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Chapter 2

Abiotic Stress in Plants and Metabolic Responses

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Additional information is available at the end of the chapter

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1. Introduction

The vast metabolic diversity observed in plants is the direct result of continuous evolutionary processes. There are more than 200,000 known plant secondary metabolites, representing a vast reservoir of diverse functions. When the environment is adverse and plant growth is affected, metabolism is profoundly involved in signaling, physiological regulation, and defense responses. At the same time, in feedback, abiotic stresses affect the biosynthesis, concentration, transport, and storage of primary and secondary metabolites. Metabolic adjustments in response to abiotic stressors involve fine adjustments in amino acid, carbohydrate, and amine metabolic pathways. Proper activation of early metabolic responses helps cells restore chemical and energetic imbalances imposed by the stress and is crucial to acclimation and survival. Time-series experiments have revealed that metabolic activities respond to stress more quickly than transcriptional activities do. In order to study and map all the simultaneous metabolic responses and, more importantly, to link these responses to a specific abiotic stress, integrative and comprehensive analyses are required. Metabolomics is the systematic approach through which qualitative and quantitative analysis of a large number of metabolites is increasing our knowledge of how complex metabolic networks interact and how they are dynamically modified under stress adaptation and tolerance processes. A vast amount of research has been done using metabolomic approaches to (*i*) characterize metabolic responses to abiotic stress, (*ii*) to discover novel genes and annotate gene function, and, (*iii*) more recently, to identify metabolic quantitative trait loci. The integration of the collected metabolic data concerning abiotic stress responses is helping in the identification of tolerance traits that may be transferable to cultivated crop species. In this review, the diverse metabolic responses identified in plants so far are discussed. We also include recent advances in the study of plant metabolomes and metabolic fluxes with a focus on abiotic stress-tolerance trait interactions.

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2. Abiotic stresses and the impact on agriculture

Today, in a world of 7 billion people, agriculture is facing great challenges to ensure a sufficient food supply while maintaining high productivity and quality standards. In addition to an ever increasing demographic demand, alterations in weather patterns due to changes in climate are impacting crop productivity globally. Warming and shifts in rainfall patterns caused an historically high $10.3 billion in crop insurance payments to cover agriculture losses in 2011 in the U.S. [1]. Unfavorable climate (resulting in abiotic stresses) not only causes changes in agro-ecological conditions, but indirectly affects growth and distribution of incomes, and thus increasing the demand for agricultural production [2]. Adverse climatic factors, such as water scarcity (drought), extreme temperatures (heat, freezing), photon irradiance, and contamination of soils by high ion concentration (salt, metals), are the major growth stressors that significantly limit productivity and quality of crop species worldwide. As has been pointed out, current achievements in crop production have been associated with management practices that have degraded the land and water systems [3]. Soil and water salinity problems exist in crop lands in China, India, the United States, Argentina, Sudan, and many other countries in Western and Central Asia. Globally, an estimated 34 million irrigated hectares are salinized [4], and the global cost of irrigation-induced salinity is equivalent to an estimated US$11 billion per year [5].

A promising strategy to cope with adverse scenario is to take advantage of the flexibility that biodiversity (genes, species, ecosystems) offers and increase the ability of crop plants to adapt to abiotic stresses. The Food and Agricultural Organization (FAO) of the United Nations promotes the use of adapted plants and the selection and propagation of crop varieties adapted or resistant to adverse conditions [6]. Global programs, such as the Global Partnership Initiative for Plant Breeding Capacity Building (GIPB), aim to select and distribute crops and cultivars with tolerance to abiotic stresses for sustainable use of plant genetic resources for food and agriculture [7].

3. Plant responses to abiotic stress

Through the history of evolution, plants have developed a wide variety of highly sophisticated and efficient mechanisms to sense, respond, and adapt to a wide range of environmental changes. When in adverse or limiting growth conditions, plants respond by activating tolerance mechanisms at multiple levels of organization (molecular, tissue, anatomical, and morphological), by adjusting the membrane system and the cell wall architecture, by altering the cell cycle and rate of cell division, and by metabolic tuning [8]. At a molecular level, many genes are induced or repressed by abiotic stress, involving a precise regulation of extensive stress-gene networks [9-11]. Products of those genes may function in stress response and tolerance at the cellular level. Proteins involved in biosynthesis of osmoprotectant compounds, detoxification enzyme systems, proteases, transporters, and chaperones are among the multiple protein functions triggered as a first line of direct protection from stress. In addition,

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activation of regulatory proteins (*e.g.*, transcription factors, protein phosphatases, and kinases) and signaling molecules are essential in the concomitant regulation of signal transduction and stress-responsive gene expression [12, 13]. Early plant response mechanisms prevent or alleviate cellular damage caused by the stress and re-establish homeostatic conditions and allow continuation of growth [14]. Equilibrium recovery of the energetic, osmotic, and redox imbalances imposed by the stressor are the first targets of plant immediate responses.

Observed tolerance responses towards abiotic stress in plants are generally composed of stress-specific response mechanisms and also more general adaptive responses that confer strategic advantages in adverse conditions. General response mechanisms related to central pathways are involved in energy maintenance and include calcium signal cascades [15, 16], reactive oxygen species scavenging/signaling elements [17, 18], and energy deprivation (energy sensor protein kinase, SnRK1) signaling [19]. Induction of these central pathways is observed during plant acclimation towards different types of stress. For example, protein kinase SnRK1is a central metabolic regulator of the expression of genes related to energy-depleting conditions, but this kinase also becomes active when plants face different types of abiotic stress such as drought, salt, flooding, or nutrient depravation [20-24]. SnRK1 kinases modify the expression of over 1000 stress-responsive genes allowing the re-establishment of homeostasis by repressing energy consuming processes, thus promoting stress tolerance[24, 25]. The optimization of cellular energy resources during stress is essential for plant acclimation; energetically expensive processes are partially arrested, such as reproductive activities, translation, and some biosynthetic pathways. For example, nitrogen and carbon assimilation are impaired in maize during salt stress and potassium-deficiency stress; the synthesis of free amino acids, chlorophyll, and protein are also affected [26-28]. Once energy-expensive processes are curtailed, energy resources can be redirected to activate protective mechanisms. This is exemplified by the decrease in *de novo* protein synthesis in *Brassica napus* seedlings, *Glycine max, Lotus japonicas*, and *Medicago truncatula* during heat stress accompanied by an increased translation of heat shock proteins [29, 30].

4. Metabolic adjustments during stressing conditions: Osmolyte accumulation

A common defensive mechanism activated in plants exposed to stressing conditions is the production and accumulation of compatible solutes. The chemical nature of these small molecular weight organic osmoprotectants is diverse; these molecules include amino acids (asparagine, proline, serine), amines (polyamines and glycinebetaine), and γ-amino-N-butyric acid (GABA). Furthermore, carbohydrates, including fructose, sucrose, trehalose, raffinose, and polyols (myo-inositol, D-pinitol) [12, 31], as well as pools of anti-oxidants such as glutathione (GSH) and ascorbate [32, 33], accumulate in response to osmotic stress. Common characteristics of these diverse solutes are a high level of solubility in the cellular milieu and lack of inhibition of enzyme activities even at high concentrations. Accumulation of compatible solutes in response to stress is not only observed in plants, it is a defense mechanism triggered in animal cells, bacteria, and marine algae, indicative of an evolutionarily conserved trait [34,

35]. Scavenging of reactive oxygen species (ROS) to restore redox metabolism, preservation of cellular turgor by restitution of osmotic balance, and associated protection and stabilization of proteins and cellular structures are among the multiple protective functions of compatible osmoprotectants during environmental stress [36-38].

A large amount of research has been done on the beneficial effects of compatible solutes on plant tolerance to environmental stress. Correlation between amino acid accumulation (mainly proline) and stress tolerance was described in the mid-1960s in Bermuda grass during water stress [39]. Since then, extensive work has proven that proline serves as an osmoprotectant, a cryoprotectant, a signaling molecule, a protein structure stabilizer, and an ROS scavenger in response to stresses that cause dehydration; including salinity, freezing, heavy metals, and drought (low water potential) [40, 41]. Proline oxidation may also provide energy to sustain metabolically demanding programs of plant reproduction, once the stress has passed [42].

Proline metabolism and its regulation are processes well characterized in plants. Proline is synthesized from glutamate in the cytoplasm or chloroplasts: Δ-1-pyrroline-5-carboxylate synthetase (P5CS) reduces glutamate to glutamate semialdehyde (GSA). Then GSA spontaneously cyclizes into pyrroline-5-carboxylate (P5C), which is further reduced by P5C reductase (P5CR) to proline. Conversely, proline is catabolized within the mitochondrial matrix by action of proline dehydrogenase (ProDH) and P5C dehydrogenase (P5CDH) to glutamate. In an alternative pathway, proline can be synthesized from ornithine in a pathway involving ornithine δ-aminotransferase (OAT). Core enzymes P5CS, P5C, P5CR, ProDH, and OAT are responsible for maintaining the balance between biosynthesis and catabolism of proline. Regulation comes at transcriptional level of genes encoding the key enzymes. Transcriptional up-regulation of genes for P5CS and P5C to increase proline synthesis from glutamate and down-regulation of genes for P5CR and ProDH to arrest proline catabolism is observed during dehydration/osmotic stress [43]. Also, post-translational regulation of core enzymes is closely associated with proline levels and environmental signals. For example, the *Arabidopsis* P5CS1 enzyme is subjected to feedback inhibition by proline, controlling the carbon influx into the biosynthetic pathway [44, 45]. Considering that proline accumulation is associated with stress tolerance, that core enzymes regulate proline biosynthesis, and that these core enzymes are likely rate-limiting steps for its accumulation, logic dictates that overexpression of biosynthetic proline enzymes might increase the levels of the compatible solute and thus improve the tolerance in plants against abiotic stress. Several studies have tested this by overexpressing genes for P5CS or P5C enzymes in different plant species, reporting the expected rise in proline levels and the associated resistance to dehydration, salinity, or freezing [46-53]. Furthermore, deletion of genes coding ProDH [54] or P5CDH [55, 56], expression of a feedback-insensitive P5CS [45], or the overexpression of OAT [57, 58] increase the cellular levels of proline and osmoprotection to some abiotic stresses.

Comparable extensive work has been done for other compatible solutes such as γ-aminobutyric acid [59], glycine betaine [60], trehalose [61], mannitol, and sorbitol [36]; these solutes are efficient protectors against some abiotic stressors. Metabolic pathways for biosynthesis and catabolism of compatible solutes, their regulation, participant enzymes, and compartmentalization are well characterized in most important plant species. This knowledge has led to

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strategies for improvement of plant tolerance involving the accumulation of those protective osmolytes in plants by expression of core biosynthetic enzymes or their improved derivatives, expression of related transporters, and deletion of osmolyte-consuming enzymes. These numerous studies have provided evidence that enhanced accumulation of compatible solutes correlates with reinforcement of plant resistance to adverse growth conditions.

5. Plant metabolomics and applications

The traditional approach of enhancing the accumulation of a specific compounds in response to a determined stimulus, as done with compatible solutes, have resulted in some degree of tolerance in plants, and also demonstrates that the ability to redirect nutrients to imperative processes and the induction of adequate metabolic adjustments are crucial for plant survival during conditions of stress. However, this is a sectioned view of how plants regulate their entire metabolism in response to stressing conditions. In order to achieve a more comprehensive understanding, we must consider that plant metabolism is an intricate network of interconnected reactions. Plants have a high degree of subcellular compartmentation, a vast repertory of metabolites, and developmental stage strongly influences metabolism. Therefore, metabolic responses are complex and dynamic and involve the modification of more than one metabolite. Also, accumulation of a specific compound is not an absolute requirement indicative of a tolerance trait; adjustment of the flux through a certain metabolic pathway might be enough to contribute to stress tolerance [62]. Recently, it has been reported that plants modulate stoichiometry and metabolism in a flexible manner in order to maintain optimal fitness in mechanisms of storage, defense, and reproduction under varying conditions of temperature and water availability [63]. Furthermore, time-series experiments in *Arabidopsis thaliana* plants subjected to temperature and/or light alterations revealed that time-resolved metabolic activities respond more quickly than transcriptional activities do [64].

Traditional molecular approaches for tracing metabolic phenotypes in plants responding to abiotic stress have identified and manipulated specific genes or groups of genes in plant models. These have primarily been genes involved in early responses or in down-stream assembly of the response reaction. With the application of new powerful tools of molecular biology and bioinformatics, large collections of genes have been subjected to complete analysis. To arrive at a complete and comprehensive knowledge of physiology in the plant response to abiotic stress, researchers are embracing ionomic profiling, transcriptomic, proteomic and metabolomic analysis. A deep dissection of the biochemical pathways in plants facing stressing conditions requires integrative and comprehensive analyses in order to identify all the simultaneous metabolic responses and, more importantly, to be able to link these responses to specific abiotic stress. In this sense, metabolomics could contribute significantly to the study of metabolic responses to stress in plants by identifying diverse metabolites, such as the by-products of stress metabolism, stress signal transduction molecules, and molecules that are part of the acclimation response [65].

The metabolome is the entirety of small molecules present in an organism and can be regarded as the ultimate expression of its genotype in response to environmental changes. Metabolomics

is gaining importance in plant research in both basic and applied contexts. Metabolomic studies have already shown how detailed information gained from chemical composition can help us to understand the various physiological and biochemical changes occurring in the plants and their influence on the phenotype. The analytical measurement of several hundreds to thousands of metabolites is becoming a standard laboratory technique with the advent of "hyphenated" analytical platforms of separation methods and various detection systems. Separation methods include gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE). Different types of mass spectrometry (MS), nuclear magnetic resonance (NMR), and ultraviolet light spectroscopy (UV/VIS) devices are utilized for detection. Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) is a specialized technique often used in direct infusion (DI) mode for metabolomics analyses, as its high mass accuracy allows a separation solely based on this parameter. Each methodology offers advantages and disadvantages, and the method of choice will depend on the type of sample and metabolites to be determined, and the combination of analytical platforms [66].

GC and MS were the first pair of techniques to be combined, delivering high robustness and reproducibility. GC-MS remains one of the most widely used methods for obtaining metabolomic data because of its ease of use, excellent separation power, and its reproducibility. The main drawback of GC-MS is that only thermally stable volatile metabolites, or non-volatile compounds that can be chemically altered to make them volatile, can be detected [67, 68]. NMR spectroscopy is a fingerprinting technique that offers several advantages over high-throughput metabolite analyses, such as relatively simple sample preparation and the non-destructive analysis of samples. NMR can detect different classes of metabolites in a sample, regardless of their size, charge, volatility, or stability with excellent resolution and reproducibility [69]. Labeling of metabolites with isotopes and subsequent NMR analysis is also useful for metabolic flux analysis and fluxomics as it allows tracking the selective signal enhancement of isotopologues [70]. Recent advances with high-throughput approaches using ultra-high-field FT-ICR-MS alone or in combination with other tools of 'first pass' metabolome analysis as electrospray ionization mass spectrometry (ESI-MS) are expected to make inventory of the entire metabolome in a single sample possible in the near future [71, 72].

In metabolomics, the implicit objective is to identify and quantify all possible metabolites in a cellular system under defined states of stress conditions (biotic or abiotic) over a particular time scale in order to characterize accurately the metabolic profile [73]. But metabolome studies have some analytical limitations. It is important to have in mind that from the total amount of metabolites in a sample, only an informative portion can be reliably identified and quantified. In addition, metabolic networks in multicellular eukaryotes, specifically in plants, are challenging because of the large size of the metabolome, extensive secondary metabolism, and the considerable variation in tissue-specific metabolic activity [74]. Therefore, experimental design and sample preparation need to be done with great care because environmental and experimental variation confer noticeable impact on the resulting metabolic profiles. This has been demonstrated in legumes in which a high proportion of nutritional and metabolic changes depend on non-controllable environmental variables [75].

Metabolomic analyses have been applied to the functional identification of unknown genes through metabolic profiling of plants in which some genes are up- or down-regulated, the discovery of biomarkers associated with disease phenotypes, the safety assessment of genetically modified organisms (GMOs), the characterization of plant metabolites of nutritional importance and significance in human health, and the discovery of compounds involved in plant resistance to biotic and abiotic stresses [76]. Metabolic profiles can be used as signatures for assessing the genetic variation among different cultivars or species of the same genotype at different growth stages and environments. The metabolite profile represents phenotypic information; this means that qualitative and quantitative metabolic measurements can be related to the genotypes of the plants to differentiate closely related individuals [77, 78]. Once the identification of individual metabolites is available, connections among metabolites can be established, and then metabolic profiles can be used to infer mechanisms of defense. Metabolic profiles will guide tailoring of genotypes for acceptable performance under adverse growth conditions and will be of help in design and development of crop plant cultivars best suited to sustainable agriculture [79, 80]. Metabolomics tools have been used to evaluate the impact of the genotype and the environment on the quality of plant growth in the study of interpecific hybrids between *Jacobaea aquatica* and *J. vulgaris* (common weeds native to Northern Eurasia). An NMR-based metabolomics profiling approach was used to correlate the expression of high and low concentrations of particular compounds, including phenylpropanoids and sugars, with results of quantification of genetically controlled differences between major primary and secondary metabolites [81]. In melon (*Cucumis melo* L.), metabolomic and elemental profiling of fruit quality were found to be affected by genotype and environment [82].

6. Plant metabolomics and drought stress

The variable and often insufficient rainfalls in extended areas of rain-fed agriculture, the unsustainable groundwater use for irrigated agriculture worldwide, and the fast-growing demands for urban water are putting extreme pressure on global food crop production. The demand for water to sustain the agriculture systems in many countries will continue to increase as a result of growing populations [83]. This progressively worsening water scarcity is imposing hydric stress on both rain-fed and irrigated crops. Water deficiency stress induces a wide range of physiological and biochemical alterations in plants; arrestment of cell growth and photosynthesis and enhanced respiration are among the early affects. Genome expression is extensively remodeled, activating and repressing a variety of genes with diverse functions [11, 84]. Sensing water deficit and activation of defense mechanisms comes through chemical signals in which abscisic acid (ABA) plays a central role. ABA accumulates in tissues of plants subjected to hydric stress and promotes transpiration reduction via stomatal closure. Through this mechanism, plants minimize water losses and diminish stress injury. ABA regulates expression of many stress-responsive genes, including the late embryogenesis abundant (LEA) proteins, leading to a reinforcement of drought stress tolerance in plants [85]. Many questions

remain unresolved concerning hydric stress-plant metabolic response: How does drought stress perturb metabolism in crop plants? How does hydric stress affect the metabolism of wild plants? What modern strategies of "omics" could be exploited to support future programs of crop breeding to lead to a more sustainable agriculture?

As previously described, one of the main mechanisms by which plants cope with water deficits is osmotic adjustment. These adjustments maintain a positive cell turgor via the active accumulation of compatible solutes. Traditionally, the analysis of metabolic responses to drought stress was limited to analysis of one or two classes of compounds considered as "role players" in the development of tolerance. Application of metabolomic approaches is providing a less biased perspective of metabolic profiles of response and also is aiding in the discovery of novel metabolic phenotypes. Unbiased GC-MS metabolomic profiling in *Eucalyptus* showed that drought stress alters a larger number of leaf metabolites than the previously reported in targeted analysis. Accumulation of shikimic acid and two cyclohexanepentol stereoisomers in response to drought stress was described for the first time in *Eucalyptus*. Also, the magnitude of metabolic adjustments in response to water stress correlates with the sensitivity/tolerant phenotype observed; drought affected around 30-40% of measured metabolites in *Eucalyptus dumosa* (a drought-sensitive specie) compared to 10-15% in *Eucalyptus pauciflora* (a drought-tolerant specie) [86]. Similarly, critical differences in the metabolic responses were observed when drought-tolerant (NA5009RG) and drought-sensitive (DM50048) soybean cultivars were analyzed by ^{1}H NMR-based metabolomics. Interestingly, no enhanced accumulation of the traditional osmoprotectants, such as proline, soluble sugars as sucrose or myo-inositol, organic acids or other amino acids (except for aspartate), were detected in the leaves of either genotype during water stress. In contrast, levels of 2-oxoglutaric acid, pinitol, and allantoin were affected differentially in the genotypes when drought was imposed, suggesting possible roles as osmoprotectants [87]. In contrast to soybean, levels of amino acids, including proline, tryptophan, leucine, isoleucine, and valine, were increased under drought stress in three different cultivars of wheat (*Triticum aestivum*) analyzed for 103 metabolites in a targeted GC-MS approach [88]. Metabolic adjustments in response to adverse conditions are transient and depend on the severity of the stress. In a 17-day time course experiment in maize (*Zea mays*) subjected to drought stress, GC-MS metabolic analysis revealed changes in concentrations of 28 metabolites. Accumulation of soluble carbohydrates, proline and eight other amino acids, shikimate, serine, glycine, and aconitase, was accompanied by the decrement of leaf starch, malate, fumarate, 2-oxoglutarate, and seven amino acids during the drought treatment course. However, as the water potential became more negative, between the 8th and 10th days, the changes in some metabolites were more dramatic, demonstrating their dependence on stress severity [89].

Accumulation of compatible solutes is an evolutionary conserved trait in bacteria, plants, animal cells, and marine algae. A recent GC-MS metabolomic analysis confirmed that the moss *Physcomitrella patens* also triggers compatible solute accumulation in response to drought stress. After two weeks of physiological drought stress, 26 metabolites were differentially affected in gametophores, including altrose, maltitol, L-proline, maltose, isomaltose, and butyric acid, comparable to metabolic adjustments previously reported in stressed *Arabidop-*

sis leaves. More interesting is the recent report of a new compound, annotated as EITTMS_N12C_ATHR_2988.6_1135EC44, with no previously mass spectra matching record, accumulated specifically in response to drought stress in this moss [90].

7. Plant metabolomics and salinity stress

A current problem for crop plants worldwide, which will become more critical in the future, is salt stress imposed by salinity in soils due to poor practices in irrigation and over-fertilization, among other causes. Salt stress induces abscisic acid synthesis; abscisic acid transported to guard cells closes stomata, resulting in decreased photosynthesis, photo-inhibition, and oxidative stress. This causes an immediate inhibition of cell expansion, visible as general plant growth inhibition, accelerated development, and senescence [91]. To cope with salt stress plants implement strategies that include lowering of rates of photosynthesis, stomatal conductance, and transpiration [92]. Sodium ion, by its similar chemical nature to potassium ion, competes with and inhibits the potassium uptake by the root. Potassium deficiency results in growth inhibition because this ion is involved in the capacitance of a plethora of enzyme activities in addition to its participation in maintaining membrane potential and cell turgor [91].

The metabolic perturbation in plants exposed to salinity involves a broad spectrum of metabolic pathways and both primary and secondary metabolism. For example, in a proteomic study in foxtail millet (cv. Prasad), 29 proteins were significantly up- or down-regulated due to NaCl stress, with great impact on primary metabolism. These proteins were classified into nine functional categories: cell wall biogenesis (lignin biosynthesis), among these were caffeic acid 3-O-methyltransferase and caffeoyl CoA 3-O-methyltransferase; photosynthesis and energy metabolism, which included proteins like cytochrome P450 71D9, phytochrome 1, photosystem I reaction center subunit IV B, and ATP synthase F1 sector subunit beta, among others; nitrogen metabolism, proteins like glutamine synthetase root isozyme 4, ferredoxin-dependent glutamate synthase, chloroplast precursor (Fd-GOGAT), and urease; carbohydrate metabolism, proteins such as UDP-glucose 4-epimerase GEPI42 (galactowaldenase) and beta-amylase; and lipid metabolism including isovaleryl-CoA dehydrogenase 2 and aldehyde dehydrogenase [93].

Studies using metabolomic tools in plant models and plant crops have shown that the physiology in salt stress courses through a complex metabolic response including different systematic mechanisms, time-course changes, and salt-dose dependence. The biochemical changes involve metabolic pathways that fulfill crucial functions in the plant adaptation to salt stressing conditions. Time-course metabolite profiling in cell cultures of *A. thaliana* exposed to salt stress demonstrates that glycerol and inositol are abundant 24 h after salt stress exposure, whereas lactate and sucrose accumulate 48 h later. The methylation cycle, the phenylpropanoid pathway, and glycine betaine biosynthesis exhibit induction as a short-term response to salinity stress, whereas glycolysis and sucrose metabolism and reduction in methylation are long-term responses. Long-term salt exposure also causes a reduction in the metabolites that

were initially responsive [94]. In tobacco plants treated with various doses of salt, 1 day of treatment with 50 mM NaCl induced accumulation of sucrose, and to a lesser extent glucose and fructose, through gluconeogenesis. Further stress (500 mM NaCl for another day) led to elevation of proline and even higher elevation in sucrose levels compared to the lower dose; at the same time, glucose and fructose levels decreased as transamination-related metabolites (asparagine, glutamine, and GABA) did. These data suggest that sugar and proline biosynthesis pathways are metabolic mechanisms for control of salt stress over one- to two-day periods (short-term). Proline continues to be observed at high levels at later stages (3 to 7 days under highly stressing concentrations of 500 mM NaCl) and sucrose decreases (although it remains at high levels compared to control). There are also significant elevations in levels of asparagine, valine, isoleucine, tryptophan, myo-inositol, uracil, and allantoin, and reductions in glucose, fructose, glutamine, GABA, malate, fumarate, choline, uridine, hypoxantine, nicotine, N-methylnicotinamide, and formate [95]. Similarly, in maize plants stressed with salt solutions ranging in concentration from 50 to 150 mM NaCl, the metabolic profile of the shoot extracts changes most dramatically compared to controls in the plants exposed to the highest salt concentration [96].

Another complexity in the metabolic perturbations in salt-stressed plants consists of tissue-specific response differences. In maize plants exposed to 50-150 mM NaCl saline solution, levels of sucrose and alanine were increased and levels of glucose decreased in roots and shoots. Other osmoprotectants exhibited differentiated behavior: GABA, malic acid, and succinate levels increased in roots, while glutamate, asparagine and glycine betaine were at higher concentrations in shoots. There were decreased levels of acetoacetate in roots and of malic acid and *trans*-aconitic acid in shoots. A progressive metabolic response was more evident in shoots than in roots [96].

In comparative ionomics and metabolite profiling of related *Lotus* species (*Lotus corniculatus*, *L. tenuis*, and *L. creticus*) under salt stress, the extremophile *L. creticus* (adapted to highly saline coastal regions) exhibits better survival after long-term exposure to salinity and is more efficient at excluding Cl^- from shoot tissue than the two cultivated glycophytes *L. corniculatus* and *L. tenuis* (grassland forage species). Sodium ion levels are higher in the extremophile than the cultivars under both control conditions and salt stress. In *L. creticus*, a differential homeostasis of Cl^-, Na^+, and K^+ is accompanied by distinct nutritional changes compared to the glycophytes *L. corniculatus* and *L. tenuis*. Magnesium and iron levels increase in *L. creticus* after salt treatment, but levels of potassium, manganese, zinc, and calcium do not. In non-stressed control plants, 41 metabolites are found at lower levels in *L. creticus* than in the two glycophytes, and 10 metabolites are at higher levels in *L. creticus*. These data demonstrate that each of these species has a distinct basal metabolic profile and that these profiles do not show a concordance with salt stress or salt tolerance. In salt stress conditions, 48 metabolites show similar changes in all species, either increasing or decreasing, with increased levels the amino acids proline, serine, threonine, glycine, and phenylalanine; the sugars sucrose and fructose, myo-inositol and other unidentified metabolites; and with decreased levels of organic acids such as citric, succinic, fumaric, erythronic, glycolic, and aconitic acid, including ethanolamine and putrescine, among others. Of note is that more than half of the metabolites affected by salt

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treatment are common among the three species, and only one-third of responsive metabolites in *L. creticus* are not shared with the glycophytes. Interestingly, the changes in the pool sizes of these metabolites are only marginal [97]. A few changes in the metabolic profile are extremophile-specific, but most salt-elicited changes in metabolism are similar. Other studies in glycophytes under salt stress indicate that organic acids and intermediates of the citric acid cycle tend to decrease [98]. Also in genus *Lotus*, model species (*L. japonicus*, *L. filicaulis*, and *L. burttii*) and cultivated species (*L. corniculatus*, *L. glaber*, and *L. uliginosus*) exhibit consistent negative correlation in the Cl^- levels in the shoots and tolerance to salinity, but metabolic profiles diverge amongst genotypes; asparagine levels are higher in the more tolerant genotypes. These results support the conclusion that Cl^-exclusion from the shoots represents a key physiological mechanism for salt tolerance in legumes; moreover, an increased level of the osmoprotectant asparagine is typical [99]. In *L. japonicus*, which has a robust metabolic response to salt stress, levels of proline and serine, polyolsononitol and pinitol, and myo-inositol increase [75].

All these studies demonstrate that the metabolic plant response to salinity stress is variable depending on the genus and species and even the cultivar under consideration. Differential metabolic rearrangements are in intimate correlation with genetic backgrounds. Furthermore, the plant physiology in salt stress with time proceeds through a complex metabolic response including different systematic mechanisms and changes. Inside a salt-stressed plant as a biological unit, different tissues respond differentially and in some cases the responses are even contrasting. From comparative ionomics studies, it is evident also that under salinity stress, differential homeostasis of ions as Cl^-, Na^+, and K^+ is correlated with distinct nutritional changes in extremophile and glycophyte species, even inside the same genus. Noticeable differences exist between plant species in the way they react to surpass the osmotic pressure imposed by high soil salt content through mechanisms such as tolerance, efficiency in salt exclusion, changes in nutrient homeostasis, and osmotic adjustment. From the aforementioned studies, metabolic markers in the response to high salinity in plants include glycine betaine, sucrose, asparagine, GABA, malic acid, aspartic acid, and *trans*-aconitic acid. In legumes, increases in levels of the amino acids asparagine, proline, and serine are notable as are increases in polyolsononitol, pinitol, and myo-inositol [75].

8. Plant metabolomics and oxidative stress

An increase in intracellular levels of ROS is a common consequence of adverse growth conditions. An imbalance between ROS synthesis and scavenging is caused in a manner independent of the nature of the stress; it is induced by both biotic and abiotic types of stress. Toxic concentrations of ROS cause severe damage to protein structures, inhibit the activity of multiple enzymes of important metabolic pathways, and result in oxidation of macromolecules including lipids and DNA. All these adverse events compromise cellular integrity and may lead to cell death [100, 101]. Normal cellular metabolic activity also results in ROS generation under regular growth conditions. Thus, cells sense uncontrolled elevation of ROS and use them as a signaling mechanism to activate protective responses [102]. In this context plants have

developed efficient mechanisms for removal of toxic concentrations of ROS. The antioxidant system is composed of protective enzymes (*e.g.*, superoxide dismutase, catalase, peroxidase, reductase, and redoxin) and radical scavenger metabolites (mainly GSH and ascorbate). GSH is an essential component of the antioxidant system that donates an electron to unstable molecules such as ROS to make them less reactive and also can acts as a redox buffer in the recycling of ascorbic acid from its oxidized form to its reduced form by the enzyme dehydroascorbate reductase [103]. Organized remodeling of metabolic networks is a crucial response that gives the cells the best chance of surviving the oxidative challenge.

In *A. thaliana*, oxidative treatment with methyl viologen causes the down-regulation of photosynthesis-related genes and concomitant cessation of starch and sucrose synthesis pathways, meanwhile catabolic pathways are activated. These metabolic adjustments avoid the waste of energy used in non-defensive processes and mobilize carbon reserves towards actions of emergency relief such as the accumulation of maltose, a protein structure-stabilizer molecule [104]. A GC-MS metabolomic study, together with an analysis of key metabolic fluxes of cell cultures and roots of *A. thaliana* treated with the oxidative stressor menadione, revealed the similarities and divergences in the metabolic adjustments triggered in both culture systems. Inhibition of the tricarboxylic acid cycle (TCA) by accumulation of pyruvate and citrate is accompanied by a decrement of malate, succinate, and fumarate pools. This early (0.5 h) response was observed in both systems. Inhibition of TCA cycle concomitantly causes a decrement in the pools of glutamate and aspartate due to the inhibition of the synthesis of TCA-linked precursors 2-oxoglutarate and oxaloacetate, respectively. Another mutual early metabolic redistribution is the redirection of the carbon flux from glycolysis to the oxidative pentose phosphate (OPP) pathway. This is also reflected by the decrement in the glycolytic pools of glucose-6 phosphate and fructose 6-P, and the increment in the OPP pathway intermediates ribulose 5-phosphate and ribose 5-phosphate. Increased carbon flux through the OPP pathway might supply reducing power (via nicotinamide adenine dinucleotide phosphate, NADPH) for antioxidant activity, since oxidative stress decreases the levels of the reductants GSH, ascorbate, and NADPH. After 2 and 6 h of stress progression, metabolic adjustments in response to oxidative stress are different in roots than in cell suspension cultures. In roots, pools of TCA cycle intermediates and amino acids are recovered. In contrast, in cell cultures, the concentrations of these metabolites remains depressed throughout the time course, indicating higher basal levels of oxidative stress in cell cultures. At the end of the treatment time (6 h), 39 metabolites, including GABA, aromatic amino acids (tryptophan, phenylalanine, and tyrosine), proline, and other amino acids, were significantly altered in roots. These results showed the broad spectrum of metabolic modifications elicited in response to oxidative stress and the influence of the biological system analyzed [105].

Redirection of carbon flux from glycolysis through the OPP pathway and subsequent increase in the levels of NADPH was also reported in rice cell cultures treated with menadione. CE-MS analysis of these rice cultures showed the depletion of most sugar phosphates resulting from glycolysis (pyruvate, 3-phosphoglyceric acid, dihydroxyacetone phosphate, fructose-6-phosphate, glucose-1-phosphate (G1P), G6P, G3P, phosphoenolpyruvate) and TCA-organic acids (2-oxoglutarate, aconitate, citrate, fumarate, isocitrate, malate, succinate) and increases

in the levels of OPP pathway intermediates (6-phosphogluconate, ribose 5-phosphate, ribulose 5-phosphate). Incremental increases in the biosynthesis of GSH and intermediates (*O*-acetyl-L-serine, cysteine, and γ-glutamyl-L-cysteine) are also observed in the menadione-treated rice cell cultures [106].

9. Perspectives

Metabolome analysis has become an invaluable tool in the study of plant metabolic changes that occur in response to abiotic stresses. Despite progress achieved, metabolomics is a developing methodology with room for improvement. From a technical perspective, further developments are required to improve sensitivity for identification of previously uncharacterized molecules and for quantification of cellular metabolites and their fluxes at much higher resolution. This will allow the identification of novel metabolites and pathways and will allow linkage to responses to specific stresses, and, therefore, increase our level of knowledge of the elegant regulation and precise adjustments of plant metabolic networks in response to stress.

Another challenging task is the integration of metabolic data with data from experiments profiling the transcriptome, proteome, and genetic variations obtained from the same tissue, cell type, or plant species in response to a determined environmental condition. Integrated information can be used to map the loci underlying various metabolites and to link these loci to crop phenotypes, to understand the mechanisms underlying the inheritance of important traits, and to understand biochemical pathways and global relationships among metabolic systems. Elucidation of the regulatory networks involved in the activation/repression of key genes related to metabolic phenotypes in response to determined abiotic stress is becoming possible. Transcription factors (TFs) are central player in the signal transduction network, connecting the processes of stress signal sensing and expression of stress-responsive genes. Thus engineered TFs have emerged as powerful tools to manipulate complex metabolic pathways in plants and generate more robust metabolic phenotypes [107, 108].

Metabolic networks are highly dynamic, and changes with time are influenced by stress severity, plant developmental stage, and cellular compartmentalization. Since metabolic profiling only reveals the steady-state level of metabolites, detailed kinetics and flux analyses will support a better understanding of metabolic fluctuations in response to stress [109]. Genome-scale models (GSM) are *in silico* metabolic flux models derived from genome annotation that contain stoichiometry of all known metabolic reactions of an organism of interest. Construction of detailed GSMs applied to plant metabolism will provide information about distribution of metabolic fluxes at a specific genotype, a determined developmental stage, or a particular environmental condition. This detailed knowledge of the metabolic and physiological status of the cell can be used to design rational metabolic engineering strategies and to predict required genetic modifications to obtain a desired metabolic phenotype such as optimized biomass production, increased accumulation of a valuable metabolite, accumulation of a metabolite of response towards abiotic stress, or modification of metabolic flux through a specific pathway of significance [110]. Recently advances have been made in this

field. For example, in rice, by using four complementary analytical platforms based on high-coverage metabolomics, molecular backgrounds of quality traits and metabolite profiles were correlated with overall population structure and genetic diversity, demonstrating that quality traits could be predicted from the metabolome composition, and that traits can be linked with metabolomics data. Results like these are opening the doors to modern plant breeding programs [111].

Once a metabotype (metabolic phenotype) is confirmed to strengthen the tolerance to a particular abiotic stressor, the next challenge will be the transfer of this metabolic trait to a non-adapted plant species of interest. Engineering of more tolerant plants will then require the efficient integration and expression of one to several transgenes in order to modify an existent metabolic pathway or reconstruct a new complete one. Development and optimization of protocols for robust transformation of nucleus, mitochondria, and chloroplasts must be made available for higher plants including economically important crops; this will open new opportunities for plant metabolic engineering [112]. Future research progress on these topics will lead to novel strategies for plant breeding and elevating the health and performance of crops under adverse growth conditions to keep up with the ever-increasing needs for food and feed worldwide.

10. Conclusions

Metabolomics is the comprehensive and quantitative analysis of the entirety of small molecules present in an organism that can be regarded as the ultimate expression of its genotype in response to environmental changes, often characterized by several simultaneous abiotic and biotic stresses. Results obtained from a number of metabolomic studies in plants in response to different abiotic stresses have shown detailed relevant information about chemical composition, including specific osmoprotectants, directly related to physiological and biochemical changes, and have shed light on how these changes reflect the plant phenotype. Metabolomic studies are impacting both basic and applied research. Metabolomic studies will generate knowledge regarding how plant metabolism is differentially adjusted in relation to a specific stress and whether metabolic adjustments are stress specific or common to different types of stress. These studies will also reveal how metabolic pathways coordinate their fluxes and enzymes activities in order to strength their cellular energy requirements under stressing conditions. In an applied context, metabolomic approaches are providing a broader, deeper, and an integral perspective of metabolic profiles in the acclimation plant response to stressing environments. This information will reveal metabotypes with potential to be transferred to sensitive, economically important crops and will allow design of strategies to improve the adaptation of plants towards adverse conditions. Ultimately, design strategies will consider plant metabolism as a whole set of interconnected biochemical networks and not as sections of reactions that lead to the accumulation of a final metabolite. The task is challenging as it must take into account that reactions to stress course through a complex metabolic response, including different systematic mechanisms, time-course changes, and stress-dose dependences. Moreover, there are differences among plant tissues, and, as expected, marked differences

between plants at the genus and species levels, exposing intimate correlation with genetic backgrounds. Nevertheless, the application of more advanced metabolomics tools will lead to new knowledge that will accelerate the design and the improvement of plant breeding projects, that surely will lead to the next generation of crops for specific applications in particular circumstances to cope with abiotic and biotic stress on agricultural crops worldwide.

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