Ghasem Hosseini Salekdeh *Editor*

Agricultural Proteomics Volume 2

Environmental Stresses

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Editor
Ghasem Hosseini Salekdeh
Department of Systems Biology
Agricultural Biotechnology Research
Institute of Iran
Karaj
Iran

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Preface

According to FAO's estimate, the number of people suffering from chronic hunger has increased to over a billion.

Because most of the extreme poor who suffer from hunger live in rural areas, the effort to enhance agricultural productivity will be a key element to reduce the numbers of the global population suffering hunger.

This goal will not be achieved unless we develop new genotypes of food crops and animals that will both improve production under suboptimal conditions. The discovery of genotypes with the capacity to cope with these problems suggests that increasing the support of breeding for fragile environments is a viable strategy for uplifting the rural poor. However, breeding for environmental stresses is a slow and inefficient process. Although several genotypes with good stress tolerance to environmental stresses have been identified or developed, it is difficult to transfer these traits to elite backgrounds because they are genetically very complex. One possibility currently being evaluated for enhancement of stress tolerance is to apply biomarkers in breeding programs to follow the inheritance of major genes that are difficult to phenotype, such as pyramids of disease resistance genes of similar effect. Proteomics is a powerful approach to identify proteins associated with stress tolerance. It offers an entry point for identifying possibly significant changes in protein levels against a background of unresponsive proteins.

The application of proteomics is usually initiated by detection of stress-responsive proteins through the comparison of proteomics data between stressed and control organisms. Identification of these expressional candidate proteins may then reveal that some of them have functions clearly consistent with the stress tolerance trait. Other relevant information including the expression pattern of mRNA and the metabolomics may help to further verify the correlation of these candidate proteins with desirable traits. The step forward from collecting proteomics data to functional prediction will pave the way for the sustainable agricultural production under unfavorable environmental conditions.

This book will cover several topics to elaborate how proteomics may contribute to our understanding of mechanisms involved in stress adaptation. The knowledge

being accumulated through a wide range of proteomics technologies may eventually be utilized in breeding programs to enhance stress tolerance. This book presents a comprehensive review about the responses of crop and farm animals to environmental stresses. Challenges related to stress phenotyping and integration of proteomics and other omics data have also been addressed.

Karaj, Iran Ghasem Hosseini Salekdeh

Contents

Contributors

Jose B. Abucay, Jr., Genetics and Biotechnology Division, Plant Molecular Biology Laboratory, International Rice Research Institute, Metro Manila, Philippines; College of Arts and Sciences, University of the Philippines Los Baños, Los Baños, Laguna, Philippines

Pooja R. Aggarwal National Institute of Plant Genome Research, Aruna Asaf Ali Marg, New Delhi, India

Owen K. Atkin ARC Centre of Excellence in Plant Energy Biology, Division of Plant Sciences, Research School of Biology, Australian National University, Canberra, ACT, Australia

Brian J. Atwell Department of Biological Sciences, Faculty of Science and Engineering, Macquarie University, Sydney, NSW, Australia

Elena Bencurova Department of Bioinformatics, Biocenter, University of Wuerzburg, Würzburg, Germany; Laboratory of Biomedical Microbiology and Immunology, Department of Microbiology and Immunology, University of Veterinary Medicine and Pharmacy in Kosice, Kosice, Slovakia

Niranjan Chakraborty National Institute of Plant Genome Research, Aruna Asaf Ali Marg, New Delhi, India

Subhra Chakraborty National Institute of Plant Genome Research, Aruna Asaf Ali Marg, New Delhi, India

Sixue Chen Department of Biology, Plant Molecular and Cellular Biology, Interdisciplinary Center for Biotechnology Research, Genetics Institute, University of Florida, Gainesville, FL, USA

Thomas Dandekar Department of Bioinformatics, Biocenter, University of Wuerzburg, Würzburg, Germany

Mehdi Dastranj Department of Systems Biology, Agricultural Biotechnology Research Institute of Iran, Agricultural Research, Education, and Extension

Organization, Karaj, Iran; Department of Plant Protection, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran

Peter David Eckersall Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK

Mohammad-Reza Ghaffari Systems Biology Department, Agricultural Biotechnology Research Institute of Iran, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

Javad Gharechahi Chemical Injuries Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

Herra L. Grajo Genetics and Biotechnology Division, Plant Molecular Biology Laboratory, International Rice Research Institute, Metro Manila, Philippines; College of Arts and Sciences, University of the Philippines Los Baños, Los Baños, Laguna, Philippines

Mohammad-Reza Hajirezaei Department of Physiology and Cell Biology, Molecular Plant Nutrition, Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany

Yue Hao Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, Beijing, China

Ante Jerkovic Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, NSW, Australia

Mudassar Nawaz Khan Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Japan; National Institute of Crop Science, National Agriculture and Food Research Organization, Tsukuba, Japan

Ajay Kohli Genetics and Biotechnology Division, Plant Molecular Biology Laboratory, International Rice Research Institute, Metro Manila, Philippines

Setsuko Komatsu Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Japan; National Institute of Crop Science, National Agriculture and Food Research Organization, Tsukuba, Japan

Sheldon Lawrence II Department of Biology, Plant Molecular and Cellular Biology, University of Florida, Gainesville, FL, USA; Genetics Institute, University of Florida, Gainesville, FL, USA

Jianke Li Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, Beijing, China

Mohsen Mardi Department of Systems Biology, Agricultural Biotechnology Research Institute of Iran, Agricultural Research, Education, and Extension Organization, Karaj, Iran

Mehdi Mirzaei Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, NSW, Australia

Hans-Peter Mock Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Stadt Seeland, Gatersleben, Germany

Kanika Narula National Institute of Plant Genome Research, Aruna Asaf Ali Marg, New Delhi, India

Mohammad-Zaman Nouri Rice Research Institute of Iran, Mazandaran Branch, Agricultural Research, Education and Extension Organization (AREEO), Amol, Iran

Ehsan Oskoueian Agricultural Biotechnology Research Institute of Iran (ABRII), Mashhad, Iran

Jennifer Parker Department of Biology, Plant Molecular and Cellular Biology, Genetics Institute, University of Florida, Gainesville, FL, USA

Ghasem Hosseini Salekdeh Department of Systems Biology, Agricultural Biotechnology Research Institute of Iran, Agricultural Research, Education, and Extension Organization, Karaj, Iran

Andrew P. Scafaro ARC Centre of Excellence in Plant Energy Biology, Division of Plant Sciences, Research School of Biology, Australian National University, Canberra, ACT, Australia

Golandam Sharifi Department of Basic Sciences, Iranian Institute for Encyclopedia Research, Tehran, Iran

Hamid Sobhanian Department of Biology, Payame Noor Universtiy, Tehran, Iran

Jennylyn L. Trinidad Genetics and Biotechnology Division, Plant Molecular Biology Laboratory, International Rice Research Institute, Metro Manila, Philippines; College of Arts and Sciences, University of the Philippines Los Baños, Los Baños, Laguna, Philippines

Katja Witzel Leibniz Institute of Vegetable and Ornamental Crops (IGZ), Grossbeeren, Germany

Paul Worden Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, NSW, Australia

Yunqi Wu Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, NSW, Australia

Chapter 1
Well-Designed Experiments Make Proteomic Studies on Stressed Plants Meaningful

Brian J. Atwell

Abstract Analysis of the impact of abiotic stresses on plants is technically demanding. The cultivation of plants, application of treatments, choice of tissues and preparation of biological samples for proteomic analysis is as important as the subsequent identification of proteins. With appropriate precautions, proteomics will greatly improve our understanding of the mechanisms of abiotic stress tolerance. Hence, this chapter summarises some of the major design faults that can compromise the interpretation of 'stress experiments'. The examples of salt, drought, thermal stress and waterlogging are taken as representative of commonly encountered stresses, with recommendations for ways to avoid artefacts in design. The importance of interactions between these stresses is then discussed, pointing out the relevance of carefully constructed time courses and attendant physiological measurements to define the degree of stress. Tissue selection is also emphasised, recognising that stresses have differential impacts on different organs. Finally, the significance of choice of plant species is discussed, with recognition of the value of model species and the importance of expanding the range of taxa used if the full range of stress acclimation responses is to be identified through proteomics.

Keywords Experimental design · Abiotic stress

1.1 Introduction

Proteomic technologies have evolved rapidly in the past two decades, becoming an indispensable tool in the analysis of gene expression [1]. Because protein complements provide qualitatively different information from transcriptomes [2], proteomics will bring important new insights to plant phenomics under stress. However, the full extent of the disjunct between transcriptome and proteome is yet

B.J. Atwell (✉)
Department of Biological Sciences, Faculty of Science and Engineering,
Macquarie University, Sydney, NSW 2109, Australia
e-mail: brian.atwell@mq.edu.au

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to be revealed. Direct evidence for abiotic stresses modifying translation of mRNAs is scarce and deserves closer attention over a range of conditions. In hypoxic *Arabidopsis* plants, much of the mRNA population remains untranslated, leading to a proteome that is defined by the demands of the stressed cell [3].

In spite of great technical strides, the opportunities afforded by proteomics still have their limits, with detection of low-abundance proteins and post-translational modifications providing continuing challenges [2]. However, deep sequencing of DNA and extensive proteomic profiles are driving the concept of 'proteogenomics'—the marriage of proteomics with genomics to develop a deeper understanding of crop phenomics [4, 5]. Initial attempts will be based on the major, well documented crop species such as rice, from which they will extend to genetically complex species such as wheat and novel crop species.

This review does not set out to appraise these technologies but rather to analyse the methodology by which *biological samples are prepared* for subsequent proteomic analysis. Because 'agricultural proteomics' will make a major contribution to our understanding of the mechanisms of abiotic stress tolerance by quantifying gene expression levels under stress in high-performing hybrids [2], special care is required to avoid flawed experimental practices that could compromise interpretation of data and their application to breeding and targeted gene transfer. The sections that follow dissect the physiological, developmental and genetic factors that influence the results of gene expression analyses. They specifically address experimental design, particularly time courses of experiments and informed sampling of biological tissues from plants. Cautionary themes are presented under three headings (experimental design related to specific abiotic stresses, time frames and sampling). All three themes should be taken into consideration during the production of biological samples for proteomic experiments.

1.2 Designing Experiments to Mimic Abiotic Stress Observed in the Field

The environmental hazards that restrict agricultural productivity are either climatic (e.g. drought, salinisation, frost, light imbalance), chemical (e.g. inorganic nutrition, salt, herbicide residues) or biotic (invertebrate, fungal or bacterial attack). This section deals with the appropriate design of experiments required to mimic four of the major abiotic stresses on crops—salinity, drought, temperature and waterlogging. Through the precise application of these stresses in controlled conditions, we can gain confidence in proteomics as a tool to inform the genetic improvement of our major crop species. With sophisticated hardware (e.g. well-lit environmental cabinets) and software (e.g. ramping of conditions rather than simple day/night settings) now available, experimentalists can nuance the application of abiotic stress in a way not previously possible. Thereby, temperature, light, humidity and inorganic nutrition can all be very closely aligned with field observations.

Abiotic stresses do not act on plants independently—they interact, as we see at the tissue and cell level. This interaction is manifested in the phenotypic responses that we observe in experiments. For example, drought and salinity are mechanistically connected, with salt affecting land plants by perturbing cell water relations, as well as via the toxic effects of ions on cell metabolism. Therefore, 'osmotic drought' caused by salinity is likely to have gene expression responses in common with 'hydraulic drought', which is caused by soil water depletion, low air humidity and/or high wind speeds. However, longer term changes in the proteome will be specific to the toxic effects of sodium and chloride and will be manifested in expression of ion transporters that are required for compartmentation and efflux. Yet, surprisingly, many publications claim to reveal gene-level responses to salinity without designing experiments to discriminate between the dual effects of water relations and toxicity. If proteomics is to be effective, careful application of treatments (in this example, salt), time courses and environmental conditions must all be managed to lead us to the most likely explanation at the cell level for the responses seen in crop species.

A further dimension is the choice of species for gene expression studies: this is inseparable from the manner in which the stress is imposed, as seen in the specific examples referred to below. One must first look to the commonly used models such as *Arabidopsis*, *Chlamydomonas*, *Brachypodium*, *Nicotiana benthamiana* and the crop species *Oryza sativa* (monocotyledons) and *Medicago trunculata* (legumes) because these species have contributed so much to our knowledge of gene-level responses to abiotic stress. However, generalising observations from these model genotypes to abiotic stress effects in all commercial crops is fraught because of the specific adaptations that might characterise particular species (Fig. 1.1). For example, the 'minimalist' deep tap-root of the dryland legume lupin contrasts with the expansive fibrous root system of wheat, in spite of both achieving efficient water use in identical dryland field conditions [6]; it is likely that each species employs some unique drought resistance strategies. Similar contrasts in root architecture can be seen for wheat and sugar beet in NMR images [7]. Dicotyledonous crop species are especially under-represented in studies aimed at identifying genes that respond to abiotic stress.

In summary, it behoves all those in the thrall of the technologies used to study gene expression to expand the range of taxa and improve the experimental designs that too often compromise abiotic stress studies. This will have the effect of creating ever larger and more reliable databases being applied to biological samples that genuinely mimic the physical constraints to yield in field crops. Relative to genomics, proteomics is a nascent science whose impact will be far deeper with rigorous application of the stresses applied (e.g. levels of stress, time courses, interaction effects). Naturally, experiments on biological extracts will always yield a proteomic profile—the challenge is to identify those protein changes that meaningfully reflect the system in which the plant normally grows. Modern proteomics based on well-executed experiments could obviate many of the criticisms that could be levelled at some earlier microarray studies.

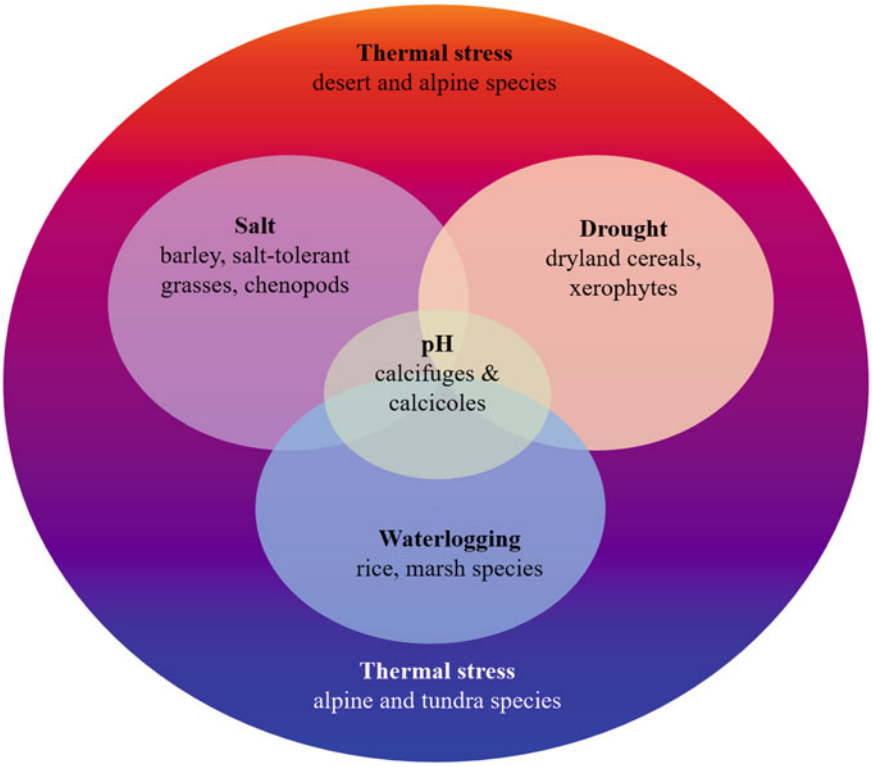

Fig. 1.1 Schematic to illustrate some ideal plant species or groups for proteomic studies on the range of abiotic stresses discussed in this chapter. The plant categories are not exclusive and the power of fully sequenced species (e.g. *Arabidopsis*, rice) as models for proteomic approaches is fully acknowledged. Calcifuges and calcicoles are plants naturally adapted to acid and alkaline soils respectively. Note that the effects of salt, drought and waterlogging interact and thus potentially produce unique proteomic responses. Extreme pH impinges especially on plants which are affected by other abiotic stresses. All these stresses are, in turn, subject to interaction with the experimental temperature regime

The following sections analyse the application of stresses individually and in combination, followed by the impact of temporal and spatial considerations in biological sampling.

1.2.1 Salt

Not uncommonly in the literature, sodium chloride is applied to plant roots in very high concentrations (100–200 mM), often in an instant, to mimic the effects of salinity on crop plants. However, only very rarely in nature is salinity visited on

crops by a sudden rise from salt-free to inundation with highly saline solution and these are circumstances in which crops generally perish because of tidal surges or tsunamis. Salinity damage in crops is more likely to be subliminal and characterised by the gradual accumulation of salts in transpiring organs (mainly leaves), with associated osmotic and toxic impacts possible [8]. Furthermore, even though sodium and chloride are the dominant ionic species in sodic soils, the distortion of normal soil chemistry means that other ions can be present in concentrations far from their optimum [9]. Moreover, calcium also plays a key role in maintenance of membrane integrity and therefore it should ideally be present at millimolar concentrations in saline solutions around roots to prevent generalised membrane dysfunction and unrestrained passive uptake of sodium [8, 10]. Similarly, other macronutrients (e.g. N, P and K) must be sufficient to maintain an adequate steady-state supply to roots, and bathing solutions containing only sodium chloride must strictly be avoided as a mimic for salinity.

A further consideration must also be the inherent salt tolerance of the test species. It is unlikely that species poorly adapted to salinity will have a concentration of novel tolerance genes. The identification of high-affinity potassium transporters (HKTs) in wheat has led to a diverse family of genes from other species that confer salt tolerance [11], underlining the importance of experiments on a broad range of species.

Arabidopsis has relatively low physiological tolerance to salt and yet concentrations of sodium chloride up to almost half that of seawater are sometimes applied to this species in the laboratory to mimic saline conditions. Such experiments are more likely to educate us about the cell senescence and death than salt tolerance. The inclusion of halophytes and salt-tolerant grasses in proteomic experiments will go a long way to realising the full benefit of gene discovery technologies. Barley is clearly a case in point, where genomic and proteomic databases have the potential to reveal insights into mechanisms of salt tolerance. Furthermore, true halophytes such as the chenopods (family Amaranthaceae) and halophytic algae offer the opportunity to discover novel salt tolerance genes that have been lost in most land plants [12].

Recommendation: Apply relatively low sodium chloride concentrations in the presence of a full nutrient complement that includes calcium over relatively long periods (days, not hours) as a standard approach. The use of plant species that have at least moderate tolerance to salinity should also be encouraged but true halophytes are likely to be most informative.

1.2.2 Drought

Drought imposes itself on plants through a succession of processes that occur well before the common symptoms of wilting and death are observed: these events normally take place over the course of days or even weeks [13]. Impaired growth and diminished yield are the ultimate result of sustained drought but the

physiological manifestations of withholding water (or dry atmospheres) are very complex. In those species which have been experimentally observed, acclimation to drought involves a suite of events in overlapping time frames—cell level changes associated with turgor loss are followed by accumulation of abscisic acid (ABA) and stomatal responses, and eventually, morphological adjustments such as thinner roots and altered root-to-shoot ratios [13]. With the benefit of many decades of information gathering on the expression of genes underlying these physiological observations, we now know that some drought responses are triggered directly by drought (e.g. ABA synthesis, biosynthesis of osmotic agents) while others are secondary or tertiary responses (e.g. accumulation of carbohydrates, senescence pathways, slower respiration).

Severe water deficits can be imposed effortlessly by removing a leaf from its parent plant, with wilting generally following quickly: naively, such an approach is sometimes thought to mimic drought. However, rapid dehydration involves little more than hydraulic shock and stomatal closure, with the more subtle adjustments to cell walls, hormone levels, tissue hydraulics and osmotic changes all masked. Thus, gradually withholding water is essential to elicit the full gamut of drought responses [13, 14] and therefore, to see changes to the proteome that represent all the acclimation processes in droughted plants. This is generally best achieved by using large soil volumes relative to plant size (see [7]), allowing soil water either to be depleted slowly [15, 16] or addition of very small volumes of water daily, enabling leaf hydration as plants acclimate to sub-optimal water supply [17, 18].

It is valuable in the analysis of abiotic stresses, including drought, to return plants to the non-stressed state by re-watering. Because re-watering immediately rehydrates plants, the pattern by which the proteomic profile returns to resemble that in continually watered plants can be re-assuring because the initial impacts of drought are likely to be reversed quickest (e.g. full cell hydration). For example, this might be seen in reduced levels of stress-inducible proteins [15]. Alternative approaches to manipulation of the proteome in response to drought ought to be employed where appropriate, including the classical split-root experiments [19]. This can effectively separate signals coming from a source (drying roots) from the hydraulic effects of drought in shoots. Another common technique used to impose drought is to add a non-permeating osmotic solute such as mannitol or polyethylene glycol to the root medium. While this achieves dehydration osmotically [20] it is unlikely to replicate the far more subtle acclimation responses of a true drought and can be hydrolysed and/or taken up by plant cells [21].

Recommendation: Drought is distinct from tissue dehydration and is most often imposed slowly in nature, leading to a wide range of acclimation responses. Therefore, in experiments drought should be mimicked by allowing plants to transpire water from large soil volumes. The effect of drought on gene expression can be further elucidated by re-watering to reverse the drought or splitting root systems into dry and wet compartments.

1.2.3 Thermal Stress

Many of the world's major crops grow and develop at temperatures outside the optimal diurnal range (say, 20–28 °C). While heat stress has frequently in the past been dismissed as little more than a subordinate of drought stress, it is a distinct phenomenon and in irrigated crops in the humid tropics, is likely to occur independently of drought. As with drought experiments, the artificial imposition of heat (and chilling) should be done using regimes that are guided by data from the field, such as those available from thermal loggers or meteorological observations. The imposition of drought and heat reported by Ashoub et al. [16] conforms to these general principles, with stress applied in graduated regimes. In that changes in the expression of stress-responsive genes are seen when temperate species are exposed to temperatures in the low thirties [22], extreme temperatures should only be imposed when justified by the habitat of the experimental species. Arguably, the most important metabolic changes occur within 5–10 °C of the optimal temperature range.

Similarly chilling must be imposed within physiological boundaries that are defined by field conditions, and at a rate that is plausible. Accordingly, chilling should be increased over timeframes of hours (simulating phenomena such as frost damage) or in some cases imposed over a period of days, as required for frost hardening in much colder environments [23]. Localised chilling of organs (e.g. roots) can be used to elicit release of mobile signals that trigger a change in the proteome of remote organs such as shoots [24]. Such an approach exploits proteomics to reveal the identity of either heat- and cold-inducible long-distance signals but has limited relevance to field plants outside those where rapid atmospheric heating accompanies evaporative cooling at the soil surface (e.g. irrigated rice in hot savanna).

Artificial growth conditions such as atmosphere-controlled glasshouses and growth cabinets have the capacity to heat and cool plants over a huge range in just minutes, further necessitating stepwise changes in temperature as a new steady-state is established. Ignoring the need for temperature ramping leads to experiments that measure how gene expression responds to thermal shock and provides no insights into acclimation to temperature shifts.

Recommendation: Impose heat stress by stepwise increases in temperature, generally during the daytime, and in accordance with the natural range of temperature stress that is likely to be experienced. Chilling should also be imposed gradually unless it is aimed at simulating sudden events such as frost in unhardened plants.

1.2.4 Waterlogging

As with drought, plants undergo a series of chemical, metabolic and structural changes during acclimation to flooding, with the primary impact being on roots, contrasting with impacts on shoots during drought and atmospheric fluctuations. The importance of care in the choice of tissues to be sampled for proteomics will be addressed in detail below (see '*The importance tissue sampling*').

Changing the oxygen supply to tissues abruptly is known to cause damage and even death of cells, especially in root apices, which are most metabolically active [25]. These authors showed that in the absence of internal ventilation in the form of aerenchyma, even flood-tolerant species such as rice are unable to withstand anoxia. In testing the effect of anoxia on plants, hypoxic pre-treatment is strongly recommended to alleviate damage from 'anoxic shock' (see [26]) as this qualitatively changes the tolerance of vulnerable tissues such as maize roots to anoxia [27]. The dissection of what constitutes shock versus steady-state stress is discussed in the final section.

Some experimenters advisedly test the recovery from low-oxygen stress by re-establishing aeration. However, just as the switch from normoxia to anoxia is very damaging, abrupt increases in oxygen supply to tissues are potentially deleterious, in this case because of the inadequacy of oxidative reactions to consume available oxygen, and subsequent release of deleterious reactive oxygen species [28]. Therefore, recovery treatments need to be applied with care, probably by hypoxic post-treatment.

Paradoxically, plant organs (e.g. roots, rhizomes) of highly flood-tolerant species largely owe their survival in low-oxygen environments to a system of aerenchyma which ventilate cells and re-supply surrounding medium with oxygen. This adaptation is highly developed in species such as rice and over-wintering wetland plants [29]. Furthermore, the rate at which oxygen diffuses out of roots varies with genotype [30]. Thus, while anoxia can be imposed on the root medium, the actual oxygen status of individual root zones from different genotypes might not be comparable at the time that they are sampled for proteomics because oxygen transport into these root systems varies with the proportion of aerenchyma and oxygen leakage rates [28]. This is particularly true for the stele of roots, which can be anoxic while the surrounding cortex is hypoxic [31]. Disparate anoxia tolerance in the dimorphic root systems of grasses [32] adds a further dimension that must be taken into account during sampling. Such subtleties require careful consideration and while in general, excision of organs should not be the first choice, there is a case where the confounding effects of long-distance transport of oxygen (or carbohydrates) make interpretation of data difficult in intact systems [33].

Choice of species is especially critical when probing the proteome of roots because some species are relatively tolerant to hypoxia/anoxia, while others are so intolerant that even hypoxia can kill them or at the least, inhibit all function [28].

This contrast is particularly pertinent when the pre-eminent plant model species (*Arabidopsis* vs. rice) represent extremes of tolerance to low oxygen, calling for low-oxygen treatments that recognise these tolerances. Broader taxonomic contrasts that include poplar and algae as well as *Arabidopsis* and rice, have been employed to identify common transcriptional responses [34] and similar metabolomic and expression profiles have also compared poplar with rice and *Arabidopsis* [35]. With gene expression having been studied in so few of the plant species which are adapted to marshes, wetlands and waterways, there is a powerful case for quantitative proteomics that encompasses more species and diverse oxygen treatments.

Anoxia severely impairs protein synthesis, even in rice seedlings [36] because most of the energy generated is used to synthesise new proteins [37]. It follows that tissues exposed to anoxia for short periods will reveal a proteomic profile dominated by proteins that were present prior to the low-oxygen treatment: this is obviously to be avoided. To discriminate the synthesis of novel proteins during the low-energy, low-oxygen period, quantitative proteins (e.g. enrichment of ^{15}N in proteins that were synthesised from labelled exogenous ammonium or amino acids) is a better approach [38].

Finally, the microbial populations that inhabit the rhizoplane of root systems that are not grown axenically are substantial; microbes have high protein concentration per unit biomass and rapid turnover rates [39]. These prokaryotic populations are clearly a confounding factor in proteome analysis and must be either eliminated or suppressed if the true root proteome is to be considered in gene expression studies. The advent of quantitative proteomics makes this even more pressing because the rates of incorporation of labelled precursor amino acids or ammonium into the microbial proteome will be so much faster that into the roots.

Recommendation: Lower (or raise) oxygen concentrations around root systems in one or more steps through the hypoxic range over at least 24-h periods in order to avoid tissue death and oxidative damage when anoxia (or normoxia) are reached. Roots should be sampled for proteomics with a clear knowledge of the actual oxygen status of the intact tissue, as well as its inherent tolerance to anoxia, developmental stage and the microbial populations that reside in the rhizosphere.

1.3 Managing Interactions Between Abiotic Stresses

Preceding sections describe how best to apply *individual* stresses to plants. However, appreciation of the more complex question of interacting abiotic events is also vitally important because the impact of one stress can exacerbate, or ameliorate, that of a second stress [40, 41]. Such interactions can be entirely abiotic, i.e. physical events external to the plant such as high temperature exacerbating oxygen deficiency. In reverse, low soil temperatures reduce root and microbial respiration and alleviate damage from waterlogging [42].

While the physiological manifestations of abiotic interactions might be obvious, there is far less certainty about the proteomic changes that are triggered as part of

the biological response. Suzuki et al. [41] refer to signalling pathways that are common to particular stress combinations. As post-transcriptional modifications (e.g. RNA processing, protein phosphorylation) are revealed, greater complexity will necessarily be added to the gene expression patterns that are observed in response to interacting stresses [40].

A few common examples of stress interactions are listed below. This is not an exhaustive catalogue—see Suzuki et al. [41] for a more complete listing—but is an indication of some abiotic stresses that interact in a non-additive manner. While the impact of these interactions cannot be predicted at the gene or protein level, they should be foreseen using extensive knowledge of the whole-plant responses documented [43].

Drought and heat: Ambient temperature can exceed the actual leaf temperature by many degrees because of transpirational cooling [44, 45]. Thus, experimental protocols should take *actual* leaf temperature into account when assessing the impact of heat on leaves. The phenomenon of leaf 'self-cooling' adds complexity to the heat × drought interaction, with leaf temperatures rising close to the ambient atmospheric temperature as transpiration rates fall but the impacts of drought lessening as water losses are constrained by stomatal closure.

Drought and salinity: The introductory section raises a classical example of the complexity of salinity stress, where the dual impacts of hydraulics and toxicity can operate on separate time courses. To some degree, osmotic effects (leading to compromised hydraulics) and cell-level toxicity can be partly managed by sampling over rigorous time courses after stress application. For example, hydraulic effects become evident within minutes of adding salts to the root medium, with lower root water potential being transduced to the xylem, and subsequently the leaves [46]. Over a longer time course, salts can accumulate in the cell walls of leaves in non-halophytes, hastening the dehydration of mesophyll cells and initiating necrosis. Some of these salts are taken up by leaf cells, triggering biochemical and metabolic responses that are ultimately deleterious in the absence of compartmentation [47]. This chronological series of events is likely to elicit shifts in the proteome, with each tissue sampled minutes, hours, days and weeks after salinisation producing qualitatively distinct protein profiles. Well-designed experiments require time-course measurements of water and ionic status of tissues and aligning these data with the proteome at each time point. The proteomes of control plants should be reported alongside tissues of treated plants.

Temperature and low oxygen: Oxygen status is strongly dependent on temperature, with high temperature reducing soluble oxygen concentration and raising respiration rates, thus exacerbating the effects of inundation. However, this example amply reinforces the importance of time as an interacting factor with multiple stresses, with plants of the same *physiological* age not exhibiting a temperature × oxygen interaction while those of the same *chronological* age showed increased damage at high temperatures [48]. It is clearly a requirement that experiments on low oxygen responses in roots take careful account of temperature,

developmental age and tissue type (see '*The importance tissue sampling*'). It is established above that temperature shifts produce major qualitative shifts in the proteome of rice leaves and cultured cells [49, 50] and low oxygen concentrations also cause a highly characteristic expression of anaerobically induced genes [51, 52]. However, the interaction of abiotic factors with oxygen supply must always be carefully considered if the full impact of stresses is to be revealed at the protein level. The best example of such an interaction comes from Waters et al. [53], who measured recovery of growth in wheat root apices as a way to assess the interaction of the various abiotic factors with oxygen deficits. Notably, root tip mortality rose dramatically as temperatures were increased from 15 to 25 °C, pH was lowered from 6 to 4 or carbohydrate supply was restricted, illustrating the importance of careful control of experimental conditions.

Low pH and various abiotic stresses: As shown above, oxygen deficits compromise the energy status of cells and in a low pH bathing medium, cell function is further impaired through cytoplasmic acidification [54]. Because regulation of proton transport, membrane potential and potassium retention have such profound implications for cell function [55], the protocols used when any abiotic stress is applied must take careful account of external pH. Moreover, as proteomics expands to tackle field-scale agricultural questions, the large range of pH observed in the natural environment must be considered, particularly for plants growing in the acid soils of many modern agricultural systems. External pH must be managed carefully in the laboratory, where acidification of the bathing medium around plant tissues is a risk if the volume of bathing solution is low and inadequately buffered.

The availability of metabolic energy lies at the core of the interaction between abiotic stresses and low external pH [55]. Specifically, metabolic energy is used to maintain membrane potential in living cells below −100 mV by extruding protons across the plasma membrane and tonoplast. Therefore, any abiotic factor that compromises ATP availability (e.g. anaerobiosis, thermal stress, phytotoxins) is likely to reduce cell membrane potentials and trigger the release of common stress sensors such as reactive oxygen species and Ca^{2+} [55]. These events are further amplified by acidification of the external medium because the free energy required for proton extrusion increases as the proton gradient becomes less favourable [56]. The expression of genes under these stress conditions is often coordinated by a series of transcription factors (e.g. AP2/ERF, B3, NAC, SBP and WRKY), many of which are common to multiple stresses such as cold, anoxia and dehydration (see [52, 57]). Transcription factors activate DNA-binding domains and trigger the transcription of a large array of proteins. Hence abiotic events, especially in combination with acidic conditions, will necessarily result in distinctive proteomes. One would expect that in acute stress, proteins typical of programmed cell death would be commonly observed [58]. It is therefore critical to control experimental conditions and the composition of bathing media very closely.

1.4 General Principles for the Design of 'Stress' Experiments

Two general principles should guide the design of experiments aimed at identifying the key processes in plant acclimation to abiotic stress—time and space. In short, one must first select a time course for the application of stress and recovery from it, compatible with the synthesis of proteins that are necessary for acclimation. Second, tissues which are sampled must be sufficient to provide a credible proteome but homogeneous enough to represent a tissue-specific response. This section is aimed at enunciating these general principles.

1.4.1 The Importance of Time

Decisions on time courses should be influenced by the intensity of stress and the rate of its imposition (Fig. 1.2). This should be guided as much as possible by whatever physiological literature is available for similar genotypes under the same stresses. For example, microarray data can be helpful in defining a physiologically meaningful time course for sampling tissues [59]. In this context, the general observation that protein turnover in plants has a half-time of 1–2 days [38] is germane; abiotic stresses applied for less than one day are unlikely to achieve a new steady state, with the proteome 'contaminated' with proteins that were present prior

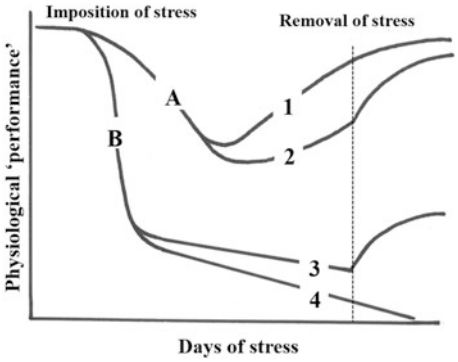

Fig. 1.2 Conceptual figure to show potential time courses of plant response to an arbitrary abiotic stress. Hence the y-axis is labelled *Physiological 'performance'* to indicate a process such as growth, development or, not of a metabolic function (e.g. photosynthesis). Scenarios *A* and *B* depict a mild and acute response to the stress; plants in Scenario *A* are likely to have been pre-conditioned to the stress (e.g. hypoxia prior to anoxia) while Scenario *B* is a shock treatment. After the physiological effects take hold, plants respond in at least four ways: (*1*) almost complete acclimation; (*2*) partial acclimation followed by rapid recovery after removal of stress; (*3*) severe decline under stress but not death—recovery on removal of stress; (*4*) irreversible damage and death—no recovery on removal of stress

to stress imposition. Therefore, sub-lethal levels of stress applied slowly enough to register a true acclimation is generally called for. There may also be cases for abrupt imposition of abiotic stress where the effect is not lethal and is believed to reflect a natural phenomenon such as flooding or frost. Furthermore, combining short and long-term stresses can help separate secondary (downstream) proteomic responses to stress from the primary effects, which might be the better targets for plant improvement.

Valuable information on appropriate sampling times under stress can often be found in transcriptomic and metabolomics studies, which can inform proteomics experiments. Alongside this, the power, efficiency and cost of the most contemporary proteomic techniques should enable far more intensive sampling and thus more detailed gene expression time courses. These promise to reveal important stages in the metabolic response to various abiotic stresses.

An excellent example of the importance of time courses is the distinct patterns of metabolite and expressed genes when oxygen was withheld from rice seedlings for up to 48 h [59]. After growing seedlings in anoxia, or aeration, some were switched to the opposite treatment and further tested up the 6 h later. This study clearly reveals the fact that gene products do not accumulate linearly over time, with a peak of 5000 transcripts being up- or down-regulated 3–12 h after imbibition but larger contrasts in transcript numbers between aerated and anoxic tissues appearing over the following 24 h. In another study on rice seedlings, Lasanthi-Kudahettige et al. [60] observed a similar disconnect between transcript levels for two isoforms of alcohol dehydrogenase, whereby one peaked at 3 h after anoxia and the other isoform after 7 h. This illustrates the distortion of gene expression data that can be caused by single, or too few, sampling times in non-steady state conditions after stress is imposed on plants [61]. Expression of genes that are induced by a variety of abiotic stresses are often analysed in detail over 24 h (e.g. [62]), revealing part of the acclimation response but almost certainly prior to the establishment of a new steady state. Moreover, changes in the proteome will generally become apparent in timeframes even slower than the transcriptional changes reported above. Processes such as carbohydrate accumulation, membrane properties and cell wall changes are typically observed over several days and ought to be more explicitly considered in experimental design.

1.4.2 The Importance of Tissue Sampling

Having designed a temporal regime for imposing abiotic stress that gives the best chance of identifying those proteins that are critical for acclimation and survival, it is then important to sample tissues judiciously in order to identify key proteins in subsequent proteomics analysis.

Higher plants differentiate into totally distinct tissue types: even apparently homogeneous tissues can have a high degree of heterogeneity (e.g. root apices, shoot apical meristems), while the functional specialisation in adjacent tissues

(e.g. stele and cortex) is inevitably reflected in the genes expressed. One of the most convenient models for studying the spatial separation of function is in root apices, where adjacent zones of cell division, elongation/expansion and maturation have distinct functions and therefore proteomes (e.g. [63]). In preparing tissues for proteomics from these various root tissues, the proteome of the membrane fraction ought to be extracted alongside the soluble fraction because of the importance of transport in root function.

Tissue sampling is further complicated by the interaction between development and abiotic stress. One must question whether tissues at the same distance from common reference point (e.g. the apex of shoots or roots) in stressed and unstressed plants are necessarily at the same stage of development. In roots, for example, drought has been shown to qualitatively alter the dynamic of cell division and expansion [64], with the result that sampling the same length of tissues from contrasting drought regimes is almost certain to confound development with stress response.

Sampling is equally important in a number of other circumstances where stress is imposed. In the case of salt applied to roots, its accumulation in shoots is broadly proportional to the time for which leaves have been transpiring. This must be recognised during leaf sampling, where developmental age might be appropriate when a range of salt concentrations are to be compared. As in the previous example of roots in drought, the slowing of growth as a result of an abiotic stress complicates comparisons of tissue samples, which might alternatively be selected at a common chronological age or developmental stage.

In one of the earliest protein studies to be published, Sachs et al. [51] reported the major proteins that are synthesised when maize roots became anaerobic. This study has led over the years to a far more complete analysis of anaerobic gene expression, including in rice and *Arabidopsis*. Notably, a recent report on the relationship between the faster and slower growing regions of rice coleoptiles that were less than 20 mm long showed that fine-scale sampling within individual organs is rewarding and should be extended to the proteomic and metabolomics levels [52].

Plant survival during and after floods is a major agronomic question. For dryland species, little progress has been made and yet it has long been known that a major adaptation to inundation for many species, particularly monocotyledons, is the formation of aerenchyma—air channels that form in the root cortex through cell degradation. The cell-level events that lead to this phenomenon are critically important to breeding for greater flood tolerance in modern crops and therefore have captured the attention of researchers in recent years [65]. Because the proportion of root tissue that undergoes lysogeny is so small and close to the cell elongation zone, it is only now that proteomics has become a credible way to tackle the exact pattern of gene expression required to break down cortical cells in such an orderly fashion. This will require fine-scale tissue sampling which is guided by the anatomy of cortical cell breakdown and the molecular clues to when this degradation process is occurring [66] but promises great rewards if proteomics can lead us to targets for breeding programs.

Summary: The advent of mass spectrometry with higher sensitivity allows for physical samples of just a few tens of milligrams, enabling tissues with ever more highly defined physiological properties to be used in experiments. This is especially true where meristems are to be compared; arguably dividing cells have hitherto been ignored in proteomic studies and their response to abiotic stresses should be more deeply investigated as the opportunities for fine-scale sampling improve.

1.5 How Do Acclimation and Shock Differ?

A conceptual question in any discussion of experimental design is the line between stress (followed by acclimation) versus tissue shock, senescence and cell death (Fig. 1.2). This can never be satisfactorily resolved but the aim of the homily above is to design better experiments that inform us about acclimation and thereby, identify targets for genotypic improvement in subsequent breeding and biotechnology [4]. There is no single criterion for differentiating acclimation from damage due to shock. Markers for cell ageing or death might include caspases and other markers of programmed cell death, oxidative enzymes (polyphenol oxidases) and DNA repair enzymes. These molecular markers should be combined with physiological observations such as respiration rates, which should be sufficient to sustain cell function, and histochemical evidence (e.g. the use of vital stains—[53]). Recovery experiments are also vitally important because the failure of, not for a (healthy) steady state to be re-established indicates permanent tissue damage and is strong evidence that shock, senescence and cell death are taking precedence over acclimation. Comprehensive proteomic analyses promise to identify new markers for irreversible cell damage which might well become molecular signatures for over-zealous application of abiotic stress and a platform for design of meaningful experiments.

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Chapter 2
Cereal Root Proteomics for Complementing the Mechanistic Understanding of Plant Abiotic Stress Tolerance

Jennylyn L. Trinidad, Herra L. Grajo, Jose B. Abucay, Jr. and Ajay Kohli

Abstract Cereals are a staple food for four billion people globally with rice, wheat and maize making up 60 % of the energy intake by the world population. Climate change-mediated increase in the extent, frequency and unpredictability of the incidences of abiotic stresses frequently lead to decrease in the yield and grain quality of cereals. Additionally, demographic and socio-economic factors call for increase in the production of quality cereal grains. It is therefore crucial to generate stress tolerant cereal varieties and understand the underlying mechanisms so as to strategize the crop cultivation agro-physiology for long term benefits. Mechanistic understanding of plant responses to stress can best be elucidated through the omics tools and techniques and smart interpretation of their results. Proteomics forms an important aspect of the omics studies in relating the transcriptome to the metabolome. While most cereal proteomics studies dwell on the plants' overall tolerance strategies, proteomics studies either specifically on roots or comparing root responses to the aerial plant parts under stress have been somewhat limited. Root proteins are relatively difficult to extract and characterize, hence the lag in the identification of stress-specific proteins and transcription factors in the roots. However, with the advancements in protein identification and quantification, several important mechanisms have been determined to be at play during abiotic stresses. Root proteins with significant roles are mainly involved in ROS detoxification, energy metabolism, cell wall metabolism, and disease and defense responses. Plasma membrane proteins, regulators of signal transductions and ion channels also contribute to increased stress tolerance. This review brings together an understanding of stress response established by the proteomic studies on cereal

J.L. Trinidad · H.L. Grajo · J.B. Abucay Jr. · A. Kohli (✉)
Genetics and Biotechnology Division, Plant Molecular Biology Laboratory, International Rice Research Institute, DAPO 7777, Metro Manila, Philippines
e-mail: a.kohli@irri.org

J.L. Trinidad · H.L. Grajo · J.B. Abucay Jr.
College of Arts and Sciences, University of the Philippines Los Baños, College 4031, Los Baños, Laguna, Philippines

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