many other salt stress-responsive proteins still need to be identified. For example, the non-cytosolic proteins such as membrane proteins and nuclear proteins are believed to play key roles in osmosensing, ion transport and signal transduction. Interestingly, Saqib *et al*., (2006) have reported that, after short-term exposure to first phase of salt stress, salt-resistant and -sensitive wheat genotypes showed similar biochemical response at protein level. This initial biochemical reaction may be a secondary effect rather than a specific adaptation. The salt stress response in pea roots for the first time was studied by Kav *et al*., (2004). Their findings suggested the possible existence of a novel signal transduction pathway involving SOD, H_2O_2, NDPK and PR10 proteins with a potentially crucial role in abiotic stress responses.

Chitteti and Peng (2007) have investigated protein differential regulation under salt stress using the Pro-Q Diamond dye in rice and the majority of proteins stained heavily by Pro-Q Diamond dye have a relatively low pI. This investigation is consistent with the fact that protein phosphorylation results in acidification of the proteins. Further, they have successful in identifying 17 of the 20 up-regulated and 11 of the 18 down-regulated putative phosphoproteins. Although some of them are typical salt stress response proteins, these 17 proteins have not been reported in previous proteome studies under salt stress in rice, suggesting that the Pro-Q Diamond in gel stain is a useful tool in identifying stress response proteins.

Recent studies by Jiang *et al*., (2007) have identified 86-differentially abundant protein spots through liquid chromatography tandem mass spectrometry in *Arabidopsis* roots under NaCl stress. The proteins identified included many previously characterized stress-responsive proteins and others related to processes including scavenging for reactive oxygen species; signal transduction; translation, cell wall biosynthesis, protein translation, processing and degradation; and metabolism of energy, amino acids, and hormones. Based on the study, a poor statistical correlation (6 h, $r = -0.13$; 48 h, $r = 0.11$) was detected between protein expression data with previous microarray results, at the resolution of individual genes and proteins, supporting the concept that post-transcriptional regulation plays an important role in stress-responsive gene expression. This study highlights the need for conducting combined transcriptomic and proteomic analyses in root proteomic studies.

A comparative root proteome studies on salt-tolerant and -sensitive barley genotypes was conducted by Witzel *et al*., (2009). Thirty-nine proteins showed cultivar-specific or stress-related expression and the protein identification was successful for 26 proteins. Candidate proteins conferring salt tolerance include peroxidase, glutathione S-transferase (GST), lactoylglutathione lyase, SAM synthase, and a carboxymethylenebutenolidase-like protein. Among those, two proteins involved in the

glutathione-based detoxification of reactive oxygen species (ROS) were more abundant in the tolerant genotype, while proteins involved in iron uptake were expressed at a higher level in the sensitive one (Witzel *et al*., 2009). This study identified some potential candidates for enhancing salt tolerance in barley and emphasizes the role of proteins involved in ROS detoxification during salinity stress.

Witzel *et al*., (2014) have conducted proteomic analysis in the roots of two contrasting barley (cv. Steptoe and cv. Morex) and seedlings were exposed to a period of 1, 4, 7, or 10-d at 0, 100, or 150 mM NaCl. Root proteome samples were analyzed based on combined two-dimensional gel electrophoresis and nano-LC-ESI-Q-TOF-MS. In their study, a number of cultivar-specific and salinity stress-responsive proteins were identified. The mass spectrometry-based identification was successful for 74 proteins, and a hierarchical clustering analysis grouped these into five clusters based on similarity of expression profile (Witzel *et al*., 2014). The rank product method was used to statistically assess the early and late responses, and this delivered a number of novel candidate proteins underlying salinity tolerance in barley. Among these few were germin-like proteins, some pathogenesis-related proteins, and numerous as-yet un-characterized proteins. Particularly, proteins involved in detoxification pathways and terpenoid biosynthesis were detected as early responsive to salinity and may function as a means of modulating growth-regulating mechanisms and membrane stability via fine tuning of phytohormone and secondary metabolism in the root.

Zhang *et al*., (2009) have studied the role of apoplastic proteins in 10-day-old rice (*Oryza sativa*) plants under imposed salt stress, and the soluble apoplast proteins extracted were resolved using two-dimensional electrophoresis for differential analysis compared with untreated controls. Ten protein spots that increased or decreased significantly in abundance were subjected to mass spectrometry identification. Among them, an apoplastic protein, with extracellular domain-like cysteine-rich motifs (DUF26), *O.sativa* root meander curling (OsRMC) has shown significant increase in abundance in response to salt stress during the initial phase. Further, OsRMC RNA interference transgenic rice has been generated to assess the function of OsRMC in the salt stress response. The results showed that knocking down the expression level of OsRMC in transgenic rice led to insensitive seed germination, improved growth inhibition, and enhanced stress tolerance to NaCl than in un-transgenic plants (Zhang *et al*., (2009). This study indicates that plant apoplastic proteins may have important roles in plant salt stress response.

Li *et al*., (2015) have applied an iTRAQ-based proteomic technique to compare the abundance of proteins in control and salt-stressed roots of cotton for 24 h. In total, 128 differentially expressed proteins (DEPs) were identified, of which 76-proteins showed increased abundance and 52 decreased under salt stress conditions. In their study, the majority of proteins have functions related to stress and defense, protein metabolism, carbohydrate and energy metabolism, membrane and transport, cell wall and cytoskeleton metabolism, transcription, signal transduction, and in addition some novel salt-responsive proteins were identified (Li *et al*., 2015). A large number of cotton root proteins were identified and quantified by using iTRAQ-based proteomic technique and the study demonstrates that iTRAQ is dependable technique.

Cheng *et al*., (2009) investigated the effects of salt stress on alteration in rice (*Oryza sativa*) root plasma-membrane-associated proteins. Two-dimensional gel electrophoresis coupled with mass spectrometry has identified 18-differentially expressed proteins, including a new leucine-rich-repeat (LRR) receptor-like protein kinase (RLK) and most of these identified membrane associated proteins are involved in important physiological processes such as membrane stabilization, ion homeostasis, and signal transduction (Cheng *et al*., 2009). Based on the study, the results indicate that different rice cultivars might have different salt stress response mechanisms.

The root proteome of salt-tolerant (DH-187) and sensitive (DH-14) lines of barley was investigated by Mostek *et al*., (2015) under imposed salt stress. This investigation revealed that the sensitive and tolerant barley lines respond to salt stress differently. The most notable differences pertaining to the proteins that are involved in signal transduction, detoxification processes, protein folding and cell wall metabolism which were up-regulated under salt stress uniquely in tolerant barley line. Further they have concluded that, the enhanced salinity tolerance of DH-187 line could be due to the increased activity of signal transduction pathways leading to the accumulation

of stress protective proteins and changes in cell wall structure.

Manaa *et al*., (2011) characterized the response of four tomato genotypes after imposing 14-day salt stress at the young plantlet stage. A common set of proteins (nine spots), up- or down-regulated by salt-stress irrespective of the genotype, was detected. The impact of the genotype effect on tomato proteome variations was more pronounced than the salt effect and 33 spots that were not variable with salt stress varied with the genotype. The remaining number of variable spots (48) shared the combined effects of genotype and salt stress, putatively linked to the degrees of genotype tolerance. The up-regulated proteins under salt stress were mainly involved in energy-related and carbon metabolism, and exhibited most-tolerant versus most-sensitive abundance variations. Unexpectedly, some antioxidant and defense proteins were also down-regulated, while some proteins putatively involved in osmoprotectant synthesis and cell wall reinforcement were up-regulated by salt stress mainly in tolerant genotypes (Manaa *et al*., 2011). They conclude that the effect of 14-d stress on the tomato root proteome and underlined significant genotype differences, suggests the importance of making use of genetic variability in tomato.

The above-mentioned investigations suggested the power of proteomic approaches in identifying functional proteins responsive to salt stress in plant roots. However, our understanding of salt stress-responsive proteins in roots is still far from complete. Clearly, there is a need to examine and analyze a much larger number of salt stress-responsive regulatory proteins in roots of various plant species, including woody species in order to understand in-depth insights of molecular complexity of salt stress.

2.2. Drought

Drought is one of the major environmental stress factors that affects the growth and development of plants through alterations in gene expression and metabolism. Despite many decades of research, drought stress is continues to be a challenging task to the agricultural scientists in general and plant breeders in particular. It is a persistent problem to agricultural production in many developing countries, and an occasional cause of losses of agricultural production in the developed ones.

Yoshimura *et al*., (2008) have reported that, root development was significantly enhanced at the early stage of drought stress compared to the irrigated wild watermelon plants. This response indicates activation of a drought avoidance mechanism in wild watermelon for absorbing water from deep soil layers. Consistent with this observation, comparative proteome analysis revealed that the early stage of drought stress induced many proteins which are involved in root morphogenesis and carbon/nitrogen metabolism, and these may contribute to the drought avoidance via the enhancement of root growth. On the other hand, lignin synthesis-related proteins and molecular chaperones, which may function in the enhancement of physical desiccation tolerance and maintenance of protein integrity, respectively, were induced mostly at the later stage of drought stress (Yoshimura *et al*., 2008). The investigation suggests that during the progression of the drought stress xerophyte switches survival strategies from drought-avoidance to -tolerance, by regulating its root proteome in a temporally programmed manner. Further, this investigation provides new insights into the complex molecular networks operate within plant roots in response to adverse environments.

Robello *et al*., (2008) have investigated the root proteome analysis of rice under drought stress and mass spectrometry identification resulted 22-proteins that were associated to putative drought tolerance. Further, they found that the proteomic analyses were complementary to the genomic data obtained. In upland rice the expression of genes associated with cell protection against oxidative damage is considered important to cope with water deficit. In the tolerant genotype, a higher expression of genes and proteins related to this function was identified. Interestingly, a higher diversity in protein profile was noticed in the susceptible genotype, enlightening more uniquely expressed proteins than the tolerant genotype. This study clearly indicates that proteomics studies can disclose important additional information and that the use of complementary approaches is useful for understanding complex biological traits in a better way, such as drought tolerance (Robello *et al*., 2008).

A proteomics approach was used to study different regions of the maize (*Zea mays*) root elongation zone under water deficit, and this study mainly focused on water soluble and loosely

ionically bound cell wall proteins (CWPs)(Zhu *et al*., 2007). The cell elongation in primary roots of maize is maintained preferentially toward the apex under water deficit, and this response involves modification of cell wall extension properties. The region-specific change in protein profiles was compared between well-watered and water-stressed roots. In total, 152 water deficit-responsive proteins were identified and categorized into five groups based on their potential function in the cell wall, reactive oxygen species (ROS) metabolism, defense and detoxification, hydrolases, carbohydrate metabolism, and other/unknown proteins (Zhu *et al*., 2007). The stress-induced changes in CWPs involve multiple processes that are likely to regulate the response of cell elongation as indicated by the study. Particularly, in the apical region of elongation zone of water-stressed roots, the ROS metabolism related protein abundance changes was predicted an increase in apoplastic ROS production (Zhu *et al*., 2007). This response could contribute directly to the enhancement of wall loosening in this region. This high throughput proteomic analysis of root under water stress conditions revealed a novel insight into the complexity of mechanisms, and highlights the spatial differences in CWPs composition in the root elongation zone (Zhu *et al*., 2007).

Drought stress caused decrease in relative abundance of metabolism-related proteins in sensitive and tolerant lines of sunflower (Ghaffari *et al*., 2013). The abundance of energy and disease/defense-related proteins were decreased in the sensitive but increased in the tolerant line. The study indicate that changes in ROS scavenging, recovery and water transport abilities in roots is an essential protective mechanism for maintaining root growth and drought tolerance of sunflower (Ghaffari *et al*., 2013).

Simova-Stoilova *et al*., (2015) have investigated the root proteome responses after subjecting 20-day-old holm oak plantlets to water limitation for a period of 10- and 20-days, each followed by 10-days of recovery. The identified proteins were classified into different functional groups such as metabolism, protein biosynthesis and proteolysis, defense against biotic stress, cellular protection against abiotic stress, and intracellular transport. Drought stress caused decrease in abundance of several enzymes of the carbohydrate metabolism in roots, while the abundance of ATP synthesis and secondary metabolism was increased. The results point at active metabolic adjustment and mobilization of the defense system in roots to actively counteract drought stress.

Two day-old soybean seedlings were subjected to drought stress for 4-days and recovered for 4-days and a gel-free proteomic technique was applied to analyse the protein samples (Khan and Komatsu, 2016). A total of 792 and 888 proteins were identified from the control and recovering seedlings, respectively. The identified proteins were related to functional categories of stress, hormone metabolism, cell wall, secondary metabolism, and fermentation. The cluster analysis of the identified proteins revealed that peroxidase and aldehyde dehydrogenase abundance was highly altered in the seedlings during the post-drought recovery (Khan and Komatsu, 2016). The peroxidase activity was decreased under drought conditions, but increased during recovery. In contrast, the activity of aldehyde dehydrogenase was increased upon drought stress, but decreased during the recovery stage. The investigation suggests that peroxidase and aldehyde dehydrogenase plays a key role in post-drought recovery in soybean by scavenging toxic reactive oxygen species and reducing the load of harmful aldehydes (http://f1000.com/prime/ext/726420332).

The comparative proteome analysis between the wild-type and transgenic DREB1A over-expressing homozygous rice roots was studied under drought stress (Paul *et al*., 2015). After 7-d of drought stress the plants were re-watered for 24 h at reproductive stage for both wild-type and transgenic rice and total proteins were analyzed by two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry (MS). The major class of identified proteins belongs to carbohydrate and energy metabolism, while stress and defense-related proteins are especially up-regulated under drought stress in both the plants. A novel protein, R40C1 was reported to be up-regulated in roots of transgenic plants which may play a key role in generation of drought tolerant plants (Paul *et al*., 2015).

Atikur *et al*. (2015) have studied Alfalfa seedlings which were exposed to water stress for 12- and 15-days, respectively, followed by 3-days of re-watering. Two proteins, namely cytosolic ascorbate peroxidase (APx2) and putative F-box protein were newly detected on 2-DE maps of water stressed plants. The identified proteins are involved

in a variety of cellular functions including calcium signaling, ABA biosynthesis, ROS regulation, transcription/ translation, antioxidant/ detoxification/stress defense, energy metabolism, signal transduction, and storage. Despite its importance as a major abiotic stress factor, very little attention has been paid to date on root proteome analysis under drought stress in different crop/ plants. Hence, more focus is required on this aspect to gain in-depth molecular insights, and to address this problem in a better way.

2.3. Waterlogging

Waterlogging is defined as prolonged soil saturation with water at least 20% higher than the field capacity (Aggarwal *et al*., 2006). It is a major problem of utmost importance, as it limits the growth and yield of many crops in humid areas. The inability of crops to withstand excess water in the rhizosphere adversely alters metabolism and leads to a major reduction in crop yield (Alam *et al*., 2010). Globally, approximately 10% of irrigated farmlands suffer from frequent waterlogging; however, values up to 20% occur in specific regions such as Eastern Europe and the Russian Federation (FAO 2002; Alam *et al*., 2010).

Ahsan *et al*. (2008) reported that in response to waterlogging, tomato roots showed 35 differentially-expressed proteins, out of which 29-proteins were identified using MALDI-TOF analysis. Of the proteins identified, 16 were up-regulated and 13 were down-regulated in waterlogged tomato roots. In addition to previously identified waterlogging-regulated proteins, in their study several novel proteins were identified such as 3-beta-hydroxylase, phenylalanine ammonia-lyase, glutamyl-tRNA reductase, xavanone 3-hydroxylase, mitochondrial ATPase alpha subunit, cysteineprotease, DWARF1, and NIM1-like protein-2. These novel proteins are involved in several cellular processes, including hormone and secondary metabolite synthesis, programmed cell death, and stress and defense mechanisms.

Alam *et al*. (2010) have investigated soybean roots in response to waterlogging stress. Two-dimensional gel electrophoresis (2-DE) technique has applied in resolving total proteins isolated from root samples. In their study, a total of 24 reproducible protein spots were resolved. Of these, 14 protein spots showed increase in abundance, five protein spots showed decreased abundance; and five were newly induced in waterlogged roots. These proteins were involved in several processes that might work cooperatively to establish a new homeostasis under waterlogging stress. The identification of novel proteins such as a translation initiation factor, apyrase, auxin-amidohydrolase and coproporphyrinogen oxidase in response to waterlogging stress may provide new insight into the molecular basis of the waterlogging-stress response of soybean (Alam *et al*., 2010). Further, the study demonstrates that through the concerted action of carbohydrate consumption and by regulating programmed cell death the soybean plant can cope with waterlogging-stress.

Mustafa and Komatsu (2014) have applied a glycoproteomic technique to study flooding responsive mechanism in early stage soybeans. In total, 69 and 111-glycoproteins were identified with and without flooding stress for 2-days, respectively. Functional categorization of these identified glycoproteins indicated that, proteins related to protein degradation, cell wall, and glycolysis were increased in abundance; while decrease in abundance was recorded for stress-related proteins under flooding stress. Also the level of accumulation of glycoproteins localized in the secretory-pathway decreased under flooding stress. Sub-cellular prediction of these identified glycoproteins indicated their localization to the cytoplasm, nucleus, and secretory-pathways.

2.4. Temperature stress

The effects of global warming will not be limited to rising mean annual temperatures around the globe. There will also be a remarkable increase in both frequency and amplitude of severe temperature events, resulting in more extreme hot and cold days, more frequently (Neilson *et al*., 2010). Thus, the degree to which temperature fluctuations deviate from long-term mean values becomes critical for productivity of all plant species, including crops (Neilson *et al*., 2010). A vast literature has been accumulated to date on stress adaptation mechanisms of the plant areal parts, whereas mechanisms of root tolerance to heat stress are rather scanty. Plant roots are more sensitive to heat stress and it was demonstrated by various studies, and suggest that high soil temperature is more detrimental than high air temperature for whole-plant growth (Xu and Huang, 2001; Liu and Huang, 2005). Roots may express different proteomes from leaves, grains, or fruits due to unique functions and

their different sensitivity to heat stress (Xu and Huang, 2008). Proteomic profiling of root tissues under temperature stress will enable molecular dissection of heat and cold tolerance mechanisms in plant roots.

2.4.1. Cold

In rice roots, a total of 27 up-regulated proteins were identified by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry or electro spray ionization-tandem mass spectrometry (ESI-MS/MS), after subjecting plants to chilling stress treatment (Lee *et al*., 2009). In this study, a group of novel proteins were identified including acetyltransferase, phosphogluconate dehydrogenase, NADP-specific isocitrate dehydrogenase, fructokinase, PrMC3, putative alpha-soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein, and glyoxalase-1, in addition to the previously identified cold-stress-responsive proteins. The identified proteins are involved in several cellular processes, including energy production and metabolism, vesicular trafficking, and detoxification. The gene expression at mRNA level of some selected proteins revealed that transcription levels are not always concomitant to the translational level.

Hashimoto *et al*. (2009) have identified 12 number of cold stress responsive proteins from the rice root plasma membrane using 2D-PAGE based proteomics method. The identified proteins were such as receptor -type protein kinase, GPI-anchored protein, leucine-rich repeat transmembrane protein kinase, water channel protein, plasma membrane integral protein, lipid transfer protein, phosphate transporter and MAP3K like protein kinase. Further, cold shock protein-1 was significantly decreased in plasma membrane of rice under cold stress.

Two pea lines (*Pisum sativum* L.) with contrasted behaviors towards chilling and subsequent frost were studied by Dumont *et al*. (2011). Following a chilling period, the Champagne line showed tolerant to frost, whereas, Terese line remains sensitive. Fifteen-root proteins were identified and these proteins were shared out in proteins related to chilling response or cold acclimation. Altogether, the investigation revealed that cold acclimation is a very complex biological process that might be linked to genetic variability within the two pea plant species (Dumont *et al*., 2011). The investigation of root proteome expression in different plants under chilling stress and identification of some novel proteins could be useful for better understanding the molecular basis of chilling stress responses in plants.

2.4.2. High temperature

Xu and Huang (2008) reported that, upon heat stress imposition seventy protein spots were altered in at least one species. Both moderate and severe heat stress caused down-regulation of majority of proteins than up-regulated, and thermal *Agrostis scabra* roots had more up-regulated proteins than *Agrostis stolonifera* roots. The mass spectrometry studies led to the identification of corresponding sequences of 66-differentially-expressed protein spots. The results suggested that up-regulation of sucrose synthase, glutathione S-transferase, superoxide dismutase, and heat shock protein stress-inducible protein (Sti) may contribute to the superior root thermo-tolerance of *A.scabra*. In addition, two isoforms of fructose-biphosphate aldolase were highly phosphorylated under heat stress as revealed by phosphoproteomic analysis, and thermal *A.scabra* had greater phosphorylation than *A.stolonifera*, suggesting that the aldolase phosphorylation might be involved in root thermo tolerance (Xu and Huang, 2008).

2.5. Heavy metal stress

It has been reported that the annual toxicity of all toxic metals mobilized exceeds the combined total toxicity of radioactive and organic wastes produced every year from all sources (Nriagu and Pacyna, 1998). Moreover, soil pollution by heavy metals currently affects about 235 million hectares (Giordani *et al*., 2005).

The root proteome changes of hydroponically grown *Arabidopsis thaliana* plants was studied after treating with 10 mM Cd^{2+} for 24h (Roth *et al*., 2006). The heavy metal stress triggers the synthesis of phytochelatins (PCs), glutathione-derived metal-binding peptides. Two-dimensional gel electrophoresis using different pH gradients in the first dimension detected on average of 1100 spots per gel type. The Cd^{2+} treatment caused significant changes in protein abundance for forty-one spots. The MALDI-TOF mass spectrometry identification was successful for 17-proteins, which were present in 25-different spots. Through western blot analysis and also selective enrichment of a protein family

(glutathione S-transferases; GST) by affinity chromatography, the selected results were independently confirmed. Most of the identified proteins belong to four different classes, metabolic enzymes such as ATP sulphurylase, glycine hydroxymethyltransferase, and trehalose-6-phosphate phosphatase; GST; latex allergen-like proteins; and unknown proteins. These results represent a basis for reverse genetics studies to better understand plant responses to toxic metal exposure and to the generation of internal sinks for reduced sulphur. Recently, Zhen *et al*. (2007) have studied the proteome analysis of soybean (cv.Baxi-10, Al-resistant) roots to Al stress. A total of 30 Al-responsive proteins were identified, among which a group of HSPs were newly identified as being induced by Al stress. This suggests that HSPs may protect root cells by re-folding proteins to their native conformations, thereby protecting against Al stress-induced protein degradation and denaturation. In another study by Chen *et al*. (2015) investigated the differences in Cu-binding protein expression between Cu-tolerant and Cu-sensitive rice varieties using a new IMAC method. In total, 27 differentially-expressed Cu-binding proteins were identified, out of which, sixteen proteins were not previously identified as Cu-IMAC-binding proteins either from plants or animals (Chen *et al*., 2015). These novel Cu-binding proteins were of four main types, proteins involved in antioxidant defense and detoxification, putative pathogenesis-related proteins, putative cold-shock domain proteins, and eukaryotic translation initiation factors.

Romeo *et al*. (2014) have performed the proteome analysis of *Populus*×*euramericana* (clone I-214) roots in response to Zn (1 mM [treatment] or 1 μM [control]). A gel-based proteomic approach coupled with morphological and biochemical analysis was used to identify differentially expressed proteins in Zn-treated roots of poplar. The results suggests that the mitochondria and vacuole are the poplar root cellular organelles most affected by excess Zn, and that the most relevant pathways modulated by Zn were the antioxidant, carbohydrate/energy and amino acid metabolisms.

Liu *et al*. (2014) utilized recently developed 6-plex Tandem Mass Tag (TMT) for relative and absolute quantitation methods to achieve a comprehensive understanding of Cu tolerance/detoxification molecular mechanisms in *Elsholtzia splendens* root cell wall, for the first time. LC-MS/MS approach was followed to analyze the Cu-responsive cell wall proteins and polysaccharides. The majority of 22-up-regulated proteins were involved in the antioxidant defense pathway, cell wall polysaccharide remodeling, and cell metabolism process. Changes in polysaccharide amount, composition, and distribution could offer more binding sites for Cu ions. The 33-down-regulated proteins were involved in the signal pathway, energy, and protein synthesis. Kumar and Majeti (2014) have studied Pb-stress effects on *Talinum triangulare* Jacq. (Willd.) after exposing the plants for 7-days and proteomic study was performed for control and 1.25 mM Pb-treated plants to examine the root protein dynamics in the presence of Pb. Twenty-three major proteins showed increased abundance, of which three proteins are new (appeared only in 1.25 mM Pb). Functional categorization of identified proteins under 1.25 mM Pb-stress have given a very clear indication about their involvement in root architecture, energy metabolism, reactive oxygen species (ROS) detoxification, cell signaling, primary and secondary metabolisms, and molecular transport systems.

The seedlings of 'Sour pummelo' (*Citrus grandis*) and 'Xuegan' (*Citrus sinensis*) were irrigated for 17-weeks with 2 (control) or 600 μM (Mn-toxic) $MnSO_4$ (You *et al*., 2014). Two-dimensional electrophoresis (2-DE) subsequent gel analysis yielded 11 up-regulated and 42 down-regulated protein spots from Mn-toxic *C. sinensis* roots, and 25 up-regulated and 14 down-regulated protein spots from Mn-toxic *C. grandis* roots. This indicates more remarkable metabolic flexibility in *C. sinensis* roots than in *C. grandis* ones. They found important differences in Mn-toxicity-induced changes in root protein profiles as well as root metabolic responses between the two species, especially in these proteins involved in protein biosynthesis and degradation, nucleic acid metabolism, carbohydrate and energy metabolism, and stress responses. The abundance of proteins related to nucleic acid metabolism, glycolysis and cell transport increases in non tolerant *C. grandis* roots in response to Mn-toxicity, and decreases in tolerant *C. sinensis* roots (You *et al*., 2014).

Aina *et al*. (2007) have investigated the responses of rice root proteome to a high concentration of Cd (100 mM). A high Cd concentration strongly induced the expression of regulatory proteins including ER1-like receptor kinase and Cytokinin

oxidase, and some metabolic enzymes such as cynnamyl-alcohol dehydrogenase, whereas in response to Cd proteins involved in ATP metabolism was decreased. At 10µM Cd concentration, it appears that rice reacts by inducing several defense responses which include increase of GSH levels, induction of transporters and degradation of oxidatively modified proteins.

A comparative proteomic study was conducted on three different *Thlaspi* accessions and the study revealed clear differences in the proteomes of three *Thlaspi* accessions, whereas the effects on each proteome of Cd exposure were less pronounced (Tuomainen *et al*., 2006). The proteomic study revealed a significant intensity differences (a total of 267-spots in roots and 246-spots in shoots) between the *Thlaspi* accessions. On the other hand, fewer spots (68-spots in root and 17- spots in shoots) showed significant differences among accessions due to the heavy metal exposures, than the differences among the *Thlaspi* accessions, suggesting that the accessions were the main sources of differences. However, it was also found that in tolerant accession some antioxidant-related proteins abundance was significantly increased, suggesting that antioxidant-related proteins may be responsible for the improved metal-tolerance in accession of *Thlaspi* (Tuomainen *et al*., 2006).

Roth *et al*. (2006) found that GSTFs 2 and 6-9 were up-regulated in *Arabidopsis* root following Cd-treatment, suggesting that they may be involved in a common response to heavy-metal stress in *Arabidopsis*; however, GSTUs 19 and 20 may have Cu-specificity. Recently, Ahsan *et al*. (2008) found that an omega-domain containing GST was markedly induced by arsenate (As) treatment, whereas its expression was unchanged on treatment with As, Cu, Al, or H_2O_2, suggesting that GST-omega may be involved in As biotransformation and metabolism in rice root cells. Taken together, the study advocate that, in general, GSTs are closely involved in the response to metal toxicity; however, some of them are specific to certain chemicals or metals and have specific functions in detoxifying particular substances.

3. Combined proteomic and transcriptomic studies under abiotic stress in roots

Transcriptome profiling, a widely used technique to identify stress-responsive genes (Jiang and Deyholos, 2006; Rabello *et al*., 2008), has contributed to our understanding of abiotic stress in species including Arabidopsis and rice. Jiang *et al*. (2007) investigated NaCl stress responses at the root proteome level, and compared these results with the transcriptome data. At the resolution of individual genes and proteins, poor statistical correlation (6 h, r= –0.13; 48 h, r=0.11) of these protein expression data with microarray results was detected in Arabidopsis roots under salt stress (Jiang *et al*. 2007), supporting the concept that post-transcriptional regulation plays an important role in stress-responsive gene expression, and highlighting the need for combined transcriptomic and proteomic analyses (Jiang and Deyholos, 2006; Jiang *et al*., 2007).

Rabello *et al*. (2008) investigated the drought stress effects in root system at reproductive stage of two up-land rice genotypes, characterized as susceptible and tolerant to drought stress, using combined genomic and proteomic approaches. Several protein families identified in the proteomic analysis were not detected in the cDNA analysis. These results clearly show that proteomics studies can reveal important additional information and that the use of complementary approaches is useful for a better understanding of complex biological traits, such as drought tolerance. It is well known that transcript levels do not always reflect protein amounts (Lian *et al*., 2006). Therefore, it is possible that the transcripts related to the proteins exclusively present in IRAT-20 2D maps were in low amounts, and not detected by the genomic analysis, or they were subtracted from the control condition in the hybridization process (Rabello *et al*., 2008).

However, transcriptome studies has some precincts, because, mRNA levels are not always correlated to those of corresponding proteins, due in part to post-transcriptional regulation. Previously, in studies involving yeast (*Saccharomyces cerevisiae*), animals, or *Arabidopsis* (Gygi *et al*., 1999; Mooney *et al*., 2006) only a poor or moderate correlation between changes in the levels of specific mRNAs and their corresponding proteins has been reported. Furthermore, post-translational modifications, such as phosphorylation and glycosylation, can result in a remarkable increase in proteome complexity without a concomitant increase in gene expression. Further, to date very few studies are exists on combined proteomic and transcriptomic analysis under abiotic stress in root tissues of different crops, and further attention is required on this aspect.

4. Proteomic methodologies used in studies of abiotic stress induced dynamic changes in the proteome

Current proteomic methods can be broadly divided into two groups, gel-free mass spectrometry-based approaches, and gel-based approaches, including conventional two-dimensional gel electrophoresis (2-DE) and differential in-gel electrophoresis. In proteomic analysis the extraction and purification of proteins from samples is a key and critical part.The reproducibility and reliability of a proteomic data relies on the quality of biological sample used. Plant tissues contain a range of various components such as proteases, polysaccharides, lipids, phenolic compounds and secondary metabolites; which will interfere with gel electrophoresis. A protocol found suitable for a particular plant of a species might not be appropriate for the other. It is improbable to suggest a single protein extraction protocol for different tissues, which are endowed with developmental-specific metabolites and similar views were proposed in earlier studies (Stalikas, 2007; Wu *et al*., 2014). Every protein extraction method and subsequent analytical procedure has their physic-chemical limitations; both should be investigated before selecting an appropriate protocol for conducting in-depth proteome studies.

To date, only classical TCA-acetone precipitation and phenol extraction methods proved useful as standard methods for protein extraction from different plant species and organs (Sebastiana *et al*., 2013; Wu *et al*., 2014; Singh *et al*., 2015; Vaganan *et al*., 2015; Surabhi *et al*., 2016). Overall, the general consensus is that TCA-acetone-based protein extraction is effective for younger tissues (Damerval *et al*., 1986; Santoni *et al*., 1997) and seeds (Carpentier *et al*., 2005; Singh *et al*., 2015) and may not be effective for isolation of proteins from fruit tissues. The phenol-based method has the potential to generate samples of higher purity for recalcitrant tissues than the TCA-acetone method, as compounds such as polysaccharides and other water-soluble contaminants are separated from the proteins that are solubilized in the phenolic layer (Hurkman and Tanaka, 1986). Further, for the recalcitrant plant materials containing high level of phenols and soluble polysaccharides, the phenol method showed a high efficiency for protein extraction and resolution (Wang *et al*., 2003; Rose *et al*., 2004, Saravanan and Rose, 2004; Carpentier *et al*., 2005; Surabhi *et al*., 2016).

4.1. Gel-based system

The most frequently used technique in abiotic stress-related proteomic studies is classical 2-DE coupled with Edman sequencing, MALDI-TOF MS, or ESI-Q-TOF MS/MS analysis (Table 1)., the gels are stained with CBB and/or silver stain in conventional 2-DE analyses. Nowadays, various fluorescent reagents are in use to label protein samples before 2-DE (2-D DIGE), which allows several samples to be separated simultaneously and visualized in one gel (Tonge *et al*., 2001), has been used to identify heavy metal-induced differentially-regulated proteins (Kieffer *et al*., 2008). The 2-D DIGE technique reduces the gel-to-gel variation considerably, that are commonly encountered in 2-D gel-based electrophoresis approach. Further, it improves the power of the quantitative analysis by the use of a common standard labeled by one of the three dyes (e.g. Cy2) and used on each gel of the experiment. This standard contains an equal amount of each sample; those are considered in the experiment. Thus, all the proteins contained in this standard will be present in the gel and will migrate at the same pI and MW values on each gel, allowing a better comparison after matching of all the standards across the gel batch during image analysis.

4.2. Gel-free system

In iTRAQ, samples are labeled at peptide level and it is an LC-based gel-free method. In contrast, in 2-D DIGE a sample are labeled at protein level and is a gel-based method. In DIGE method, higher number of biological replicates can be run within fewer gels; in this, pooling of samples into one analysis has the aim of reducing experimental variation and it raises the confidence level of protein changes. The iTRAQ method progressively replaces the gel-based DIGE and comparison studies of both techniques have demonstrated that these two techniques are complementary (Alvarez *et al*., 2009). All the proteins present in requisite amounts will be systematically quantified and identified in iTRAQ method, and at the end it provides a more comprehensive map of the protein content of a sample. Secondly, the iTRAQ can identify proteins outside the pH range of commonly used gels and distinguish between proteins that would co-migrate on a gel, whereas DIGE resolves only soluble proteins included in a pH range of 3 to 11(Alvarez *et al*., 2009). The differential expression of post-translationally modified proteins, different

isoforms, and gene families can be detected using DIGE by resolving spots at different pI and MW. Among a group of isoforms, most peptide sequences are identical in iTRAQ method and these isoforms may not be distinguished. Thus, common peptides shared by different isoforms will be grouped into the same pro-group by ProteinPilot and they will not be used for protein quantification. Further, this can lead to delusion of protein changes and mask specific changes in isoform abundance resulting from treatment. In a study by Alvarez *et al*. (2009) used both iTRAQ and DIGE as complementary approaches to study the Cd stress on *Brassica juncea* roots. They demonstrated that iTRAQ able to detect and quantify a much larger number of proteins and appear to be a more robust technique, but, DIGE provided useful complementary information to the iTRAQ experiment. DIGE will be more suitable for certain types of analyses, such as those focusing on PTM. These different strengths emphasize the importance of technical diversity in revealing the complexity of biological systems (Alvarez *et al*., 2009).

Recent advancement in LC-MS based quantitative techniques such as isotope-coded affinity tags (ICAT) (Gygi *et al*., 1999), stable isotope labeling by amino acids in cell cultures (SILAC) (Schutz *et al*., 2011), and isobaric tags for relative and absolute quantification (iTRAQ) (Alvarez *et al*., 2009) showed advantages for relative quantification of proteins or peptides on a large scale. Advances in these techniques and in the MS field can allow the analysis of complex proteomes at organ/tissue and whole plant levels in different crops. This technical advancement in gel-free proteomics could further expand our scope of understanding of abiotic stress sensing mechanisms in plant roots.

5. Mass spectrometry in plant root proteomics

The mass spectrometers have become increasingly attractive analytical instruments for biologists, over the last decade, due in part to new ionization methods coupled with major improvements in sensitivity, mass accuracy, resolution and ease of use, which have extended the scope of applicability of MS to characterize large intact macromolecules such as proteins. MS is now firmly ingrained as the method of choice for both protein identification and characterization of post-translational modifications. In the recent years, the development of Orbitrap and new dissociation methods such as electron-transfer dissociation (ETD), have opened up new possibility in proteome analysis (Jorrin-Novo *et al*., 2009). However, it is necessary to choose the appropriate instrument for the purpose and as there is no MS that can be useful for all fields of proteome analysis. MALDI-TOF/MS is often used for high-throughput identification of the protein by peptide mass fingerprinting (Witzel *et al*., 2009; 2010). In the analysis of amino acid sequence and post-translational modification, MS/MS such as ESI-IT and ESI-Q-TOF/MS are used. The proteins are analyzed after digestion with protease into the peptides in most of the MS analyses such as MALDI-TOF/MS, ESI-Q-TOF/MS and ESI-IT/MS, because large molecular weight of proteins cannot be analyzed directly. This approach is called 'bottom-up proteomics'. In contrast, the whole protein can directly be applied in FT-MS analysis to obtain information.

FT-MS allows analyzing the sequence information and post-translational modification of proteins automatically without preparation by 2-DE and liquid chromatography (LC) (Hirano *et al*., 2004). This approach is called 'top-down proteomics' or top-down mass spectrometry (Hirano *et al*., 2004). It is nevertheless important to note that one of the major achievements of last generation instruments, such as orbitrap or FT-ICR mass spectrometers, has the ability to measure masses with very high resolution (up to 150000 for the FT-ICR) and very high mass accuracy (up to 1 ppm) (Bachi and Bonaldi, 2008). This allows approaching the elemental composition of peptides, at least for peptide masses commonly measured in 'bottom-up proteomics' experiments (Zubarev and Mann, 2007).

Despite these technological innovations and advancements, the analysis of a full proteome is still a challenging task, mainly because of the high complexity of protein samples (Bachi and Bonaldi, 2008). To overcome this difficulty, several separation techniques such as multidimensional chromatography, MudPit (Washburn *et al*., 2001) or specific enrichment/ depletion techniques, Tandem Affinity Purification (Gavin *et al*., 2002) and equalizer beads (Guerrier *et al*., 2008) can be applied prior to mass spectrometric analysis. These approaches increase the proteome coverage and the dynamic range of large-scale proteomics analysis.

Further, several software algorithms are available to compare the observed peptide masses against those predicted from theoretical tryptic

fragments for every protein in a sequence database. Protein identification is usually based on the *in silico* match between experimentally determined peptide masses and those theoretically derived from the database (Chen and Harmon, 2006). MALDI-TOF PMF is a relatively inexpensive, rapid, and high throughput technique with the potential to analyze hundreds of samples a day (Chen and Harmon, 2006).

6. Achievements and challenges

Root proteomics studies were conducted under drought (Ghaffari *et al*., 2013; Simova-Stoilova *et al*., 2015), salinity (Witzel *et al*., 2009; Mostek *et al*., 2015), heavy metal (Chen *et al*., 2015; Xue *et al*., 2015), waterlogging (Guerrier *et al*., 2008) and temperature stress (Xu and Huang, 2008; Lee *et al*., 2009) conditions. Recently, Cheng *et al*. (2009) studied rice root plasma-membrane-associated proteome under salt stress. Most of those identified membrane associated proteins are involved in important physiological processes such as membrane stabilization, ion homeostasis, and signal transduction. A phosphoproteome study under salt stress in rice roots was conducted by Chitteti and Peng (2007). Root proteome studies were conducted under different abiotic stresses alone on different crops such as rice (Cheng *et al*., 2009), barley (Witzel *et al*., 2009; Mostek *et al*., 2015), maize (Zorb *et al*., 2010), fruit (Ahsan *et al*., 2008; Yoshimura *et al*., 2008) and oilseed crops (Ghaffari *et al*., 2013; Mustafa and Komatsu, 2014). Most of the root proteomic studies were focused on crop plants, and woody plant root proteomics need to be conducted. Sensitive proteomic approaches are capable of identifying low-abundance proteins (especially transcription factors and regulatory proteins) involved in the initial stress response in crops. However, combination of multiple abiotic stresses on roots needs to be conducted on crops.

The primary challenge when dealing with root tissues are their multi-cellular complexity. It is also tricky to harvest root parts from soil-grown plants. Because, while harvesting roots, either part of root will get damaged or lose of root hair and root-tip region may occur. The majority of the root proteomic studies are based on gel-based separation methods, with this, identification of low abundant proteins such as transcription factor (regulatory proteins), signaling proteins, ion channels and transporters was limited (Ghosh and Xu, 2014). Most of the experiments are based on one-time measurements, and time course experiments are required in order to get reliable data on protein expression under different stress conditions. Further, majority of the studies are based on single stress treatment alone, and imposition of multiple abiotic stress factors on crops and identification of candidate proteins under these conditions will provide more valuable information (Li *et al*., 2011).

Focuses on early responsive proteins are required in order to identify regulatory and signaling proteins. Currently, crop proteomic changes are often analyzed after several hours, even days after a stress onset (Witzel *et al*., 2009; Meng *et al*., 2014). Thus, early (the initial) proteome changes of crops under abiotic stress conditions should be further studied. These types of studies will give important insights in the signaling cascade activated immediately in crops in response to abiotic stresses. Advanced LC based separation technique significantly improves detectability of low abundant proteins such as transcription factors, kinases, ion channels and transporter proteins (Ghosh and Xu, 2014).

7. Conclusion

The peptide fragmentation and recent advances in mass spectrometry significantly improve the coverage of root proteomic profiles and opens up new perspectives for the dissection of molecular mechanisms underlying adaptive responses to abiotic stressors (Schmidt, 2014). Recent advancement in proteomic methodologies, such as multi-dimensional protein fractionation, ICAT, iTRAQ, SILAC, DIGE, and high-resolution tandem mass spectrometry has facilitated a more accurate comparison of crop stress responses and can detect more deferentially abundant proteins than prior analyses. Particularly, when working with the root samples, an appropriate sampling method needs to be developed in order to obtain relatively pure sub-cellular fractions to enhance the probability of identifying stress proteins (genes) from specific cells or tissues. A promising sampling method which can sort specific cell types of interest from sectioned specimens of heterogeneous tissues under direct microscopic visualization with the assistance of a laser beam is laser capture micro-dissection (LCM) (Longuespée *et al*., 2014). The LCM technique has been successfully applied in rice (Suwabe *et al*., 2008; Kubo *et al*., 2013) and in maize transcriptome and microarray studies (Nakazono *et al*., 2003; Rajhi *et al*., 2011). Hopefully, combined with high sensitive

protein staining technologies coupled with recent advancement in mass spectrometers, LCM has the potential to promote crop stress proteomics at a cellular level. Another promising technique is free flow electrophoresis (FFE), which can isolate much purer membrane fractions and/or organelles. In plant proteomics studies FFE technique has been successfully applied to isolate different cell organelles such as tonoplast, mitochondria, plasma membranes and Golgi apparatus (Barkla *et al.*, 2013). Integration of proteomics result with findings from other large scale 'omics' and bioinformatics applications will surely facilitate the establishment of molecular networks underlying abiotic stress response and tolerance in plant root system.

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Abbreviations

CWPs, cell wall proteins; DEPs, differentially expressed proteins; DIGE, Differential in Gel Electrophoresis; ESI, electrospray ionization; FFE, free flow electrophoresis; FT-ICR, Fourier transform ion cyclotron resonance; ICAT, isotope-coded affinity tags; IMAC, immobilized metal affinity chromatography; iTRAQ, isobaric tags for relative and absolute quantification; LC, liquid chromatography; LCM, laser capture micro-dissection; MALDI-TOF, Matrix-assisted laser desorption/ionization-time of flight; MS/MS, tandem mass spectrometry; MudPit, Multidimensional Protein Identification Technology; PTM, Post-translational modification; SILAC, stable isotope labeling by amino acids in cell cultures.

Conflict of interest

The authors declare no conflict of interest.

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