REVIEW

Proteomic analysis of temperature stress in plants

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In this review we examine current approaches used for proteomic analysis of temperature stress in plants. Rapid advances in this field in recent years are discussed, including metabolic, chemical and isotopic labeling, and label-free quantitative techniques. These are compared and contrasted with well-established methods such as 2-DE approaches. Examples of applications of various methods are presented, and technical difficulties and limitations of each are also considered. Results of previous studies are examined in detail, and commonly occurring temperature stress response proteins are collated. We conclude that technical advances, and improvements in genome sequence availability, will have an ever increasing impact on our understanding of molecular mechanisms of stress response in plants.

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1 Introduction

1.1 Why investigate temperature stress in plants?

Water and nutrient availability, salinity, and temperature fluctuations have marked influences on agricultural production around the world. Climate change is set to increase the frequency and severity of environmentally limited production as global warming will cause more frequent extreme temperature events. Climatologists generally agree that a doubling of CO_2 levels since the onset of the Industrial Revolution will lead to an increase of 5°C in the mean temperature of the Earth over the next half-century, with severe climatic consequences. Increases in average temperature worldwide culminated with the last decade being the hottest in the last 1000 years, and 1998 the warmest individual year recorded [1]. The effects of global warming will not be limited to rising mean annual temperatures around the globe. There will also be a dramatic increase in both frequency and amplitude of severe temperature events, resulting in more extreme hot and cold days, more frequently. These fluctuations will have profound impacts on natural ecosystems and agriculture. Higher plants have a finite capacity to acclimate to temperature shocks, even though long-term adaptation is well established in populations from extreme biomes. Thus, the degree to which temperature fluctuations deviate from long-term mean values becomes critical for productivity of all plant species, including crops.

1.2 Temperature stress in rice and wheat

Reproductive development is a critical and sensitive process in the life cycle of flowering plants; the direct output of reproductive development in crop plants is food for human consumption. Rice and wheat are the most important cereals providing staple food for about 80% of the world's

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Abbreviations: APX, ascorbate peroxidase; **BR,** brassinosteroid; **DAA,** days after anthesis; **HILEP,** hydroponic isotope labeling of entire plants; **ICAT,** isotopically coded affinity tagging; **iTRAQ,** isobaric tags for relative and absolute quantitation; **OsCIA,** *Oryza sativa* cold-induced anther protein; **Rubisco,** ribulose-1,5-bisphosphate carboxylase/oxygenase; **sHSP,** small HSP; **SILAC,** stable isotope labeling of amino acids in culture; **SILIP,** stable isotope labeling *in planta* population, and prices for rice and wheat have risen to record highs due to global demand in recent years. Male gametophyte development in many crops including rice and wheat is extremely susceptible to various stresses such as cold temperature, heat and drought, which directly cause grain yield reduction. Understanding the molecular basis for such phenomena will have great impact on engineering or breeding of crops tolerant to stresses at the reproductive stage.

In addition to its economic importance, rice has become an attractive model system for cereal genomic research because of its relatively small genome, a high degree of genomic synteny with other cereal crop plants, availability of tens of thousands of searchable insertion lines (*e.g.* transferred DNA, Tos17, Ds), compatibility with genetic transformation and availability of a sequenced genome that contains approximately 32 000 genes. Wheat genome and expressed sequence tag sequencing is catching up rapidly, aided by next-generation sequencing technologies. Although it is important to study rice as both a model species and an essential crop, it is equally valuable to investigate wheat, to compare and contrast the response of different species to abiotic stresses and exploit the research findings in agriculture.

In recent years, transcriptomics and proteomics have been applied to identify stress-responsive genes and proteins that are regulated by elevated temperatures, salinity, cold and water deficit in several vegetative tissues in rice and wheat [2–5]. Proteomic approaches were also applied to find general stress related proteins [6–12]. Stress-related genes and proteins involved in several pathways have been identified, such as abscissic acid and jasmonic acid biosynthesis and signaling, redox homeostatis, energy metabolism, polysaccharide and cell wall metabolism, and defense. These include key factors such as dehydration-responsive element/C-repeat binding factors, basic leucine zipper proteins, NAC, MYB and MYC type transcription factors, and microRNAs, just to name a few.

1.3 Differences between stress responses in vegetative and reproductive tissues

The reproductive stage of crop development is required for grain production. The early stages of male gametophyte development are particularly susceptible to abiotic stress damage caused by cold, drought, salinity, heat, flooding and nutrient deficiency. This damage occurs in many important crops such as rice, wheat, maize, barley, sorghum and chickpea [13]. Cool conditions during the reproductive stage limit crop yield in all temperate rice-growing areas of the world. In Australia, for example, this causes an average annual yield reduction of 5–10%, which amounts to losses of up to $A44 million; unpredictable cold snaps occurring on average every 3–4 years can result in yield losses of 20–40% [13, 14]. Drought is another major abiotic stress limiting plant production worldwide, as plants are most susceptible to water stress at the reproductive stage.

In rain-fed rice and wheat, a brief episode of water stress during meiosis disrupts subsequent male microsporogenesis, leading to male sterility and a reduction in grain set [15–18], believed to be linked to a disturbance in carbohydrate metabolism [19]. Heat stress also causes sterility and induces abnormalities in the spikelets in rice production [20], and heat stress above 30°C during floret formation causes complete sterility in wheat [21, 22]. The biochemical regulatory mechanisms responsible for these observed phenotypes are not fully understood. Male sterility induced by drought in wheat shows common features with that induced by cold in rice: the accumulation of non-reducing sugars and the failure of starch accumulation in the pollen grains. All of these stresses act specifically at the young microspore stage, when the tapetum is functioning at maximum capacity. In both species, conditions causing stress-induced male sterility have no damaging influence on female sexual development, suggesting that the female gametophyte has greater stress tolerance [22]. In the process of cold damage, for instance, anthers become smaller and cells in the tapetal layer in the anthers, which surrounds and is responsible for transferring nutrients to the developing pollen, undergo hypertrophy and eventual breakdown. In both rice and wheat, tapetum degradation, microspore callose wall and exine formation, and carbohydrate metabolism all seem to be perturbed by stress conditions which ultimately cause male sterility [13, 23, 24].

Only a small number of stress responsive genes have been identified in both the vegetative and reproductive stages. For instance, two genes, *OsSALT* (encodes a 15-kDa mannose-binding lectin protein) and *OsNac6* (encodes an apical meristem transcription factor) are induced by abiotic stresses in rice [25]. However, these stress responses and their impacts at the reproductive stage are different to stress responses at the vegetative stages, and it is clear that abiotic stresses affect grain-yield more than vegetative growth [26].

2 Sample preparation and experimental design

Sample preparation techniques used in mammalian, bacterial or yeast proteomic studies are often not applicable to plant samples. Plant tissues are difficult to disrupt due to the rigidity of cell walls, which are made of a complex assembly of polysaccharides. Protein extraction is made difficult because of the presence of secondary metabolites, mainly polyphenolic compounds, which can cause protein precipitation, streaking or artificial spots on 2-DE gels, and charge heterogeneity [27, 28]. There are two common approaches for plant protein extraction, each usually beginning with mechanical disruption of the tissue by grinding in a mortar and pestle under liquid nitrogen [29]. Proteins can then either be precipitated with trichloroacetic acid-acetone, which removes interfering compounds, and solubilized in a buffer containing a mixture of chaotropes, reducing agents and detergent, or alternatively proteins can be extracted with phenol followed by ammonium acetate-methanol precipitation. Since its development, the phenol extraction method [30, 31] has become the preferred technique for use with recalcitrant tissues and has been used successfully to study olive leaves [32], grape berries [33] and wood from Poplar [34].

Each plant tissue has diverse biophysical properties and hence sample preparation may need to be optimized for each type of tissue [35]. Each tissue has its own inherent difficulties: it is difficult to obtain clean root material from roots grown in soil; seeds have high starch content and contain abundant storage proteins; and leaves have extremely high ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) content. To avoid contaminated root samples, plants can be grown hydroponically [36, 37] or aeroponically [38]. These alternatives serve as good laboratory models with minimal mechanical damage, direct visualization and accessibility for accurate growth measurements, but these artificial media cannot replicate conditions of plants grown in the field. Therefore, a limited number of root proteomic studies have been performed in comparison to studies using other vegetative tissues.

Extremely abundant proteins can mask the detection of lower abundance proteins by MS, but reliable and robust methods to remove highly abundant proteins from plant tissue samples are still under development. Rubisco is the plant analogue of albumin in mammalian physiological fluids, constituting approximately 30% of protein in leaves [39]. There are two main methods for the removal of Rubisco during sample preparation: use of an anti-Rubisco antibody affinity column, either produced commercially [40] or in-house [39], and PEG fractionation [41, 42]. Both of these methods have shown considerable promise but are yet to become widely adopted.

Few proteomic studies using fruits or flowers have been reported. Most fruits have a high carbohydrate content with minimal protein; for example, mature grape berries contain 26% sugar [33]. Some work has been published on the proteomic analyses of grape proteins despite the fact that grapes contain very high amounts of polyphenols, proanthocyanidins and condensed tannins [43]. Most plant studies are restricted to using seedlings because sufficient leaf material can be obtained after a relatively short growth period, typically weeks to months. However, a study involving protein expression in bark in peach trees used material from trees that had been grown for more than a year prior to sampling [44].

Studies of fruits and flowers are further restricted by the annual reproductive cycle of most plants. As an example, a proteomic analysis of light stress in tomatoes involved harvesting material from plants grown outdoors during a three-month optimal window during the Northern spring [45]. Repetition of such a study would involve waiting up to 12 months until the subsequent growing season, which represents a stark contrast to the relative ease with which experiments in simple laboratory based organisms, such as yeast or *E. coli,* can be replicated.

Despite all of these various difficulties, there are an increasing number of plant proteomics papers published in scientific literature in recent years. Researchers in the plant field are becoming aware that the results produced by well-designed proteomic studies justify the amount of work and technical difficulty involved.

3 Quantitative proteomics techniques

Traditional 2-DE techniques have been used as the core method for comparative quantitative proteomic studies in many different plant species. However, due to certain limitations of these techniques, a number of higher throughput and more sensitive alternatives have been developed and applied. In this section, we present selected examples of some of the major recent developments in quantitative proteomic techniques. This is not intended to be comprehensive; more detailed information on recent trends in quantitative proteomic techniques in general [46–52], and specific to plants [53–58], can be found in recent reviews.

The classical 2-DE-based proteomic method provides a visual output for protein profiling and comparative mapping of expressed proteins between biological samples. Applications of 2-DE have been reported extensively in various plant species; recent examples include studies of Arabidopsis [59, 60], soybean [61, 62], rice [63–65], wheat [66], barley [67], potato [68, 69], tomato [70] and many others. 2-DE and 2-D DIGE techniques can be applied to all plant species and are not necessarily limited to plants with a fully sequenced genome. For example, 2-D DIGE has been used in characterization of the proteome of strawberry [71] and sunflower [72], and to examine proteome expression changes of bark tissues of peach [44] and leaf tissues of tomato [45, 57].

Alternatives to 2-DE-based methods are generally subdivided into two categories: stable isotope labeling approaches such as stable isotope labeling with amino acids in cell culture (SILAC), ^{15}N labeling, isotope coded affinity tagging (ICAT) and isobaric tags for relative and absolute quantitation (iTRAQ); and label-free approaches, including peak area integration and spectral counting.

In metabolic labeling, isotopic labels can be incorporated during cellular metabolism or during protein synthesis, either as exogenous amino acids in cell culture or as ^{15}N, often in the form of $K^{15}NO_3$ as a nitrogen source. SILAC uses *in vivo* corporation of isotope labels during protein synthesis and cellular metabolism [73].

The SILAC technique has been developed and applied with significant success in yeast and mammalian cells [74, 75]. However, the application of SILAC in plant proteomic studies is problematic due to the ability of autotrophic plant cells to synthesize all amino acids. It has also been reported that complete labeling could be hindered due to carbon fixation of atmospheric CO_2 by plants [76, 77]; for example, in a study of *in vivo* stable isotope labeling of Arabidopsis cell culture, only 80% efficiency in labeling has been achieved by using exogenous amino acids. It is possible to apply SILAC in proteomic studies using plant cell cultures, such as one report using SILAC in Arabidopsis cell culture to study changes in protein expression caused by addition of salicylic acid [76]. Drought stress or insect colonization in plants are good examples of stresses which cannot be modeled in a cell culture system.

The second major approach in metabolic stable isotope labeling involves the introduction of a heavy isotope such as ^{15}N, which can be applied to both cell culture [78, 79] and whole plant systems [80, 81]. Hydroponic isotope labeling of entire plants (HILEP) is a quantitative proteomics method used to determine the changes in protein expression of whole plants and has been shown to give 100% efficiency in labeling of Arabidopsis proteins by growth in hydroponic media containing ^{15}N salts [82]. Another new approach, stable isotope labeling *in planta* (SILIP) demonstrates ^{15}N isotope labeling of the tomato plant proteome carried out in soil based medium [83], with confirmation of 99% labeling efficiency shown by LC-MS/MS of ^{15}N labeled peptides.

In chemical labeling, isotopically distinct labels can be added to proteins after protein extraction and preparation. A few applications of ICAT in plant proteomics have been reported, focused on characterizing the endoplasmic reticulum and Golgi apparatus in Arabidopsis [84], and mitochondrial membrane proteins [85] and chloroplast soluble stromal proteins in maize leaves [86]. iTRAQ is another chemical labeling technique used in stable isotope quantitative proteomics [87]. The application of iTRAQ has been reported in numerous recent plant studies; for example, protein abundances were compared among two different boron-tolerant and intolerant barley lines [88]. One disadvantage of this technique has been the requirement to use a high resolution mass spectrometer to analyze the diagnostic fragment ions, but this has been mitigated by the development of a fragmentation algorithm and appropriate software for analyzing iTRAQ fragment ions on an ion trap mass spectrometer [89].

Label-free quantitation encompasses several techniques for identification and quantitation of differentially expressed proteins in two or more complex biological samples. One type of label-free quantitation uses measurement of peptide ion chromatogram peak intensities to measure the changes in expression pattern between multiple samples, with each sample analyzed individually and their peptide ion chromatograms re-constructed and compared [90, 91]. Another type of label-free quantitation, known as peptide spectral counting, uses the number of MS/MS spectra matched to a given protein within an experiment as a measure of relative protein abundance. Calculation of normalized spectral abundance factors is the latest related development in the spectral counting approach [92, 93]. In a recent study both of these label-free approaches were examined, and the authors found that the techniques were equivalent in reproducibility and linearity aspects. However, spectral counting was preferred as it was simpler and faster to conduct, and was subsequently applied to an investigation of major allergens in transgenic peanut lines [94].

The combination of shotgun proteomics and spectral counting promises researchers the ability to examine changes in protein expression patterns in various cell types, without labeling limitations. It is thus possible to use such approaches in analysis of multiple data points in a quantitative study of abiotic stress in plants, such as a variety of time points or temperature levels.

Given the large number of quantitative proteomic techniques available, the large number of plant species studied and the range of abiotic and biotic stress under investigation, there is a myriad of combinations of techniques and biological systems in the scientific literature. It is worth noting that several studies have shown that application of multiple quantitative proteomics techniques to answer a single question is highly beneficial, as the approaches yield complementary datasets [95, 96]. It is not possible to list all of the methods and systems involved, but in Table 1 we have included examples of plant stress proteomics studies using the techniques referred to in this section.

4 Genomic information in plants

Regardless of the specific techniques used, proteomics relies heavily on the availability of complete genome sequence information for use in protein identification. Plants typically have very complex genomes with high levels of redundancy and large multi-gene families, such as the kinases and HSPs in Arabidopsis [97–99]. Stress response mechanisms in plants are also characterized by multivariate signaling networks with crosstalk between pathways, making their deconvolution even more difficult [100]. Much of this degree of genetic complexity is thought to be driven by evolutionary imperatives; as sessile organisms, plants need to have multiple defense mechanisms to guard against biotic and abiotic threats.

Complete genome sequences in plants are still relatively uncommon, especially when compared to the large number of complete genome sequences available for other microorganisms and mammalian species. This stands in direct contrast to their distribution in the naturally occurring biome, where there are estimated to be 4500 species of mammals and 375 000 species of plants. It is always difficult to define exactly when a genome sequence is considered ‘‘finished,’’ and even those which are considered to be complete are subject to ongoing revision, as illustrated by

Table 1. Quantitative proteomic techniques and applications in plant stress

Species	Biological study	Technique	Reference
Agrostis spp.	Heat stress	2-DE	[121]
Oryza sativa	Cold stress	2-DE	[63]
Oryza sativa	Cold stress	2-DE	[64]
Glycine max	Salt stress	2-DE	[61]
Oryza sativa	Drought stress	2-DE	[122]
Arabidopsis thaliana	Cold stress	2-D DIGE	[109]
Prunus persica	Cold stress	2-D DIGE	[44]
Lycopersicon esculentum	Shade-avoidance	2-D DIGE	[45]
Lycopersicon esculentum	Salt and osmotic stress	2-D DIGE	[123]
Arabidopsis thaliana	Salt and osmotic stress	2-D DIGE	[124]
Populus spp.	Ozone exposure	2-D DIGE	[125]
Arabidopsis thaliana	Subcellular compartment proteomics	ICAT	[84]
Arabidopsis thaliana	Mitochondrial proteomics	ICAT	[85]
Zea mays	Chloroplast proteomics	ICAT	[86]
Arabidopsis thaliana	Salicylic acid stress	SILAC	[76]
Arabidopsis thaliana	Heat stress	^{15}N differential metabolic labeling	[82]
Arabidopsis thaliana	Oxidative stress	HILEP (^{15}N metabolic labeling)	[82]
Arabidopsis thaliana	Quantitative phosphoproteomics	^{15}N metabolic labeling	[126]
Lycopersicon esculentum	Whole plant labeling in soil-based medium	SILIP (^{15}N metabolic labeling)	[83]
Arabidopsis thaliana	Plant defense response to pathogens	iTRAQ	[127]
Arabidopsis thaliana	Chloroplast proteomics	iTRAQ	[128]
Oryza sativa	Defense response to insect infestation	iTRAQ	[129]
Hordeum vulgare	Boron toxicity stress	iTRAQ	[88]
Lycopersicon esculentum	Defense response to insect herbivory	Label-free quantitation	[130]
Arabidopsis thaliana	Sucrose-induced phosphorylation changes	Label-free quantitation	[131]
Arachis hypogaea	Seed allergen profiling	Label-free quantitation	[94]
Arabidopsis thaliana	Cell dedifferentiation	Label-free quantitation	[132]

Table 2. Plant genome projects at or near completion

Arabidopsis thaliana (Thale Cress)	[133]
Oryza sativa (Rice)	[102, 134]
Brassica rapa (Mustard)	[135]
Carica papaya (Papaya)	[136]
Glycine max (Soybean)	[137]
Gossypium hirsutum (Cotton)	[138]
Hordeum vulgare (Barley)	[139, 140]
Lotus japonicus (Lotus)	[141]
Medicago truncatula (Barrel medic)	[142]
Agrostis stolonifera (Grass)	[143]
Physcomitrella patens (Moss)	[144]
Populus trichocarpa (Poplar)	[145]
Solanum lycopersicum (Tomato)	[146]
Sorghum bicolor (Sorghum)	[147]
Triticum aestivum (Wheat)	[148]
Vitis vinifera (Wine Grape)	[149]
Zea mays (Maize)	[150, 151]

the use of large-scale shotgun proteomics data to significantly improve gene identification in Arabidopsis using an approach known as proteogenomics [101]. Despite these difficulties, plant genome sequencing has made great progress in recent years. In Table 2 we have included 17 plant species for which relatively complete genome sequences, suitable for use in proteomics studies, are freely available.

5 Cold stress

5.1 Cold stress in rice

Rice plants provide one of the most important food sources with over half of the world's population relying on its grain as a staple food. The rice genome has been sequenced yet nearly one third of proteins remain uncharacterized despite recent efforts in rice proteome research [102, 103]. Proteomic analysis of temperature stress in rice is primarily focused on variations of cold stresses yet the molecular mechanisms of cold stress perception, signaling and tolerance remain unresolved.

Cold damage at the early microspore development stage in rice results in pollen sterility, also known as cold-induced male sterility, which is caused by incomplete ripening of pollen grains. In two studies, the effects of mild cold stress on the proteome of anthers of cultivar (cv.) Doongara have been explored after exposing plants at the early microspore phase of development to 12°C for 96 h [104] or for 24, 48 and 96 h [105]. The plants were subsequently allowed to reach the trinucleate microspore stage in control conditions before microscopic and proteomic analyses of anthers took place. Anthers of cold treated plants showed abnormal physical changes including reduction in both grain size and pollen content. Four days of cold treatment in cv. Doongara plants resulted in 86% sterility [105]. Proteins extracted from anthers were separated by 2-DE and, in both studies, the majority of the anther proteome showed no change. In the two studies, a total of 105 proteins displayed differential spot patterns on 2-DE gels in response to cold stress, 11 protein spots were common between the two studies, 85 spots were analyzed by MS or amino-terminal sequencing, and 31 proteins were successfully identified. In the earlier study, there was evidence of increased partial protein degradation in proteins that were up-regulated as a result of cold treatment. Similar protein degradation was not observed in control samples and peptides that were matched in PMF analysis were the *N*- or *C*-terminal regions of identified proteins, which further supports a distinct degradation process occurring in response to cold stress. It appears that the cold-induced degradation was different to normal programmed cell death pathway degradation as the cold-induced protein degradation resulted in stable products, visible on 2-DE gels and identifiable by sequencing. In programmed cell death degradation, proteins are degraded to small peptides or individual amino acids. Several proteins identified were novel cold-responsive proteins, including *Oryza sativa* cold-induced anther protein (OsCIA) [105]. This protein was present in panicles, leaves and seedlings under control conditions and was induced in anthers after cold stress. However it was observed that OsCIA mRNA levels were not altered in anthers, panicles, leaves or seedlings after cold treatment. OsCIA is an example of a protein that has tissue-specific differential expression which is regulated by post-transcriptional modifications.

To distinguish cold stress responsive proteins from proteins that were up-regulated due to cellular damage, Cui *et al*. [106] exposed rice seedlings to progressively low temperatures. This approach also allowed the examination of dynamic protein expression patterns in response to increasing cold stress intensity. Two-week-old plants were exposed to decreasing temperatures of 15, 10 and 5°C each for 24 h, leaves were harvested after each treatment and fractionated proteins from control and treated samples were separated by 2-DE for spot volume comparison. The fractionation procedure resulted in two fractions: fraction one contained soluble proteins, which included Rubisco, and the second fraction contained non-soluble, structure-associated proteins. Of 1700 protein spots, 60 showed an increase in abundance after progressively low cold treatment and 41 of these proteins were identified by PMF or MS/MS. Over 43% of proteins identified were resident in the chloroplast, which highlights the susceptibility of this organelle to cold stress, a common observation among cold stress research involving leaf protein expression. Three main functional groups of proteins were found to be up-regulated with metabolic proteins representing the largest group showing altered expression. Ten proteins identified were involved in protein synthesis, folding, assembly or degradation. Proteins that contribute to cell wall biosynthesis were the second-most represented category, followed by proteins that are associated with antioxidant reactions. Intriguingly, these functional groups of proteins have been identified independently in subsequent cold stress studies in rice.

Yan *et al.* [12] studied the effects of chilling temperatures of 6°C on rice seedlings where leaves were sampled after 0, 6, and 24 h of stress and also after a 24-h recovery period. Leaf proteins were extracted and separated by 2-DE, which reproducibly resolved over 1000 protein spots with 31 found to be down-regulated and 65 up-regulated. The majority of spots that displayed differential expression were consistently up- or down-regulated across all time points, but were less pronounced after 24 h of recovery. Differentially expressed proteins were analyzed by PMF or MS/MS and functionally classified. Functional categories that each contributed over 5% to total cold responsive proteins included energy metabolism, carbon metabolism, photorespiration, and translation. The functional group that was most affected by cold stress was photosynthesis with 35.3% of cold-responsive proteins residing in this category. Partial protein degradation was also observed in this study with photosynthesis appearing to be the most affected pathway; fragments of photosynthesis-related proteins (including 19 fragments of Rubisco large subunit) were detected on 2-DE gels, which were subsequently confirmed by western blotting. The authors suggest that stress-induced ROS may be affecting these proteins and thereby flagging them as targets for proteolytic degradation. Proteins capable of controlling ROS levels were also evident, attributed by 5.9% of total cold-responsive proteins, including the commonly cold stress induced ascorbate peroxidase (APX).

In a separate study by Hashimoto and Komatsu, a 2-DE analysis also elucidated the degradation of Rubisco large subunit in leaf blades of 2-wk-old seedlings following a cold stress treatment of 5°C for 48 h [39]. Other proteins located in the chloroplasts were also decreased in response to low temperature treatment including the energy metabolic proteins, H^+-transporting ATP synthase and vacuolar ATPase B subunit, again indicating damage to this organelle and a decrease in photosynthetic rate in suboptimal temperature conditions. In addition to analyzing leaf blade proteins, these authors also considered the effect of cold stress on other organs of the rice plant. Proteins from leaf sheaths and roots were also analyzed by 2-DE in an attempt to dissect organ-specific changes in response to cold stress. In roots 12 protein spots displayed differential expression (eight were identified by PMF), in leaf sheaths nine proteins were changed in response to cold (eight were identified by PMF), and in leaf blades changes in 18 proteins were detected (13 were identified by PMF and three by MS/MS). Besides only minimal overlap in the proteins identified in different tissues, there was little evidence to support organ-specific changes in protein expression in response to cold stress. UDP-glucose pyrophosporylase, a protein involved in cellulose synthesis, was identified in all three tissues and displayed an up-regulation in roots and leaf blades but a down-regulation in leaf sheaths. However, UDP-glucose pyrophosphorylase has previously been shown to be up-regulated in response to cold stress in the leaf blade of rice seedlings [106], in anthers of trinucleate stage rice plants [104], and in bark of peach tree (*Prunus persica*) [44]. UDP-glucose pyrophosphorylase was also up-regulated in response to heat in rice leaves [9]. It appears that UDP-glucose pyrophosphorylase is not an organ-specific protein in response to cold and is more likely a general stress response protein up-regulated in several different tissues. The authors also performed a Rubisco depletion step using an antibody-affinity column and re-analyzed the samples. After Rubisco depletion four previously unseen protein spots showed a response to cold stress and two down-regulated chloroplastic proteins were identified by MS/MS.

An alternative approach for depleting Rubisco from samples was taken by Lee *et al.* [107] in the analysis of leaf proteins that showed differential expression in response to extreme and moderate cold stress. Rice seedlings were grown for 3 wk then subjected to 5°C for 12, 24 and 36 h or 10°C for 24 and 72 h. Leaves were harvested after each time point and protein extracts were fractionated with PEG, an alternative method of sample preparation for removing Rubisco from leaf material [41, 42, 108]. This is a more refined method of fractionation for Rubisco removal in comparison to the fractionation performed by Cui *et al.* [106]. Fractionated proteins were separated by 2-DE and 14 spots were found to be up-regulated by cold stress across all time points in both temperature stresses and 12 were identified by either PMF or MS/MS. Seven proteins were newly identified as cold stress responsive proteins, perhaps owing to the absence of Rubisco in the samples, as non-depleted samples were not analyzed in conjunction with Rubisco-depleted samples. In support of the theory of ROS accumulation, four of the 14 up-regulated proteins (APX, glutathionine *S*-transferase, thioredoxin *h*, and thioredoxin peroxidase) were identified as antioxidant enzymes and showed a marked accumulation after the 5°C exposure period. These enzymes have been previously identified as either cold stress response proteins or proteins induced by oxidative stress caused by ROS. One of the novel cold-induced proteins identified in this study was a RING zinc finger protein-like protein, which showed to be significantly responsive only to extreme cold stress. Previously, over-expression of a C2H2-type zinc finger gene in soybean enhanced cold tolerance in plants [41]. The authors suggest the RING zinc finger protein identified in this study could be used as a cold stress marker in plants.

In a similar study the effect of chilling was analyzed in rice roots after seedlings were exposed to 10°C for 24 and 72 h [64]. Proteins were PEG-fractionated to obtain high resolution 2-DE gels with an increased number of discrete spots. Thirty-seven protein spots showed differential accumulation after low temperature exposure and 27 proteins were identified by MS. The majority of proteins identified were involved in energy production and metabolism. Despite using PEG-fractionation during sample preparation, only one protein identified as being regulated by cold stress was considered to be low in abundance.

Recently, Komatsu *et al.* [63] looked at not only the protein abundance changes in cold stressed plants, but also the effects cold stress on *N*-glycosylation patterns. This was determined by lectin blot analysis where proteins that were separated by 2-DE were probed with the plant lectin, concanavalin A, which was conjugated with peroxidase. In this study, proteins were extracted from the basal parts of the leaf sheath of 2-wk-old seedlings that were exposed to 5°C for 48 h or from plants that were cold stressed and allowed to recover for 24 h. Initially, 12 protein spots displayed a change in lectin reactivity after cold stress however, upon comparison with coomassie stained 2-DE gels, this figure was reduced to seven as the increase or decrease in lectin reactivity was correlated with a respective change in protein abundance. Cold-responsive glycoproteins, two of which could not be identified, were identified by Edman degradation or PMF. The majority of proteins that were affected by cold stress were considered to be associated with energy production. To further investigate post-translational modifications in cold stress responsive proteins, 2-DE gels were exposed to [γ-^{32}P]ATP to study phosphorylation events. Two calreticulin protein spots were identified; one spot was shown to be phosphorylated by cold stress while the other spot was dephosphorylated. Both spots were identified as being glycosylated but did not change in abundance.

5.2 Cold stress in Arabidopsis

Arabidopsis is the first plant genome that was sequenced and is important for plant research as a model plant for dicotyledons. Numerous studies have been carried out to understand the proteomic responses of Arabidopsis to cold temperature stress. A study investigating the response of Arabidopsis plants to cold temperature stress for a prolonged time subjected 5-wk-old Arabidopsis plants to cold temperature stress (6 and 10°C) for 1 wk [109]. Following treatment, plants were transferred back to control conditions (20/18°C day/night) for a further week. Proteins extracted from rosette leaves of control and treated plants were analyzed using 2-D DIGE. In plants grown at 6°C, at least 22 spots showed a twofold change in expression when compared with the control. Out of these 22 protein spots, 18 were up-regulated while four were down-regulated. Further, 14 out of these 22 spots were identified by PMF and ESI-MS/MS, and three of them were dehydrins, a subset of late embryogenesis abundant proteins. Dehydrins are believed to play a protective role during dehydrative stress conditions. In plants grown at 10°C, 18 proteins showed differential expression, with 16 up-regulated and two down-regulated. Cellular reactions after recovery from cold stress were also studied, and revealed that most of the spots that showed protein accumulation under cold stress treatment returned to levels observed under control conditions.

Studies have also been conducted to analyze the effect of cold temperature stress at the subcellular level. The nuclear proteome of Arabidopsis was characterized and its response to cold temperature stress was analyzed [59]. Plants were grown at 22°C for 3 wk and then subjected to cold stress at 4°C for 6 h in the dark. Nuclear protein extracts were analyzed using 2-DE and PMF. A total of 184 proteins were identified from the nuclear proteome and 54 showed at least a twofold change in expression in response to cold stress. Of these 54 proteins, 40 were up-regulated while 14 were down-regulated. Out of the 40 up-regulated proteins six were further examined by Northern blot analysis. These proteins were involved in protein synthesis, RNA metabolism, protein folding and transcriptional regulation. RNA analysis of these six proteins in a time-course experiment revealed that the RNA accumulation pattern was different in comparison to the protein expression observed after 6 h of cold treatment.

The response to cold temperature stress of the proteomes of chloroplast lumen and stroma of Arabidopsis have also been examined [110]. Arabidopsis plants were grown at 23/18°C (day/night) for 30 days before being transferred to a stress temperature of 5°C. Leaves were harvested 1 day (cold shock), 10 days (short-term acclimation) and 40 days (long-term acclimation) after cold stress treatment. The two soluble chloroplast proteomes of the stroma and lumen were analyzed by 2-D DIGE. The stromal proteome did not show any major changes after 1 day of cold treatment but 52 protein spots changed in abundance after 10 days of treatment. Of these, eight were newly induced and 31 increased in abundance while 13 decreased. After 40 days of cold treatment an additional 23 protein spots changed in abundance while two were newly induced. Changes in abundance in the stromal proteome were classified into three groups: (i) proteins responding to cold shock that increased in abundance after 10 days of cold treatment but returned to control levels after 40 days of cold treatment, (ii) proteins responding to long term cold acclimation that increased after 10 days of treatment and remained enriched even after 40 days of treatment, or only increased in abundance after 40 days of treatment and (iii) proteins that showed reduced abundance both after 10 and 40 days of treatment. A total of 35 proteins that responded during cold acclimation were identified in the stromal proteome. The lumenal proteome was less sensitive to cold temperature treatment in comparison to the response shown by the stromal proteome. While both proteomes responded similarly 1 day after treatment, only four lumen protein spots increased in abundance after 10 days of cold treatment. The major changes in expression in the lumen proteome were observed after 40 days of cold treatment. A total of eight proteins were identified which showed a twofold change in abundance during cold acclimation. Long term acclimation changed both lumen and stromal proteomes but, in general, new proteins appeared in the lumen while protein abundances changed in the stromal compartment. Altogether this study identified 43 differentially expressed proteins in stroma and lumen which participate in photosynthesis, other plastid metabolic functions, hormone biosynthesis and stress sensing, and signal transduction.

5.3 Cold stress in woody plants

The majority of temperature stress proteomics studies have been performed with herbaceous plants. Members of the genus *Populus* are used as model organisms for molecular biology studies in trees, are valuable for their wood, are ecologically important and grow in temperate forests. When temperate plants are exposed to low temperatures metabolic changes occur that allow the plants to withstand chilling stress. The subsequent ability of the plant to endure freezing temperatures is known as cold acclimation. Renaut *et al.* found that after 3-month-old Poplar plants were exposed to 4°C for 14 days, plants were able to tolerate freezing temperatures as low as −9.8°C compared to non-acclimated plants that were only able to withstand temperatures of −5.7°C [111]. Exposure to 4°C did not affect plant survival; leaf necrosis was not observed, but cessation of growth occurred. Proteomic analysis was used to analyze the nature of molecular changes in leaves during this period of cold acclimation.

Over 800 leaf protein spots were reproducibly visualized on 2-DE gels with 60 spots showing significant changes in abundance following cold stress. Using PMF, 26 proteins were identified with homologous proteins from a composite database of *Viridiplantae* sequences as the *Populus* genome had not been sequenced at the time of publication. Similar to cold stress responses in herbaceous plants, Poplar responded to low temperature by up-regulating the expression of proteins associated with ROS defense, such as the detoxifying enzymes ascrobate peroxidise, thioredoxin and peroxiredoxin. In response to low temperature, a large proportion of up-regulated proteins (10 out of 21) were found to be molecular chaperone-like proteins involved in protein folding and stabilization. Other proteins identified as being cold stress responsive were involved in signaling and water stress. Although many of the protein spots could not be identified due to database limitations, this was not a hindrance when compared to other studies where few spot identifications were made in spite of a complete genome sequence being available. In contrast to many other 2-DE gel studies, all of the spots that displayed differential expression were identified by MS in a study focused on shade-avoidance in tomato plants, even though a genome sequence was not complete [45]. Poplar now has a fully sequenced genome and proteomic studies on this species should be easier in the future [112].

A recent study of cold stress responses in woody plants was conducted using the bark tissue of peach (*P. persica* L. Batsch) plants. In woody plants, cold acclimation is usually initiated by low, non-freezing temperatures accompanied by short photoperiod, so 1-year-old peach trees were exposed to 5°C with 8/16 h (day/night) cycles for 5 wk. 2-D DIGE was used to examine the response of proteins in bark tissue to either low temperature or day length, and also a combination of these treatments. The majority of proteins identified by PMF were associated with low temperature alone or in association with low temperature, indicating that temperature had a greater affect on the physiology of peach tree than photoperiod. Proteins that increased in abundance following cold stress were functionally classified as being involved in stress response, lignin metabolism, glycolysis, amino acid metabolism, protein catabolism, or acting as chaperones. Proteins that were down-regulated in response to low temperature were involved in plant hormone response, cytoskeletal organization, defense mechanisms and photosynthesis.

5.4 Cold stress in other plant species

Mitochondria of pea (*Pisum sativum)* have been analyzed to study the proteomic effects of three common stresses in plants: low temperature, drought and herbicide application [113]. Mitochondria were isolated to focus analysis on oxidative stress damage; cold and drought treatments are mild causes of oxidative damage while herbicide treatment is known to cause irreversible oxidative stress. Ten-day-old plants were exposed to 36 h of 4°C, restricted access to water for 7 days or treated with the herbicide paraquat for 12 h prior to harvesting. Leaf mitochondrial protein were separated by 2-DE and 33 protein spots were found to be reproducibly changed by at least one treatment. All but one protein were identified by MS and three proteins were found to be chloroplast contaminants. Proteins were categorized as belonging to five different groups: (i) glycine decarboxylases and serine hydroxymethyltransferases, (ii) Tricarboxylic acid cycle proteins, (iii) oxidative phosphorylation complexes, (iv) HSPs, and (v) miscellaneous proteins. Many protein identifications were found to be degraded proteins and this was supported by a decrease in abundance of corresponding intact proteins. Interestingly, group (i) of proteins were particularly susceptible to degradation in cold-stressed leaves compared to drought-affected plants. Drought stressed leaves showed an increase in induction of defense-related proteins rather than displaying damage to proteins.

Mung bean (*Vigna radiata*) epicotyls were studied to analyze the effects of brassinosteroid (BR) treatment in combination with cold stress [114]. A gene from mung bean (*CYP90A2*) had previously been found to be strongly suppressed by low temperatures and was found to have an involvement in BR synthesis. The authors hypothesized that treatment with BR could allow plants to recover from the growth-inhibiting effects of cold stress. Seedlings were germinated for 5 days, sprayed with EBL (a biologically active BR) and transferred to a 28 or 10°C chamber for 3 days. Epicotyl growth was enhanced by EBL treatment at both 28 and 10°C when compared with non-treated plants and was more effective than other phytohormones tested. To assess which proteins had a recovery effect after BR treatment and cold stress, proteins from epicotyls were analyzed by 2-DE. Seventeen protein spots that were down-regulated by cold stress were re-up-regulated with EBL treatment while one protein remained repressed. Ten proteins that recovered after cold stress as a result of EBL treatment were identified and found to be involved in cell growth, wall formation, ATP production, stress response and methionine assimilation. Three proteins were identified as belonging to methionine assimilation pathways. Intriguingly, exogenous application of methionine also promoted epicotyl elongation in control conditions and during cold treatment of 10°C.

A summary of commonly occurring proteins that were altered in abundance in response to low temperature stress in various studies is presented in Table 3.

6 Heat stress

Heat stress is one of the main abiotic stresses that have a major impact on the yield and quality of crops in many parts of the world. Global mean temperature is increasing with time and thus studying how plants respond to high temperature stress at the protein level is important to develop heat tolerant cultivars. To this end, several studies have been carried out that include a variety of different designs of heat stress experiments, which vary even among studies on a particular species. The experimental designs vary according to the species and tissues studied, the temperature treatment and duration, the age of the plant, and the control plants which the treated plants were compared with. Proteomic responses to heat stress have been studied on cereals such as rice, wheat and barley, and non-cereal species such as Arabidopsis, *Populus euphratica* and Norway spruce (*Picea abies*).

6.1 Heat stress in rice

Heat stress response studies have been performed on rice (japonica rice, cv. Dongjin) leaves [9] and caryopses [10]. In the former, 2-wk-old rice seedlings grown at 28°C for 2 wk were subjected to a temperature of 42°C for 12 h or 24 h. Proteins extracted from leaves were pre-fractionated with 15% PEG in order to identify low abundant proteins. Proteins in the PEG supernatant and the pellet were then analyzed separately by 2-DE. When both PEG supernatant and pellet fractions were considered, 73 protein spots were differentially expressed at one time point or more. Thirty-four of these were from the PEG supernatant fraction while 39 were from

Table 3. Proteins differentially expressed in response to cold stress

Name	Species – tissue	Reference
Proteins up-regulated in response to cold stress		
2,3-bisphosphoglycerate-independent phosphoglycerate mutase	*Arabidopsis thaliana* – leaf, *Oryza sativa* – anther, leaf	[12, 104, 109]
20S proteasome (α subunit B, β-6 subunit)	*Oryza sativa* – anther, *Prunus persica* – bark	[44, 105]
Aconitate hydratase	*Oryza sativa* – leaf, root	[64, 106]
Adenylate kinase A	*Oryza sativa* – anther, root, leaf	[12, 39, 105]
Ascorbate peroxidase	*Oryza sativa* – anther, leaf, *Populus* spp. – leaf	[12, 104, 107, 111]
ATPase (α subunit, -like protein, T517 family)	*Arabidopsis thaliana, Oryza sativa* – leaf	[12, 59, 106]
ATP synthase CFI (α, β, δ, ε, γ chains)	*Arabidopsis thaliana*, *Oryza sativa* – leaf	[12, 106, 110]
Calreticulin	*Oryza sativa* – basal leaf sheath, root	[63, 64]
Catalase	*Arabidopsis thaliana, Oryza sativa* – leaf	[39, 110]
Chaperonin (60 kDa, 20 kDa, precursor, α and β subunits)	*Oryza sativa, Populus* spp. – leaf	[12, 106, 111]
Cysteine proteinase	*Oryza sativa* – root, leaf	[39, 107]
Cysteine proteinase inhibitor	*Oryza sativa* – anther, leaf	[12, 105]
Elongation factor (1 β′, Tu)	*Oryza sativa* – leaf, basal leaf sheath	[63, 106]
Enolase	*Oryza sativa* – leaf, basal leaf sheath	[12, 63]
Glutathione *S*-transferase	*Arabidopsis thaliana, Oryza sativa* – leaf	[106, 107, 110]
Heat shock protein (26a, 70, 90-like protein)	*Arabidopsis thaliana, Oryza sativa* – leaf, *Oryza sativa* – anther	[12, 59, 104, 106]
Isoflavone reductase	*Oryza sativa* – root, *Prunus persica* – bark	[44, 64]
Late embryogenesis abundant protein (10 kDa, drought-inducible)	*Oryza sativa*, *Populus* spp. – leaf	[107, 111]
Malate dehydrogenase (NADP, NAD-dependent)	*Arabidopsis thaliana, Oryza sativa* – leaf, *Prunus persica* – bark	[12, 44, 110]
Nucleoside diphosphate kinase 1	*Oryza sativa* – anther, leaf, leaf sheath	[39, 104, 107]
Oxalyl-CoA decarboxylase	*Oryza sativa* – leaf, root	[12, 106, 64]
Oxygen evolving complex protein (Photosystem II)	*Oryza sativa* – leaf	[12, 39, 106]
Phosphogluconate dehydrogenase	*Oryza sativa* – leaf, root	[64, 106]
RAB protein (2A, 24)	*Oryza sativa*, *Populus* spp. – leaf	[12, 111]
Ribonucleoprotein (29 kDa, U2 small nuclear)	*Arabidopsis thaliana* – leaf	[59, 110]
Ribosomal protein (40S S12E, 60S P0, P2-A, P2-B, P3-A, L12, protein-like)	*Arabidopsis thaliana, Oryza sativa* – leaf, *Oryza sativa* – anther	[12, 59, 105, 109]
RNA/mRNA binding protein (precursor)	*Arabidopsis thaliana, Oryza sativa* – leaf	[59, 106, 109]
Rubisco (large and small chains, precursor)	*Oryza sativa* – leaf	[12, 39]
S-adenosylmethionine synthetase (1, 2)	*Oryza sativa* – leaf, *Prunus persica* – bark	[12, 44, 106]
Thiazole biosynthetic enzyme	*Arabidopsis thaliana* – leaf, *Prunus persica* – bark	[44, 110]
Thioredoxin (*h*-type, *m*-type 1,)	*Arabidopsis thaliana, Oryza sativa*, *Populus* spp. – leaf	[12, 107, 111]
UDP-glucose pyrophosphorylase	*Oryza sativa* – anther, root, leaf	[39, 104, 106]
Proteins down-regulated in response to cold stress		
Ascorbate peroxidase	*Oryza sativa* – anther, leaf	[12, 104]
ATPase (α subunit, vacuolar β subunit, mitochondrial F1)	*Oryza sativa* – leaf, basal leaf sheath	[12, 39, 63]
ATP synthase (β subunit)	*Oryza sativa*, *Populus* spp. – leaf	[39, 111]
Calreticulin (precursor)	*Oryza sativa* – anther, basal leaf sheath, leaf sheath	[39, 63, 105]
Elongation factor (G, 1-β′)	*Oryza sativa* – leaf	[12, 39]
Heat shock protein (70, 70B, 90)	*Oryza sativa* – leaf, basal leaf sheath	[12, 39, 63]
Rubisco (large and small chains, precursors)	*Oryza sativa* – leaf	[12, 39, 111]
Rubisco subunit binding-protein α subunit	*Oryza sativa* – leaf sheath, basal leaf sheath	[39, 63]
UDP-glucose pyrophosphorylase	*Oryza sativa* – leaf sheath, basal leaf sheath	[39, 63]

the PEG pellet fraction. Of these 73 protein spots, 47 were up-regulated, 18 were down-regulated and 8 were newly induced after heat treatment. These differentially expressed protein spots were analyzed by PMF and 48 proteins were identified. Out of these 48 proteins, 18 were HSPs, which were exclusively identified from the PEG pellet fraction. HSPs are involved in protein stability and folding, indicating that protein denaturation and regulation are greatly affected by high temperature stress. This study revealed a group of newly induced low molecular small HSPs (sHSPs) including a low molecular weight mitochondrial sHSP. In addition, many other differentially expressed proteins were identified including proteins that related to energy production, photosynthesis, cell wall biosynthesis and stress related proteins. In summary the results of the study suggest that plants respond to high temperature stress in a complex manner and HSPs play a major role in the response.

Another study using two rice cultivars, indica type Taichung Native 1 (TN 1) and japonica type heat-sensitive Tainung 67 (TNG 67), analyzed the effect of high temperature on expression of proteins related to rice quality during caryopsis development [10]. Plants exposed to high temperature treatment were grown at 35/30°C (day/night) while the control plants were grown at 30/25°C (day/night). The method used for identifying proteins that were differentially expressed was 2-DE followed by LC-MS/MS. The results revealed that kernels of both cultivars had lower levels of an allergen-like protein under high temperature treatment as opposed to the control plants. TNG 67 produced lower amounts of an unknown protein and formed chalky grains under high temperature treatment. It also showed a lower level of elongation factor 1β under treatment in comparison to the control kernels 12 days after anthesis (DAA). Moreover, the results showed that the high temperature treatment increased the abundance of low molecular weight sHSPs but had little effect on the expression of high molecular weight HSPs. Further, glyceraldehyde-3-phosphate dehydrogenase, an enzyme involved in the glycolytic pathway, was increased in abundance in TNG 67 kernels at 12 DAA, while prolamine, a storage protein, was increased in expression especially in TN 1. The authors also investigated the relationship between sHSP expression and chalky endosperm using 10 cultivars that showed different levels of chalkiness. The results showed a positive correlation between the expression of sHSPs and chalky kernels because a lower level of sHSP expression was observed in so-called premium quality rice varieties than in chalky varieties.

6.2 Heat stress in wheat

Wheat is another important cereal on which several heat stress response studies have been carried out. These studies mainly focused on the grain because heat stress would cause deleterious effects on the dough quality if the grain experienced heat stress during the grain filling period. The effect of heat shock on the protein expression patterns of two cultivars, heat susceptible Wyuna and heat tolerant Fang, have been examined [115]. The aim of the study was to identify proteins associated with heat tolerance. Plants were grown at 24/18°C (day/night) during development and were subjected to 40/25°C (day/night) on 15, 16 and 17 days post-anthesis. Sampling of grains was done 17 DAA and at maturity (45 DAA). Differentially expressed proteins were identified by 2-DE, PMF and MS/MS. In addition to the proteome analysis the effect of heat stress on dough quality was also studied and it was found that heat shock caused weaker dough properties in Wyuna when compared to the control. The dough properties of heat tolerant cv. Fang on the other hand were not affected by heat shock. The proteomic results showed that Fang expressed more diverse as well as more abundant HSPs in comparison to the Wyuna. The majority of these proteins were identified as sHSPs. Furthermore, some isoforms of sHSPs showed differences in expression levels between the two cultivars. This study also identified some sHSPs uniquely expressed in Fang when subjected to heat stress conditions.

Two further studies have been carried out to examine the effect of heat stress on protein composition of hexaploid wheat during grain filling: one characterized the heat-responsive proteins from total endosperm [116], while the other characterized heat-responsive proteins from a non-prolamin fraction [66]. Plants used for the study were grown in field conditions until flowering. Control plants were transferred to 18/10°C (day/night) and the treatment plants were transferred to 34/10°C (day/night). Harvesting was done during the post-anthesis period, based on thermal time corresponding to the cumulative averages of daily temperatures. Proteins extracted from whole grains were analyzed by 2-DE and compared based on the spot volumes in order to identify the differentially expressed proteins in response to high temperature stress. Further, these proteins were identified by PMF and LC-MS/MS.

The study of the response of proteins from total endosperm identified 36 protein spots to be up-regulated and one spot to be down-regulated [116]. Among the heat induced proteins were several HSPs belonging to HSP90, HSP70 and sHSP families, other stress related proteins and enzymes involved in general cellular metabolism. The down-regulated protein was related to glucose-1-phosphate adenyltransferase, which is known to play a role in starch synthesis. In the study of the non-prolamin, or water soluble, fraction, 24 protein spots were up-regulated while 19 were down-regulated [66]. Among these proteins were sHSPs and a HSP belonging to HSP90 family, proteins involved in important metabolic pathways, granule-bound starch synthase and β-amylase, and other proteins related to plant defense, signal transduction, stress and translation. In this study glucose-1-phosphate adenyltransferase was identified to be down-regulated showing similar results to their previous study [116]. However, in addition several other proteins including ATP synthase β chain were down-regulated as well.

6.3 Heat stress in barley

Proteomic analysis of heat stress response has been performed on barley shoots [117]. Two barley (*Hordeum vulgare*) cultivars, Mandolina (abiotic stress tolerant, spring type) and Jubilant (abiotic stress susceptible, spring type), were used for the study. They were germinated and grown at 24°C and, after 5 days, half of each group was subjected to 40°C for 2 h in order to impose a short-term heat shock following which protein extraction was performed immediately. Analysis of proteins by 2-DE followed by PMF or LC-

MS/MS identified several proteins to be differentially expressed in both cultivars. Among them were distinct isoforms of several sHSPs. In addition, the expression of *S*-adenosylmethionine synthetase (SAM-S) was found to be up-regulated in cv. Mandolina compared to cv. Jubilant. Expression of SAM-S is known to increase under some stress conditions. Two sHSPs were unique to cv. Jubilant, the stress susceptible cultivar. One protein that decreased in abundance under heat stress was a 23-kDa oxygen evolving protein of photosystem II, which is important for photosynthesis.

6.4 Heat stress in Arabidopsis

Several studies that analyzed the proteomic response to heat stress have been carried out on Arabidopsis. The effect of a combination of drought and heat stress on Arabidopsis was studied recently [118], as it is one of the most frequently occurring stress combinations in the field. Plants were subjected to drought, heat or a combination of both. They were grown at 14/10°C (day/night) and were transferred to 42°C for 6 h for the heat treatment. Soluble proteins were extracted from plants and analyzed by 2-DE, and 45 proteins were identified that were unique to the combined heat and drought stress. These included enzymes involved in ROS detoxification, malate metabolism and the Calvin cycle. Interestingly, out of these 45 proteins, 16 were chloroplast proteins, which suggested that the chloroplast plays an important role in plant response to a combination of heat and drought stress. Further investigations were performed on the protein APX1, which accumulated in response to the combined stress but not under the individual stresses of heat or drought. This study involved nine APX transcripts and the results showed that transcripts of APX1 and APX2 accumulated in response to heat stress, whereas transcripts of APX1, APX2, APX3 and APX6 increased in expression under combined stress. When an APX1-deficient mutant (*apx 1*) was exposed to combined stress, it was found to be more sensitive than wild type to the stress combination. The results from the overall study suggested that cytosolic APX1 is an important enzyme in the process of acclimation to combined drought and heat stress.

Another recent heat-shock response study involved multiplexed quantitative proteomics using differential metabolic labeling [82]. Heat stress was imposed on 7-wk-old seedlings that were grown at 20°C by transferring to 38°C for 1, 2 and 4 h. In this study a number of known HSPs as well as some proteins previously not associated with heat shock were detected. For instance, NADP-malic enzyme and ribosomal protein L3-1 were found to be associated with heat stress for the first time.

6.5 Heat stress in other plant species

The mechanisms involved in survival under heat stress were examined in *Populus euphratica*, an ideal plant species for abiotic stress response studies as it can survive extreme temperatures, drought and salt stress [119]. In this study, plants were grown at 30/20°C (day/night) and subjected to heat stress at 42/37°C (day/night), both with 16/8 h (day/night) cycles. However, unlike in other studies, the change in temperature during stress period was applied gradually. Harvesting of mature leaves was done after 6, 30 and 54 h from the beginning of the heat stress treatment. Differentially expressed proteins were visualized by 2-DE, and identified by MALDI-MS and MALDI-MS/MS. Proteins were grouped based on their protein accumulation pattern and several distinct groups were observed. These groups were proteins that showed short-term up-regulation, long-term up-regulation, short-term down-regulation and long-term down-regulation. The results showed that short-term up-regulated proteins were related to membrane destabilization and cytoskeleton restructuring, sulfur assimilation, thiamine and hydrophobic amino acid biosynthesis, and protein stability. Proteins that remained up-regulated for longer periods were involved in redox homeostasis and photosynthesis. The results also suggested that protein synthesis was affected at early stages of heat stress but recovered afterwards. The only protein that showed long-term down-regulation was FtsH-like protein.

Protein expression of two ecotypes of Norway spruce was investigated in response to high temperature stress [120]. The importance of these two ecotypes (low elevation and high elevation) was that the high elevation ecotype showed lower levels of thermotolerance and higher levels of tolerance to oxidative stress compared to the low elevation ecotype. Seedlings of these two ecotypes were grown at 16°C and were subjected to heat stress when they were 2 months old. In the heat stress treatment, a 15-min pulse heat shock at 42°C was applied and it was followed by a 3-h recovery period at 16°C, after which 1 h heat-shock treatment was applied at 42°C. In this study plants were preconditioned with a short heat shock, allowing the plants to maximize their response to subsequent heat-shock treatment. Control plants and plants subjected to heat stress were harvested at four time points; immediately after heat-shock treatment and 5, 10 and 30 h after treatment. Protein analysis was performed using 2-DE and LC-MS/MS. The analysis of needles showed an accumulation of sHSPs during the recovery from heat stress, specifically in the low elevation ecotype (higher level of thermotolerance). In the high elevation ecotype glyceraldehyde-3-phosphate dehydrogenase was increased in expression during the recovery and decreased later.

Roots were examined in a heat stress study of two C_3 grasses that vary in thermal tolerance: *Agrostis scabra*, a geothermal grass species capable of surviving in soils heated at 45–50°C, and *Agrostis stolonifera*, a heat-sensitive grass species [121]. By contrasting the proteomic response of these two grasses to heat stress the authors aimed to dissect the mechanisms of superior thermal tolerance in *A. scabra*. Sixty-day-old plants were exposed to 30 or 40°C and

Table 4. Proteins differentially expressed in response to heat stress

Name	Species – tissue	Reference
Proteins up-regulated by heat stress		
ATP sulfurylase (APS)	*Agrostis scabra* – root, *Populus euphratica* – leaf	[119, 121]
ATP synthase (F1 mitochondrial β subunit, F1 chloroplastic α chain, vacuolar subunit B and E)	*Oryza sativa, Populus euphratica* – leaf, *Triticum aestivum* – grain	[9, 66, 116, 119]
Chaperonin (60 β subunit, precursor, 10)	*Oryza sativa, Populus euphratica* – leaf	[9, 119]
Elongation Factor EF-Tu	*Oryza sativa* – leaf, *Triticum aestivum* – grain	[9, 66]
Glutathione S-transferase (34, PM24)	*Agrostis scabra*, *Agrostis stolonifera* – root, *Arabidopsis thaliana* – leaf	[9, 82, 121]
Glyceraldehyde-3-phosphate dehydrogenase	*Agrostis scabra*, *Agrostis stolonifera* – root, *Oryza sativa* – caryopsis, *Populus euphratica* – leaf	[66, 119, 121]
GTP-binding nuclear protein RAN (1B, 2, small)	*Agrostis scabra*, *Agrostis stolonifera* – root, *Populus euphratica* – leaf, *Triticum aestivum* – grain	[66, 119, 121]
Small heat shock protein (precursor, isoforms, 17, 17.3, 17.4, 17.6A, 17.6B, 17.7, 17.9, 18, 18.1, 19.2, 21, 22 kDa, low molecular weight)	*Arabidopsis thaliana* – leaf, *Hordeum vulgare* – shoot, *Oryza sativa* – caryopsis, leaf, *Triticum aestivum* – grain	[9, 66, 82, 115, 116, 117]
Heat shock protein (66.2, 70, 70b, 79.5, 80, 82, 83, 90, cognate 80 kDa)	*Arabidopsis thaliana*, *Oryza sativa, Populus euphratica* – leaf, *Triticum aestivum* – grain	[9, 66, 82, 116, 119]
Heat shock protein, others (ClpB3, ClpB4, 101 kDa, class I, putative)	*Arabidopsis thaliana* – leaf, *Oryza sativa* – caryopsis, leaf	[9, 66, 82]
Thioredoxin *h*	*Oryza sativa, Populus euphratica* – leaf	[9, 119]
UDP glucose pyrophosphorylase	*Agrostis scabra* – root, *Oryza sativa* – leaf	[9, 121]
Proteins down-regulated by heat stress		
Adenosylhomocysteinase	*Agrostis stolonifera* – root, *Populus euphratica* – leaf	[119]
ATP synthase (beta and epsilon chains)	*Oryza sativa*, *Populus euphratica* – leaf, *Triticum aestivum* – grain	[9, 66, 119]
Methionine synthase	*Agrostis stolonifera* – root, *Populus euphratica* – leaf	[119, 121]
Peroxidase	*Agrostis scabra* – *Agrostis stolonifera*, root, *Oryza sativa* – leaf	[9, 121]
Pyrophosphatase (ADP glucose, inorganic)	*Hordeum vulgare* – shoot, *Oryza sativa* – leaf	[9, 117]

harvested after 2 and 10 days. Seventy protein spots showed differential accumulation in at least one species when analyzed by 2-DE. More spots were down-regulated as a result of heat stress, but *A. scabra* exhibited more up-regulated protein spots under both heat stress regimes. Of the 70 protein spots excised, 66 were identified by MS and most proteins affected by heat stress were categorized as being related to energy production. The two grasses displayed different proteomic profiles in response to heat stress. Proteins uniquely regulated by heat in *A. scabra* included sucrose synthase, superoxide dismutase, glutathione *S*-transferase, and stress-inducible HSP. This suggests that these proteins may contribute to increased survival of *A. scabra* under high temperature conditions.

A summary of commonly occurring proteins that were altered in abundance in response to high temperature stress in various studies is presented in Table 4.

7 Conclusions and future perspectives

In this review we have shown that a wide range of plant stress proteomics studies are currently in progress, using numerous methodologies, plant species and stress conditions. As more results are published, it is becoming increasingly clear that high temperature and low temperature stresses cause distinct molecular responses in plant tissues. High temperature stress response is characterized by an abundance of HSPs, while low temperature stress response is characterized by significant effects on chloroplast components, ROS detoxification, and energy production. As more data is generated in such studies, it provides suitable candidates for selective breeding programs aimed at enhancing stress tolerance in ecologically and economically important plant species.

Plant cells are fundamentally different to those of mammalian species, and these biological differences cause inherent difficulties in plant proteomics studies. Recent progress has overcome some of these obstacles and therefore plant proteomics studies are flourishing. This has also been aided by the recent completion of genome sequences of a number of additional plant species, with more expected in the near future.

Advances in techniques and approaches will change the way plant stress proteomics studies are conducted in future. The majority of studies in current literature are based on pairwise comparisons, typically involving 2-DE of protein extracts and exhaustive image analysis. The advent of quantitative shotgun proteomics approaches, whether based on stable isotope labeling or label-free counting, facilitates the design of studies involving comparison of multiple variables. This will allow more detailed dissection of stress-response pathways at the molecular level.

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