Mohammad Anwar Hossain
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Drought Stress Tolerance in Plants, Volume 2

Molecular and Genetic Perspectives

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Preface

In nature, plants are frequently exposed to various abiotic stresses such as drought, extreme temperatures, submergence, high salt levels, heavy metals, mineral deficiency, and toxicity. Plants are seldom exposed to a single abiotic stress, but are more frequently exposed to multiple stressors. For instance, high temperatures and drought are commonly encountered together, and the impact of these two stressors on plants can be aggravated by mineral toxicities. It has been projected that abiotic stressors may adversely affect yields in up to 70 % of staple food crops. Among the various abiotic stresses, drought is the most common and lethal for plants, particularly in critical growth stages, and may result in the complete failure of crops. Conventional breeding approaches, such as selection, hybridization, hybrid breeding, wide hybridization, and ideotype breeding, have been used in the past and have resulted in the development of abiotic stress-tolerant crop varieties. However, given the increasing demand of food due to an increasing world population, particularly in Asia, the pace of conventionally bred varieties is very slow. Drought is a complex trait and is governed by a number of genes with complex interactions and low heritability, and thus is hard to investigate. Additionally, the utilization of wild relatives for the development of drought-tolerant crop varieties has been slow due to cross incompatibility, the complex genetic nature of drought resistance, and cumbersome breeding and phenotypic procedures. Advances in molecular genetics have revealed complex cascades of events at the cellular level that control the adaptation of crop plants to drought, and that numerous genes are involved in the initiation of abiotic stress-related defenses. These genes can be divided into three major categories. The first group consists of genes concerned with direct protection of essential proteins and membranes, such as osmoprotectants, free radical scavengers, late embryogenesis abundant (LEA) proteins, heat shock proteins, and chaperones. The second group comprises membrane transporters and ion channels, involved in water and ion uptake. The third group consists of regulatory proteins, including kinases and transcription factors that are involved in transcriptional regulation of stress-related genes. These transcription factors are distributed in several families, such as the MYB, bHLH, bZIP, NAC, and AP2/EREBP families.

In the past few years, considerable effort has gone into deciphering the mechanisms underlying plant responses to water deficit with the aim to develop crop plants resilient to drought stress. In this book *Drought Tolerance in Plants, Vol 2: Molecular and Genetic Perspectives* we present a collection of 21 chapters written by experts in the field of drought tolerance in plants. It is a timely contribution to a topic that is of great importance for future food security. Chapter 1 describes our current understanding of plant drought responses from the gene to the whole plant. In this chapter, the authors review, in depth, morphological, physiological, biochemical, and molecular mechanisms associated with drought responses and adaptations of crop plants. Chapter 2 describes the genetic basis of cellular and developmental mechanisms and traits conferring drought stress tolerance. Chapter 3 aims to uncover the process of drought signal perception, amplification, and transduction, and to help readers understand the convergent signaling networks in plants exposed to multiple stressors. Chapter 4 deals with the molecular adaptation strategies of plants under drought stress. The authors discuss signaling molecules, transcription factors, drought-responsive genes, and the regulation of gene expression associated with the modulation of drought stress tolerance. Chapter 5 unravels the recent advances in decoding the ABA signaling pathways in plant cells that are involved in drought tolerance. Chapter 6 provides a comprehensive overview of plant responses to drought at the genetic level. The authors critically discuss plant transcriptomic studies investigating drought responses in model plants and how transcriptomic data can be used to evaluate drought tolerance in plants. Chapter 7 provides an in-depth overview of metabolomic studies related to drought responses, discussing preparation techniques for metabolomics, analytical advances, metabolic responses to drought stress, and metabolic engineering of compatible solutes for drought tolerance in plants, as well as the future of metabolomics as a tool to study drought tolerance. Chapter 8 summarizes the importance of microRNAs, including drought-responsive miRNAs and their targets, and the strategies to use miRNAs to enhance plant drought tolerance. Chapter 9 deals with the chloroplastic proteomics of plants in response to drought, salinity, heat, light, and ozone stress. The authors critically discuss the importance of alteration of protein structure under various abiotic stresses. Chapter 10 is concerned with the metabolic responses of plants under drought and other abiotic stress conditions and concentrates on the importance of stress duration and intensity, as well as the importance of developmental stage. Chapter 11 provides an overview of the various definitions of drought, basic principles of plant water relations, and the similarities and differences of drought responses and tolerance in wheat and barley genotypes at different developmental stages, at physiological, biochemical, and molecular levels. In this chapter, the authors provide a brief account of breeding strategies and the potential of molecular approaches to enhance drought tolerance in wheat and barley. Chapter 12 discusses common stress-responsive transcription factors, their interaction networks and epigenetic control, bioinformatics studies, and the molecular modification of transcription factors involved in abiotic stress tolerance. Chapter 13 addresses the historical development of mutation breeding, mutation breeding strategies for various plant species, including tilling and eco-tilling, the

application of mutation breeding for the improvement of quantitative traits, breeding strategies for developing drought-tolerant crop plants, and future perspectives for mutation breeding. Chapter 14 describes various methods to identify candidate genes for drought responses and tolerance, and provides a list of candidate genes from model to cultivated crop plants, which might be used for the improvement of drought tolerance by genetic engineering. Chapter 15 deals with the principles of microarray, gene expression profiling, and gene ontology enrichment analysis under drought stress and the future applications of these techniques. Chapter 16 deals with system biology approaches for drought stress tolerance, which include transcriptome reprogramming under drought, proteomic insights, the crucial roles of metabolomics and transcriptomics, and the quest for systems biology approaches that can be used to understand plant adaptation to drought. Chapter 17 represents a comprehensive overview of oxidative stress and reactive oxygen species (ROS), ROS signal transduction pathways, the effects of ROS on plant growth and metabolism, transgenic plants with higher enzymatic and nonenzymatic defense systems, and their tolerance to drought stress and future perspectives. Chapter 18 investigates the potential for engineering glycine betaine (GB) metabolism for drought tolerance. In this chapter, the authors summarize the biosynthesis of GB, genetically engineered biosynthesis of GB for drought tolerance, the roles of GB in drought tolerance, and GB-induced expression of genes associated with drought tolerance. Chapters 19 and 20 overviews the transgenic approach to produce drought-tolerant plants, including past achievements, challenges, and perspectives. In these chapters, the authors discuss the examples of genetically engineered crops for drought tolerance, including the environmental and food safety assessment of genetically modified crops. Chapter 21 discusses chromatin and drought tolerance. In this chapter, the authors present a comprehensive discussion of chromatin, transcriptional control of drought stress via chromatin modifying genes, and future strategies for chromatin control of sustainable drought tolerance in crop plants.

We hope that this volume will be helpful in building approaches to combat drought stress in plants. This volume will, it is hoped, serve as a key source of information and knowledge to graduate and postgraduate students, teachers, and abiotic stress researchers around the globe. We also believe that it will be of interest to a wide range of plant scientists, including plant breeders, biotechnologists, molecular biologists, agronomists, and physiologists who are interested in drought responses and tolerance of crop plants. This book would not have been possible without the contributions of the experts who were eager to share their knowledge in molecular and genetic perspectives in drought stress, and our heartiest gratitude to all of them. We would like to extend thanks to Dr. Kenneth Teng, the editorial staff of Springer, New York in enabling this book project. Finally, our special thanks to all of the staff members of Springer, Switzerland who are directly or indirectly

associated with us in the book project for their steady support and efforts for the timely publication of this volume. We strongly believe that the information covered in this book will make a sound contribution to this fascinating area of research.

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Chapter 1
Understanding How Plants Respond to Drought Stress at the Molecular and Whole Plant Levels

Nezar H. Samarah

Abbreviations

A	Photosynthetic rate
ABA	Abscisic acid
ABFs	ABRE- binding factors
ABRE	ABA-responsive element
ACC	1-aminocyclopropane-1-carboxylic acid
AP2	APETALA 2
APX	Ascorbate peroxidase
AREB	ABRE-binding protein
AsA	Reduced ascorbate
ASH	Ascorbic acid
bZIP	Two basic leucine zipper transcription factors
CAT	Catalase
CBF	CRT binding factor
CRT	C-repeat
DA	Drought avoidance
DAG-PP	Diacylglycerol-pyrophosphate
DE	Drought escape
Dehydrin	Dehydration-induced
DHAR	Dehydroascorbate reductase
DRE	Dehydration-responsive element
DREB	DRE-binding protein
DT	Drought tolerance
E	Transpiration rate
ERF	Ethylene-responsive element binding factor
ETC	Electron transport chain
EUW	Effective use of water
GOPX	Guaicol peroxidase

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GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione-S- transferase
H_2O_2	Hydrogen peroxide
HI	Harvest index
LEA	Late embryogenesis proteins
LOX1	Lipoxygenase 1
LWP	Leaf water potential
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MDAR	Monodehydroascorbate reductase
MDHAR	Monodehydroascorbate reductase
NAC	NAM, ATAF, CUC
NACR	NAC recognition sequence
NO	Nitric oxide
$O_2^{\cdot-}$	Superoxide anion
$^1O_2$	Singlet oxygen
OA	Osmotic adjustment
$^{\cdot}OH$	Hydroxyl radicals
P5CS	Delta (1)-pyrroline-5-carboxylate synthetase
PA	Phosphatidic acid
PCK	Phosphoenolpyruvate carboxylase
PEG	Polyethylene glycol
PLD	Phospholipase D
POD	Peroxidase
PP2C	2C-type protein phosphatase
PPDK	Pyruvate orthophosphate dikinase
PSI	Photosystem I
PSII	Photosystem II
PYL	Pyrabactin-resistance like
PYR	Pyrabactin resistance
QTL	Quantitative trait locus
RAB	Response to ABA
RCAB	Regulatory component of ABA receptor
ROS	Reactive oxygen species
Rubisco	Ribulose 1,5 bisphosphate carboxylase/oxygenase
RuBP	Ribulose 1,5 bisphosphate
SnRK2	Sucrose non-fermenting 1-related protein kinase
SOD	Superoxide dismutase
WUE	Water use efficiency

1.1 Introduction

With climate change and abnormal weather events, more frequent drought and other stresses such as heat and salinity are likely to occur all over the world [169, 185, 229]. Drought stress reduces plant growth and crop production [40, 90].

Drought stress induces a range of morphological, physiological, biochemical, and molecular changes in plants [178]. Plant growth (leaf expansion and size, leaf area index, plant height, plant branching, and plant tiller numbers) has been reduced when drought stress was imposed on plants during vegetative growth stages [92]. Under severe drought stress, plants senesce their leaves to reduce transpiration rate and water consumption [8, 165]. Drought has been reported to enhance plant growth and development, shorten the duration of the seed filling period, and remobilize the reserves in plant parts to growing seeds, resulting in a great reduction in the duration of the photosynthetic capacity of plants and lowering seed yield [34, 77, 198].

At the physiological and biochemical level, drought stress induces stomatal closure and consequently decreases photosynthetic rate, stomatal conductance, and transpiration rate [54, 121, 154]. Drought also inhibits the biochemistry of photosynthesis [141, 214]. Soluble solutes accumulate in plants under drought stress to maintain plant turgor pressure at a lower leaf water potential (LWP) in a process known as an osmotic adjustment (OA) [56, 208]. Production of reactive oxygen species (ROS) under drought stress can cause oxidative damages to lipids, cell nucleic acid, and proteins [22, 106]. Plants respond to the increase in ROS by producing nonenzymatic antioxidants or enzymatic defense (detoxification and scavenging enzymes) to prevent or reduce the oxidative damages of the ROS under drought stress [10, 106, 122, 132, 161, 240]. Protein synthesis is also changed in response to drought. Dehydrin proteins (Class II of LEA proteins) are induced in response to drought stress and have been shown to function in drought tolerance (DT) [38, 94, 123].

Plants also respond to drought at the molecular level. Drought stress induces the accumulation of ABA [131]. Drought stress and ABA induce drought-responsive and ABA-responsive genes mediated through ABA-independent and ABA-dependent regulatory pathways [23, 41, 211]. Other important signaling transduction pathways mediate drought-related gene expression such as strigolactone hormone [118], ROS [15, 113], lipid derived signaling [113, 167], soluble sugars, and others [55, 99]. Transcription factors such as ABREB/ABF, DREB1/CBF, DREB2, and NAC play an important role in the regulatory network of drought-related genes [169]. The drought-inducible genes include genes encoding important functional and regulatory proteins [189, 211, 212]. Genetically engineering plants to express the drought-inducible genes has shown drought stress tolerance and can be a promising tool to improve DT in plants [138, 229, 238].

Development of crops for enhanced drought resistance requires knowledge of morphological and physiological responses and genetic control of the contributing traits at different plant developmental stages [92]. This chapter summarizes

whole plant growth and yield performance under drought to understand plant DT at the physiological and molecular levels. Understanding plant responses to drought at the morphological, physiological, and molecular levels and how these changes ameliorate the effect of drought stress on plant productivity is needed to improve plant stress tolerance using biotechnology, while maintaining the yield and quality of crop [28, 178]. Using a genetic engineering approach to produce transgenic plants by transferring a specific drought-related gene can improve DT in plants.

1.2 Drought Resistance and Adaptation Mechanisms

Drought resistance is a broad term applied to both wild species and crop plants with adapted traits that enable them to cope with water shortage [19]. Drought resistance mechanisms in plants have been classified into: (1) drought escape (DE) (the ability of plants to complete their life cycle in the presence of water before the onset of drought stress); (2) drought avoidance (DA) (the ability of plants to maintain tissue hydrated; DT at high water potential); (3) DT (the ability of plants to function while dehydrated; DT at low water potential; desiccation tolerance) [90, 220]. Plants that escape drought, such as desert ephemerals, annual crops, and pasture plants, exhibit earlier flowering, shorter plant life cycle, and developmental plasticity [20, 210]. *Brassica rapa* plants escape drought through early flowering rather than avoid drought through increased water-use efficiency [100]. *B. rapa* plants grown from seeds collected from natural populations after five consecutive years of drought had an evolutionary shift to a DE mechanism in which plants had an earlier flowering [100]. This mechanism of DE was related to lower water-use efficiency (high transpiration and inefficient water use), leading to rapid development and shortened growing season [100]. With respect to the DA mechanism, plants have two important strategies: enhancing water uptake from soil (roots traits); and reducing water loss from plants (stomatal characteristics and morph-anatomical traits such as leaf rolling, dense leaf pubescence, thick cuticle and epicuticular wax layer, heavily lignified tissue, smaller mesophyll cell and less intercellular spaces, reduced plant growth, and leaf senescence) [20]. Root growth rate, root volume, root depth, and root dry weight are traits related to DA [237]. In the third mechanism of plant resistance to drought (DT), plants can tolerate drought through OA, antioxidant defense mechanisms, dehydrins and late embryogenesis abundant (LEA) proteins, ABA response, desiccation tolerance [237], and water-use efficiency [measured as photosynthetic carbon gain (A) over transpiration water loss (stomatal conductance, g)] [20, 100]. Two water use strategies may be employed by woody plants: prodigal water use behavior (beneficial in a short interruption of water supply), and conservative water use behavior (beneficial in a long-term drought) [20]. In the first strategy (prodigal water use), plants had a high stomatal conductance, high carbon exchange rate, low water use efficiency, and plants grow faster [20]. In the second strategy (conservative water use), plants have higher water use efficiency, high capacity for drought resistance, and slow growth rate [20]. Thus higher

drought resistance is sometimes linked to even or lower water use efficiency; however, in other cases, higher drought resistance is associated with high water use efficiency, indicating that drought resistance and water use efficiency are not synonymous terms [20]. It is impossible to assess the relative contributions of DE, DA, and DT to overall drought resistance at the whole-plant level in rice [237]. DT and DA traits had a distinct genetic basis [237]. The genetic variation in DE and avoidance in natural herbaceous populations is complex and controlled by many quantitative trait loci (QTL) of small effect, and gene × environment interactions, indicating genetic constraints limit the concurrent evolution of both DE and avoidance strategies [136].

In arid and semiarid regions where rainfall occurs during winter and spring and no rainfall during summer (dry condition), plants use different drought resistant mechanisms to cope with drought stress in these regions. In the DE mechanism, rapid phenological development of plants ensures that flowering and grain filling occur before the onset of water shortage and high temperature, which prematurely terminate the plant life cycle and reduce yield. Improved reproductive success by DE also includes better partitioning of assimilates and stored reserves from stem and root to developing fruits and seeds [20]. The acceleration of maturity (phenological adjustment), coupled with a high seed filling rate (shoot biomass distribution), reduced the negative effect of drought stress in the drought-resistant common bean cultivars (*Phaseolus vulgaris* L.) [196]. Despite the advantage of DE during the water shortage seasons, DE limits yield in years with plentiful rainfall. Other drought resistant mechanisms in arid and semiarid regions including an early vigor, a greater number of fertile flowers, a longer duration of seed growth, an increased harvest index (HI), an OA, a high assimilate transfer to the seed, a rapid grain growth, and high water-use efficiency have been shown to improve cereal yield in these water-limited regions [16, 19]. The ability of plants to save water and retain some residual soil moisture to the end of the season may also contribute to better yield [19]. In a Mediterranean legume crop such as lupine (*Lupinus luteus* L.), contrasting adaptive strategies to terminal drought-stress have been reported. Long-season, high-rainfall habitats select for traits (competitive traits) that are related to delayed phenology, high biomass, and productivity, leading to high water use and early stress onset, whereas terminal drought-prone environments select for the opposite traits (ruderal traits) that facilitate DE/avoidance but limit reproductive potential [30].

In subhumid regions where rainfall occurs during summer, the ability of summer crops to develop a rapid growth and flowering over an extended period (indeterminate growth habit) may reduce their vulnerability to drought stress compared with crops that set their flowering at a specific period (determinate growth habit). Indeterminate cultivars of soybean were better able to recover from water stress imposed preflowering and during early pod development and had greater seed yield after recovery from stress treatment than were determinate ones [228]. This increase in the yield of the indeterminate cultivars was associated with more pods per node rather than with other yield components, or with leaf area index or leaf area duration differences [228]. The indeterminate cultivars of cotton (*Gossypium hirsutum* L.)

had higher lint yields and irrigation water-use efficiency than did the determinate cultivars at the intermediate moisture level, suggesting that cotton cultivars with relatively indeterminate growth habit are better adapted to environments with limited soil moisture than cotton cultivars with relatively determinate growth habit [188].

1.2.1 Morphological Adaptation

An early response to drought stress is a reduction in plant growth. Plant growth is an irreversible process including cell division, cell elongation, and differentiation. Leaf elongation occurs as a balance among cell turgor pressure, cell wall threshold, and cell wall extensibility [220]. Under drought stress, cell turgor is less than cell wall threshold, which results in a reduction in leaf elongation and size and a retardation of plant growth [220]. Drought stress reduces leaf size, stem extension, and root proliferation [92]. Impaired enzyme activities, loss of turgor, and decreased energy supply under drought stress results in a reduction in both cell division and elongation [93, 220]. Loss of turgor under drought decreased growth and productivity of sunflower (*Heliantus annuus* L.) due to reductions in LWP, rate of cell division, and elongation [129]. Drought significantly reduced shoot and root dry weights in Asian red sage (*Salvia miltiorrhiza* L.), but the shoots were more affected than roots [147]. Although severe drought stress terminated root growth earlier and significantly decreased the rate of root growth as a result of inhibition of both cell elongation and cell production in a Sonoran desert cactus (*Pachycereus pringlei*, Cactaceae), the total number of lateral roots and primordia was the same under severe and well-watered conditions [81]. These results indicated that lateral root formation is a stable developmental process resistant to severe water stress and that water stress accelerates the determinate developmental program of the primary root, which are two important features for successful seedling establishment in a desert [81].

Another plant response to drought stress is leaf senescence and abscission [8] which is also an important factor in determining seed yield. Leaf senescence, mediated by enhancing the synthesis of endogenous plant hormone ethylene under drought stress [9, 12], determines the duration of photosynthesis and the seed filling period [13]. When drought stress occurred during late reproductive growth, leaf senescence was accelerated [67, 165]. Although leaf senescence under drought can be an adaptive mechanism to drought by reducing plant water consumption, leaf senescence reduces plant yield under drought.

Pandey et al. [182] reported that the increase in drought intensity decreased growth parameters such as leaf area index, leaf area duration, crop growth rate, and shoot dry matter of grain legumes. The reduction in leaf area under drought was due to the loss of turgor and reduced leaf number [91]. Seed yield of grain legumes was positively correlated with leaf area index, leaf area duration, crop growth rate, and shoot dry matter [182]. Black bean (*Phaseolus vulgaris* L.) exposed to water stress during vegetative growth had lower plant height and leaf area index than did

well-watered plants [173]. Dry conditions resulted in 68 % reduction in leaf area index of soybean plants compared to irrigated conditions [70]. Drought decreased leaf expansion, tiller formation, and leaf area due to early senescence [129, 137, 175]. This reduction in leaf expansion and growth can be a way to adapt to drought by reducing plant water consumption (plant survival), but the reduction in plant growth under drought is related to the reduction in seed yield.

Drought stress has been reported to reduce seed yield and yield components and the intensity of this reduction depends on the growth stage at which drought occurs, showing that plants are most susceptible to water stress at the reproductive growth stage [198]. Drought stress imposed on soybean during vegetative growth decreased internode length and plant height [76]. When drought occurred during vegetative stages of rice, drought had only a small effect on subsequent plant development and grain yield, resulting in up to 30 % reduction in yield due to reduced panicle number per unit area or reduced number of spikelets per panicle [34]. Drought stress early during the reproductive stage can delay or inhibit flowering. In most studied species, the most stress-sensitive reproductive stage is the stage of meiosis [198]. In the mitotic stage of flowering, drought causes pollen sterility and only affects female fertility when stress is severe [14, 198]. When drought stress was imposed during the reproductive growth stage of wheat, pollen fertility was most affected [187]. The most sensitive stage of wheat yield to drought stress is in the early spikelet development (5 d after jointing) [187]. The rice and maize plants are highly susceptible to drought during flowering (anthesis) and early grain initiation, causing pollen sterility, failure of pollination, spikelet death, and zygotic abortion due to changes in carbohydrate availability and metabolism [172, 198]. Drought stress conditions modify source-sink relations by inducing premature senescence in the photosynthetic source tissues of the plant, by reducing the number and growth of the harvestable sink organs, and by affecting the transport and use of assimilates, thereby influencing plant growth, adaptive responses, and consequently crop yield [12, 13]. Drought stress strongly reduced the cell wall invertases (cwInv) activities, key metabolic enzymes regulating sink activity through the hydrolytic cleavage of sucrose into hexose monomers [12]. The most sensitive stage to drought in rice was observed when stress occurred during panicle development [34, 187], resulting in delayed anthesis, reduced number of spikelets per panicle, decreased percentage of filled grains, and consequently reducing grain yield to less than 20 % of the control (irrigated plants) [34]. Drought stress during pod lengthening (R_3–R_5) of soybean decreased the number of pods per vegetative dry matter unit [76]. Drought stress during grain development and filling shortened the grain filling period (prematurely terminated) and reduced seed size and weight [198]. Drought stress during early seed-fill of soybean [*Glycine max* (L.) Merr.] reduced the number of seeds per pod, whereas late stress during the seed filling period (after the abortion limit stage) decreased seed weight [76]. Drought stress during grain filling of rice hastened the plant maturity and reduced the percentage of filled grain and individual grain weight [34]. Genetic control of yield under reproductive-stage drought stress in rice showed that a QTL (*qtl12.1*) had a large effect on grain yield under stress, increased HI, biomass yield, and plant height

while reducing the number of days to flowering [31]. In barley, drought stress during the grain filling period enhanced the grain growth rate, shortened the grain filling duration, decreased grain yield by reducing the number of tillers, spikes, and grains per plant, and individual grain weight, indicating that postanthesis drought stress was detrimental to grain yield regardless of the stress severity [201, 202, 205]. Late-terminal drought stress (rainfed) shortened reproductive growth duration and fastened maturity of chickpea plants, resulting in a decrease in seed yield by 49–54 % compared with irrigated plants, indicating that drought during the reproductive growth stage was detrimental to all genotypes studied (desi and kabuli) [206]. Other researchers have shown that desi chickpea genotypes (smaller, dark seeds) were generally more drought and heat tolerant than kabuli chickpea genotypes (larger, pale seeds) [44]. In muskmelon (*Cucumis melo* var. *reticulatus*) plants grown under three irrigation levels [0.5, 0.75, 1.0 actual evapotranspiration (AET)], decreasing the irrigation level decreased the length, diameter, weight, Brix, flesh firmness, seeds, and fertile seeds of melon fruit [11]. The best irrigation level for getting the highest total fruit yield was at 0.75 AET [11]. Drought stress during reproductive stages reduces photosynthetic rate, assimilates partitioning to expanding cells, which increases flower and pod abortion and decreases vegetative growth, duration of the seed filling stage, seed number, seed size, and consequently decreases total seed yield [127].

1.2.2 Physiological and Biochemical Adaptation

1.2.2.1 Controlling Guard Cell Behavior and Leaf Water Status

An early response of plants to drought stress is the closure of their stomata to prevent transpiration water loss and leaf dehydration [52, 54, 154], affecting all plant water relations. The stomata closure under drought stress can result from a decrease in leaf turgor and water potential [152] or from low relative humidity of the atmosphere [156]. Under drought stress, stomata closure is considered as a first step to adapt to drought by maintaining cell turgor to continue plant metabolism [146] and to prevent the risk of losing its water transport capacity [133]. Drought stress decreased the relative leaf water content, the LWP, and the transpiration rate and concomitantly increased the leaf temperature in wheat and rice [92, 213]. Drought stress also reduced the relative leaf water content and transpiration rate in other crop species such as barley, soybean, and triticale [146, 195]. The stomatal closure and the decrease in stomatal conductance and transpiration rate under drought stress have been related to higher water-use efficiency (the ratio of dry matter produced to water consumed) in wheat [1], clover (*Trifolium alexandrium*) [143]), and alfalfa (*Medicago sativa*) [142]. Drought tolerant species improve the water-use efficiency by controlling the stomatal function to allow carbon fixation at stress and reduce the water loss [92, 236]. However, in the case of severe drought stress where plant growth and biomass accumulation are greatly diminished, the water-use efficiency is

also reduced [69]. Most plants tend to show an increase in water-use efficiency when drought stress is mild [54]. Although water-use efficiency is often considered an important determinant of yield under stress and even as a component of crop drought resistance, selection for high WUE in breeding for water-limited conditions most likely leads, under most conditions, to reduced yield and reduced drought resistance [33]. Because biomass production is closely linked to transpiration, breeding for effective use of water (EUW; implies maximal soil moisture capture for transpiration, reduced nonstomatal transpiration, and minimal water loss by soil evaporation) is the most important target for yield improvement under drought stress by improving plant water status and sustaining assimilate partitions and reproductive success (HI) [33].

Stomatal closure is mediated by the plant hormone ABA [73, 163]. Biosynthesis of ABA is triggered by a decrease in soil water content and plant turgor [78]. During soil drying, ABA is synthesized in the roots and transported by the xylem to the shoot to inhibit leaf expansion and induce stomatal closure before the change in leaf water status [78, 114, 209]. ABA abundance in the xylem sap of field-grown grapevines was correlated with stomatal conductance [218]. The expression of genes associated with ABA synthesis (the 9-*cis*-epoxycarotenoid dioxygenase), *NCED1* and *NCED2*, was higher in the roots than in the leaves, especially when soil moisture declined and vapor pressure deficit increased [218]. Their expression in roots was correlated with ABA abundance in the roots, xylem sap, and leaves [218]. The results provide evidence that ABA plays an important role in linking stomatal response to soil moisture status [218].

Other hormones in addition to ABA are involved in the regulation of stomatal closure. Increased cytokinin concentration in the xylem promoted stomatal opening by decreasing the stomatal sensitivity to ABA [230], whereas the decrease in the root cytokinins was concomitant with the increase in xylem ABA and the reduction in stomatal conductance [219]. Plant hormones such as ABA, auxin, cytokinins, ethylene, and gibberellins have been shown to be involved in plant response to different environmental stresses [78, 209].

A hydraulic signaling also contributes to plant response to drought stress. Stomata respond to the rate of water loss from the leaf (evaporation demand), the change in the rate of water supply from soil, the change in xylem conductance, and to the change in leaf turgor, which can be translated to a signal to regulate stomatal aperture [43, 59, 156, 170]. The decline in root water uptake and then water potential and turgor in the leaves can lead to stomatal closure and a decrease in leaf elongation [43, 59].

1.2.2.2 Modulation of Photosynthetic Behavior Under Drought

Photosynthesis is another primary process to be inhibited by drought stress [52]. The reduction in photosynthesis under drought can result directly from stomatal and nonstomatal limitation of photosynthesis, and/or indirectly as a result of oxidative stress [53, 105, 155] (Fig. 1.1). Studies have shown that stomatal limitations could

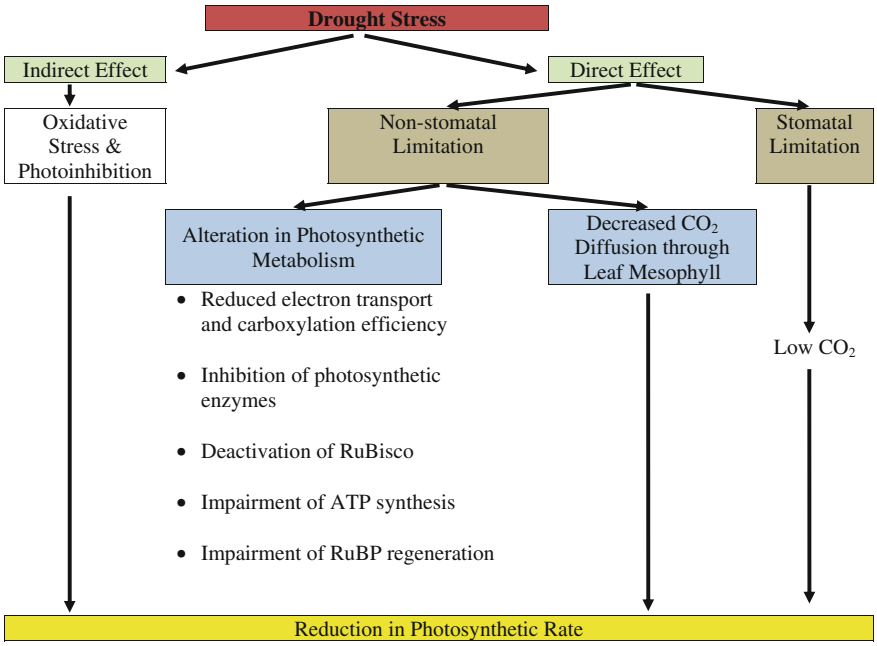

Fig. 1.1 Direct and indirect effect of drought stress on photosynthetic rate in plants. *RuBP* Ribulose 1,5 bisphosphate; *RuBisco* Ribulose 1,5 bisphosphate carboxylase/oxygenase

largely reduce CO_2 assimilation under mild to moderate drought, whereas non-stomatal limitations could account for a larger part under severe drought stress [95, 105, 116]. In stomatal limitation of photosynthesis, stomata closure occurs in response to the decline in leaf turgor and results in a decrease in CO_2 availability in the leaf intercellular air spaces [95, 96, 105].

In addition to stomatal limitation of photosynthesis, nonstomatal limitation by the decrease in CO_2 diffusion through the leaf mesophyll (a reduced mesophyll conductance) [95, 96] or alteration in photosynthetic metabolism [66, 141, 214] plays a role in determining the photosynthesis capacity under drought stress. Mesophyll conductance was depressed under drought stress [102]. The change in mesophyll conductance may be linked to alteration in the structure of the intercellular spaces due to the leaf shrinkage [141] or to the internal resistance to CO_2 diffusion in the liquid phase inside cells [103]. Other studies suggested that biochemical factors also induce changes in mesophyll conductance such as exogenous application of ABA, temperature, light, and CO_2 concentration [97, 98]. The other nonstomatal limitation of photosynthesis under drought is attributed to the alteration in photosynthetic metabolism in response to the lowered supply of CO_2 under prolonged stress [55]. These alterations in photosynthetic metabolism under drought stress include the inhibition of the photosynthetic enzymes and the synthesis of ATP and the deactivation of carboxylation enzyme RuBisco by the low intercellular CO_2 [37, 159, 191]

or by the presence of tight-binding inhibitors [186]. The ATP synthesis and regeneration of ribulose 1,5 bisphosphate (RuBP) impair photosynthesis under mild drought stress [140, 141, 224]. The decrease in the activity of enzymes of the Calvin cycle has been observed in plants that are slowly exposed to a prolonged drought stress [155]. Drought stress suppresses photochemical efficiency of photosystem PSII by decreasing electron transport [150]. Meyer and Genty [160] reported that dehydration and ABA treatment decreased the electron transport rate, which was mediated by the Rubisco deactivation. Drought stress caused only small differences in the maximum efficiency of photosystem II (F_v/F_m), an indicator of the intactness of the photosynthesis electron transport [214], but had a more pronounced effect on carboxylation velocity of RuBisco ($V_{c,\max}$) in selected grassland species, decreasing the $V_{c,\max}$ of *Phleum pratense* by 20 % [214]. In addition to stomatal limitation, drought stress caused damage to photosystem II (PSII), reduced electron transport and carboxylation efficiencies of barley plants under mild drought stress [105]. Under severe drought stress, there were structural and biochemical impairment of light-dependent reactions as well as carboxylation process of photosynthesis and an acceleration of photoinhibition [105].

Stomatal limitation of CO_2 availability under drought stress could possibly lead to increased susceptibility to photodamage [66] and oxidative stress caused by the accumulation of ROS [191]. Plant mechanisms to protect the photosynthetic apparatus from the photodamage under drought are by diverting the absorbed light from photochemistry to thermal dissipation involving the xanthophyll cycle [75], or by photorespiration as suggested by its increase under drought stress [54, 124]. The rate of photorespiration in the tolerant C_3 plant *Pancratium maritimum* L. was clearly higher under moderate salt and drought stresses than under severe stresses [3]. The oxidative stress, as measured by malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), was higher under severe stresses than under moderate stresses [3]. In spite of the upregulation of photorespiration under moderate stresses, the level of the oxidative stress was lower than the severe stresses because of the efficient upregulation of detoxification enzymes, catalase (CAT), and peroxidase (POD), under moderate stresses, indicating that photorespiration may not be a major contributor to the oxidative load under salt and drought stresses [3].

Photosynthetic capacity might be a factor in determining plant tolerance to water stress. Working with two cotton cultivars, TAMCO HQ95 and G&P 74 +, Gerik et al. [104] reported that the ability of HQ95 to yield more under drought stress could be due to its higher intrinsic photosynthetic capacity and carbon partitioning to the boll (higher HI). In maize, accumulated net photosynthesis across new and old hybrids was lower in drought stress treatments than well-watered treatments [174]. New maize hybrids had higher accumulated net canopy photosynthesis under drought stress and recovered faster after rehydration than old hybrids, indicating that new hybrids were more drought tolerant than old hybrids [174].

1.2.2.3 Osmotic Adjustment Through Synthesis of Osmolytes Under Drought

OA is a biochemical mechanism that helps plants tolerate water stress and saline conditions [56, 208]. In osmotic adjusted plants, the accumulation of organic solutes, known as compatible solutes, in the cell such as proline, glycine betaine, sugar alcohol, and sorbitol can improve cell hydration, maintaining cell turgor at lower water potential so the plant can survive longer and maintain metabolic processes in drying soil [27, 56, 208, 220]. Other inorganic ions mainly Na^+, K^+, Ca^{2+}, and Cl^- make a great contribution in OA by ion transport processes with related ion antiporters and ion channels [56]. Drought-stress treatments increased concentrations of phosphorus (P), potassium (K), calcium (Ca), molybdenum (Mo), manganese (Mn), copper (Cu), and zinc (Zn) in mature soybean seeds above well-watered treatment [204]. The K(+) uptake transporters 6 (KUP6) are regulated directly via an ABA signaling complex and act as key factors in OA by balancing potassium homeostasis in cell growth and drought stress responses [177]. Other regulatory processes of plant adaptation to drought stress involve control of cell OA by synthesis of osmoprotectants [7, 49]. Under various stresses, the concentration of proline in plants increased up to 80 % of the amino acid pool compared with a concentration of less than 5 % in plants under unstressed conditions [158]. Drought stress treatment increased the accumulation of compatible solutes including proline, glycine betaine, and free amino acid in *Panicum sumatrense* at 70 d after seeding compared to the control [10]. Drought stress altered sugar metabolism and increased the concentration of hexose sugars, cyclitol (scylloinositol), and proline by 3.8-, 1.5-, and 25-fold expressed on unit dry weight, respectively, suggesting that altered solute partitioning and OA may be an important factor in DT of *Ziziphus mauritiana* [60]. Gradual and severe drought reduced the concentration of sucrose in the full, rounded large seeds of soybean compared with well-watered treatment [203]. During gradual drought stress, there was a 65 % increase in bulk tissue elastic modulus (wall rigidity) coupled with OA [60]. This increase in bulk tissue elastic modulus resulted in turgor loss at the same relative water content in both stressed and unstressed leaves [60]. The OA enables plants to have a higher transpiration rate at lower LWP and to extract more water from drier soil [220]. OA also enables plants to sustain a high photosynthetic rate and expansion growth under drought stress [47]. Aside from maintaining cell hydration and turgor, organic solutes, such as proline and glycine betaine, can also assist in keeping functional macromolecules in solution, protect the cell membrane, stabilize enzymes and proteins, and protect against oxidation [18, 57, 162, 199, 215, 226, 232]. Proline plays a role as osmolyte, a reservoir of carbon and nitrogen, and has been shown to protect plants against radical-induced damage [158].

In particular crop species, OA has positively affected growth and yield under drought stress [208]. Under drought stress conditions, tolerant genotypes of sunflower had delayed wilting and maintained turgor at lower LWP than sensitive genotypes [181]. OA was observed in grass species [32] and soybean plants grown under water stress [68]. OA in wheat plants was evaluated based on plotting turgor

pressure as a function of LWP in a linear slope except in the region of high water potential [164]. The results indicated that wheat cultivars with steeper slope had greater OA. The OA was greater at the tillering stage than at the heading stage for all cultivars tested [164]. It was not clear; however, whether the differences among cultivars in allocation of biomass to grain under water stress were due to OA [164]. A drought-tolerant soybean genotype PI416937 (PI) maintained turgidity during the seed-filling stage [46] and had higher transpiration and net carbon exchange rates, and higher yield [217] under drought stress. DT in the PI genotype was associated with greater mass, volume, and surface area of root [128], lower solute potential, higher pressure potential and relative water content of leaf (OA) [217], and more tolerance to high level of soil Al [112]). Genotypic variation among inbred lines of rice for the maintenance of LWP and OA under drought stress suggested that variation in OA was not related to grain yield nor yield components; however, traits contributing to the maintenance of high LWP, minimized the negative effects of water deficit on spikelet sterility, and consequently improved grain yield [134]. OA was not associated with higher yield for soybean plants grown under water stress [68]. Recent advances in biotechnology have shown that transgenic plants with a high accumulation of proline and glycine betaine had enhanced DT mainly due to protection mechanisms against oxidative stress and not necessarily caused by OA [57, 162, 226, 232].

1.2.2.4 Antioxidative Defense and Mitigation of Oxidative Stress Under Drought

Drought stress accelerates the production of ROS such as singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$), and nitric oxide (NO) [122]. Excess generation and accumulation of ROS cause oxidative damage in the apoplastic compartments and damage of cellular membranes by lipid peroxidation [22] and cause damage to proteins, carbohydrates, and DNA [106]. The production of ROS results from impairment of the electron transport process in the chloroplasts and mitochondria [106, 122]. The major sites for the production of singlet oxygen ($^1O_2$) and superoxide anion ($O_2^{\cdot-}$) in the chloroplast are photosystem I and II (PSI and PSII), whereas the major site for the generation of superoxide anion ($O_2^{\cdot-}$) in the mitochondria are complex I, ubiquinone, and complex III of the electron transport chain (ETC) [106]. The plant's strategy to reduce or prevent the damage of the ROS is to enhance and strengthen the defense mechanisms of naturally occurring nonenzymatic antioxidants (ascorbic acid, ASH; glutathione, GSH; phenolic compounds, alkaloids, nonprotein amino acids, and α-tocopherols) and enzymatic mechanisms (ROS detoxification and scavenging; superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione reductase, GR; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione peroxidase, GPX; guaicol peroxidase, GOPX; glutathione-S- transferase, GST; and lipoxygenase, LOX1 [106, 122, 132, 161, 240]. Alpha-tocopherol played an important protective role against

drought stress in a relatively drought-tolerant genotype of barley [105]. Drought stress treatment caused an increase in activity of antioxidant enzymes such as superoxide dismutase, catalase, and peroxidase in *Panicum sumatrense* [10]. Drought stress upregulated the expression of *CAT2*, *SOD*, and *APX* genes and increased the activity of antioxidant enzymes of SOD and APX, indicating a unique pattern of activity and gene expression of the antioxidant enzymes in two barley genotypes under controlled severe drought [120]. Other researchers suggested a link between proline accumulation under stress and the quenching of singlet oxygen species [158]. Polyamines, citrulline, and several enzymes act as antioxidants and reduce the adverse effects of water deficit [92]. A drought-tolerant wheat cultivar had a higher membrane stability index (MSI), lower accumulated H_2O_2, and higher activity of antioxidant enzymes such as catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), and superoxide dismutase (SOD) than the drought-sensitive genotype under drought stress imposed during plant vegetative growth (18–32 d after sowing) [123]. The antioxidant defense mechanisms protect the plant cell from oxidative damage by scavenging of ROS [106]. The ROS also affects the expression of numerous genes and therefore is involved in many processes such as growth, cell cycle, programmed cell death, abiotic stress responses, pathogen defense, systemic signaling, and development [106].

1.2.2.5 Synthesis of Inducible Proteins Under Drought

Increases and decreases in protein synthesis under water stress have been reported in the literature. Although water stress resulted in an overall decline in protein synthesis, an increase in synthesis of several proteins detected by two-dimensional gel electrophoresis has been shown in soybean [29]. In maize, water stress increased the expression of 50 proteins, decreased the expression of 23 proteins, and induced 10 proteins only in the stressed plants out of 413 spots detected by two-dimensional gel electrophoresis [192]. The various proteins identified were known to be involved in plant response to water stress such as RAB17 (response to ABA) protein, and enzymes involved in metabolic pathways such as glycolysis, Krebs cycle, and lignin synthesis pathways [192] or dehydrin (dehydration-induced) proteins [62].

Proteins synthesized in response to water stress or ABA are identified as dehydrin and RAB (RAB17) proteins. Dehydrin is a family of proteins reported to be induced in response to various environmental stresses such as water, salt, cold, and ABA [27, 35, 62, 115] to reduce cellular damage [94]. Expression of dehydrin proteins (class II LEA proteins) was induced in response to water stress [38], salt [89], low temperature [171], and ABA [38]. Dehydrin proteins (DHN-5) in wheat (*Triticum durum*) have been shown to be induced by salt and abscisic acid [42]. Exposure of wheat to several stresses, such as cold, drought, and wounding, induced the expression of the dehydrin gene (*Cor410b*) by several-fold compared to the nonstressed plants [85]. Drought and salt stresses induced the expression of five *StLEA* classified as dehydrins in potato (*Solanum tuberosum*) [51]. Dehydrin proteins are present in cyanobacteria [64] and several plant species [63].

Immunolocalization studies showed that dehydrin proteins might be present in the cytoplasm [23, 166], in the nucleus [108], in the plasma membrane, or the mitochondria [35]. Dehydrin proteins were expressed in cotton during seed desiccation along with LEA proteins (LEA proteins) [119]. RAB and dehydrins are classified as class II LEA proteins [115, 144].

Many dehydrin proteins were identified as Wcs200 [222] and RAB21 [166] in rice, Wcs200 in wheat [180], G50 [48] in maize, TAS14 [110], LE4 and LE25 [41] in tomato, and CAP85 (cold and dehydration acclimated protein) in spinach leaf [171].

Dehydrins have been characterized by three major conserved amino acid sequences [62]. A highly conserved lysine-rich sequence of EKKGIMDKIKEKLPG amino acids (K segment) has been reported to exist near the carboxyl terminus of dehydrin proteins and repeated one to many times within the protein [62]. Many dehydrins contained a tract of serine residue (S segment) [62]. However, a dehydrin protein without a serine residue was characterized [65]. Another sequence of amino acid DEYGNP (Y segment) of dehydrin protein has been found near the amino terminus of many dehydrins [62]. The three sequences were called "YSK" segments [27, 62, 115].

Other characteristics of dehydrin and RAB proteins are their hydrophilic nature, resistance to denaturing by heat, lack of cysteine and tryptophan, responsiveness to ABA, and the presence of a lysine-rich sequence [35, 62, 63, 109, 166, 227]. Dehydrin proteins, detected in corn and barley seedlings in response to dehydration, were hydrophilic, glycine rich, and free of tryptophan and cysteine amino acids, and contained a conserved, repeating lysine-rich sequence of amino acids occurring twice at the carboxyl terminus and throughout the proteins [63]. Dehydrin proteins in corn and barely seedlings were similar to ABA-induced rice proteins and, to some extent, similar to cotton embryo proteins [63].

Many researchers have studied the expression of dehydrin proteins under water stress in several plant species. In soybean, dehydrin proteins (28 and 32 kDa) were detected 18 d after R_5 ($R_{5.8}$) in developing seeds from drought-stressed plants but not in seeds from the well-watered plants [207]. In the mature seeds, dehydrin proteins (28, 32, and 34 kDa) accumulated similarly in the large, round and small, shriveled seeds from drought-stressed plants as well as the well-watered plants [203, 207]. In two genotypes of barley (*Hordeum vulgare* L.), the dehydrin gene (*HvDHN1*) was upregulated at the early stage (2 d) and late stage (16 d) of drought treatment in the Rum genotype (drought tolerant genotype) and at later stages of drought (9 and 16 d) in Yarmouk genotype, but dehydrin gene (*HvDHN9*) was not expressed under drought stress at all stages of drought treatments in both genotypes [121]. Dehydration of barley and maize seedlings increased the expression of dehydrin mRNAs and induced the synthesis of dehydration-induced proteins (dehydrin proteins) [61, 63]. Polypeptides from dehydrating wheat and barley seedlings reacted immunologically with antibodies raised against maize dehydrin, indicating that dehydrin proteins were similar among grasses [61]. Water stress induced dehydrin expression in peach [17]. Transcripts of six drought-stress genes involved in essential biochemical and physiological function in plant cells were induced by drought stress in both drought-tolerant and -sensitive sunflower genotypes of

sunflower but three of the six transcripts were expressed in higher levels in the tolerant genotype than the sensitive genotype [181]. Transcripts of dehydrin-like proteins (*sdi* –8) accumulated in sunflower leaves of both genotypes in response to drought stress but were at a higher level in the tolerant genotypes [181]. The expression of dehydrin genes (*dhn*, *wcor*, and *dreb*) was highly induced in leaves of the tolerant genotype of wheat under severe water stress [123]. Overexpression of the dehydrin gene (*OsDhn1*-OXs) in rice improved tolerance to salt and drought stresses via scavenging ROS [139]. The overexpression of a wild chickpea (*Cicer pinnatifidum*) dehydrin in tobacco plants had positive effects on their dehydration tolerance [27]. Subjecting two maize (*Zea mays* L.) genotypes, with contrasting sensitivity to dehydration, to moderate drought condition caused upregulation of protective and stress-related proteins (mainly chaperones and dehydrins) in both the sensitive and tolerant genotypes, but the number and levels of upregulated protective proteins were generally lower in the sensitive genotype [28]. The results indicated that the sensitive genotype had a reduced level of photosynthesis as indicated by specific changes in the components of the translation machinery [28]. Cryotolerant species such as wheat and rye accumulate more heat-stable dehydrins than cryosensitive species such as maize [35].

Regulation of gene expression by ABA suggests that ABA may have a role regulating the plant response to dehydration [72] and in mediating the DT in plants [50]. Under water deficient conditions, ABA regulated the expression of a group of drought-induced genes [41]. Dehydrin-like proteins in pea seedlings were induced in response to ABA [23]. In contrast, the expression of dehydrin and LEA proteins in developing seeds might not be regulated by ABA [193]. Expression of RAB/dehydrin proteins in the immature embryo of barley in response to an osmotic stress imposed using mannitol was independent of the ABA pathway [88]. Two ABA-response proteins, ABR17 and ABR18, were synthesized in *Pisum sativum* seeds during desiccation [26]. Characterization of the two proteins revealed that they were similar in amino acid composition and 56 % identical in their N-terminal sequence, but the two proteins had no homology in their N-terminal sequence to any of the LEA or dehydrin proteins [26]. Several factors such as developmental stages, hormones, and dehydration, can regulate the expression and activities of LEA proteins including dehydrins [51]. Dehydrins mRNA (DHN14) rapidly accumulated in winter wheat in response to drought and ABA [58]. In an investigation on sunflower (*Helianthus annuus* L.), the relationship between abscisic acid and accumulation of a dehydrin mRNA (*HaDhn1a*) has shown that there are two regulation pathways of *HaDhn1a* transcript accumulation, an ABA-dependent and an ABA-independent one, which may have cumulative effects [107].

A proposed role of dehydrins, LEA, RAB, and other stress proteins has been in protecting cells from dehydration stress [17, 39, 61, 83]. The highly conserved lysine-rich sequence (K segment) within dehydrin proteins forms a secondary structure (an amphiphilic α helix), which suggests that the K segment is an essential part for dehydrin function under dehydration stress [63, 83, 84, 111, 194]. The hypothesized role for the K segment of dehydrin is to form a hydrophobic interaction with DNA [109], partially denatured proteins, and damaged membranes, thus

acting as a chaperone to stabilize protein folding under dehydration [62, 94, 109]. It has been supposed that dehydrin's function is to stabilize proteins in the membrane or in the matrix of the mitochondria [35]. Although dehydrin proteins are hydrophilic proteins, G50 (dehydrin protein) in maize was able to form a hydrophobic interaction in vitro [48]. Dehydrin may also have a role similar to compatible solutes (such as proline, sucrose, and glycine betaine) in OA. Another possible role of dehydrins is to bind with the accumulated ions (ion sequestering) under water stress and to control solute concentration in the cytoplasm [82]. Dehydrin may have a cryoprotective role in macromolecular stabilization by binding water molecules on their hydrophilic surfaces which reverses or prevents further denaturation of cellular proteins [45, 62]. Maturation proteins, which were induced in response to ABA or dehydration, might protect the plant under stress by stabilizing cell membranes [83]. Evidence from biochemical assays and localization experiments suggests multiple roles for dehydrins, including membrane protection, cryoprotection of enzymes, and protection from ROS [115].

1.3 Molecular Adaptation Under Drought Stress

1.3.1 Signaling Pathway Under Drought and Their Crosstalk

Plant tolerance to drought stress is triggered by complex multicomponent signaling pathways to restore cellular homeostasis and promote survival [113] (Fig. 1.2). The plant phytohormone abscisic acid plays a role in plant response to drought and DT by activating stress-responsive genes and regulating stomatal conductance [179,

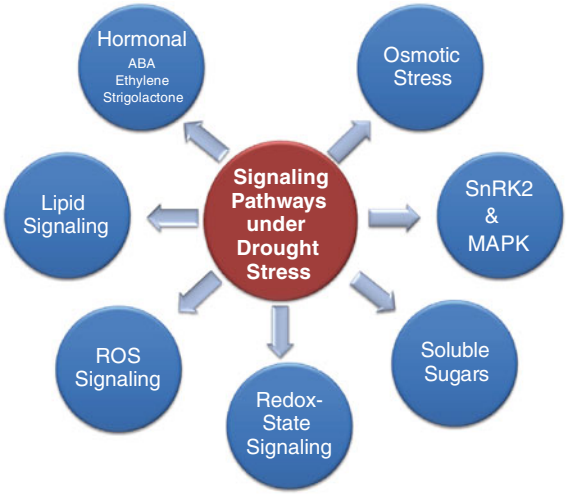

Fig. 1.2 Signaling pathways under drought stress. *ROS* Reactive oxygen species; *SnRK2* Sucrose nonfermenting 1-related protein kinase 2; *MAPK* Mitogen-activated protein kinase

231] and has a vital function as a growth inhibitor [71]. The ABA is synthesized de novo in response to drought stress, which induces the expression of 9-*cis* epoxy-carotenoid dioxygenase (NCED) gene involved in ABA biosynthesis in *Arabidopsis* [131]. Transgenic *Arabidopsis* plants overexpressed *AtNCED3* gene increased the endogenous ABA, increased the transcription of drought- and ABA-inducible genes, showed a reduction in transpiration rate from leaves, and enhanced DT [131]. The ABA triggers the transcriptional changes in genes related to carbohydrate and lipid metabolism, indicating its function at the interface of plant response to stress and cellular metabolism [125]. Exogenous application of ABA could effectively alleviate damages caused by multiple abiotic stresses, including drought, salt, heat, and cold by inducing the accumulation of osmoprotectants and antioxidants, keeping cell membrane integrity, increasing photosynthesis, and keeping ion homeostasis, which protected Bermuda grass from damages caused by abiotic stresses [49]. Previously, ABA was thought to be a long-distance messenger of stress traveling from roots to shoot [179]. However, recent studies have shown that ABA is produced in the veins of the leaves themselves, where it acts on nearby stomata, as well as in the specialized "guard" cells, which close and open the stomata [179].

The abscisic acid signal is perceived by different cellular receptors [113] (Table 1.1). Recent research suggested that there are three cellular ABA receptors: nucleocytoplasmic [153, 184]; plasma membrane [183]; and chloroplast (receptors) [79] (Table 1.1). The ABA binds with the nucleocytoplasmic receptor (PYR/PYL/RCARs), which inhibits the type 2C protein phosphatases (PP2Cs) [153, 190] (Fig. 1.3). Inactivation of PP2Cs activates accumulation of active sucrose nonfermenting 1-related protein kinase 2 (SnRK2 s; [153, 190]). The SnRK2 s regulates ABA-responsive transcription factors (AREB/ABF; ABA-responsive element binding protein/ABRE-binding factor) [two basic leucine zipper (bZIP) transcription factors] by binding with the ABA-responsive *cis* element (ABRE) of the ABA-regulated genes [234]. Other transcription factors such as MYC, MYB [2], RD26, and NAC [101] are involved in the induction of drought-inducible genes mediated through ABA-dependent pathway (Fig. 1.3).

In addition to the ABA-dependent pathway, the drought stress signal is mediated through an ABA-independent pathway (osmotic stress signal) to regulate the

Table 1.1 ABA cellular receptors

Cellular compartments	Receptors	Name	References
Nucleocytoplasmic	PYR/PYL/RCARs	PYR Pyrabactin resistance PYL Pyrabactin resistance like RCARs Regulatory component of ABA receptors	[153, 184]
Plasma membrane	GTGs (GTG1/GTG2)	G protein-coupled receptor-type G protein	[183]
Chloroplast	CHLH/ABAR	H subunit of Mg-chelatase	[79]

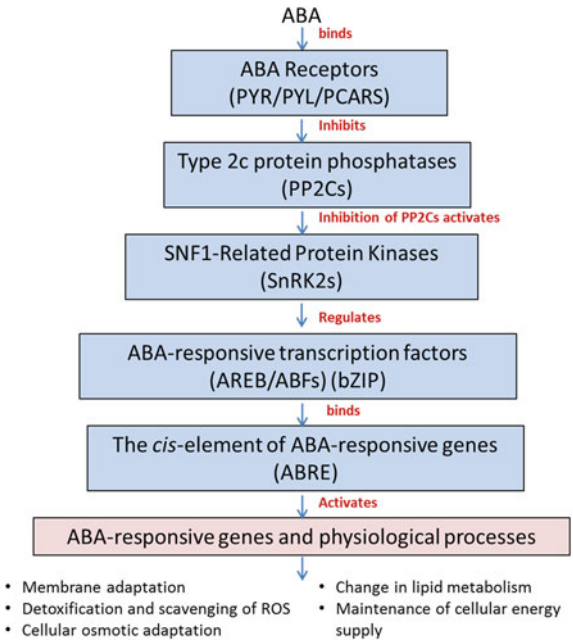

Fig. 1.3 ABA signaling pathways in regulating ABA-responsive genes and physiological processes

expression of different drought-inducible genes [54, 211] (Fig. 1.4). Transcription factors DREB1/CBF and DREB2 (belonging to AP2/ERF transcription factors) bind with the *cis*-acting element DRE/CRT (dehydration-responsive element/C-Repeat) of the drought-inducible genes and induce the expression of the DT genes [148, 211, 233]. DREB and ERF proteins regulated the expression of wheat (*Triticum durum*) dehydrin gene (*TdCor410b*) through a single functional C-repeat (CRT) element [85]. Another ABA-independent pathway regulating the dehydration response is through a transcription factor identified as NAC which binds with the *cis*-element of the drought-inducible gene (*ERD1*) [216, 225] (Fig. 1.4).

Recently, Ha et al. [118] reported that the hormone strigolactone positively regulates drought response and tolerance in *Arabidopsis*, reporting a new signaling pathway. The synthesis of strigolactone is controlled by a more axillary growth (Max) gene family [118]. *Arabidopsis* plants with Max mutant genes were more sensitive to drought- and salt-stress compared with the wild types and exhibited increased stomatal density, increased leaf water loss rate, and slower ABA-induced stomatal closure, suggesting that strigolactone acts by regulating stomatal transpiration rate [118]. They also suggest that strigolactone and ABA interact and play an important role in integrating stress signals to stomatal development and function [118].

Current models emphasize the function of ROS as a signaling interface in plant drought and salt adaptation that links stress signals to regulation of metabolism and cellular energy balance (ROS signaling) [15, 113] (Fig. 1.2). The transcription factor (*WRKY*) is suggested to be involved in linking osmotic and oxidative stress

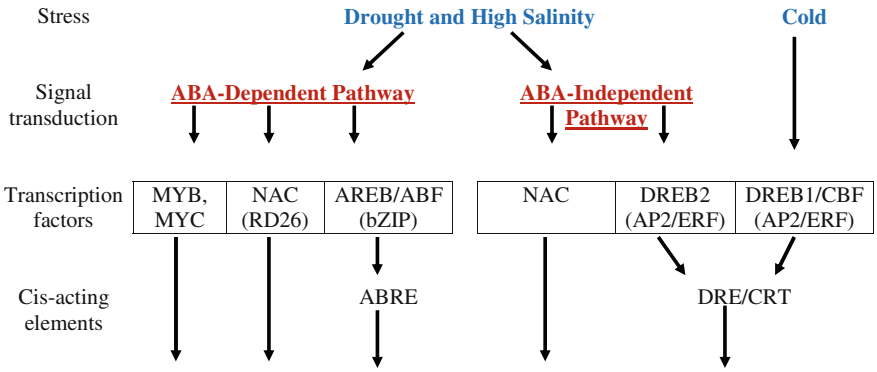

Fig. 1.4 Transcriptional regulatory networks of abiotic stress signals and gene expression. There are three ABA-dependent and three ABA-independent pathways. In the ABA-dependent pathway, the transcription factors (MYB, MYC, NAC, and AREB/ABF) function in ABA-inducible gene expression. AREB/ABF binds with the *cis*-acting element (ABRE) of the ABA-inducible genes. In the ABA-independent pathway, the transcription factors (NAC, DREB1/CBF, and DREB2) function in this process. The DREB1/CBF and DREB2 bind with the *cis*-acting element (DRE/CRT) of the ABA-independent stress inducible genes. Modified from Shinozaki and Yamaguchi-Shinozaki [211] with permission of Oxford University Press

defense and in ROS-mediated signaling crosstalks [113]. The transcription factor of *WRKY33* controls genes that function in detoxification of ROS such as glutathione *S*-transferase (*GSTU11*), peroxidases, and lipoxygenase (*LOX1*) in *Arabidopsis* [132]. The transcription factor *ThWRKY4* controls the genes related to ROS scavenging such as superoxide dismutase and peroxidase in *Tamarix hispida* [240]. These data suggest that the transcription factor *WRKY* has a role in controlling cellular ROS levels in abiotic stress signaling. Plants with overexpression of *ThWRKY4* were referred to *ThWRKY4*-mediated cellular protection against toxic ROS levels [240]. Recent advances suggest that there is a crosstalk of ROS signaling with other stress-triggered hormonal signaling such as ABA and other endogenously induced redox and metabolites signaling [113].

Increasing evidence indicates that lipid signaling is an integral part of the complex regulatory network in plant response to drought and salinity [167, 197] and is involved in primary sensing of abiotic stresses and in triggering and regulating cellular hormonal signaling [113]. Modification of lipids produces different signaling messengers such as phosphatidic acid (PA), diacylglycerol-pyrophosphate (DAG-PP), and phosphoinositides [36]. The production of PA, the key lipid messenger in plant response to environmental stresses, is regulated by phospholipase D (PLD) [126]. The activity of PLD in plants increases in response to abiotic stress such dehydration, drought, and salinity [126]. Recent studies indicated that PLD and PA play an important role in plant drought and salt stress tolerance [25, 151]. PLDα1 promotes stomatal closure and reduces water loss [126, 151]. PA binds with

PP2C (a protein phosphatase 2c; ABI1, ABA-insensitive 1) and inhibits the negative effect of PP2C in the ABA signaling pathway, thus promoting the ABA effect on stomatal closure [239], indicating that the modification of cellular lipid (through the biosynthesis of PA by *PLDα*) is essential for regulating abiotic stress-related ABA signaling [113, 151]. Introducing Arabidopsis *PLDα* under the control of a guard-cell–specific promoter $AtKatl_{pro}$ into two canola (*Brassica napus*) cultivars ($AtKatl_{pro::}$ *PLDα*) decreased water loss and improved biomass accumulation under drought and salt stresses in canola [151]. These results support the evidence of the involvement of lipid signaling in plant response to drought and DT.

Other signal pathways trigger plant response to drought stress such as SnRK2 (sucrose nonfermenting 1-related protein kinase 2) and MAPK (mitogen-activated protein kinase) signaling, possibly stomatal signaling [113, 145], soluble sugars (namely sucrose, glucose, and fructose) signaling [55], and redox-state of the photosynthetic electron components and the redox active molecules acting as regulatory agents [99] (Fig. 1.2).

1.3.2 Activation of Transcriptional Factors Under Drought

As indicated in the previous section, drought stress induces the expression of DT genes through ABA-dependent and -independent pathways. There are four major transcription factors involved in the regulatory network in plant response to drought, cold, and heat stresses [169] (Table 1.2). AREB/ABFs are bZIP transcription factors that regulate ABA-dependent gene expression under osmotic stress by binding with the *cis*-element of the ABA-responsive (ABRE) gene expression [157]. The transcription factors DREB1/CBF and DBEB2 in *Arabidopsis* regulate ABA-independent gene expression by binding with the *cis*-element of DRE/CRT of drought-responsive genes [5, 6, 148]. Other transcription factors (NAC genes) have been identified in *Arabidopsis* and rice that have important functions in stress responses [168]. The NAC transcription factors (SNAC group) bind with the

Table 1.2 Major transcription factors for abiotic stress-responsive gene expression to improve drought tolerance

Abiotic stress	Transcription factors	*Cis*-acting element of stress-responsive gene	References
Osmotic (ABA-dependent)	AREB/ABF	ABRE	[157]
Cold	DREB1/CBF	DRE/CRT	[130, 148]
Osmotic and Heat	DREB2	DRE/CRT	[87, 149, 200]
Drought	NAC	NACR	[168, 223]

NACR (NAC recognition sequence) of the stress-responsive genes in *Arabidopsis* [168, 223]. The drought-response transcription factors such as AREB/ABF, DREB1/CBF, DREB2, and NAC function in DT and in transcriptional regulatory networks and crosstalk in abiotic stresses including drought, salt, cold, and heat [87, 149, 169, 200]. The *TaWLIP19* (wheat version of bZIP), *TaWRKY10*, *TaMYB33*, and *TaNAC69* transcription factor genes were induced in *Triticum aestivum* and *Triticum turgidum*, indicating the involvement of these transcription factors in drought stress responses [24].

1.3.3 Drought Induced Genes and Their Expression in Transgenic

Molecular analysis using microarray identified the drought-inducible genes [211, 212]. The products of these genes in *Arabidopsis* and rice were classified into functional and regulatory proteins [189, 212]. The functional proteins include those proteins involved in abiotic stress tolerance such as protection factors of macromolecules (such as LEA proteins, chaperones, and dehydrins), detoxification enzymes, enzymes for the biosynthesis of osmolytes (proline and sugar), water channel transporter, and proteases. The regulatory proteins include transcription factors (DREB2, AREB, MYB, MYC, bZIP, and NAC), protein kinases, phosphatases, lipid metabolism, and ABA biosynthesis.

With the advancement of biotechnology and increasing genomic tools, the transgenic approach has become an attractive strategy for genetically engineering plants for improving DT [138, 229, 238]. Molecular control mechanisms of the transgenic plants for stress tolerance are based on activation, regulation, or overexpression of specific drought-related genes [229]. These genes are either functional proteins (osmolyte synthesis enzyme, raffinose oligosaccharides biosynthesis enzyme, dehydrin proteins, key photosynthetic enzymes, ROS detoxification and scavenging enzymes), or regulatory proteins (signaling and transcriptional factors) [7, 211, 212, 229]. In the last decade, several attempts using genetic engineering have been made to enhance plant tolerance to drought through producing transgenic plants with a "single-function" gene as well as a transcription factor for abiotic stress tolerance [7]. Because the tolerance to abiotic stress in nature is controlled by many genes; the recent trend is shifting towards genetic transformation of multiple genes or transcription factors [7].

Transgenic plants with expressed functional proteins improved DT. Transgenic soybean expressing *AtP5CR* gene (encoding L-Δ1-Pyrroline-5-carboxylate reductase) had higher relative water content and enhanced DT [74]. Wheat plants transformed with the *Vigna aconitifolia* [Delta (1)-pyrroline-5-carboxylate synthetase (P5CS) cDNA that encodes the key regulatory enzyme in proline biosynthesis] showed an increase in the accumulation of proline and enhanced tolerance to drought mainly due to protection mechanisms against oxidative stress and not caused by OA [226]. Proline accumulation in other transgenic plants acts as a component of

antioxidative defense and enhanced DT [162, 215, 232]. Transgenic *Arabidopsis* plants with the *Go1S* gene (encoding galactinol synthase, a key enzyme involved in raffinose oligosaccharides biosynthesis) was shown to enhance DT [221]. Transgenic rice plants with overexpression of the dehydrin gene (*OsDhn1*-OXs) showed an enhanced tolerance to drought and salt stress as indicated by the chlorophyll fluorescence (Fv/Fm), fresh and dry weight, water and chlorophyll content, and survival ratio, and had an increased tolerance to oxidative stress and maintained a relatively low level of H_2O_2 under salt and drought stress as compared to wild-type plants [139]. Transgenic plants of *Arabidopsis thaliana* with ectopic expression of dehydrins (Dhn-5) exhibited stronger growth under high concentrations of NaCl or under water deprivation, and showed a faster recovery from mannitol treatment, had more negative water potential, higher proline contents, and lower water loss rate under water stress compared to wild-type plants [42]. Transgenic plants with Dhn-5 had an improved tolerance to salt and drought stress through OA [42]. Plant adaptation to drought stress can be markedly improved in tomato (*Solanum lycopersicum* L.) by overexpression of the cell wall invertase (cwInv) gene *CIN1* (a key enzyme regulating sink activity and playing a role in plant growth and development) from *Chenopodium rubrum* [12]. Transgenic plants with *CIN1* overexpression reduced water consumption by limiting stomatal conductance during the drought period, whereas photosynthetic activity was maintained, delayed senescence, leading to higher water use efficiency and improving DA strategy [12]. *CIN1* overexpression was accompanied by the increase in the concentrations of the senescence-delaying hormone trans-zeatin and the decrease in the senescence-inducing ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) in the leaves [12].

Improvement in photosynthetic capacity under drought stress can be achieved by engineering transgenic plants with the overexpression of the genes of a key C_4 photosynthetic pathway to C_3 plants. Two transgenic rice lines were produced by overexpression of the maize-specific pyruvate orthophosphate dikinase (PPDK) independently or in combination with maize-C4–specific phosphoenolpyruvate carboxylase (PCK) [117]. Transgenic rice lines had an increased leaf photosynthetic rate, higher leaf water content, stomatal conductance, transpiration efficiency, and root oxidation activity, and a stronger active oxygen scavenging defense than the untransgenic line (wild type) under well-watered and drought-stress treatments [117].

Transgenic plants with overexpressed genes coding antioxidant enzymes showed enhanced tolerance to drought stress. Transgenic tobacco plants with cytosolic Cu/Zn-superoxide dismutases (SOD) from *Oryza sativa* showed enhanced tolerance to oxidative stress and enhanced tolerance to salt, water, and polyethylene glycol (PEG) stresses as measured by net photosynthesis over the wild type [21]. These results suggested that the overexpressed Cu/Zn-SOD enhanced the chloroplast antioxidant system [21]. Developing transgenic tobacco plants overexpressing *Arabidopsis thaliana* Monodehydroascorbate reductase (MDAR gene; *AtMDAR1*) exhibited higher MDAR activity and a higher level of reduced ascorbate (AsA) compared to nontransformed control plants [86]. The transgenic plants

showed enhanced stress tolerance in term of significantly higher net photosynthesis rates under ozone, salt, and PEG stresses [86]. Tobacco (*Nicotiana tabacum* L.) plants transformed and constitutively overexpressed with the *Arabidopsis thaliana* gene for ascorbate peroxidase 3 (APX3) exhibited greater photosynthetic capacity, higher fruit number, and seed mass [235].

Overexpressing regulatory proteins in transgenic plants has also shown an improvement in DT. Transgenic *Arabidopsis* with overexpression of *AtNCED3* gene (9-*cis*-epoxycarotenoid dioxygenase enzyme involved in ABA synthesis) had an increase in endogenous ABA level, higher transcription of drought- and ABA-inducible genes, lower transpiration rate, and enhanced DT [131]. Overexpression of the DREB1/CBF transcription factor in transgenic plants increased stress tolerance to drought, freezing, and salt stresses [148]. Overexpression of the rice gene OsDREB1A transcription factor (homologues of DREB1/CBF) in *Arabidopsis* resulted in stress-responsive gene expression and stress tolerance [80]. Similarly, overexpression of the rice transcription factor gene OsDREB1 or *Arabidopsis* transcription factor gene (DREB1) enhanced drought and chilling tolerance in rice [130]. Overexpression of ABF3 or AREB2/ABF4 transcription factor genes increased the sensitivity to ABA, reduced transpiration rate, and improved the DT of *Arabidopsis* [135]. Overexpression of *AP37* (subgroup of AP2 transcription factor) in rice under the control of the constitutive promoter *OsCc1* (the *OsCc1*:*AP37* plants) significantly enhanced DT in the field, which increased grain yield by 16–57 % over controls under severe drought conditions, yet exhibited no significant difference under normal growth conditions, suggesting the *AP37* gene has the potential to improve DT in rice without causing undesirable growth phenotypes [176]. Overexpression of the Arabidopsis HARDY gene [belongs to the stress-related AP2/ERF transcription factor (APETALA2/ethylene responsive element binding factors)] in *Trifolium alexandrinum* L. (transgenic plants) improved the instantaneous WUE under drought stress by reducing transpiration (E), improved the growth of drought-stressed transgenic plants by delaying water depletion from soil and preventing rapid decline in photosynthesis (A), and significantly increased the transgenic plant fresh and dry weights under the combined drought and salt stresses in the field compared to the wild-type plants [4]. The results proved the efficiency of overexpression of a single gene in improving tolerance to abiotic stress under field conditions [4].

Recently, *Arabidopsis* plants were engineered with a modified ABA receptor [Pyrabactin Resistance 1 (RYR1)] that can be activated by a common agrochemical fungicide (mandipropamid) [185]. The transgenic plants with re-engineered receptor can withstand 12 days of water withholding and survive after being rewatered when plants are sprayed with mandipropamid agrochemical compared with the control plants (wild type). This result demonstrated that mandipropamid was efficient in controlling ABA responses and DT [185].

1.4 Concluding Remarks

Plant responses to drought are complex interacting processes including morphological, physiological, biochemical, and molecular changes that allow plants to cope with drought stress. Plant strategies to cope with drought can be through escape, avoidance, or tolerance. The contribution of each of these strategies for drought resistance varies among species and interacts with environmental conditions and depends on many factors. In the escape mechanism, plants end their life cycle before the onset of drought stress at the end of the growing season, which results in earlier maturity and in most cases lower yield to survive the terminal drought stress. In the avoidance mechanism, two important processes are involved: plants uptake more water from the soil and conserve moisture by regulating the stomatal aperture to maintain leaf water hydration. In the last strategy, plants trigger mechanisms to withstand water dehydration including OA, accumulation of dehydrins, scavenging the ROS, repairing the damage in proteins and membranes. To regulate these responses, drought signaling pathways are involved in mediating the expression of many drought-responsive genes. These pathways include hormonal signals (such as ABA, ethylene, and strigolactone), ROS signal, lipid-derived signal, soluble sugar signal, and others. These signaling pathways are either ABA-dependent or ABA-independent. These signal pathways are crosstalk in mediating plant responses to drought and other stresses. Major transcription factors such as ABREB/ABF, DREB1/CBF, DREB2, and NAC play important roles in the crosstalk of these signaling pathways. Drought stress regulates expression of many drought-responsive genes including functional proteins (such as osmolyte, antioxidant enzymes, and dehydrins) and regulatory proteins (such as transcription factors). In the last decade, many studies have been done to transfer a single or multiple genes from one plant to another or overexpressing them in the same plants to produce transgenic plants. Transgenic plants have shown enhanced DT and can be a promising tool for further understanding the functions of these drought-responsive genes in plants under drought stress. Researchers reported that transgenic plants with overexpressing genes encoding to photosynthetic enzymes, osmolyte synthesis, dehydrins, antioxidant enzymes, enzymes involved in ABA synthesis, ABA receptors, and enzymes involved in source-sink regulation have shown improved DT. Because drought stresses in the field are very complex phenomena involving many interacting factors and coincide with many other stresses, future research is needed to focus on the transfer of more than one gene and to test these transgenic lines under natural growing conditions in the field.

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Chapter 2
Genetics of Drought Stress Tolerance in Crop Plants

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2.1 Introduction

Ensuring stable crop yields in an era where climate change threatens traditional agricultural practices through altered rainfall patterns and increased urban consumption has become a vital concern in global food security. Projected freshwater availability for irrigation indicates that between 20 and 60 Mha of irrigated cropland may have to be reverted to rainfed management [57]. Formerly irrigated crops would become entirely dependent on rainfall and vulnerable to yield loss due to drought. Biotechnology and mining of germplasm of numerous crop species has resulted in discovery of traits that control water use efficiency (WUE) and improve drought tolerance, but only a few of these traits have been implemented in the field [116]. "We need a Blue Revolution in agriculture that focuses on increasing productivity per unit of water—more crop per drop," Secretary-General Kofi Annan called for a "Blue Revolution" in agriculture [153]. The Green Revolution drastically increased agricultural productivity by remediating nutrient-poor soils with fertilizer, developing irrigation methods and systems, instigating integrated control strategies for weeds and pests, and selected crops that responded to these changes with high yields. The Blue Revolution proposes to address decreasing water availability and climate change. This requires not just drought-tolerance traits, but a high level of control of WUE while still maintaining high yields required by both industrial agriculture and smallholders. An integrated research approach has emerged as a valuable strategy to tackle these necessities. One component of this approach is the mining and introduction of traits from landraces and wild relatives in order to improve drought

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tolerance and WUE in crop species. In some cases, this has been combined with modern biotechnology in an integrated approach. Recently, drought-tolerant maize hybrids have been released in the United States as a result of biotechnology and introduction of traits from distant relatives [140].

This chapter gives a broad overview of the genetic basis of developmental and cellular responses known in crops to enhance drought tolerance. A number of key processes and genes have been discovered in model systems that show great promise for use in crop species. Furthermore, this chapter also addresses the potential for wild relatives of five major crop species (tomato, potato, wheat, rice, and corn) as sources for genetic improvement. These landraces and wild relatives are often remarkably well adapted to their environment through natural selection and traditional breeding. These relatives provide a resource for mining novel traits for the genetic improvement of cultivated crops that are vulnerable to environmental stress.

2.2 Cellular Mechanisms and Traits Conferring Drought Tolerance

2.2.1 Genes Controlling Primary and Secondary Metabolism

Plants respond to environmental stresses through various physiological and biochemical changes. Exposure to drought, high salinity, and low temperature leads to cellular dehydration. This removal of water from the cytoplasm results in a decrease of cytosolic and vacuolar volumes. In response, plants increase the production of specific sets of primary and secondary metabolites that act as osmoprotectants, osmolytes, antioxidants, and stress signals. The net accumulation of these solutes lowers the cellular osmotic potential and draws water into the cell to maintain turgor pressure. Osmoprotectants preserve the cellular apparatus from the damage caused by dehydration, without interfering with the normal metabolic processes at the cellular level. These solutes include amines (polyamines and glycinebetaine), amino acids (proline), soluble sugars (glucose, sucrose, trehalose), and polyols (mannitol, sorbitol and inositol; [172]). Because some crops have low levels of these compounds, the manipulation of genes involved in osmoprotectant biosynthesis pathways is one of the strategies to improve stress tolerance in plants [158].

Polyamines (PAs) are small aliphatic nitrogen compounds that are ubiquitous in all organisms. The biological function of PAs is associated with their cationic nature. In plants, PAs act as regulatory molecules implicated in fundamental cellular processes, including embryogenesis, floral development, and pollen tube growth [185]. Significant accumulation of the three most common PAs, putrescine (Put), spermidine (Spd), and spermine (Spm), occurs during biotic and abiotic stress [198]. Modulation of the PAs biosynthetic pathway by overexpression of ornithine and arginine decarboxylases (*ODC*, *ADC*), S-adenosyl-methionine-decarboxylase

(*SAMDC*), and Spermidine synthase (*SPDS*) resulted in enhanced tolerance to different environmental stresses in rice (*Oryza sativa*; [37, 54]), tobacco (*Nicotiana tabacum*) and tomato (*Solanum lycopersicum*; [7]).

Glycine betaine (GB) is a quaternary ammonium derivative of glycine and is considered a major osmolyte involved in cell membrane protection. In response to various abiotic stresses such as drought and salinity, GBs are accumulated in chloroplasts and other plastids of many plant species. One of the principal roles of GB is that it encourages water influx into cells for maintaining the intracellular osmotic equilibrium and regulates the cascade of signal transduction [156]. The overproduction of GB in various plants including maize (*Zea mays*) [155] and cotton (*Gossypium hirsutum*) [131] by modulation of two key genes involved in GB biosynthesis, *betA* (encoding choline dehydrogenase) and *CMO* (choline monooxygenase), results in improved yield production under stressful field conditions.

In addition, the accumulation of proline under stress conditions in many plant species has been correlated with stress tolerance. Proline (Pro) is a versatile amino acid that is essential both as a component of protein and as a free amino acid. To avoid cellular dehydration, proline facilitates water uptake and reduces the accumulation of Na^+ and Cl^- [12]. In plants, the Pro biosynthesis takes place in the cytosol and in the plastids. The principal precursor, glutamate, is converted to proline by two consecutive steps catalyzed by pyrroline-5-carboxylatesynthetase (*P5CS*) and P5C reductase (*P5CR*). The degradation of proline occurs in mitochondria by the reverse action of proline dehydrogenase (*PDH*) and pyrroline-5-carboxylate dehydrogenase (*P5CDH*; [113]). The modulation of expression of these genes significantly enhances endogenous levels of proline and increases drought stress tolerance in wheat (*Triticum aestivum*; [193]) as well as in rice [177], promoting growth, antioxidant defense, and decreasing uptake of Na^+ and Cl^-.

The manipulation of osmoprotectant accumulation was also successfully used with the reduced forms of sugars such as glucose, sucrose, fructose, and trehalose. Sugars provide carbon for cellular metabolism and regulate growth and development in plants. During salinity and drought stresses, sugars and sugar alcohols act as osmoprotectants regulating the osmotic adjustment, protecting the membrane by interacting with protein complexes and enzymes, and scavenging toxic ROS [191]. The regulation of trehalase activity occurs by expression of trehalose synthesis-related genes: trehalose phosphate synthase (*TPS*) and trehalose-6-phosphate phosphatase (*TPP*), improve tolerance to abiotic stresses in rice [66] and alfalfa (*Medicago sativa*; [178]), decreasing aggregation of denatured proteins [13, 112]. Similarly, sugar alcohols including mannitol, sorbitol, and inositol improve stress tolerance of plants. Mannitol is the most common polyol in nature and is synthesized in mature leaves from mannose-6-phosphate by the combined action of mannose-6-phosphate reductase (*M6PR*) and a mannose-6-phosphate phosphatase. It is then translocated through the phloem and oxidized to mannose by mannitol dehydrogenase (*MTD*) or stored and used as a carbon source. During abiotic stress, mannitol is accumulated in the cytosol to act as a scavenger of hydroxyl radicals and to stabilize macromolecules [47].

Metabolic plasticity as well as biosynthesis and accumulation of osmoprotective compounds are promising mechanisms of plant acclimation to stress. Polyamines,

glycine betaine, amino acids, polyols, and some classes of sugar are active participants in the response to drought and salinity. Therefore, their biosynthetic pathways represent interesting targets for future breeding applications.

2.2.2 Stress-Induced Regulatory Genes

Plants respond and adapt to water deficit at both the cellular and molecular levels, by accumulating osmolytes and proteins specifically involved in stress tolerance. Drought induces the biosynthesis of the phytohormone abscisic acid (ABA), which in turn causes stomatal closure and induces expression of stress-related genes. There are two important transcriptional networks activated under abiotic stress conditions in Arabidopsis: an abscisic acid (ABA)-dependent signaling pathway and an ABA-independent regulatory network. Transcription factors (TFs) activated by ABA include the AREB/ABF (ABA-responsive *cis*-element binding protein/ABA-responsive *cis*-element binding factor). The AREB/ABF TFs have a bZIP domain and four conserved domains containing SnRK2 phosphorylation sites. Upon phosphorylation, AREB/ABFs are activated and bind to the ABA-responsive *cis*-element (ABRE; PyACGTGG/TC), enriched in the promoter regions of drought-inducible genes. AREB/ABFs function as master transcriptional activators regulating ABRE-dependent gene expression in ABA signaling under drought stress conditions.

Other important transcriptional regulators, such as the MYC and MYB proteins, function as activators in the ABA-dependent regulatory systems [2, 190]. A MYC TF, RD22BP-1 (AtMYC2), and AtMYB2 have been shown to bind *cis*-elements in the RD22 promoter and cooperatively activate RD22 [1]. These MYC and MYB proteins are synthesized after the accumulation of endogenous ABA, indicating that their role is in a late stage of the stress responses. SnRK2 functions upstream of ABA-responsive expression of RD22 and RD29B. In particular, two ABRE motifs are important in the expression of RD29B. Instead, ABRE-like motifs are not involved in the ABA regulation of RD22. Several drought-inducible genes do not respond to ABA treatment, suggesting the existence of an ABA-independent pathway in the dehydration stress response. This pathway is mediated by dehydration-responsive element-binding (DREB)-type TF. DREB2 proteins are members of the AP2/ERF family of plant-specific TFs; they bind to dehydration-responsive element/C-repeat (DRE/CRT) and their conserved DNA-binding motif is A/GCCGAC. Among the eight DREB2 genes in Arabidopsis, DREB2A and DREB2B are highly induced by drought, high salinity, and heat stress. Evidence for interaction between the AREB/ABFs and DREB/CBFs has been reported. Lee et al. [119] showed that the DREB1A/CBF3, DREB2A, and DREB2C proteins interact physically with AREB/ABF proteins. These data suggest crosstalk between elements of the ABA-dependent and -independent response pathways.

It has also become clear that changes in gene expression patterns and in RNA processing are involved in stomatal movement. The first TFs for which a role in

stomatal opening/closure has been clearly demonstrated were the Arabidopsis AtMYB60 [46] and AtMYB61 proteins [122]. AtMYB60 is specifically localized in guard cells and its expression is upregulated by signals that induce stomatal opening, such as white and blue light, and downregulated by darkness, desiccation, and ABA treatment, signals that promote stomatal closure. In contrast to AtMYB60, the AtMYB61 gene is mainly expressed in guard cells in the darkness, when stomata are closed.

Two other Arabidopsis MYB have been described for their involvement in stomatal movements: AtMYB44 [103] and AtMYB15 [53]. AtMYB44 expression was induced by ABA and by different abiotic stresses. It was highly expressed in guard cells. AtMYB44 negatively regulates the expression of genes encoding a group of serine/threonine protein phosphatases 2C (PP2Cs) that have been previously described as negative regulators of the ABA signaling. The AtMYB15 gene has a role in the regulation of stomatal closure. In Arabidopsis three other TFs involved in stomatal movements have been characterized: AtERF7 [175], NFYA5 [121], and NPX1 [106]. AtERF7 belongs to the APETALA2/ethylene-responsive element binding proteins (AP2/EREBP) family. This protein binds to the GCC box located in the promoter of its target genes and acts as a repressor of transcription. NFYA5 is a member of the Arabidopsis NF-YA family. Nuclear factor Y (NF Y) is a TF that binds to the CCAAT box, a *cis*-element present in about one fourth of eukaryotic gene promoters. The expression of NFYA5 is upregulated by ABA and drought and the gene is highly expressed in vascular tissues and guard cells.

A novel Arabidopsis transcriptional regulator involved in stomatal movement is nuclear protein X1 (NPX1). This protein is a nuclear factor, without a functional DNA binding motif. It acts as a negative regulator of transcription, probably through the interaction with other proteins that bind DNA.

Two TFs, SNAC1 and DST, involved in the regulation of stomatal movements have been identified in rice [83, 86]. Stress responsive NAC1 (SNAC1) is a member of the plant-specific NAC (NAM, ATAF, and CUC) family of TF that includes 149 members in rice. SNAC1 expression is induced in response to abiotic stresses and is predominant in guard cells under drought conditions. Drought and salt tolerance (DST) is a C2 H2-type zinc finger-containing protein. DST is unique in that its single zinc finger motif is required for both its DNA-binding and transactivation. Huang and colleagues [86] found that DST is involved in a novel H_2O_2-mediated pathway for stomatal closure that is ABA-independent.

2.2.3 Regulation of ROS

An important cellular mechanism conferring drought tolerance is the regulation of reactive oxygen species (ROS). ROS are chemically reactive molecules containing oxygen and they are formed in the metabolism of oxygen and have important roles in cell signaling and homeostasis. It has been established that 1–2 % of oxygen absorbed by plants is used to produce ROS in plants [24]. Overaccumulation of

ROS from abiotic stress contributes to major losses of crop productivity and is an important economic problem for cultivated plants worldwide [69]. Accumulation of ROS causes oxidative stress, which in turn results in oxidative damage to proteins, DNA, and lipids [69]. Increased levels of ROS have been reported during biotic and/or abiotic stresses, such as pathogen attack, wounding, UV irradiation, high light, drought, salinity, and chilling [170]. Acclimation of plants to drought and salinity is often associated with increased levels of ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (HO), and singlet oxygen (O_2), which are toxic for the cell [74]. These products are produced in chloroplasts by the electron transport chain when CO_2 is limited, in mitochondria during overreduction of the electron chain transport and in peroxisomes when glycolate is oxidized to glyoxylic acid during photorespiration [144]. In addition, the plasma membrane together with the cell wall and apoplast can make an important contribution to drought-induced ROS production. During nonstress conditions, ROS are efficiently eliminated by nonenzymatic and enzymatic antioxidants. However, during drought and salt stress, the production of ROS exceeds the capacity of the antioxidative systems to remove them, causing oxidative stress [192]. In these conditions, the elimination of ROS is a key response to tolerate drought stress. This is mainly achieved by antioxidant compounds such as ascorbic acid, glutathione, thioredoxin, and by oxyreductant enzymes as glutathione peroxidase, superoxide dismutase, and catalase (Table 2.1).

The antioxidant compounds play different roles: ascorbic acid (AsA) is used as a substrate by ascorbate peroxidase to reduce H_2O_2 to H_2O in the ascorbate–glutathione cycle and generates monodehydroascorbate, which further dissociates to ascorbic acid and dehydroascorbate [65]. α-Tocopherol is a lipid soluble antioxidant that acts as lipophilic antioxidant and interacts with polyunsaturated acyl groups of lipids. This in turn reduces the deleterious effects of ROS by stabilizing the membrane and acts as a modulator of signal transduction [60]. Glutathione (GSH) is a tripeptide (γglu-cys-gly) that reduces disulfide bonds formed within cytoplasmic proteins to cysteines by serving as an electron donor. It has been reported that the conversion ratio of reduced glutathione to its oxidized (GSSG) form during the detoxification of H_2O_2 is an indicator of cellular redox balance. This has been widely reported in plants under various abiotic stresses [81]. The defense against ROS is maintained by detoxifying enzymes such as superoxide

Table 2.1 ROS scavenging antioxidant enzymes, their substrates and products

Enzymatic antioxydant	Reaction catalyzed
Superoxide dismutase (SOD)	$O_2^- + O_2^- + 2H^+ \rightarrow 2H_2O_2 + O_2$
Catalase (CAT)	$H_2O_2 \rightarrow H_2O + ½\ O_2$
Ascorbate peroxidase (APX)	$H_2O_2 + AsA \rightarrow 2\ H_2O + DHA$
Monodehydroascorbate reductase (MDHAR)	$MDHA + NAD(P)H \rightarrow AsA + NAD(P)^+$
Dehydroascorbate reductase (DHAR)	$DHA + 2GSH \rightarrow AsA + GSSG$
Glutathione reductase (GR)	$GSSG + NAD(P)H \rightarrow 2GSH + NAD(P)^+$

dismutase, ascorbate peroxidase, glutathione peroxidase, and catalase. Superoxide dismutase converts superoxide to H_2O_2, and ascorbate peroxidase and glutathione peroxidase detoxify H_2O_2 to water, and catalase converts H_2O_2 to oxygen [9]. Several studies have been published about tolerant transgenic plants overexpressing scavenging antioxidant enzymes. Transgenic rice plants overexpressing *OsMT1α*, a gene coding for superoxide dismutase, showed enhanced tolerance to drought together with an increase in catalase and ascorbate activity [203]. Overexpression of ascorbate peroxidase in tobacco chloroplasts enhanced plant tolerance to salt stress and water deficit [15]. Tobacco plants overexpressing *Prosopis julifora* glutathione S-transferase (*PjGSTU1*) had increased survival over controls under 15 % PEG stress [68]. Tobacco cells silenced in the PDH gene showed an accumulation of proline and enhanced osmotolerance with respect to the wild-type cells [182].

2.3 Developmental Mechanisms and Traits Conferring Drought Tolerance

2.3.1 Genes Controlling WUE: Stomatal Sensitivity and ABA

Stomata are the key organs regulating plant gas exchanges with the environment, responsible for controlling over 98 % of the CO_2 and H_2O exchanged by plants with the outside air [117]. Several endogenous and environmental cues regulate stomatal movements, including hormonal stimuli, atmospheric CO_2 concentrations, presence and wavelength of light, and pathogen attack [46]. Integration of all these signals determines stomatal conductance and therefore photosynthesis and transpiration rate, resulting in plant growth and control of dehydration. WUE is defined by the ratio of water loss to carbon gain [105]. At the leaf level, WUE is determined by the net CO_2 assimilated by photosynthesis divided by the water lost through transpiration [180]. Changes in climatic conditions result in a modulation of WUE. For example, the recent rise in atmospheric CO_2 has resulted in increased CO_2 uptake and reduced transpiration in temperate and boreal forests of the northern hemisphere [105]. An increased WUE is a main target of breeding programs for several important crops and genetic variation has been used to identify quantitative trait loci governing WUE, often using carbon isotope discrimination as a simple measure to quantify WUE. This approach has been used in tomato [137], alfalfa [101], and sunflower [4]. In some instances, quantitative trait loci (QTL) isolation has been followed by identification of candidate genes. This was the case in a recent study in Pinus, where the first WUE QTL for which the responsible gene identified was an orthologue of the Arabidopsis Erecta gene [50]. This gene encodes a receptor-like kinase involved in the determination of stomatal density and patterning [138]. The identification of genes involved in regulation of stomatal movements has extensively relied on Arabidopsis. Several components of the signal transduction cascades activated by high/low CO_2 concentrations and ABA have

been identified and signaling pathways have been elucidated. Responses appear specific to the different stimuli in the early stages and subsequently merge in the downstream transduction pathways [107].

Several plasma-membrane–associated ion transporters are responsible for membrane polarization and depolarization events necessary to induce water exit/entrance in the guard cells that cause stomatal closure/opening, respectively. Slow anion channel-associated 1 (SLAC1) is an S-type anion channel, which, upon activation, transports anions such as malate to the apoplast [67, 189]. Membrane depolarization in turn stimulates the efflux of K^+ ions, followed by osmosis of H_2O. In an opposing role to SLAC1, ABC transporter B family member 14 (AtABCB14) transports malate into the guard cells, thereby preventing stomatal closure [118]. Gated outwardly-rectifying K^+ channel (GORK) is a K^+ outward-rectifying channel expressed in guard cells and largely responsible for the K^+ efflux caused by ABA [56, 82]. Knockout mutants in which *GORK* expression is abolished display an increased water loss caused by defects in stomatal closure [82]. Potassium channel in *Arabidopsis thaliana* 1 (KAT1) is a hyperpolarization-activated inward-rectifying potassium channel that mediates potassium influx into guard cells leading to stomatal opening [21]. Another essential component of stomatal opening is AHA1/OST2 (open stomata 2), the guard-cell plasma membrane H^+-ATPase responsible for the plasma-membrane hyperpolarization, which initiates stomata opening [142]. Plasma membrane located NADPH oxidases AtRBOHD/AtRBOHF (respiratory burst oxidase homologue D and F) determine the ABA-triggered production of second messenger ROS [115, 173] that activate plasma membrane Ca^{2+} channels, and cause ABA-induced stomatal closure [115].

Regulation of these effectors to induce stomatal closure when water scarcity conditions are perceived is achieved through an increase in ABA concentration within guard cells. This intracellular increase is the result of biosynthesis, translocation from other tissues, or the mobilization of inactive,glycosylated forms. ABA is perceived by the pyrabactin resistance (PYR)/PYL (PYR1-like)/regulatory component of ABA response (RCAR) family of intracellular ABA receptors [133, 151]. Initially identified in Arabidopsis, the ABA receptors have since been described in many species such as tomato [179, 71], beechnut [164], strawberry [39], rice [108], sweet orange [163], and soybean [16]. Upon binding to ABA, the receptors undergo a conformational change that enables them to bind to and inactivate protein phosphatase 2C (PP2Cs), a family of major negative regulators of ABA responses including ABA insensitive 1 (ABI1), ABA insensitive 2 (ABI2), homology to ABI1 (HAB1), and PP2CA [64]. When the PP2Cs are bound to the ABA receptors, their activity is inhibited and downstream components are released from PP2C-operated inactivation. In guard cells, the main target of PP2C inhibition is OST1 (open stomata 1, also known as SnRK2.6/SRK2E), a Ser/Thr kinase that constitutes a major hub for the regulation of immediate and transcriptional responses to ABA and is also involved in CO_2 responses. When OST1 is released from PP2C inhibition, several downstream effectors such as TFs (see section above) and proteins located on the plasma membrane get phosphorylated, resulting in the activation of stomatal closure promoters such as SLAC1 and AtRBOHF [67, 109, 173]. By contrast,

OST1-induced phosphorylation results in the inactivation of inhibitors of stomatal closure. For example, phosphorylation at Thr306 results in reduction of the activity of KAT1 [168].

Early signaling components of the CO_2 response pathway have also been isolated. The first identified negative regulator of CO_2-induced stomatal closure is the kinase high temperature 1 (HT1) [76]. *ht1–2* mutants show a constitutive high CO_2 response but still retain the ability to respond to variations in light wavelengths and to ABA, placing HT1 upstream of the merging point of these different signal transduction cascades. Stomatal closure induced by high CO_2 concentrations is promoted by two β-carbonic anhydrases expressed in the guard cells, CA1 and CA4. Analysis of *ca1ca4* double mutants, which retained the ability to respond to shifts in light wavelength and ABA, indicated that carbonic anhydrases act early in CO_2 perception [84]. Triple mutants in which *HT1*, *CA1*, and *CA4* were inactivated showed a constitutive high CO_2 response phenotype similar to that of *ht1–2*, indicating that *HT1* is epistatic to *CA1/CA4*. An upstream regulator of HT1 kinase was recently identified in resistant to high carbon dioxide 1 (RHC1), a mate-like protein [184]. By examining genetic interactions and biochemical properties of the different CO_2 sensing and signal transduction machinery, Tian and colleagues [184] proposed that RHC1 senses carbonic anhydrase-generated increases in carbonic acid and undergoes a conformational change that enables the inhibition of HT1. Inactivation of HT1 removes inhibition of stomatal closure by releasing OST1 from inhibition through phosphorylation.

2.3.2 Architectural: Roots, Stomatal Density, Cuticle and Waxes

2.3.2.1 Roots

The root is an important organ providing water, nutrients, and hormones to the aboveground tissues as well as mechanical support. Root architecture is a term that describes the distribution of roots within the soil profile through space and time [132] and defines the zone of water and nutrient availability to plants. This plays an important role in abiotic stress tolerance, crop performance, and yield. The interactions between developmental programs and the responses to abiotic and biotic environmental stimuli determine the architecture of the roots through which the plant explores the soil [135]. In response to environmental changes, root architecture is modified to increase water uptake efficiency. Therefore, understanding the development and architecture of roots has potential for the exploitation and manipulation of root characteristics to optimize growth in unfavorable environmental conditions [51]. Drought has a major effect on root architecture, with many plants preferentially increasing primary root elongation and suppressing lateral root branching in response to stress. Many plants adapted to drought, such as sorghum, have a naturally more vertically oriented root structure [171]. Recent studies have

identified many genetic components that contribute to root architecture; some of these have the potential to limit crop loss due to adverse environmental conditions [102].

In rice, several genes related to root architecture that confer a yield advantage in conditions of water deficit have been identified [41, 42, 93, 98]. In particular, the NAC family of TFs was characterized with regard to root architecture and some members of this family were overexpressed [211]. One of these, OsNAC9, alters the root architecture enhancing drought resistance and grain yield under field conditions [157]. The authors evaluated the overexpression of the TF OsNAC9 under the control of a constitutive or root-specific promoter in transgenic rice under both normal and drought conditions. In suboptimal water availability, grain yield was found to be improved in both transgenic lines, which showed a reduced lateral root density. Zhan et al. [208] reported that maize recombinant inbred lines with few, but long, lateral roots had substantially deeper rooting, greater leaf relative water content, greater stomatal conductance, and 50 % greater shoot biomass than lines with numerous short roots. In water stress conditions, these recombinant lines had 144 % greater yield than controls.

Hormone balance also plays an important role in the definition of root architecture. Seo and colleagues [169] reported that an Arabidopsis R2R3-type MYB transcription factor, MYB96, regulates lateral root meristem activation under drought conditions via ABA-auxin signaling crosstalk. In this signaling scheme, the MYB96-mediated ABA signals are incorporated into an auxin signaling pathway that involves a subset of *GH3* genes encoding auxin-conjugating enzymes. The activation-tagged mutant overexpressing *MYB96* had a dwarf phenotype and reduced lateral root formation while exhibiting enhanced drought resistance. Expression of the *GH3* genes was significantly elevated, which is consistent with the reduced lateral root formation. In contrast, the *MYB96*-deficient knockout mutant produced more lateral roots and was more susceptible to drought stress. The authors speculate that *MYB96* is a molecular link that integrates ABA and auxin signals that auxin homeostasis during lateral root development, especially under water-deficit conditions. Cao and Li [36] showed that autophagic programmed cell death (PCD) happens in the region of the root apical meristem in response to severe water deficit. They emphasized that ROS accumulation may trigger the cell death process of the meristematic cells in the stressed root tips. Analysis of the Arabidopsis mutant *atbi1-1*, BAX inhibitor-1 (AtBI1), under severe water stress revealed that AtBI1 and the endoplasmic reticulum stress-response pathway modulate water stress-induced PCD. These mutants develop thick and short lateral roots that result in increased tolerance to the water stress. Under severe drought conditions, plants activate the PCD program in the root apical root meristem, so that apical root dominance is removed. In this way, plants can remodel their root system architecture to adapt to the stress environment.

In conclusion, aboveground components of plants are well studied because they are accessible. Roots are less well studied because they are not readily visible and replicating the conditions in which they grow can prove difficult. Root traits, especially root length, density, and depth, have long been seen as critical traits in

order to improve crop adaptation to water stress. It is important to note that drought tolerance is a complex composite resulting from the interaction of root and shoot traits [130]. The size and activity of the root system determines the rate at which the shoot system can produce photosynthates. It is evident that improving yield under drought conditions will require a whole plant growth and functioning approach. Drought adaptation must be evaluated in relation to the timing and severity of drought stress. This may vary according to soil water-holding capacity, moisture availability at crop sowing, timing and quantity of in-season rainfall, and in association with other major abiotic stresses, such as high temperature and salinity. Drought stress frequently occurs along with high-temperature stress, and crosstalk occurs between the responses to these stresses at various levels.

2.3.2.2 Stomatal Density

Stomata are pores on leaf epidermis for both water and carbon dioxide fluxes and play a crucial role in photosynthesis and transpiration processes. The best compromise between photosynthesis and transpiration would maximize CO_2 uptake and minimize water loss, and ultimately achieve the possible maximal WUE. Stomata are, therefore, the primary determinants of plant drought tolerance because crop water loss directly involves stomata [206]. Leaf gas exchanges are greatly affected by stomatal density, defined as pore size or number. Environmental factors, such light, CO_2, temperature, humidity, and drought, can affect plant stomatal density during the development of plants. Recent advances have identified a number of genes regulating stomatal density and this has made it possible to generate plants with modified stomatal densities and analyze the effect of stomatal density on plant WUE. In Arabidopsis it has been demonstrated that angustifolia3 (AN3) functions as a focal regulator of water stress tolerance and WUE by a mechanism that involves transcriptional repression of YDA, a MAPKK kinase gene that negatively regulates stomatal development, stomatal density, and transpiration [141]. Arabidopsis plants lacking AN3 activity have high drought stress tolerance because of low stomatal densities and improved root architecture. Such plants also exhibit enhanced WUE through lower transpiration without a reduction in biomass accumulation. The AN3 was associated with a region of the YDA promoter in vivo. Mutation in YDA significantly decreased the stomatal density and root length of *an3* mutant, thus proving the participation of YDA in *an3* drought tolerance and WUE enhancement. These components form an AN3–YDA complex, which allows the integration of water deficit stress signaling into the production or spacing of stomata and cell proliferation, thus leading to drought tolerance and enhanced WUE.

Also in Arabidopsis, Franks et al. [63] found that a reduction in stomatal conductance via reduced stomatal density in epidermal patterning factor (EPF2)-overexpressing plants increased both instantaneous and long-term WUE without altering significantly the photosynthetic capacity. Conversely, plants lacking both EPF1 and EPF2 expression exhibited higher stomatal density, higher stomatal conductance, and lower instantaneous and long-term WUE. Arabidopsis plants with

lower stomatal densities that have reduced transpiration and greater drought tolerance have been found to have little or no loss of nutrient uptake [78].

In rice it has been reported that an Arabidopsis homeodomain–leucine zipper transcription factor enhanced drought tolerance/homeodomain glabrous11 (EDT1/HDG11) was able to confer drought tolerance and increase grain yield in transgenic plants. The improved drought tolerance was associated with a more extensive root system, reduced stomatal density, and higher water use efficiency [207]. Heterologous expression in tobacco of *SlERF36*, a tomato EAR motif-containing transcription factor, leads to a 25–35 % reduction in stomatal density but without any effect on stomatal size or sensitivity [187]. Reduction in stomatal density leads to a marked reduction in stomatal conductance (42–56 %) as well as transpiration and is associated with reduced CO_2 assimilation rates, reduction in growth, early flowering, and senescence. *SlERF36* overexpressing plants have constitutively high nonphotochemical quenching (NPQ) that might function as a protective measure to prevent damage from high excitation pressure. The high NPQ leads to markedly reduced light utilization and low electron transport rates even at low light intensities. Taken together, these data suggest that *SlERF36* exerts a negative control over stomatal density and modulates photosynthesis and plant development through its direct or indirect effects. In Arabidopsis, the expression of *Medicago truncatula* cold-acclimation specific protein 31 (MtCAS31) in response to NaCl, ABA, cold, and drought stress was analyzed [200]. MtCAS31 was significantly upregulated following drought stress. Overexpression of MtCAS31 markedly increased drought tolerance and decreased stomatal density of transgenic plants.

In rice, Liu et al. [123] observed that phytochrome B (*phyB*) mutants exhibited enhanced drought tolerance, suggesting that *phyB* may be involved in the regulation of tolerance to drought stress. They demonstrated that *phyB* mutants exhibited reduced stomatal density and length and showed a decreased transpiration per unit leaf area that contributed to the improved drought tolerance. In these plants, the expression of genes related to stomatal such as Erecta and Expansin gene families were upregulated in the *phyB* mutants by comparison. This suggests that this increased expression in the leaves of the *phyB* mutants probably resulted in the enlarged epidermal cells and therefore the reduced stomatal density without changing the stomatal index. The Arabidopsis GT-2 LIKE 1 loss-of-function mutations (*gtl1*) result in increased water deficit tolerance and higher integrated WUE by reducing daytime transpiration without a reduction in biomass accumulation [206]. The *gtl1* plants had higher instantaneous WUE that was attributable to about 25 % lower transpiration and stomatal conductance but CO_2 assimilation. Lower transpiration was associated with higher expression of stomatal density and distribution1 (SDD1) and an about 25 % reduction in abaxial stomatal density. GTL1 expression occurred in abaxial epidermal cells where the protein was localized to the nucleus, and its expression was downregulated by water stress. GTL1 interacts with a region of the SDD1 promoter that contains a GT3 box, necessary for the interaction between GTL1 and the SDD1 promoter. These results establish that GTL1 negatively regulates WUE by modulating stomatal density via

transrepression of SDD1. Using two cultivars with contrasting responses to salinity has been able to demonstrate that reduced stomatal density increased salinity tolerance and WUE under salt stress. Constitutive low transpiration fluxes associated with reduced stomatal density may uncouple plant adaptation and yield reduction under saline stress in a specific agricultural context [18, 150]. It is plausible that through genetic modification of stomatal density, by breeding selection and/or genetic methods, improving crops for better WUE and drought tolerance is achievable. Understanding how multiple signals contrast with the components of stomatal development will be the next challenge. Knowledge of such molecular interactions will elucidate the significance of stomata to whole-plant growth, development, and physiology. Lastly, as genome sequence information of more plant species becomes available, it will also become possible to understand the conservation and uniqueness of the evolution of gene regulatory networks specifying stomatal development.

2.3.2.3 Cuticle and Waxes

The plant cuticle is a hydrophobic coating composed of a cutin polyester membrane impregnated and overlaid with free waxes that provides the last barrier over essentially all aerial plant organs [72]. The cuticle is synthesized by the epidermal cells and it can protect plants from nonstomatal water loss, dust deposits, pollen, and air pollutants as well as biotic and abiotic stresses such as UV radiation damage and bacterial and fungal pathogens [97, 114, 161, 162]. The mechanical structure and chemical composition of cuticle lipids vary considerably between plant species, and in response to environmental stimuli and stresses. Several studies have indicated that drought can induce increased wax deposition on the leaf surfaces of different plant species, including Arabidopsis [204], cotton [28], peanut [166], and tree tobacco [34]. The importance of cuticle function is highlighted by studies using mutants defective in cuticle biosynthesis, which often do not survive when germinating under normal conditions but can be rescued by high humidity [203].

Increased levels of cuticular waxes have been associated with enhanced drought tolerance in oat [22], rice [92], and sorghum [100]. A mutant of wild barley, *eibi1*, with a very thin cutin layer, was hypersensitive to drought [40]. Breeding for greater tolerance and yield under drought conditions led to increased amounts of cuticle waxes, further confirming the connection between drought tolerance and cuticle properties [70]. Thus, the activated biosynthesis of cuticle waxes appears to be an established plant response to dry conditions.

Many genes coding for enzymes involved in the biosynthesis of cuticle components have been isolated and characterized [99, 167, 169].

Transcription factors (TFs) are involved in the regulation of biosynthesis and accumulation of cuticle components. Most of these belong to one of three different families: ethylene responsive factors (ERFs), myeloblastosis family (MYB) TFs, and homeodomain–leucine zipper class IV (HD-Zip IV) factors [5, 31, 45, 96, 169, 209]. Overexpression of these TFs leads to changes in cuticle accumulation and/or

composition often increasing stress tolerance. In many cases, overexpression of these TFs negatively affects plant growth and yield [5, 209]. Recently, Wang et al. [195] isolated a *CER1* homologue *CsCER1*, a gene involved in alkane biosynthesis, in cucumber. They showed that abnormal expression of *CsCER1* in transgenic cucumber plants had dramatic effects on very-long–chain (VLC) alkane biosynthesis, cuticle permeability, and drought resistance. In Arabidopsis, the mutant, *shine* (*shn*) displays characteristics of plant surface defects [5]. When compared with the wild type, leaves of *shn* show a deep shiny green appearance, with a curled structure. They also have altered cuticle permeability, cuticular wax load and structure, and epidermal differentiation. The *SHN* gene encodes an AP2/EREBP transcription factor, and the characterization of two of its homologues suggests that this clade of genes acts in the regulation of lipid biosynthesis required for protection of plants from the environment, including organ separation processes and wounding. The tomato orthologue, SlSHN1 transcription factor, was also isolated and the expression analysis indicated that it is induced in response to drought conditions [6]. Overexpression of SlSHN1 in tomato produced plants that showed mild growth retardation with shiny and dark green leaves. Expression analysis indicated that several wax-related synthesis genes were induced in transgenic lines overexpressing SlSHN1. Transgenic tomato plants showed higher drought tolerance compared to wild-type plants; this was reflected in delayed wilting of transgenic lines, improved water status, and reduced water loss.

Zhou et al. [212] conducted a functional analysis of OsGL1-6 in rice. OsGL1-6 is homologous to CER1 in Arabidopsis and *Wda1* in rice, universally expressed in vegetative and reproductive organs, and especially highly expressed in leaf epidermal cells and vascular bundles. A phenotypic characterization and drought sensitivity experiments on OsGL1-6 antisense-RNA transgenic plants indicated that OsGL1-6 is involved in cuticular wax accumulation and drought resistance. The drought susceptibility was in agreement with their deficient cuticles and positively correlated with the reduced accumulation of the leaf cuticular wax, implying its role in drought stress resistance. Thus, genetic modification of OsGL1-6 may have great potential for improving the drought resistance of rice. Studies on mutation of the eceriferum9 (CER9) gene in Arabidopsis showed extreme alteration in the cuticular wax profile (especially on leaves) toward the VLC free fatty acids tetracosanoic acid (C24) and hexacosanoic acid (C26; [129]). Relative to the wild type, *cer9* mutants exhibit elevated cuticle membrane thickness over epidermal cells and cuticular ledges with increased occlusion of the stomatal pore. CER9 is the first described cuticle biosynthesis gene whose deficiency improves both plant response to water deficit and WUE, indicating that CER9 may encode an important new cuticle-associated drought tolerance determinant. These studies provide evidence that the CER9 protein is a negative regulator of cuticle lipid synthesis via its putative role as an E3 ubiquitin ligase, similar to Doa10 in yeast. Due to its novel impact on plant water status, elucidation of CER9's cellular function may reveal new molecular breeding and transgenic strategies to improve the drought tolerance and WUE of crop plants.

Systematic studies of the large collection of diverse wax mutants now available should highlight the specific contribution of single wax compounds in

plant/environment interactions as well as in the organization of waxes, together with cutin, in the highly structured cuticle. Clearly, a coregulation of cutin and waxes is required for both environmental and developmental purposes of the cuticle. The transcriptional regulators controlling the deposition of both lipophilic materials throughout plant development must be investigated [23].

2.4 Landraces and Wild Relatives as Sources for Drought Tolerance Traits

2.4.1 Tomato

Global demand for tomato is steadily increasing, particularly in developing areas prone to drought cycles [174]. Therefore, it is necessary to develop more drought-tolerant tomato varieties to address this need. Although there are a growing number of studies designed to investigate the mechanisms of drought response in tomato, most modern varieties are sensitive to a wide range of abiotic stresses [62]. The efforts of the International Solanacee genome project (SOL) and of the 100 Tomato Genome Sequencing Consortium et al. [183], have recently provided a tremendous genomic resource for the research community and the possibility successfully to exploit for tomato breeding for stress tolerance the enormous reservoir of adaptive traits present in wild species and in some *S. lycopersicum* landraces locally adapted to arid environments. There are a number of tomato relatives, including *Solanum chilense* and *S. peruvianum*, adapted to growth under water restriction imposed by their habitat, that are known to be very drought tolerant, but only a few studies have examined in detail drought tolerance-related morphophysiological traits [181, 199]. Here, we focus on two promising wild relatives that have been already extensively characterized for their adaptive features and for which a wide collection of genetic and genomic tools are available. The wild relative *Solanum pennellii* is native to the Andean area of South America and is evolutionarily adapted to arid conditions [160]. Comparative transcriptomics between *S. pennellii* and *S. lycopersicum* showed distinct patterns of evolution. Domesticated tomato was selected for a number of fruit traits and postharvest quality, however, the wild relative retained a number of gene expression patterns more suited for environmental response and stress tolerance [110]. For example, several genes involved in wax deposition were highly expressed in *S. pennellii*, possibly accounting for the thicker cuticle of the wild species compared to cultivated tomato. By contrast, one developmental regulator contributing to the definition of the stomatal index had a lower expression in *S. pennellii*, which has a different stomatal density compared to *S. lycopersicum* [110]. This selective pressure driven by adaptation to an arid environment has resulted in rapidly evolving genes not selected for in the artificial selection that shaped domesticated tomato. The recently completed genome of *S. pennellii* makes it an ideal source for mining novel traits [27]. A number of introgression lines (ILs) are available, where the whole genome of *S. pennellii* is represented in the genetic

background cultivar, M82 of *S. lycopersicum* [58]. Some of these segments have been shown to increase remarkably the agronomic performance of *S. lycopersicum* and some lines showed increased yield and brix units under drought stress [75]. Adaptation to arid conditions in *S. pennellii* is also accompanied by increased salt tolerance over cultivated tomato. It appears that the antioxidative systems in *S. pennellii* are more robust than cultivated tomato, particularly under salt stress [145]. Genomic studies, between *S. lycopersicum* and *S. pennellii*, of QTLs associated with drought or salt tolerance have correlated these QTLs with gene copy number, allelic polymorphisms, and polymorphisms within the promoters of key genes [27]. Traits from *S. pennellii* have already been shown to increase drought tolerance in cultivated tomato. The universal stress protein (USP) is involved in ABA responses to abiotic stress but is not well characterized. Transgenic tomato plants expressing the *S. pennellii* USP gene were more tolerant to drought stress as seedlings and adults [128]. It is certain that *S. pennellii* will provide a number of traits for the genetic improvement of tomato under adverse stress conditions.

The second species of interest is *Solanum habrochaites*, which is highly tolerant to drought and low temperatures [41, 42]. Comparative transcriptomic studies between *S. habrochaites* and *S. lycopersicum* have been done on secondary metabolism [25], freezing tolerance [41, 42], glandular trichomes [139], and disease resistance [165]. As with *S. pennellii*, near isogenic lines and backcross recombinant inbred lines have been developed in the *S. lycopersicum* background [61, 146]. These lines provide a valuable resource for discovery of novel traits and map important quantitative trait loci. Under root chilling stress *S. habrochaites* exhibits tight control of stomatal closure and retention of water whereas *S. lycopersicum* is unable to prevent water loss in these conditions [11]. This implies that *S. habrochaites* exhibits tighter control in water limiting conditions. Introduction of the *S. habrochaites* cold-induced SK3-type dehydrin gene increased both cold and drought tolerance in cultivated tomato. These transgenic plants also grew better under osmotic and salt stress and were more tolerant to oxidative stress [125]. Adaptations to the environment on slopes of the Andes of Ecuador and Peru have already provided novel traits for cold tolerance and will doubtlessly provide more for the improvement of drought tolerance in tomato.

2.4.2 Potato

Potato production worldwide is strongly affected by water stress, either because of insufficient rainfall or due to inadequate irrigation. Improving drought tolerance is consequently becoming a priority for potato breeders, particularly in the perspective of climate change. Modern potato varieties are highly sensitive to drought stress [197]. In contrast, landraces of Andean potato species and wild potatoes occurring in the Americas, from the United States to Chile and Uruguay [79] are better adapted to harsh environments and regularly exposed to water-deficit conditions. Moreover, primitive forms of cultivated potato and their wild relatives provide a rich, unique,

and diverse source of genetic variation, which could be a source of various traits for potato breeding. This may be because of their adaptation to a broad range of habitats and niches varying in latitude, altitude, habitat, soil, and precipitation regimes.

The many wild relatives and primitive cultivars of potato have proven to be valuable in breeding programs for improvement of disease resistance, abiotic stress tolerance, and other agronomic traits and qualities of interests [17, 19, 49, 80, 95, 149, 176]. As potato has gained importance as a food source in developing countries [90], the breeding has shifted to adaptation to the conditions of these countries, generally hot and dry environments.

Therefore genes from wild relatives and landraces should be explored to the improvement of potato tolerance [79]. The major problem in this case is undesirable effects of genes linked to the introgressed trait or gene [91, 136, 32]. In addition, photoperiod requirements of modern varieties compared with native potato are different, which could explain why plants from interspecific crosses and back-crosses often have lower yield, small tuber number, late maturity, poorer foliage, and altered tuber appearance when grown under nonsuitable photoperiods [104].

Drought-tolerant accessions identified in Andean potatoes [188] have been barely used in breeding programs because of their adaptation to the short day conditions prevalent in the low latitudes. In this context, some attempts have been made to transfer drought-tolerance genes from wild to cultivated potato species via traditional breeding. In addition, potato breeders have used somatic fusion, embryo rescue, and bridging strategies to overcome the natural barriers from interspecific crossing between wild and cultivated species. Screening for drought tolerance in potato landraces has been performed by Cabello et al. [33, 32]. A high proportion of accessions combining drought tolerance with high irrigated yield were found in Andean landraces, particularly in the species *S. curtilobum* in the *S. tuberosum* L. cultivar groups Stenotomum, Andigenum, and Chaucha. Watanabe et al. [196] identified *S. chillonanum*, *S. jamesii*, and *S. okadae* as potential drought-tolerant species by screening 44 accessions of wild species selected based on their drought habitats derived from GIS information. Climate change and other factors that additionally increase pressure on ecosystems are threatening the existence of many wild relatives. The establishment and maintenance of gene banks is intended to narrow the loss of this diversity in varieties. The genetic resources of the potato are preserved in the form of true potato seeds, vegetative tubers, and in vitro seedlings. In particular, conservation under in situ conditions is considered an important strategy to preserve the genetic resources. In situ conservation involves exposing the varieties in question to natural conditions in the field. Sustainably increasing productivity in a changing climate is one of the most important challenges for people conducting research on potato worldwide to ensure food security. Primitive cultivars and wild relatives of potato have been used as sources of desirable traits, such as resistance or tolerance to diseases, pests, and environmental stresses, and of tuber qualities, for potato breeding. Tools for incorporating useful alleles from its wild relatives into cultivated potato have been developed so that there remains a

broad gene pool to be more effectively exploited. Currently, large amounts of potato germplasm containing useful alleles are available in gene banks around the world; however, re-collection may reveal novel genes. Precise identification of species is essential for making decisions for effective utilization of germplasm collections; therefore, taxonomic research and updating taxonomical descriptions of the gene bank collections in potato are indispensable [134].

2.4.3 Wheat

The Green Revolution resulted in high-yielding semi-dwarf wheat (*T. aestivum*) and rice (*O. sativa*) cultivars with improved responsiveness to fertilizer and irrigation [29]. This selection for aboveground traits and focus on yields under optimal environments may have overlooked traits that enhance growth under limited water [194]. Over the previous decades, a number of studies have examined the drought tolerance of wheat landraces [26, 52, 55, 152]. Later studies used DNA fingerprinting to reveal diversity and divergence among wild relatives and landraces [159]. Larger high-throughput studies of these wheat relatives have been able to assess over 9000 SNPs amongst 2994 accessions comprising both modern cultivars and landraces [38]. Such studies that establish genomic diversity maps are valuable tools for finding relevant traits in landraces and wild relatives.

Allelic diversity in wheat has been increased with landrace accessions from extreme environments through crossing or interspecific hybridization. This hybridization uses ancestral genomes to produce synthetic hexaploid-derived wheat lines (SYN-DER) [159]. Synthetic hexaploid wheats (SHWs) and their synthetic derivative lines (SDLs) provide a way of introducing genetic diversity from ancestor genomes into cultivated wheat varieties [127]. These SYN-DER lines have been used to study novel drought tolerance alleles from wheat relatives [8, 48]. Landraces of wheat can also be used to improve traits controlling root architecture and drought avoidance. Many modern wheat varieties have smaller root masses, optimized for shallow irrigation and absorption of added fertilizers, compared to some landraces. Some of the landraces have mapped traits, such as 1RS that can improve root architecture and drought avoidance [194]. When the chromosome segment 7DL from the wild relative *Agropyron elongatum* was introduced into cultivated wheat, the translocation line showed improved root and shoot biomass, improved water stress adaptation, and enhanced access to water for growth [154].

Nutrient assimilation and growth under limiting conditions is an additional target for genetic improvement. Landraces and wild relatives have the potential to improve assimilation, particularly under limiting conditions. Studies with *Brachypodium distachyon* have revealed heritable traits that maintain growth, even in limiting conditions [89].

Proteomic studies using contrasting wheat landraces, N49 and N14, under drought stress revealed key proteins that are involved in oxidative stress response, senescence, and mobilization of carbohydrate reserves [20, 59]. Further studies of drought-tolerant landraces with proteomes and subproteomes under stress can elucidate the role of drought-responsive proteins and their expression, abundance, and posttranslation modifications [85].

2.4.4 Rice

O. sativa is thought to have been domesticated over 6000 years ago, but since domestication introgression of wild germplasm from cross-compatible species has been a natural and ongoing process [14]. Over 120,000 genotypes of *O. sativa* and *Oryza glaberrima* exist in gene banks, but very little genetic diversity exists within these accessions [14]. More than 22 wild species of *Oryza* are known and the genetic diversity within these wild relatives can provide novel traits for drought tolerance. Transfer of genes from wild relatives into cultivated rice has had several impediments to making crosses, such as low crossability and limited recombination between chromosomes [30]. With the discovery of the killer–protector system at the S5 locus encoded by three tightly linked genes, open reading frame 3 (ORF3), ORF4, and ORF5, it may be possible to overcome reduced fertility in hybrids [205]. The research community is striving to build tools to utilize the existing diversity. Two of the goals of the International Oryza Map Alignment Project are to sequence reference genomes and transcriptomes for all species and generate advanced mapping populations for functional and breeding studies [94]. Sequencing of 517 rice landraces revealed approximately 3.6 million SNPs that were used to construct a high-density haplotype map of the rice genome [87]. Genomewide association studies using such resources can identify novel traits that contribute to agronomic improvement. Such association studies can reveal the role of known drought tolerance genes, such as OsDREB1F, and the presence of variant proteins within drought-tolerant wild relatives [172]. Generation of introgression lines using elite cultivars and a wild rice Dongxiang accession (*O. rufipogon* Griff.) was used to generate and identify a drought-tolerant introgression line [210]. Comparative analysis of cultivated rice and the drought-tolerant landrace, Nagina 22 (N22), revealed differential regulation of both primary and secondary metabolism genes [120]. Of the three major cereal crops, rice is the most sensitive to drought stress, primarily due to the shallow roots of most cultivars [111]. Improvement in root architecture and root depth is a key focus in rice improvement [73]. The locus deeper rooting 1 (DRO1) has been definitively shown to contribute to drought avoidance in rice. DRO1 alters root system architecture by controlling root angle growth [186]. DRO1 was successfully introduced into IR64, a commonly grown shallow-rooting cultivar, and the near isogenic line containing DRO1 demonstrated deeper rooting and improved drought avoidance [10].

2.4.5 Corn

When researchers analyzed field-level data for 17 years of maize productivity in the American mid-west, they found that absolute yields have increased over that time period. However, sensitivity of maize yields to drought stress had increased. Selection and breeding for traits clearly increased overall yield, but failed to decrease yield sensitivity to drought stress [126]. Although yield potentials have drastically increased in maize through traditional and molecular breeding, much less emphasis has been placed on breeding for WUE or drought tolerance. QTLs associated with tolerance have been identified, however, they may be of limited utility for applied breeding due to their dependency on genetic background and a lack of understanding of the biophysical basis of these traits [35].

In maize, tropical landraces and inbred lines possess numerous potential traits for increasing WUE and drought tolerance. A number of these lines have been assessed for their drought tolerance [3, 88, 143, 147, 148, 202]. Traits controlling root anatomy and morphology can also play a key role in maize drought tolerance. When lines with contrasting cortical cell sizes were subjected to water-stress conditions, those with large cortical cells showed 21 and 27 % deeper rooting, 50 % greater stomatal conductance, and 59 % greater CO_2 assimilation [43]. The group who reported these finding proposes that the increased tolerance is due to a reduction in the metabolic cost of soil exploration by roots and facilitates greater exploration to increase water acquisition. The same group found that lines with a reduced cortical cell file number have a similar benefit under water stress [44]. Recombinant inbred lines with contrasting root number and length have been assessed in a number of water-limiting conditions. Lines with fewer, but longer roots showed greater stomatal conductance and 50 % more shoot biomass and up to 144 % great yield under water-limiting conditions [208]. Identification of hormonal regulators associated with QTLs, such as members of the CYP707A subfamily responsible for ABA catabolism and ARR, a negative regulator of cytokinin signaling, have revealed control points in regulating hormonal responses to drought stress [201]. Genomewide analysis of 368 varieties was used to evaluate DREB transcription factors in conjunction with the cloning of 18 *ZmDREB* genes present in the maize B73 genome. Analysis indicated a significant association between *ZmDREB2.7* and drought tolerance at early developmental stages. Natural variation in the promoter region of *ZmDREB2.7* correlated with varying levels of drought tolerance [124]. Tolerant landraces demonstrated higher stomatal conductance and rates of photosynthesis under drought stress. Upregulation of ABI3 and *HVA22* exclusively in drought-tolerant lines indicated ABA-responsive genes may play a key role. The investigators also found a number of other differentially regulated genes, AP2, bHLH, C2C2, C2H2, C3H, zinc finger, CCAAT binding factor (HAP2), and WRKY gene families, expressed in tolerant lines, but not in the sensitive line [77].

2.5 Conclusions

As discussed in this chapter, there are a number of key genes that control or contribute to drought responses in crop species. Understanding the mechanisms behind these traits is essential for the genetic improvement of crops. Although new genomes and transcriptomes emerge daily for these species, model systems, such as *Arabidopsis thaliana*, remain our best system for dissecting these traits. Wild relatives and landraces represent a vast pool of traits that can be utilized for novel stress tolerance traits, but a thorough functional characterization is necessary in order to take advantage of the benefits they offer. The challenges posed by drought and water deficit are not insurmountable; they can be overcome with sufficient understanding of the genetic basis of tolerance and the resources available to the research community.

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Chapter 3
Tolerance to Drought Stress in Plants: Unravelling the Signaling Networks

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3.1 Introduction

The ever-increasing human population and food demands share a linear statistical relation. Food security is the major threat in the milieu of increasing population and devastating climate regimes. In the current era of increasing uncertainty for crop production, climate smart agriculture assumes importance. Risks associated with climate change demand technical and technological advances in the agricultural sector. Among the various abiotic stresses that limit plant growth and productvity, drought is the most crucial factor in tropical regions. Because drought tolerance is a complex trait, for targeted crop improvement an understanding of the traits and molecular mechanisms linked to tolerance to drought assumes significance. A trait-based approach is the current strategy to unravel and tackle the complexity of drought stress response in plants. Many cellular tolerance mechanisms starting from signal perception to activation of multiple downstream processes are being examined and employed for targeted manipulation of plants for stress tolerance. Plants, being sessile, have evolved diverse adaptive mechanisms that allow them to survive in an ever-changing environment. They have the ability to sense their environment and undergo changes in their physiology and developmental processes, whether they are adverse or beneficial. Adjustments under stressful condition depend upon the signal perception, signal transduction, and activation of acclimation response in a coordinated manner.

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3.2 Generalized Signal Perception and Transduction Pathway: Signaling Cadres

Plants respond to environmental variables or stress by complex, well-coordinated mechanisms. The signaling cascade is under the control of multiphase regulation involving various gene products, protein complexes, and metabolites. There exists a tightly regulated signaling network starting from stress signal perception up to downstream functional gene/protein activation, and the genes that regulate their activity [1]. A general signal transduction pathway includes the journey right from the signal perception by the stress-specific receptor followed by signal amplification by secondary messengers, signal transduction leading to activation/disruption of regulatory protein activity that governs functional gene activation or expression involved in stress response (Fig. 3.1).

3.3 Drought Signal Perception: Receptors and Their Relevance

External stimuli activate the receptor molecules and initiate complex downstream signaling networks that exhibit crosstalk in order to respond to various environmental and developmental cues in an appropriate and integrated manner [2]. Endogenous stimuli alter the molecular and biochemical mechanisms that adjust overall plant growth and development for better survival [3–8]. The signals involved in intercellular signaling control molecular processes that play important roles during growth and development and stress responses. The pivotal role of cell–cell communication has been identified in cell fate determination and organ

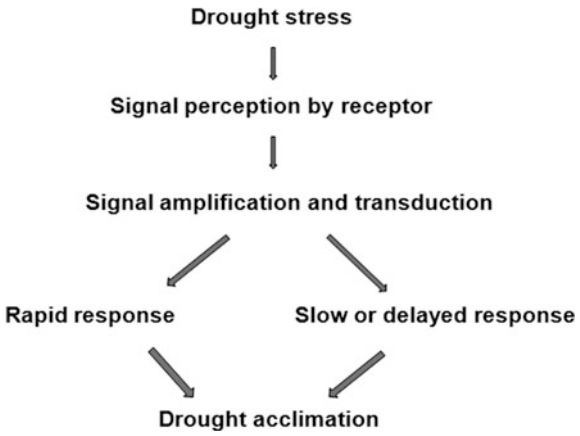

Fig. 3.1 Schematic representation of generalized signaling network associated with drought signaling in plants

development [9]. Signaling molecules involved in the cell–cell communication include small organic molecules, small peptides, ions, and physical stimuli. These signals are initially received by receptor proteins and sequentially transmitted to target signaling elements. Phosphorelay involving the activation of protein phosphorylation cascades is very crucial for early stress response. Several protein kinases act as signal transducers under drought [10, 11]. The reported receptors involved in drought stress response include receptor-like kinases (RLKs), receptor-like cytoplasmic kinases (RLCKs), and histidine kinases (HKs).

3.3.1 Receptor-Like Kinases (RLKs)

RLKs play important roles in perceiving external and sensing internal signals, and also activating downstream signaling responses in plants. Perception of extracellular signals through plasma membrane-bound RLKs in plants are known to alter the concentrations of cellular ions and molecules that activate protein phosphorylation pathways, thus assisting in converting external signals to intracellular cytoplasmic signals. RLKs belonging to the serine/threonine protein kinase family are involved in phosphorylation and dephosphorylation of the Ser/Thr residues [12, 13]. They contain Ser/Thr kinase as a cytosolic domain and have structural elements similar to animal receptor tyrosine kinases (RTKs). This major gene family in plants contains 610 members in *Arabidopsis* and about 1132 members in rice [12, 14].

RLKs convey the signal to their target proteins in the cytoplasm by catalytic processes of protein kinase activity. In plants, RLKs are grouped into multiple subfamilies on the basis of N-terminal extracellular domains, and the proteins containing a leucine-rich repeat domain (LRR-RLKs) are reported to be the largest subfamily. RLKs perceive peptidic ligands or phytohormones to trigger the signaling cascades, leading to an adaptation to the adverse environmental changes. In a well-studied crop, rice, about 309 LRR-RLK genes have been reported that are classified into five subgroups [15], and there are indications on the role of LRR-RLKs in regulating different environmental stress responses. LRR-RLKs involved in stress response have been identified, and salt-induced receptor-like kinase, Srlk (encoding an LRR kinase), regulates salt stress response in roots [16].

The importance of RLKs in abiotic stress response has been well recognized in both monocots and dicots and the LRR-RLK proteins seem to play a key role in regulating drought response. The LRR-RLK family genes, OsSIK1 and OsSIK2, affecting drought response, have been well studied in rice [17, 18] and more vital information on RLKs in drought response are being reported, indicating that these are major components in drought signaling in plants. OsSIK2, which is expressed mainly in rice leaves and leaf sheaths [17], is induced by NaCl, drought, cold, dark, and ABA treatment. OsSIK2 overexpressing transgenic plants exhibit enhanced tolerance to salt and drought stress [17]. The existing information indicates that RLKs are capable of perceiving abiotic stress signals and activating downstream responses in plants.

3.3.2 Receptor-Like Cytoplasmic Kinases (RLCK)

Membrane localized RLKs are known for their early stress-responsive roles wherein they perceive external stimuli and convert them into meaningful cellular responses as discussed previously [2, 19]. RLKs possess an extracellular domain, a transmembrane domain, and an intracellular kinase domain [20], which function by homodimerization or heterodimerization, upon signal perception, followed by phosphorylation cascades resulting in stress responses [2, 21]. In addition, there are plant-specific RLKs without an extracellular domain with only the transmembrane domain containing an intracellular kinase domain or only an intracellular kinase domain. These RLKs are termed RLCKs [22–26]. About 200 and 379 RLCK encoding genes have been reported in the *Arabidopsis* and rice genomes, respectively [22, 24, 26]. In the rice genome, 82 RLCKs were differentially expressed under abiotic stresses [26]. A list of functionally characterized abiotic stress-associated RLCKs is given in Table 3.1.

3.3.3 Histidine Kinases (HKs)

HKs are the important receptors involved in a wide range of cellular responses in bacteria, fungi, and plants. They are part of two-component signaling systems, and can phosphorylate a specific target protein, termed the response regulator, and modulate its interactions with downstream elements [27]. Similar to RLKs, plant

Table 3.1 List of functionally characterized receptor-like cytoplasmic kinases (RLCKs) involved in abiotic stress response

Sl. No.	RLCK	Stimulus	Reference
1	ARCK1 (*Arabidopsis thaliana*)	ABA- and osmotic stress-inducible; Interacts with cysteine-rich receptor-like kinase 36 (CRK36)	[104]
2	GsCBRLK (*Glycine soja*)	High salinity and ABA inducible; Calcium-binding RLCK	[105, 136]
3	CRCK1 (*Arabidopsis thaliana*)	Calmodulin-binding RLCK	[105, 136]
4	PSTOL1 (Traditional rice)	Phosphate deficit soil; Improved root growth and grain yield of rice and drought response	[106]
5	OsRLCK253 (Rice)	Interacts with stress-associated proteins (OsSAP1/11) to mediate drought and salt stress responses	[107]
6	OsGUDK (Rice)	Drought stress signaling by activation of stress genes by OsAP37	[25]

HKs have been shown to mediate responses to endogenous and exogenous stimuli. In plants HKs play a crucial role in hormone signaling, and also abiotic and biotic stress responses. Nine HKs namely, ETR1, ETR2, EIN4, CKI1, AHK1/AtHK1, AHK2, AHK3, AHK4/CRE1/WOL, and AHK5 have been reported in *Arabidopsis*. The ETR1, ETR2, EIN4, CKI1, AHK2, AHK3, and AHK4/CRE1/WOL HKs mediate hormone signaling, whereas AHK1/AtHK1, AHK2, and AHK3 are associated with drought signaling [28, 29]. The AHK5 was initially shown to act as a negative regulator of ABA/ethylene-mediated root growth inhibition and AHK5 negatively regulates ROS levels during abiotic stress [29].

3.4 Drought Signal Amplification

3.4.1 Secondary Messengers in Signaling and Their Complex Interactive Effects

The activation of a signal transduction pathway is initiated by the perception of a signal by a specific receptor, in any case. This elicits the production of secondary signals, which can mediate protein phosphorylation cascades such as mitogen-activated protein kinase (MAPK) signaling. In principle, both second messengers and hormones can be regarded as secondary signals [19]. Calcium (Ca^{2+}) and reactive oxygen species (ROS) are well-studied second messengers along with others such as phospholipids [30]. Some of the secondary signals are involved in multiple pathways because of the intricate crosstalk networks among different signaling systems. A single stressor such as drought can activate several signaling pathways that could serve the purpose under different stress scenarios.

The perceived stress signal is conveyed to the nucleus, by an array of secondary messengers such as inositol 1, 4, 5-trisphosphate (IP3), diacylglycerol (DAG), and Ca^{2+} which are generated by phospholipid signaling systems. On signal perception, by stabilization of calcium mobilizing signals, Ca^{2+} levels spike or oscillate within a cell. The cellular Ca^{2+} signals are detected and further transmitted by sensor molecules. The three main classes of calcium sensors identified in plants are calmodulin (CaM) and CaM-related proteins, calcium-dependent protein kinases (CDPKs), and calcineurin B-like proteins (CBLs) [31, 32]. The stress signal can first activate phospholipase C (PLC), which generates IP_3 and DAG by hydrolysis of PIP_2 leading to an increase in cytosolic Ca^{2+}, which is sensed by Ca^{2+} sensors. These sensors further transmit the message by phosphorelay signal transduction leading to the expression of multiple stress-responsive genes, the products of which can directly or indirectly impart stress tolerance.

Plants possess a variety of phospholipid-based signaling pathways when subjected to abiotic stress, which changes the phospholipid composition of the plasma membrane [30]. PLC, phospholipase D (PLD), phospholipase A2 (PLA2), diacylglycerol pyrophosphate (DGPP), and phosphatidylinositol 3,5-bisphosphate (PI

(2, 3)P2) are the major enzymes involved in major phospholipid signaling pathways [33]. The transient production of phosphatidic acid (PA), either by the phosphorylation of DAG in the PLC signaling cascade or by the PLD signaling cascade, activates specific MAPK pathways [34]. Drought stress rapidly induces the PLD-mediated production of phosphatidic acid [35, 36]. Under oxidative stress, which could be an indirect aftereffect of drought stress, phosphoinositide-dependent kinase 1 (PDK1), a potential PA target, activates OXI1, which lies upstream of MPK3 and MPK6 [37]. The serine/threonine protein kinase Pto-interacting 1-4 (PTI1-4) is now identified as a downstream target of OXI1 which physically interacts with MPK3 and MPK6 [38]. The PA, PDK1, OXI1, and MPK3/MPK6 could be part of the same signaling pathway under stress. The significance of PLD under drought stress is aptly emphasized by the increased sensitivity of *pldα3* knockout plants to salt and dehydration stresses [39].

Reactive oxygen species (ROS) are widely reported to regulate water-deficit–induced stomatal closure [40, 41], which is an important water conservation mechanism reducing transpirational water losses. This indicates its significance under drought, which makes it a powerful signaling molecule involved in the drought acclimation response. There is an increased ROS production in photosynthesis during drought. Chloroplast production of ROS is a major driver of redox signaling or damage during drought. The mitochondrial electron transport chain is another possible source of superoxide and H_2O_2 during drought [42]. In spite of their detrimental nature, ROS are considered to be key signaling partners in response to stress stimuli [43, 44]. Any deviation from cellular homeostasis leads to an imbalance in the steady-state ROS levels which acts as a signal. Extracellular H_2O_2 is thought to be important in systemic long-distance signaling [45]. Plants possess certain defensive mechanisms to get rid of excess ROS by employing scavenging enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, or glutathione reductases [46]. ROS and several antioxidant molecules are tightly linked to integrate the metabolic and physiological status in various organelles to regulate complex interorganellar signaling pathways leading to cellular response [44, 46–48]. Recently, ROS has been given the status of rapid long-distance autopropagating signals, ROS waves, which are transferred throughout the plant to coordinate systemic plant stress responses [44]. There are many antioxidative and redox-homeostatic mechanisms functional under drought. The removal of superoxide and H_2O_2 are regulated by low levels of antioxidants such as ascorbate and reduced glutathione (GSH). Catalases (CATs) and ascorbate peroxidases (APXs) are the main enzymes involved in H_2O_2 removal. Simultaneously, glutathione peroxidases (GPX) [49], glutathione S-transferases (GST) [50], and peroxiredoxins (PRX) [51] reduce H_2O_2 and organic hydroperoxides by ascorbate-independent thiol-mediated pathways. This information suggests that plants have fine-tuned inbuilt mechanisms to regulate the levels of ROS, and ROS-mediated signaling.

3.5 Drought Signal Transduction

3.5.1 MAPK Kinase Cascades and Signal Transduction

As mentioned above, the drought stress signaling network in plants is complex, composed of multiple interacting proteins where the function of a molecule is dependent on the interaction and the activation of another protein. The cell surface receptors sense the stimuli and convey stress signals through downstream pathways and phosphorylation events regulated by MAPK and secondary messengers. Signal transduction via MAP kinases seems to be layered and complex, as the MAPKs are multigene families involved in diverse functions such as development, defense, hormones, and biotic and abiotic stress signaling [52]. Some of the MAPKs may play dual roles as scaffold protein and kinase enzyme at the same time. For example, the oxidative stress-activated MAP triple kinase 1 (MAPKKK, OMTK1) has a role as an adapter and a phosphate transporter [53]. Many MAPKs are reported to be positively regulated under abiotic stresses [52]. However, some MAPKs play negative roles in stress tolerance. For example, overexpression of OsMAPK33 in rice [54] and MKK9 in *Arabidopsis* [55], causes high sensitivity to salt stress in rice and *Arabidopsis*, respectively.

Several members of MAPK modules are involved in salt, drought, or cold tolerance in plants. For example, overexpression of MKK2 that targeted both MPK4 and MPK6 results in upregulation of several stress-induced genes, leading to increased freezing and salt tolerance [56]. There are indications on the direct role of MAPK in drought signaling. MAPK is upregulated in three apple species with high activity in the drought-tolerant species *Malus sieversii* suggesting the protein is associated with the natural drought tolerance in trees [57]. *Arabidopsis* MPK and MKK members can activate the promoter of a dehydration-responsive gene, RD29, indicating that MAPK cascades are involved in drought signaling. *Arabidopsis* MPK2, MPK3, MPK4, MPK5, MPK12, and MAPKKK4 are induced by drought [58, 59]. *GhMPK2*, a cotton MAPK was found to have role in reducing water loss and adjusting osmotic pressure under drought conditions [60, 61]. *GhMPK16*, another cotton MAPK, belonging to the MAPK group D, was also reported to be a drought-responsive gene [62].

Many other MAPKs are also reported to be involved in drought response. MPK3 and MPK6 play a vital role in the control of stomatal movement, which is essential for water conservation. These two kinases together with their upstream activators MKK4 and MKK5 are also involved in stomata development and patterning [63]. They operate in close cooperation with hydrogen peroxide and abscisic acid (ABA) and hence they probably control stomatal movements [64, 65]. Many other kinases, including *OsMSRMK2* and *OsMSRMK3* have been found to be induced by multiple stresses such as wounding, salinity, drought, heavy metals, fungal elicitors, or UV irradiation [66, 67]. Similarly, *OsMAPK5* was found to have multiple roles in both biotic and abiotic stress responses. Downregulation of this kinase results in constitutive expression of several pathogen-related genes and enhanced

Table 3.2 Some MAP kinases involved in drought stress responses in different plant species

Plant species	MAPK name	Accession number	Reference	Remarks
Arabidopsis	AtMEKK1	NM_100771	[56, 59, 108]	In addition to dehydration, the protein is responsive to cold and salinity
	AtMPK1	AAD32871	[109–111]	Cold, dehydration, hyperosmolarity, salinity
	AtMKK7	AAF25995	[59]	Dehydration, hyperosomolarity, salinity
Rice	OsMPK3	DQ826422	[66, 67]	Drought, temperature, salinity
	OsMKK6	DQ779790	[112, 113]	Cold, drought, salinity
	OsMPK4	FJ621301	[68, 114]	Cold, drought, salinity
	DSM1	Os02g50970	[115]	Drought
Alfalfa	MMK4	X82270	[116]	Cold, drought
Maize	ZmMPK3	EU130900	[117]	Cold, drought, salinity
Cotton	GhMPK2	FJ966890	[61, 63]	Cold, drought, salinity
	GhMPK16	FJ966889	[62]	Drought

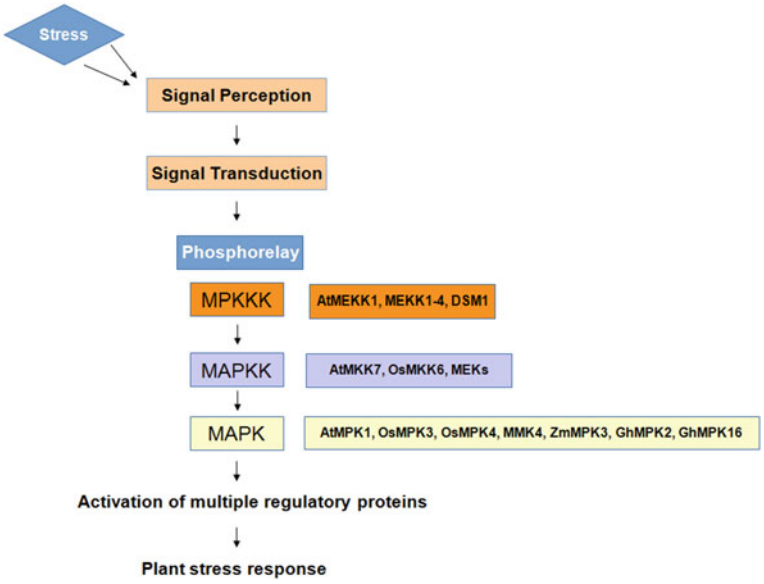

Fig. 3.2 Schematic representation of MAPK cascade under drought stress

resistance to both fungal and bacterial pathogens. The downregulated plants exhibit reduced tolerance against cold, salt, and drought. Conversely, over-expression of *OsMAPK5* in transgenic plants leads to the increased multiple-stress tolerance [68].

Many different MAPK have been identified in response to multiple environmental stresses (Table 3.2). As discussed above, MAPK serve as signal carriers and scaffolding proteins, and thus help in linking complex arrays of interacting molecules in developmental and drought adaptive responses (Fig. 3.2).

3.6 Hormonal Signaling Pathways: Multiple Stress Interactions

Phytohormones are yet another group of secondary signal integrators that help in signaling. They are key regulators of plant physiology with respect to growth and development, as well as being mediators of environmental stress responses [8]. For example, overexpression of CRK45 (a stress-inducible kinase involved in ABA signaling) results in enhanced drought tolerance by fine-tuning endogenous ABA levels [69]. Similarly, stress-inducible expression of IPT (isopentenyltransferase mediating the rate-limiting step in cytokinin biosynthesis) leads to an increase in cytokinin content, antioxidant scavenging, and changes in root growth, which contributes to improved grain yield under drought conditions [8, 70, 71]. These indicate that multiple hormonal crosstalks are critical in contributing to overall drought tolerance levels. Among the different phytohormones, because of the direct physiological influential role, abscisic acid (ABA) is discussed in detail in this chapter.

3.6.1 ABA Signaling Pathways

3.6.1.1 Signal Perception and Transduction Modules

Abscisic acid is a major phytohormone directly involved in drought stress response. ABA-mediated signaling plays an important role in plant responses to abiotic and biotic stresses. Hence, over the past few years, efforts from different directions have been put together to elucidate and confirm the ABA signaling mechanism. The plant genes for ABA biosynthesis and sequence of the pathway have been elucidated [72]. Recently, a new family of proteins was reported as candidate ABA sensors known as PYR/PYL/RCAR proteins (*PYRABACTIN RESISTANCE 1*/PYR1-like/regulatory component of ABA receptor), which were found to bind ABA and inhibit the activity of specific protein phosphatase enzymes, the type 2C plant PP2Cs [73]. In the absence of ABA, PP2Cs constitutively inactivate SnRK2s by physically interacting with and dephosphorylating serine residues in the kinase activation loop. Without activation, SnRK2s are unable to transmit the signal to downstream targets. ABA-bound PYR/PYL/RCAR receptors bind and inhibit PPC2s, thereby allowing activation of SnRK2s. Active SnRK2 kinases phosphorylate downstream target proteins, including AREB/ABF transcription factors, anion channels, and NADPH oxidases, to induce ABA responses [74, 75].

3.6.1.2 ABA Dependent and Independent Regulatory Signaling

Of late, more emphasis is on examining and unraveling the complex signaling cascades during drought stress response, mainly to identify specificity and crosstalk in ABA-dependent and ABA-independent gene expression cascades. ABA signaling leads to large changes in gene expression, which may involve changes in transcription, transcript processing, and stability [76]. ABA-responsive elements, ABREs (*cis*-element-PyACGTGG/TC) are the key nucleotide sequences to which ABRE-binding (AREB) or ABRE-binding factors (ABFs) bind to activate ABA-dependent gene expression. AREB1 is a key positive regulator of ABA signaling in vegetative tissues under drought stress. ABA-induced modification of AREB1 is required to activate ABRE dependent downstream gene expression [77]. Phosphorylation/dephosphorylation events play important roles in ABA signaling wherein AREB/ABF proteins are phosphorylated for activation [78, 79]. Similarly, a *cis*-element, dehydration-responsive element/C-repeat (DRE/CRT), and DRE-/CRT-binding protein 2 (DREB2) transcription factors play key roles in ABA-independent gene expression in response to osmotic stress.

The MAP kinase MKK1/MPK6 module is an important component of the ABA-dependent signaling pathway that is responsible for H_2O_2 production and subsequent stress responses by enhancing catalase CAT1 expression [80]. ABA signaling is important in stomatal guard cells by controlling the turgor and volume of the guard cells by promoting the efflux of anions and potassium cations, and by favoring the conversion of malate into starch. This results in stomatal closure thereby protecting the plant from excessive water loss. It has been shown that the MAP kinase cascade partners, MPK4, MPK9, MPK12, MPK15, and MKK2 are involved in ABA-mediated guard cell signaling [81], among which MPK9 and MPK12, the positive regulators seem to act upstream of ABA-dependent anion channel activation and downstream of ROS signaling [82]. In summary, ABA is the major player in drought acclimation as it has roles in diverse signaling networks.

3.7 Small RNAs and Small Signaling Peptides as Signals Under Stress

3.7.1 Small RNAs: Role Under Drought

The general concept of a genotype driving a phenotype through protein expression does not hold true in all cases. It is very crucial to understand the regulation exerted by all the transcribed regions of the DNA. The transcriptome repertoire comprises a huge array of RNA molecules varying with respect to their size, abundance, and protein coding capacity. Transcripts can be either coding (mRNAs) or noncoding (nc) which includes the house keeping ncRNAs as well as the regulatory ncRNAs. Ribosomal RNAs, t-RNAs, and small nuclear and small nucleolar RNAs constitute the house keeping ncRNAs. The regulatory ncRNAs enact their roles in the form of

short or small (siRNAs, miRNAs, piwiRNAs; <200 nucleotides) and long ncRNAs (lncRNAs; >200 nucleotides) [83]. In the recent years efforts have been made to understand the evolution [84], possible mechanisms [85], and plant developmental roles of lncRNAs [86].

Recently, two independent research groups have reported the role of lncRNA in regulating male sterility in rice. Ding et al. [87] reported that the *pms3* locus regulating photoperiod sensitive male sterility (PSMS) in the *japonica* rice line Nongken58S, encodes an lncRNA named long-day–specific male-fertility–associated RNA (LDMAR). A spontaneous mutation causing an SNP in the locus alters the secondary structure of LDMAR, leading to its reduced transcription due to methylation by itself at the promoter region, as a consequence of which male sterility occurs under long-day conditions. Soon after, Zhou et al. [88] identified and reported the photo- or thermosensitive male sterility (TSMS) regulating locus, *p/tms12-1*, which confers PSMS in the *japonica* rice line, Nongken58S, and TSMS in the *indica* rice line, Peiai 64S. The research results suggest that the locus encodes a likely unique lncRNA which produces a small RNA named *osa-smR5864 m*, which is responsible for the male sterile phenotype, the mechanism of which is not known. These two studies provide a starting point for figuring out how this fascinating ncRNA locus is responsible for the male fertility regulation. Hence, it is evident that lncRNAs could play potent roles in regulating various plant developmental processes and respond to diverse environmental cues.

Micro RNAs (miRNAs) are a class of small RNAs some of which are involved in plant defense mechanisms to cope with diverse environmental cues by reprogramming of gene expression. Drought stress alters the expression of many genes/metabolites, including ABA-inducible genes, helicases, dehydrins, vacuolar acid invertase, glutathione S-transferase (GST), proline, and carbohydrates [89]. miRNAs are gene regulators that modulate the expression and activity of these drought-responsive genes/gene products. There are many miRNAs reported to participate in a number of gene regulatory networks important for drought tolerance (Table 3.3).

The mechanisms of miRNA involvement in stress tolerance and their target regulatory networks are not well understood. This is mainly because endogenous miRNA regulates multiple genes and each gene can in turn be regulated by multiple miRNAs. It is also critical to characterize the *cis*-regulatory elements in the miRNA genes to determine the corresponding transcription factors and to describe how the

Table 3.3 A few reported miRNAs involved in drought stress responses in different plant species

miRNA	Function	Reference
miR159	ABA response, NaCl stress response	[118–123]
miR169	Response to different abiotic stresses	[60, 61, 98, 99, 119, 123–130]
miR171	Response to abiotic stresses	[119, 121, 123, 126, 129–132]
miR394	Abiotic stress-response pathway	[98, 125, 126, 133]
miR1444	Probable role for improving plant water stress	[133–135]
miR2118	Response to drought stress	[72, 129, 130]

miRNAs are regulated by drought. However, the targets of these drought-regulated miRNAs are largely unknown although they have been partly understood by developing miRNA transgenic plants for drought tolerance [90]. Many of these small RNAs act as short- and long-distance signals to perpetuate the message of stress advent and possible precautionary measures to withstand the harsh stressful conditions.

3.7.2 Peptide Signaling Under Drought

In plants, there are several ways of intercellular communication, which include phytohormones, mobile transcription factors, noncoding RNAs, and secreted signaling peptides. Until the 18 amino acid systemin was discovered by Pearce et al. [91] peptides were not known as regulators of signaling events in plants. Peptide-signaling molecules create diverse modular signals in animal systems, but it is only recently that an array of signaling peptides has been identified in plants [92]. The secreted signaling peptides discovered in plants can be grouped into two: small posttranslationally modified peptides (SPTMPs) and Cys-rich peptides (CRPs). The SPTMPs are a group of peptides characterized by small (<20 amino acid) mature peptides, whereas CRPs are larger than the former (<160 amino acids) [93]. Several secreted peptides have been recognized as cell–cell communicators in plants, coordinating and integrating cellular functions linked to development and biotic stress responses. Recently, a few reports indicated the role of peptide signaling in fertilization EMBRYO SAC1–4 (ES1–4) [94–96], programmed cell death [97], and other developmental events. However, no exhaustive reports are available to demonstrate the role of secreted peptides in abiotic stress signaling.

A recent study showed that a plant natriuretic peptide, *AtPNP-A*, acts as an apoplastic and paracrine stress-response molecule. Abiotic stimuli such as osmoticum and salt enhance *PNP-A* expression. *PNP-A* can be exported from the cell where it can act on itself (autocrine) or adjacent cells (paracrine) as it moves through the apoplast. *PNP-A* transiently increases intracellular cGMP levels, regulates stomatal opening, and induces changes in ion fluxes which directly enhances water uptake and photosynthesis [98, 99]. It is speculated that *AtPNP-A* may increase dark respiration as an early response to stress in order to provide energy for more delayed stress-specific responses. Thus, *AtPNP-A* systemically modulates plant physiology and thus homeostasis that may form an important part of the plant's communication system in response to stress [100]. Very recently, a salt-induced 11-amino–acid peptide in *Arabidopsis*, AtCAPE1 (related to the cysteine-rich tomato immune regulator CAPE1), which is a novel peptide balancing immune and salinity responses was identified [101]. The new signaling peptide candidates are being unraveled and those involved in salinity stresses could also possess some function under drought stress.

In conclusion, peptidomics is a relatively new field in plant science that in the coming years may help to unravel the plant *peptidome*. This may promote our understanding of signaling pathways and will provide a new layer for analysis in systems biology under biotic and abiotic stresses.

3.8 Inter-organellar Signaling

Plants have a unique coordination between the nuclear and organellar genomes residing in them to manifest their phenotype. Plant stability is vested with the coordinated performance of the nuclear, mitochondrial, and chloroplast genomes, the relationship among which is not well defined. However, the signaling between the nucleus and organelles is highly regulated. In anterograde regulation, the nucleus lays a control on organellar gene expression via its own regulators. In retrograde signaling, nuclear gene expression is regulated as a consequence of signals generated from organelles [102]. Hence the pathways of communication between various organelles of a plant cell are complex and interdependent.

A recent research study has shown that dual targeting of a nucleoid protein encoded by nuclear gene, *MSH1* (MutS homologue 1), results in specific plastid and mitochondrial manifestations thereby affecting overall developmental events. It has been reported that *MSH1* disruption can result in green- white leaf variegation. The protein localizes to mitochondria as well as plastids and genetic complementation studies have revealed the distinct functions of the protein in both organelles. The gene disruption has also resulted in altered cellular and organelle morphology and associated physiology. Experimental evidence for leaf variegation was provided by showing altered plastid redox state in the white tissue sectors in variegated leaves in *msh1* mutants and was also considered as an adaptive strategy under high light conditions [103]. Nucleus–organelle crosstalk via *MSH1* enhances the plant's repertoire for environmental response and interorganellar coordination. Early eukaryotes and mitochondria generate acetate and hydrogen peroxide (H_2O_2) to induce various signaling mechanisms to communicate to the rest of the cell. At present mitochondria are thought to function by releasing metabolites and ROS, activating 5' AMP-activated protein kinase (AMPK) and peptides, as well as changing inner mitochondrial membrane potential and calcium concentrations. Mitochondrial-associated membranes (MAMs) along with other organelles such as the endoplasmic reticulum serve as crucial platforms for interorganellar signaling. These findings help us to conclude that plant phenotypic manifestations occur in coordination between retrograde and anterograde signaling. Hence, the multiorganellar genomes present in a living cell are tightly coordinated under diverse environmental cues to regulate plant growth.

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Chapter 4
Plant Molecular Adaptations and Strategies Under Drought Stress

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4.1 Introduction

Growth and development of plants can be significantly influenced by several environmental factors. Among them, drought is one of the main abiotic factors limiting the productivity of crops. Furthermore, as an aggravate aspect, drought is increasingly growing in dimension of severity in many regions of the world [1]. Thus, the development of crops tolerant to drought will be significantly advantageous in regions where such stress frequently occurs.

Stress is an altered physiological condition caused by factors that tend to disrupt the equilibrium of an organism. In plants, the water deficit caused by drought reduces growth and development, arising from the reduction of water content, diminished leaf water potential and turgor loss, closure of stomata, and decrease in cell enlargement and growth (Fig. 4.1a) [2]. Other effects of drought that limit plant growth and crop productivity include the reduction of photosynthesis, osmotic stress-imposed constraints on plant processes, and interference with nutrient availability as the soil dries [3].

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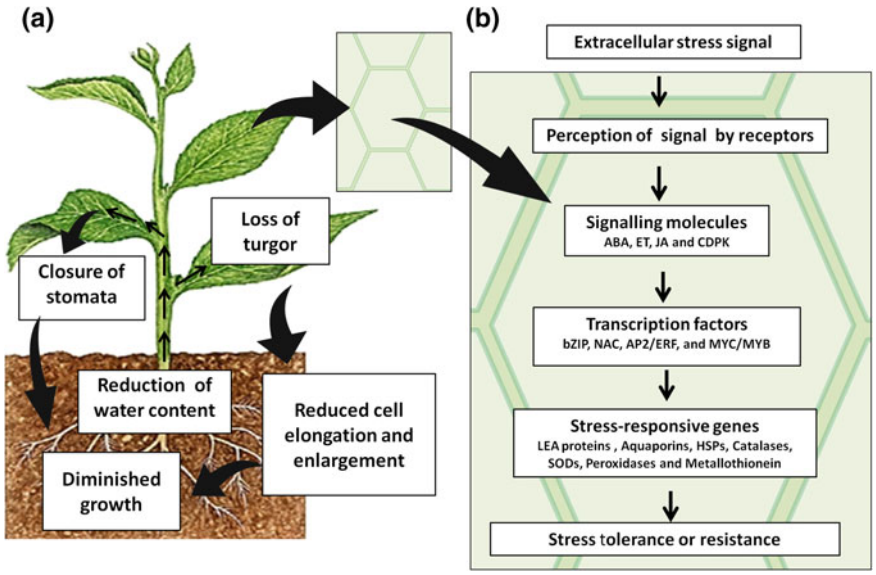

Fig. 4.1 Adaptation of plant to drought involves morphological, cellular, and molecular alterations to prevent injury to the plant. **a** Some important causes of growth reduction in plants under drought stress. **b** Molecular mechanisms that regulate the expression of stress-responsive genes of plant under abiotic stress

As a response to stress caused by diverse environmental factors the higher plants have evolved adaptive mechanisms at the physiological, cellular, and molecular levels [4]. The response of a plant to abiotic stress, first involves the perception of the extracellular stress signal by receptors of the cell, followed by many stress regulatory networks, including signal transduction and transcriptional regulation of stress-responsive gene expression that result in physiological response of tolerance or resistance of the plant to stress [3]. Thus, as depicted in Fig. 4.1b, at the molecular level the response of the plant to abiotic stress, such as drought, comprises the participation of signaling molecules such as hormones, transcription factors, and stress-responsive genes coding for proteins with protective roles against stress, including LEA proteins and peroxidases. Therefore, the elucidation of a molecular pathway for plant response to stress is essential to understanding how plants respond and adapt themselves to diverse abiotic stress.

Molecular knowledge of stress regulatory networks is likely to pave the way for engineering plants that can withstand and give satisfactory economic yield under drought stress. The specific importance to crop plants is not whether they survive stress, but whether they show significant yields under stress conditions [1, 2].

Comprehensive research has been done on identification of many molecules involved in regulatory networks of plant response to stress, such as signaling molecules (hormones, phosphatases, and protein kinases), transcription factors (bZIP, NAC, AP2/ERF, and MYB/MYC), and stress-responsive genes (LEA

proteins, aquaporins, HSPs, catalases, SODs, peroxidases, and metallothioneins; Fig. 4.1b) [5]. Furthermore, some key genes of these stress regulatory networks have been important candidates for the development of transgenic plants tolerant to drought [6].

4.2 Transcriptional Regulation of Gene Expression

According to the mechanism of plant response to stress, the stress signal is detected by the cell and transduced by different transduction components resulting ultimately in transcription of stress-responsive genes [3]. Therefore, as part of the molecular adaptive mechanisms of plants to stress, the regulation of gene expression involves changes in transcript levels of gene-coding proteins that can directly or indirectly provide stress tolerance to the plant. Stress-responsive genes with potential for engineering of plants tolerant to drought include genes coding for LEA proteins [7, 8] and aquaporins [9, 10]. The main roles of these proteins in providing drought tolerance are presented in this chapter.

It is well known that the transcriptional regulation in eukaryotic organisms involves the interaction of *cis*-acting regulatory elements that are conserved DNA sequences found in the promoter gene with regulatory proteins, also known as transcription factors. By interaction with *cis*-acting elements, these regulatory proteins can activate and/or repress the transcription of the target gene, whose product can play roles in various biological processes, including tolerance to abiotic stresses. Thus, due to essential roles of certain transcription factors in regulating downstream stress-responsive genes, their genes have also been useful in providing stress tolerance in transgenic plants [6, 11, 12, 13, 14, 15].

In addition to the binding transcription factors, some c*is*-acting regulatory elements can also act as response elements for signaling molecules of stress regulatory networks, such as hormones. An example is the ABA Response Element (ABRE), a *cis*-acting element found in the promoter gene responsive to abscisic acid (ABA), which is accumulated under osmotic stress conditions caused by drought, and has a key role in stress responses and tolerance [14, 15]. Other signaling molecules of stress regulatory networks comprise protein kinases that can regulate the activity of transcription factors by mechanisms of phosphorylation [16, 17].

Within the intricate and complex stress regulatory networks, several signaling molecules, transcription factors, and *cis*-acting elements found in drought regulons have been identified in many plants. In the last decade, *Arabidopsis thaliana*, a genetic model plant, has been extensively used for unraveling the molecular basis of stress tolerance. Arabidopsis also proved to be extremely important for assessing functions for individual stress-associated genes due to the availability of knockout mutants and its amenability for genetic transformation [4, 5, 18].

Advances have been made in the development of drought-tolerant transgenic plants, including rice, tomato, soybean, maize, barley, and Arabidopsis [6, 19, 20, 21], among others. Such genetically engineered plants have generally been developed

using gene-encoding proteins that control drought regulatory networks. Stress signaling networks in drought responses are composed of intracellular signaling systems, transcriptional regulatory complexes, and intercellular communication systems [22, 23]. These proteins include transcription factors, protein kinases, receptor-like kinases, enzymes related to osmoprotectant or plant hormone synthesis, and other regulatory or functional proteins [6]. Major transcription factor families of plants, such as bZIP, NAC, AP2/ERF, and MYC orchestrate regulatory networks underlying drought stress tolerance [5].

4.2.1 Signaling Molecules

Acclimation of plants to changes in their environment requires a new state of cellular homeostasis achieved by a delicate balance between multiple pathways. Hormones, phosphatases, and protein kinases are crucial components within the stress-induced signaling network that regulates a multitude of biochemical and physiological processes [24].

The hormone ABA is a major molecule facilitating signal transduction during drought stress response. This master ABA-responsive transcription factor regulates a diverse array of genes that coordinate cellular responses to the drought stress. Such cellular responses include stomatal closure, induction of stress proteins, and accumulation of various metabolites for the protection of cells against water-deficit stress [23, 25]. Kuromori et al. [25] have demonstrated that specific cells in vascular tissue synthesize ABA and transport the molecule to target cells. Bauer et al. [26] have proposed that ABA is autonomously synthesized in guard cells. Drought stress signals can also be propagated through ABA-independent pathways [23]. Plant genes responding to ABA contain the ABRE in their promoters. ABRE binding factors (AREB/ABF) are basic leucine zipper (bZIP) transcription factors that bind to ABREs and regulate osmotic stress tolerance in an ABA-dependent manner [14, 15].

Other hormones, such as jasmonic acid (JA) and ethylene (ET), are also involved in facilitating signal transduction during drought stress [27]. Major JA and ET signaling hubs such as Jasmonate Zim (JAZ) proteins, Constitutive Triple Response1 (CTR1), Mylocytomatosis Oncogene Homologue 2 (MYC2), Ethylene Insensitive 2 (EIN2), EIN3, and several members of the APETALA 2-Ethylene Response Factor (AP2/ERF) transcription factor gene family have complex regulatory roles during stress adaptation [6, 27, 28]. JA is implicated in promoting stomatal closure. It was proposed that drought stress prevents the conversion of precursor 12-oxo-phytodienoic acid (OPDA) to JA. OPDA then acts either independently or together with ABA to promote stomatal closure, leading to increased drought tolerance [27, 29]. In contrast, the ET has been implicated in both stomatal opening and closure [30].

Drought stress signaling can be triggered by accumulation of calcium-dependent protein kinase (CDPK). Often this process is a result of early osmotic stress-induced

Ca^{2+} spiking/oscillation, which leads to CDPK activation and drought-responsive gene transcription. Additionally, they can be a consequence of stress-responsive selective proteolysis or phospholipid hydrolysis [31]. A positive regulatory effect of CDPKs in drought stress signaling may be explained by the enhanced expression of ABA-responsive genes [32].

4.2.2 Transcription Factors

Genes induced during stress conditions not only protect cells from stress by the production of important metabolic proteins (functional proteins), but also regulate the genes for signal transduction in the stress response (regulatory proteins), such as the transcription factors (TFs). TFs are sequence-specific DNA-binding proteins able to activate and/or repress transcription. They are responsible for the selectivity in gene regulation and are often expressed in tissue-specific, development-stage–specific or via stimulus-dependent pathway. Overexpression of key TF genes has been shown to impart stress-tolerant phenotypes in several studies [6, 13, 14, 15].

4.2.2.1 AP2/ERF

The APETALA2/ethylene responsive element (AP2/ERF) superfamily is a large group of plant-specific transcription factors containing at least one DNA binding domain, named the AP2 domain and divided into three separate families, namely the ERF, AP2, and RAV families [33, 34]. This domain was first identified in the Arabidopsis homeotic gene APETALA 2 [35], and a similar domain was found in tobacco ethylene-responsive element binding proteins (EREBPs) [36].

The conserved DNA binding domain characteristic of the AP2/ERF superfamily is composed of 60 amino acid residues that confer a typical three-dimensional conformation organized into a layer of three antiparallel beta-sheets followed by a parallel alpha helix. Following a general rule, AP2-containing TFs can be roughly classified as activators or as repressors depending on whether they activate or suppress transcription of specific target genes [33].

AP2/ERF genes were identified in tobacco [37], rice [38, 39], grape [40], Arabidopsis [41], wheat [42], apple [43], and potato [44]. These genes resulted in improved tolerance against pathogen attack and osmotic stress [37], drought, low temperature, salinity [39], cold, and heat [41]. Due to their plasticity and specificity of individual members of this family, AP2/ERF transcription factors represent valuable targets for genetic engineering and breeding of crops [33].

Dehydration responsive element binding proteins (DREB2) proteins are members of the AP2/ERF family of plant-specific transcription factors. Among the eight *DREB2* genes in Arabidopsis, *DREB2A* and *DREB2B* are highly induced by drought, high salinity, and heat stress, and function as transcriptional activators in the ABA-independent pathway [6]. The yield of transgenic rice plants expressing

DREB1A under drought stress conditions was increased in comparison to the nontransgenic plants [38]. Likewise, in transgenic potato plants overexpressing *StDREB1* and *StDREB2*, the level of drought tolerance was significantly greater than in the wild-type control plant.

The results suggest that the StDREB1 and StDREB2 as AP2/ERF transcription factors may play dual roles in response to drought stress in potato [44].

4.2.2.2 bZIP

The basic leucine zipper (bZIP) is an important group of transcription factors in plants [45]. In plants, they are involved in important processes such as pathogen defense, abiotic stress signaling, hormone signaling, and energy metabolism, as well as development, including flowering, senescence, and seedling maturation [46, 47]. bZIP genes were identified in Arabidopsis [48], soybean [49], tomato [50], sorghum [51], maize [47], and rice [46].

The name of the bZIP family is derived from the basic region/leucine zipper domain found in all its members. This domain consists of an uninterrupted α-helix comprising a basic region (BR) which is necessary and sufficient to bind the DNA, followed by a C-terminal leucine zipper (LZ) motif responsible for the dimerization. The bZIP family was subdivided according to sequence similarities and functional features resulting in 10 groups. Although many bZIPs can form homodimers, bZIP members classified in different groups can be combined through heterodimerization to form specific bZIP pairs with distinct functionalities [47, 52].

About 75 members of the bZIP TFs family were identified in Arabidopsis, and they were divided into more than 10 groups. Many of the well-studied group A bZIP TFs play a central role in ABA signaling. The ABA-responsive element binding protein (AREB) subfamily of bZIPs is upregulated by drought stress. For example, the ABA responsive element (ABRE) binding proteins/factors (AREBs/ABFs) *AREB1/ABF2*, *AREB2/ABF4*, *ABF1*, and *ABF3* are mainly expressed in vegetative tissues and all except *ABF1* are key regulators of ABA signaling that respond to drought stress [53]. Overexpression of *AREB2/ABF4* or *ABF3* in Arabidopsis conferred ABA hypersensitivity, reduced transpiration, and enhanced drought tolerance [54], whereas overexpression of an activated form of *AREB1/ABF2* also showed increased ABA sensitivity and drought tolerance [55].

bZIP regulators have been explored as potential candidates for application in the improvement of drought tolerance in crops [53]. For example, the Group A TF OsABF1 from rice [50] and SlAREB from tomato [56] both enhanced tolerance to drought and salt stress. In maize, the expression level of ZmbZIP37 was increased under drought stress, implying a possible regulatory role in response to such stress [47].

4.2.2.3 MYB/MYC

The proteins of MYC/MYB families are found in both plants and animals playing many varied functions. In plants, these families participate in the ABA-dependent pathway of stress signaling for the upregulation of the abiotic stress responsive genes. Many *MYB* and *MYC* genes have been studied for their involvement in the regulation of abiotic stress response, such as drought stress [28, 57].

MYB TFs contain the MYB domain involved in DNA binding. A MYB domain is usually composed of one to three imperfect repeats, each with about 52 amino acid residues which form three α-helices; the second and the third ones are involved in the formation of a helix–turn–helix (HTH) fold [58]. MYC TFs are members of the basic helix–loop–helix (bHLH) domain that is a highly conserved amino acid motif. This motif defines these groups of transcription factors. The bHLH domain consists of 50–60 amino acids that form two distinct segments: a stretch of 10–15 predominantly basic amino acids (the basic region) and a section of roughly 40 amino acids predicted to form two amphipathic α-helices separated by a loop of variable length (the helix–loop–helix region) [59].

MYC and MYB proteins play important roles in many physiological processes under normal or stress conditions and both MYC/MYB TFs participate in the ABA-dependent pathway of stress signaling for the upregulation of the abiotic stress-responsive genes [28, 60, 61]. MYB is a large TF family in plants. There are over 198 and 183 MYB genes in *Arabidopsis* and rice, respectively, where many of them are regulated by drought [62, 63].

Katiyar et al. [62] reported that 65 % of *MYB* genes expressed in rice seedlings were differentially regulated under drought stress. In Arabidopsis, 51 % of *AtMYB* genes were upregulated by drought whereas 41 % are downregulated by such stress [62, 64].

In *Arabidopsis*, many MYB genes are responsive to abiotic stress. For example, *AtMYB2* functions in the ABA-mediated drought stress response and *AtMYB102* is a key regulatory component in responses of *Arabidopsis* to osmotic stress, salinity stress, and ABA application [65]. In addition, *AtMYB96* modulates ABA signaling in response to abiotic stresses in *Arabidopsis* [66].

The rice *OsMYB4* was reported to play a positive role in cold and drought tolerance in transgenic plants of Arabidopsis, tomato, and apple [67–69]. *OsMYB55* was shown to be involved in tolerance to high temperature through enhanced amino acid metabolism [70]. In a recent study, molecular characteristic features of *OsMYB2* have clearly been indicating its regulatory role in salt, cold, and dehydration tolerance in rice [71].

Among *MYC* genes, *MYC2* is an ABA- and drought-responsive gene and therefore earlier studies have focused on the role of MYC2 in ABA signaling. Indeed, MYC2 overexpressing plants and the myc2 mutant show increased and reduced ABA sensitivity, respectively. Furthermore, transactivation assays show that MYC2 is capable of activating the expression of the ABA response gene Responsive to Dessication22 (*RD22*), showing that MYC2 is a positive regulator of ABA signaling [72].

Transgenic plants overexpressing both MYC2 and MYB2, a drought-inducible MYB TF, showed reduced electrolyte leakage following mannitol treatment, suggesting that MYC2 can contribute to stress tolerance [72]. In contrast, a recent study found an increased drought tolerance in the myc2 mutant based on smaller relative biomass reduction observed under drought conditions than in wild-type plants [73]. Therefore, the role of MYC2 in abiotic stress tolerance is not as conclusive as its role in ABA signaling [28]

4.2.2.4 NAC

The NAC family of plant-specific TFs is one of the largest in the plant genome [74]. The NAC transcription factor contains a highly conserved N-terminal DNA-binding domain and a diversified C-terminal domain [75] and based on the motif distribution, the NAC domain can be further divided into five subdomains (A–E) [76]. The NAC domain was originally characterized from consensus sequences from petunia NAM and Arabidopsis ATAF1, ATAF2, and CUC2. Therefore, NAC was derived from the names of the first three described TFs containing the NAC domain, namely no apical meristem (NAM), ATAF1-2, and cup-shaped cotyledon (CUC2) [77].

NAC family genes have been identified by genome-wide analysis from various plant species, such as Arabidopsis [76], rice [78], poplar [79], and soybean [80]. NAC proteins play essential roles in diverse aspects of plant development, such as pattern formation in embryos [81] and lateral root development [82]. The NAC TFs function as important components in complex signaling progresses during plant stress responses. Considering the relatively large number of NAC TFs from different plants and their unknown and diverse roles under complex environmental stimuli, it remains a considerable challenge to uncover their roles in abiotic stress [83].

There is increasing evidence demonstrating that NAC family transcription factors are involved in responses to various biotic and abiotic stresses, including drought, salinity, cold, bacterial and fungal pathogens, and low-oxygen stress [83, 84]. The expression of three Arabidopsis NAC genes, *ANAC019*, *ANAC055*, and *ANAC072 (RD26)*, was induced by drought, high salinity, and ABA, respectively. Overexpression of these three genes remarkably enhances tolerance to drought stress [85].

Others Arabidopsis NAC genes, such as *ATAF1* (*ANAC002*) and *ATAF2* (*ANAC081*), together with *ANAC102* and *ANAC032* were phylogenetically classified into a small subfamily (ATAF) [76, 86]. *ATAF1* was initially reported to play a negative role in response to drought stress by functional analysis of *ataf1* null mutants [87]. However, studies reported by [88] showed that the overexpression of *ATAF1* conferred an enhanced drought tolerance, revealing a positive role of *ATAF1* in plant drought response.

Understanding the complex mechanism of drought and salinity tolerance is important for agriculture production. Many *NAC* genes have been shown to be

involved in plant responses to drought and salinity stress. In transgenic rice, the *Os01g66120/OsNAC2/6* and *Os11g03300/OsNAC10* genes were found to enhance drought and salt tolerance [16, 89], and *Os03g60080/SNAC1* increased grain yield (21–34 %) under drought stress [90].

Plant response to abiotic stresses is via both ABA-dependent and ABA-independent signal transduction pathways, where ABA can act as a signaling molecule of regulatory networks of plant response to stress. Arabidopsis overexpressing *MlNAC5* exhibited hypersensitivity to exogenous ABA and enhanced tolerance to dehydration stress. A higher ABA sensitivity may stimulate stomatal closure to retain water and increase drought tolerance in plants [91], as was found in Arabidopsis and maize [88, 92].

Much progress in NAC TF functional research has been attained over the past decade. However, most of these advances are related to the involvement of biotic stress. Thus, the identification of NAC functions in biotic and abiotic stresses will remain a substantial challenge in the coming years.

4.3 Drought-Responsive Genes

4.3.1 Late Embryogenesis Abundant Proteins

A class of proteins widely involved in plant response to drought is called late embryogenesis abundant (LEA) proteins, first discovered in late stages of embryo development in plant seeds [93]. Under dissection conditions, there is an improved accumulation of mRNA molecules coding for LEA [94]. The increase of this expression is correlated to an improvement in abscisic acid levels, whose induction is associated with increased drought tolerance [95].

LEA proteins are members of a large group of glycine-rich proteins that act in ion sequestration [96]. There are several groups of LEA proteins distributed through different classifications based mainly on different motifs present in the amino acid sequence of each protein [97, 98]. In *Arabidopsis thaliana*, 51 genes encoding LEA proteins clustered into nine families [99].

LEA proteins are mainly low molecular weight (10–30 kDa) proteins and are mainly composed of hydrophilic amino acids ordered in repeated sequence (e.g., Gly and Lys) in higher plants, forming hyperhydrophilic domains and allowing thermal stability [100]. Most LEA proteins are randomly coiled in solution, cytoplasmic, and hydrophilic proteins [97], although some called atypical present a preponderance of hydrophobic content. These proteins may also be included in the group of intrinsically disordered proteins [101].

The differential expression of genes coding for LEA proteins in response to drought has been reported in several species of plants [102–104]. Furthermore, studies have detected overexpression of the LEA protein contributes to the resistance of *E. coli* cells against drought [105, 106].

Changes in conformation of these proteins have been reported at the cellular level, considering increased expression of LEA proteins under dehydrating conditions [107, 108]. Other reports have assessed their role in water retention as hydration buffers [109], as molecular chaperone [110], in the protection of cell membrane [111, 112], and sequestration of reactive oxygen species [113]. The heat tolerance is a common feature of all proteins of this family [105]. Several in vitro studies have shown activity of the LEA proteins in protecting other enzymes against dissecting-induced aggregation [114–116].

The role of LEA protein expression in generating drought-tolerant plants has also been ratified. For instance, [8] verified the role of LEA protein in water-stress protection by overexpression of the *HVA1* gene from barley into rice plants. In this study, an increase of growth rate stability under conditions of water deficit was detected in transgenic plants, as well as better recovery of growth, compared to control plants. Also in rice, the *OsLEA3-1* gene was overexpressed in lineages at field conditions, which had higher grain yield than the wild-type under drought stress [7]. A higher survival rate in transgenic *Arabidopsis* plants for *BnLEA4-1* gene [117] was also observed, and the expression of the *TaLEA* gene improved cell membrane protection in transgenic poplar [118].

Transgenic plants of *Salvia miltiorrhiza* overexpressing the *SmLEA* gene showed reduction of water loss under dehydrating conditions [106]. Overexpression of the *SiLEA14* gene of foxtail millet improved resistance to osmotic stress, as well as contributed to the increase of free proline and soluble sugar content, which are metabolites related to defense against water stress in plants [119]. Under conditions of drought stress, [120] observed that, compared to control plants, Arabidopsis transgenic plants overexpressing the *JcLEA* gene had higher relative water content and less damage to the cell membrane, as well as a higher increase in glucose accumulation, which contributed to the stability of the internal milieu of the plant cells.

These results demonstrate that the prospecting of genes coding for LEA proteins is fundamental to better understanding of endogenous mechanisms of plant defense against dehydration conditions and molecular breeding.

4.3.2 Aquaporins

The protein family called major intrinsic (MIP) includes aquaporins that constitute a family of proteins that act to regulate the movement of water through intracellular and plasma membranes of plants and animals. Aquaporins may also be referred to as water channels and contribute to the translocation of water molecules [121, 122], as well as solutes (urea, boric acid, and silicic acid) and gases (ammonia and carbon dioxide) [123].

These proteins are expressed in nearly all plant tissues and their high expression occurs in organ development and contributes to the maintenance of cell turgidity [124]. The activity of aquaporins in membranes and the change of their abundance

can control the rate of water transport along the transcellular pathway, influencing the movement of guard cells or cell expansion [125].

There are five existing subgroups for aquaporins, which vary according to cell location: plasma membrane intrinsic proteins (PIP), vacuolar membrane (tonoplast) intrinsic proteins (TIP) [126], nodulin-26–like intrinsic membrane proteins (NIPs), small basic intrinsic proteins (SIPs) [127], and the X intrinsic proteins (XIPs) [128].

The aquaporins' molecular weight ranges from 21 to 34 kDa, consisting of six membrane-spanning α-helices connected by five loops (A to E) and N- and C-termini facing the cytosol [129]. Several studies performed thus far have reported the important role of aquaporins in response to drought in plants. For instance, Xu et al. [130, 131] demonstrated that TaTIP2;2 acts as a negative regulator of salinity and drought stress. Moreover, these authors observed the response of this protein is independent of abscisic acid, in accordance with the expression of other TIP proteins, whose expression is generally not induced by hormonal regulation.

According to Khan et al. [132], the overexpression of JcPIP2;7 might help in faster water uptake through outer water channels, leading to faster imbibition thus accelerating germination even under normal conditions. The JcTIP1;3 probably is internally localized to the vacuolar membrane. Thus, as with other TIPS, such protein functions more in maintaining cell turgidity and might interact intricately with the cellular developmental and stress signaling machinery.

Li et al. [60, 61] observed through *GoPIP1* overexpression that the protein GoPIP1 could modify the water movement, changing the stomatal aperture (faster water loss through leaves). Therefore, its overexpression had a negative impact on plant growth under drought stress, supporting the proposition that, under drought stress, a general increase in water transport is harmful in most plant tissues and cells, as observed for studies reporting overexpression of other aquaporins [133, 134].

On the other hand, Lian et al. [9] detected that, compared to the wild-type plant, the transgenic lowland rice (overexpressing the RWC3 aquaporin) exhibited higher root osmotic hydraulic conductivity, leaf water potential, and relative cumulative transpiration, improving drought tolerance and corroborating other studies reporting aquaporins upregulate drought tolerance [135–137].

Zhou et al. [10] verified that *TaAQP7* generates an increase in drought stress tolerance in transgenic tobacco by improving the ability to retain water, reduce reactive oxygen species accumulation and membrane damage, and enhance the antioxidants' activities. In *Arabidopsis thaliana,* [138] detected that MaPIP1;1 contributed to increased drought tolerance associated with decreased membrane injury and improved osmotic adjustment (MaPIP1;1-overexpressing transgenic plants have maintained higher levels of proline).

The information presented above confirms the importance of studies related to aquaporins, which are essential for plant breeding focused in the maintenance of water balance in plants.

4.3.3 Heat Shock Proteins

Heat shock proteins (HSPs), also known as heat stress proteins, were identified initially in response to high temperatures and are present in prokaryotes and eukaryotes. The increased expression of HSPs is related to defense mechanisms against injuries caused by dehydration, as the decrease in cellular volume that promotes the crowding of cytoplasmic components. This crowding generates an increase of molecular interactions that can cause protein denaturation and membrane fusion [139].

The association of HSPs with membranes can contribute to drought-induced changes in cellular architecture and help in the maintenance of normal membrane-associated processes during drought stress [140, 141]. It is known that, at the cellular level, these proteins respond to various stresses and act in normal cells as molecular chaperones. This role was confirmed by means of heterologous expression in *E. coli* and subsequent purification of recombinant protein, [142–144]. Thus, HSPs contribute to reducing the impact of protein denaturing conditions and contribute to the maintenance and/or restoration of protein structure and its homeostasis [145].

According to the molecular weight (15–42 kDa), there are five major families of HSPs: the Hsp70 (DnaK), chaperonins (GroEL and Hsp60), the Hsp90, the Hsp100 (Clp), and the small Hsp (sHSP) family [146]. Studies have shown differential expression in response to drought for HSPs of different molecular weights [104, 123, 147]. There is a significant increase in induction of HSP expression when there is a combination of different stresses, such as the combined effect of drought and high temperature [148].

The regulation of genes encoding HSPs is strongly related to the heat stress transcription factors (HSTF). The overexpression of genes coding for these transcription factors have been reported to induce drought resistance in transformed plants [11, 12]. These regulatory proteins are usually located in the cytoplasm, where they are found inactivated. The activation takes place by means of stress conditions and consequent oligomerization, as well as recompartmentation to the nucleus. This enables the occurrence of binding to promoter sequences of genes encoding HSPs [149].

Several studies have reported the widespread importance of HSP expression in response to drought. Sun et al. [150] and Cho and Hong [151] verified that AtHSP16.6A and NtHSP70-1, respectively, can participate in the regulation of water flow during drought. Moreover, Sato and Yokoya [141] detected that rice transgenic seedlings with higher expression levels of sHSP17.7 showed growth recovery potential after submission to drought.

In addition, *GHSP26* gene product might act as a factor in the signal transduction pathway of a drought stress response from the nature of early induction [140]. In *Arabidopsis thaliana*, Zhang et al. [136, 137] verified that ectopic expression of cytosolic sHSP 17.1 generated more biomass and less water loss, as

well as flowered earlier and recovered more quickly and robustly after being rewatered.

Recently, it was found that in transgenic sugarcane plants overexpressing the EaHSP70 protein there was a drastic increase (2000-fold or more) in the upregulation of the HSP70 gene compared to the control plants. In this study, in addition to increased tolerance to drought, the upregulation of abiotic stress-responsive genes (DREB2, DNA helicase 45, LEA, RD29, ERD, ERF, Cor15, and BRICK) was more than 100-fold in each transgenic event when compared to control plants. Thus, the authors suggest that the expression of these genes might be one of the reasons for its enhanced drought tolerance [152].

4.4 Molecules with Antioxidative Activity (Protection Against ROS)

Drought stress drastically affects various physiological traits in plants. It is known that the downregulation of photosynthesis due to drought stress is mainly the result of a reduction in stomatal conductance, although the photosynthetic apparatus is not significantly affected [153]. It is generally observed from the first stages of water shortage due to limited CO_2 diffusion through stomata. The limitation of CO_2 assimilation in water-stressed plants causes the overreduction of the photosynthetic electron chain. Consequently, plants are exposed to an excess of light energy that leaves cannot dissipate and which cannot be converted into biochemical energy. Then there is a redirection of photon energy and that leads to the production of reactive oxygen species (ROS) and finally to a substantial oxidative damage [154, 155]. This state is so-called oxidative stress.

ROS are partially reduced forms of atmospheric oxygen and under normal conditions their production in plant cells is tightly controlled by the scavenging system [156]. The main ROS are superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and singlet oxygen (O). They are present in all plant cells because of aerobic lifestyle [157, 158].

In plant cells chloroplasts, mitochondria, and peroxisomes are important intracellular generators of ROS. It is now widely accepted that the production of these species at a higher level results in a loss of balance between the production ROS and their removal [159, 160]. If not effectively and rapidly removed from plants, excessive levels of ROS are responsible for various stress-induced damages to macromolecules and cellular structure including RNA and DNA damage, enzyme inhibition, protein oxidation, membrane lipid peroxidation, and ultimately cell death. Then, their scavenging is necessary to protect the subcellular components and for maintenance of normal growth and development [154, 157, 160, 161, 162].

Plants have evolved a complex system of antioxidant molecules to prevent oxidative injury. The antioxidant defense mechanism plays an important role by delaying or preventing the oxidation of cellular oxidable substrates. Antioxidants

exert their effects by scavenging ROS, activating a battery of detoxifying proteins, or preventing the generation of ROS [157]. This network is composed of over 150 genes encoding ROS-producing proteins, with enzymatic and nonenzymatic molecules [163]. The well-known enzymatic antioxidants comprise superoxide dismutase (SOD), catalase (CAT), and peroxidases (glutathione peroxidases, GPX; ascorbate peroxidase, APX). These enzymes are present in practically all subcellular compartments. Usually, an organelle has more than one enzyme able to scavenge a single ROS [164]. Furthermore, they also possess numerous low molecular weight antioxidant nonenzymatic compounds. Glutathione, flavonoids, alkaloids, carotenoids, and polyamines are the main nonenzymatic components [154, 155, 157, 160, 161, 165]. Together, all these molecules act as the main defense against ROS produced in various parts of plant cells [162].

The extent of oxidative stress in a cell is determined by the amounts of superoxide, H_2O_2, and hydroxyl radicals. Therefore, the balance of SOD, APX, and CAT activities will be crucial for suppressing toxic ROS levels in a cell. Changing the balance of scavenging enzymes will induce compensatory mechanisms. For example, when CAT activity was reduced in plants, scavenging enzymes such as APX and GPX were upregulated. Unexpected effects can also occur. When compared to plants with suppressed CAT, plants lacking both APX and CAT were less sensitive to oxidative stress [158, 166].

However, it is known that ROS also act as signaling molecules that can trigger cell responses. In this context, focus has been on H_2O_2, the most stable ROS. H_2O_2 generated in chloroplasts can function directly as a signaling agent. To act in this function, H_2O_2 must be able to rise rapidly to a threshold concentration and remain high enough for a sufficient time so that it can oxidize the molecules involved in the cell-signaling events. Then enzymes that scavenge ROS must play two roles: in an active state, they keep ROS concentrations at safe levels. In a deactivated state, ROS concentrations reach critical levels for activation of signaling components [167].

4.4.1 Enzymatic Molecules

4.4.1.1 Superoxide Dismutase

Superoxide dismutases belong to a family of metalloenzymes that protect cells from the harmful effects of superoxide radical (O_2^-) by catalyzing its dismutation into molecular oxygen and hydrogen peroxide (H_2O_2) [168, 169]. Depending on the metal in their active site, SODs are classified into four groups: CuZnSODs, NiSODs, FeSODs, and MnSODs. Each SOD group displays a distinct subcellular distribution and structural features [170, 171].

They are important for early metabolic cellular defense, acting as the first line of defense against ROS. The resultant H_2O_2 can be detoxified to oxygen and water by CAT or APX, which occur mainly in peroxisomes [130, 131, 153]. The balance

between SODs and the different H_2O_2-scavenging enzymes in cells is considered to be crucial in determining the steady-state level of O_2^- and H_2O_2. This balance, together with the sequestering of metal ions by ferritin and other metal-binding proteins, prevents the formation of the highly toxic HO radical [165].

Recent studies have demonstrated the main role of different SODs under drought conditions. In *Pennisetum glaucum,* different abiotic stresses were able to induce a CuZnSOD. In addition, when expressed in bacteria, it conferred enhanced tolerance to oxidative stress [172]. Under salinity stress, *Arabidopsis* increased the expression of two FeSODs [173]. Sales et al. [174], studying sugarcane plants, observed that SOD improves the metabolism of plants subjected to water deficit. In transgenic plums with the overexpression of CuZnSOD, the tolerance to salt and drought stress was enhanced [175].

Some studies reported to SODs a role against structural damages. The overexpression of CuZnSOD in transgenic tobacco improved tolerance against drought stress, alleviating the cellular and tissue damages produced by water stress conditions [176]. Shafi et al. [177] reported in *Arabidopsis* that the expression of CuZnSOD genes positively regulates secondary cell wall biosynthesis and promotes plant growth and yield under salt stress, showing the importance of SOD to the development of plants under stress conditions. Ambiguous results were also reported. Drought stress triggered in Arabidopsis a downregulation of CuZnSODs, but an upregulation of FeSODs [171].

4.4.1.2 Catalases

Catalases (H_2O_2 oxidoreductase; CAT) are tetrameric heme-containing enzymes, mostly localized in peroxisomes that are bound by a single membrane and contain hydrogen peroxide-generating oxidases. They are also localized in glyoxysomes and mitochondria and are apparently absent in the chloroplast. They serve as efficient scavengers of ROS, mainly in the removal of excessive H_2O_2 generated during developmental processes or by environmental stimuli into water and oxygen in all aerobic organisms [162, 178]. Catalases play an important role in biotic/abiotic stress, to avoid oxidative damage. Plant catalases are composed of a multigene family and have been reported in many plant species [179]. Plant peroxisomal proteins including catalases require particular peroxisomal targeting signal (PTS) for import into peroxisomes. The catalase activity levels is inversely correlated with the cellular H_2O_2 amounts of plants [178].

There are two main routes for H_2O_2 metabolism in cells: its removal by peroxidases and by catalases. Peroxidases require a small reducing molecule to act as a regenerating cofactor. On the other hand, catalases mainly catalyze a dismutation reaction in which a first oxidizing molecule of H_2O_2 is transformed to water and a second reducing H_2O_2 is then converted to O_2. Thus, no additional reductant is required. Catalases are encoded by three genes [172].

Considering the key role of CAT in photorespiration, many authors focused on the role of the CAT catalysis pathway under both drought and salt stress. Indeed,

the maintenance of CAT activity in leaves of drought-stressed plants likely allowed the removal of photorespiratory H_2O_2 produced when plants were subjected to the water deficit of salinity [175].

Transgenic plants expressing CAT had increased tolerance against drought stress. However, studies have reported that the expression of either SOD or CAT alone led to no change in response to drought stress. These contradictory findings may be due to the complex network of plant antioxidant defenses and raise the possibility that a higher tolerance to oxidative stress might be achieved by pyramiding or stacking genes in a single genotype. The antioxidant effects of the two enzymes are directly linked through their converting superoxide to H_2O_2 and H_2O_2 to oxygen and water, sequentially [130, 131]. The combination of the two upregulated genes was reported as improving the drought stress responses. In cassava, the removal of ROS was enhanced by overproduction of both CAT and CuZnSOD, delaying the postharvest physiological deterioration of storage roots [130, 131].

In potato, the single overexpression of a catalase controlled the H_2O_2 levels and delayed the leaf senescence (related to oxidative damage) [178]. The catalase induction can also be triggered by other abiotic stresses, such as heavy metal and hyperosmotic stresses, as shown by [179].

On the other hand, low levels of catalases allow the accumulation of H_2O_2 in cells. Michelet et al. [167], working with knockdown mutants of *Chlamydomonas reinhardtii*, downregulated catalase activity and reported high levels of H_2O_2. For the authors, this concentration seems to be necessary to activate H_2O_2-dependent signaling pathways stimulating the expression of H_2O_2 responsive genes. However, high levels of H_2O_2 cannot be maintained for long periods, because the deficiency in catalases can also promote cell death, as reported by [180] in *Arabidopsis*.

4.4.1.3 Peroxidases

Plant peroxidases can be grouped into three classes based on their structural and catalytic properties. Class I peroxidases include intracellular enzymes, such as microbial cytochrome C peroxidase, bacterial catalase-peroxidases, and APX in plants, bacteria, and yeast. Class II peroxidases, including lignin peroxidase, are extracellular fungal peroxidases. Class III peroxidases are secreted into the cell wall or the surrounding medium and the vacuole [181].

In the complexity of the regulation network of plant antioxidant defenses, APX is an antioxidant enzyme that plays a key role in drought stress responses and following recovery from drought [175]. They are found in higher plants, chlorophytes, red algae, and members of the protist kingdom [164]. They have multiple locations and are among the most important key enzymes that scavenge potentially harmful H_2O_2 from the chloroplasts and cytosol of plant cells [176]. There are two main isoforms: APX1 and APX2. APX1 is constitutively expressed in roots, leaves, stems, and other plant tissues, and its expression is significantly upregulated in response to a large number of biotic and abiotic stresses [163]. APX2 is also involved in the response of plants to abiotic stress. Expression of APX2 is almost

undetected in many plant tissues and is significantly upregulated in roots in response to wounding and oxidative stress and in roots and shoots in response to salinity and osmotic stress [163]. The main hydrogen peroxide-detoxification system in plant chloroplasts is the ascorbate–glutathione (ASC–GSH) cycle, in which APX is a key enzyme. APX utilizes AsA as specific electron donor to reduce H_2O_2 to water [164].

The ROS-scavenging enzymes in plants have been widely studied and the results have demonstrated that, in response to environmental stress, APX activity generally increases along with other enzyme activities, such as CAT and SOD. In addition, the balance of APX, GPX, and CAT activities, representing the main enzymatic H_2O_2 scavenging mechanism in plants, is crucial for the suppression of toxic H_2O_2 levels in a cell. As reported above, the enzymes APX, GPX, and CAT are able to scavenge H_2O_2 with different mechanisms. If the balance of scavenging enzymes changes, compensatory mechanisms are induced (i.e., APX and GPX are upregulated when CAT activity is reduced in plants) [162, 175]. Weisany et al. [162] reported in soybean that abiotic stresses, such as salinity and drought, could increase the production of both enzymes: CAT and peroxidases (APX and POD).

In transgenic plums with the overexpression of APX, the tolerance to salt and drought stress was enhanced (Xing et al. 2015). Sales et al. [174], studying sugarcane plants, observed that APX improves the metabolism of plants subjected to water deficit. In *Arabidopsis*, [163] showed that deficiency in APX2 resulted in a decreased tolerance to light stress, which is related to drought stress. Also, these plants produced more seeds under heat and drought stresses, suggesting the activation of protection mechanisms of reproductive tissues from heat and drought damage. Some peroxidases are strongly induced by both abiotic and biotic stresses. Choi and Hwang [181] reported it in *Capsicum annuum*: PO2 was induced by drought, salt, cold, and infection by a fungal pathogen. Plants without PO2 were more susceptible to these stresses. In *Arabidopsis*, when the peroxidase was overexpressed, the plants were more tolerant to all stresses.

On the other hand, in wheat, different isoforms can be expressed in different levels under abiotic stress (up- and downregulated), as well the same isoform can also be differentially regulated in different wheat genotypes [182].

4.4.2 Nonenzymatic Molecules

4.4.2.1 Glutathione

Glutathione (γ-glutamyl cysteinyl glycine) is an abundant, ubiquitous, and main low molecular weight thiol in all aerobic organisms. The presence of cysteine confers its biological properties mainly as antioxidant function through its involvement in cell redox homeostasis [183, 184]. It is a tripeptide constituted of glutamate (Glu), cysteine (Cys), and glycine (Gly), and is represented by the formula g–Glu–Cys–Gly. GSH exists either in a reduced form (GSH) with a free thiol

group or in an oxidized form (GSSG) with a disulfide between two identical molecules. The presence of Cys in the chemical reactivity and high water solubility of the thiol (-SH) group of GSH confer its biological properties and make it a crucial metabolite to perform multiple functions including growth, development, and plant responses to drought stress [185, 186]. GSHs also function with GSTs to detoxify a range of herbicides by tagging electrophilic compounds for removal during oxidative stress [178]. Among the nonenzymatic antioxidants, GSH is considered the most important intracellular defense against ROS and/or their reaction products-induced oxidative damage in plants [187].

It has long been recognized that GSH is oxidized by ROS as part of the antioxidant barrier that prevents excessive oxidation of sensitive cellular components. Unlike the oxidized forms of many other primary and secondary metabolites that can also react with ROS, GSSG is rapidly recycled by the glutathione reductases (GRs) in key organelles and the cytosol. A main characteristic of glutathione is its high concentration in relation to other cellular thiols. In general, glutathione accumulates to millimolar concentrations, with tissue contents well in excess of free cysteine. A second key characteristic of the glutathione is its high reduction state. In the absence of abiotic stresses, tissues maintain measurable GSH [184].

Plants of *Arabidopsis* treated with GSH showed more tolerance to drought stress [178]. On the other hand, when a GR gene (the GSH recycler) is not expressing, plants increase their sensitivity to abiotic stress, as reported by Wu et al. [188]. Also, GSH has supplemental functions in plants. Ramírez et al. [189] showed its protective role against iron deficiency in the same above-mentioned species.

4.4.2.2 Flavonoids

Flavonoids are a vast class of plant polyphenolic secondary metabolites encompassing more than 10,000 structures, showing a common three-ring chemical structure (C6–C3–C6). The main classes of flavonoids are anthocyanins (red to purple pigments), flavonols (colorless to pale yellow pigments), flavanols (colorless pigments that become brown after oxidation), and proanthocyanidins (PAs) or condensed tannins. These compounds are widely distributed in different amounts, according to the plant species, organ, developmental stage, and growth conditions [190]. The multiplicity of the functional roles of flavonoids in plant–environment interactions is consistent with their presence in a wide array of cells and subcellular compartments [191].

Flavonoids have the capacity to absorb the most energetic solar wavelengths (i.e., UV-B and UV-A), inhibit the generation of ROS, and then quench them once they are formed [192]. There is evidence corroborating the hypothesis that they have antioxidant functions in higher plants that are challenged with a range of environmental stresses, constituting a secondary ROS-scavenging system in plants suffering from severe excess excitation energy to the photosynthetic apparatus [191, 193] and also preventing the generation of ROS [190].

The biosynthesis of flavonoids is upregulated as a consequence of UV radiation and in response to a wide range of other abiotic and biotic stresses, ranging from nitrogen/phosphorus depletion to salinity/drought stress [194]. For instance, Agati et al. [191] have reported that root-zone salinity stress had a very similar effect on flavonoid metabolism to that exerted by UV radiation in *Ligustrum vulgare*. This shows that flavonoid pathway genes (*FLS*-flavonol synthase and *F3'H*-flavonoid 3′-hydroxylase) are also upregulated under salt/drought stress conditions.

The biosynthesis of antioxidant flavonoids increases more in stress-sensitive species than in stress-tolerant species; stress-sensitive species present a less effective first line of defense against ROS under stressful conditions and are subsequently exposed to a more severe "oxidative stress" [191].

Some studies have shown the antioxidant function of flavonoids. It was reported in wheat leaves that the expression of flavonoid biosynthesis genes and accumulation of flavonoid in response to drought stress improve the stress tolerance [195]. In addition, flavonoids with radical scavenging activity mitigate against oxidative and drought stress in *Arabidopsis thaliana* [196].

4.4.2.3 Carotenoids

Carotenoids are isoprenoids containing 40 carbon atoms and 3–13 conjugated double bonds in their skeleton [197]. They are synthesized by photosynthetic organisms and some nonphotosynthetic bacteria and fungi. In plants, they are synthesized in plastids, where they play different functions [198]. Carotenoids such as b-carotene, lycopene, and lutein are important in the food and oil industries because of their powerful antioxidant activities [199].

They play a main dual role in photosynthetic organisms: first, they serve as accessory pigments in the photosystems, increasing light absorption in the blue spectral domain (420–500 nm). Carotenoids are synthesized in plastids and accumulate as red, orange, and yellow pigments in flowers, fruit, and roots [197, 199]. Second, they protect the photosynthetic apparatus against toxic ROS produced by plant abiotic stresses, especially singlet oxygen ($^1O_2$), being considered to be the first line of defense of plants against O_2 toxicity. The second occurs either via a physical mechanism involving thermal energy dissipation or via a chemical mechanism involving direct oxidation of the carotenoid molecule. The latter mechanism can produce a variety of products (aldehydes, ketones, endoperoxides, and lactones) resulting from their direct oxidation by O_2, all being potential antioxidant candidates [197, 200].

One such molecule, the volatile β-carotene derivative β-cyclocitral, triggers changes in the expression of $^1O_2$-responsive genes and leads to an enhancement of photo-oxidative stress tolerance [197]. Additional protective functions of carotenoids include stabilization of membrane lipid bilayers, scavenging of free radicals and protection against membrane lipid peroxidation [201].

In sweet potato, the expression of the *Or* gene, responsible for the accumulation of carotenoids, increased in response to abiotic stress. Also, plants transformed with this gene exhibited increased antioxidant activity, showing the possible role of carotenoids in oxidative stress [199]. In *Arabidopsis*, oxidative stress induces the oxidation of carotenoids, and its products change the expression of many responsive genes to this kind of stress, increasing the tolerance of these plants [197].

Ruiz-Sola et al. [198], studying *Arabidopsis*, reported that the presence of high concentrations of salt in the growth medium rapidly triggers a root-specific activation of the carotenoid pathway. It shows the probable participation of carotenoid molecules in salt/drought stress. Finally, in *Brassica rapa* and *Brassica oleracea*, enzymes involved in carotenoid oxygenase (a key enzyme involved in the metabolism of carotenoids) pathways may be activated as multifunctional stress signaling factors under abiotic stress treatment conditions [199].

4.4.2.4 Polyamines

Polyamines (PAs) are essential solute compounds for cell survival. They are small, flexible, organic polycationic compounds of low molecular weight that are present in almost all cells and most living organisms, except some archaeal methanogens and halophiles [202]. PAs are present in all compartments of the plant cell, including the nucleus. The main PAs are: diamine putrescine (Put), triamine spermidine (Spd), tetramines spermine (Spm), and its isomer thermosspermine (tSpm). They can all be found in free and conjugated forms [203].

PAs have key roles in a variety of regulatory and cellular processes such as cell division and elongation, root growth, flower and fruit development, replication, transcription, translation, membrane and cell wall stabilization, chromatin organization, ribosome biogenesis, and programmed cell death [204]. Current evidence points to the occurrence of intricate crosstalk between polyamines, stress hormones, and other metabolic pathways required for their function. The identification of molecular mechanisms suggests that some PAs conjugate to hydroxycinnamic acids, and the products of PA oxidation (hydrogen peroxide and γ-aminobutyric acid) are required for different processes in plant development pathways during the lifespan of plants and participate in abiotic and biotic stress responses [205].

Among the different classes of compatible solutes, polyamines stand as one of the most effective against extreme environmental stresses, which include drought, salinity, low temperature, oxidative stress, and metal toxicity. In tobacco, the response to heat/drought stress involved a transient increase in the levels of free and conjugated Put and in the levels of free Sp, norspermidine (N-Spd) and Spm [206].

PAs partially reversed the NaCl-induced phenotypic and physiological disturbances in *Citrus aurantium*. The expression of PA biosynthesis and catabolism genes was systematically upregulated by PAs. In addition, PAs altered the oxidative status in salt-stressed plants as inferred by changes in ROS production and redox status accompanied by regulation of transcript expression and activities of various

antioxidant enzymes [207]. In *Arabidopsis*, the polyamine spm protects from heat stress-induced damage by increasing expression of heat shock-related genes [203].

4.5 Conclusions and Future Perspectives

The understanding about cellular and molecular mechanisms of plant response to stress is essential to development of genetically engineered plants focused on acquisition or increasing of tolerance against abiotic stresses such as drought, allowing better insights into the high complexity of molecular strategies used by plants to their adaptations under adverse conditions. These adaptations contribute to a considerable reduction of productivity losses of plants with agronomical importance. Thus, this chapter presented efforts of scientific research to unveil the participation of several genetic components (such as signaling molecules, transcription factors, stress-responsive genes, and enzymatic and nonenzymatic molecules with antioxidative activity) in the drought stress response and its relationship with endogenous defense mechanisms of plants.

How the manipulation of gene expression, mainly by the overexpression of certain genes, can contribute to reach the plant tolerance to drought was also discussed. There is great importance in the achievement of more scientific studies for further identification of new components involved in the drought response. Such studies will promote a better understanding about the role of different genes and proteins whose contribution to tolerance is already well described, especially knowledge about the combined effect of several gene products related to this response, culminating in the generation of tolerant plants at the field level with high-yield production.

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Chapter 5
The Role of Abscisic Acid in Drought Stress: How ABA Helps Plants to Cope with Drought Stress

Agata Daszkowska-Golec

Abbreviations

7′HO ABA	7′-hydroxy ABA
8′HO ABA	8′-hydroxy ABA
9′HO ABA	9′-hydroxy ABA
AAO	Aldehyde oxidase
ABA	Abscisic acid
ABA2	Short-chain dehydrogenase/reductase
ABA-GE	ABA glucosyl ester
ABCG	ATP-binding cassette (ABC) protein G subfamily
ABI5	ABA-insensitive 5
ABRE	ABA responsive element
ADP	Adenosine diphosphate
AIP2	ABI3-interacting protein 2
AIRP1	Arabidopsis ABA-insensitive RING protein 1
AIT	ABA-IMPORTING TRANSPORTER 1
At	*Arabidopsis thaliana*
ATP	Adenosine triphosphate
AtrbohF	NADPH oxidase
BAK1	BRI1-associated receptor kinase 1
BG	Glucosyltransferase
BIN2	BRASSINOSTEROID INSENSITIVE 2
BR	Brassinosteroids
CE	Coupling element
CUL4	Cullin4
CYP707A	Cytochrome p450
DIS1	Drought-induced SINA protein 1
DOR	Drought tolerance repressor

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DPA	Dihydrophaseic acid
DSG1	Dwarf and small grain1
DTX50	The detoxification efflux carriers/multidrug and toxic compound extrusion 50
DWA1/2	DDB1-BINDING WD40 PROTEIN1/2
FRET	Förster resonance energy transfer
HvSNAC1	STRESS-RESPONSIVE NAC 1
KAT1	POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1
KEG	Keep on going
NCED	9-*cis* epoxycarotenoid dioxygenase
neoPA	Neophaseic acid
OE	Overexpression
Os	Oryza sativa
OsABF1	ABI-like factor
OsDRO1	Deeper rooting 1
OsLEA4	Late emrbryogenesis protein
OsRK1/SAPK6	STRESS ASSOCIATED PROTEIN 5
OST1	Open Stomata 1
P	Phosphorylation
PA	Phaseic acid
PIP21	PLASMA MEMBRANE INTRINSIC PROTEIN 2;1
PKS5	PROTEIN KINASE SOS2-LIKE 5
PP2C	PROTEIN PHOSPHATASE 2C
PUB19	U-box domain-containing protein 19
PYR/PYL/RCAR	PYRABACTIN-RESISTANCE 1/PYRABACTIN RESISTANCE LIKE/ REGULATORY COMPONENT OF ABA RECEPTOR
QUAC1/ALMT12	QUICK ANION CHANNEL 1/ALUMINUM-ACTIVATED ANION CHANNEL 12
RING-1	Really interesting protein finger 1
RHA2a	RING finger E3 ligase
Rma1	RING finger motif
ROS	Reactive oxygen species
S	Sumoylation
SAP5	STRESS ASSOCIATED PROTEIN 5
SDIR1	Salt- and drought-induced RING finger 1
SIZ1	Small ubiquitin-related modifier 1/2 (AtSUMO1/2)
SLAC1	SLOW ANION CHANNEL ASSOCIATED 1
SnRK	Sucrose nonfermenting related kinase 2
TaERA1	Enhanced response to ABA1
U	Ubiquitination
VIGS	Virus induces gene silencing

WUE	Water use efficiency
ZEP	Zeaxanthin epoxidase
ZF1	RING Zinc finger 1
Zm	Zea mays
ZmCPK11	Calcium-dependent protein kinase 11

5.1 Introduction

Discovered at least 50 years ago as a growth inhibitor accumulating in abscising cotton fruit, ABA (abscisic acid) turned out to be the main regulator of drought stress response. ABA is famous for its stress-related properties but it also regulates many development and growth processes. Under drought stress ABA elicits two distinct responses: rapid and gradual. The earliest and most rapid plant reaction, regulated mainly by ABA, is stomatal closure which minimizes the water loss through limited transpiration. Exposure to ABA triggers guard cells to decrease their volume and close across the airway pore. This is achieved via changes in ion fluxes within the guard cell. ABA gradually increases hydraulic conductivity and promotes cell elongation in the root, enabling the plant to recover after water-deficit stress. ABA induces accumulation of osmotically active compounds, which protects cells from damage.

ABA signaling under drought stress is extremely complicated and has been gradually elucidated using a wide spectrum of forward and reverse genetics methods (reviewed in [24]). Recent progress in plant functional genomics accelerated the process of identification and characterization of genes involved in ABA-dependent drought stress response in different species. In 2009 ABA receptors were identified by four independent teams using different approaches. It was an exciting breakthrough in the field of decoding ABA signaling and has further completed this complicated network [75, 88, 96, 108]. The class of soluble PYR/PYL/RCAR receptors (PYRABACTIN-RESISTANCE 1/PYRABACTIN RESISTANCE LIKE/REGULATORY COMPONENT OF ABA RECEPTOR) act at the initial step of the ABA "core signaling pathway", linking together the previously identified and characterized PP2Cs (PROTEIN PHOSPHATASES 2C) and SnRK2s (SUCROSE NONFERMENTING RELATED KINASES 2; [27, 29, 129, 135]). Hitherto, the important regulatory role of PYR/PYL/RCAR receptors was reported in achieving drought stress tolerance in several species including Arabidopsis, rice, and tomato [9, 31, 33, 51, 90, 99].

The last decade enabled a better understanding of the molecular basis of ABA metabolism and transport, and using the high-throughput technologies brought us closer to elucidation of ABA signaling in response to stress. This knowledge has been already implemented in practical breeding for traits with agronomic importance, such as drought tolerance in crops. The impact of ABA signaling on the plant

phenotype is multilayered. For example, such traits as improved water balance through stomatal closure, deeper roots, cuticular resistance due to a thicker layer of waxes, and reduced leaf area due to inhibited growth, or the osmotic adjustment, antioxidant defense, and membrane thermostability may serve (reviewed in [13]). Physiological and morphological adaptations to environmental constraints are associated with wide transcriptional changes controlled by complex molecular mechanisms. Therefore, elucidation of ABA signaling under drought stress can be further implemented in the development of drought-tolerant crops.

5.2 Recent Highlights on Integration of Signaling, Metabolism, and Transport of ABA

The sensitivity of the tissue to ABA will determine the level of response to this phytohormone. The concentration of ABA in a specific tissue reflects a balance of ABA biosynthesis and inactivation by turnover or conjugation, further modifies compartmentation and transport (reviewed in [24]). Taking into account that developmentally programmed and conditionally induced ABA accumulations control diversity of physiological responses in multiple types of plant cells, the important question to address is how cellular ABA concentrations are patterned across tissues and over time. The answer is crucial for fully understanding how ABA promotes abiotic stress tolerance and regulates key developmental processes, such as seed germination and senescence. For ABA action, the accumulation of biologically active ABA at the site of perception is necessary. Although ABA synthesis is required for the regulation of ABA catabolism, processes of ABA de/conjugation and in addition the long-distance transport are also of the utmost importance. The property of a long-distance movement of ABA has made it a critical signal messenger for plant in many developmental processes and in response to different abiotic stresses [53–55, 148]. Growing evidence suggests that different ABA levels have an impact on ABA perception and affect water-use efficiency (WUE) in crop plants by ABA-dosage-dependent responses [112]. Here, we discuss tightly connected processes of ABA biosynthesis, inactivation, and transport. Their high coordination, together with the proper signal transduction, enables plants to respond to stress early enough to survive under unfavorable conditions.

5.2.1 Boosting ABA Biosynthesis in Response to Drought

A local level of active ABA results from the precise coordination among ABA biosynthesis, catabolism, conjugation, and transportation. Early biochemical studies and subsequent genetic and physiological analyses resulted in isolation of a series of ABA-deficient mutants providing the information and tools on the ABA

biosynthetic pathway together with identification of crucial enzymes. Further studies revealed that ABA metabolism is subjected to the feedback regulation; it turned out that some genes encoding ABA metabolic enzymes are regulated by environmental cues such as drought.

ABA is primarily synthetized in plastids with the exception of the last two steps where xanthoxin is converted to ABA in the cytosol (Fig. 5.1; [79, 114, 122]). Biosynthesis of ABA in higher plants is followed in an "indirect" pathway that begins with IPP (isopentenyl pyrophosphate), the biological isoprene unit. IPP is the precursor of all terpenoids as well as many plant hormones. A more specific pathway to ABA biosynthesis starts from the conversion of zeaxanthin to trans-violaxanthin, a two-step epoxidation process catalyzed by ZEP/AtABA1 (zeaxanthin epoxidase; [5, 79]). Overexpression of *ZEP* in transgenic plants confers enhanced drought and salt tolerance [95]. The next step of ABA biosynthesis is catalyzed by NCED (9-*cis* epoxycarotenoid dioxygenase). It is the oxidative cleavage of 9-*cis*-violaxanthin and/or 9-*cis*-neoxanthin to produce 15C compound, xanthoxin. This reaction is considered to be rate-limiting in ABA biosynthesis and is the last step of ABA biosynthesis performed in plastids [121]. Arabidopsis *NCED3* expression is highly regulated by abiotic stresses, especially water deficit. A significant increase in *NCED* transcript levels can be detected within 15–30 min after leaf detachment or dehydration treatment [100], which indicates that the activation of *NCED* genes can be fairly quick. Similar to Arabidopsis, the *NCED3* gene was significantly induced by water stress in rice [142]. Moreover, *NCED3* expression was also reported in roots, indicating their function in response to environmental cues in the ABA-dependent manner [121]. The product of NCED reaction—xanthoxin is exported to the cytosol and converted to ABA via an intermediate—abscisic aldehyde by a series of oxidative steps performed by SDR/AtABA2 (short-chain dehydrogenase/reductase; [16, 17, 32]). Abscisic aldehyde is then oxidized to ABA by AAO/AO (aldehyde oxidase; [113]).

Gene and protein expression analyses in Arabidopsis revealed that ABA biosynthesis enzymes are the most abundant in parenchyma cells in vascular bundles under both drought and nonstressed conditions [16, 17, 23]. Further studies showed that the activity of ABA biosynthetic enzymes is also high in phloem companion cells of vascular tissues [55]. However, of the utmost importance is the autonomous ability of guard cells to perform ABA biosynthesis as shown recently by Bauer et al. [7]. Guard cell-autonomous ABA synthesis allows the plant to respond more rapidly to changing environmental conditions and facilitates the maintenance of water status homeostasis. Kuromori et al. [55] suggested that ABA synthesis in both vascular and guard cells may be related to stomatal closure/opening regulation, however, the process remains to be fully elucidated.

Although ABA synthesis is required for a proper response to stresses, it is still unclear which of the processes (biosynthesis, catabolism, or de/conjugation) is the main factor in controlling the level of ABA accumulation under stress. When compared with the ABA biosynthesis pathway, ABA catabolism is much simpler. Abscisic acid can be hydroxylated at three different methyl groups in the ring structure (C-7′, C-8′, and C-9′; [152]). Although the hydroxylation does not reduce

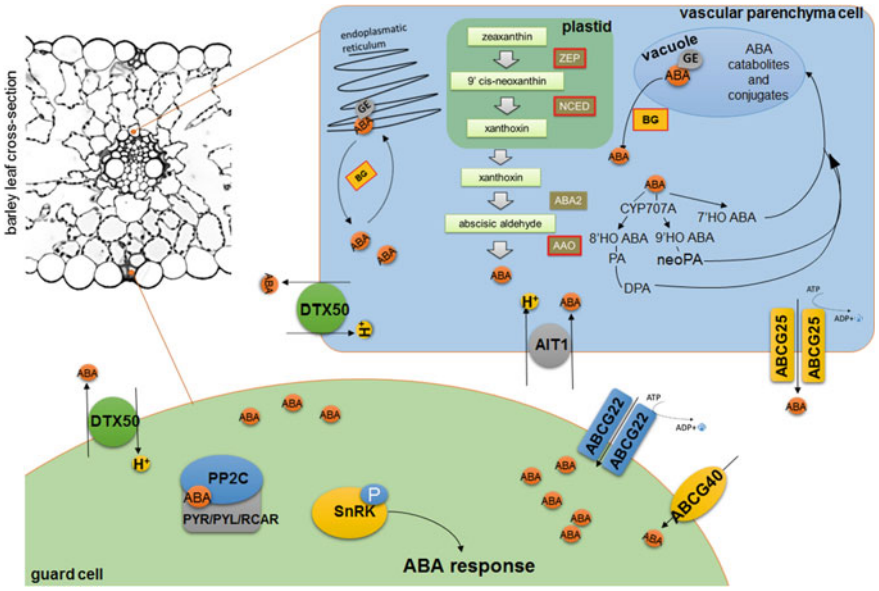

Fig. 5.1 The complex coordination of ABA biosynthesis, catabolism, conjugation, deconjugation, transport, and signaling processes. *ZEP* Zeaxanthin epoxidase; *NCED* 9-*cis* epoxycarotenoid dioxygenase; *ABA2* short-chain dehydrogenase/reductase; *AAO* aldehyde oxidase; *ABA-GE* ABA glucosyl ester; *BG* glucosyltransferase; *CYP707A* cytochrome p450; *8′HO ABA* 8′-hydroxy ABA; *7′HO ABA* 7′-hydroxy ABA; *9′HO ABA* 9′-hydroxy ABA; *PA* phaseic acid; *DPA* dihydrophaseic acid; *neoPA* neophaseic acid; *DTX50* detoxification efflux carriers/multidrug and toxic compound extrusion 50; *AIT* ABA-IMPORTING TRANSPORTER 1; *ABCG25* ATP-binding cassette (ABC) protein G subfamily 25; *ABCG22* ATP-binding cassette (ABC) protein G subfamily 22; *ABCG40* ATP-binding cassette (ABC) protein G subfamily 40; *PP2C* protein phosphatase 2C; *PYR/PYL/RCAR* PYRABACTIN-RESISTANCE 1/PYRABACTIN RESISTANCE LIKE/REGULATORY COMPONENT OF ABA RECEPTOR; *SnRK* sucrose nonfermenting related kinase 2; *ATP* adenosine triphosphate; *ADP* adenosine diphosphate

the biological activity of ABA, it can trigger further inactivation steps. Among the hydroxylated products, only the 8′-hydroxy ABA can be changed into PA (phaseic acid) by cyclization and then into DPA (dihydrophaseic acid) by further reduction [86]. DPA is the end product of ABA catabolism which does not exhibit any ABA-like activity (Fig. 5.1).

In barley expression analyses of genes involved in ABA catabolic pathway showed that only *HvABA8′OH-1* was upregulated during drought stress. ABA8′ hydroxylase (ABA8′OH) encodes the cytochrome p450 (belonging to CYP707 family) which degrades active ABA into 8′hydroxy-ABA. When ABA level increases in response to drought stress, it is also important to decrease the excess of ABA rapidly to fine-tune ABA concentration, thus, transcripts of ABA catabolic genes, such as *HvABA8′OH-1* increase under these conditions [111]. Also the transcript levels of *CYP707As* (ABA 8-hydroxylase) were induced by dehydration and exogenous ABA treatment in Arabidopsis [56, 106].

Apart from ABA biosynthesis and catabolism, ABA de/conjugation was also reported to act as a quick mechanism for releasing active ABA in response to abiotic stresses [63, 138]. Inactivated ABA at the C-1 hydroxyl group, known as ABA glucosyl ester (ABA-GE) accumulates in vacuoles or apoplastic space. The conjugation reaction is catalyzed by BG1 (ABA glucosyltransferase; [63]). Analyses of Arabidopsis BG1 protein showed that ABA conjugation plays a pivotal role in creating an active ABA pool that allows for plants to adjust to changing physiological and environmental conditions [63]. *Atbg1* knockout mutant exhibited a lower ABA level and ABA-defective phenotype, whereas in overexpression lines the increased levels of drought-induced ABA accumulation were observed. Further study showed that the BG1 in WT Arabidopsis under water stress was much higher than in normal condition, indicating the importance of ABA de/conjugation processes in controlling the ABA levels in response to stress ([133]; Fig. 5.1).

5.2.2 *ABA on the Move—Recent Highlights in Transport of ABA*

The increase of the amount of ABA available for intracellular receptors requires its fast and efficient transport. ABA is a weak acid; it exists in an anionic form (ABA–) or protonated (ABAH). When the protonated form of ABA is uncharged, it is able to diffuse through the plasma membrane. Therefore, the movement of ABA has long been understood to occur via pH-dependent diffusion [6, 139]. However, this type of ABA transport has limitations. An increase of pH caused by drought stress can drastically decrease the pool of apoplastic-diffusible ABAH, which indicates the necessity of ABA transporters for delivery of ABA during drought [42]. In Arabidopsis, the identification of ABA transporters focused attention on the ABC protein family (ATP-binding cassette) which is divided into 8 major subfamilies: A–H, according to protein architecture. The largest known family involved in ABA transport is the G subfamily. This subfamily (ABCG) is divided into two groups: the half-sized transporters (formerly known as WBC white–brown complex) and full-sized transporters (known also as a pleiotropic drug resistance transporters, PDRs; [132]). ABCG25, the half-sized transporter, expressed mainly in vascular tissues, was characterized as an ABA efflux carrier. The Arabidopsis overexpression line with the *ABCG25* gene showed a reduced water loss from leaves under drought stress due to delivery of ABA to guard cells resulting in limitation of evaporation [53, 55]. Another ABA transporter, ABCG40, the full-sized transporter, imports ABA into stomatal cells and is mainly expressed in guard cells. Knockout mutants of Arabidopsis in the *ABCG40* gene exhibited reduced ABA sensitivity and thus were more sensitive to drought stress [46]. Kanno et al. [49] reported that the *abcg40* mutant had increased stomatal aperture and decreased surface temperature in stems. Kuromori et al. [54] reported another ABA importer, ABCG22, the half-sized transporter, which enhanced ABA influx intro guard cells.

Similarly to *abcg40*, a mutant in the *ABCG22* gene showed increased transpiration and thus drought susceptibility.

In addition to the primary ABA transporters described above, there is also a group of proteins known as secondary ABA transporters. One of them is a nitrate transporter (NRT1.2) belonging to the NRT1/PRT (nitrate transporter1/peptide transporter) family known also as AIT1 (ABA-IMPORTING TRANSPORTER) identified by Kanno et al. [49]. The *AIT1* promoter was active in vascular tissues of the inflorescence stems, leaves, and roots. Analysis of loss-of-function *ait1* mutants revealed that AIT1 is important for the regulation of stomatal closure in inflorescence stems by the engagement of AIT1 in ABA import. Recently, another ABA transporter was identified by Zhang et al. [147] during detailed studies concerning the action of DTX50 belonging to DTX/MATE (detoxification efflux carriers/multidrug and toxic compound extrusion) family. The highest activity of DTX50, as ABA exporter, was noticed when apoplastic pH was 7.0, which is typical for drought conditions (Fig. 5.1).

Apart from ABA transporters engaged directly in drought response, there are two more ABA transporters, ABCG30 and ABCG31, identified recently in Arabidopsis by Kang et al. [48]. Thus far it was shown that they perform the action during ABA-dependent regulation of seed germination.

5.2.3 The Core ABA Signaling that Triggers Stomatal Closure in Drought Response

During drought-induced stomatal closure, guard cells can adjust their volume up to 40 % in a few tens of minutes with accompanying reshuffling of their vacuolar apparatus [26]. This rapid response to changed environmental cues is mainly regulated by ABA. Thus, deep characterization and understanding of its ABA complex signaling network is crucial for further modulation of plant response to drought stress focused on stomata action.

5.2.3.1 The Most Significant Ion Channels, Transporters, and Pumps in Stomatal Movements

During the opening of the stomata, the most important is the H^+-ATPase pump mediating the efflux of H^+ from the guard cells and K^+ inward-rectifying channels. In the guard cells, the action of H^+-ATPase activity is positively regulated by blue light and auxins, whereas Ca^{2+} and ABA act as negative regulators. The efflux of H^+ hyperpolarizes the plasma membrane and leads to K^+ uptake via activation of inward K^+ rectifying channels, such as KAT1 (POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1), KAT2 (POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 2), and AKT1 (ARABIDOPSIS THALIANA K + TRANSPORTER 1; [98, 110, 118, 119]). K^+ uptake is balanced by counter-ions, mainly Cl^- obtained from the apoplast,

NO_3^-, or $malate^{2-}$ that is derived from starch breakdown. Ions supplied into the guard cells together with water transported via aquaporins generate the turgor necessary to keep stomata open.

When the conditions are not favorable, stomata closure is activated by a cascade of events leading to the efflux of solutes from the guard cell. First, the inhibition of H^+-ATPase and the activation of anion channels together result in membrane depolarization. Anion channels such as rapid channels (R-type) and slow channels (S-type) facilitate the efflux of $malate^{2-}$, Cl^-, and NO_3^- [101, 103]. The decreased level of $malate^{2-}$ in guard cells is also linked with the gluconeogenic conversion of $malate^{2-}$ into starch [140]. Membrane depolarization creates a driving force for the efflux of K^+ via K^+ outwardly rectifying channels such as GORK (GUARD CELL OUTWARDLY RECTIFYING K^+ CHANNEL) [43]. Another event that accompanies stomatal closure is an elevation of the cytoplasmic Ca^{2+} concentration as a result of Ca^{2+} release via channels situated in both the plasma membrane and in the tonoplast [76]. Ca^{2+} channels are encoded by genes from three gene-families: TPC1 (TWO-PORE CHANNEL 1; [97]), CNGC (CYCLIC NUCLEOTIDE GATED CHANNEL; [25]) and GLR (GLUTAMATE RECEPTOR; [59]). Taken together, the efflux of solutes from the guard cells leads to a reduced turgor and stomatal closure (reviewed in [20]).

5.2.3.2 The Core Signalosome of ABA–Receptor–PP2CA–SnRK Complex

A great advance in decoding elements of the ABA signaling network occurred when several groups using different approaches independently identified key ABA soluble receptors [75, 88, 96, 108]. Chemical genetics emerged as the solution of the problem of the identification of receptor. Pyrabactin (4-bromo-*N*-[pyridine-2-yl methyl] naphthalene-1-sulfonamide) is a synthetic compound that partially mimics the inhibitory effect of ABA during seed germination and seedling development. Using a series of pyrabactin-resistant mutants and the map-based cloning approach, several genes encoding ABA-binding proteins, including PYR1 (PYRABACTIN-RESISTANCE 1) have been identified [108]. PYR1 is one of the 14 homologues (PYL, PYRA BACTIN RESISTANCE LIKE) present in the Arabidopsis genome [75, 88, 96, 108]. The PYR/PYL/RCAR–ABA receptor complex binds ABA and forms ternary complexes inhibiting the ability of clade A of PP2Cs to dephosphorylate SnRK2. PP2Cs are the negative regulators of ABA signaling, among them are key players in "core ABA signaling" (Fig. 5.1) such as ABI1 (ABA INSENSITIVE 1), ABI2 (ABA INSENSITIVE 2), and HAB1 (HYPERSENSITIVE TO ABA1; reviewed in [21]). The ABA signal flows further to the downstream targets and activates the kinases (SnRK2), such as SnRK2.2/D, SnRK2.3/E, and SnRK2.6/OST1/E which are the positive regulators of ABA signaling [27, 29, 129, 135]. With the increase of drought-induced ABA accumulation, the PYR/PYL–PP2C–SnRK2 signalosome activates guard cell anion channels, leading to plasma membrane depolarization and stomata closure. This part of ABA signaling with the focus on the key regulator, OST1 (OPEN STOMATA 1) kinase is reviewed below. The constitutive overexpression of

ABA receptors improved drought tolerance in Arabidopsis, rice, and tomato [9, 31, 90, 151] but on the other hand, in nonstressed conditions it negatively influenced the yield [51]. These results suggest that precise regulation of activity of individual or multiple receptors, taking into account their high redundancy, will be required for field-level drought tolerance.

5.2.3.3 Early ABA Signaling Kinase SnRK2.6/OST1/E Acts as a Central Regulator of Stomata Action

The kinase SnRK2.6/OST1/E (hereafter referred to as OST1), displays dominant kinase activity during drought stress response when the ABA signal is relayed to the guard cells. Mutants in *OST1* showed a wilty phenotype under water-deficit conditions and were insensitive to ABA-induced stomatal closure [83]. The physical interaction between OST1 and main regulators of solute fluxes (inward K^+ channel, the anion channel SLAC1 (SLOW ANION CHANNEL ASSOCIATED 1), and the NADPH oxidase AtrbohF) leading to stomatal closure were identified [60, 64, 109, 116, 131]. Here we summarize the action of the selected regulators that are under control of OST1 kinase, identified as a key player in ABA-induced stomata closure (Fig. 5.2).

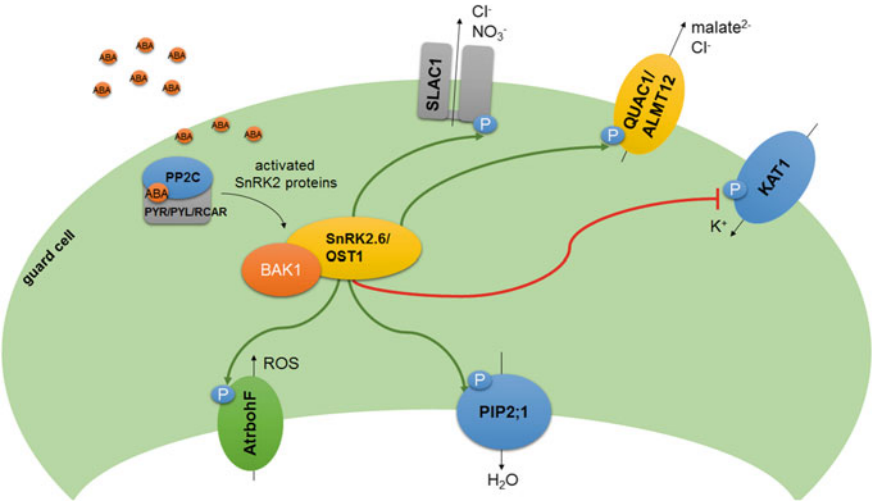

Fig. 5.2 The pivotal role of OST1 kinase in coordinating pumps, iosn channels and signaling events in order to close stomata under drought stress in ABA-dependent manner. *PP2C* PROTEIN PHOSPHATASE 2C; *PYR/PYL/RCAR* PYRABACTIN-RESISTANCE 1/PYRABACTIN RESISTANCE LIKE/REGULATORY COMPONENT OF ABA RECEPTOR; *SnRK2* sucrose nonfermenting related kinase 2; *OST1* Open Stomata 1; *BAK1* BRI1-associated receptor kinase 1; *SLAC1* SLOW ANION CHANNEL ASSOCIATED 1; *QUAC1/ALMT12* QUICK ANION CHANNEL 1/ALUMINUM-ACTIVATED ANION CHANNEL 12; *KAT1* POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1; PIP2;1; *AtrbohF* NADPH oxidases; *ROS* reactive oxygen species

ABA-induced inhibition of ABI1 protein phosphatase from clade A (PP2CA) prompt no longer inactive OST1 to play its role in ABA signaling. OST1 binds to and activates the S-type anion channel, SLAC1, by phosphorylating multiple amino acids on the N-terminus end of the SLAC1 protein [60, 64, 131], and the R-type anion channel QUAC/ALMT12 (QUICK ANION CHANNEL 1/ALUMINUM-ACTIVATED ANION CHANNEL 12; [39]) in the plasma membrane of guard cells. SLAC1 is a plasma membrane (PM) S-type (slow-activating sustained) anion channel with a preference for Cl^- and NO_3^- [30, 60, 64]. This protein is mainly stimulated by ABA, CO_2, Ca^{2+}, NO, and H_2O_2 [130]. Mutants in *SLAC1* show disruption in stomatal closure, and also are characterized by excessive accumulation of anions, such as Cl^-. Efflux of anions through the anion channel is essential for stomatal closure [93, 102]. In addition to the role of OST1 in regulation of slow anion currents, OST1 also influences the rapidly activating anion currents in guard cells through activation of QUAC1/ALMT12. It has been identified as a malate-sensitive R-type (rapid-transient) anion channel. Loss-of-function *quac1* mutant exhibited impairment of stimulus-induced stomatal closure [81].

In addition to anion channels, OST1 phosphorylates the Arabidopsis inward-rectifying K^+-channel KAT1 (POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1; [109]) and NADPH oxidases AtrbohD and AtrbohF involved in ROS (reactive oxygen species) production [116]. KAT1 is one of six K^+ inward channels identified in Arabidopsis and expressed in guard cells ([2, 35, 65, 92, 118, 119]). The inward-rectifying K^+ channel KAT1 is of a great importance inasmuch as it has a key role in stomatal opening through K^+ uptake, and the inhibition of KAT1 channel activity is one of the requirements to enable stomatal closure [35, 118, 119, 65]. Therefore, the negative regulation of KAT1 mediated by OST1 phosphorylation might be a key for drought stress response via stomatal closure resulting in action of limit transpiration [107].

Reactive oxygen species function as the secondary messengers in positive regulation of ABA signaling [19]. It was shown that in the Arabidopsis genome two of the 10 NADPH oxidases, AtrbohD and AtrbohF, are responsible for ABA-induced ROS production and subsequent events leading to stomatal closure [58]. The *ost1* mutant showed reduction of ABA-triggered ROS production in guard cells, suggesting that OST1 acts upstream of NADPH oxidases in the stress-signaling network [83]. The study performed by Sirichandra et al. [116] on AtrbohF, the major NADPH oxidase in ABA regulation of stomatal movements, showed that AtrbohF interacts with and is phosphorylated by OST1.

It can be concluded that OST1 is a very important regulator of stomatal movement because it links signals from ABA receptors and further downstream elements of the ABA response cascade. Another part of the OST1 regulatory pathway showed research performed by Shang et al. [115] using Arabidopsis mutant related to brassinosteroids signaling, *bak1* (BRI1, associated receptor kinase 1). *bak1* lost more water than wild-type plants and showed ABA insensitivity in stomatal closure. Moreover, ABA-induced OST1 expression and reactive oxygen species production were also impaired in *bak1*. Their study revealed that BAK1 forms a complex with OST1 near the plasma membrane. Moreover, this interaction is enhanced by ABA

and inhibited by ABI1. It clearly showed that BAK1 is a positive regulator of stomatal closure under drought conditions.

Surprisingly, in stomata action, which is triggered in response to environmental stimuli and controls the water status of plants, the membrane water transport mechanism is still unclear and highly hypothetical. This problem was partly resolved by Grondin et al. [34] who showed that ABA-triggered stomatal closure requires increased permeability to water and possibly to hydrogen peroxide, through OST1-dependent phosphorylation of PIP2;1 (plasma membrane intrinsic protein 2;1) in guard cells. PIP2;1 is the predominant PIP in Arabidopsis and the second highly expressed member of the PIP2 subclass in guard cells [65]. The involvement of PIP2 in water transport is significantly more robust than that performed by members of the PIP1 subfamily. The *pip2;1* mutant of Arabidopsis showed impairment in stomatal closure in the presence of ABA. Grondin et al. [34] proved that PIP2;1 is not engaged in guard cell water permeability in control conditions whereas in response to ABA its activity is significantly increased. Next, they showed that ROS accumulation in response to ABA in guard cells is dependent on PIP2;1 action. Therefore, *pip2;1* mutant is unable to accumulate enough ROS and it is not able to close its stomata properly in response to ABA. While deciphering the mechanism of regulation of PIP2;1 in accordance with ABA and stress response the authors identified a novel regulator of PIP2;1, the OST1 kinase.

5.2.4 *The Mysterious Part of Stress Signaling—ABA-Dependent Posttranscriptional and Posttranslational Regulations*

Both posttranscriptional and posttranslational modifications contribute significantly to the fine-tuning of the complex network of plant stress response. Posttranscriptional modifications such as alternative splicing and RNA-mediated silencing control the amount of specific transcripts. Posttranslational modifications change the activity, subcellular localization, and half-life of proteins. Here, we focus on this type of modification in the ABA-dependent stress response.

For decades processes of phosphorylation and dephosphorylation were recognized as the major mechanisms for protein modification. Phosphorylation and dephosphorylation are reversible biochemical processes mediated by protein kinases and phosphatases, respectively. Phosphorylation mostly happens at the hydroxyl group of serines (S), threonines (T), and, infrequently, at tyrosines (Y) of proteins. Protein phosphorylation and dephosphorylation also affect other types of protein modification, such as ubiquitination and sumoylation. Ubiquitination is the covalent modification of target proteins through the conjugation of monoubiquitin

or polyubiquitin chains of variable length and has a profound bearing on the fate and function of its substrates. Sumoylation of a substrate involves sequential catalytic reactions by a cascade of enzymes: SUMO E1, E2, and E3. Recently, *S*-nitrosylation [104] has emerged as a posttranslational modification significantly important in stress response. During the *S*-nitrosylation the covalent attachment of a nitrogen monoxide group to the thiol side chain of cysteine occurs. Interactions across these modification mechanisms ensure temporally and spatially appropriate patterns of downstream gene expression.

Proteins involved in ABA signaling under stress conditions which are under posttranslational modifications are summarized in Table 5.1.

The most attention in ABA-dependent response to stress (mainly during seed germination) in the light of posttranslational modifications is focused on *ABI5* (*ABA-insensitive 5*; Fig. 5.3).

Posttranslational regulation of ABI5 is currently the best-understood mechanism in plant signaling. *ABI5* encodes a member of bZIP transcription factors that is directly activated by kinases from the "core ABA signalosome". Seven members of the bZIP family have been functionally characterized, among them ABF1, ABF2, ABF3, and ABF4 have important roles in ABA-dependent stress signaling, either independently or via combined interactions [28, 41, 144]. Their engagement in ABA and stress signaling was first identified in Arabidopsis and then confirmed in crops such as rice [154] and barley [10, 11].

It was found that ABI5 protein is activated by phosphorylation performed by kinases SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1, and SRK2I/SnRK2.3. It was also reported that PKS5 (SOS2-like protein kinase 5/CIPK11) protein kinase can phosphorylate ABI5 in order to regulate ABA inhibition of seed germination in Arabidopsis [84, 137, 153]. In addition to the SnRKs and PKS5/CIPK11, glycogen synthase kinase 3-like kinase BRASSINOSTEROID INSENSITIVE 2 (BIN2) and calcineurin B-like interacting protein kinase 26 (CIPK26) were also reported to phosphorylate ABI5 [36, 74].

According to the aforementioned interactions activity of ABI5 is regulated by phosphorylation, which positively or negatively correlates with the total protein level of ABI5 under ABA treatment. The question then emerges of how the protein level of ABI5 is regulated. Most transcription factors in eukaryotes are degraded via the ubiquitin-26S proteasome pathway. An increased amount of ABI5 protein observed in seedlings upon treatment with 26S proteasome inhibitors reflected the fact that the degradation of ABI5 is dependent on the ubiquitin proteasome system (UPS). A really interesting new gene (RING)-type E3 ligase keep on going (KEG), identified as a negative regulator of ABA signaling, is required for maintaining low levels of ABI5 [117]. KEG directly interacts with and ubiquitinates ABI5 [69]. In the presence of ABA enhanced stability of ABI5 was observed due to ABA-induced KEG ubiquitination and proteasomal degradation [67]. The RING type E3 ligase KEG-mediated ABI5 degradation occurs in the cytoplasm, trans-Golgi network, and early endosome [69]. Taking into account that ABI5 is a

Table 5.1 Posttranslational modifications (ubiquitination) with known or predicted functions in ABA and drought stress signaling

Enzyme	Protein name	Species	Biological function	Target	References
E2	PUB9	At	ABA signaling	nd	[107]
	PUB22/23	At	Drought and salinity stress tolerance	RPN12a	[18]
	PUB19	At	Drought stress tolerance, ABA signaling	nd	[71]
E3	AIP2	At	ABA signaling	ABI3	[149]
	CUL4	At	ABA signaling and drought tolerance	PYL4, PYL8, PYL9	[40]
	AIRP1	At	ABA-dependent drought stress tolerance	nd	[105]
	KEG	At	ABA signaling	ABI5	[117]
	KEG	At	ABA signaling	CIPK26	[74]
	RHA2a	At	ABA signaling	nd	[8]
	Rma1	At	Drought stress tolerance	PIP2;1	[60, 64]
	SAP5	At	Drought and salinity tolerance	MBP-1	[47]
	SDIR1	At	Drought and salinity tolerance, ABA signaling	SDIRIP1	[145]
	XERICO	At	Drought stress tolerance, ABA biosynthesis	nd	[52]
	DIS1	Os	Drought stress tolerance	Nek6	[87]
	BIRF1	Os	Drought and oxidative stress tolerance	nd	[68]
	DSG1	Os	ABA signaling	ABI3	[94]
	RING-1	Os	Drought and heat tolerance	nd	[80]
	ZF1	Zm	Drought and salinity stress tolerance	nd	[38]
U-box	DWA1/2	At	ABA signaling	ABI5	[61, 62]
	DOR	At	Drought stress tolerance	nd	[150]

AIP2 ABI3-Interacting Protein 2; *CUL4* Cullin4; *AIRP1* Arabidopsis ABA-insensitive RING protein 1; *KEG* KEEP ON GOING; *RHA2a* RING finger E3 ligase; *Rma1* RING finger motif; *SAP5* STRESS ASSOCIATED PROTEIN 5; *SDIR1* salt and drought induced RING Finger 1; *DIS1* drought-induced SINA protein 1; *DSG1* dwarf and small grain1; *RING-1* RING finger 1; *ZF1* RING zinc finger 1; *DWA1/2* DWD hypersensitive to ABA 1; *DOR* drought tolerance repressor; *PUB9* U-box domain-containing protein 9; *PUB22/23* U-box domain-containing protein 22/23; *PUB19* U-box domain-containing protein 19; *At* Arabidopsis thaliana; *Os* Oryza sativa; *Zm* Zea mays; *nd* no data

transcription factor, the discovery of E3 ligase or ligase complex(es) in the nucleus seemed to be important to understand the ABI5 degradation mechanism. It was

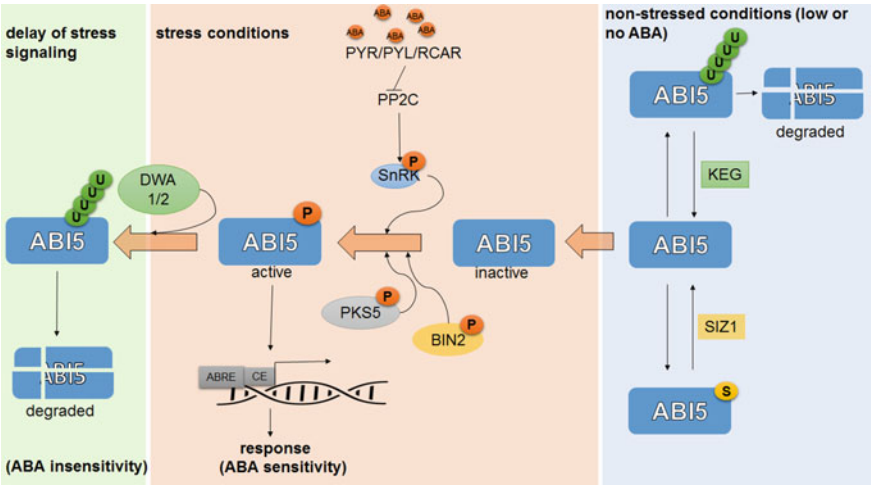

Fig. 5.3 Posttranslational modifications of ABI5 under stress. *ABI5* ABA-insensitive 5; *PYR/PYL/RCAR* PYRABACTIN-RESISTANCE 1/PYRABACTIN RESISTANCE LIKE/REGULATORY COMPONENT OF ABA RECEPTOR; *PP2C* PROTEIN PHOSPHATASE 2C; *SnRK* sucrose nonfermenting related kinase 2; *DWA1/2* DDB1-BINDING WD40 PROTEIN1/2; *PKS5* PROTEIN KINASE SOS2-LIKE 5; *BIN2* BRASSINOSTEROID INSENSITIVE 2; *ABRE* ABA responsive element; *CE* coupling element; *KEG* keep on going; *SIZ1* Small ubiquitin-related modifier 1/2 (AtSUMO1/2); *S* sumoylation; *U* ubiquitination; *P* phosphorylation

shown that the degradation of ABI5 in the nucleus occurred by DWA1/2-DDB1 CUL4 E3 ligase complex [61, 62].

Sumoylation was also found to regulate ABI5 activity competitively. Biochemical evidence demonstrated that ABI5 was sumoylated by SIZ1 (small ubiquitin-related modifier 1/2 (AtSUMO1/2)), and that SIZ1-mediated ABI5 sumoylation increased the stability of ABI5, implying that sumoylation of ABI5 by SUMO E3 ligase SIZ1 protects ABI5 from ubiquitin-mediated ABI5 degradation [73, 82].

It was shown that S-nitrosylation of ABI5 facilitates its degradation through CULLIN4-based and KEG E3 ligases, and promotes seed germination [3].

The fine-tuned regulation of the ABI5 protein could serve as a model to study the stability and activity of other key components affecting PTMs (posttranslational modifications). It should be stressed that most PTMs of ABI5 were described during seed germination under abiotic stress conditions. Nevertheless, taking into account ABI5 engagement in drought stress tolerance, similar modifications should be considered. Numerous important components in the stress signaling network undergo the PTMs that result in changed drought response suggesting their importance in modulating the response to stress (Table 5.1).

5.3 ABACUS, ABAleons—Chemical Miracles in Monitoring the Action and Distribution of ABA—When Biology Meets Top Chemical Research

It is clear that only precisely performed coordination of biosynthesis, de/conjugation, and catabolism together with transport and signal transduction of ABA is crucial for the proper plant response to unfavorable environmental conditions. The coordination is both spatially and temporally regulated. The question is how these patterns integrate into the proper ABA level within the particular cell. The knowledge of ABA levels and dynamics in cells over time is of the greatest importance. Recently, Jones et al. [44] and Waadt et al. [136] made use of FRET technology (Förster resonance energy transfer, FRET [91]) in the construction of fluorescent biosensors for ABA, respectively: ABAleons and ABACUS (abscisic acid concentration and uptake sensors). FRET relies on the distance-dependent transfer of energy from a donor molecule to an acceptor molecule. The donor molecule is the dye or chromophore that initially absorbs the energy and the acceptor is the chromophore to which the energy is subsequently transferred. The transfer of energy leads to a reduction in the donor's fluorescence intensity and excited state lifetime, and an increase in the acceptor's emission intensity. A pair of molecules that interact in such a manner is often referred to as a donor/acceptor pair.

Basically, these FRET sensors of ABA are fusion proteins of ABA receptor PYR/PYL/RCAR (more precisely the ABA sensory domain) linked to PP2C (ABI1) sandwiched by fluorescent proteins of the FRET pair. ABA induces an intramolecular interaction that alters the efficiency of energy transfer from FRET donor (CFP, cyan fluorescent protein variant) to FRET acceptor (YFP, yellow fluorescent protein variant). During the excitation of the donor, the ratio of acceptor emission over donor emission will vary according to ABA binding (Fig. 5.4). Therefore, ABA biosensors based on the FRET phenomenon can be used to report ABA concentrations in the solution, cell-type, or subcellular compartment containing the FRET biosensors [45, 136].

ABAleon biosensors have a high affinity for ABA that correspond well to the affinities of their intrinsic PYR1 domains in the presence of ABI1 [136] whereas ABACUS is a more engineered platform that allows for screening libraries of constructs after expression in bacteria or yeasts. Its affinity for ABA is medium; it corresponds to the intrinsic PYL1 domains in the absence of ABI1 [44]. The authors of these FRET biosensors of ABA underlined that this was the first generation of biosensors with a high potential but needed re-engineering. The main reason is the ABA-related phenotype resulting from ABAleon and ABACUS expression *in planta*. ABACUS-expressing plants exhibit ABA hypersensitivity which is consistent with PYL1 overexpression, but ABAleon-expressing plants showed ABA-hyposensitivity potentially related to overexpression of the intristic domain of ABI1 [136].

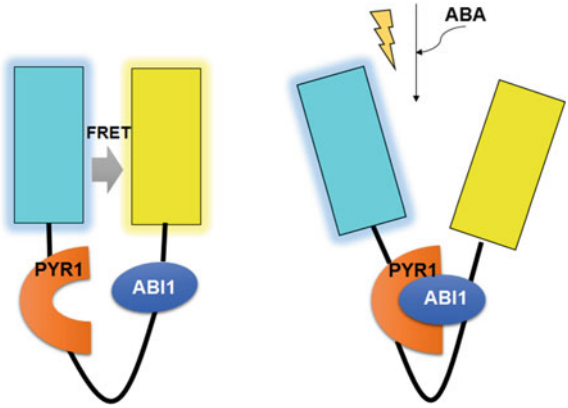

Fig. 5.4 The basics of FRET sensors of ABA (ABACUS, ABAleons). *PYR1* PYRABACTIN-RESISTANCE 1; *ABI1* ABA INSENSITIVE 1; *FRET* Förster resonance energy transfer

In the future, current and next-generation ABAleon and ABACUS systems for biosensing of ABA will allow for a higher spatiotemporal resolution of ABA accumulation and elimination rates that are extremely dynamic under drought stress conditions.

5.4 Drought-Resistant Crops—Taking Advantage from Basic Studies

Recent progress made in understanding the mechanism of ABA signal transduction, based on the identification of novel genetic components, resulted in a wide range of genetic targets for manipulation and genetic engineering to obtain drought-tolerant crops. Due to the evolutionary conservation of the core of ABA signaling in plants, the genes identified as major ABA signaling components in Arabidopsis can be used as targets for genetic manipulation of agronomical important crop species.

Successful applications of the core ABA signaling components, especially derived from *Hordeum vulgare, Oryza sativa*, *Zea mays*, *Triticum aestivum* along with characterization of drought stress responses in transgenic plants are summarized in Table 5.2.

It should be noted that better performance of most of these plants was shown under severe water-limited conditions in growth chambers and greenhouses, whereas in field experiments drought tolerance was exhibited together with lower yield. Although many of the manipulated stress tolerance factors also showed promise in the field, none of the examples summarized in Table 5.2 has been shown to provide durable drought tolerance that translates to the agriculture production process.

One of the genes that has been successfully introduced into a crop plant and that gave improved drought tolerance in field trials together with not decreased yield was

Table 5.2 Transgenic drought-tolerant crops mainly achieved by modulation of the core ABA signalosome

Species	Gene	Mutant OE/loss-of-function/T-DNA/VIGS	Drought tolerance-related plant phenotypes	Reference
Barley (*Hordeum vulgare*)	*HvSNAC1*	OE	Enhanced drought responses	[1]
Maize (*Zea mays*)	*ZmCPK11*	OE	ABA-induced ROS production	[22]
	ZmPP2C	OE	Drought sensitive responses	[70]
	ZmOST1	Loss-of-function	Stomatal phenotypes	[134]
	ZmbZIP72	OE	Enhanced drought responses	[143]
Rice (*Oryza sativa*)	*OsPYL11/RCAR5*	OE	Enhanced drought responses	[50]
	OsPYL/RCAR5	OE	Enhanced drought responses	[51]
	OsLEA4	OE	Enhanced drought responses	[37]
	OsRK1/SAPK6	OE	Reduced ABA response	[14]
	OsDRO1	Loss-of-function	Enhanced drought tolerance	[126, 127, 128]
	OsSLAC1	Loss-of-function	Increased stomatal conductance	[57]
	OsbZIP16	OE	Enhanced drought responses	[15]
	OsbZIP23	OE	Enhanced drought responses	[141]
	OsbZIP46CAI	OE	Enhanced drought responses	[123, 124]
	OsbZIP71	OE	Enhanced drought responses	[66]
	OsbZIP72	OE	Enhanced drought responses	[72]
	OsABF1	T-DNA	Drought-sensitive responses	[4]
	OsNAC5	OE	Enhanced drought responses	[120]
	OsNAC6	OE	Enhanced drought responses	[85]
	OsWRKY45-1	OE	Drought-sensitive responses	[125]
	OsWRKY45-2	OE	Enhanced ABA responses	[125]

(continued)

Table 5.2 (continued)

Species	Gene	Mutant OE/loss-of-function/T-DNA/VIGS	Drought tolerance-related plant phenotypes	Reference
Wheat (*Triticum aestivum*)	*TaSnRK2.5*	OE	Enhanced drought responses	[78]
	TaSnRK2.8	OE	Enhanced drought responses	[146]
	TaERA1	VIGS	Drought tolerant responses	[77]
	TaWRKY2	OE	Enhanced drought responses	[89]
	TaWRKY19	OE	Enhanced drought responses	[89]
	TaNAC2a	OE	Enhanced drought responses	[123, 124]

HvSNAC1 STRESS-RESPONSIVE NAC 1; *ZmCPK11* calcium-dependent protein kinase 11; *ZmPP2C* protein phosphatase 2C; *ZmOST1* Open Stomata 1; ZmbZIP72; *OsPYL11/RCAR5* PYRABACTIN RESISTANCE LIKE 11/REGULATORY COMPONENT OF ABA RECEPTOR 5; *OsPYL/RCAR5* PYRABACTIN RESISTANCE LIKE/REGULATORY COMPONENT OF ABA RECEPTOR 5; *OsLEA4* late emrbryogenesis protein; *OsRK1/SAPK6* STRESS ASSOCIATED PROTEIN 5; *OsDRO1* DEEPER ROOTING 1; *OsSLAC1* slow anion channel associated 1; *OsABF1* ABI like factor; *TaSnRK2.5* sucrose nonfermenting kinase 2.5; *TaSnRK2.8* sucrose nonfermenting kinase 2.8; *TaERA1* enhanced response to ABA1; *OE* overexpression; *VIGS* virus induces gene silencing; *ROS* reactive oxygen species

the gene encoding cold shock protein B (CspB) RNA chaperone from *Bacillus subtilis*. The CspB gene is important in the ability of bacteria to adapt to cold, and its overexpression in plants was shown to provide drought tolerance in Arabidopsis, rice, and maize [12]. Results from field experiments showed that a maize line overexpressing the CspB gene had a higher yield under water-deficit conditions than nontransgenic plants and expressed a yield equivalent to the nontransgenic plants under nonstressed conditions. To date, the first and only commercial available drought-tolerant transgenic crop is maize overexpressing CspB named DroughtGard™ Hybrids (http://www.monsanto.com/products/pages/droughtgardhybrids.aspx?WT.mc_id=1_droughtgard ; http://www.genuity.com/corn/Pages/Genuity-DroughtGard-Hybrids.aspx; [12]).

5.5 Conclusions and Outlook

Here, the outline of several aspects of plant drought response controlled by ABA is presented to highlight processes in which ABA act as a main regulator such as stomatal closure in response to drought stress.

It can be concluded that recent findings with regard to ABA's biosynthesis, catabolism, transport, and control of gene expression further complete knowledge about the mechanism of ABA action and simultaneously enable advanced and more applied studies. Only very recently have we begun to understand the mechanisms of ABA perception by the plant cell, thus further research is needed in order to achieve a full understanding of drought-response mechanisms in plants and to implement them for production of crops with improved drought tolerance.

There is a need to combine the data derived from different basic studies. Detailed analyses of the networks of protein interactions, the coexpression of genes, metabolic factors, and the like should provide insights into the key regulators of drought response. Furthermore, genetic tools, such as advanced pipelines for genome studies, mutation detection, gene discovery and expression, and the complex "omics" databases updated frequently are needed. The targeted editing of genomes with TALEN (TRANSCRIPTION ACTIVATOR-LIKE EFFECTOR NUCLEASE) and CRISPR-Cas9 technologies is also likely to enable a precisely designing of alleles that aid stress tolerance which seems to accelerate further adopting it in breeding programs. It should be underlined that the essential step in development of new drought-tolerant plants/varieties is a proof-of-concept experiment: field assessment of yield.

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Chapter 6
Drought Stress Tolerance in Plants: Insights from Transcriptomic Studies

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6.1 Introduction

Cellular organization and the need for liquid water are characteristics that define all living organisms [143]. However, water resources are limited and variable throughout the regions of the world. Although the demographic growth rate has been trending down, the world population for 2050 is estimated to exceed 9 billion people [131]. Population growth increases demand for food production, which is reliant on water resources. The situation does not get better if we consider climate changes that have been progressing nowadays. Therefore, efficient handling of these reserves has become crucial.

The future of agriculture implies the need to be more productive and sustainable at the same time. Plant breeding programs around the world seek the development of less demanding materials for cultivation traits, energy, and water, and that can favorably respond to biotic and abiotic stresses. Traditional breeding has been responsible for releasing improved cultivars and varieties during the last four decades. Many methods, such as introgression breeding, somatic hybridization, and mutagenesis, were developed and some of them incorporated by breeding programs in an attempt to improve crops. Also phenotypic characterization of segregating progenies has been used to improve production and resistance/tolerance to stresses [86, 106].

Throughout evolution, plants have developed molecular mechanisms to adjust physiologically to abiotic stress conditions, of which drought is the most damaging, causing significant yield losses. Thus, plants have a cellular machinery acting at different levels, from the perception of stress, transduction of signals, and activation of responsive genes to the initial stimulus [13]. To unravel this genetic network of

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molecular interactions is a challenge for researchers, but this knowledge has the potential to generate applications in biotechnology and plant breeding with socioeconomic consequences.

With the development of molecular biology, DNA began to be sequenced applying the Sanger method, but this technique was very laborious, with low yield and high cost. With the advent of the EST (Expressed Sequence Tag, [1]) technique, transcriptomics had its first boost, allowing the grant of knowledge about the genes through their transcripts. The NGS (Next Generation Sequence; [90]) technologies, in which high-performance platforms sequenced large amounts (typically millions) of short DNA sequences (25–400 pb) substantially reducing costs, multiplied this boost. Thus, a multiplicity of biological processes may be addressed by transcriptomics showing how genetic information is reorchestrated in an organism after an initial stimulus. In this way, transcriptomics allows the global understanding of relevant processes leading to the identification of molecular candidates with potential for use in breeding programs. This chapter provides diverse content covering transcriptomics studies on plants under drought stress, also addressing the current outlook of the methodologies applied and the identified genes responding to the stress, going through an overview of some trangenic plants and patents.

6.2 Plant Strategies Facing Drought Stress

In nature, plants are subject to the simultaneous occurrence of different stresses (abiotic and biotic), resulting in a high degree of complexity of adaptive responses to the environment [6, 122]. Drought and salinity are among the major environmental factors that adversely affect plant growth and development, resulting in a negative impact on agricultural productivity and yields [95, 133]. Concerning drought stress, plants can suffer extended periods of gradual water scarcity (days, weeks, or months, for example) or may undergo short periods of significant dehydration (hours or days). Consequently, the response of the plant as a whole to drought stress involves the development of a series of changes at different levels, including molecular, physiological, and morphological modifications, allowing plants to adapt to environmental stresses [6, 11, 24].

In general, drought adaptation is conferred in higher plants through a combination of different strategies:

(a) *Drought escape*: The plant's ability to complete its life cycle or adjust its phenological development during periods of sufficient water supply before water deficit becomes severe enough to cause damage [24, 36]. Some plants are likely to have metabolic regulation adjusted for rapid growth [136].
(b) *Drought avoidance*: The ability of some plants to minimize water loss, therefore preventing or retarding the negative impact of the drought stress [62] by maximizing water absorption from their roots or reducing evapotranspiration from their aerial parts. Systems associated with drought avoidance

include the improvement of the water uptake mechanisms, storage in plant cells, and stomatal closure [6, 24, 36] among others.

(c) *Drought tolerance*: The ability of the plants to maintain a certain level of physiological activity even under severe drought stress conditions, being able to achieve acceptable yield [124].

The drought tolerance mechanism involves a rapid regulation of a variety of genes involved in several metabolic pathways associated with repair or reduction of damage due to stress [98]. Such genes aim not only to protect the plant from the stress by the production of metabolic proteins, such as compatible solutes, water channel proteins, and membrane transporters, but also to participate in signal transduction regulation [36, 111].

Because of the intricate nature of drought tolerance and also the fact that the expression of this characteristic is dependent on the interactions with the environment and other genes/characters [62], traditional breeding presents less success than expected [82]. An additional strategy is selecting for secondary traits (e.g., grain yield) related to the applied stress. Also, the development of high-density linkage maps may provide some tools for dissecting the genetic basis underlying this complex trait, into individual components [148].

The new genomic platforms for DNA sequencing in association with bioinformatics analysis have added new insights into the genetic basis of drought tolerance [129]. Some genomic regions associated with drought tolerance or responsiveness has been identified by genome-wide analysis [66]. Thus, many plant molecular mechanisms for drought tolerance involve changes in the Omics profiles, including transcriptomics, proteomics, and metabolomics levels [158]. The goal is to find out how to combine these approaches to explain the drought-tolerant physiological response. The exploration of these concepts will lead to our better understanding of how plants can adequately tolerate drought [11]. Transcriptomics is the first step, in which the virtual transcripts' abundance of a given gene that is probably responding to the stimulus supports the results.

6.3 Generating Transcriptomics Data

Advances in the knowledge of drought response molecular mechanisms are of great importance to understand how such mechanisms affect both plant growth and development [136]. As mentioned by Mir et al. [86] plant physiology studies need to be developed for precise phenotyping of drought response before implementing molecular strategies, to unravel the drought-tolerance complex mechanism and further exploration through breeding approaches. Thus, different methods attempting to simulate drought stress in plants have been developed. Below there is a brief description of some of them, but how to simulate the real drought in a controlled experiment is still a reasonable question. Frequently, stress is estimated in crops by comparing an arbitrary stress treatment with a negative control,

although plants in nature undergo a wide variety and level of stresses at the same time, which requires a range of different responses.

6.3.1 Simulating Drought Stresses in Plants

Although currently there is still a limited understanding of the molecular networks underlying plant responses to stress, some parameters such as stress severity, organ/cell identity, and developmental stage should be considered in a drought-stress experiment [26]. However, much of our knowledge of the effects of water deficit comes from studies exposing plants to severe dehydration or by withholding water from plants for a period until they show severe wilting, many times addressing only the wild-type or the stress-sensitive type, without the evaluation of some tolerant crop. Another common practice is the addition of mannitol, sorbitol, or polyethylene glycol (PEG) inducing osmotic shock in plants and thereby reducing the water potential of the tissue, and so promoting drought-stress simulation [26]. The effects of these strategies will probably not be the same considering the plant transcriptomes to be generated.

According to Munns et al. [94], over the timescale of days to months, water loss is determined by leaf area and stomatal conductance. Over the timescale of minutes to hours, it is regulated by stomatal behavior. Stomatal conductance responds rapidly to changes in soil water potential and provides the main limitation for photosynthesis and growth, two characteristics well documented in such experiments [63, 140]. Thus, as the applied stress needs to be defined, setting the exposure time is also crucial.

Responses to drought simulated in different ways have been used for different purposes in transcriptomic studies. They include the identification of genes differentially expressed in *Populus* [22]; in the molecular mechanism of water regulation in cotton [67], drought-induced microRNAs in rice [152], and drought-responsive cDNA libraries of chickpea [133], among others.

6.3.1.1 Root Dehydration Stress

Root dehydration stress has been carried out by withholding watering or by the removal of hydroponic solution (or substrate) and subjecting it to dehydration for different periods of time (minutes, hour) at room temperature. In general, plants are grown in a greenhouse under controlled conditions of temperature, humidity, and photoperiod, in the hydroponic system or using vermiculite with normal watering. After a period of plant acclimatization, the stress treatment is applied. In the case of hydroponics, plants are removed from the hydroponic solution and left exposed to air during the defined period of time until sampling. Ferreira Neto et al. [37] applied this methodology with two contrasting drought-responsive soybean cultivars. In the case of plants growing on a solid substrate, plants need to be carefully removed,

gently washed, and allowed to dry on filter paper during the time of exposure to the stress. The greenhouse conditions (humidity, temperature, and light intensity) need to be recorded and the stress severity can be measured by estimating the relative water content (RWC) of some leaves. After the time period of stress treatment, roots are collected from dried plants. This methodology was applied by Ha et al. [43] also comparing two contrasting drought-responsive soybean cultivars. Other examples of crops used in such experiments are: *Phaseolus acutifolius* and *P. vulgaris* [84], chickpea [88], barley [42], and *Brassica napus* [72].

6.3.1.2 Progressive Drought Stress

Gradual and progressive soil-water deficit has been a particular strategy commonly used when the loss of water (through transpiration) is calculated as the difference between the pot weight of the current day and that of the previous day until the stage defined as the endpoint for the water-deficit treatment. For example, transpiration of drought stressed plants reaching <1 % of the control plants, or when the RWC has reached about 50–60 %. When the drought-stressed plant hits this stage, the plant tissues are harvested. Kawaguchi et al. [56] applied this method to an *Arabidopsis* microarray study where plants were subjected to progressive soil-water deficit with RWC measured at intervals and leaves collected after 8 days when the RWC reached 65 %. Another comparative analysis of expressed sequence tags (ESTs) from chickpea genotypes was conducted in the same way (Deokar et al. [30]).

6.3.1.3 Withholding Watering/Rewatering

Drought can vary in severity, timing, and duration, and from year to year due to changes in rainfall patterns. Consequently, many plant aspects (from the molecular/genetic level, biochemical and physiological processes) are altered to survive or adapt to periodic drought. This way, exposing plants to moderate drought and, then, rewatering before moving forward is a strategy used to simulate specific natural field conditions.

In this strategy, plants are, in general, submitted first to slow and moderate drought stress (approximately days to a few weeks after withholding watering) and after that, plants are rewatered and maintained for more days for root response to rewatering. However, extent and magnitude of the rewatering stimulation may depend on predrought (intensity and duration) and species [145].

Progressive drought and rewatering treatments have been reported in *Medicago* [151], which is a model legume, as well as in many important crops, such as wheat [118], maize [153], alfalfa [55], and common bean [149], among others.

6.3.1.4 PEG

Plant water-deficit stress is induced using a solution containing polyethylene glycol. In general PEG is applied to the watering solution, and the drought stress effect is measured on a timescale of hours or days of PEG addition. The PEG concentration varies and many times it is necessary to test a range of concentrations.

Responses to drought induced by PEG have been measured in *Arabidopsis* [61], rice [152], *Populus* [22], cotton [67], chickpea [133], and wheat [156], among others.

6.3.2 Applying Current Molecular Methods

Over the past years, advances have been made regarding elucidation of the molecular mechanisms associated with drought tolerance. Transcriptomics or mRNA expression profiling is one of the most used approaches to the identification and quantification of spatial and temporal gene expression under specific conditions [127]. Consequently, several technologies have been developed to evaluate transcriptome profiling, including hybridization or sequence-based approaches. Currently, there are around three dozen different methods described for gene expression analysis. These methods have been extensively reviewed (for details see: [54, 112]) and are not discussed here. Mainly, they can be classified into two broad categories: (a) hybridization of complementary nucleotide strands to immobilized sequence targets such as cDNAs, amplicons, or oligonucleotides, with the microarray technology as its principal representative; and (b) sequencing and transcript counting, such as tag-based approaches and RNA-Seq.

A simple search was done to give an idea of the techniques applied, in transcriptomic studies published in 2015 (January through August), covering plants under drought stress, using the keywords *transcriptomics* AND *plants* AND *drought*, in the PubMed database (NCBI). The results showed 78 scientific articles, but only 48 addressed plant transcriptomes under drought stress. Table 6.1 shows an extract of this search, indicating the method of choice for obtaining the data, the high-throughput sequencing method, the species, and the sampled tissue. Other aspects also included (number of biological replicates, the technique used to validate the data, the number of replicates used in validation) are described elsewhere.

6.3.2.1 Microarray Technology

This technology was developed in the mid-1990s [110] and has contributed enormously to transcriptome analysis. The method is based on a DNA "chip", which analyzes the hybridization of targets (marked by fluorescence) to groups of specific cDNA/gene probes, which are immobilized on a support (spot). An information system, comprising a reader (scanner with a set of lasers), a special microscope, and

Table 6.1 Some scientific articles (published from January to August 2015[a]) covering transcriptomics of plants subjected to drought stress. Data provided: high-throughput method and number of biological replicates[b], plant species, tissues analyzed, data validation method and biological replicates[c], and reference

High-throughput method	Biological replicates[b]	Plant species	Tissue	Validation method	Replicates[c]	RG	Reference
Microarray	n.i.	*Arabidopsis thaliana*	Root	RT-qPCR	3^B, 9^T	1	[5]
Microarray	3	*Arabidopsis thaliana*	Seeds	n.i.	n.i.	n.i.	[27]
RNA-Seq	n.i.	*Miscanthus lutarioriparius*	Leaves	RT-qPCR	3^T	1	[35]
RNA-Seq	n.i.	*Solanum tuberosum*	Stolon	RT-qPCR	n.i.	1	[39]
Microarray	3	*Oryza sativa*	Leaves	RT-qPCR	n.i.	1	[69]
RNA-Seq	2	*Triticum aestivum*	Leaves	RT-qPCR	3^B	1	[72]
RNA-Seq	3	*Oryzasativa*	Leaves	RT-qPCR	n.i.	1	[123]
RNA-Seq	2	*Brassica napus*	Leaves/seedling	RT-qPCR	2^B,3^T	3	[135]
RNA-Seq	n.i.	*Alhagi sparsifolia*	Root	RT-qPCR	3^T	1	[144]

[a]Based on search in the PubMed database with the keywords *Plant* AND *Transcriptomic* AND *Drought*
[b]Relative to the treatments in the experiment
[c]Number of replicates in RT-qPCR ([B]biological; [T]technical); *n.i.* No information available; *RG* Number of reference genes

Table 6.2 Some scientific articles (published from January to August 2015[a]) applying Microarray technology to plants under drought stress. Data provided: plant species, tissues analyzed, transcriptomic analysis, drought stress simulation, and references

Plant species	Tissue analyzed	Transcriptomic analysis	Drought stress simulation	Reference
Triticum aestivum	Root and leaves	microRNAs	PEG 6000 (20 % w/v)	[2]
Lotus japonicus	Leaves	Whole transcriptome	Progressive drought[c]	[38]
Glycine max	Roots	Whole transcriptome	Plants drying on filter paper[c]	[43]
Solanum lycopersicum	Roots	Whole transcriptome	Removing water from the container	[46]
Brassica rapa	Leaves	MADS-box TFs	Plants drying on filter paper	[109]
Zea mays	Leaves	TIFY transcription factors	Progressive drought[b]	[12]
Oryza sativa	–	NAC transcription factors	Plants drying on paper towels	[120]
Catharanthus roseus	Roots	Kinase family	Removing water from the container	[96]

[a]Based on search in PubMed database using the keywords *Microarrays* AND *Drought* AND *Stress*
[b]Based on soil water content measurements
[c]Monitoring the relative water contents; *PEG* Polyethylene Glycol. *TFs* Transcription factors

a camera, analyzes the probe–target hybridizations. The technique is specially applied in model species and economically important crops, for which there are available chips or financial support for their manufacture, such as *Arabidopsis thaliana*, *Oryza sativa*, *Lotus japonicus*, *Glycine max*, and *Triticum aestivum*, among others (Table 6.2).

However, although considered to be a gold standard technique for gene expression analysis, it presents recognized limitations, such as nonspecific hybridizations, insufficient sensitivity to quantify rare transcripts, and analysis restricted to those transcripts immobilized on the support [9]. Deyholos [31] presents a critical review covering the use and limitations of microarrays. Methods applying NGS technology overcome most of these constraints.

In addition to the methodological limitations of microarrays described above, this technique is still expressively used in transcriptomic studies with plants under drought stress. The performed search showed 14 scientific articles (from 48) using microarrays (almost 30 %; data not shown); Table 6.2 presents some of them.

6.3.2.2 Tag-Based and RNA-Seq Approaches

Historically, the principal methods of this group comprise EST (expressed sequence tags, [1]), SAGE (serial analysis of gene expression, [134]) and its derivates, MPSS (massively parallel signature sequencing, [17] and RNA-Seq [89]).

The EST was a standard method to determine gene expression profiles at the beginning of automated DNA sequencing, in the mid-1990s. Currently, its primary use is to generate gene sequences [83]. The method is based on the sequencing of thousands of cDNAs that have been cloned into bacterial plasmids (insert with 400–600 bp). Transcripts of many genes were identified by bioinformatics tools and analyzed to achieve a global expression profile of the sample [1]. However, some limitations became evident. As a result of the methodology, the quality of the sequences is not always that desirable, which restricts the informative ESTs. Also, depending on the library, the redundancy of the transcripts relating to the same information can be very meaningful, which combined with a low coverage of the transcriptome, in general, minimizes the chances of finding rare transcripts [73]. Finally, a single EST represents only one transcript. Thus, the technique is considered low yielding [125].

The SAGE technique overcame some of these limitations. This method extracts, from each cDNA in a library, one tag with 11–14 bp length. Then two tags are attached (ditags), and several ditags are concatenated and cloned into a plasmid vector, which is sequenced. Subsequently, the tags are extracted, their frequencies counted/normalized in libraries, and finally annotated by BLAST alignments [4]. The statistical variations in the frequencies of tags in different libraries allow the generation of gene expression profiles of the samples under study [134]. Thus, the SAGE technique provides higher yield when compared to the EST method, because for each insert there is information related to dozens of transcripts [125]. However, this technique also presents limitations. The size of the tag is small for an unambiguous identification of the gene, which is even more critical if the studied organism/species does not have a representative EST database.

The tag length was sometimes improved [LongSAGE (21 bp; [108]) and SuperSAGE (tag 26 bp; [78])], enabling a more appropriate annotation. With SuperSAGE tags it is possible to study two eukaryotes present in the same sample, as in a pathogen–host relationship [79]. With the development of NGS technologies, there was no need for concatamers and cloning in plasmids, so the SuperSAGE method was simplified, which increased the level of system information.

Furthermore, some features of the SAGE and derived methods overlap those observed for microarrays. Tag-based data are digital and simple to use, making it easier to compare the generated gene expression profiles [60, 125]. Also, they are considered to be of “open architecture”, not requiring prior knowledge of the sequences evaluated, favoring the discovery of novel transcripts, in opposition to the “closed architecture” of the microarray technique [79, 125].

In the search performed through the PubMed database (restricted for January to August 2015), only two scientific articles were observed presenting tag-based

methods applied in plants but they addressed cold and freeze stresses (data not shown).

The MPSS method was developed based on the individual cloning of cDNAs in microbeads with the subsequent parallel sequencing of tags in substantial numbers. Tags (16–20 nt long) are obtained from the *Sau*3A restriction site (or *Dpn*II) situated closest to the 3'-end of the transcript. This technique presents two advantages over the original SAGE: the length of the tag, which diminishes the ambiguity in the tag-gene annotation, and the automated process that produces millions of tags, allowing better sampling and possibilities to identify rare transcripts of biologically significant genes [103]. However, unlike SAGE and its derivatives, the methodology is more complicated and challenging to perform [83]. Other limitations concern the lack of *Sau*3A recognition sites on the cDNA and some ambiguity in tag-gene annotation.

Nowadays, the RNA-Seq method has been highlighted and is the method of choice for transcriptomic studies [92]. The method uses the fragmentation of RNAs and deep-sequencing technology in a very high-throughput manner [138], allowing a broad coverage of the transcriptome. Depending on this coverage, the discovery of rare transcripts is possible, which may go unnoticed with other methodologies. Because the method is quantitative, there are no limits for gene expression detection, and a range of expression levels can be revealed, providing a digital gene expression in genomic scale.

The RNA-Seq method has provided a complete characterization of RNA transcripts of a particular tissue [140], especially in nonmodel plants [33] inasmuch as RNA-Seq does not require a reference genome to obtain some useful transcriptomic information [119]. However, each sequence can be unambiguously mapped onto a reference genome if available. Another advantage is the possibility of a reading informing how two exons connect to each other or even the relationship between multiple exons.

The search in the PubMed database (restricted from January to August 2015), with the keywords *transcriptomics* AND *plants* AND *drought*, showed that the method was applied in 32 of 48 scientific articles retrieved (almost 70 %; data not shown). Some of these articles are shown in Table 6.1.

Comparing transcriptional patterns generated from RNA-Seq and HT-SuperSAGE libraries (High-Throughput SuperSAGE or deepSuperSAGE), both created from samples of the same experiment, [116] observed that both techniques showed similar levels of sensitivity in a study of appressorium development by the blast fungus *Magnaporthe oryzae* on rice. This result was in agreement with [10], which estimated 90 % coverage of the human transcriptome, the need for about 40 million RNA-Seq reads, and around 5 million of 3'-tag digital gene expression (DGE), using the Illumina Genome Analyzer. DGE is similar to HT-SuperSAGE, but generating smaller tags (20–21 bp), because the tagging enzyme is the same used in LongSAGE (*MmeI*).

Despite all these technologies, and improvements including sequencing chemistry, hardware, software, and methods of analysis, the expectations for transcriptomics studies will continue to increase [139].

6.4 Plant Transcriptomic Studies Covering Drought Response

6.4.1 Transcriptomic Studies in Crops Under Drought Stress

Transcriptome studies covering plant drought responses have used model and nonmodel plant species, including economically important crops. Considering model plants, *Arabidopsis* is certainly the most studied due to their numerous advantages: short generation time, small genome fully sequenced, and many mutants identified, among others. These characteristics granted several programs involving gene expression studies and mutant generation for an understanding of the processes involving their estimated 25,498 genes [126]. Concerning the search through the PubMed database (January to August 2015), from the total of 48 articles (29 species represented), at least ten scientific articles addressed drought stress in *A. thaliana*, of which eight used microarrays and two applied the RNA-Seq method (data not shown). Logically these studies provide a lot of valuable information. However, the transcriptome analysis of wild-type model plants not tolerant to drought stress can hinder the understanding of useful biological processes because they may involve senescence or even death [31]. The second species most represented was soybean with seven articles, most of them applying the RNA-Seq method. Briefs of transcriptomic studies, addressing drought in some crops, as examples, are given below.

6.4.1.1 Soybean [*Glycine max* (L.) Merr.]

In a microarray analysis of soybean leaf tissues, under drought stress during the vegetative (V6) and reproductive (R2) stages, [63] observed that a significant number of genes respond to drought stress in a stage-specific manner, suggesting that both conserved and unconserved pathways might take part in the regulation of drought response during different stages of plant development. In soybean plants under drought stress, the downregulation of many photosynthesis-related genes (which contribute to growth retardation under drought stress) is an example of an adaptive mechanism for plant survival [63]. Wang et al. [140] also verified that water deficit in rice plants significantly inhibits photosynthesis.

The analysis of HT-SuperSAGE libraries, from drought-tolerant and sensitive soybean accessions (root dehydrated by air), identified 36 differentially expressed genes associated with the biosynthesis of osmoprotectants and in silico mapping them (25 loci) in the soybean genome [59]. Using the same libraries, Ferreira Neto et al. [37] tried to characterize the early transcriptional responses (25–150 min after stress) of two contrasting accessions cultivated in hydroponic solution. The authors identified candidate genes related to gene ontology (GO) categories “hormone response”, “water response”, “salt stress response” and “oxidative stress response”, figuring them among the most promising genes for future studies. In addition,

validated data by RT-qPCR showed that after 25 min transcriptional reorganization started and the transcriptomes responding to this stress pointed out some differences between accessions.

The same two accessions using the designed experiment also generated subtractive libraries and RNA-Seq data [76]. Based on that, the authors demonstrated that drought stress in soybean affects diurnal oscillation of both drought-responsive and circadian clock genes. Furthermore, Rodrigues et al. [105] showed that time of day, as well as light and temperature oscillations considerably affect the regulation of water-deficit stress response in these soybean plants, demonstrating how it is important to analyze different time intervals to characterize plants responding to drought stress.

6.4.1.2 Chickpea (*Cicer arietinum* L.)

ESTs were generated by suppression subtraction hybridization (SSH) to identify differentially expressed genes in drought-tolerant and susceptible accessions in chickpea [30]. More than 50 % of the genes identified were associated with drought stress in chickpea for the first time. Also, it provided a comparative overview of genotype-specific expression patterns of more than 830 unigenes in root tissues of chickpea responding to drought.

Molina et al. [88], applying SuperSAGE to the analysis of gene expression in chickpea roots responding to drought, observed a strong transcriptional remodeling six hours after applying the drought stress. Among the differentially expressed genes, downregulation of genes involved in photosynthesis, energy metabolism, and many other stress-responsive genes was verified. Among the upregulated genes were those involved in early responses to biotic and abiotic stresses. Also, regulatory genes encoding transcription factors/signal transduction proteins showed both up- and downregulation. Drought-responsive genes were also identified from drought-challenged root tissues of two contrasting chickpea accessions [48]. Mapping of the drought-responsive genes onto various pathways allowed exploring gene categories activated during drought response, with the prominence of those related to energy metabolism, secondary metabolism, transcription regulators, and stress responses, which are well documented as responsive to a wide array of stresses.

6.4.1.3 Rice (*Oryza sativa* L.)

In rice, recent studies have revealed dozens of genes identified as drought responsive, including several transcription factor and protein kinase-encoding genes, suggesting that phytohormones may mediate a crosstalk among regulatory pathways under drought, salt, and cold stresses [44]. In a whole genome microarray analysis, Zhou et al. [154] identified drought-responsive genes of rice in the shoot, flag leaf, and panicle, under drought and high salinity stress. The expression profiles found in many different organs revealed a broad variety of organ-specific patterns of

regulation. Furthermore, either stress seems to influence the expression patterns of a significant number of genes involved in transcription and cell signaling processes, in an organ-specific manner. In turn, Wang et al. [140] carried out a genome-wide profiling (microarray) in three developmental stages of three rice tissues (leaf, root, and young panicle) under drought stress and control conditions. Their work revealed that most of the drought differentially expressed genes were under temporal and spatial regulation, suggesting crosstalk between various development cues and environmental stimuli. Using the Affymetrix Rice Genome Array, Lenka et al. [64] observed that the drought-tolerant rice (Nagina 22) and drought-sensible rice IR64 exhibited a diverse transcriptional response under both control and drought stress conditions. Drought tolerance was found to be associated with enhanced enzymatic activity, whereas susceptibility was governed by significant downregulation of transcriptional regulatory protein-encoding genes; some of them have already been shown to confer drought tolerance in transgenic plants.

6.4.1.4 Barley (*Hordeum vulgare* L.)

The analysis of differentially expressed barley genes between drought-stressed and normal growth conditions at the reproductive stage was reported by Guo et al. [42]. Seventeen genes were expressed exclusively in two drought-tolerant genotypes under drought stress. In a recent study, using RNA-Seq to drought-tolerant and -sensitive wild barley accessions under drought, also during reproductive development, Hübner et al. [50] revealed similar gene expression patterns when studying drought-tolerant accessions with varied genetic background and geographic origin. Furthermore, it was observed that reproductive success under drought, defined as the relative grain loss between treated and untreated plants, during flowering and early maturation appears to be a physiological adaptation in the evolutionary history of wild barley.

6.4.2 Plant Drought-Responsive Genes

Plants growing in unfavorable environmental conditions, such as drought, provide changes in their internal environments, trying to adjust to the drought conditions and survive. Drought tolerance comprises quantitative inheritance that is dependent on the joint action of various genes in line with environmental factors. In this sense, a significant amount of transcriptome sequences has become available, which has provided valuable information regarding drought-responsive genes [155, 158]. A large number of these genes work not only in protecting the plant from dehydration but also in regulating transcription and signal transduction in response to drought [102]. According to Shinozaki and Yamaguchi-Shinozaki [114], the products of these regulated genes during stressful situations such as drought can be primarily classified (Fig. 6.1) into two major groups: functional proteins that work directly for the protection of proteins and membranes, and regulatory proteins

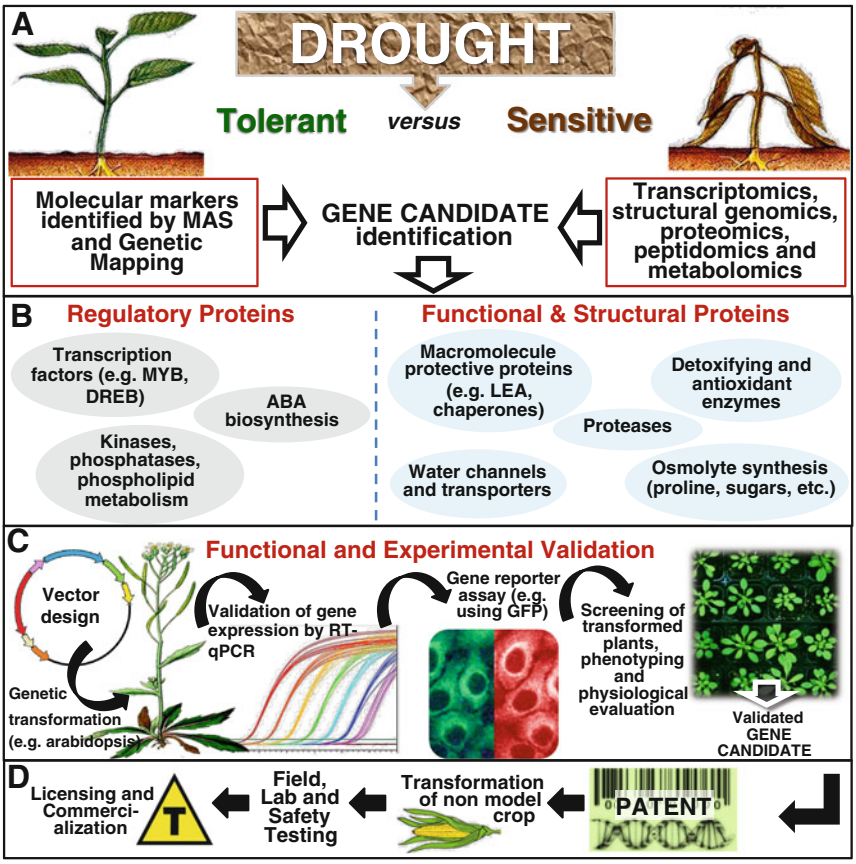

Fig. 6.1 Plant transcriptomic studies covering drought tolerance. **a** Contrasting accessions are subjected to drought stress evaluation trying to identify useful candidate genes by transcriptomics. **b** Candidate genes responding to the drought stress are annotated and basically classified into two broad groups. **c** Expression of gene candidates is validated by RT-qPCR or transgenesis. **d** GMO generation and further steps: patent protection, biosafety, licensing, and commercialization

involved in the perception, signaling cascade, and transcriptional control. Analysis of several organisms has emphasized these two categories [14, 49]. Additionally, a group of molecules comprising noncoding RNAs (ncRNAs) are also briefly presented due to their role in stress responses.

6.4.2.1 Protective Genes

This group includes genes that encode proteins with continuous protection against drought stress. It is represented by LEA proteins (late embryogenesis abundant proteins), heat shock proteins, chaperones, osmoprotectants, free radical scavengers, proteins involved in the capture and transport of water and ions, such as

aquaporins/water channel proteins and ion exchangers/carriers, as well as active enzymes involved in the osmolyte biosynthesis, in the reactive oxygen species (ROS) detoxification, among other metabolic processes, some of them still unknown. For example, the overexpression of LEA proteins was associated in many cases with desiccation tolerance, but the exact mechanism behind this tolerance is still unclear [137]. In turn, some osmolytes have a dual function: to help maintain the osmotic balance of the plant cell under drought [52], and acting in stabilizing the three-dimensional structure of proteins [147].

The water transport into the cells is essential for maintaining plant cell physiology, especially during drought periods. In plant–water relations, the water channel proteins (such as aquaporins) are central pieces [3]. A tobacco plasma membrane aquaporin, NtAQP1, showed its importance in the cell and its function in the plant when silenced by an RNA antisense technique [115]. The antisense plants, when compared with the control, presented low hydraulic conductivity at the root and lowered water-stress resistance.

ROS are relevant signaling molecules in periods of stress [87, 128], but they can cause oxidative damage to cells if there is an imbalance in their production [23]. It is important to keep them within tolerable levels [85]. Mitigation of oxidative damage by the action of antioxidants and ROS scavengers may enhance plant tolerance to drought. The induction of AtALDH3 (an aldehyde dehydrogenase gene) in *A. thaliana* resulted in tolerance to drought and salt stress [121].

6.4.2.2 Genes Encoding Regulatory Proteins

This group includes genes that code for regulatory proteins that act on expression of stress-responsive gene, such as transcription factors (TF superfamily comprising HSF, DREB, bHLH, bZIP, ERF, among others), proteins involved in signal transduction, such as protein kinases and MAP kinases, and also enzymes involved in the phospholipid metabolism.

TFs have emerged as relevant actors to manipulate complex metabolic pathways in response to environmental stresses in plants [51], due to their ability to induce other signal transduction networks, even if their initial expression has been discreet. Their action permeates steps ranging from the perception of stress signals to control multiple pathways, and allowing the eukaryotic cells to change their growth patterns in a variety of ways, including under drought stress [146]. Myriad important TFs associated with drought tolerance have been identified (e.g., [111]. Within the many TF families used in transgenic approaches for drought tolerance, the DREB1 genes deserve special mention. They include a conserved drought-responsive element (DRE) in their promoters [113]. In *A. thaliana* several transcripts encoding DRE-binding proteins (as DREB1A, DREB2A) were identified precisely activating transcription of genes containing the DRE domain [70].

Kinases, in turn, mediate reversible protein phosphorylation, which is a cell-signaling mechanism [97]. Their activity is highly regulated, sometimes by autophosphorylation, activating or inhibiting proteins, or binding to small

molecules, as a calcium sensor (i.e., CDPKs, calcium-dependent protein kinases). They recognize several environmental stimuli, involving both biotic [107] and abiotic [20] stresses, and they have a role in signal transduction. The overexpression of a CDPK (OsCPK4) provided drought tolerance in rice, acting as a positive regulator, to protect cell membranes from damage resulting from oxidative stress [20]. Searching for kinases in SuperSAGE libraries (more than 13 million tags), comprising biotic and abiotic stresses carried out using cowpea (leaves/roots), [57] identified a total of 713 potential kinases, covering 13 families. From these, 169 were differentially expressed ($p < 0.05$), being 100 up- and 69 downregulated when comparing different libraries.

Other important signaling molecules in plant cells (eukaryotes in general) are phospholipids and among them the phosphatidylinositols. Such compounds have important signaling actions in plants under osmotic stress [93], which is a significant side effect of drought. Overexpression of a phosphatidylinositol synthase gene influenced the membrane lipid composition and increased the ABA synthesis in corn [71], leading to an increase in drought tolerance.

6.4.2.3 Noncoding RNAs, NcRNAs

When referring to plant stress tolerance, attention turns primarily to protein-signaling mechanisms and protein networks, but nowadays some attention has been given to noncoding RNAs (ncRNAs). They are very abundant and diverse [91], including small RNAs, which are associated with RNAi mechanisms (e.g., miRNAs and siRNAs, 18–25 nt in length; [21]) and epigenetic processes (e.g., cytosine methylation; [80]) and the long noncoding natural antisense transcripts—lncNATs (over 200 nt in length; [141]).

The lncRNAs can act directly or be processed into shorter miRNAs and siRNAs [151]. They may regulate the expression of their target genes by some possible mechanisms. They are collision of the transcriptional machinery; competition for TFs; silencing by RNAi; disruption of posttranscriptional modifications and translation of the sense transcript; and forming RNA/RNA duplexes, masking specific signals in the sense RNA needed for splicing, stability, or degradation (reviewed in [34, 65]).

In an *A. thaliana* survey, in which 70 % of annotated mRNAs were in association with antisense transcripts, the authors identified 37,238 lncNATs pairs [141]. Using a strand-specific RNA sequencing approach in *A. thaliana* some identified lncRNAs induced by *Fusarium oxysporum* infection allowed finding new transcriptionally active regions (TARs; [157]). These lncRNAs were transcribed from intergenic or intragenic regions or even from introns presented in genes encoding proteins.

Studies indicate lncRNAs regulating plant adaptation to stress acting on complex regulatory networks of genes that encode proteins. ncRNAs acting in response to drought/osmotic stresses were also documented in the recent scientific literature for *A. thaliana* [77], corn [151], and *Medicago truncatula* [142].

Thus, it is observed that the plant cell has an arsenal of strategies to minimize the effects of stresses. In this context, the plants aim to explore their genetic capabilities, taking advantage of their genes and regulations (at different levels, such as transcriptional and posttranscriptional), working together harmoniously, and seeking to adapt the physiology of these plants to the new condition.

6.4.3 Transcriptomic Data Validation

The whole transcriptome sequencing uncovers myriad genes that need to have their expression validated by at least one additional separate experiment (Fig. 6.1), especially those differentially expressed that may be potential candidates for transgenesis. Thus, one way to reinforce the reliability of transcriptomics data is to use an additional technique to analyze the same population of transcripts in a data validation process. The Northern Blot technique was used for this purpose [40, 130]. Today, the preferred method for validation of genes differentially expressed based on their transcripts is the reverse-transcription quantitative PCR (RT-qPCR, Fig. 6.1). However, to validate by qPCR is not that trivial. Thus, to increase transparency and reliability of the results obtained with qPCR it is imperative to follow the MIQE guidelines (The Minimum Information for Publication of Quantitative Real-Time PCR Experiments; [18]. In this guide, the use of biological and technical replicates is expected, and their importance is discussed hereafter.

6.4.3.1 Biological and Technical Replicates

In a transcriptomic study trends that threaten the representativeness of the transcripts need to be avoided to ensure the reproducibility and accuracy of the data. Therefore, it is necessary to take some precautions at every stage of the process (Fig. 6.1). It includes selection of the biological materials, application of the stress, preparation and sequencing of cDNA libraries [139], and transcriptome analysis [45], among others, in order to ensure data reliability. Thus, predicting the use of replicates in the experimental design is necessary to ensure better accuracy of the estimated gene expressions. In this context, the replicates [25] can be: (a) biological, with two or more experimental units (individuals) representing the same biological condition (comprising the natural variation of the experimental group going through analysis); or (b) technical, when the same sample of one experimental unit, which is processed more than once (comprising the experimental variation of the test).

The ideal situation requires the use of both replicates in a transcriptomic assay, but costs can limit their use. Thus, the choice of which type of replicate will be used should first consider the existence, or not, of biological variation in the system. If the studied organism is an allogamous plant (one that performs cross-fertilization), biological replicates should be provided due to the genetic variability among seeds.

If the accessions are clones, there is not, in principle, natural variation among individuals, except those of epigenetic origin. Some examples of experiments using biological replicates in the transcriptomic studies covering plants under drought stress are shown in Table 6.1. In the same table, the numbers of replicates applied to validate the expression data by a second technique are also indicated. According to Table 6.1, the biological replicates for the libraries were not always present; whereas both the biological and the technical replicates required for validating the expression data (RT-qPCR) were always there.

6.4.3.2 RT-qPCR Studies

As mentioned before, RT-qPCR is considered to be the gold standard and the choice method for validation of gene expression data in transcriptomics analysis [18]. These analyses require specific formatting regarding the generation of experiment analysis and assembly of RT-qPCR reactions to ensure that the results are statistically confirmed, making them more reliable. Thus, after an explosion of data from transcriptomics and the perception that the published results of genic expression could not be easily analyzed, checked, compared, and interpreted by the scientific community, researchers have developed the idea of standards with the minimum of information (MI). In this way, they established some guidelines, including the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE; [18]). Thus, the MIQE Guidelines was proposed with emphasis on the steps to generate reliable data and results. It describes experimental design, sample, nucleic acid extraction, reverse transcription, qPCR (target, oligonucleotides, protocol, validation), and data analysis, including the statistical methods [18, 53]. During the submission of an article to a scientific journal, the authors need to send this guide (for authors, reviewers, and editors) compiled in a checklist form together with the manuscript. It provides accurate information, classified as Essential (E) or Desirable (D), so the reviewers can assess the quality of the work or to repeat the experiments. Its use also promotes consistency between laboratories and experimental transparency and shall be published as a supplement.

To validate expression data of genes identified and selected from transcriptomics studies it is necessary to define reference genes and to use them correctly. That is, its condition of reference gene as internal control must be confirmed (validated) by each research group and for the experiment in question. A simplified MIQE, called MIQE Précis [19], provides practical guidance on the main parameters of a qPCR assay, its documentation, and communication results, including some comments about reference genes.

Reference genes should not vary in the tissue or samples under investigation or response to the treatment. The reference genes are used in a normalization data process, which is performed against multiple reference genes. The normalization is necessary because it ensures that the samples are compared at the same quantitative level in relation to the analyzed population of transcripts. Therefore, changes in the expression of target genes with regard to normalizing genes consistently indicate

that there is a change in the orchestration of such genes and no changes resulting from other variables, including the amount of starting material and/or enzymatic efficiencies, among others [132]. The choice of normalization genes is based on statistical analysis of their estimated stability, performed by software such as GeNorm [132], NormFinder [7], and BestKeeper [99]. Silva et al. [28] proposed a novel set of reference genes for RT-qPCR in drought-stressed sugarcane, the reference genes of which went through evaluation by all the mentioned software and were later used in the normalization process. The data normalization process should be performed on no less than three reference genes. It needs to be noted that according to Table 6.1, most of the scientific articles used only one reference gene in the RT-qPCR validation process.

6.4.3.3 Transgenic Approach for Functional Studies

The achievement of cisgenic (genetic modification with genes from the same species) or transgenic plants (GMO, genetically modified organism) is another way to strengthen the reliability of transcriptomics results. If one considers the generation of transgenic plants aiming for the acquisition of tolerance to drought stress, some successful approaches have been reported based on transcriptomic data. These methods take the assumption that the up- or downregulation of a given gene is associated with the tolerant phenotype. Thus, after molecular candidates have been identified, selected, and validated based on their differential expression showed by plants under stress versus the negative control, they can be used in transgenesis trying to analyze their impact on plant physiology (Fig. 6.1). An example is reported by Zhang et al. [150]. The authors observed an accumulation of miR169, induced by drought stress in tomato (*Solanum lycopersicum*). The transgenic lines overexpressing miR169c when compared with the wild-type showed an increased stress tolerance, being attributed to a decrease in the stomatal opening, the lower transpiration rates, and lower water loss per leaf. In consequence, the miR169c targets [three nuclear factor Y subunit genes (SlNF-YA1/2/3) and one multidrug resistance-associated protein gene (SlMRP1)] were significantly suppressed during stress. In this particular case, although miR169 belongs to a conserved family of miRNA, there is contradicting evidence on behaviors in different species. Transcriptome analysis of *M. truncatula* under drought stress showed miR169 expression being suppressed during the stress period [150]. The authors explained the conflict with the literature as a difference in the stress level to which the plants were exposed. In the present case, the samples include tissues under different stress conditions (6, 8, 10, and 12 days after withholding water). Thus, experimental procedures involving the stress treatment, including the exposure time, can also influence the detection or not of a particular candidate/target because the transcriptome is dynamic and constantly renovated.

Genetically modified plants producing artificial miRNAs (amiRNA) also include examples of some success. For instance, higher drought tolerance was observed in potato after amiRNA-based silencing of CBP80, a protein involved in RNA

processing. Improved tolerance to water stress was correlated with increased leaf stomata and trichome density and ABA-hypersensitive stomatal closure. Transcriptomic analysis of silenced CBP80 plants revealed the presence of a cascade of effects, including downregulation of miR159 and upregulation of ABA-related TF genes MYB33 and MYB101 [100].

Despite the advances comprising genetic transformation associated with a single gene, it is believed that tolerance to drought (and also to salinity) should be more efficient and lasting for metabolic engineering projects involving multiple genes and pathways, extrapolating tolerance induced by a single gene [11]. An interesting example was given for peanut (*Arachis hypogaea* L.) with the simultaneous transformation and coexpression of three stress-responsive TFs (*At*ABF3, *At*DREB2A, and *At*HB7) associated with downstream gene expression [101]. Transformed peanut plants expressing the mentioned TFs presented increased tolerance to drought, salinity, and oxidative stresses when compared to wild-type, also bearing increased total plant biomass, indicating that this strategy is promising and should be further evaluated in field trials in more advanced generations.

Another form of validation occurs through functional studies, by processing plants to assess the expression of the gene candidate in association with a reporter gene that identifies their spatiotemporal expression throughout the development of transformed individuals. In this regard, the most widely used model plant has been *A. thaliana*. Also, it stands out for its ease of genetic transformation and heterologous expression for use in high-throughput assays [29]. An example of analysis and validation studies of gene function through heterologous expression regards bZIP factors, known to play important roles in the ABA signaling pathway in *Arabidopsis*. Lu et al. [74] identified evolutionarily conserved bZIP orthologues between *Arabidopsis* and rice, indicating that they may share similar functions, also indicated by their induction under ABA, ACC, and abiotic stresses. OsbZIP72, a member of this group, was proved to be an ABRE-binding factor in rice using the yeast hybrid systems. Transgenic rice plants overexpressing OsbZIP72 exhibited hypersensitivity to ABA, elevated levels of ABA response products (as LEAs), and an enhanced ability of drought tolerance.

Both genetic manipulation, based on heterologous gene expression, and natural/induced diversity could be exploited for increased drought tolerance because the loss-of-function mutations or overexpression of specific components of posttranscriptional or posttranslational mechanisms may generate genotypes with increased tolerance [81]. The overexpression of a rice SUMO E3 ligase OsSIZ1 in *Agrostis stolonifera* L. (creeping bentgrass) improved fitness under heat stress and water deficit, possibly associated with a higher photosynthesis rate and overall plant growth, more robust roots, increased cell membrane integrity, and water retention [68]. SUMO E3 ligase is a central enzyme in the sumoylation process, and this process modulates protein stability, subcellular localization, and activity. A growing number of translational and posttranslational processes have been identified in association with drought and other types of environmental stresses [41]. Even if improved tolerance of these engineered plants is sometimes associated

with penalties in plant growth and/or yield, thus limiting their effective exploitation, these examples are very promising.

6.5 Overview on Patents Related to Drought-Tolerant Plants

In a scenario where crop breeding is crucial to improving food production, biotechnological approaches, including molecular breeding and genetic engineering —also associated with conventional breeding—are the most straightforward strategies to develop crops more tolerant to abiotic stresses, with emphasis on drought. Traditionally, the discovery of potential candidate genes for biotechnological use could be based on molecular markers for use in marker-assisted selection (MAS) or in genetic mapping, associating them with quantitative trait loci (QTLs) and phenotypic features [86] or based on analysis of comparative differential expression by evaluating contrasting accessions (Fig. 6.1). In this way, structural genomics (especially when focused on regulatory elements), proteomics, and metabolomics are used to recognize the most promising candidate genes, among the large number of changing molecular components under drought stress [47, 104]. After the functional validation of the selected candidate gene, this "invention" could be patented. However, a crucial point is whether the assignment of a function or process to a DNA sequence could be characterized as an "invention". At first it was believed that this patent should involve some technical innovation, with a distinction drawn between patentable and nonpatentable discoveries. In the 1980s, a majority decision of the US Supreme Court [32] defined that a genetically modified bacteria able to degrade crude oil was an invention. This decision provided the legal basis for US biotech discoveries. In 1998, the European Parliament enacted a Biotechnology Directive in which Article 3.2 deliberated that "Biological material isolated from its natural environment or produced by a technical process can be considered an invention, even if it previously existed in nature" [16].

Therefore, there are many patents including different genes, gene parts, or their products deposited throughout the world. For example, by searching the US Patent and Trademark Office (PTO) databank using the keywords *drought* AND *transgenic* AND *plant* almost 10,000 results were retrieved (in August 2015). However, most of them do not regard plants that are already on the market (or on the way to be), being associated with experimental assays, reproductive processes, or microorganisms, among others. For comparative purposes, with the search conducted in the same PTO database in June 2011 by Kido et al. [58], with the keywords *stress* AND *sugarcane* OR *monocot*, the authors retrieved 110 results, most of them related to genes and their products from plants or even microorganisms in some association with plant stress tolerance. Of these, only 67 were related to plant genes, of which 21 were TFs, 20 related to signal transduction (mainly kinases), and 26 with diverse functions. Concerning these 110 results, the

largest set of patented genes (and their products) included TFs, protein kinases and phosphatases, protein methyltransferases, GTP-binding proteins, and DNA binding proteins. Regarding the TFs, AP2 (APETALA2) domain, the MYB transcription factor family, the WRKY protein family, the zinc finger protein (Z) family, the CAAT-element binding proteins, the bZIP family, and the scarecrow (SCR) family, were the most represented families. At that moment, not many genes were associated with differential expression profiles using open architecture methods or in association with NGS techniques. The same keywords (*stress* AND *sugarcane* OR *monocot*) in a search realized in August 2015, in the same PTO database, retrieved almost 6000 results. When replacing the keyword *stress* by "*drought stress*" around 4870 results were retrieved. This vertiginous increment (from 110 to 6000 or even 4870) reflects the numerous genetic studies performed at the wide-genome/transcriptome level involving plant responses to stress. Of course, as pointed out by Kido et al. [58], not all retrieved results are related to genes or their products from plants in some association with drought tolerance. However, many plant genes are expected to be classified in the same categories covering the 67 patents pointed out by Kido et al. [58] or into those described by Somvanshi [117]. This author performed a detailed evaluation of patents (30) associated with drought-tolerant genes, classifying them into five categories: (a) proline biosynthesis; (b) dehydration responsive element binding factors (DREB) and C-repeat sequences binding factors (CBF); (c) protein kinases; (d) TFs; and (e) miscellaneous drought-tolerance genes.

Despite many genes mentioned in this chapter having been already subjected to patent, due to their potential use in the development of drought-tolerant plants, one crucial point is that many studies reporting increased drought tolerance comprised transformed plants growing under laboratory and greenhouse conditions, with few evaluations under field conditions. An interesting case study carried out by Lu et al. [75] regarded advanced field trials for poplar transgenic hybrids [(*Populus tomentosa* Carrière × *Populus bolleana* Mast.) × *P. tomentosa*] carrying the DREB1 gene from *Atriplex hortensis* L. Transformed plants have been cultivated in 10 fields since 2005 and presented improved performance under saline and alkali stress conditions. The still current field trials have been accessed to verify if the AhDREB1 continue present in the transgenic trees and if it is regularly expressed. The results of qPCR have shown that some decrease in the transcription levels occurred compared with those from four years before, whereas no foreign gene was found in the genomic DNA of microorganisms in the soil near the transformed poplars. In another work [15], peanut plants transformed with a DREB1A gene were tested in the laboratory and also in four field trials under various water-stress regimes. The transformed plants showed substantial improvement (24 %) in yield under drought in field conditions, presenting significantly higher seed filling and 20–30 % lower pod yield as compared with untransformed plants also under drought. In addition to this agreement, cultivation in field conditions needs to be done, carefully observing the biosafety issues (e.g., following same guidelines for environmental risk assessment of GMO as proposed by Andrade et al. [8].

Steps including crop transformation, field trials (under different environmental conditions), licensing (including attendance to biosafety norms), and commercialization require significant investment for the installation and up-to-date maintenance of the genotyping pipeline, an efficient data collection and processing data system, the DNA evaluations, and the achievement of licensing, all of these demanding a multidisciplinary well-trained team.

6.6 Conclusion and Perspective

Considering the expected population growth, increased demand for food, limited water resources, and the limitations of traditional plant breeding programs, the use of molecular tools should be encouraged to accelerate the release of elite materials that respond more readily to environmental stresses. Among abiotic stresses, drought is one of the most injurious, causing significant economic damage. In addition, drought tolerance has a very complex character. However, to understand how plants respond to drought stress at the molecular level is essential for the identification of useful genes in breeding programs. Transcriptomics studies of plants under stress allow us to evaluate global gene expression profiles of contrasting phenotypes, helping to identify genes related to drought tolerance. Different methods can be applied in plant transcriptomics, considering the application of the drought-stress treatment on plants, generation and sequencing of libraries, and validation of the transcriptomic data by a second technique. Some of those required methods were presented and their importance discussed in this chapter. Also, results from a search in the PubMed database looking for scientific articles published from January to August 2015, covering drought stress in plants and transcriptomic illustrate several of the comments.

Transcriptomics studies covering plants in response to drought stress basically report the same genes, showing a decrease of transcripts related to primary energy metabolism and photosynthesis, and an increase in stress signaling, and proteins with antioxidative and osmoprotective functions. This is reflected even in the patents, but this feeling is not so clear due to the significant increase in patents deposited in the PTO database, for example, in the last decade, which should be a consequence of this large amount of data available from transcriptomics, due to NGS technology and bioinformatics analysis.

Still the fact that transcriptomics studies covering plants responding to drought report basically the same genes is partially due to the limitations of the methodologies applied. One of these limitations is just the difficulty in simulating the plant stress in the way it occurred in nature. Also, most of the genes identified showed no major influence on drought tolerance, despite some successful examples of transgenic plants, but almost always without field evaluations over several years. On the other hand, research in the Omics are directed by discoveries and not by hypotheses, which makes the discussion of the results being directed by the results presented in the literature, thus replicating simplified models, rather than getting

deeper answers that should be addressed by the plant physiology. In addition, another transcriptomic limitation is that transcript abundance does not mean active products of genes, therefore only when the results of the several Omics integrate in the so-called systems biology, will a better understanding of plant responses to environmental stresses be more evident.

Nevertheless, without doubt, transcriptomics of plant responses to drought (stresses in general) still have a lot to offer and plant breeding programs also need such information so they can continue to make progress in improving our crops.

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Chapter 7
Drought Stress Tolerance in Plants: Insights from Metabolomics

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7.1 Introduction

Crop losses due to variable weather patterns associated with climate change have risen over the past decades, and climate models predict an increased incidence particularly of droughts, floods, and extreme temperatures [1–4]. This situation together with the growing food demand is anticipated to pose a real threat to global food security [5, 6]. It is therefore imperative to develop strategies to improve food availability substantially in variable environments, and ultimately, transfer this knowledge to farmers in the timeframe needed. This has been progressively accomplished through breeding [7] and biotechnology [8] approaches that use innovative methods to develop plants with enhanced abiotic stress tolerance. Over the past years, much progress has been made in the area of plant metabolomics applied to abiotic stress research [9–11], however, the mechanisms defining plant resilience to changing environments are still far from being completely understood.

One approach to improving this knowledge is to reveal the underlying central metabolic pathways that might play an important role in modulating plant growth, development, and stress responses to adverse situations that in the natural environment are always a combination of several stress factors [12, 13]. When a certain metabolic pathway is activated, precursors and intermediates are channeled to produce a bioactive molecule, an antioxidant, a signaling compound, a cell structure biosynthesis intermediate, or even a storage compound. The production of these compounds, in turn, can be regulated by other compounds, signalling molecules, such as plant hormones, that can feed back, activate, or inactivate different metabolic steps so that, as a whole, the actual metabolite composition of a given plant species is the result of a particular gene expression profile. Considering the meta-

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bolome as the balance between defense, signalling and damage, metabolites can be used to assess plant tolerance to a certain stress situation [14, 15].

Water deficit causes many adverse effects on plants making drought the most serious environmental factor limiting the productivity of agricultural crops worldwide with devastating economical and sociological impact [16]. The production of reactive oxygen species (ROS) is one of the primary responses to stress causing cell damage following the decline in photosynthesis [17–19]. Cellular responses to drought include adjustments of the membrane system, modifications of the cell wall architecture, changes in cell cycle and cell division, and in addition plants alter metabolism in various ways, including the accumulation of several amino acids such as proline, valine, leucine, and isoleucine, along with water-soluble carbohydrates and polyols, known to have an osmoprotective role [20, 21].

The twentieth century marked the dawn of molecular biology, and recently, after the publication of the *Arabidopsis thaliana* and human genomes [22], a number of strategies have been developed to cover the entire aspects of an organism's biology and to monitor and control cellular responses to genetic perturbations or environmental changes. To understand the organisation of cellular functions at different levels, an integrative approach with large-scale experiments, so-called 'omics' data, that includes transcriptomics [23, 24], proteomics [25–27], and metabolomics [23, 28, 29] is required.

The full suite of metabolites, low-molecular–weight molecules synthesised by any biological system at any specific physiological state, comprises its metabolome, described as bridges between genotypes and phenotypes deflecting different biological endpoints as the downstream result of gene expression [30]. The extensive knowledge on metabolic flows therefore allows the assessment of genotypic or phenotypic differences between plant species [31], and can help us to characterise unknown gene functions [32], identify diurnal changes in metabolite levels [33], observe responses to biotic or abiotic stresses [34], as well as to discover target metabolites as important nutritional or agronomical biomarkers [35].

Metabolomics has therefore emerged as a complementary tool to functional genomics that aims to quantify the complete set of metabolites in a given organism at a given developmental stage and in a given organ, tissue, or cell type [36], providing the approach to a signature of the functional metabolic phenotype.

The concept of profiling the metabolome, however, has not been as eagerly engaged as its parallel omics counterparts due to the technical complexity of metabolomics. Studies of the plant metabolome, as compared with genomics (nucleic acids) and proteomics (peptides and proteins), include the analysis of a wide range of chemical species with diverse physical properties, from ionic inorganic compounds to biochemically derived hydrophilic carbohydrates, organic and amino acids, and a range of hydrophobic lipid-related compounds [37, 38], and this is a challenging analytical task due to the wide array of molecules with different structures and chemical properties. For instance, more than 200,000 different metabolites are estimated to exist in the plant kingdom [36, 39] and a single accession of Arabidopsis contains more than 5000 metabolites over a large dynamic

range in concentrations that can vary from femtomolar to millimolar [40]. Consequently, there is no single analytical technique that is suited to the precise and accurate identification and quantification of all metabolites of interest. Due to their chemical diversity and broad dynamic range in cellular abundance, different extraction techniques and combinations of analytical methods are thus often employed to achieve adequate metabolite coverage [11]. Regardless of the analytical setup, plant metabolomics studies can be divided into two main approaches: nontargeted, used for global metabolome analysis, that is, comprehensive analysis of all the measurable analytes in a sample including identification of unknown signals; and targeted, where predefined metabolite-specific signals are used to determine metabolites precisely and accurately. Due to its high selectivity and sensitivity, mass spectrometry (MS) is widely used in targeted metabolomics, generally coupled to different separation techniques such as liquid chromatography–mass spectrometry (LC-MS; Sect. 7.3.1), gas chromatography–mass spectrometry (GC-MS; Sect. 7.3.2), and capillary electrophoresis–mass spectrometry (CE-MS; Sect. 7.3.3) [11, 37, 41–48].

The development of drought-tolerance strategies in plants is necessary for productive sustainable agriculture in arid and semiarid areas of the world with very limited precious resources such as fresh water. At present, it is also desirable to improve the yield of crops grown in infertile soils based on crops that are tolerant to low soil fertility. Therefore, understanding the mechanisms underlying stress tolerance in plants is not only an academic interest, but has also countless socioeconomic implications. Plant metabolomics offers the opportunity to gain deeper insights into the fundamental biochemical basis of food, as a truly interdisciplinary field of science which combines analytical chemistry and platform technologies that use MS coupled to sophisticated data analysis [49] to evaluate the impact of environmental and genetic factors on the plant metabolome [50, 51]. In this chapter, metabolomics as a tool to investigate drought stress in plants is discussed, from sample preparation and analytical technologies to the analysis of metabolic responses. This information will ultimately provide the knowledge basis to facilitate our understanding of the plant's flexibility to reconfigure metabolic pathways to sustain cellular homeostasis thereby ensuring their survival in adverse conditions, such as drought stress.

7.2 Sample Preparation for Plant Metabolomics

Plants constantly change their metabolism to support their growth and development when exposed to environmental changes, that is, abiotic and biotic stresses. Metabolic reactions are continuously occurring in the different plant tissues within seconds, and the changes in the metabolite levels are highly dependent of different plant physiological processes, such as photosynthesis and respiration activity. When aiming to conduct a plant metabolomics study, a good experimental design must be prepared in advance, and each sample preparation step should be

previously defined: (i) plant tissue harvest, (ii) metabolic quenching, and (iii) metabolite extraction. In addition, randomisation procedures throughout all the experimental workflow should be envisaged to minimise potential sources of experimental errors [11, 52, 53].

7.2.1 Harvesting

Herein, the plant or organ under study should be harvested under the conditions defined in the experimental design, that is, plant developmental stage, time of the day, and so on [11, 52]. In addition, a careful sampling method must be carried out to reduce biological variation. A minimum of six independent biological replicates should be used to provide representative samples of the plant system under study, and to increase the reproducibility of the metabolomics analysis [52–54]. During the harvesting step, it is also important to monitor environmental variations, including small variations that might occur in the greenhouse, because these variations might influence the biochemical status of the plant material. Furthermore, prelabelling of all sample containers (e.g., aluminium foil or polypropylene tubes) and a clear sample identification file should also be prepared in advance as these will simplify the subsequent sample preparation steps. As mentioned before, because metabolic reactions are subject to rapid enzymatic turnover, plant harvest should be done as quickly as possible to prevent changes in metabolite levels and minimise loss of high turnover rate metabolites, such as glycolytic intermediates [55].

7.2.2 Quenching

The quenching step aims to inactivate the enzymatic activity and consequently stop metabolism. To achieve this purpose, the plant tissue is rapidly frozen in liquid nitrogen (i.e., shock freezing) and stored at –80 °C. In field conditions where liquid nitrogen is not usually available, harvested plant tissues are stored and transported in dry ice until they can be transferred to a liquid nitrogen container. Furthermore, fresh-frozen plant tissues are finely homogenised with a cooled pestle and mortar filled with liquid nitrogen or with a ball mill and stored at –80 °C until extraction [11, 52, 54].

7.2.3 Extraction

Many factors must be considered in the extraction protocol, namely solvent characteristics and solvent/sample ratio, as well as duration and temperature of the

extraction to ensure that (i) metabolites are completely extracted, (ii) variability in the metabolite levels as a result of extraction is minimised, and (iii) metabolites are not modified [55]. Because the metabolome consists of a wide variety of compounds at very different levels and with very different polarities, no single solvent is capable to dissolve the whole range of compound diversity in routine metabolomics studies. It is therefore desirable to perform several extractions with different solvents to have a better view of the metabolome.

The solvent can be selected based on its physicochemical properties, such as selectivity and polarity, the last playing a critical role because solvents will extract metabolites according to the 'like-dissolves-like' principle: polar metabolites are generally extracted with polar organic solvents such as methanol and methanol–water mixtures that are directly added to fresh frozen tissues at low temperature (4 °C); nonpolar solvents such as chloroform are used to extract lipophilic metabolites [38, 39]. Nonetheless, extraction from a biological matrix cannot be simplified according to purely chemical solvation logic and metabolomics studies must be designed to detect as many metabolites as possible, thus preliminary experiments need to be performed to understand which solvents or solvent mixtures are the most efficient for the extraction and are capable of extracting diverse groups of metabolites [11]. The two-phase solvent system consisting of chloroform, methanol, and water (2:1:1, v/v) is a well-established solvent system for the extraction of polar and nonpolar metabolites due to the advantage of being able to fractionate metabolites from a single sample into a polar aqueous phase (water:methanol) and a lipophilic organic phase (chloroform), that can be analysed separately [44]. For LC-MS-based metabolomics studies, ideally the extraction solvent is miscible and preferable similar to the liquid chromatography (LC) mobile phase used, typically aqueous eluents with up to 50 % of an organic solvent such as methanol or acetonitrile for typical reverse-phase separations (Sect. 7.3.1). For GC-MS analysis, on the other hand, the range of possible solvents is limited to volatile compounds and the analysis of polar compounds must undergo derivatisation (Sect. 7.3.2).

7.3 Plant Metabolomics Analytical Technologies

Because of the vast chemical diversity of the plant metabolome, modern plant metabolomics studies often combine multiple MS-based platforms in an effort to attain more complete metabolite coverage from a complex biological plant sample. Nuclear magnetic resonance (NMR) is also widely recognised as a powerful tool in plant metabolomics to assist structure elucidation of metabolites and detailed analysis of the biomolecular composition of a plant extract with the advantage of relatively simple sample preparation. Nonetheless, due to its poor sensitivity and poor dynamic range relative to MS, NMR-based plant metabolomics only allows detection and quantification of the most abundant metabolites; thus, NMR is most commonly used as a metabolic fingerprinting technique [56, 57]. In the next section

we discuss in more detail three powerful MS-based platforms currently used in plant metabolomics studies, namely LC-MS, GC-MS, and CE-MS.

7.3.1 LC-MS

Since the late 1960s, improved chromatographic methods have made peak identifications possible relying solely on chromatography [58], however, the introduction in the late 1990s of the electrospray ionisation (ESI) source has offered a robust and versatile interface to connect liquid chromatography and mass spectrometry [59, 60], thereby helping to overcome the drawbacks of directly injecting complex samples to profile several hundreds of compounds in a single crude plant extract [36].

Liquid chromatography can reduce ion suppression caused by coeluting compounds and often can separate isomers. In addition, a good analytical separation will result in better detection limits and MS data quality due to reduced background noise. Further improvement of the potential to identify metabolites and to provide an even more detailed metabolite profile of plant extracts could be obtained by combining LC with ultra-high resolution mass spectrometry (HRMS) such as Fourier transform ion cyclotron resonance mass spectrometry or FT-ICR-MS [61], LC-NMR-MS [62], or ultra-performance LC (UPLC) coupled to MS [63].

LC-ESI-MS is suitable for the analysis of semipolar metabolites, resulting in protonated $[M + H]^+$ (in the positive ion mode) or deprotonated $[M - H]^-$ (in the negative ion mode) molecular masses [64]. In plant metabolomics studies, LC-MS data are usually acquired with reversed phase liquid chromatography using C-18 narrow bore columns with a gradient elution program. However, very polar metabolites are not retained on classical reversed phase stationary phases and elute with the void volume making the separation often insufficient in the case of complex biological samples. Alternative LC-MS methods have been reported for the analysis of polar compounds based on new column chemistries. Hydrophilic interaction liquid chromatography (HILIC) and porous graphitic carbon (PGC) stationary phases are gaining acceptance in the analysis of highly polar metabolites typically found in the plant metabolome.

HILIC is orthogonal to reversed phase chromatography that uses either silica or derivatised silica with low-aqueous/high-organic solvent systems, and although similar to normal phase chromatography, HILIC requires a substantial amount of water in the mobile phase forming a stagnant water layer on the stationary phase surface into which polar analytes can be partitioned and retained. Therefore HILIC uses typical MS-compatible mobile phases (water-miscible polar organic solvents such as acetonitrile or methanol) without the need for ion-pairing reagents that allow efficient online coupling with ESI-MS. The application of HILIC-LC-MS in metabolomics studies has been reviewed by Cubbon and coworkers, who also discuss the retention mechanism involved in HILIC separations [65].

The first coverage of a broader range of the plant metabolome was achieved by Tolstikov and Fiehn with a HILIC-LC-ESI-MS method that detected a wide range

of highly polar metabolites, including raffinose family oligosaccharides (RFOs), amino acids, amino sugars, and sugar nucleotides from *Cucurbita maxima* phloem tissues [66]. Following a similar approach, Antonio and coworkers developed a HILIC-LC-ESI-MS method for the targeted analysis of carbohydrate-related metabolites present in Arabidopsis leaf extracts [67]. Using this method, an efficient separation and identification of glucose, sucrose, raffinose, stachyose, and verbascose was achieved in only 12 min, a distinct improvement for these analytes over the more broadly targeted method developed by Tolstikov and Fiehn [66].

The porous graphitic carbon stationary phase has also shown to be suitable for the analysis of highly polar metabolites. Its unique surface is composed of a planar network of hexagonally arranged carbon atoms that provide stable and reproducible LC-MS analyses throughout the entire pH range 0–14. The PGC retention mechanism is described elsewhere [68, 69]. It is defined as the polar retention effect on graphite (PREG) and essentially this retention mechanism is based on two main factors: dispersive interactions of the analyte between the mobile phase and graphite surface, by which retention increases as the hydrophobicity of the molecule increases; and charge-induced interactions of a polar analyte with the polarisable surface of graphite. Overall, the combination of these two factors increases the retention of analytes with higher molecular area in contact with the graphite surface, and analytes in which the type and position of the functional groups enable more points of interaction with the graphite surface. Consequently, planar molecules are more retained on the PGC surface than highly structured, three-dimensional, and rigid molecules, thereby allowing the separation of structural related metabolites, such as water-soluble carbohydrates.

Applications of the PGC stationary phase in the plant metabolomics field include the analysis of a range of oligosaccharides from *Triticum aestivum* stems [70], phosphorylated carbohydrates from Arabidopsis leaves [71], RFOs from *Lupinus albus* stems [72], and from leaves of the resurrection plant *Haberlea rhodopensis* [73], and nucleotide carbohydrates in Arabidopsis tissues [74].

7.3.2 GC-MS

Gas chromatography coupled to mass spectrometry is a key analytical technique used in plant metabolomics [11, 40, 75, 76]. This technique integrates the robust and reproducible chromatographic process of GC that separates a wide range of metabolites with the MS ability to provide structural information of these metabolites. Consequently, identification and quantification of a few hundred metabolites is achieved in a GC-MS analysis of a single plant extract [75].

The reproducibility of the GC-MS analysis is due to the electron ionisation (EI) method in which molecules interact with kinetically activated electrons at an accepted average standard energy of 70 eV. Moreover, improved mass analyser technologies such as orthogonal time-of-flight (oTOF) with faster acquisition times, higher mass accuracy, and higher deconvolution power allow high-throughput

instrumental analysis. One requisite to analyse plant polar metabolites with GC-MS is to perform a derivatisation step. Herein, a chemical modification in the metabolite's polar functional groups is obtained by adding a derivatisation agent that helps decrease the metabolite's polarity, and consequently, increases the volatility and thermal stability. For GC-MS plant metabolomics studies, a well-established derivatisation protocol is composed of two chemical reactions, namely methoxyamination followed by silylation. Detailed information regarding these two reactions is found in the literature [11, 77].

Stable isotope-labelling experiments (e.g., ^{13}C, ^{15}N, $^{13}CO_2$) coupled to conventional GC-MS analysis have become indispensable in modern plant metabolomics studies. This methodology known as steady-state metabolic flux analysis (MFA) allows us to determine the multiple metabolite fluxes through the different pathways of the plant metabolism and helps us to better understand the regulation of the metabolic networks. The concepts of this methodology have been extensively reviewed in the literature [78] and some applications of this methodology to different plant tissues include: potato (*Solanum tuberosum*) tubers [79]; Arabidopsis cell cultures [80, 81], leaves [82]; tomato (*Solanum lycopersicum*) leaves [83]; *Medicago truncatula* seedlings [84, 85]; *Catharanthus roseus* cell cultures [86]; developing *Glycine max* embryos [87], and roots under hypoxia [88].

7.3.3 CE-MS

Capillary electrophoresis coupled to mass spectrometry provides a viable alternative for metabolite profiling due to its ability to detect ionic metabolites, such as sugar phosphates and nucleotides, in addition to amino acids and organic acids [89, 90]. It has also the advantage of high resolving power, low sample volume requirements, and the possibility to separate cations, anions, and uncharged molecules simultaneously [91] on the basis of their mass-to-charge ratio. It has been reported that more than 200 metabolite signals could be determined in Arabidopsis analyses in which up to 70–100 metabolites were identifiable [92, 93].

Separations can be achieved in a fast and highly efficient way without the need for extensive sample pretreatment or high organic solvent consumption and the use of simple fused-silica capillaries instead of more expensive LC columns. However, the poor concentration sensitivity due to the limited sample volume that can be introduced into the capillary is a drawback of the technique even though it can be improved by combining CE with MS, making CE-MS an attractive complementary technique for metabolomics studies.

ESI is the main ionisation technique used for CE-MS in metabolomics [89] and the coupling of CE to ESI-MS can be performed via a sheath–liquid interface [94], the most widely used. In this configuration the separation capillary is inserted in a tube of larger diameter, in a coaxial setting, and via that tube the conductive sheath liquid to which the CE terminating voltage is applied is administered and merges with the CE effluent at the capillary outlet. A gas flow is applied via a third coaxial

capillary in order to facilitate spray formation in the ESI source [94]. Coupling can also be performed using a sheathless interface, in which case the CE voltage is directly applied to the CE buffer at the capillary outlet, by applying a metal coating to the end of a tapered separation capillary or by connecting a metal-coated, full metal, or conductive polymeric sprayer tip to the CE outlet [94].

CE-MS has been used to analyse intermediates of plant primary metabolic pathways, including glycolysis, tricarboxylic acid (TCA) cycle, and pentose phosphate pathway. Takahashi and coworkers directly quantified levels of nicotinamide nucleotides that play important roles as cofactors in multiple enzymatic reactions in the photosynthetic process in Arabidopsis [95]. The same authors performed untargeted CE-TOF-MS metabolite analysis for completely unbiased analysis of Arabidopsis mutants [93], and also analysis of Arabidopsis mutants that overexpress the NAD kinase 2 (*NADK2*) gene and its effect on carbon and nitrogen metabolism using CE-MS [96]. Watanabe and colleagues quantified carbohydrates, amino acids, and primary metabolites using CE-MS to examine responses of plant respiration to elevated CO_2 [97], and recently Maruyama and coworkers performed an integrated analysis to clarify relationships among cold- and dehydration-responsive metabolites, phytohormones, and gene transcription in rice plants [98], but several other applications can be found in the literature with *C. roseus* [99] and *Nicotiana tabacum* leaves [100]. In any case the technique requires several parameters, such as cone voltage, sheath liquid flow rate, capillary alignment, and nebuliser gas pressure, to optimise ion responses in CE-ESI-MS [101], and even so, each sample matrix can have a huge effect on the migration behavior of analytes potentially to compromise its reproducibility.

The major obstacle in plant metabolomics studies is that the diversity and variability of the physicochemical properties encountered in the metabolome is so high that none of the currently available MS techniques can analyse all metabolites simultaneously. Moreover, the quantification of the metabolites encountered is challenging because signal intensity in MS is not only a function of concentration or mass but also depends on the chemical structure of the analytes and can be influenced by matrix interferences, as observed in ESI. Overall, the high selectivity of mass spectrometers in combination with low-detection limits, as well as their compatibility with separation techniques and their ability to deliver quantitative data, makes MS an ideal tool for metabolomics applications [11].

7.4 Metabolite Responses to Drought Stress

Plants are sessile organisms, regularly threatened by the failure to escape unfavourable environmental perturbations such as drought stress. To maintain homeostasis, several response mechanisms must be activated in parallel with fine adjustments in plant growth and development to allow rapid adaptation and survival [102]. The adjusting pathways require extensive reprogramming of metabolism and gene expression, and comprise stress sensors or receptors, signaling cascades that

include an extensive complex of protein–protein interactions, transcription factors, and promoter elements, and ultimately, output proteins and metabolites [103–107].

Drought is considered one of the main environmental factors limiting plant growth and yield. Predictions have recently suggested that in the near future climate changes will most likely be associated with even worse problems, leading to frequent periods of drought as well as threats to both natural and agricultural ecosystems [108]. Accordingly, drought leads to several morphological and physiological changes in plants. Such alterations occur at both temporal (e.g., vegetative and reproductive stages are distinctly affected) and spatial scales (e.g., distinct organs and tissues present different behaviors; [109]. Those changes include reduction in shoot growth [110], while root growth is maintained [111], decreases in photosynthesis and transpiration rates as a direct consequence of the closure of leaf stomata mediated by abscisic acid (ABA [112, 113], changes in signalling pathways [112], activation of detoxification processes [114], reduction in tissue water potential, and transcriptional and posttranscriptional regulation of several stress-related genes [115, 116]. To survive, almost all organisms, ranging from microbes to animals and land plants, have evolved to synthesise high amounts of osmolytes also known as compatible solutes (e.g., amino acids, water-soluble carbohydrates; Sect. 7.4.1) in response to water stress [117].

Advances in the understanding of the metabolic reprogramming in plants under stress conditions have largely benefited from the contribution of highly sensitive MS-based platforms, and modern plant metabolomics programs now use MS as a powerful tool for dissecting the complex adaptive mechanisms underlying drought stress as well as for studies of gene function for genetic engineering programs targeting water-stress tolerance. In the subsequent section, we discuss recent applications of metabolomics to study plant responses to drought stress, including the accumulation of key drought-stress—responsive metabolites.

7.4.1 Compatible Solutes and Osmotic Adjustment in Drought-Stress Tolerance and Desiccation

One strategy that plants have adopted to cope with drought-stress situations is to fine-tune their metabolism to mobilise metabolites for the synthesis of vital protective compounds for osmotic adjustment. This process involves the net accumulation of osmolytes in the cell to save or even stimulate the water uptake into the cell by osmosis to maintain turgour pressure. As a consequence of this osmolyte accumulation, a decrease in the osmotic potential of the cell is observed and the turgour pressure is maintained as the cell uptakes water. Consequently, osmotic adjustment is commonly recognised as an effective factor of drought tolerance in several plants to enable water uptake and the maintenance of plant metabolic activity, hence, growth and productivity as the water potential decreases [112, 113].

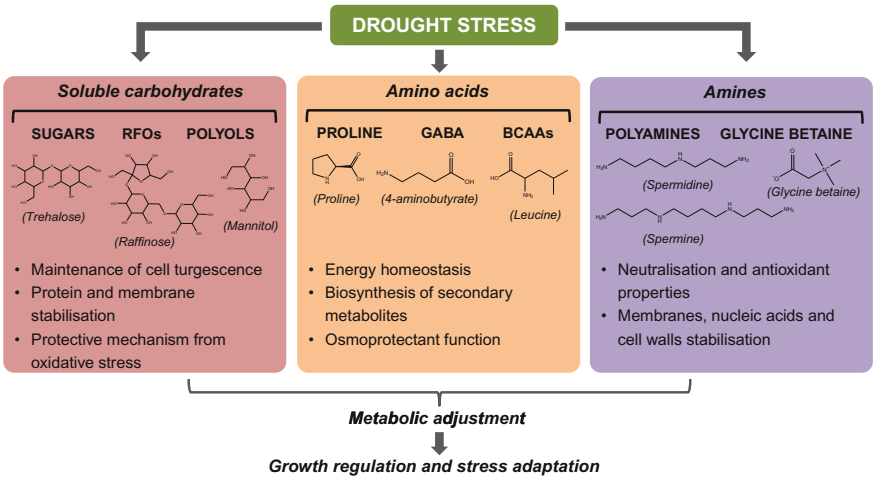

Fig. 7.1 Osmolyte compound classes. In response to drought stress, plant growth and development undergo fine metabolic adjustments as an adaptive response for stress tolerance

Osmolytes or compatible solutes are highly soluble, small-molecular–weight organic compounds that do not inhibit cellular metabolism, thus 'compatible', even at high concentrations, and exhibit osmoprotective properties on cells and membranes [21, 114]. Common osmolytes include soluble carbohydrates (e.g., glucose, sucrose, trehalose), the RFOs (raffinose, stachyose, and verbascose), polyols (e.g., mannitol, sorbitol), amino acids (e.g., proline, branched-chain amino acids), quaternary ammonium compounds (e.g., glycine betaine), as well as polyamines (e.g., putrescine, spermidine, and spermine; Fig. 7.1).

The accumulation of compatible solutes in plant cells is generally recognised to be of pivotal importance not only because compatible solutes help in stabilising membranes, enzymes, and proteins, or maintaining cell turgour by osmotic adjustment, but also because their accumulation confers protection against oxidative damage by decreasing the levels of ROS which in turn helps re-establish cellular redox balance. It has long been known that drought increases the risk of oxidative stress by increasing the production of ROS in different cellular compartments [118]. It has also been demonstrated that degradation of proteins damaged by oxidative stress generate specific peptides that can act as secondary ROS messengers and might contribute to retrograde ROS signaling during stress situations [119]. Several ROS forms have the ability to interact with proteins, lipids, and DNA during abiotic stress episodes, and thus impair the normal function of cells [17, 19, 120].

Although the osmoregulatory mechanisms involved in drought-stress tolerance and desiccation are still far from being completely understood, it is generally accepted that the increase in the levels of osmolytes must always be coordinated with both the osmotic and metabolic needs required for plant survival under drought stress [114].

7.4.1.1 Soluble Carbohydrates (Trehalose, RFOs, and Polyols)

As previously mentioned, carbohydrate metabolism as long been known to play an important role in osmoprotection during dehydration. Dehydration-treated plants have a greater need to adjust osmotically to alleviate the loss of cell turgour, and consequently, the levels of some water-soluble carbohydrates and some polyols increase in plant cells [9, 102, 113, 114].

Trehalose is a nonreducing sugar commonly synthesised in nature via consecutive enzymatic reactions: (i) trehalose-6-phosphate synthase (TPS) generates the intermediate trehalose-6-phosphate (T6P) from UDP-glucose and glucose-6-phosphate followed by (ii) a dephosphorylation reaction to trehalose catalysed by trehalose-6-phosphate phosphatase (TPP; [121]. In nonvascular plants, trehalose is known to act as a stress protectant against cellular damage caused by unfavourable environmental conditions as well as to be particularly strongly involved in mechanisms of protein and cellular membrane stabilisation in response to drought [122]. However, the majority of angiosperms, except for those highly desiccation-tolerant or resurrection plants [123, 124], accumulate insignificant amounts of trehalose, and abiotic stress periods only slightly modified their content; under these conditions, a direct role of this osmolyte in mediating osmotic adjustment has been excluded [125, 126].

Fructose and sucrose are also known to function as osmoprotectant molecules, and when related to elevated concentrations of raffinose, the levels of these osmolytes were shown to act jointly in the dehydration tolerance of wheat seedlings [127]. Similarly, Urano and coworkers observed increased levels of fructose in Arabidopsis plants under drought [34]. On the other hand, sucrose was shown to replace proline as the key osmolyte during a more severe combined drought and heat-stress treatment in Arabidopsis leaves [128].

Another important class of nonreducing sugars widely distributed in the plant kingdom is the raffinose family oligosaccharides, namely raffinose, stachyose, and verbascose. RFOs biosynthesis begins with the production of galactinol from *myo*-inositol and UDP-galactose, a reaction catalysed by galactinol synthase (GolS). Sequential additions of one, two, or three galactose units linked to the glucose moiety of sucrose via α(1–6) glycosidic linkages leads to the production of raffinose, stachyose, and verbascose, respectively [67]. An LC-MS targeted metabolite method was established by Antonio and coworkers to investigate the influence of drought stress on carbohydrate metabolism of *Lupinus albus* stem tissues [72]. This approach allowed the separation and detection of a set of 12 water-soluble organic osmolytes that ranged from mono-, disaccharides, and RFOs to polyols. This study revealed a general accumulation of these molecules, including raffinose, during drought stress, and although the role of RFOs in drought tolerance is not entirely understood, increased evidence has been reported for the strong correlation between accumulation of RFOs and the development of desiccation tolerance [72, 73, 129–133]. Interestingly, the predominant water-soluble carbohydrate in the roots of the resurrection plants of the genus *Craterostigma* subjected to drought is the tetrasaccharide RFO stachyose [134]. During drought episodes, stachyose was shown to be mobilised to sucrose in the roots, but has also been suggested that

stachyose is translocated to other tissues to support carbohydrate metabolism during desiccation [134]. Furthermore, stachyose has been reported to be the major phloem-mobile water-soluble carbohydrate in *Craterostigma plantagineum* [135]. A recent study demonstrated that stachyose accumulation in the leaves of *Craterostigma* species undergoing desiccation is likely to be due to de novo synthesis (along with other RFOs, such as raffinose and verbascose) to exert a further mechanism of protection in desiccated leaves [136].

Wide-ranging analyses with several omics approaches have been the subject of current research of plant responses induced by drought [73, 137–139]. One example is the work developed by Gechev and collaborators who merged transcriptomics and metabolomics tools to examine the mechanisms of desiccation tolerance of the resurrection plant *Haberla rhodopensis* in four different dehydration states (well-watered, partially dehydrated, desiccated, and rehydrated [73]. In this study, the most abundant transcripts in fully hydrated and rehydrated plants encoded proteins involved in carbohydrate metabolism (e.g., genes that encode galactinol synthases and a stachyose synthase, as well as genes that encode sucrose synthases and sucrose-6-phosphate synthases); those results are likely to indicate the importance of carbohydrate metabolism to protect cells during water shortage situations. In addition, genes encoding proteins that prevent stress-related cellular damage and participate in antioxidant defense (e.g., late embryogenesis abundant proteins or LEA proteins) were found to be the most abundantly upregulated group of genes in response to desiccation. In this study, GC-TOF-MS metabolite profiling combined with two different LC-MS targeted metabolite approaches allowed extensive coverage of the metabolome of *H. rhodopensis*. Altogether, results revealed that upon dehydration, sucrose, maltose, and the RFOs stachyose and verbascose accumulated in *H. rhodopensis* to significantly high levels; those data suggest an adaptive feature to survive dehydration [73].

Yobi and collaborators achieved a global unbiased metabolic profiling in the desert plant *Selaginella lepidophylla* with a combination of two autonomous platforms based on ultra-high–performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) and GC-MS [140]. In that work, the metabolite profile of *S. lepidophylla*, a resurrection species renowned for its ability to survive almost complete desiccation, was examined at several hydration levels during a rehydration/dehydration cycle. The carbohydrate profile revealed that trehalose, sucrose, and glucose accumulated the most. However, although the relative x-fold levels of trehalose were higher than sucrose and glucose, they did not change upon desiccation. This observation might exclude a direct role of trehalose to mediate osmotic adjustment [114]. Amongst the several metabolite changes observed during the rehydration/dehydration cycle in *S. lepidophylla*, some specific metabolites related with glycolysis/gluconeogenesis (e.g., glucose-6-phosphate, fructose-6-phosphate, pyruvate), TCA cycle (e.g., 2-oxoglutarate, succinate, fumarate, oxaloacetate), and polyols (e.g., sorbitol, myo-inositol, and mannitol) were more abundant in the hydrated state. The highest levels of polyols in the hydrated treatment were suggested to be of pivotal importance for resurrection plants to slow the rate of water loss during desiccation as well as water uptake during rehydration [140].

Polyols, such as the straight-chain mannitol and galactinol, are also commonly associated with desiccation protection, essentially due to their primary function as macromolecule stabilisers and ROS scavengers, thus protecting cells from oxidative damage during stress [141]. Abebe and coworkers reported that, in mild concentrations, mannitol might increase tolerance to both drought and salt stress in wheat plants by facilitating the development of biomass under stress conditions [142]. In addition, Nishizawa and coworkers observed that a sixfold increase in the levels of galactinol shows a protective mechanism of cellular metabolism from oxidative stress caused by several types of abiotic constrains including drought [143]. On the other hand, increased mannitol levels usually lead to severe abnormalities in plants, such as sterility and growth arrest [142].

7.4.1.2 Amino Acids (Proline, GABA and Others)

Amino acids are amongst the most important metabolites within living systems. Not only do they serve as the basic components of proteins, but they are also intermediates of metabolic pathways leading to the synthesis of multiple primary and secondary metabolites serving diverse functions including energy homeostasis and plant responses to various abiotic and biotic stresses [144].

Accumulation of several amino acids has been observed in many plants exposed to drought [34, 73, 106, 107, 145, 146, 147]. The overall accumulation of amino acids upon stress is likely to stem from amino acid biosynthesis and/or from increased protein breakdown induced by biotic or abiotic stress situations [9]. Although the increased levels of amino acids might denote cell impairment in certain species, such as barley [148], increased contents of particular amino acids should have a positive effect during stress adaptation [9].

Under several abiotic stress conditions (e.g., cold, salt, and drought) proline accumulates and functions as an osmoprotectant [149, 150]. In addition, several works reported that proline may act as a ROS scavenger and/or a molecular chaperone protecting cells from structural damage by protein stabilisation [20, 150, 151]. It is also well known that proline levels are usually determined through the balance between biosynthesis and catabolism [151]. In this sense, proline synthesis in the cytosol is stimulated by stress conditions whereas situations of stress recovery are able to increase proline catabolism in mitochondria. However, it has been suggested that plants that were engineered to overaccumulate proline to enhance their tolerance to abiotic stress [152–155] might not be resistant to field conditions [156, 128], especially due to the fact that proline can be toxic to cells in some situations, mainly if it is not appropriately removed from the cell system [153, 156–158]. Moreover, it has been reported that the effect of proline during dehydration tolerance might be lower than that of some water-soluble carbohydrates [159].

It is well established that both aspartate and glutamate are mainly used as N and C donors for the biosynthesis of amino acids and organic acids [105, 160]. Those amino acids are central regulators of C/N metabolism, interacting with multiple metabolic networks [145]. Accordingly, recent studies have suggested interplay between the so-called γ-aminobutyric acid (GABA) shunt and the TCA cycle through several bypasses [160]. It is well known that the nonprotein amino acid GABA is mostly metabolised via succinic semialdehyde dehydrogenase to succinic acid, therefore fueling the TCA cycle via the GABA shunt [83, 161]. Antonio and coworkers have recently used ^{13}C- and ^{15}N-labeled substrates for studying metabolism under hypoxia using wild-type roots of the crop legume soybean (*Glycine max*). ^{13}C-pyruvate labelling was performed to compare flux through the TCA-cycle, fermentation, alanine metabolism, and the GABA shunt, whereas ^{13}C-glutamate and ^{15}N-ammonium labelling were performed to address the flux via glutamate to succinate. An efficient alternative carbon flux that would explain the accumulation of alanine, GABA, and succinate upon hypoxia via pathways mediated by alanine metabolism and the GABA shunt was suggested to be of pivotal importance for plant survival under hypoxia [88]. In contrast, the stimulation of the GABA shunt in response to an increased requirement for TCA cycle intermediates has been shown to play a central role to support the biosynthesis of secondary metabolites for plant survival under drought-stress conditions [162]. Furthermore, abiotic stress seems to increase cytosolic Ca^{2+} levels, which stimulates calmodulin-independent GABA decarboxylase (GAD) activity and further GABA synthesis [163].

7.4.1.3 Branched-Chain Amino Acids (Valine, Leucine, and Isoleucine)

Several recent studies have suggested an important role of branched-chain amino acids or BCAA (classified by their small branched hydrocarbon residues) in the metabolism of plants under water-deficit conditions. BCAAs (e.g., leucine, isoleucine, and valine) levels increase under water deficit in a widespread range of species including Arabidopsis [138, 164], tomato [165], barley [106], maize [107], the resurrection glacial relic *H. rhodopensis* [73], legumes of the genus *Lotus* [105], and grasses of the *Sprobolus* family [166]. For instance, Urano and coworkers have observed that in Arabidopsis the level of BCAA increased under drought conditions and that such increase seems to be regulated at a transcriptional level [34]. Similarly, the activities of some enzymes related to the catabolism of BCAA showed rapid increase in response to abiotic stresses [145], and therefore it is reasonable to suggest that those enzymes might play an important role in the metabolism of BCAA under stress situations.

In addition, transgenic maize plants overexpressing a gene that encodes an ABA-, stress-, and ripening-induced protein (*ZmASR1*) showed higher tolerance to water deficit in comparison to the WT counterpart [147]. Remarkably, those plants presented a significant decrease in the levels of BCAA under conditions of limited water availability. It was suggested that the increased biomass observed in those plants is related with the transcriptional regulation of genes involved in the biosynthesis of BCAA [147], which might indicate that the degradation of such amino acids is intimately related with increased water-stress tolerance in those plants. Finally, Pires and coworkers have recently used transgenic and GC-MS approaches to study the potential role of BCAA in Arabidopsis plants under water-deficit episodes. The results highlighted that BCAA catabolism, but not the accumulation of BCAAs per se, seem to play an important role in the tolerance mechanisms to short-term episodes of drought, most likely by delaying the stress onset [167]. In agreement, Malatrasi and coworkers reported that an increased degradation of BCAA under drought could provide an alternative carbon source for the TCA cycle or detoxification mechanism by maintaining the pool of free BCAA at levels compatible with cellular homeostasis during stress situations [168].

Isoleucine biosynthesis is highly coordinated with both leucine and valine biosynthesis [145]. Although there is now evidence for a further mitochondrial BCAA transaminase (BCAT) [169], the BCAA biosynthesis seems to occur exclusively in plastids where valine and isoleucine are formed in two parallel pathways using four common enzymes, namely acetohydroxy acid synthase (AHAS), ketolacid reductoisomerase (KARI), dihydroxy-acid dehydratase (DHAD), and BCAT, whereas leucine synthesis branches off from 2-oxoisovalerate, the last intermediate of the valine biosynthetic pathway, to follow a three-step chain elongation catalysed by isopropylmalate synthase (IPMS), isopropylmalate isomerase (IMPI), and isopropylmalate dehydrogenase (IPMDH), which ends with a transamination catalysed by a BCAT [170, 171]. Interestingly the transcripts of the *ZmAHAS1*, *ZmKARI1*, *ZmKARI2*, and transaminase *ZmBCAT4* were upregulated in maize under drought [147], indicating a fine-tuning of biosynthetic genes under the abiotic stress condition.

7.4.1.4 Amines (Polyamines and Glycine Betaine)

Polyamines (e.g., putrescine, spermidine, spermine) are small nitrogen-containing compounds ubiquitous in nature and also involved in plant responses to several types of stress, such as drought [172–178]. Polyamines can occur as free bases, as conjugates to small molecules such as phenolic acids (conjugated forms), and as conjugates to macromolecules such as proteins (bound forms [179, 180]). These metabolites are well known by their antisenescence and antistress effects due to the neutralisation ability and antioxidant properties, as well as the capability of stabilising membranes, nucleic acids, or cell walls [175].

Do and collaborators used a GC-TOF-MS platform to identify and quantify the relative pool sizes of metabolites related to polyamine metabolism in 21 rice cultivars (*Oryza sativa* L. ssp. *indica* and *japonica*) exposed to moderate long-term drought stress. The combination of transcriptomics and metabolomics analysis data was consistent with a synchronised adjustment of polyamine synthesis for accumulation of spermine under drought conditions. In addition, the pool size of the amino acid arginine, the main substrate for polyamine synthesis, increased under water-deficit episodes, indicating that this pathway was not substrate downregulated. Altogether those results are in good agreement with a role of polyamine metabolism to protect plants against drought stress [178].

Several genes related to the metabolism of polyamines have been identified and expression profiles have been analysed under different stress conditions [181]. Many authors have reported the increased expression of arginine decarboxylase 2 (*ADC2*), spermidine synthase 1 (*SPDS1*), and spermine synthase (*SPMS*) genes in plants under limited water availability conditions [178, 182, 183]. Furthermore, transgenic rice plants overexpressing *ADC* and S-adenosylmethionine decarboxylase (*SAMDC*) genes presented a significant increase in putrescine levels, the polyamine precursor of spermidine and spermine [184, 185]. Interestingly, increased expression of *ADC2*, *SPDS1*, and *SPMS* genes after water deficit seems to be an ABA-dependent response [183], once gene expression induction was not observed in Arabidopsis deficient (*aba2*) or insensitive (*aba1*) mutants to ABA. Furthermore, the same authors have also observed the presence of dehydration-responsive element-binding as well as ABA-responsive element-binding in promoter regions of *ADC2*, *SPDS1*, and *SPMS* genes. Such results indicate that ABA regulates expression of many genes related to polyamine biosynthesis, particularly under environmental stress conditions [183].

Finally, the quaternary ammonium compound glycine betaine is widespread in the plant kingdom, albeit it presents sporadic distribution among plant species [186]. Some authors have reported the natural glycine betaine accumulation in response to drought stress [187–189]. It has been widely accepted that glycine betaine is able to protect plant structures by stabilising proteins and membranes in vitro [190, 191], as well as to function as a molecular chaperone [191, 192].

7.5 Metabolic Engineering of Compatible Solutes for Drought Tolerance in Plants

The ability to synthesise most of the osmoprotectants that are commonly accumulated by stress-tolerant plants is not usually reported in major crops. In this sense, the development of engineered stress-sensitive crop plants for osmolyte accumulation traits through conventional plant breeding, marker-assisted selection, or genetic engineering is expected to offer novel and exciting strategies to develop new cultivars with enhanced abiotic stress tolerance [155, 193–197].

Although traditional breeding and marked-assisted approaches have been long employed, and several drought-tolerant genotypes have been generated over the years [198, 199], those strategies are limited by the complexity of drought-tolerance traits, low genetic variance of yield-related components, and the absence of effective selection methods [200, 201]. In contrast, metabolic engineering strategies seem to be a more effective and rapid methodology to improve drought tolerance [202]. Metabolic engineering is the directed improvement of cellular properties through the modification of specific biochemical reactions or the introduction of new pathways for the biosynthesis of osmoprotectants into plants, using the recombinant DNA technology [202, 203]. Genetic modification for enhanced desiccation tolerance is commonly based on the manipulation of transcription factors, signalling-related enzymes or genes related to biosynthesis of osmoprotectants [204].

In recent years, metabolomics approaches have been employed in the search of metabolic markers for drought tolerance due to their close relationship with yield phenotypes [205–207]. In this sense, the identification of promising metabolite marker candidates jointly with the well-established techniques of identification of drought-related genes can be expected to provide more prospects for drought-tolerance engineering [207–209]. Manipulation of biosynthetic/catabolic genes involved in osmoprotectant accumulation (e.g., trehalose, mannitol, proline, and glycine betaine) has been the major target to engineer plants that have enhanced drought tolerance [204]. In this section, we illustrate a few examples of how metabolic engineering might help in the accumulation of osmoprotectants in plants.

Efforts to increase trehalose biosynthesis in tobacco [210], rice [125, 211], potato [212], and tomato [213, 214] led to the generation of drought-tolerant transgenic plants overexpressing genes encoding key biosynthetic enzymes of trehalose metabolism, namely TPS and TPP, under the control of tissue-specific, stress-responsive, or constitutive promoters. Transgenic Arabidopsis plants overexpressing *AtTPS1* (one of the eleven putative *TPS* genes identified in Arabidopsis) also exhibited drought-stress tolerance as well as ABA and glucose-insensitive phenotype [215]. Similarly, it was recently demonstrated that the overexpression of *TPP* in maize ears improved yield in mild (from 9 to 49 % of grain yield) and especially severe (from 31 to 123 % of grain yield) drought conditions relative to yields from nontransformed controls [216]. Transgenic plants overexpressing the trehalose biosynthetic genes present, however, some negative pleiotropic effects, such as stunted growth, low stomatal density, reduction in sucrose levels, lancet-shaped leaves, and aberrant development of roots [215, 217]. Nevertheless, Garg and coworkers reported drought tolerance in rice by engineering trehalose overproduction without the negative pleiotropic effects observed in other works. The expression of a bifunctional *TPS* fusion gene resulted in elevated glucose, fructose, and sucrose levels, confirming the pivotal role of trehalose in sugar sensing, carbon metabolism, and desiccation tolerance [125].

Some polyols such as mannitol, myo-inositol, and sorbitol have been targeted for the engineering of osmoprotectant accumulation. Heterologous expression of

mannitol-1-phosphate dehydrogenase (*mtlD*) from *Escherichia coli*, which is responsible for the reversible conversion of fructose-6-phosphate to mannitol-1-phosphate in wheat led to improved tolerance to water deficit [142]. However, the levels of accumulated mannitol in transgenic plants were not significant to account for its drought-tolerance effects as an osmolyte, indicating that the beneficial effect of mannitol resulted from protective mechanisms such as scavenging of ROS or stabilisation of macromolecules [142]. Furthermore, in a recent field-scale effort, Obata and coworkers reported that myo-inositol is a quite promising metabolic marker for breeding of drought-tolerant maize by analyses of metabolite profiles of leaves and comparison with grain yield in field trials [207].

The increase of proline levels in plants under drought is caused by both the activation of its biosynthesis and by the inhibition of its degradation. Transgenic approaches have shown that the overexpression of the gene that encodes Δ-1-pyrroline-5-carboxylate synthetase (P5CS) in tobacco, soybean, and petunia, an enzyme directly related to proline biosynthesis, led to increased levels of proline as well as drought tolerance [152, 218–220]. On the other hand, Arabidopsis *p5cs1* knockout mutants and antisense Δ-1-pyrroline-5-carboxylate reductase (*P5CR*) transgenic soybean plants were compromised in proline biosynthesis and resulted in increased sensitivity to drought [221, 222].

Metabolic engineering for glycine betaine biosynthesis in nonaccumulating organisms has been commonly reported [223, 224]. Transgenic approaches have shown that the introduction of glycine betaine biosynthesis related-genes (e.g., betaine aldehyde dehydrogenase, *BADH*; choline oxidase, *COD*) into nonaccumulator plants, such as tomato [225], potato [223], and Arabidopsis [226], led to the accumulation of glycine betaine, and consequently, tolerance to drought stress improved.

In addition to the use of drought-responsive promoters for engineering osmolyte biosynthesis, the manipulation of some stress signal sensing-related genes is a powerful tool for the development of drought-tolerant plants. Furthermore, inasmuch as oxidative stress is a component of drought, improvement of the ROS scavenging system, the stimulation of chaperone-like activities that protect macromolecule structure and metabolic detoxification are expected to confer tolerance to drought in plants [227]. This could be achieved either via reiterative engineering or by crossing and selecting transgenic plants engineered for different traits [208]. Altogether, those strategies aim to improve yields thereby leading to a substantial impact on worldwide food production.

7.6 Future Perspectives

When the environment is adverse and plant growth is affected, metabolism is profoundly involved in signalling, physiological regulation, and defense responses. In parallel, abiotic stress conditions also affect the biosynthesis, transport, and storage of primary and secondary metabolites, and to sustain cellular homeostasis in

response to such adverse conditions, plants undergo fine metabolic adjustments at the central metabolism level. A common defensive mechanism activated in plants exposed to abiotic stress conditions is the production and accumulation of highly soluble compatible solutes. Recent advances in our understanding of the metabolic pathways for biosynthesis and catabolism of compatible solutes, their regulation, and participant enzymes, has led to novel strategies for the improvement of plant tolerance that involve the net accumulation of those protective compounds. In this chapter, a large amount of research has been described which provides evidence that enhanced accumulation of compatible solutes correlates with reinforcement of plant resilience when availability of water in the soil is limited. Future progress on this research topic will lead to novel strategies for plant fitness under adverse growth conditions to sustain the ever-growing needs for food and feed worldwide.

Metabolomics is one omics approach that can be used to acquire comprehensive information on the composition of a metabolite pool providing a functional screen of the cellular state. By quantifying the changes taking place inside cells at specific times and under specific abiotic stress conditions, metabolomics offers new insight into cellular biology and a new path of research into the development of abiotic stress-tolerant crops. Although MS-based metabolomics has proven to be a valuable tool due to its high sensitivity and selectivity compared to other technological platforms, we still face huge challenges in terms of the dynamic range of current commercially available MS systems. Innovative approaches are required to elevate the coverage of metabolite analyses to a truly metabolomics (comprehensive) scale, and therefore, enhance our ability to identify stress-responsive metabolites in a high-throughput manner.

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Chapter 8
MicroRNAs: A Potential Resource and Tool in Enhancing Plant Tolerance to Drought

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8.1 Introduction

Drought is the single most devastating environmental stress limiting crop productivity. According to the Food and Agriculture Organization of the United Nations (FAO), more than 11 million people have died since 1900 as a consequence of drought (http://www.fao.org/docrep/017/aq191e/aq191e.pdf). Just in Australia in 2006, 46 % of wheat yield was lost to drought (http://www.fao.org/docrep/017/aq191e/aq191e.pdf). Increases in global temperature and human population will put further demands on food production systems. Therefore, it is of the highest priority to develop drought-tolerant cultivars with high yield potential and stability. The following strategies can be adopted to provide information to help achieve this target. Firstly, using plant physiology to understand the complex network of drought-related traits; secondly, using molecular genetics to discover quantitative trait loci (QTLs) that affect yield under drought or the expression of drought-tolerance–related traits; and finally using molecular biology to identify drought-responsive genes which can represent candidates for the genes controlling tolerance QTLs as well as genes that can potentially be used in transformation for engineering drought tolerance [14]. Because drought tolerance is a genetically complex trait, a successful transgenic strategy would likely rely on transformation with gene regulators that affect key processes. MicroRNAs (miRNAs) are one such class of gene regulator and are expected to play a pivotal role in drought tolerance.

MiRNAs are small single-stranded noncoding RNA molecules, 20 to 24 nucleotides (nt) in size, and are widely distributed in various organisms. Some

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miRNAs are evolutionally conserved across plant species. MiRNAs are a quite a different class of gene regulator from transcription factors (TFs). They regulate gene expression at the levels of translation, transcription, and posttranscription, and participate in almost every aspect of biology, including developmental, physiological, and pathological processes [90, 139]. Many genes including TFs, such as HD-ZIP III [11, 20, 166], SCARECROW-like (SCL) [83], APETALA2 (AP2) and AP2-like [5, 18, 161], the auxin response factor (ARF) family [88, 123, 155], the squamosa promoter binding protein (SBP) family, and CUP-SHAPED COTYLEDON (CUC1) and CUC2 [6, 39, 65], are controlled by miRNAs. Several miRNAs (e.g., miR159, miR160, miR164, miR167, miR393, miR397b, miR402, and miR413) also modulate key components of hormone signalling pathways, hormone homeostasis, and plant responses to hormones [78, 111, 119, 128, 157]. However, the overall regulatory scheme has yet to be completely defined. This chapter presents an overview of miRNA biogenesis and functional mechanisms in plants, miRNA responses to drought, and regulatory networks of drought-responsive miRNAs and their targets, and suggests some strategies for using miRNAs to genetically improve plant tolerance to drought stress.

8.2 Discovery, Biogenesis, and Functional Mechanisms of miRNAs

8.2.1 Discovery of miRNAs

MiRNAs were first discovered in 1993 in the nematode *Caenorhabditis elegans* [67]. At that time miRNAs were only considered as small temporal RNAs. In 2001, they were formally named and recognised as a distinct class of RNAs with regulatory functions [63, 64, 66]. Plant miRNAs were first discovered in *Arabidopsis* in 2002 [110]. Currently, 6843 miRNAs have been identified in 64 plant species (http://mirbase.org/, Release 20: June 2013). The rate of miRNA identification in plants is increasing rapidly with the availability of complete genome sequences, high-throughput sequencing methods, and improved computational and experimental protocols [48, 53, 59, 68, 72, 74, 77, 120, 126, 134, 156, 158, 162].

MiRNAs are well conserved in plants and animals, and negatively regulate gene expression [67, 110, 122]. Nt 2–8 of the miRNA, called the miRNA 'seed', determine the target specificity [124]. In contrast to most animal miRNAs which have partial complementarity to their messenger RNA (mRNA) targets, plant miRNAs have almost perfect Watson–Crick pairing with their mRNA targets. MiRNAs regulate gene expression at the levels of transcription, posttranscription and translation, and affect many biological processes [8, 116, 133]. MiRNAs play a pivotal role in plant adaption to various stresses including drought [34, 82, 127].

8.2.2 Biogenesis of miRNAs

MiRNAs are encoded by miRNA genes that are usually transcribed into primary miRNAs (pri-miRNAs) by RNA polymerase II [148]. Some miRNA genes, especially those with upstream Alu sequences, transfer RNAs (tRNAs), and mammalian wide interspersed repeat (MWIR) promoter units, are transcribed by RNA polymerase III (Pol III) [31]. Pri-miRNAs have a modified nt at the 5′ end (cap structure), multiple adenosines at the 3′ end (poly(A) tail), and stem-loop structures. They are subsequently processed into stem-loop molecules (pre-miRNAs) in the nucleus by the microprocessor complex consisting of the dsRNA-binding protein DiGeorge Syndrome Critical Region 8 (DGCR8 or 'Pasha' in invertebrates) and the RNase III enzyme Drosha (in animals) or Dicer (in plants) [24, 36]. Some miRNAs such as mirtrons originating from introns can bypass the microprocessor complex and directly enter the miRNA maturation pathway as pre-miRNAs [167]. Furthermore, a few miRNAs can be generated independently of the splicing pathway. However, their mechanisms of maturation have not yet been fully characterised [50].

Pre-miRNAs are further processed into a miRNA/miRNA* duplex with a two-nt overhang at the 3′ end by Dicer in both plants and animals [8, 52]. In plants, this process occurs in the nucleus where Dicer is confined, but in animals it occurs in the cytoplasm. Apart from Dicer, a double-stranded RNA (dsRNA) binding protein HYPONASTIC LEAVES1 (HYL1) and a C2H2 Zn-finger protein SERRATE (SE) also participate in processing to release the miRNA/miRNA* duplex [8, 52]. Before the miRNA/miRNA* duplex is transported from the nucleus to the cytoplasm by Hasty (HST), an Exportin 5 homologue [107], the 3′ overhang is methylated by RNA methyltransferase Hua-Enhancer1 (HEN1) to prevent the duplex from being degraded by SMALL RNA DEGRADING NUCLEASE (SDN) [71]. In the cytoplasm, one strand of the duplex with a less thermodynamically stable 5′ end is loaded into the Argonaut protein (AGO1) to form the miRNA-induced silencing complex (miRISC) [131]. This strand is termed the guide strand as it guides AGO1 to recognise mRNA targets and silence expression of these targets. In general, the other strand, termed the passenger strand or miRNA star (miRNA*), is rapidly degraded. However, some miRNA* can also be incorporated into the miRISC, where it is stabilised and contributes a function [42, 104]. In addition, some miRNAs can be derived from the loop sequences of the stem-loop structures and are functional [42, 103, 140]. Because of these exceptions, miRNA and miRNA* are currently widely replaced by the '3p' and '5p' suffixes according to their positions in pre-miRNAs.

8.2.3 Functional Mechanisms of miRNAs

The regulatory function of plant miRNAs is exerted by the following mechanisms: transcriptional gene silencing (TGS), posttranscriptional gene silencing (PTGS),

and translational inhibition (TI). All of these mechanisms rely on the base-pairing between miRNAs and their targeted mRNAs.

8.2.3.1 Transcriptional Gene Silencing (TGS)

TGS refers to the repression of transcription, and can result from the silencing of TFs, DNA methylation of promoter sites, or histone modification. The last two mechanisms prevent access of transcriptional machinery such as DNA-dependent RNA polymerase and TFs to target genes. MiRNA-mediated DNA methylation was first reported in plants in 2004 [7]. In that case, coding sequences of the two *Arabidopsis* TF genes *PHABULOSA (PHB)* and *PHAVOLUTA (PHV)* were heavily methylated downstream of the miR165/166-complementary site. However, it is still unclear how the block of methylation downstream of the miRNA binding site could influence the transcription of *PHB* and *PHV*. Other reported cases of miRNA-directed DNA methylation have generally been limited to approximately an 80-nt region around the miRNA and target binding site [145]. This differs from small interfering RNA (siRNA)-directed DNA methylation, which involves methylation of cytosine in all sequence contexts, across regions of several hundred to several thousand nt [16, 93].

MiRNAs have been found to direct AGO1 in a sequence-specific manner to their complementary target sites in the promoter regions of target genes, thereby preventing accesses by transcriptional machinery. Recently, a novel miRNA population of 23–27 nt generated by DCL3 was found to be specifically associated with the AGO4 protein and to mediate cytosine DNA methylation in both cis and trans [4, 17, 46, 58, 132, 144]. A plant-specific SNF2-like chromatin remodelling protein, DEFECTIVE IN RNA DIRECTED DNA METHYLATION1 (DRD1), is involved in the DNA methylation (Kanno et al. 2004). DRD1 enhances the target DNA methylation by reconfiguring chromatin [92].

8.2.3.2 Posttranscriptional Gene Silencing (PTGS)

PTGS involves degradation of transcribed targets, and is initiated by miRNA-guided cleavage executed by AGO1 in the miRISC at a position between nt 10 and nt 11 with respect to the miRNA. AGO1 is one of the AGO family members that contains four characteristic domains: N- terminal, PAZ, Mid, and a C-terminal PIWI domain [47]. The initial interaction between the PAZ domain, which contains a typical single-stranded nucleic acid binding motif [15], and the miRNA is essential for efficient cleavage [136]. In addition, a continuous A-form helix between miRNA and the targeted transcript is also required for target cleavage [21, 43]. After cleavage the generated 3′ and 5′ fragments characteristic of RNAse H activity [91, 121] are subsequently degraded via a natural degradation pathway. It is likely that the 3’ fragment is degraded by Xrn1 independently of the exosome components Rrp4 or Ski2, whereas the 5′ fragment is degraded by the exosome.

8.2.3.3 Translational Inhibition (TI)

TI results in the inability of the mRNA to be translated into a protein. This process also takes place in the cytoplasm. However, unlike PTGS, TI does not require a perfect sequence match between the miRNA and its target mRNA [8]. Although the role of miRNAs in TI remains unclear, miRISC, which contains miRNAs, has been shown to affect eukaryotic translation initiation factor 4F cap recognition, 40S small ribosomal subunit recruitment, and/or incorporation of the 60S subunit and the formation of the 80S ribosomal complex. Recent studies showed that GW182 in the miRISC is required for miRNA-mediated TI [37]. This protein directly interacts with AGO proteins and possibly mediates deadenylation [30], which is a primary mechanism of TI. In addition, GW182 may block translation initiation by preventing the 80S ribosome complex from forming, or by competing with the translation initiation factor 4F, which is required for efficient translation initiation [142]. However, it is noteworthy that targeted mRNAs without poly-A tails can still be subjected to miRNA-mediated TI [30]. This suggests that some other as yet unknown factors are involved in TI. Kong et al. [62] reported that miRNA-mediated TI might depend on the promoter of the targeted mRNA because different promoters determine whether TI happens at an early or late stage [62]. In view of these facts, the mechanism that a miRNA will adopt in mediating gene silencing will be determined by multiple factors such as cis and trans factors, the specific AGO protein and its associated proteins.

8.3 Responses of miRNAs to Drought Stress

Drought has been shown to affect the expression of many genes including miRNAs. Thus far a large number of miRNAs have been found to be differentially expressed under drought conditions. In *Arabidopsis*, some miRNAs such as miR156, miR159, miR167, miR168, miR171, miR172, miR319, miR393, miR396, miR397, and miR408 were upregulated, whereas others such as miR169, miR170, and miR417 were downregulated [32]. Interestingly, members of the same miRNA family could be influenced by drought differently. For instance, only two out of 14 members in the miR169 family in *Arabidopsis* (miR169a and miR169c) were substantially downregulated under drought conditions [73]. In rice, only one out of 17 members in the miR169 family (miR169 g) was induced by drought, and furthermore the induction of this member was more prominent in roots than in shoots [161]. All the other members of the miR169 family were not regulated by drought in these two plant species. In addition, the same miRNAs could have different expression patterns, depending on the plant species. For example, drought upregulated miR1510 and miR396 in *Glycine max* but downregulated them in *Medicago truncatula* [89]. Drought upregulated miR156 in *Vigna unguiculata, G. max, Triticum dicoccoides, Hordeum vulgare, Populus euphratica, Prunus persica*, and *Panicum virgatum* but downregulated it in rice, maize, and *Populus tomentosa*. Drought upregulated miR167 in *P. tomentosa* but

downregulated it in *P. Persica*. Drought upregulated miR408 in *M. truncatula* but downregulated it in rice, *P. persica*, *P. tomentosa*, and *Populus trichocarpa*. Drought upregulated miR398 in *T. dicoccoides* but downregulated it in *P. Persica*. Drought upregulated miR394 in *P. tomentosa* and *G. max* but downregulated it in *P. trichocarpa*. Drought downregulated miR395 in *P. tomentosa* and *P. Persica* but upregulated it in rice. Similarly, drought did not influence expression of miR160, miR164, miR166, miR394, miR395, miR398, miR399, miR474, miR1450, and miR2111 in *Arabidopsis* but did in other plant species.

MiRNA expression can also depend on the specific drought conditions. For example, in *M. truncatula*, miR398a/b was upregulated when the relative water content (RWC) was between 30 and 50 % [130] but was downregulated when it was between 68.3 and 87.4 % [135]. The sensitivity of some miRNAs to the type of drought may reflect the limited capability of miRNAs in response to drought stress or sensitivity of miRNA-regulating genes. It may also be the result of different spatiotemporal manner. The above examples indicate that the regulation of miRNAs by drought involves a lot of factors. This could be true because plants respond to drought via a range of physiological and biochemical mechanisms [10].

8.4 Correlation of Expression of Drought-Responsive miRNAs and Their Targets

Because miRNAs are gene regulators, the discovery that many miRNAs are differentially expressed under drought suggests that many genes are involved in the drought response. Indeed in *Arabidopsis*, 1700 out of the 22,500 genes analysed using a microarray were induced by drought stress, including 600 that were induced more than fivefold [13]. By employing methodologies such as quantitative real-time PCR (qRT-PCR), Western Blotting, 5′ rapid amplification of cDNA ends (5′ RACE), degradome libraries, and complementary experiments, many genes differentially expressed by drought have been identified to be targets of drought-responsive miRNAs. In plants these targets are mostly TFs, which can also in turn regulate the expression of miRNAs [41]. Some important TFs that are targeted by drought-responsive miRNAs are listed in Table 8.1. SQUAMOSA promoter binding protein-like (SBP or SPL) TF is one of them. This TF plays central roles in plant phase transition, tissue and architecture development, gibberellin signalling, sporogenesis, and response to copper and fungal toxins [19]. Previous study showed that this TF was targeted by miR156 [149]. Interestingly, among 10 miR156-targeted SPL genes (*SPL2, SPL3, SPL4, SPL5, SPL6, SPL9, SPL10, SPL11, SPL13*, and *SPL15*) in *Arabidopsis*, *SPL9* can in turn positively regulate the expression of miR172 by binding to the miR172b promoter, whereas other SPL genes such as *SPL10, SPL11*, and *SPL15* act redundantly in the transcriptional regulation of miR172 [144]. The targets of miR172 are *APETALA2* (*AP2*) and AP2-like TFs such as target of *EAT1* (*TOE1*) *TOE2, TOE3, SCHLAFMUTZE* (*SMZ*), and *SCHNARCHZAPFEN* (*SNZ*) [5, 18, 22, 161]. *AP2* is a floral organ

Table 8.1 Transcription factors targeted by miRNAs

Transcription factor	miRNA	References[a]
SPL	miR156/miR157	[125]
MYB	miR159, miR828	[1, 76]
ARF	miR160	[39]
NAC	miR164, miR319	[112, 164]
NFY	miR169	[102]
TCP	miR159, miR319	[1, 128]
AP2	miR172	[5]
AST	miR395	[2]
GRF	miR396	[128]
CSD	miR398	[38, 99]

[a]Only one reference per miRNA is given in the table due to the space limitation

identity gene [12, 27] whereas AP2-like genes mainly act as flowering repressors [5, 55, 90, 118]. Overexpression of AP2-like genes in *Arabidopsis* results in late flowering, whereas the loss of AP2-like genes promotes early flowering [5, 55, 118]. Early flowering is considered as a drought escape mechanism [44]. Thus, miR172 is expected to be able to contribute drought tolerance. Indeed, transgenic overexpression of miR172 has been shown to reduce water loss in *Arabidopsis* [44]. However, it is worth pointing out that overexpression of miR172 does not affect the expression of miR156, although overexpression of miR156 reduces the expression of miR172 [144]. In addition, overexpression of AP2-like genes or SPL genes in plants elevated the expression levels of both miR172 and miR156. These results indicate that miR156 and miR172 have a sequential action in regulating flowering time in which miR156 negatively regulates the expression of miR172 via *SPL9*, and miR172 only acts downstream of miR156. Overall, a possible feedback loop exists among miR156, miR172, and their targets.

SPL genes were also found to activate the transcription of several TF families including the MYB family. The MYB family of TFs are present in all eukaryotes, and in plants they are key factors in regulatory networks controlling development, metabolism, and responses to biotic and abiotic stresses. In *Arabidopsis* two MYB TFs (MYB33 and MYB101) regulate ABA-dependent germination [111]. Overexpression of cleavage-resistant MYB genes resulted in hypersensitivity to ABA during germination [111]. However, overexpression of miR159a reduced the levels of *MYB33* and *MYB101* transcripts and increased seed hyposensitivity to ABA [111]. This indicates that miR159 targets the MYB TFs [94] and negatively regulates ABA responses during seed germination [111]. ABA is known to have a role in the instigation and maintenance of seed germination and dormancy [115]. Thus, the miR159-guided cleavage of MYB TFs could possibly be a homeostatic mechanism to reset hormone signalling during germination under drought stress. However, this mechanism may also involve participation of other factors, because the MYB TFs

have been identified to bind to the *cis*-elements in dehydration-responsive gene *RD22* promoter and trigger *RD22* expression [26]. In addition, MYB genes respond to gibberellic acid (GA) during flowering [1]. Furthermore, a transcription regulator, AUXIN RESPONSE FACTOR 10 (ARF10), also modulates the response to ABA during germination [81]. It is worth noting here that *ARF10* is targeted by miR160. Overexpression of an ARF10 mutant gene which was resistant to miR160 cleavage displayed an ABA-hypersensitive phenotype during germination. This indicates that miR160-guided auxin inhibition has a role in the regulation of ABA-responsive genes. This in turn suggests that crosstalk may exist between auxin and ABA signalling during seed germination [81].

Another miRNA, miR319, also mediates cleavage of MYB TFs, but not significantly due to its low expression [105]. In vitro, miR319-mediated cleavage of MYB TFs was efficient [105]. The main targets of miR319 are TCP (TEOSINTE BRANCHED, CYCLOIDEA and PROLIFERATING CELL FACTORS (PCF)) TFs, which are involved in growth, cell proliferation, and setting organ identity in plants [98]. These targets cannot be regulated by miR159 despite that it is identical to miR319 in 17 out of the 21 nt [105]. A recent study showed that miR319 also targets a NAC gene, *AsNAC60* [164]. The NAC family, whose acronym is derived from three genes *NAM*, *ATAF*, and *CUC2*, is one of the largest plant TF families [113] and plays important roles in many processes including the response of plants to abiotic stresses such as drought [163]. Overexpression of NAC genes has resulted in enhanced drought tolerance [46, 97, 129, 152, 163]. Further studies demonstrated that NAC TFs directly bind to the promoters of drought-responsive genes [129, 152]. However, not all NAC genes can enhance drought tolerance. For example, *ATAF1* negatively regulates plant drought tolerance [84], and overexpression of *GmNAC11* increased sensitivity to drought stress [45]. Thus, whether miR319 can contribute drought tolerance would depend on which NAC gene is targeted. In a recently study, overexpression of osa-miR319a increased leaf wax content and water retention [164], indicating that miR319 itself can improve drought tolerance.

An MDR-like (*MDR1*) ABC transporter and NAC (*NAC1*) were found to be the targets of miR164 [147]. *NAC1* acts downstream of TRANSPORT INHIBITOR RESPONSE1 (*TIR1*) and is required to transmit auxin signals that enhance lateral root emergence [147], whereas *MDR1* helps determine auxin levels and controls lateral root growth rate [33]. The higher the auxin concentration in lateral roots the more *MDR1* is produced [141]. Expression of a mutant *NAC1* transgene resistant to miR164 in *Arabidopsis* increased the number of lateral roots [39]. By contrast, overexpression of miR164 reduced lateral root numbers, indicating that miR164 inhibits auxin signalling and lateral root formation by downregulating *NAC1* and *MDR1*. Inhibition of lateral root development is considered as an adaptive mechanism to drought stress [150]. Delaying leaf senescence was found to be another function of miR164 [114], which is considered as an alternative drought-tolerance mechanism. Leaf senescence is closely linked to ethylene, whose production enhances leaf senescence [3, 86, 87].

SPL7, which is not the target of miR156, was found to regulate miR398 positively, whose overexpression in rice increased sensitivity to drought and salinity [85]. In vitro SPL7 directly binds to GTAC motifs in the miR398 promoter, which is essential and sufficient for the response to copper deficiency in vivo [154]. *SPL7* together with *SPL9* is also required for the expression of miR397, miR408, and miR857 involved in copper homeostasis and the expression of genes encoding several copper transporters and CCH (a copper chaperone) [154]. In addition to drought response, miR398 also responds to ABA and salt stress, but differently between plant species [49]. For example, under ABA treatment miR398 expression increased in poplar, but decreased in *Arabidopsis* [49]. The targets of miR398 are *CSD1* (copper/zinc superoxide dismutase 1), *CSD2* (copper/zinc superoxide dismutase 2), *CCS* (a copper chaperone of CSD1 and CSD2), and *COX5b–1* (a zinc-binding subunit of cytochrome c oxidase) [49, 51, 128]. Under oxidative stress these targets are upregulated, whereas miR398 is downregulated [127]. These regulatory patterns promote oxidative stress tolerance, which can be a component mechanism of drought stress tolerance [135]. Previous studies showed that miR398 also responds to many other factors such as Cu^{2+}, sucrose, ozone, pathogens, and herbicides [28, 48, 49, 127]. These data suggest that the involvement of miR398 in the drought response may be complicated.

Unlike miR398, miR408, which is activated by SPL7 and SPL9, targets the conserved plastocyanin-like protein family, whose expression levels are correlated with drought tolerance. The level of plastocyanin-like gene transcript decreased in tolerant rice cultivars relative to intolerant cultivars, whereas miR408 was the opposite [96]. This suggests that miR408 is involved in drought tolerance.

Five miRNAs, miR393, miR168, miR528, miR167, and miR169, are involved in the ABA and auxin signalling pathways. Of them, miR393 is upregulated by ABA [128]. Two auxin receptor genes, transport inhibitor response 1 (*TIR1*) and auxin signalling F-box 2 (*AFB2*), are the targets of miR393 [146]. *TIR1* positively regulates auxin signalling via degradation of Aux/IAA proteins [25]. Suppression of *TIR1* and *AFB2* reduced the expression of an auxin transporter (*OsAUX1*), thereby downregulating *OsTB1*, a tillering inhibitor [146]. Overexpression of osa-miR393 in rice increased tillering and inhibited horizontal root proliferation, which is an adaptive response against drought stress. This adaptive response is partially mediated by ABA, whose concentration in the dehydrating roots controls lateral root growth [150]. These data imply that miR393 is responsible for both cellular homeostasis and morphological adaption under drought stress.

Mitogen-activated protein kinase (MAPK) is a target of miR168. This protein kinase is a signalling molecule important for transmitting stress signals and ensuring plants' adaptive response to drought. In tomato, silencing *MAPK* resulted in reduced drought tolerance caused by impaired ABA and hydrogen peroxide (H_2O_2) signalling [69]. ABA induces antioxidant defence for scavenging reactive oxygen species (ROS) [137]. Another target of miR168 is the AGO1 gene, whose protein product is the key component of the RISC used to cleave miRNA-guided targets. Therefore, miR168 would not be suitable as a transgene for crop improvement.

Superoxide dismutase (*SOD*) and peroxidase (*POD*) are the two targets of miR528 in maize [137]. These two enzymes play essential roles in plant stomatal movement and antioxidant defence. In drought-treated maize leaves, the levels of SOD and POD increase with ABA, which is a result of the downregulation of miR528 [137]. Another target of miR528 may be plantacyanin which regulates plant reproduction [57], although this target has not been experimentally confirmed.

The ARFs that positively regulate adventitious rooting [40] can also be targeted by miR167 [82, 143]. Overexpression of miR167 in transgenic rice has been shown to reduce ARF gene expression and tillering number [82]. Sequence analysis showed that the ABA-responsive element (ABRE) shared by most ABA-responsive genes [95, 151] is enriched in the promoter region of miR167 [79]. Because ABRE has antagonistic action in regulation on auxin signalling for growth retardation of plants and ABA-responsive miRNAs have many target genes involved in the auxin signalling cascade [26], miR167 may have a regulatory action in drought-induced ABA-auxin signalling. Another target of miR167 in maize is phospholipase D (*PLD*). Downregulation of miR167 accumulated PLD in maize leaves at the early stage of drought [137]. PLD produces phosphatidic acid (PA) that binds to ABI1 protein phosphatase 2C (ABI1 PP2C) to achieve ABA-induced stomatal closure [137]. Both PLD and PA participate in the H_2O_2-induced activation of a MAPK cascade [137]. Therefore, the regulation of *PLD* by miR167 is via the ABA signalling and/or a MAPK cascade.

The miR169 family, especially the miR169g member, was one of the first miRNAs found to be drought-regulated. This family contains 397 members identified from various plant species (http://mirbase.org/, Release 20: June 2013). The targets of miR169 are nuclear factor Y (NF-Y) TFs composed of three unique subunits, NF-YA, NF-YB, and NF-YC. NF-Ys are present in all eukaryotes and are crucial for the expression of a number of drought-responsive genes [23]. A multidrug resistance-associated protein (SIMRP1) gene is also a target of miR169 [159]. Members of the NF-Y family of TFs are regulated in various ways by drought and ABA [73]. For example, of the five barley NF-YBs, only *HvNF-YB3* is upregulated by drought, and *HvNF-YB1* and *HvNF-YB4* were downregulated by ABA [75]. This variety in expression patterns of NF-Y TFs may reflect the degree of cleavage efficiency of miR169, whose expression also differs between members. In addition, it is also possible that miR169 cleaves transcripts of only certain NF-Y TFs. This has been the case for miR399, which cleaves its target, *PHO2*, via an imperfect base pairing complementarity [42]. Another possibility is that the differential expression may be due to the dehydration-responsive element (DRE) and/or ABRE present in the promoter regions of NF-Y TFs and miR169 genes [102, 161]. DRE is a major *cis*-acting element that functions in ABA-independent gene expression during abiotic stress [153]. This could in turn suggests that the expression of NF-Y TFs is involved in both ABA-dependent and ABA-independent pathways.

Over-expression of several individual NF-Y subunits such as *AtNFYB1, ZmNFYB2, AtNFYA5, HvNF-YB1, OsNFYA2*, and *GmNFYA3* has given rise to ABA hypersensitivity and drought tolerance [73, 75, 100, 102]. Their regulators,

Table 8.2 Contribution of transgenic miRNAs to stress tolerance

Transgenic miRNA	Transgenic plant	Stress tolerance	References
miR169	Tomato	Drought	[159]
miR319	Bentgrass	Drought/salinity	[164]
miR394	Arabidopsis	Drought	[101]
miR395	Arabidopsis	Drought/salinity	[60]
miR402	Arabidopsis	Drought/salinity/cold	[61]
miR417	Arabidopsis	Salinity/ABA	[54]

such as TFs AtAIB, ABF3, ABF4, and AtSDIR1, also increased sensitivity to ABA [56, 70, 160], indicating that the NF-Y TFs cooperate with other genes to prevent water loss. Unlike the NF-Y TFs, the contribution of miR169 to drought tolerance differs between plant species. Although overexpression of miR169 resulted in drought tolerance in tomato [159], overexpression of miR169 in *Arabidopsis* and *Medicago* decreased drought tolerance [73, 135]. This could be related to different drought modified expression of the native miR169 among these plant species; in tomato, drought stress upregulates miR169, but in *Arabidopsis* and *Medicago* it downregulates miR169, fitting the inverse relationship observed between the levels of the miR169s and their NF-Y targets. These data provide examples where elements that are upregulated by drought as native genes, such as miR169 in tomato and NF-Y TFs in *Arabidopsis* and *Medicago*, can enhance drought tolerance when transgenically overexpressed.

Intriguingly, some NF-Y TFs can also influence flowering time. In *Arabidopsis*, overexpression of *NF-YB1* and *NF-YC*, but not *NF-YB3*, resulted in early flowering [9, 75], whereas overexpression of *NF-YA4* or *AtNF-YB1* delayed flowering [80, 138]. Taken together, these data indicate that drought-responsive miRNAs may function in drought tolerance by regulating the genes involved in ABA-dependent, ABA-independent, and auxin signalling pathways and that the miRNA-regulated genes such as *SPL, MYB, NAC, AP2*, and *NF-Y* may interact with each other. Table 8.2 summarises reports of drought-enhancing effects arising from transgenically overexpressing miRNAs, and also refers to cases where the transgenic plants showed enhanced tolerance to other abiotic stresses such as salinity and cold. This information shows that miRNAs could offer a valuable source and tool for enhancing plant tolerance to drought as well as to other stresses.

8.5 Strategies to Use miRNAs for Enhancing Plant Drought Tolerance

As described above, miRNAs have potential for use in engineering plant drought tolerance. The choice of the miRNA transgene, and the way it is expressed, will be critical for success of this approach. Generally, when expression of the native miRNA is positively correlated with drought tolerance, this miRNA needs to be overexpressed

in transgenics to achieve drought tolerance. Alternatively, the target genes of this miRNA need to be knocked out or knocked down to give tolerance. However, when the native miRNA expression is negatively correlated with drought tolerance, suppression of this miRNA, or overexpression of its target genes, should be considered. In some cases, drought-inducible or tissue-specific promoters may be needed to drive transgene expression to avoid negative side effects on plant performance. MiRNA expression can be inhibited using miRNA inhibitors, but it is not efficient. TALEN technology can also be used to knock out individual miRNAs [35, 109].

The knockdown of target genes can be achieved using their regulating miRNAs. To avoid unintentional targeting of additional genes by the transgene, which may otherwise produce unwanted side effects, artificial miRNAs (amiRNAs) have been adopted. AmiRNAs were first used in plants in 2004 for knocking down expression of specific genes [106]. AmiRNAs can also be used to knock down the expression of miRNAs [29]. The use of amiRNAs to improve drought tolerance of potato has been reported [108]. AmiRNA technology therefore has potential as a tool for engineering plant drought tolerance. Compared to siRNAs, which are also commonly used for knocking down the expression of particular genes, miRNAs and amiRNAs have the advantage that they can be made to be more specific [165] and furthermore, their silencing activities are stable through generations [117].

8.6 Conclusions and Perspectives

Plants have evolved physiological and biochemical mechanisms to minimise the damaging effects of drought, and these mechanisms can potentially be enhanced in strategies to engineer improved crop varieties. MiRNAs play a central role in such mechanisms. However, identification of the specific miRNAs conferring drought tolerance is challenging because the expression of miRNAs is often drought-regulated in a member-, species- and/or drought condition-dependent manner. This calls for further research with careful consideration of these factors. Two innovative strategies can be adopted to identify specific miRNAs with potential in engineering drought tolerance. Firstly, sequencing of miRNAs from drought-treated and -untreated samples using high-throughput next-generation sequencing (NGS) technologies can enable detection of drought-induced miRNAs to an unprecedented depth and accuracy. NGS can also be applied to identify miRNA-mediated cleavage sites across the whole transcriptome, an approach termed ‘degradome sequencing’. Secondly, the miRNA fraction of RNAs of drought stressed and unstressed tissues can be hybridised to cDNA microarrays to identify miRNA targets and identify differentially expressed miRNAs on a transcriptome-wide scale. For accurate detection and quantification of individual miRNAs and their targets, qRT-PCR is the best option. qRT-PCR can also be done on crude lysates of a single cell, and does not involve any complex downstream processing. *Cis*-regulatory elements in miRNA genes and their corresponding TFs, as well as regulatory networks of miRNAs and their targets, also need to be characterized.

These data could shed further light on mechanisms of miRNA-mediated drought tolerance, and could assist in the identification of the best miRNAs or miRNA targets to use in transgenic approaches for engineering plant drought tolerance.

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Chapter 9
The Response of Chloroplast Proteome to Abiotic Stress

Fen Ning and Wei Wang

9.1 Introduction

The chloroplast is the most studied plastid type harboring the pigment called chlorophyll and carries out photosynthesis as its main function. Besides, the chloroplast also participates in biosynthesis of fatty acid and lipid, hormone, amino acid, vitamin, secondary metabolites such as alkaloids and isoprenoids, as well as reduction of nitrite and sulfate [1]. All the autotrophs cannot live without the products of photosynthesis. However, photosynthesis efficiency is considerably dependent on the plant's ability to adapt to environmental conditions under which it lives. It has been well-known that abiotic stresses, such as drought, salinity, ozone, light, and high/low temperature tend to reduce the average yields for most crops extensively. The chloroplast is one organelle that is highly sensitive to abiotic stress and it plays a leading role in the modulation of stress responses [2]. Therefore, to understand the molecular mechanism of chloroplast response to abiotic stress for increased photosynthesis efficiency, more attention needs to be paid to the chloroplast.

In the chloroplast, the cellular signals identified thus far can be linked to certain stress conditions, and the mainly characterized signals are intermediates of the tetrapyrrole biosynthesis pathway, the redox state of the thylakoid membrane, and ROS [3]. Abiotic stress is thought to considerably alter the ultrastructure of the chloroplast and concentration of various pigments and metabolites including enzymes and other photosynthesis-related proteins thus hampers the process of

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photosynthesis in most plants [4]. For better understanding the intrinsic molecular mechanism of how plant cells modulate their protein expression network to cope with abiotic stress, a further study on the chloroplast proteome is of great value toward development of stress-tolerant crops, in the background of increasing world population and deficient crop productivity.

Recent advances in the proteomic field, especially the use of mass spectrometry (MS), have allowed high-throughput experiments to be conducted on chloroplast samples and to provide additional information about functional compartmentalization [5, 6]. Proteome research focused on the whole chloroplast has been well studied [7]. A previous study has proposed that about 3,000 different types of proteins are present in the mature chloroplast that have specialized distributions and functions [7]. In addition, the different subcellular chloroplast compartments have also been investigated, aiming to establish the proteome repertoire of subplastidial compartments, including the envelope membranes [8], the thylakoid membranes and lumen, the stroma [9], and the plastoglobule [10]. With the continuous development of genome sequencing programs and proteomic technologies, huge amounts of plastid proteomics data have been gathered in several databases such as the Plant Protein Database (PPDB) [11], the Subcellular Proteomics Database SUBA [12], the Plastid Protein Database (Plprot) [13], AT_Chloro [8], and the MASCP Gator [14].

Both the new technologies and the plant plastid proteomics database have provided a strong foundation to identify a more complete set of chloroplast proteins (the chloroplast proteome) as well as their expression levels and posttranslational modifications (PTMs) in a global manner. Simultaneously, the chloroplast proteome can provide fundamental information of plant response to a given stress at the functional level and thus enhance our knowledge about the plant stress-related molecular mechanism. Although numerous reviews have been conducted on the chloroplast proteome, review articles specifically dedicated to the chloroplast proteome under abiotic stress are really scant. The present chapter attempts to provide an overview of the current significant achievements about the chloroplast proteome in response to abiotic stress for better understanding of the abiotic stress-tolerance mechanism of the plant at the protein level.

9.2 Function and Biogenesis of the Chloroplast

From an evolutionary perspective, it is now widely accepted that the chloroplast is descended from cyanobacteria through endosymbiosis [15]. The chloroplast is a kind of semiautonomous organelle, and the vast majority of chloroplast proteins are encoded by the nuclear genome, which are synthesized in the cytosol and post-translationally targeted to the chloroplast [16]. Not all of the encoded proteins seem to be imported into the plastid due to the lack of predictable N-terminal transit peptides [7].

The chloroplast plays an essential role in cellular metabolism and has many unique roles in processes of global significance, such as photosynthesis, the main function of the chloroplast. Photosynthesis is a complex and highly integrated biological process involving the coordinated functioning of chloroplast compartments, including thylakoids, stroma, and the chloroplast envelope [8]. The thylakoids are a highly organized internal membrane network formed of flat compressed vesicles, and they are the center of oxygenic photosynthesis. The thylakoid membrane system includes four abundant multisubunit protein complexes, PSI, PSII, the ATP synthase, and the cytochrome b6f complex (Cytb6f). Here solar energy is collected and converted into chemical energy (ATP and NADPH). The stroma, an aqueous fluid that permeates the internal components and several solutes, is the main place for the conversion of carbon dioxide into carbohydrates. In addition, several catalytic reactions also occur in the stroma, which allow the synthesis of compounds such as amino acids. The envelope is a double-membrane structure surrounding the chloroplast and controls the metabolic dialogue between the chloroplast and the rest of the cell. In addition, the envelope also plays a key role in other metabolic reactions such as pigment, lipid, or vitamin synthesis [1]. To characterize the chloroplast proteome fully, a research on each compartment of the chloroplast is essential, which has its own specific subset of proteins, or subproteome. Only then can we characterize most chloroplast proteins, including those that are hydrophobic, of low abundance, or transiently expressed.

9.3 How Photosynthesis Is Affected Under Abiotic Stress

Abiotic stress tends to affect photosynthesis by stress-induced stomatal or non-stomatal limitations [17]. The regulation of leaf stomatal conductance is a critical phenomenon in plants, for an important role in both prevention of desiccation and CO_2 acquisition [18]. In an initial phase of stress, or relatively mild stress, the effects of salinity and drought on photosynthesis are mainly attributed to the stomatal limitations for diffusion of gases, which ultimately alter photosynthesis and the mesophyll metabolism [19]. Severe drought stress limits photosynthesis by inhibiting the acquisition of CO_2 and lowering biochemical activity, such as a decline in ribulose bisphosphate carboxylase oxygenase (Rubisco) activity [20], which subsequently will result in relatively high photorespiration. The relatively higher photorespiration caused by drought would accelerate the consumption of fixed CO_2 and increase the alternative sink of electrons through the oxygenase reaction of Rubisco, leading to wasteful use of electrons and lower CO_2 use efficiency, finally leading to inhibited photosynthesis.

In the chloroplast, the accumulation of ROS is an unavoidable factor affecting photosynthesis due to the spatial and temporal concurrence of electron and energy transfer reactions with photosynthetically generated molecular oxygen [21]. The production of ROS can cause oxidative damage to lipids, proteins, and DNA [22].

Excess ROS generation in the chloroplast can result in damage to thylakoidal membranes, stromal and membrane-bound proteins, causing functional loss of various components of the photosynthesis electron transport chain. To cope with stressful conditions plants have evolved a complex system of enzymatic and nonenzymatic antioxidants, such as superoxide dismutase (SOD), glutathione peroxidase (GPX), ascorbate peroxidase (APX), and ascorbic acid and glutathione. The antioxidant system showed high abundance under salt stress, as is evidenced by proteomic profiles of salt-responsive proteins generated in the chloroplast of wheat [23], maize [24], and soybean [25].

9.4 Chloroplast Proteome Under Abiotic Stress

Generally, abiotic stress affects photosynthesis by limiting CO_2 fixation and reducing the generation of $NADP^+$ through the Calvin cycle. As a consequence, the photosynthetic electron transport chain is overreduced, which generates superoxide radicals and singlet oxygen in the chloroplast. The enhanced production of ROS is harmful to the function of chloroplast protein. As the plant stress response is a dynamic process, it depends considerably upon stress intensity and duration, as well as plant genotype, therefore the chloroplast proteome changes are investigated under different conditions, either different stress treatments on the same plant or in reverse.

9.4.1 Drought Stress

Drought stress is a major factor influencing plant chloroplast. The chloroplast protein response to drought stress is dependent on the genotype of the plant. Changes in the chloroplast proteome of tall fescue (*Festuca arundinacea*) with different levels of drought stress tolerance have been studied [20]. The results showed that 10 proteins were differentially accumulated among the 82 identified proteins. All the identified proteins were involved either directly in photosynthetic reactions (include ATP synthase, oxygen-evolving enhancer protein 2, and ferredoxin-$NADP^+$ oxidoreductase, FNR), or in the protection of the photosynthetic apparatus against different components of drought stress (include ATP-dependent zinc FtsH metalloprotease, peptidyl-prolyl *cis-trans* isomerase cyclophilin 38, Rubisco, chloroplast lipocalin, and fibrillins). The accumulation patterns of the selected proteins clearly indicated that the drought–tolerance genotype accumulated significantly higher amounts of the crucial proteins than the drought–sensitive genotype, which suggests that some of these proteins could be involved in regulation of drought tolerance in tall fescue.

The abscisic acid (ABA) pathway is a primary signaling pathway that mediates maize adaptation to drought stress [26]. The ABA regulation of the synthesis of

chloroplast proteins in maize (*Zea mays* L.) has been studied by comparing leaf proteome differences between maize ABA-deficient mutant *vp5* and wild-type Vp5 seedlings in response to drought stress. In maize leaves, the identified proteins were primarily involved in processes such as ATP synthesis, protein synthesis, chlorophyll synthesis, CO_2 fixation, gluconeogenesis, antioxidant defense, and signal transduction. Most of the proteins that differentially accumulated in leaves were localized in the chloroplast and functioned in an ABA-dependent manner in response to drought and dim light stress. These results show an important role of ABA in regulating the synthesis of drought-induced proteins.

In wheat chloroplast proteome response to drought stress, a number of Rubisco subunits were unequally expressed after drought treatment [27]. Isoforms of Rubisco activase are thought to play an essential role in stabilizing and controlling proteolysis and in maintaining chloroplast functioning in response to drought stress. Cytb6f mediates electron transfer between PSI and PSII, and cyclic electron flow around PSI and state transitions. The Cytb6f iron–sulfur subunit was increased at first 3 d but later decreased, which was possibly caused by water deficiency, and directly affected photosynthesis degradation, altered the chloroplast structure, and promoted leaf senescence [28]. The abundance of chloroplast oxygen-evolving enhancer protein 1 was increased under drought stress.

9.4.2 Salt Stress

Salt stress is thought to have a strong influence on the components of photosynthesis apparatus, such as enzymes, photosynthetic pigments, thylakoid membrane proteins, and membrane lipids [29]. Several salt-responsive proteins have been identified in maize chloroplasts for 4 h NaCl (25 mM) treatment using a comparative 2-DE proteomics approach [24]. In the moderate salt-stressed maize seedlings, enhanced abundance of three photosynthesis-related proteins (i.e., ferredoxin NADPH reductase, 23 kDa polypeptide of PSII, and an FtsH-like protein) might reflect a mechanism to attenuate detrimental effects of Na^+ on the photosynthetic machinery. Additionally, protoporphyrinogen IX oxidase was affected by salinity. This enzyme is involved in heme and chlorophyll biosynthesis, and its substrate is the target of salt toxicity leading to massive oxidative stress. The author speculated the increment of protoporphyrinogen IX oxidase would help to alleviate oxidative stress in the salt-stressed maize chloroplast. Monogalactosyl diacylglycerol synthase and calcium-sensing receptor were also identified in the maize chloroplast, which were involved in membrane maintenance and Na^+ sensing, respectively. The results provide valuable information for future studies on the molecular mechanisms of salt tolerance in maize chloroplasts.

Salt-stressed proteins in wheat chloroplasts were analyzed by 2-DE and shotgun methods coupled with high-throughput MS [27]. Wheat seedlings were treated with 150 mM NaCl, and a total of 60 proteins were identified in the chloroplast. Salt stress had unequal impacts on the subunit composition of the thylakoid CF1-CF0

complex. Four subunits of ATP synthase—α, β, γ, and ε—were affected by salt treatment, similar to that reported in the maize chloroplast [24]. There is growing evidence that the ATP synthase is also a target for a damaging effect caused by salt stress and singlet oxygen. The observed first increased and subsequently decreased and then increased again of chlorophyll a-b binding proteins might reflect changes in the patterns of carbon flux in response to reduced photosynthesis and a higher demand for osmotic adjustment. Here, the Rubisco was strongly inhibited in salt-stressed wheat chloroplast. The upregulated fructose-bisphosphate aldolases suggested they might play a role in acclimating wheat seedlings to anaerobic conditions due to oxidative stress [30]. In addition, some other proteins such as sucrose synthase 4, malate dehydrogenase, PSI reaction center subunits II and IV, glutamate dehydrogenase 1 (GDH), and glutamine synthase (GS) were also identified under salt stress in wheat chloroplast. In general, the most known proteins involved in photosynthesis and salt stress have been well revealed in wheat chloroplast by proteomics in this research.

9.4.3 Heat Stress

Heat stress is associated with the incorrect protein folding and denaturation of several intracellular proteins and membrane complexes in plants. Heat stress can lead to increased expression of several proteins with chaperone functions, especially several members of heat-shock proteins (HSPs). It is well known that sHSPs localized in the chloroplast can protect thermolabile PSII in isolated chloroplasts and are important for heat acclimation [31]. For example, overexpression of OsHSP26 confers enhanced tolerance against oxidative and heat stresses in tall fescue [32]. Recently, it was demonstrated that chloroplast sHSP26 is abundant in maize leaves under heat stress and potentially involved in maize heat tolerance [33]. Four proteins, including ATP synthase subunit β, chlorophyll a-b binding protein, oxygen-evolving enhancer protein 1, and PSI reaction center subunit IV, strongly interacted with sHSP26 and their abundance greatly declined after RNAi of sHSP26 under heat stress.

Heat stress also leads to oxidative damage in plants. Upregulation of several enzymes involved in redox homeostasis such as chloroplast precursors of SOD were reported. SOD is known to have a high melting temperature and this may be one reason behind its themostability [34]. A study in *Chenopodium album* showed that SOD isozymes in the chloroplast were Cu/Zn-SOD whereas in mitochondria were Mn-SOD, and the chloroplast contained more heat-stable isozymes of SOD and APX than mitochondria [35]. The loss in chloroplastic Cu/Zn-SOD reduced by heat would result in increased damage to PSII in *Lotus japonicas* [36].

9.4.4 Cold Stress

Cold stress is associated with significant alterations in energy metabolism. It can decrease the rate of enzyme-catalyzed reactions resulting in metabolic imbalances associated with oxidative stresses. Cold also causes membrane damage due to severe cellular dehydration caused by ice formation in inter- and intracellular spaces [37]. 2-DE based proteomic analysis was used to obtain insights of the *Arabidopsis thaliana* chloroplast proteome under short- and long-term cold stress [38]. Cold shock induced minor changes in the plastid proteome, whereas both short- and long-term acclimation to cold resulted in important changes including modulations in protein abundance occurring in the stroma. In total, 43 differentially displayed proteins were identified to participate in photosynthesis, other plastid metabolic functions, hormone biosynthesis, and stress sensing and signal transduction, providing new insights into the cold sensing and acclimatization of plants.

It has been repeatedly reported that under cold stress, *Arabidopsis* chloroplast RNA binding proteins CP31a and CP29a were upregulated; the proteins could be regulated by phosphorylation and were involved in plastid mRNA processing [39, 40].

9.4.5 High/Low Light Stress

Light intensity has a significant impact on photosynthetic light reactions, which occur in the thylakoid membrane system of the chloroplast. Structural changes of the thylakoid membrane network induced by high light (HL) stress in plant chloroplast have been studied [41]. Proteins responding to light treatment in *Arabidopsis* are mainly related to metabolic pathways (mainly carbon metabolism), protein synthesis, and energy production [3]. FNR-2 plays a critical role in the redistribution of photosynthetically derived electrons to various reducing pathways such as carbon fixation, nitrogen metabolism, and chlorophyll biosynthesis; under HL stress, the abundance of FNR-2 was increased [42]. ATP synthase in the chloroplast is regulated by light and metabolite factors, and its activity is particularly influenced by ROS [43–45]. Two aldolase isoforms (FBA1 and FBA2) were detected, indicating some functional specialization. Epimerase and isomerase decreased significantly in response to HL. The study showed that phosphoribulokinase (PRK), which plays an essential role in regulating the flow of sugar through the Calvin cycle, was upregulated by light. Rubisco activase and beta-carbonic anhydrase 1, with a defined role in carbon fixation modulation, also increased under light treatment. The results of this study also supported the participation of the proteins in signaling and controlling of chloroplast metabolism, and in the regulation of plant response to environmental changes.

The quantitative response of the thylakoid-associated proteome of *Arabidopsis* under HL has been investigated [46]. After 5 d under HL, both wild-type and *vtc*2-2 plants accumulated anthocyanins, increased their total ascorbate content, and

decreased PSII efficiency. The significant change of the identified proteins results from genotype, light treatment, and/or their interaction. The most important response was the upregulation of thylakoid YCF37 (likely involved in PSI assembly or oligomerization [47]), specific fibrillins, fru-biphosphate aldolase-1 and a flavin reductase-like protein, which all located in thylakoid-associated plastoglobules. A steroid dehydrogenase-like protein also showed a systematic upregulation in *vtc*2-2. Fe-SOD was downregulated in *vtc*2-2, whereas Cu/Zn-SOD was upregulated. Other lumenal- and thylakoid-associated proteins did not change, although PsbS containing an extra transmembrane helix increased in wild type upon light stress.

9.4.6 Ozone Stress

Ozone exposure is thought to have great influence on the soluble Calvin cycle proteins, and it also has an effect on the subunits of the photosynthetic complexes, PSI and PSII, as well as the reaction center proteins (e.g., D1, D2, or PSI core complex) or light-harvesting complexes (LHCI and LHCII) [48]. Proteome analysis of soybean chloroplast responding to ozone stress was conducted [49]. The experiment result revealed 32 differentially expressed chloroplast proteins. Proteins associated with photosynthesis, including PSI/PSII and carbon assimilation were downregulated under stress, and this is possibly one of the main causes of reduced photosynthesis under O_3 stress. The initial decline of Rubisco and other photosynthesis proteins may be the cause of decreased CO_2 fixation, thus ultimately reducing the products of the Calvin cycle [50]. On the contrary, proteins associated with the antioxidant defense mechanism and carbon metabolism were mostly upregulated under O_3 stress. The authors pointed out that not only the degradation of starch and higher amounts of sucrose in response to short-term acute ozone exposure fed the tricarboxylic acid (TCA) cycle but the availability of sucrose may also play a key role in oxidative stress signaling and regulation pathways of antioxidative processes.

Thylakoids isolated from poplar leaves revealed a negative impact of ozone exposure on membrane proteins [51]. After the first 7 d of ozone exposure, FNR was upregulated, leading to an increase in the production of NAPDH for reducing power, and detoxifying the generated ROS inside and outside of the chloroplast. Later on, extrinsic photosystem proteins and ATP synthase subunits were detected to decrease in abundance after 14 d, indicating that protective measures became overwhelmed, and the chloroplast response processes could no longer counteract the severe ozone stress. A few examples of chloroplast proteomic studies under abiotic stress are presented in Table 9.1.

Table 9.1 Examples of chloroplast proteome changes under abiotic stress

Plant	Abiotic stress	Methods	Number of identified proteins	Major findings	Reference
Arabidopsis	Cold	2-DE/MALDI TOF MS	43	Identified proteins mainly participating in photosynthesis, other plastid metabolic functions, hormone biosynthesis and stress sensing and signal transduction, providing new insights for plants in cold sensing and acclimation	[38]
	High light	2-DE/ESI MS/MS	99	The thylakoid YCF37, a flavin reductase-like protein, specific fibrillins, and an aldolase increased in abundance, all located in thylakoid-associated plastoglobules, highlighting the enzymatic role of plastoglobules in chloroplast under stress	[46]
	High light	2D-DIGE/MS	28	Several photosynthesis- and carbon metabolism-related proteins as well as proteins involved in plastid mRNA processing were identified under high light stress	[3]
Maize	Salt	2-DE/MALDI-TOF MS	20	Sodium ions accumulate quickly and excessively in maize chloroplast on moderate short-term salt stress. In addition, a defined set of specific chloroplast proteins in maize changed instantaneously under salt stress	[24]
	Drought and low light	2-DE/MALDI-TOF MS	24	Identified proteins were primarily involved in processes such as ATP synthesis, protein synthesis, chlorophyll synthesis, CO_2 fixation, gluconeogenesis, signal transduction, and antioxidant defense, functioning in an ABA-dependent manner under drought and light stress	[26]
	Heat	2-DE/MALDI-TOF MS	45	Four proteins, including ATP synthase subunit β, chlorophyll a-b binding protein, oxygen-evolving enhancer protein 1, and PSI reaction center subunit IV, strongly interacted with sHSP26, and finally improved chloroplast performance under heat stress	[33]
Soybean	Ozone	2-DE/MALDI TOF MS	32	Proteins involved in antioxidant defense and carbon metabolism increased under O_3 stress	[49]

(continued)

Table 9.1 (continued)

Plant	Abiotic stress	Methods	Number of identified proteins	Major findings	Reference
Poplar	Ozone	2-DE/MALDI TOF MS	98	Under a long-term ozone exposure, the extrinsic photosystem proteins and ATP synthase subunits decreased in abundance, indicating that protective measures had became overwhelmed	[51]
Tall fescue	Drought	2-DE/MALDI-TOF/TOF MS	82	All the identified proteins were involved directly in photosynthetic reactions or in the protection of photosynthetic apparatus against different components of drought stress	[20]
Wheat	Salt and drought	2-DE/MALDI TOF MS	65 (salt) and 22 (drought)	Most known proteins involved in photosynthesis and salt and drought stress were identified in chloroplasts	[27]

9.5 Summary and Outlook

The advances in sample preparation techniques, MS instrumentation, and bioinformatic tools in proteomic fields have paved the way for high-throughput analysis to be conducted on chloroplast samples as well as their subcellular compartments. In this chapter, we tried to summarize the effects of different abiotic stresses on the plant chloroplast proteome. Quite a number of chloroplast proteins and photosynthesis proteins responding to different abiotic stresses have been efficiently identified by proteomics for better understanding of the photosynthesis and identifying the stress-responsive proteins. However, as the photosynthetic machinery and metabolic mechanisms are so complicated, we cannot investigate the mechanism by just using quantitative protein profiles. Specialized protein complexes, protein–protein interaction, and PTMs have been proposed to play key roles in photosynthesis. Thus, further proteomic studies should focus on the analysis of large-scale protein modifications and interactions under abiotic stress to enhance our understanding of the protein networks in photosynthesis. We hope this chapter provides new insights into the plant stress response mechanisms. In addition, an understanding of the mechanisms that stressful conditions affect photosynthesis would aid the improvement of growth conditions and crop yield and provide useful tools for future genetic engineering.

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Chapter 10
Metabolomics on Combined Abiotic Stress Effects in Crops

Karin Köhl

10.1 Sampling Defines the Picture

When metabolite profiling methods are applied to elucidate the response of plants to abiotic stresses, the strategy employed when taking samples from the plant has a major effect on the result. Decisions on the material sampled for the analysis and the timing of the analysis with respect to the diurnal cycle, the developmental stage of the plant, and the duration of the stress will affect the metabolite pattern significantly.

10.1.1 Organs

Leaves are the organs most frequently sampled for analysis, as they contain the tissues, in which photosynthesis as a basis for biomass production takes place. Leaf metabolism, however, changes significantly throughout the life cycle of a leaf, as the leaf or part of a leaf changes from a sink to a source to senescing tissue [2, 44, 67, 94, 110, 141]. Likewise, the leaf's response to stress may change with age [115]. Leaves that developed after stress application differ in their response from leaves that developed before the onset of stress [45, 87]. Sun and shade leaves of the same age, grown in parts of the canopy receiving different amounts and qualities of light, differ morphologically and physiologically [22, 69]. Surprisingly, transcript-level differences between sun and shade leaves were small compared to seasonal effects [91]. Furthermore, leaf samples taken from small species often

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contain stem material, when complete rosettes instead of leaf blades (with or without petioles) are sampled. As leaf age and leaf position affect metabolite composition, careful definition of the sampled leaf specimen with respect to its age and canopy position is required to ensure reproducibility of sampling and thus comparability of samples taken by different persons or at different times.

Flowers and flower organs are sampled in studies dealing with stress effects on fertility and seed development. Relevant stresses are high or low temperature or drought during or after flowering [73, 146]. The metabolic patterns of flowers change during their development [10]. Metabolite profiling on fruits is mainly performed on fruit crops such as tomato, pepper, apple, and grape. These studies focus on genetic and environmental effects on the sensory and nutritional quality of the harvest and postharvest product [26, 38, 83, 97, 113, 126, 127]. Root sampling is almost exclusively restricted to material grown on agar plates or in hydro- or aeroponics [5, 102, 119, 136]. Obtaining representative root samples from soil-grown material is laborious; thus, metabolite profiles of soil-grown roots are less frequently measured. The effort is, however, worthwhile to gain insight into root response to drought or mycorrhiza, which are best studied in soil-based systems [39, 40, 105].

10.1.2 Timing

10.1.2.1 Developmental Stage

The developmental stage of the plant influences the metabolic pattern in addition to the effect of the individual organ's age [42, 68, 92, 141]. The leaf metabolism changes especially at the onset of flowering as remobilisation of nitrogen and carbohydrates from leaves is enhanced due to remobilisation at the onset of flowering and during fruit growth [30, 66]. Thus, if genotypes differ substantially in their development speed, comparing them at the same age after sowing can be very misleading. In Arabidopsis, accessions vary significantly in the age of bolting, which may lead to comparing vegetative plants with plants that have already bolted. Ideally, samples are taken at a defined developmental stage, identified by the Zadok or BBCH scale [80, 92].

10.1.2.2 Diurnal Rhythm

Leaf metabolism changes with a diurnal rhythm, which is regulated by the plant's clock and entrained by light and temperature [52, 112]. In consequence, the concentrations of many metabolites change during the diurnal cycle. Diurnal changes are substantial in metabolites that serve as intermediate storage products of

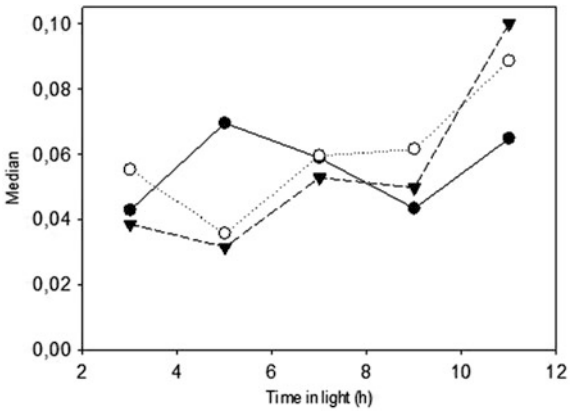

Fig. 10.1 Median of the relative change of concentration for 123 metabolites measured in leaves of *Arabidopsis thaliana* that were cultivated at 50 (*dot*), 150 (*circle*), or 550 (*triangle*) µmol m^{-2} s^{-1} (data from [35] #2999). The relative change was calculated as absolute [$(c(t) - c(t + 2h))$/mean($c(t)$, $c(t + 2h)$)] and plotted against the midpoint of the time interval. Four outliers (relative change >1) were removed from the dataset

photoassimilates, such as starch, sucrose, and raffinose family oligosaccharides. Likewise, amino acid concentrations change diurnally [35, 132, 147]. Light intensity affects the amplitude of diurnal changes in starch and total amino acid concentration. Furthermore, the shape of the curve, the time, at which the concentrations of, for example, glucose or glutamine peak, depends on the light intensity [35]. As the process of sampling takes a certain time, circadian change of metabolite concentration can influence the result if samples are not taken randomly. To identify the ideal time for sampling, the change of metabolite concentration within 2 h was calculated for 123 metabolites (see Fig. 10.1). The median change of metabolite concentration was between 3 and 7 % for the time between 2 and 10 h after the onset of light but tended to increase at the end of the day.

A most important finding for abiotic stress research is the interaction between diurnal rhythm and environmental response. The plant's response to the same stimulus depends on the time within the diurnal cycle (gating), which may result from an effect of the clock on the signalling pathway [147]. The diurnal change in the plant's response may also be the consequence of an interaction between a clock-regulated pathway (e.g., stomatal opening), and the effect of an environmental stimulus (e.g., increased vapour pressure deficit (VPD) outside the leaf) on leaf tissue, for example, change in VPD in the leaf aerenchym. An interaction between diurnal rhythm and the response to drought has been found in potato [7]. In 20 % of (detected) leaf metabolites, their concentration was simultaneously affected by drought, CO_2 concentration, and the diurnal cycle. Drought increased the amplitude of diurnal changes for hexoses and starch compared to control samples [7].

10.1.2.3 Duration of Stress

In many studies, response to stress is measured at a predefined time point after the application of stress. Measurements after a few minutes to several hours of stress generally aim at the discovery of components of the signal transduction pathway triggered by the stress [108]. Measurements after hours or days of stress target the effect of stress on the metabolism in the 'shocked' and in the 'acclimated' or 'damaged and declining' stage. When temperatures or light conditions were abruptly changed from optimal to stress levels, metabolite levels changed in less than an hour and more rapidly than transcript levels [20]. Moderate temperature decrease resulted in larger changes in transcript levels after 6 h than after 78 h. In contrast, metabolites changed rapidly in few pathways and more slowly for a larger group of metabolites, among them stress-responsive metabolites such as proline, raffinose, and polyamines [133]. The kinetic component of the response thus has a significant effect on the results. When observing the kinetics of transcript and metabolite response to temperature and light stress within the first 24 h after the change of conditions, the change of many metabolite concentrations is not continuous [20]. When *Arabidopsis thaliana* is transferred to 32 °C, the maltose concentration first increases (first 3 to 5 time points) and then decreases below the initial concentration. In contrast, the concentration of several amino acids, among them valine, asparagine, and ornithine, first decreases and then increases compared to time zero. In consequence, in studies where stress effects on metabolites are studied within 24 h, the effect of the diurnal cycle on metabolite concentrations and potential effects of the stress on the diurnal cycle have to be taken into account [33]. However, even after several days of stress, the diurnal cycle interacts with stress response [7]. When leaf metabolites in potato were determined predawn and midday after 5 or 11 days of drought stress, the time of day affected the magnitude of a metabolite's change but not its direction [7]. In contrast to many other stresses, drought stress cannot be imposed suddenly unless done in a fairly artificial way, for example, by removing the plant from its medium or by adding an osmolyte. When drought stress is imposed by withholding water from a plant in a pot or in soil, the amount of available water and the soil water potential decrease with time. The speed of change depends on the ratio between available water at the beginning of the experiment and the evapotranspiration rate of the pot–plant system. The latter is affected by the VPD of the atmosphere, the temperature of the plant leaf, and the surface of the plant and the pot. Thus plants of very different size will experience different drought stress when grown in the same pot size, which may account for the seemingly high drought tolerance of some dwarf mutants. The amount of water available at the beginning of the experiment depends on the size of the pot, the water capacity of the substrate, and its relative water content. The availability of the water depends on the soil water potential, which depends on the soil type and its water content. Large plants in relatively small pots will experience an onset of drought that may be much faster than in soil-grown plants. Furthermore, plants grown in dim light may change their leaf water content much more slowly than plants grown in high light, as leaf transpiration increases with light intensity [84].

Realistic climatic conditions are also required to assess the salt tolerance of a species, as salt accumulation is affected by transpiration [87]. Thus, ideally, the plant's water status during the experiment or when samples are taken is characterised by measuring the leaf water potential.

10.2 Drought Stress Combinations

When drought stress effects on metabolism and metabolite concentrations are studied, drought stress is predominantly applied in controlled environments where all parameters except water supply are kept constant. Under field conditions, drought stress is generally combined with other biotic and abiotic stresses [82]. Although many of these stressors combine randomly, the combinations of drought with salt, heat, and cold stress occur frequently due to constraints caused by environmental physics. Together, drought, salt, and extreme temperatures account for 70 % of yield loss in global crop production [17].

10.2.1 Drought and Salt

In arid climates, drought stress is often combined with salt stress. Under arid conditions, annual evapotranspiration exceeds annual precipitation. Thus, soil water moves predominantly towards the surface and thereby transports soluble soil compounds such as sodium, potassium, chloride, and sulphate towards the uppermost soil layer. In consequence, the osmotic potential of the soil solute decreases and salts may precipitate within the soil or at the soil surface (Fig. 10.2). This process can make arable land unfit for plant cultivation, especially over saline

Fig. 10.2 Salt precipitates on the soil surface in arid regions of the Himalaya (Nepal, October 2012)

outcrops or close to the sea. Salinisation is aggravated by insufficient irrigation or extensive use of fresh water from rivers [36, 46].

As soil salinisation took place in arid climates before man invented agriculture, so called xero-halophytes have evolved. Xero-halophytes are adapted to the combination of low matrix potentials resulting from low soil water content, low osmotic potential resulting from high solute concentrations in the soil water, and toxic concentrations of sodium and chloride [87, 104]. These plants thus evolved efficient strategies to exclude and sequester toxic ions. The challenges posed by low osmotic potentials of the soil solution and low matrix potentials in dry soils differ. In a wet saline soil, water moves easily towards the root surface. Thus, as long as the plant can reduce its water potential below the soil water potential, water uptake is not impaired. However, extensive water uptake increases the salt load of the shoot. In dry saline soils, water conductance is considerably reduced, resulting in depletion zones around the roots that force the root to grow towards new water sources. However, this concept has been challenged [27]. When water flow resistance in soil is high, maintaining root growth is paramount, which means that root turgour has to be maintained by osmotic adjustment and photosynthesis has to continue supplying chemical energy to fuel root growth. The same holds true for a plant on a dry soil without increased salt levels.

The demand on root water uptake can be alleviated by increased water use efficiency of the shoot, which is relevant for drought and salinity tolerance and can be achieved by an increase of cuticula resistance and reduced stomata density [109]. The reduction of the plant's water potential below the soil water potential relies on the accumulation of so-called compatible solutes. Compatible solutes do not interfere with metabolism at high concentrations and act as chaperons or scavengers of reactive oxygen species (ROS). Among these compounds are amino acids, especially proline, quaternary ammonium compounds including glycine betaine, soluble carbohydrates such as sugars and sugar alcohols, and raffinose family oligosaccharides [14, 41, 49, 144]. The most commonly accumulated compounds proline and glycine betaine accumulate under salt and drought stress. Salt tolerance and drought tolerance increase with the intracellular level of compatible solutes [13, 55, 60, 75, 139]. Studies on short-term response of plants to NaCl or dry soil in a system, where soil water potential was 'clamped' with a pressure bomb, indicated that reduced leaf expansion rate under stress is not brought about by altered water pressure, but by hormonal signals [85]. The plant's response to salt stress and drought are very similar [85], but not identical. Drought and salt stress lead to accumulation of aspartate, fumarate, and sucrose in *Medicago truncatula* roots, but proline and glucose accumulation was restricted to drought-treated plants [117]. Thus, salt-resistant plants are not necessarily drought resistant and vice versa.

10.2.2 Drought and Cold

The water vapour deficit at given atmospheric water content and thus the evapotranspiration rate increase with temperature. Thus, even very low annual precipitation rates of 250 mm result in humid climate conditions in high latitudes where average air temperatures are low [64]. However, there are situations where low water availability is combined with air or soil temperatures low enough to cause cell damage. The temperature limits, below which plant tissue is damaged, are highly species-specific and can be reduced by hardening (see Priming). Many tropical plant species such as rice, cucurbits, and tomato suffer from so-called chilling damage at temperatures well above freezing [65]. Species from Mediterranean climates or tropical mountains tolerate temperatures down to a few degrees Celsius below zero as long as they can prevent ice formation inside the cell by accumulation of osmolytes. These can prevent freezing down to temperatures of −5 °C. Some species that grow in regions, where temperatures change between freezing and thawing each day, acquire meta-stable states, in which supercooled cell sap remains liquid down to temperatures of <−30 °C [143]. Species from temperate regions and high latitudes tolerate freezing of leaf tissue and wood, in the extreme down to temperatures of below −40 °C and show a pronounced change of cold tolerance during the year [65].

In cold and arid climates, plants are subjected to low temperatures and low humidity or low precipitation. Cold arid and semi-arid regions are found in northern Asia, especially in the Himalaya, in northwest China, northern Iran, and Afghanistan, but also on the Colorado plateau and in Alberta (Canada; [32, 50, 74, 114]). In many cold and dry areas, all (trans-Himalaya) or a substantial percentage (Colorado) of precipitation falls as snow during the cold months, alleviating low temperature effects. Plants thus are most likely affected by a combination of cold and drought when they are not or no longer covered by snow. When frozen leaves are exposed to the sun, their water potential can be higher than that of the surrounding air, which results in water loss by evaporation. At the same time, water uptake from the soil and water transport in the xylem is restricted by high water flow resistance in frozen soil and xylem [129]. In consequence, leaf cells can be damaged by low leaf water content. Evergreen trees adapted to very low winter temperatures reduce water loss from the leaves by producing thick cuticula. In chilling-sensitive plants such as tomato or pumpkin, chilling stress is often combined with drought stress as temperature-dependent inhibition of water uptake by the roots and transport in the xylem occurs, when temperatures fall below 15 °C [64].

10.2.3 Drought and Heat

Drought stress is frequently combined with heat stress as a large amount of energy is required to convert liquid water into water vapour. This large latent energy of

water renders a wet evaporation object that is exposed to radiation cooler than a dry object. Both on the global scale as on the level of a single plant, evaporation of water consumes most of the solar energy that reaches the atmosphere, respectively, the plant, compared to less than 3 % used by photosynthesis [37]. Thus, if water is scarce, plant temperature tends to increase. This may explain why when both stresses are combined experimentally, the metabolic response tends to be more similar to the drought response than to the heat response [90].

Plants that are subjected to drought stress reduce transpiration by closing stomata. As energy consumption by phase transition from liquid to vapour decreases, absorbed radiation is converted into heat and results in a temperature increase of the leaf. In consequence, leaf temperatures can be 10 °C higher than air temperature [21]. Many species limit this effect by changing the leaf surface exposed to radiation and thus the amount of intercepted radiation by leaf rolling or altering the angle between leaf surface and direct radiation. The radiation that is not intercepted by the plant canopy will reach the soil surface and will increase the soil temperature. This increase depends on the soil's albedo, its moisture content, and evaporation rate. Thus temperatures increase very rapidly at the surface of dry, dark, sandy soil (e.g., derived from volcanic ash or dry peat) and much slower in wet clay soils. In consequence, the soil surface can be much hotter than the air during the day. This fact is very important for the germination and establishment of seedlings under drought stress. Rain or irrigation water can transport heat to lower soil layers, which results in an increase of root temperatures that cannot be compensated by water evaporation. These environmental physics constraints do not only define the selection pressure, to which plants in hot arid environments were subjected. Environmental physics also affects the degree of stress a plant is subjected to in a drought or heat stress experiment under controlled conditions. In consequence, plants kept at the same, high air temperature (e.g., *Arabidopsis thaliana* at 30 °C) can experience leaf temperatures much lower than 30 °C when grown in moist soil and at moderate light intensities, whereas leaf temperatures can be much higher than 30 °C when plants are grown in dry soil, in the dark, or at high light intensities, or in a very moist atmosphere [56]. Furthermore, small pots with a dark surface or closed containers (Petri dishes) exposed to sunlight can heat up to temperatures well above the atmospheric temperature. Thus, judging the degree of stress, to which an experimental plant was exposed, requires detailed meta-information on atmosphere and soil conditions. Without these often under-reported meta-data, comparison of metabolite profiles of different drought and heat experiments is very difficult.

10.3 Stress Duration and Intensity Affect the Metabolic Response

The plant's physiological and molecular response to adverse abiotic conditions depends on the duration and the intensity of the stress [87]. Short-term stress treatments study the plant's response within a few minutes to several hours after the onset of stress. These studies mainly focus on the identification of components of stress perception, signal cascading, and subsequent regulation of gene expression [15, 89, 125, 145]. In long-term stress treatment, timing and duration of the stress are adjusted to the developmental phase and the time at which the stress typically hits the species or—especially in agricultural research—at which stress causes the largest yield loss. In temperate regions, droughts typically last a few weeks and are followed by periods of ample precipitation. Under these conditions, fitness or yield depends on the genotype's ability to recover from stress. In semi-arid climates, wet seasons are often followed by several months of drought, during which the plant may die (terminal stress) with or without completing its life cycle. Under these conditions, yield or fitness depends on the species' ability to make the most out of available water. In Indian pearl millet varieties, variation in grain yield under terminal drought depends on small temporal differences in water uptake and use during the plant's life cycle [134].

The intensity of a stress treatment needs to be judged with respect to the stress resistance of the respective species. Stress resistance can be quantified by the cardinal points of stress intensity, which specify the highest or lowest value of an environmental parameter acceptable to the respective species. In the case of temperature stress, these are the minimum and maximum temperatures of survival, growth, photosynthesis, and so on. Resistance to chemical stress can be given by the toxic concentration, at which survival or growth declines in a logit-like manner. This logit curve can be parameterised by the no observed effect concentration (NOEC), which is the highest concentration, at which no effects on growth or survival are observed, or the lethal dosis, at which 50 (LD50) or 100 % (LD100) of the specimens die. Although the NOEC is an ideal concentration to study stress effects without secondary effects, it is rarely used, as its determination requires an even higher number of replicates than LD50 measurements.

In many short-term stress treatments, short stress duration is combined with high stress intensity and fast increase of stress intensity (shock treatment; [121]. Short-term drought stress treatments are often dehydration treatment, in which the plant is removed from its water supply (e.g., out of hydroponics) or the root system is treated with PEG. Only 10 % of genes with significant change in expression upon dehydration shock also show altered expression after several days of moderate drought stress [121]. In conclusion, dehydration shock treatments seemed to be suitable as a fast screening method with the necessity to validate the obtained results under more realistic conditions [121]. For salt-tolerance studies, the predictive value of assays based on the survival in short, hard treatment for performance under agricultural conditions has been disputed. In short treatments, the salt

effect is restricted to the osmotic effect, whereas salt toxicity effects develop considerably later [86, 87].

10.3.1 Response Types A: Tolerance, Resistance, and Avoidance

The plant's response to stress is quantified by determining parameters related to fitness (for wild plants) or yield (for crops). As growth and seed yield determination are laborious, proxies such as shoot biomass, photosynthesis, or leaf survival are used to assess resistance. However, it has to be taken into account that shoot biomass is not necessarily correlated to seed production or yield even in a population of closely related genotypes as shown by the wide variation in harvest index [78]. Stress response is classified as tolerant or sensitive, which are the extremes of a response continuum. In a tolerant plant, fitness and yield change less with increasing stress intensity than in a sensitive plant. As illustrated in Fig. 10.3, high tolerance is thus not necessarily linked to a high yield under stress conditions. Stress tolerance often occurs in crop cultivars of low yield potential, which means that they show low yield under optimal conditions. There are even indications for a negative correlation between yield potential and stress tolerance (yield penalty), although it has not yet been shown that tolerance implies metabolic costs [13, 87, 116].

In its strict definition [71], stress tolerance is one strategy, and stress avoidance the other strategy to achieve stress resistance. Whereas a stress-tolerating organism tolerates stress in the sense defined above, a stress avoider employs a mechanism to

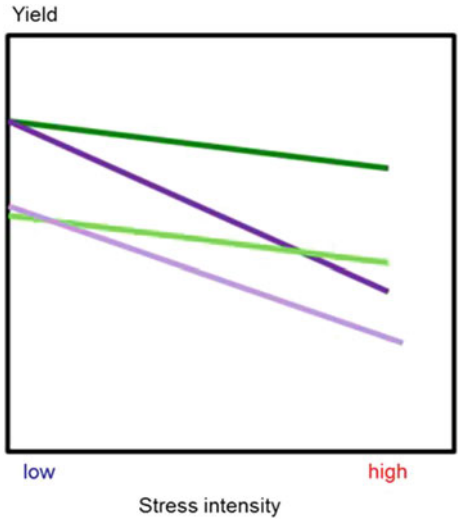

Fig. 10.3 Relationship between yield and stress intensity. In highly tolerant genotypes (*green*) yield is less affected by stress intensity than in sensitive genotypes (*violet*), but tolerance can be combined with high (*dark green*) or low (*light green*) yield potential

prevent the organism from being confronted with the stress. Classical examples are winter annuals and bulb plants in climates with dry hot summers and moist mild winters (Mediterranean climate), which are in a dormant state as seeds or bulbs when heat and drought stress prevail [64]. Likewise, summer annuals avoid cold and drought stress during winter by germinating in spring and terminating their life cycle within a few months. However, when looking more closely at the mechanism employed by plants that grow and produce seeds during the stress period, it becomes obvious that avoidance and tolerance are not sharply distinct classes but rather extremes in a continuum. With respect to drought tolerance this can be illustrated by deep-rooting trees on one end and dehydration-tolerant resurrection plants on the other end of the strategy continuum. In arid climates, some tree species grow deep roots to tap into deeper water layers containing water from precipitation during wet seasons. This strategy is discussed as one possible adaptation mechanism in crops for semi-arid environments [122]. Obviously, this mechanism is only sustainable if the reservoirs these plants tap into are refilled by precipitation eventually. In some cases, the rain that feeds these water reservoirs fell in distant areas or even a few years ago; in the worst case, the water reservoirs are historic. This mechanism resembles the strategy employed by water-storing plants such as succulent cacti and euphorbia but also the savannah tree baobab, which take up water during rainy seasons and store it within bulky shoot organs. Water-storing and deep rooting plants may thus suffer less from water shortage than co-occurring plants without these adaptations. However, they have to invest carbon and energy into the production and maintenance of heterotrophic tissues. In ecosystems similar to those of water-storing avoiders, desiccation-tolerant resurrection plants survive dry periods by dehydration. These plants tolerate dehydration of the shoot down to 10 % of their saturated water content [12]. This group contains a number of higher plants, including some grass species, and most mosses. The cytoplasm of their leaf cells tolerates extreme dehydration in a dormant state due to various protective mechanisms. This strategy resembles the process in ripening seeds, which also acquire the ability to survive dehydration and thus avoid times of water shortage and extreme temperatures. In contrast to seeds, leaves of resurrection plants can resume net-carbon fixation within very short times after rehydration.

The majority of plants and almost all crops can neither draw on large internal or external water stores nor do they tolerate complete dehydration of their leaf cytoplasm. These plants experience stress when water uptake and transport into the shoot no longer match the water loss by transpiration. In this case, many physiological and metabolic changes can be observed, some of which are adjustments to prevent damage, and others are the consequence of cell damage. Unfortunately, it is not trivial to distinguish between both. An illustrative example for this problem is the role of proline accumulation in stress response. Proline accumulates in the leaves of many plant species, when plants are subjected to drought, salt, oxidative stress, or extreme temperatures [120]. Proline concentration in unstressed mature leaves is fairly low (<2 mmol kg^{-1}), but proline accumulates by a factor of >20 to concentrations above 50 mmol kg^{-1} when plants are subjected to drought or extreme temperatures [58, 88]. Thus, proline has been suggested to act as a

protective compound (see below) within a stress- tolerance mechanism [120, 137]. However, when comparing sensitive and tolerant cultivars of crop species, proline concentrations under stress are reported to be higher in sensitive than in tolerant genotypes. This pattern suggests that proline accumulation is linked to damage, either as a consequence of damage (like scattered glass after a car accident) or part of the mechanism to prevent further damage (in the image of the accident: the warning triangle at the roadside). The association of proline accumulation pattern and tolerance in single genotypes that vary widely in their genetic background is inadequate to prove causality, as tolerance and metabolic change may be chance associations. To unravel the role of compounds in stress tolerance, metabolite concentrations are observed in time-series or in genetically similar genotypes, in which metabolite concentrations are modified by—ideally inducible and tissue-specific—overexpression or repression of genes coding for relevant enzymes [87]. Modification of proline concentration by constitutive overexpression of Delta (1)-pyrroline-5-carboxylate synthase (P5CS) resulted in proline accumulation and affected salt and osmolyte tolerance in a species-specific manner [137]. Similar effects were observed when proline concentrations were increased by inhibition of proline degradation [120, 137]. Thus, although proline is accumulated in response to various stresses and likely to be involved in the tolerance mechanism, proline accumulation is no requirement for tolerance [120]. A similar conclusion was drawn for raffinose, which increases during cold acclimation, but is, as mutant studies showed, not required for freezing tolerance [148]. Thus, compounds that are accumulated by plants under stress are likely to be involved in the tolerance mechanism without being a requirement for tolerance.

10.3.2 Response Types B: Disruption, Adjustment, Senescence, and Chaos

Physiological studies concentrate on the abiotic stress effects on leaves in light as the site and situation, in which carbon is fixed as a basis of growth. Drought, extreme temperatures, and salt result directly or indirectly in a change of water status and temperature of the leaf towards an unfavourable range [14]. Under drought conditions, water potentials of soil and atmosphere decrease. Heat decreases air water potential; frost immobilises water in the soil and increases water flow resistance in the shoot [21, 84, 129]. As the plant is basically clamped between soil and atmosphere in a water-flow continuum, decreased soil or atmosphere water potentials rapidly lead to a decreased water potential of the leaf [95]. By closing stomata, the plant can increase the resistance of water flow between leaf and atmosphere to reduce water loss and increase leaf water potential at the cost of increasing leaf temperatures. Decrease of water potential of plant cells results in a loss of turgour pressure, which is a prerequisite for cell expansion in growing tissues and thus required for any morphological adaptation of the plant. As

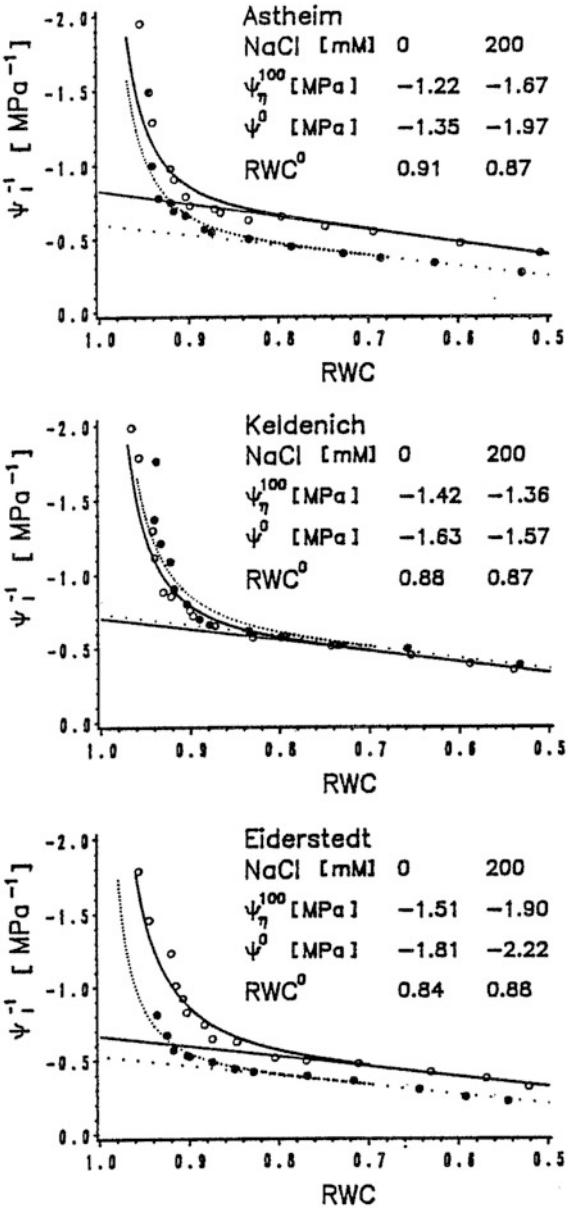

Fig. 10.4 Pressure volume curve of leaves from three different populations of *Armeria maritima* grown at salt concentrations of 0 (*open circles*) or 200 mmol l^{-1} NaCl (*dots*) [58]. Curve fitting and calculation of osmotic potential Ψ at 100 and 0 % turgour according to Andersen et al. [3]

explained above (see Drought and Salt), high water flow resistance in dry soils requires maintenance or even increase of root growth to obtain the water required to compensate for water losses from the plant to the atmosphere. By accumulating osmolytes within the cell, the plant maintains turgour and thus continues growth at lower tissue water potentials (see Fig. 10.4).

Several metabolite groups have been identified as so-called compatible solutes that can be accumulated to high concentrations either in the vacuole or in the cytoplasm without affecting metabolism by adverse effects on enzymes. Proline, quaternary ammonium compounds, polyamines, polyols, and sucrose and oligosaccharides were grouped among compatible solutes based on the observation that (a) they accumulate when leaves are subjected to abiotic stress, and (b) they can be present in high concentrations without inhibiting enzyme activities or bacterial growth in vitro [6, 14, 48, 49, 144]. Furthermore, many of the above-mentioned compounds have been supposed to protect macromolecules and membranes from damage under stress conditions [1, 6, 47, 51, 101, 137].

10.3.2.1 Oxidative Stress

Stomatal closure results in reduced CO_2 uptake and low CO_2/O_2 ratios, which decreases photosynthesis and increases photorespiration. Photosynthesis itself is a process which is most sensitive to abiotic stress [100]. High leaf temperatures as a consequence of stomatal closure or high external temperatures damage the oxygen-evolving complex and cofactors in PS II, enhance oxygenase activity of Rubisco, and damage the ATP generating system [1]. In consequence, most abiotic stresses lead to oxidative stress [90]. Some of the metabolites accumulated under drought, heat, or salt stress were shown to act as antioxidants or to protect enzymes that detoxify ROS. Proline scavenges ROS by acting as singlet oxygen quencher and by stabilising among others superoxide dismutase and catalase [120]. ROS scavenger functions have also been assumed for mannitol or polyols in general [14]. However, when Arabidopsis is transferred to heat or high light conditions, no rapid accumulation of proline, mannitol, or myo-inositol is observed within the first 6 h [20], although all these metabolites have been shown to accumulate after prolonged heat, salt, or drought stress [18, 34, 77]. Another compound with protective function against ROS is tryptophan. It is assumed to protect proteins, especially the D1 protein required for the repair of PS II. In cereals, which do not accumulate proline, tryptophan concentrations increase under drought within a few days [16]. In addition, oxidative stress causes a change of carbon flow away from glycolysis to the oxidative pentose phosphate pathway and a perturbation of the tricarboxylic acid (TCA) cycle [90]. This is assumed to reduce the levels of amino acids that are derived from intermediates of glycolysis or the TCA cycle. The TCA-cycle-derived amino acids aspartate (Asp) and glutamate (Glu) indeed decrease under drought and salt stress. However, glycolysis-intermediate-derived amino acids leucine (Leu), isoleucine (Ile), and valine (Val) increase under drought, salt, and heat stress [90]. The pattern of amino acid changes thus resembles less the situation under oxidative stress than the changes observed under C-starvation.

10.3.2.2 Energy Crisis?

Carbohydrate limitation results in a concentration increase of Ile, Leu, Val, arginine (Arg), phenylalanine (Phe), tryptophan, tyrosine (Tyr), and proline as a consequence of protein degradation. The induction of genes coding for protein and amino acid degradation under carbohydrate starvation and abiotic stress suggests that proteins and amino acids are used to fuel energy metabolism [29, 93, 98, 111]. Under drought, released branched chain amino acids are used by the alternative respiration pathway [90, 142]. The central carbohydrate metabolism is also considered a most important component in the adjustment of metabolome to low temperature stress [47]. Caldana et al. furthermore showed that transfer of *A. thaliana* into low light or the dark elicits a metabolic change similar to that after transfer to high temperatures [20]. Several facts thus suggest an energy shortage under abiotic stress.

The hypothesis is consistent with the observation that N-metabolism is inhibited by C-starvation and by abiotic stress, as energy for N reduction is saved when energy is limited and reduced N is released from degraded amino acids. Under C-starvation and abiotic stress, changes in Glu, glutamine (Gln), Asp, and asparagine (Asn) indicate the inhibition of N assimilation [35, 90, 93]. Altogether, these findings led to the hypothesis of an energy crisis as the common denominator of abiotic stresses [66]. Abiotic stress may cause carbon and energy limitation as a result of decreased photosynthesis in consequence of stomatal closure or damage to the photosynthetic apparatus. When drought requires enhanced root growth to forage for additional soil water, C-allocation to the root meristems has to be increased. Salt stress increases respiratory metabolism [62], and the synthesis for heat shock proteins and osmolytes in response to extreme temperatures requires C-resources. Thus, energy and carbon demand may increase under abiotic stress, while C fixation is limited. The change of TCA cycle intermediates under drought, salt, and temperature stress [28, 43, 106], the upregulation of TCA enzyme activity [62], and the induction of the respective genes [29] support the assumption that energy metabolism is enhanced by abiotic stress. The carbohydrate catabolism is fueled by starch degradation leading to increased concentrations of soluble carbohydrates under salt and extreme temperature stress. Drought stress leads to increased or decreased concentrations of mono- or disaccharides depending on stress duration and intensity. However, increased concentrations of soluble carbohydrates under salt stress and extreme temperature stresses contrast with the finding that concentrations of mono- and disaccharides, organic acids, and polyols decrease under carbon limitation. It may be speculated that in those experiments where soluble carbohydrate concentrations decreased, carbon export from the leaves was reduced to allow maintenance metabolism of the source leaf.

10.3.2.3 Senescence

In contrast, a high concentration of soluble carbohydrate in the leaves may indicate a remobilisation of resources from the leaf to provide sink tissue with energy, carbon, and nitrogen. Remobilisation means that macromolecules are degraded to export their monomers, for example, sugar or amino acids via the phloem system. Sugars such as raffinose or sucrose that are found to accumulate in leaves under stress are major transport forms of carbohydrates. The amino acids Glu and Asp and their respective amides are the main nitrogen transport form [63, 135]. Remobilisation typically occurs during senescence. This process occurs as leaf tissue ages but is indeed enhanced during drought, salt, and extreme temperature stress. Starch and protein concentrations decrease during leaf senescence and stress. The concentrations of the amino acids Val, Leu, and Ile and the polyol concentrations (myo-inositol, sorbitol, galactinol) increase with the age of a leaf [141] and under abiotic stress. Likewise, the concentrations of soluble carbohydrates (sucrose, fructose, glucose, trehalose) rise with age as under salt and extreme temperature stress. In contrast, hexoses get depleted in stress hypersensitive autophagy mutants, whereas glutamate and glutathione increase [79]. The change in proline concentration with age is less consistent than the stress-induced increase. The Gln/Glu ratio and the Asn/Asp ratios increase with age, whereas the concentrations of all four amino acids as well as alanine and arginine generally decrease with age to peak again in the final stage. Low Gln/Glu ratios are markers for nitrogen limitation, as Gln and Asn concentrations decrease in nitrogen-limited *Arabidopsis thaliana* [70, 130]. Furthermore low Gln, Glu, Asp, and Ala concentrations indicate an inhibition of nitrogen metabolism, or, if co-occurring with a decreased level of minor amino acids (e.g., Val, Ile, Arg), restart of protein synthesis, for example, after growth inhibition in consequence of C starvation [93]. Taking the observations together, there are no indications for nitrogen limitation or carbon starvation in senescing leaves; carbohydrate and amino acid concentrations are high, likewise ready for export to sink organs. This metabolic pattern of senescing leaves—high abundance of nitrogen, carbohydrates, and amino acids—resembles frequently reported metabolic patterns in leaves under stress.

Altogether, metabolic patterns in leaves subjected to abiotic stress show similarities to leaves subjected to carbon starvation, but even more so to aging leaves. An abiotic stress-induced energy crisis due to photosynthesis inhibition and potentially increased energy demand of roots may enhance senescence in source leaves to remobilise carbon and nitrogen. Stress-induced senescence of leaves has indeed been discussed as an adaptive feature for many decades [64]. Time-series analysis on the leaf and the sink organs depending on it may allow distinguishing whether stress-induced changes in the leaf metabolome reflect senescence or defence of a single leaf. This analysis should be enhanced by physiological performance measurement of the entire plant system. Detailed flow analysis may reveal whether the senescence of source leaves indeed enhances fitness of the entire plant and thus is of adaptive value or just reflects chaotic breakdown.

10.4 Priming

A plant that encounters abiotic stress will upon perceiving the environmental signal activate a signal cascade that alters gene expression and thus leads to changes in the plant's morphology, physiology, and biochemistry. These changes, that increase the plant's chance to survive stress, are called acclimation to distinguish them from evolutionary adaptation that is brought about by changes in gene sequences in subsequent generations due to natural selection. The concept of acclimation assumes that stress resistance mechanisms are not constitutively expressed, but have to be induced by an external signal [61]. The classic example of this acclimation concept is the cold acclimation process undergone by cold-resistant plants in autumn [47, 65]. In *Arabidopsis thaliana*, cold acclimation reduces the lower cardinal point of temperature (estimated as the LT50) by 3–5 °C [150]. Low temperature acclimation in the extremely cold-tolerant *Picea obovata* develops in three steps, preacclimation, early acclimation, and late acclimation to a fully acclimated state, during which the cardinal temperature decreases from −10 to below –35 °C. [4]. Resistance mechanisms are supposed to be induced rather than constitutively expressed, as they are assumed to incur costs or are incompatible with sexual reproduction [87, 116, 149]. This concept has, however, been challenged [13]. Especially species with high resistance towards abiotic stress constitutively express tolerance mechanisms that are induced by stress in less-resistant relatives [8, 33, 59, 60].

For those species that acclimate to abiotic stress, a stress memory mechanism has been postulated that primes the organism to subsequent stresses [11]. Priming is shown by treating the organisms with a mild stress, removing the stress for a certain time and then comparing the organisms' response to a more severe stress to the response of organisms that have not received the first mild stress treatment. When priming occurs, resistance to the later-occurring stress is increased in consequence of a faster and stronger response to stress signals [19, 96]. Mechanistically, priming-induced changes are linked to DNA modifications by methylations that alter DNA accessibility to RNA polymerase [31]. Priming has been shown for various abiotic stresses. When *Arabidopsis thaliana* seedlings are pretreated with high temperatures (60 min at 37 °C plus 45 min 44 °C), they will survive 80 min at 44 °C, whereas seedlings that have not received the priming treatment will die at that temperature [118]. In *Triticum aestivum*, exposure to moderate water deficits during vegetative growth increases grain yield when plants are subjected to drought or heat in the generative stage compared to plants that have not received the priming treatment [140]. Likewise, pretreatment with heat or waterlogging increased resistance to the respective stresses during grain filling [72, 140]. In all three cases, pretreatment alleviated the negative effect of abiotic stress on photosynthesis and membrane composition, whereas increased abscisic acid (ABA) concentrations modified the sink strength of developing grains.

Agriculturally, priming treatments can be employed in deficit irrigation schemes [103, 138]. In peanut, early to mid-season drought treatment maintains the amount

and quality of peanut yield when plants are additionally subjected to late-season drought, while reducing water use in semi-arid areas [103]. For agricultural application, seed priming is of special interest as it can be applied in a standardised way by the seed producer and potentially induces cross-tolerance [24]. For priming, seeds are treated with water, but also with solutions of potassium dihydrogen phosphate, sodium molybdate dihydrate, mannitol, polyethylene glycol, potassium nitrate, and salicylic acid [81, 131]. Seed priming has been shown to enhance germination and root development under suboptimal temperatures and improve early drought resistance by modification of the antioxidant system [23, 124].

During plant growth, priming by moderate abiotic stress can be replaced by treatment with hormones or xenobiotics, a phenomenon that is highly interesting for agricultural applications. The senescence-delaying effect of strobilurin fungicides has been known for more than a decade. Pyraclostrobin treatment increases drought resistance of maize under field conditions [9]. Beta-aminobutyric acid (BABA) prime pathogen and abiotic stress resistance in plants by enhancing the salicylic (SA)- or the ABA-signalling pathway [25, 54, 76, 128]. Direct SA application has a positive effect on freezing tolerance of wheat [123] and drought tolerance of maize [99]. Exogenous application of ABA has been shown to elicit PSII protection from photoinhibition [53]. Furthermore, applications of beneficial microorganisms have been shown to improve growth and abiotic stress tolerance, presumably by priming stress acclimation [107, 117].

10.5 Conclusion

Although plant resistance to drought does not necessarily imply resistance to extreme temperatures or salt, the plant's responses to these stresses resemble each other in many aspects, namely accumulation of osmolytes, protection against ROS, adjustment of energy metabolism, and processes related to altered carbon allocation and senescence. This may result from the fact that many of these stresses occur in combination [82] in consequence of constraints imposed by environmental physics. The evolutionary selection of the plant's stress response under combined stress conditions may explain some of the similarities in signalling and regulation of stress response (crosstalk) elicited by different abiotic stresses [57].

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Chapter 11
Drought Stress Response in Common Wheat, Durum Wheat, and Barley: Transcriptomics, Proteomics, Metabolomics, Physiology, and Breeding for an Enhanced Drought Tolerance

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11.1 Introduction

Drought represents the major environmental constraint limiting agricultural production worldwide [5]. There are several definitions of drought depending on different viewpoints such as meteorological drought (a deficit in rainfall with respect to average values at a given time in a given area), hydrological drought (water deficit in surface and subsurface water reservoirs), socioeconomic drought (a reduction in water consumption recommended by local authorities), physiological drought (an excessive water release by shoot with respect to water uptake by root resulting in plant wilting), agronomical drought (a water deficit period leading to reduced yield), and so on. Common wheat (*Triticum aestivum*) is the most common crop grown on the largest growing area worldwide of approximately 215 million ha and revealing the third highest grain production of 715.9 million tons after rice and maize [26]. Due to its largest growing area worldwide, wheat is grown in diverse environments with different timing and severity of drought stress with respect to the plant life cycle. It is estimated that about 65 million ha of the global wheat production area are affected by drought [26]. Common wheat (*T. aestivum*), but also durum wheat (*T. durum*) and barley (*Hordeum vulgare*) originate from the area of so-called Fertile Crescent ranging from Israel, Jordan, Syria, Iraq, to southeastern Turkey and northwestern Iran. Moreover, there are several old landraces adapted to

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harsh environments such as Ethiopia, Iran, and so on which are adapted to specific types of drought stress and can represent valuable genetic resources in breeding programs aimed at an adaptation to specific drought conditions [5].

Regarding general plant responses to a stress factor, the following types of response were distinguished by Levitt [52]: passive mechanisms including (1) drought escape to survive regular drought periods throughout year in metabolically inactive state such as seeds; drought escape is usually associated with an early vigour in cereals; active mechanisms of drought resistance including (2) drought avoidance, that is, mechanisms aimed at maintenance of sufficient cellular hydration under environmental drought stress; and (3) drought tolerance, that is, active mechanisms aimed at an adjustment of cell metabolism to a decreased intracellular water content (cellular dehydration).

Globally, drought can occur at any stage of wheat and barley development. According to Reynolds et al. [63], basically three types of drought stress can be distinguished:

1. A preflowering water deficit when drought occurs in plant vegetative stage, a type of stress common in regions of South America
2. Water deficit in grain-filling period (terminal drought stress), a common type of stress in regions of Mediterranean, South Asia (India), and Australia
3. Continuous water deficit, some regions of Middle East and South Asia.

An efficient active response to each kind of drought stress is associated with specific physiological and molecular adaptations depending on timing and severity of drought stress. There are several summarising books, book chapters, and papers dealing with drought tolerance, for example, a recent book by Blum [11], reviews of Chaves et al. [18], Cattivelli et al. [17], Komatsu et al. [41], Kosová et al. [44], and Shanker et al. [69] on the problem of complex breeding to improved drought stress tolerance in crops, or Kosová et al. [45] on breeding approaches and genetic mapping studies aimed at an enhancement of drought tolerance in wheat and barley. The aim of this chapter is to provide a complex summary of recent knowledge on wheat and barley physiological and molecular responses to water deficit as well as breeding strategies which can be utilised to improve wheat and barley adaptation to a given environment. In the following two sections, wheat and barley physiological and molecular (transcriptomic, proteomic, metabolomic) adaptations to various types of drought are discussed. Differential responses to water deficit stress between genotypes with differential strategies of drought stress response are discussed. In the next section, utilisation of knowledge on the major physiological and molecular mechanisms underlying an enhanced drought tolerance in the modern breeding programs is discussed. Genetic resources and breeding approaches using knowledge from genetic mapping studies and utilising genetic markers are summarised. Search for novel selection markers such as proteins revealing differential abundance between genotypes with contrasting tolerance is discussed.

11.2 Wheat and Barley Adaptations to Drought at Physiological Level

Water content in plant cells is affected by two contrasting processes: water uptake from soil by root and water release into air by shoot. Water movement in the soil–plant–atmosphere continuum represents a passive process which is governed by gradients in water potential: that is, water moves from sites with a higher (less negative) water potential to sites with a lower (more negative) water potential (plain water placed at earth surface occurring in a balance with water vapour has zero water potential). A generalised scheme of water movement in the soil–plant–atmosphere continuum is shown in Fig. 11.1.

To ensure water movement from soil into plant cell (water uptake), cell water potential has to be lower than soil water potential. Soil water potential is dependent on soil water content (%); however, experimental measurements have shown that the relationship between soil water potential and soil water relative content (RWC) is exponential, that is, soil water potential decreases exponentially with a decreasing soil RWC (summarised in [51]). Plants can decrease cell water potential via decreasing osmotic potential, a major component of cell water potential, due to an accumulation of osmotically active compounds in a process known as osmotic adjustment (OA). Osmotically active compounds represent hydrophilic low-molecular compounds

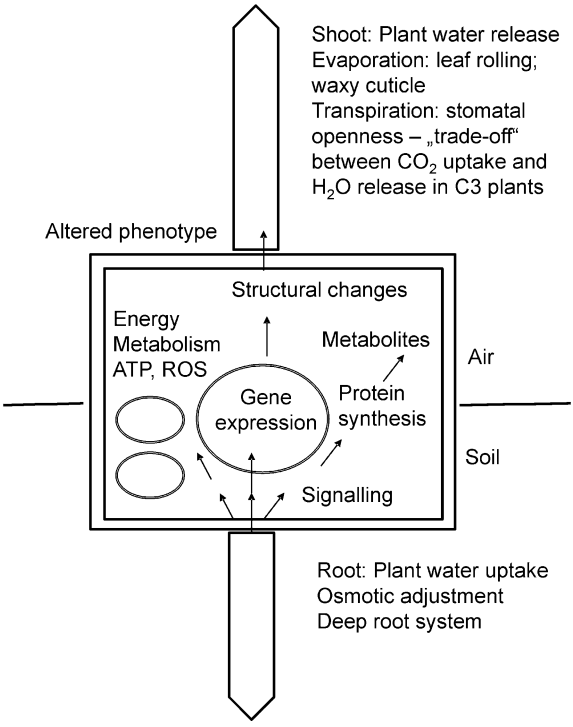

Fig. 11.1 A generalised scheme of water movement via the soil–plant–atmosphere continuum showing critical factors affecting plant water uptake from soil and plant water release into air with a focus on dehydration-induced response in plant cells

(sugars, linear and cyclic sugar alcohols, polyamines, proline, quaternary ammonium compounds, betaines) as well as hydrophilic proteins (e.g., proteins from the COR/LEA superfamily) which form transient noncovalent hydrogen bonds with water molecules thus protecting other biomolecules and supramolecular complexes from loss of hydration envelopes and denaturation [8, 42]. It is known that an accumulation of several types of osmolytes is species-specific: some plants prefer one kind of osmolyte while avoiding another; cereals from the tribe Triticeae (common and durum wheat, barley) accumulate proline and glycine betaine as the most common N-containing osmolytes and glucose and sucrose as the most common sugars in cytoplasm [36]. It has been shown that OA can efficiently help to maintain water uptake from soil only upon a relatively low decline in soil RWC; under more profound RWC decline, OA becomes inefficient due to an exponential relationship between soil RWC and water potential [51, 68]. Therefore, the development of a sufficiently deep root system which enables the plant to utilise water from deeper soil layers with relatively higher soil RWC values represents an adequate plant adaptation to soil water deficit [63]. It should be noted that water movement from soil into plant cell via plasma membrane is basically a passive process governed by water potential gradients between plant cell and soil; however, a diffusion of water molecules via the amphiphilic phospholipid bilayer can be facilitated by several integral membrane proteins forming transport channels. The most known proteins involved in water transport via plasma membrane are aquaporins (AQPs; PIP) whose relative abundance responds to changes in water deficit. Plant water uptake from soil can be determined gravimetrically (in pot experiments) or lysimetrically by determination of water extraction rate in millimeters of water column [83].

Plant water release from cell to ambient air also represents a passive process which is governed by a gradient between cell and air water potential. Water is lost from whole plant surface due to passive water evaporation which can be minimised by waxy cuticle layers or rolling leaf lamina to minimise leaf surface. However, the major way of water release is transpiration via leaf stomata, but stomatal openness does not affect only transpiration, but also CO_2 assimilation. In C3 plants including wheat and barley, stomatal closure leads to a decrease in CO_2 levels in plant mesophyll cells which leads to a shift in RubisCO activity resulting in a relative increase of oxygenase activity over carboxylase activity and decreased CO_2 assimilation. A lack of internal CO_2 (c_i) available for photosynthesis can be indirectly monitored by RubisCO preference of $^{12}CO_2$ over $^{13}CO_2$, a mechanism known as ^{13}C discrimination ($\Delta^{13}C$). Under the lack of c_i, $\Delta^{13}C$ activity of RubisCO decreases and this fact can be used as an indirect sign of water deficit. It can be concluded that there are 'trade-off' mechanisms between stomatal transpiration and photosynthesis in C3 plants because stomatal closure results not only in decreased transpiration, but also decreased CO_2 assimilation and net photosynthesis rate (P_N). Therefore, complex physiological parameters are used for a description of the relationships between water consumption and biomass production such as transpiration efficiency (TE), water use efficiency (WUE), harvest index (HI), and others. Water use efficiency represents the amount of water which is consumed for production of one unit of biomass. It has been shown that in C3 plants, a negative

relationship between WUE and stomatal openness does exist and that WUE can be indirectly monitored by $\Delta^{13}C$. Therefore, determination of $\Delta^{13}C$ in plant biomass can serve as an indirect indicator of stomatal openness and the severity of drought stress [27].

Stomatal closure can enhance WUE under severe water deficit; however, under milder stress, stomatal closure decreases CO_2 assimilation in C3 plants thus leading to a decline in biomass (and yield) production. Therefore, alternative concepts of water-saving strategies have been outlined in recent years; for example, Blum [10] proposed a concept of 'effective use of water (EUW)' which means that a plant tries to maximise water uptake from soil and to minimise water release from shoot by all other ways except for stomatal transpiration. The trade-off between stomatal transpiration and photosynthesis represents a crucial problem in breeding of C3 crops for improved drought tolerance. The crucial question is which of the alternative plant strategies—a conservative, water-saving strategy based on minimisation of water loss via transpiration under water deficit conditions resulting in stomatal closure, or a water-consuming strategy based on open stomata and maintenance of high photosynthesis rates under water deficit conditions—sresults in higher final crop yield. The answer to this crucial query lies in ambient environmental conditions, especially timing and severity of drought stress, and management practices used in the given area including costs and benefits.

Plant water loss is also affected by several characteristics related not only to individual plants, but also to the whole canopy. For example, to minimise water evaporation from soil surface, soil shading by leaves and parameters such as leaf area index (LAI) are crucial, especially in areas where drought occurs during the plant vegetative stage and where irrigation is applied [51]. A negative feedback loop was described between leaf transpiration rate and canopy temperature because an enhanced transpiration rate results in leaf cooling, a phenomenon known as canopy temperature depression (CTD) which in turn leads to a decreased transpiration [6].

Drought affects not only plant water uptake from soil and plant water release via transpiration, but it also affects water availability in plant cells which results in profound cellular responses at transcriptomic, proteomic, and metabolomic levels.

11.3 Wheat and Barley Adaptations to Drought at Molecular Level: Omics Approaches

Water represents an universal medium for biochemical reactions occurring in plant cells. Therefore, water deficit leading to cellular dehydration does not only significantly affect water potential and cell turgour, but it also profoundly affects cell homeostasis. In order to maintain functional biochemical processes in plant cells under altered environmental conditions, plants try to establish novel homeostasis in an active process of stress acclimation [46, 52]. Drought acclimation represents a

complex process including signalling and signal transduction, alterations in gene expression, protein metabolism including both protein biosynthesis and degradation, and energy metabolism as well as biosynthesis of novel metabolites and cell structural components resulting in altered phenotype. Similarly to other abiotic stresses, wheat and barley response to drought stress represents a dynamic process where several stages can be distinguished (alarm, acclimation, resistance, exhaustion, and recovery phases; [43, 50, 52], each of which can be characterised by unique transcriptome, proteome, and metabolome composition.

During the past two decades, a boom of high-throughput separation techniques including predominantly modifications of two-dimensional electrophoresis and liquid chromatography 2DE [57], 2D-DIGE [81, 82], HPLC, nanoLC coupled protein identification via tandem mass spectrometry approaches, as well as gene and whole genome sequencing projects using next-generation sequencing approaches [79, 80] have enabled researchers to study the complexity of biological processes underlying active plant stress acclimation. So-called omics approaches have enabled researchers to study plant stress response at complex transcriptome, proteome, and metabolome levels and to complement the omics results with the data obtained in physiological experiments. In the following sections, basic molecular mechanisms underlying wheat and barley stress response at the transcriptome, proteome, and metabolome levels are discussed. An overview of omics papers published regarding drought responses in common wheat, durum wheat, barley, and their wild relatives (wild emmer) is given in Table 11.1.

11.3.1 Signalling and Signal Transduction Pathways

Initially, water deficit represents an environmental signal sensed by the plant cell. Unlike other stresses such as cold or salinity revealing specific plasma membrane sensors (two-component histidine kinases as a cold sensor in cyanobacteria, [56, 71]; SOS1/SOS2/SOS3 protein complex as Na^+ sensor in *Arabidopsis thalina*, [90]), no specific drought sensor was found in plant cells to date. However, it can be proposed that drought sensing is primarily associated with a decrease in cell turgour, especially in stomatal guard cells, which is associated with alterations in cellular pH, K^+, and ABA levels. ABA receptors PYR1/PYLs/RCARs interact and inhibit phosphatases PP2CA and thus decrease stomatal aperture via an interaction with anion channel SLOW ANION CHANNEL1 and kinase OPEN STOMATA1 [60]. Similarly to other stresses, plasma membrane phospholipids and their residues resulting from phospholipase activity (diacylglycerol DAG, phosphatidylinositol-4,5-diphosphate) are involved in drought signalling. The initial signal is transduced from the plasma membrane to the nucleus using several stress-associated signalling pathways such as MAPK kinase cascade and calcium signalling (calmodulin, calnexin, calreticulin, caleosin, annexin), among others, which ensure the initial signal transduction and amplification. An enhanced level of several transcripts involved in calcium signalling such as the calcineurin B-like (CBL) protein-interacting protein kinase (CIPK)

Table 11.1 An overview of studies on drought-treated wheat and barley cultivars including wild relatives using omics (transcriptomics, proteomics, metabolomics) approaches

Plant material and drought treatment	Method	Major results	Reference
Common wheat (*Triticum aestivum*)			
Common wheat Chinese Spring, Chinese Spring deletion line CS_5AL-10; durum wheat Creso 28 % SWC (control); 12.5 and 18 % SWC (drought)	Transcriptomics: microarray (wheat genome array); qRT-PCR	3056 differentially expressed genes—functional categories: proline metabolism; osmotic and salt stress response; ethylene response; ABA response Up: transposons and retrotransposons (CS_5AL-10)	[3]
Iranian landraces N14, N49—stem (senescence stage) 80–90 % FWC (control), 50 % FWC (drought)—10, 20, 30 days after anthesis	Proteomics: 2DE MALDI-TOF/TOF; Q-TOF	135 DAP (82 identified)—redox (GST, Prx-5, Trx M), chaperonin, protein metabolism (chloroplast 50S ribosomal protein L4, proteasome regulatory subunit 14, Clp protease)	[9]
Australian wheats Kukri (S), Excalibur, RAC875 (T)—leaf Water withholding until leaf wilting in Kukri (S)	Metabolomics: GC-MS	Up: amino acids I, L, P, V, W Down: organic acids—only in T	[12]
Spring genotypes Ninchun 4, Chinese Spring—grain 14 days after flowering	Proteomics: 2DE MALDI-TOF; MALDI-TOF/TOF	152 DAP (96 identified)—Up: TCTP, Down: APX, RubisCO	[29]
Spring wheats Arvand, Khazar-1, Kelk Afghani—grain Field conditions (Azarbayjan) plus artificial irrigation	Proteomics: 2DE MALDI-TOF/TOF	121 (57 identified) Up: Trx *h*, 1-Cys peroxiredoxin, GST; PDI; LEA, sHSP17, HSP70	[32]
Cv Yumai 34—leaf Hoagland solution, 15 % PEG-6000 (3 d); 0.5 mM SA pretreatment (3 d)	Proteomics: TCA/acetone; 2DE MALDI-TOF/TOF	82 (76 identified proteins), of which 35 SA-responsive proteins Up: 14-3-3; APX, GST, SA-responsive proteins: GS1c, GST1, PDI; ATP synthase CF1 α,β	[37]

(continued)

Table 11.1 (continued)

Plant material and drought treatment	Method	Major results	Reference
Cvs Hanxuan 10 (T) and Ningchun 47 (t)—seedling leaf Hoagland solution, 20 % PEG-6000 (−075 MPa) for 48 h	Proteomics: TCA/acetone/phenol; phosphopeptide enrichment via TiO_2 microcolumns; LC-MS/MS	173 (T) and 251 (t) phosphoproteins identified: signalling (SnRK2 kinase, protein phosphatase 2C, CDPK, calmodulin 2-2); transport (AQP, MSSP2; H^+-ATPase); LEA proteins (WCOR719, WCOR825, WRAB17)	[88]
Durum wheat (*Triticum durum*)			
Durum wheat cv. Kiziltan (S), emmer (*T. dicoccoides*) lines TR39477, TTD22 (T)—leaf 9 d water withholding	Proteomics: 2DE nanoLC-ESI-MS/MS	75 identified proteins, 11 candidates for drought tolerance Genotypic differences: TPI, ATP synthase CF1 (efficient carbohydrate metabolism and ATP production)—higher in T; β-1,3-glucanase, β-1,4-glucanase, XET (cell wall remodelling for osmotic adjustment and energy source); methionine synthase—higher in S	[15]
Durum wheat cv. Ofanto—leaf 70 % FWC for 7 d (control); 57 % FWC for 7 d (stress)	Proteomics: 175 mM Tris-HCl, pH 8.8, TCA-acetone; 2DE MALDI-TOF	36 identified proteins Up: carbonic anhydrase, RubisCO LSU Down: RubisCO SSU, Calvin cycle enzymes (ALDO, PRK); ATP synthase CF1 α; plastidic GS2a,b,c	[16]
Wild emmer (*Triticum dicoccum* var. *dicoccoides*)			
Genotypes R (resistant) and S (susceptible) 8 d water withholding	Transcriptomics: Affymetrix Gene Chip; qRT-PCR	Signalling and TFs: Up: bZIP, MAPK, NAC, WRKY; sugar sensing: SnRK (R↑); down: bHLH; RNA processing: WD-40 repeat; ABA metabolism (NCED); energy transfer (FNR2); lignin metabolism; fatty acid metabolism; GLP (R↑, S↓)	[47]
Genotypes R (resistant) and S (susceptible) 7 d water withholding	Transcriptomics: Affymetrix Gene Chip; qRT-PCR Metabolomics: GC-MS	Transcriptome: ABA, GA, IAA-biosynthesis and response-related genes (PP2C/ABI1; bZIP/AREB; GA2OX1, ID1L2); MADS-box26 (R↑); Metabolome: Krebs cycle acids (R↑), 2-oxoglutarate (↑)	[48]

(continued)

Table 11.1 (continued)

Plant material and drought treatment	Method	Major results	Reference
Barley (*Hordeum vulgare*)			
Cv. Morex—lemma, palea, awn, seed 4 d water withholding	Transcriptomics: Affymetrix Barley1 GeneChip; qRT-PCR (6 selected genes)	Up: HSPs (HSP82, HSP70, sHSP26); ROS scavenging genes (CAT, class III POX); awn: chl degradation (chlorophyllase, pheophorbide *a* oxygenase)	[1]
8 Egyptian accessions, 2 selected for proteome analysis 15141 (T), 15163 (S)—leaf 24 °C; 70 % FWC (control); 5 days at 10 % FWC (stress)	Proteomics: TCA/acetone; 2D-DIGE MALDI-TOF	Up: PDI, Hsp90, Hsp100 (Clp protease), chloroplastic ATP synthase CF1 α; Genotypic differences: PPDK, Hsp70, zinc metalloprotease—higher in T than S; proteins involved in osmolyte biosynthesis (betaine aldehyde dehydrogenase, methionine synthase, SUS1)—higher in S than T	[7]
Cv. Golden Promise—leaf Ca 100 % FWC (control); 25 % FWC (stress)—28 d—combined effect of drought and *Piriformospora indica*	Proteomics: TCA/acetone; 2DE MALDI-TOF/TOF	45 identified proteins Up: RubisCO activase A, RubisCO SSU, CCOMT Down: PRK, ACP Effect of *P. indica*: RubisCO SSU, PSI Fe-S, chl *a*/*b* binding protein; CCOMT, APX, 30S ribosomal protein 3, V-ATPase, 2-Cys Prx—higher in inoculated than control plants under stress	[30]
H. vulgare cv. Martin, *H. spontaneum* 41-1 (T); Moroc9-75 (S); 70 % AWC (C), 10 % AWC (D)—0, 1, 3, 5 d	Transcriptomics: 22 K Affymetrix Barley 1 microarray; qRT-PCR	Up:P5CS, PP2c, chaperones (in T and S); Up: NADP-ME, PDH, CSMO, ALDH, ADOR, HSP17.8, DHN3 (in T only) Constitutive expression in T: CDPK, MSBP, GDA2, GST,	[31]
Lines 004186 (S), 004223 (T)—shoot 20 % PEG; 3 d water withholding	Proteomics: 2DE MALDI-TOF, nanoLC-MS/MS	Up: V-ATPase, MDH, OEE (T), HSP70—up (T), down (S) Down: RubisCO activase (S)	[38]
Cv Tokak—D: 6, 10 h after plant removal from growth medium	Transcriptomics: EST microarray with 1463 cDNA elements	Up: LEA, metallothionein-like, P5CS Down: photosynthesis-related genes	[58]
Syrian landrace Arta (T), Australian cv Keel (T)—leaf (heading stage) Drought: 50 % FWC (control), 15 % FWC (stress) for 3d; Heat: 36 °C (4 h)	Proteomics: TCA/acetone; 2DE, 2D-DIGE MALDI-TOF/TOF	99 DAP Heat—up: FBP ALDO, chaperones, proteases, eEF-G, eIF4A, RubisCO activase B Genotypic differences (14 proteins): photosynthesis-related proteins (LHCII type III Lhcb3, OEE1 PsbO, RubisCO activase B)—higher in Keel than Arta	[66]

(continued)

Table 11.1 (continued)

Plant material and drought treatment	Method	Major results	Reference
Er/Apm—leaf D: 6 h (shock); 7, 11 d (gradual D); recovery	Transcriptomics: EST microarray with 1654 cDNA elements	Up: JA metabolism (AOS), metallothionein-like (both shock andgradual D), LEA (gradual D), P5CS (shock); cytochrome P450 (recovery)	[72]
Cv. Amulet—crown 30, 35 % SWC (14 d)	Proteomics: 2D-DIGE MALDI-TOF/TOF	105 DAP—82 identified—Up: GPX, GST, chitinase II Down: cdc48, GID1L2, OEE1,2	[84]
Cvs Basrah (T) and Golden Promise (S)—leaf, root 7 d water withholding control: 80 % RWC; drought: 70 % RWC (T), 60 % RWC (S)	Proteomics: 10 mM PBS, TCA-acetone; 2D-DIGE MALDI-TOF	Identified proteins: 24 (leaf), 45 (root) Up: ABA-induced protein r40c1, small G-protein Rab2, Myb-like protein, 14-3-3 protein Down: GST, GPX Genotypic differences: Enhanced regulation of ROS (APX, CAT, LOX, class III POX) and protein folding in T than in S	[87]

Proteomic studies aimed at comparison of a differential drought response between at least two common wheat cultivars are summarised in Table 11.2

Abbreviations: ↑—an increased abundance (transcript, protein, metabolite); ↓—a decreased abundance (transcript, protein, metabolite); *2D-DIGE*—two-dimensional differential gel electrophoresis; *2DE*—two-dimensional electrophoresis; *ABA*—abscisic acid; *ALDH*—aldehyde dehydrogenase; *ALDO*—fructose-1,3-bisphosphate aldolase; *AOS*—allene oxide synthase; *APX*—ascorbate peroxidase; *AQP*—aquaporin; *AWC*—available water content; *C*—control (treatment); *CAT*—catalase; *CDPK*—calcium-dependent protein kinase; *CSMO*—C-4 sterol methyl oxidase; *Cv(s)*—cultivar(s); *D*—drought (treatment); *DAP*—differentially abundant proteins; *DHN*—dehydrin; *FNR*—ferredoxin-NADP reductase; *FWC*—field water capacity; *GC-MS*—gas chromatography mass spectrometry; *GDA2*—G2 pea dark accumulated protein; *GLP*—germin-like protein; *GS*—glutamine synthetase; *GST*—glutathione-S-transferase; *HSP*—heat shock protein; *JA*—jasmonic acid; *LEA*—Late embryogenesis-abundant; *LOX*—lipoxygenase; *MALDI-TOF*—matrix-assisted laser desorption/ionization time-of flight; *MDH*—malate dehydrogenase; *MSBP*—membrane steroid binding protein; *NADP-ME*—NADP malic enzyme; *OEE*—oxygen evolving enhancer (protein); *P5CS*—Δ^1-pyrroline-5-carboxylate synthetase; *PBS*—phosphate buffer saline; *PDH*—pyruvate dehydrogenase; *PDI*—protein disulphide isomerase; *PRK*—phosphoribulokinase; *POX*—peroxidase; *PP2C*—protein phosphatase 2C; *Prx*—peroxiredoxin; *S*—sensitive (genotype); *SnRK*—sucrose nonfermenting-related protein kinase; *SWC*—soil water capacity; *T*—tolerant (genotype); *t*—genotype less tolerant than T; *TCA*—trichloroacetic acid; *TCTP*—translationally-controlled tumor protein; *TPI*—triosophosphate isomerase; *Trx*—thioredoxin; *V-ATPase*—vacuolar ATPase; *WCOR*—wheat cold-regulated (protein); *WRAB*—wheat responsive to abscisic acid; *XET*—xyloglucan *endo*-transglycosylase

network involved in a crosstalk between ABA-dependent and ABA-independent pathways was found in a resistant wild emmer (*Triticum dicoccum* var. *dicoccoides*) genotype with respect to the sensitive one [47]. Reversible phosphorylation plays an important role in signalling events. Altered abundance of several phosphoproteins including not only proteins involved in signalling (Ca^{2+}—dependent protein kinase CDPK; mitogen-activated protein kinase MAPK, protein phosphatase 2C PP2C, sucrose nonfermenting1-related protein kinase SnRK), but also several transcription factors (TaABI5) and structural proteins (aquaporins, phosphorylation of AQP8 at Ser16, H^+-ATPase, monosaccharide sensing protein 2 MSSP2, LEA-III proteins WCOR615 and WRAB17), were found by Zhang et al. [88] in drought-treated wheat.

11.3.2 Regulation of Gene Expression

In the nucleus, the initial signal is transformed into activation or repression of several transcription factors resulting in alterations in gene expression. Transcription factors and regulatory proteins regulate the expression of effector proteins directly involved in plant stress response (chaperones, hydrophilic proteins, reactive oxygen species (ROS) scavenging enzymes, enzymes involved in osmolyte biosynthesis, PR proteins; Kosová et al. [44]). Upon drought, both ABA-dependent (AREB/ABF, CBF4, MYB, MYC) and ABA-independent (CBF/DREB1, DREB2, NAC, NAM) transcription factors are activated, some of which (MYC2, NAC) are also regulated by JA indicating a crosstalk between dehydration- and pathogen-induced signalling pathways [70]. In wheat, an exogenous ABA treatment was found to enhance levels of enzymes involved in JA biosynthesis such as 12-oxophytodienoate reductase indicating a positive effect of ABA on JA levels [2]. Similarly, salicylic acid (SA) was found to induce several drought-responsive proteins in common wheat thus leading to an enhanced drought tolerance [37]. Moreover, transcription factors involved in developmental processes such as MADS-box 26 involved in root development were found to be induced upon dehydration [48]. Several transcription factors can bind to multiple *cis*-regulatory elements in gene promoter sequences thus forming large regulons of the given TF. On the other hand, activation of gene expression of a given stress-responsive gene is usually ensured by multiple regulatory elements in its promoter sequence and by formation of a complex of homo- and hetero-oligomers of several TFs. It can thus be concluded that both signalling and gene expression processes represent complex networks of overlapping regulons regulated by multiple proteins and protein isoforms with differential posttranslational modifications (PTMs) thus ensuring adequate activation or repression of the target genes. Under a sudden drought treatment (drought shock), several regulatory transcripts such as several protein kinases and DnaJ-like proteins were also found decreased [58].

11.3.3 Protein Metabolism

Drought-induced alterations in gene expression result in alterations in protein biosynthesis and the whole translational machinery. A comparative proteomic analysis of ABA-regulated proteins found an increase in 51 proteins related to translation in drought-tolerant wheat variety Nesser whereas 40 translation-related proteins were decreased in drought-sensitive Opata [2]. Similarly, an increase in translation elongation factor eEF-1α was found in a drought-tolerant Egyptian barley accession with respect to a drought-sensitive one [7]. Alterations in several ribosomal proteins belonging both to cytosolic ribosomes (40S, 60S) and organellar prokaryote-type ribosomes (30S, 50S) were also found in several studies on drought-treated wheat and barley plants [30, 38]. Alterations in components of protein degradation machinery, namely proteasome subunits and enzymes of ubiquitin ligase complex (E1 to E3 components), were found in several proteomic studies dealing with drought in wheat and barley [2, 30].

11.3.4 Amino Acid Metabolism

Changes in protein metabolism are tightly linked to changes in amino acid metabolism because amino acids represent protein components. However, several amino acids do not only represent protein components, but also osmolytes and precursors of other metabolites in plant cells. For example, proline is a major osmolyte in cereal cells, glutamic acid is an important precursor for proline and chlorophyll, and glutamic acid together with glutamate plays a major role in nitrogen assimilation. Glycine is a precursor of an osmolyte glycine betaine. Methionine is a precursor of *S*-adenosylmethione (SAM) which is not only an universal methyl donor in plant cells (methylation as a regulatory process in DNA activity; euchromatin vs heterochromatin transitions; methylation of monolignols as an important step in cell wall lignification), but also a precursor of a wide array of stress-related compounds such as polyamines, ethylene, and phytosiderophores (Yang cycle). Increased *S*-adenosylmethione synthetase (SAMS) levels were found in several proteomic studies on drought-treated wheat and barley plants (e.g., [25]). Phenylalanine is a precursor of a wide array of aromatic compounds including flavonoids and lignin components (monolignols) which are synthesised via the phenylpropanoid pathway regulated by phenylalanine ammonia lyase (PAL). Several amino acids can also be simply deamined to yield oxoacids which are intermediates of the Krebs cycle. Moreover, apart from protein amino acids, several nonprotein amino acids such as γ-aminobutyric acid (GABA) also play an important role in drought response. In a metabolomic study on three Australian wheats with different drought tolerance, increased levels of several amino acids, namely proline, aromatic amino acids tryptophan, tyrosine, and phenylalanine, and branched amino acids leucine, isoleucine, and valine were found under drought [12]. The increase in aromatic amino

acids indicates an enhanced biosynthesis of a wide array of secondary metabolites (terpenoids, flavonoids, glycosides, lignin). In addition, tryptophan may act as a ROS scavenger.

11.3.5 Hormone Metabolism

Under drought, an upregulation of several stress-related hormones, namely ABA, JA, and SA, was found in several studies. In a study on wild emmer (*Triticum turgidum* ssp. *dicoccoides*) comparing a drought-resistant and a drought-sensitive genotype, a relatively higher upregulation of several transcripts involved in ABA, GA (gibberellin) biosynthesis (GA2OX1), and response (gibberellin receptor GID1L2 binding active GA, interacting with DELLA proteins and leading to their degradation) as well as in auxin (IAA) transport (auxin efflux carrier PIN3) was found in the roots of the resistant genotype with respect to the sensitive one which corresponded to higher ABA levels [48]. In a transcriptional profiling study on drought response in durum wheat cv. Creso, common wheat cv. Chinese Spring, and a Chinese Spring 5A_10 deletion line, drought treatment led to a significant induction of 9-*cis*-epoxycarotenoid-dioxygenase (NCED) gene encoding a crucial enzyme involved in ABA biosynthesis, in all genotypes studied [3].

11.3.6 Energy Metabolism

An active plant stress acclimation represents an energy-demanding process. However, alterations in plant cellular environments cause an enhanced risk of imbalances between the individual reactions resulting in an enhanced ROS formation. Therefore, imbalances in energy metabolism-related processes are observed in omics studies aimed at drought effects which are associated with both an increase and a decrease in several photosynthesis-, respiration-, and ATP biosynthesis-related proteins.

11.3.6.1 Photosynthesis

Drought leads to discrepancies between the rates of primary electron transport processes on thylakoid membranes (light reactions) and enzymatic reactions of the Calvin cycle in chloroplast stroma (dark reactions) resulting in an enhanced ROS formation and photoinhibitory damage of electron transport complexes. In transcriptomic studies on dehydration shock treatment in barley and terminal drought in wild emmer wheat, respectively, a decrease in several photosynthesis-related transcripts such as PSII LHC protein, RubisCO LSU, and SSU was found [47, 72]. At the protein level, a decrease in thylakoid-located chlorophyll *a*/*b* binding protein

and PSI Fe-S center and in stroma-located RubisCO subunits and several Calvin cycle enzymes such as phosphoglycerokinase (PGK) and phosphoribulokinase (PRK) was found in drought-treated barley [30] and durum wheat [16], respectively. In addition, enhanced levels of enzymes with supporting functions such as carbonic anhydrase and RubisCO activase as well as chaperones such as RubisCO large and small subunit binding proteins were found in drought-treated plants [16, 66] although genotypic differences between tolerant and sensitive lines revealing an increase and a decrease in photosynthesis-related proteins (OEC, RubisCO LSU), respectively, were also found [28, 29, 38]. Analogously, an increase in some OEC proteins (OEE1) upon mild drought (35 % soil water capacity; SWC) indicating stress acclimation followed by a decrease (OEE1, OEE2) upon a more severe drought treatment (30 % SWC) indicating stress damage was found [84]. Moreover, in photosynthesising spike organs, enhanced levels of transcripts encoding chlorophyll degradation enzymes (chlorophyllase, phaeophorbide *a* oxidase) were found under terminal drought indicating enhanced chlorophyll degradation as a potential prevention against ROS formation [1].

11.3.6.2 Aerobic and Anaerobic Respiration

Similarly to photosynthesis, an adaptation of respiratory processes aimed to minimise cellular oxidative damage was found in drought-treated plants. Due to an enhanced risk of ROS-induced cell damage, glycolysis and several alternative pathways seem upregulated whereas mitochondrial respiratory chain reactions seem downregulated in drought-treated cereals. Glycolytic enzymes were found increased in several transcriptomic and proteomic studies [16, 29]. Regarding the Krebs cycle, increased levels of citrate, aconitate, and isocitrate and their synthesising enzymes citrate synthase, aconitate hydratase, malate dehydrogenase, and succinate dehydrogenase, respectively, were found in drought-treated wild emmer in a joint transcriptomic and metabolomic study [48]. Citrate, aconitate, and isocitrate are precursors of 2-oxoglutarate which can be transaminated into glutamate, a precursor of proline. An increase in alternative oxidase (AOX) and a decrease in cytochrome *c* oxidase was found in barley spike organs under drought indicating a minimisation of ROS production in cytochrome *c* oxidase complex [1]. Consistent with an increased risk of ROS formation due to imbalances between various reactions of aerobic metabolism, a shift from aerobic to anaerobic respiration under severe drought indicated by an enhanced abundance of several glycolysis- and alcoholic fermentation-related enzymes such as 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, pyruvate kinase, and alcohol dehydrogenase and a decrease in respiratory chain-related enzymes such as NADH dehydrogenase were found in some studies [15, 84].

A decrease in catalytic subunits of ATP synthesising enzymes such as a β-subunit of CF1 ATP synthase was also found under drought indicating a decreased ability to produce ATP as a consequence of impaired photosynthesis and mitochondrial respiration [1, 16].

11.3.7 Stress-Responsive Proteins

11.3.7.1 Chaperones and Hydrophilins

Regarding cellular metabolism, drought stress represents a dehydration stress as well as an oxidative stress. A dehydration stress represents a direct result of an excessive water loss by shoot over water uptake by roots. Plants adapt to cellular dehydration by an enhanced accumulation of osmolytes and hydrophilic proteins with chaperone functions (proteins from COR/LEA superfamily, heat-shock proteins, RubisCO large and small subunits binding proteins CPN60-α and CPN60-β, and protein disulfide isomerase PDI) which help to preserve water envelopes around biomolecules and prevent the target biomolecules from denaturation. It has also been shown that some chaperones such as HSP70/HSP90 complex are involved in processes associated with stomatal closure. An enhanced abundance of several proteins with chaperone functions such as HSP70, HSP90, HSP100, PDI, and others, was reported in several proteomic studies dealing with drought ([7, 15, 38, 87]; and others). Moreover, an enhanced phosphorylation level of chaperones HSP60 and HSP90 was found in drought-tolerant winter wheat cultivar with respect to the drought-sensitive one [34]. Regarding dehydrins, an association of dehydrin transcript levels with plant RWC was found in Triticum and Aegilops seedlings. In addition, resistant genotypes revealed higher *Dhn* transcript levels even upon relatively high tissue hydration (RWC) resulting in a reduced water loss rate, thus indicating dehydrin involvement in water retention [61]. A differential level of phosphorylation of drought-induced dehydrin protein DHN5 (Y_2K) was found in two Tunisian durum wheat cultivars revealing differential drought tolerance [13].

11.3.7.2 Osmolytes

Sugars represent the major osmolytes accumulating in cereal cells upon drought. Under extreme dehydration, nonreducing sugars and oligosaccharides may substitute water in protein hydration envelopes. Under drought, enhanced levels of glucose, fructose, galactose, and mannose were found [12]. Major N-containing osmolytes accumulating in cereals include glycine betaine and proline. An enhanced expression of genes involved in glycine betaine synthesis, C-4 sterol methyl oxidase, was found in drought-tolerant *H. vulgare* ssp. *spontaneum* when exposed to drought in the reproductive stage [31]. Proline can be synthesised via two alternative pathways, the first one from glutamic acid where the rate-limiting step represents pyrroline-5-carboxylase synthase (P5CS) whose activity is regulated by its terminal product, proline, in a negative feedback loop. Overexpression of P5CS leads to enhanced proline levels in transgenic plants [40]. The other proline biosynthetic pathway starts with ornithine. An ornithine-based proline biosynthetic pathway (arginase, ornithine aminotransferase) was found to be activated in barley spike organs under terminal drought [1]. Osmolytes may play multiple roles in

cellular response to dehydration; an accumulation of osmolytes decreases osmotic potential of cell cytoplasm thus allowing water uptake from ambient environment. Moreover, osmolytes can also substitute water molecules in hydration envelopes of several biomolecules due to their polar nature and formation of hydrogen bonds. In addition, some osmolytes such as γ-aminobutyric acid (GABA) and polyamines (spermidine), but also sugars and sugar alcohols (e.g., inositol trisphosphate), may play a regulatory role in stress signalling thus affecting other stress-responsive processes.

11.3.7.3 ROS Scavenging Agents

An oxidative stress represents an indirect result of discrepancies between several reactions of aerobic metabolism, for example, light-dependent and light-independent photosynthetic reactions, Krebs cycle reactions, and mitochondrial electron transport chain, which result in an enhanced formation of reactive oxygen species. ROS can act as stress signals; however, due to their high reactivity, ROS cause uncontrolled damage of several biomolecules, and thus, the ROS level has to be precisely regulated by networks of both enzymatic and nonenzymatic ROS scavenging agents. Major nonenzymatic ROS scavenging agents include small molecules involved in direct reactions with ROS such as carotenoids, tocopherols (vitamin D), ascorbate (AsA), and tripeptide glutathione (GSH). ROS scavenging enzymes include a wide array of more or less specific proteins usually with metal cofactors in enzymatically active sites which can work in coordinated pathways, for example, ascorbate–glutathione cycle, or form different isoforms specific for subcellular compartments (e.g., isoforms of superoxide dismutase SOD, Cu/Zn-SOD (cytosolic), Mn-SOD (mitochondrial), Fe-SOD (chloroplastic); classes of glutathione-S-transferase (GST), thioredoxin isoforms, thioredoxin F, H, M, etc.). A precise regulation of abundance of redox metabolism-related proteins was found in proteomic studies dealing with drought stress by Hajheidari et al. [32], Ford et al. [28], Wendelboe-Nelson and Morris [87], Faghani et al. [25], and others. A risk of oxidative damage is also enhanced by the presence of free metal ions in cell cytoplasm. Therefore, an enhanced expression of metallothionein-like proteins involved in metal ion binding and sequestration has been observed in transcriptomic and proteomic studies dealing with drought and salinity [58, 72]. Drought also affects abundance and activity of enzymes controlling cellular pH homeostasis. An enhanced abundance of NADP malic enzyme (NADP-ME) in stomatal guard cells may contribute to stomatal closure due to its effect on malate degradation thus resulting in enhanced WUE [49].

11.3.7.4 Pathogenesis-Related Proteins and Others

Drought also induces an expression of several other stress-responsive proteins including many pathogenesis-related (PR) proteins. Lipid transfer proteins (LTPs)

may deliver lipids for formation of a thick waxy cuticle on leaf lamina as a protection against water evaporation from leaf surface. LTPs were found upregulated under drought [1]. Other PR proteins chitinases, amylases, protease inhibitors, lipoxygenases, and others, which are induced under pathogen attack; however, they are also induced upon drought inasmuch as drought stress enhances a risk of damage caused by a potential pathogen attack [2]. Other drought-responsive proteins include stress-associated proteins (SAPs) containing A20-like and AN1-like zinc finger domains which reveal enhanced phosphorylation at Ser^{106} upon drought [88].

11.3.8 Cellular Transport

In drought-treated barley, alterations in protein components of both cytoplasmic (actin) and membrane (annexins) transport were found [84]. In drought-treated wheat, phosphorylation regulation of aquaporins as transmembrane water channels was described [88].

11.3.9 Cell-Wall–Related Metabolism

Changes in cellular metabolism also affect the resulting cell wall structure. Under drought, there have been found significant changes in abundance in enzymes involved in both cell wall polyglucan and lignin metabolism which result in a decrease in cell wall extensibility. Differential levels of cell-wall–related transcripts including β-galactosidase, extensin, glycine-rich protein, and germin were found in wild emmer wheat under terminal drought [47]. An increase in xyloglucan 1,4-β-*endo*-transglycosylase (XET) involved in cleavage of β 1–4 bonds of polyglucans indicates substantial cell wall remodelling under drought. An enhanced abundance of several enzymes involved in the phenylpropanoid pathway and lignin biosynthesis such as phenylalanine ammonia lyase (PAL), caffeic acid O-methyltransferase (COMT), caffeoyl-CoA O-methyltransferase (CCOMT), as well as other enzymes involved in lignin biosynthesis (SAMS) induced by drought and ABA indicates an enhanced cell wall lignification under drought which indicates cell growth cessation [2, 15].

11.3.10 Recovery After Stress

Recovery after drought cessation (rewatering) is still seldom studied in omics studies despite the fact that establishing novel homeostasis during recovery treatment is crucial for further plant growth and development. A comparison of a dehydration shock, a gradual dehydration, and a rewatering treatment at transcriptome level was carried out by Talamé et al. [72]. The transcripts induced by both shock and gradual

drought treatments included four genes encoding metallothionein-like proteins, one sugar transporter, and genes involved in jasmonate and osmolyte biosynthesis (P5CS). Transcripts induced upon recovery treatment include cytochrome P450 involved in xenobiotics detoxification, proteins involved in ABA degradation, proteins involved in protein degradation (polyubiquitin), ROS scavenging enzymes (peroxidases), and regulatory proteins (protein kinases), whereas transcripts decreased upon recovery treatment include many drought-inducible genes such as sugar transporters and protein kinases. Ford et al. [28] studied proteome response in three Australian wheat cultivars at 24 h after rewatering following drought treatment until wilting point. In wheat, proteome analysis of a rewatering response has revealed an increased abundance of 8 out of 12 glycolysis enzymes in tolerant cultivar Excalibur indicating an enhanced need for energy during a recovery treatment. HCF136 which is a protein involved in repair and assembly of OEC and PSII complexes significantly increased in tolerant Excalibur under rewatering, indicating a quick photosystem II (PSII) repair after stress cessation. In contrast, dehydration-induced proteins COR410 and SDi-6 revealed a significant decrease at rewatering indicating a cessation of the adverse impacts of a stress treatment. Similarly, a decrease in stress- and defence-related proteins such as HSP60 and CCOMT and an increase in growth- and photosynthesis-related proteins such as tubulin α-2 and OEE2 were found after 48 h recovery treatment in a drought-tolerant wheat cultivar with respect to a drought-sensitive one [34].

11.3.11 Drought Response During Grain-Filling Period

Most omics studies on the effects of drought are focused on young plants in the vegetative stage of development. However, drought during a grain-filling period (terminal drought) can adversely affect the final grain yield. Molecular mechanisms underlying assimilate reallocation from stem to grain in two contrasting Iranian wheat landraces were investigated in a proteomic study by Bazargani et al. [9]. The tolerant landrace was able to remobilise its stem reserves efficiently due to an upregulation of several senescence-associated proteins and ROS scavenging-related proteins and a breakdown of photosynthetic proteins thus enhancing stem reserves reallocation and stem senescence. A comparative transcriptomic study of the effects of drought (4 d of water withholding) on transcriptome composition in barley spike organs (palea, lemma, awn) during a grain-filling period has revealed a relatively drought-induced damage of processes associated with photosynthesis and carbohydrate metabolism in lemma and palea with respect to awn suggesting a major role of lemma and palea in carbohydrate supply to the developing grain [1]. Expression of genes involved in both light-dependent and light-independent (Calvin cycle enzymes) reactions of photosynthesis was downregulated under drought in all spike organs with respect to control; however, the decrease was more profound in awn than in lemma and palea. Consistent with a decrease in photosynthesis-related transcripts, an increase in transcripts encoding enzymes involved in chlorophyll

degradation (chlorophyllase, pheophorbide *a* oxygenase) was found in spike organs indicating a minimisation of oxidative damage potentially caused by relatively high chlorophyll levels with respect to decreased rates of photosynthetic reactions.

11.4 Differences Between Wheat and Barley Genotypes Revealing Differential Strategies to Cope with Water Stress

In several omics studies, responses of wheat and barley with different drought tolerance strategies are compared.

Complex physiological [35], proteomic [28], and metabolomic [12] studies were carried out in three Australian wheat cultivars employing differential strategies of drought stress response. Regarding cyclic drought stress during preanthesis, postanthesis and grain-filling periods, RAC875 and Excalibur revealed 24.3 and 18 % higher yield, respectively, than Kukri thus showing Kukri as a relatively drought-sensitive cultivar with respect to RAC875 and Excalibur (tolerant). At the physiological level, RAC875 revealed relatively thick leaves with thick waxy cuticle and relatively high chlorophyll content under drought. RAC875 also revealed a conservative strategy with respect to plant water regime, showing reduced leaf area, low stomatal conductance, relatively low transpiration, a stay-green phenotype, high stem water-soluble carbohydrates (WSC), moderate OA, and slower recovery after stress. In contrast, Excalibur revealed a more dynamic strategy of drought stress response including leaf rolling upon drought, relatively high OA, relatively high stomatal conductance, low endogenous ABA, and a rapid recovery after stress [35]. At the proteome level, all three wheat cultivars revealed a decrease in photosynthesis-related proteins with the largest protein number found in Kukri [28] in comparison to RAC875 [19] and Excalibur [17]. In Kukri, a decrease in four LHC proteins was found. The largest decrease (5.4-fold) in photosynthetic proteins was found in PSI subunit VII protein in the two tolerant cultivars with respect to Kukri. All three cultivars also revealed enhanced levels of CAT and SOD involved in ROS scavenging whereas aldehyde dehydrogenase (ALDH) involved in detoxification of toxic aldehydes from lipid peroxidation revealed increased levels in both tolerant cultivars, but not in Kukri. In addition, Kukri revealed a decrease in glyoxalase I levels [28]. Metabolomic analysis revealed an increase in several amino acids, namely proline, tryptophan, and branched amino acids leucine, isoleucine, and valine, under drought in all three cultivars. A relatively small, but significant, decrease in organic acid levels under drought in the two tolerant cultivars resulting in an increase of cytosolic pH may have a signalling effect inducing drought response in the two tolerant cultivars which is absent in the sensitive one [12].

Comparative transcriptomic and metabolomic studies on wild emmer (*T. turgidum* ssp. *dicoccoides*) have revealed higher levels of several hormone (ABA, gibberellins, auxin), RNA binding, calcium (calmodulin, caleosin, annexin), and

phosphatidylinositol signalling-related genes as well as higher ABA levels in resistant (R) genotype with respect to sensitive (S) one both in well-watered (control) and drought-treated plants [48]. Consistent with the upregulation of drought-related signalling pathways, an enhanced abundance of several genes involved in GA biosynthesis (GA2 oxidase 1) and response (XERICO) as well as GA-regulated downstream genes such as *MADS*-box26 transcription factor was found in R genotype with respect to the S genotype. Similarly, several ABA-regulated downstream genes such as *LEA* genes (*RD22*) as well as an increase in ABA-regulated drought-related metabolites such as proline and glycine betaine were found in the R genotype with respect to the S one [48].

In several omics studies (transcriptomics, proteomics, metabolomics), one of the major aims is to compare differences between genotypes with a differential response to stress (differential level of tolerance, wilting point, etc.). There have been found both quantitative and qualitative differences in transcriptome, proteome, and metabolome composition as well as differences in protein PTMs between genotypes revealing differential tolerance to stress. In a large 22K Affymetrix microarray study on barley transcriptome response to drought including two drought-tolerant (Martin and *H. vulgare* ssp. *spontaneum* 41-1) and one drought-sensitive (Moroc9-75) barley cultivar, 18 genes upregulated by drought with respect to control in all three genotypes and 17 genes upregulated by drought only in the drought-tolerant genotypes were identified [31]. The drought-responsive genes common to all barley cultivars examined included P5CS, PP2C (protein phosphatase 2C), and several chaperones and stress-protective proteins (HSP17.9, HSP70; nonspecific lipid transfer protein nsLTP, LEA, PDI), that is, genes involved in drought-responsive biological mechanisms activated in barley regardless of tolerance. The 17 genes induced by drought in tolerant genotypes only included enzymes involved in stomatal closure via carbon metabolism (NADP-malic enzyme, pyruvate dehydrogenase PDH), signalling (calcium-dependent protein kinase CDPK, membrane steroid binding protein MSBP, GABA A receptor), cell-cycle regulation (G2 pea dark accumulated protein), synthesis of glycine betaine as osmoprotectant (C-4 sterol methyl oxidase), ROS scavenging enzymes (ascorbate-dependent oxidoreductase ADOR, GST), and protective proteins with chaperone functions (HSP17.8, DHN3). At the proteome level, it was shown by Peng et al. [59] and Cheng et al. [19] that a drought-tolerant wheat cultivar revealed enhanced levels of photosynthesis-related proteins (OEE2, RubisCO large subunit-binding protein), chloroplast protective (fibrillin-like), redox metabolism-related (2-Cys peroxiredoxin), and chaperone (HSP70) proteins in comparison to a drought-sensitive cultivar. Moreover, an interactomic analysis revealed complex interactions of several proteins enhanced in drought-tolerant cultivar (2-Cys peroxiredoxin, 50S ribosomal protein L1, GAPDH, HSP70) with several other proteins related to amino acid metabolism, carbon metabolism, energy metabolism, signal transduction, stress and defence-related proteins, protein folding, and nucleotide metabolism indicating that not only enhanced protein abundance but also novel protein–protein interactions may contribute to increased drought tolerance [19].

A brief overview of proteomics studies in common wheat genotypes with differential drought responses is given in Table 11.2.

Table 11.2 An overview of proteomic studies carried out on common wheat (*Triticum aestivum*) cultivars with differential drought response (sensitive vs. tolerant cultivars)

Drought treatment	Measured parameters	Tolerant genotype(s)		Sensitive genotype(s)	Notes	Reference
Two wheat cvs Drought tolerant Nesser Drought sensitive Opata Controls and ABA medium (treatment) Proteomics of root tissues iTRAQ 4-plex	**Growth**	• Faster (and plants are higher)		• Slower	• Greenhouse • 16/8 h light/dark • 21 °C • 300 W/m^2 light intensity and 40 % humidity • Photosynthetic parameters on 4-week-old leaves (second open leaf from the top) • For root proteomic profiling, seedlings were hydroponically cultivated • Stress treatments imposed by adding ABA (100 μM) for 6 h	[2]
	Initial head emergence	• 41 ± 3 days		• 53 ± 5 days		
	Fully emerged heads visible	• 50 ± 4 days		• 65 ± 5 days		
	Yield	• Higher		• Lower		
	Stomatal conductance	Consistently higher rates		Lower		
	Transpiration	Consistently higher rates		Lower		
	Root phenotypes	Improved growth under stress More lateral root numbers upon control and stress		Lower primary root length Lower lateral root density upon controls and stress		
	Protein categories differentially respond to ABA	Higher protein abundance: • Amino acid, lipid, and carbon metabolic process • Photosynthesis • Isoprenoid and lignin biosynthesis • Salicylic acid process • Oxylipin metabolic process • Terpenoid metabolic process • Auxin mediated signalling • Protein folding and transport • RNA metabolic process • Translation • Cytokinesis • Xylem development		Higher protein abundance: • Thylene biosynthetic process • Cellular homeostasis • Apoptosis • Defense response		
	ABA responsive proteins, genotype specific	Accumulated—135 (!) Proteins, e.g.: • HSP90 • HSP70 • Ca-dependent and mitogen-activated protein kinases • Phosphatases • GTP-binding proteins • 14-3-3 protein homologues • V-ATPase • Flavonoids, gibberelins, lignin, phytoalexin, polyamine • Caffeoyl-CoA O methyltransferase • *S*-adenosylmethionine (but very high in Opata controls) Down-accumulated—31 proteins		Accumulated—33 Proteins, e.g.: • Brassinosteroid signalling pathways • Bri1-kd interacting protein • β-expansin precursor • Porin proteins Down-accumulated—34 Proteins		

(continued)

Table 11.2 (continued)

Drought treatment	Measured parameters	Tolerant genotype(s)		Sensitive genotype(s)	Notes	Reference
Two wheat cvs Sensitive Chinese Spring Tolerant Ningchun 47 Five different concentrations of PEG 6000 (0, 15, 20, 25, and 30 %) in Hoagland solutions for 48 h Linear pH 4–7 SDS-PAGE CBB staining MALDI-TOF/TOF	**RWC**	Higher for 20–30 % PEG		Lower for 20–30 % PEG (no statistical analysis present in the graphs)	• Growth chamber • 26/18 °C (16/8 day/night) • Hydroponics • Three-leaf stage seedlings • Osmotic stress (PEG 6000) • Proline related to fresh weight • No specifications about sampling • No statistics (homogeneous groups) present in the graphs	[19]
	Soluble sugar content	Lower in controls, 25, and 30 %; higher in 15 and 20 %		Higher in controls, 25 and 30 %		
	Free proline content	n.s. Between cvs (but dramatic increase for both cvs in 30 %)				
	Proteomics (77 unique DAPs)	57 unique DAPs		62 unique DAPs		
	Accumulation in both cvs: • OEE2 • Carbon metabolism related proteins (TPI, GAPDH, NADP-dependent malic enzyme) Down-accumulation in Both: • Ferredoxin-NADP(H) oxidoreductase • Chlorophyll a-b binding protein • Aldolases • ATP synthase beta unit • Methionine synthase 1 enzyme • Cp31BHv	Accumulation: • Ribulose-phosphate 3-epimerase • Phosphoglycerate mutase • 6-Phosphogluconate dehydrogenase • Beta-amylase • Enolase • Stress/defense/detoxification related proteins (APX,Cu/Zn SOD, 2-Cys Peroxiredoxin BAS1, fibrillin-like protein) • Vacuolar atpase • Chitinase 2 • 50S ribosomal protein L1 • HSP70 Down-accumulation: • Polyamine oxidase		Accumulation: • Polyamine oxidase • Aconitase (cytosolic) Down-accumulation: • Ribulose-phosphate 3-epimerase • 6-Phosphogluconate dehydrogenase • Beta-amylase • RuBisCO activase • Leucine aminopeptidase		

(continued)

Table 11.2 (continued)

Drought treatment	Measured parameters	Tolerant genotype(s)		Sensitive genotype(s)	Notes	Reference
Two wheat cvs sensitive SW89.5193/kAu2, and tolerant SERI M 82 Two watering conditions (20 and 80 % FC) Linear pH 4–7 SDS-PAGE CBB staining NanoLC-MS/MS (LTQ XL Orbitrap) Real-time PCR	**Physiological and biochemical parameters**	• Higher shoot and root biomasses • Longer roots • Accumulated higher ABA in leaves • More efficient in water absorption presumably by stomata control • Higher RWC under control and stress		• Very low RWC • Higher leaf dry weight and leaf area in controls (and vice versa in stress)	• Greenhouse • 23 °C (16/8 day/night) • Silt/loam mixture in 120 cm deep 7 cm diameter plastic pipes • Two leaf stage seedlings • Two weeks after sowing • Two water regimes • Drought stress imposed by water withholding until the FC reached 20 % • Samples collected from fully expanded leaves and from roots	[25]
	Accumulation of Proteins Related to defense and oxidative stress in both cvs: • GLPs • GST • SOD • small HSPs In tolerant genotype, proteins related to cell wall biogenesis and degradation were accumulated, while down-accumulated in a sensitive one	Accumulation of proteins (in roots—R, leaves—L) uniquely in tolerant cv • Glucan endo 1-3 beta-glucosidase (R) • Peroxidase (R) • APX (R; L) • S-adenosylmethionine synthase (L) • Malate dehydrogenase (R) • Ferredoxin-NADP(H) oxidoreductase (L) Down-accumulation of proteins (in roots—R, leaves—L) Uniquely in Tolerant cv • cysteine synthase (L)		Accumulation of proteins (in roots—R, leaves—L) uniquely in sensitive cv • Harpin binding protein 1 (L) • Cold shock domain protein (R) • Protein disulfide isomerase (L) • Proteasome subunit alpha type (L) • TPI (L) • GA3PD (L) • Inorganic pyrophosphatase (L) • Gamma-glutamylcysteine synthetase (L) Down-accumulation of proteins (in roots—R, leaves—L) uniquely in sensitive cv • Glucan endo-1,3-beta-glucosidase (R) • Peroxidase (R) • 60S ribosomal protein L13 (L) • Ribosomal protein L17 (L) • *S*-adenosylmethionine synthase (L) • Adenylate kinase A (L) • Malate dehydrogenase (L) • 14-3-3 protein (R; L)		

(continued)

Table 11.2 (continued)

Drought treatment	Measured parameters	Tolerant genotype(s)		Sensitive genotype(s)	Notes	Reference
Three Australian bread wheat cvs Kukri (intolerant) Excalibur (tolerant) RAC875 (tolerant) Cvs differ in tolerance based on grain yield two watering regimes (cyclic) determined by weighing four time-related samplings iTRAQ 8plex	**Genotypes**	**Excalibur**	**RAC875**	**Kukri**	• Growth room • Plants grown in 6 kg watertight bags • 16/4 °C day/night for first 4 weeks • 17/6 °C day/night for next 4 weeks, then 23/10 °C for the remain of the experiment • Stressed plants were watered do FC until emergence of the first flag leaf (reducing the quantity of water added each day, until the least tolerant Kukri wilted) • Four cyclic sampling (day 5—water stress, day 14 and day 21—wilting, and then rewatering)	[28]
	General information	Much slower response to water stress than RAC875	Responded early to moderate stress (74 protein changes compared to controls)	Intermediate number of responding proteins		
	Mechanism of drought tolerance	High osmotic adjustment during dehydration	More water-soluble carbohydrates in the stem, thicker and more waxed leaves			
	RWC	Similar to Kukri except for day 24	The highest levels in all samplings	Low levels in all drought stress samplings		
	Excalibur lacked changes in proteins during the initial onset of stress in contrast to RAC875 that had a large number of changes At day 5 RAC875 had the largest protein changes, followed by Kukri at day 14 and finally Excalibur at day 21. Excalibur has higher osmotic adjustment which allows this cv to maintain longer function under drought before response All three cvs increased ROS scavenging capacity (SOD, CAT) as well as ROS avoidance through decreases in photosynthesis and Calvin cycle related proteins	**Day 5** • Very few changes (12) compared to controls • Increase in cell redox homeostasis, response to stress (COR410), transport **Day 14** • 24 changes • Large decrease in photosystem I subunit VII, large increase in COR410 • Increase in glycolysis, protein degradation, protein folding • Decrease in transport, translation, and photos **Day 21** • 74 protein responses • Coordinate decrease of Calvin cycle proteins • Significant decrease of photosystem I subunit VII	**Day 5** • 74 protein changes • Downregulation of the Calvin cycle proteins, glycolysis, amino acid metabolisms and photorespiration **Day 14** • Only 20 changes, almost opposite to day 5 • Mixed response in stress-related category **Day 21** • 65 changes • Largest decrease in Calvin cycle and photorespiration	**Day 5** • Very few changes (11) • Decrease in Calvin cycle, metabolic processes, photos. machinery • Increase in stress related proteins, catalase and COR410 **Day 14** • 69 changes • Decrease in Calvin cycle, Rubisco large subunit, glycolysis, photorespiration amino-acid metabolism • Increase in stress related proteins (COR 410) **Day 21** • 48 changes • Decrease in Calvin cycle, photosynthetic machinery • Increase in cell redox homeostasis, metabolic processes, prot. folding, stress proteins and translation **Rewatering** • 40 changes		

(continued)

Table 11.2 (continued)

Drought treatment	Measured parameters	Tolerant genotype(s)		Sensitive genotype(s)	Notes	Reference
		• Increase in cell redox homeostasis (Cu-Zn SOD), glycolysis **Rewatering** • 67 changes different to changes in day 21 • Increase in glycolysis, photorespiration, prot. degradation and redox	• Significant decrease of photosystem I subunit VII • Increase in amino acid metabolism, gluconeogenesis, protein degradation, protein folding and translation **Rewatering** • 47 changes similar to changes in day 21 (but no longer changes in amino acid metabolism)	• Trends very similar to day 21		
Two bread wheat cvs Drought-tolerant Hanxuan 10 (HX-10) and sensitive Chinese Spring (CS) Drought imposed by adding PEG into hydroponics solution for 48 h Integrative analysis of different tissues pH 4–7 SDS-PAGE stained by CBB MALDI-TOF/TOF Phosphorylation modifications were identified by LTQ-Orbitrap Elite MS	**The third leaf length**	Higher till recovery	Lower till recovery		• Hydroponics • Drought stress imposed by adding 20 % w/v PEG 6000 to full-strength Hoagland's solution • After 48 h of stress seedlings transferred to 0 % PEG solution • Three-leaf stage • Samples of seedling roots, leaves and intermediate sections between roots and leaves (ISRL) 0, 6, 12, 24 and 48 h after stress and 48 h after recovery	[34]
	The main root length	n.s. between cvs				
	Root number	Significantly higher for all samplings				
	MDA (degree of peroxidation of membrane lipids)	Did not change significantly until 48 h drought treatment	Decreased slightly in the early phase of drought and then increased rapidly up to 48 h; significantly higher from 12 h of stress			
	Soluble sugar content	High levels	Significantly lower from 24 h of stress			
	RWC	n.s. between cvs				
	Free proline	Significantly higher from 12 h of stress				
	DAP (root, IRSL, leaf)	Larger number of unique DAPs (58,56, 56)	Fewer unique DAPs (52, 51, 49)			
	Accumulation in both cvs.: • HSP90 • 23.5 kDa HSP	Accumulation: • 14-3-3 protein (decreased after recovery, in CS unchanged) • Calreticulin-like protein	Down-accumulation: • RuBisCO large subunit • HSP70			

(continued)

Table 11.2 (continued)

Drought treatment	Measured parameters	Tolerant genotype(s)		Sensitive genotype(s)	Notes	Reference
		• RuBisCO large subunit • HSP-70 • 4 peroxidases (e.g. dehydroascorbate reductase) • Polyamine oxidase • Ketol-acid reductoisomerase • OEE2 • V-ATPase • Tubulin α-2 chain Down-accumulation: After recovery—HSP60 and HSP70 (but unchanged in CS)	• Polyamine oxidase • In roots caffeoyl-CoA O methyltransferase (unchanged in HX-10) OEE2			
Seedlings of somatic hybrid wheat cv. Shanrong no. 3 and its parent bread wheat cv. Jinan 177 Osmotic stress by PEG 6000 solution for 24 h exposure Root (R) and leaf (L) linear pH 5–8 SDS-PAGE Silver staining MALDI-TOF/TOF	**Shanrong no. 3**	Shanrong No. 3 is salinity and drought tolerant. Shanrong No. 3 is a wheat introgression line containing alien chromatin from tall wheatgrass via asymmetric somatic hybridization between bread wheat Jinan 177 and its wild relative tall wheatgrass *Thinopyrum ponticum* Podp. *Thinopyrum* is one of the most salinity-tolerant of all monocotyledonous species			• Grown in hydroponics • Two-leaf stage seedlings (no further data about seedlings age, etc.) • Drought imposed by 18 % PEG 6000 to the half-strength Hoagland's culture solution • Stress imposed also by adding 200 mM NaCl (both stresses are osmotical) • 24 h exposure • No further information about cultivation parameters	[59]
	Physiological parameters	• Higher fresh and dry weight in controls, drought and salinity • Higher root length • Higher chlorophyll *a* and *b* in controls and under drought • Ion uptake and water balance more stable	• Lower photosynthetic rate • Higher transpiration rate in controls and salinity • Lower soluble sugars in leaf and roots (lower osmoprotectants and possible reserves of carbohydrates) • Lower sucrose in leaf and roots			

(continued)

Table 11.2 (continued)

Drought treatment	Measured parameters	Tolerant genotype(s)		Sensitive genotype(s)	Notes	Reference
	Proteomics and transcriptomics The leaf proteomes included fewer cultivar-specific differentially accumulated proteins (DAP) then RT-PCR. Both the non-stressed and stressed proteomes differed from one another. Nine of the 34 cultivar-specific DAPs were antioxidants. Among the leaf stress-responsive DEPs, 38 were up-regulated in Jinan and 29 in Shanrong	In controls 49 specific root and 30 leaf proteins were specific to tolerant cv Specific root DAPs (missing in sensitive cv): • Glutathione transferase F4 • Peroxidase • Proton pump associated proteins • Peroxidase precursor The expression of catalase (L), chlorophyll *a*/*b* binding apoprotein CP24 precursor (L), and DWARF3 (R) was up-regulated under salinity and PEG Higher presence of SOD and peroxidases	Accumulated root and leaves DAPs (missing in tolerant cv): • G protein β-subunit-like protein • Ethylene receptor Larger presence of the large subunit of Rubisco (L) Higher presence of the fragmented Rubisco subunits under both stresses			

Abbreviations: *ABA*—abscisic acid; *APX*—ascorbate peroxidase; *CAT*—catalase; *cv(s)*—cultivar(s); *COR*—cold-regulated (protein); *DAP*—diferentially accumulated proteins; *FC*—field capacity; *GAPDH*—glyceraldehyde-3-phosphate dehydrogenase; *GLP*—glycine-like protein; *GST*—glutathione-S-transferase; *h*—hour; *HSP*—heat-shock protein; *IRSL*—intermediate sections between roots and leaves; *L*—leaf; *MDA*—malondialdehyde; *n.s.*—non significant; *OEE*—oxygen-evolving (protein); *PEG*—polyethylene glycol; *R*—root; *ROS*—reactive oxygen species; *RubisCO*—ribulose-1,5-carboxylase/oxygenase; *SOD*—superoxide dismutase; *V-ATPase*—vacuolar ATPase

11.5 Breeding for an Enhanced Drought Tolerance in Wheat and Barley

11.5.1 Classical Breeding Approaches

Genetic resources of cultivated cereal crops common wheat (*T. aestivum*), durum wheat (*T. durum*), and barley (*H. vulgare*) come from the Middle East, a region known as the Fertile Crescent where they had to adapt to relatively harsh environmental conditions including cold and drought. Both wheat and barley genera have very large and diverse gene pools including primary gene pool (lines, varieties, and landraces within a given species), secondary gene pool (genetic materials within the genera *Triticum*, *Hordeum*, and *Aegilops* which form genomes of modern wheat and barley cultivars), and tertiary gene pool (wild relatives of wheat and barley such as *Thinopyrum* species which can be used in crossing and can yield interspecific hybrids). Several genetic materials from primary, and especially secondary and tertiary gene pools, reveal enhanced tolerance to several environmental stresses including drought and salinity (drought-tolerant wild barley *H. vulgare* ssp. *spontaneum*, halophytic species *H. Marinum*, and *Thinopyrum ponticum*). Moreover, durum wheat and common wheat are allotetraploid and allohexaploid species, respectively, containing two or three homeologous chromosomal sets which underlies large plasticity of their genomes and easy preparation of chromosomal deletion or substitution lines and formation of intra- and interspecific hybrids without significant adverse effects on plant viability [63].

Long-term breeding aimed at high yield and appropriate grain quality in modern wheat and barley cultivars represents a bottleneck for several alleles associated with an enhanced tolerance to several stresses including drought. Therefore, there are efforts to omit the breeding bottleneck via a de novo preparation of synthetic hexaploid wheats from its progenitors, *Triticum durum* (AB) and *Aegilops tauschii* (D). A programme on preparation of synthetic hexaploid wheats is run by the International Maize and Wheat Improvement Centre (CIMMYT), Mexico, and it is aimed at preparation of synthetic hexaploid wheat lines containing several alleles underlying an enhanced stress resistance which were lost during selection for high yield. Similarly, chromosomal substitutions were used for an introduction of resistant alleles into breeding materials; for example, a substitution of durum wheat chromosome 4A by *A. tauschii* chromosome 4D led to an improvement of salt tolerance [24]. A comparison of transcriptional profiling of drought response in durum wheat, common wheat cv. Chinese Spring, and Chinese Spring deletion line having a deletion of a terminal part of 5A chromosome has revealed a significant effect of the D genome as well as chromosome 5A on the expression of several hundreds of drought-responsive genes located either directly on the 5A and D genome, respectively, or regulated by 5A and D genome-located regulons [3].

For improvement of genetic materials via an introduction of novel QTLs underlying enhanced drought tolerance, precise mapping of the QTLs represents a crucial step. Genetic mapping studies have been carried out in order to identify

QTLs underlying an enhanced drought tolerance. Mapping populations are derived from crosses between the genotypes with contrasting values of the given phenotypic trait associated with drought tolerance (e.g., a modern high-yielding cultivar is crossed with a landrace or even a wild material, e.g., in barley, modern barley cultivars are crossed with Tadmor, a genotype derived by the International Center for Agricultural Research in Dry Areas (ICARDA) from Syrian landrace Arabi Aswad, or with wild barley *H. vulgare* ssp. *spontaneum*) to yield sets of doubled-haploids (DH), near-isogenic lines (NILs), or recombinant inbred lines (RILs) which represent suitable genetic materials for QTL mapping. Recently, crossing approaches using multiple parental lines such as backcross-nested association mapping (BCNAM) are becoming used in the breeding programs of some cereal crops such as sorghum. Phenotypic traits associated with enhanced tolerance to drought and improved grain yield under drought conditions are mostly quantitative traits of polygenic nature whose final phenotypic effect is underlined by several genes and loci with often complicated genetic interactions (additive, dominant, and heterosis effects regarding one locus; epistatic effects regarding at least two different loci). QTLs for several morphological (plant growth habit: erect, prostrate, number of tillers), physiological ($\Delta^{13}C$), biochemical (WSC: a content of water-soluble carbohydrates), agronomical (days to heading), and yield-related characteristics (harvest index, thousand grain weight) have been characterised. However, it should be taken into account that the effect of a given QTL on a given phenotypic trait is usually dependent on genetic background of the given material since there are usually genetic interactions such as epistatic effects between different genes underlying a complex quantitative trait. Moreover, regarding the final phenotypic effect, the effect of a given QTL for a given phenotypic trait is often dependent on a given environment as well as on other phenotypic traits of the given plant material. For example, QTL affecting endogenous ABA level can reveal a beneficial effect on drought tolerance only when combined with QTL underlying superior root length in the given genetic material [20]. Regarding environmental stability, QTLs can be divided into 'constitutive' QTLs which are stable over a wide range of environments, and 'adaptive' QTLs which can be detected only upon specific conditions. Phenotypic stability of a given QTL over a range of diverse environments should also be tested in order to qualify the given QTL as a promising source for breeding.

Moreover, mapping of a given QTL to a given genetic marker is necessary for the QTL transfer into novel genetic material. QTL mapping to sequences of genetic polymorphism (RFLP, AFLP, RAPD, SSR, SNP) usually does not reveal absolute genetic linkage with the given QTL due to some genetic distance and frequency of recombination events (imperfect markers). However, due to advances in genome sequencing and sequence mapping, some QTLs have already been mapped to some candidate genes underlying the given QTL and revealing absolute linkage (perfect markers or functional markers): for example, in a set of 167 F_8 RILs derived from Tadmor × Er/Apm cross, a QTL for OP (osmotic potential) and RWC on 7H chromosome cosegregated with known candidate *Acl3* locus coding for barley acyl carrier protein III [74, 75, 77] which is known to encode a cofactor of plant fatty

acid synthase involved in a de novo biosynthesis of fatty acid chain, thus affecting cell membrane fluidity [33]. Other RWC QTLs in the same mapping population were mapped in the vicinity of *Ss1B*, *Dhn4*, and *KG1348* loci coding for sucrose synthase, dehydrin 4, and thionin. A QTL for TGW (thousand grain weight) was mapped on 5HL near to the *Dhn1* locus [76]. QTLs for grain yield were mapped on 1HL in the vicinity of the photoperiodically-regulated flowering gene *Ppd-H1* and dehydration-related *Lea* gene *HVA1* indicating an important role of flowering timing and accumulation of protective LEA proteins for final grain yield [85]. The locus BM816463b on chromosome 3H coding for blue copper-binding protein involved in suppression of Al uptake from soil cosegregated with QTLs for RWC, WSC_{100}, and OP, whereas the loci BM816306a, BM816242, BM816474b, BM817178, BM816381 (a, b), and BM816122a coding for oxalate oxidase (OAA), GST-1, cathepsin B, isocitrate dehydrogenase, endopeptidase Clp, and hypothetical protein C18B2.4, respectively, cosegregated with several QTLs for RWC, OP, WSC, and WSC_{100} [22]. An overview of genetic mapping studies dealing with an identification of drought-related QTLs in barley, common wheat, durum wheat, and in crosses with their wild relatives is provided in Kosová et al. [45]. Identification of candidate genes underlying QTLs associated with drought-related traits enables an identification of genetic markers revealing 100 % linkage to a given QTL thus eliminating adverse effects of linkage drag.

Breeding using genetic markers, so-called marker-assisted selection (MAS), has become a routine part of the breeding programs globally. Moreover, combination of multiple QTLs for multiple phenotypic traits, so-called QTLs pyramiding, is becoming widely used in cereal breeding. Precise mapping and identification of genetic markers associated with candidate genes underlying QTLs for drought-tolerance–related traits could enable the breeders to eliminate adverse effects of linkage drag, that is, an introduction of adverse alleles found in the original wild material which are linked to the desired allele transferred to breeding material. Adverse effects of linkage drag can also be eliminated using a backcross selection (marker-assisted recurrent selection (MARS) approach).

11.5.2 Transgenic Approaches to Improve Drought Tolerance

In addition to classical breeding approaches, several experiments utilising transgenesis have been undertaken in order to manipulate plant physiology to reach enhanced drought tolerance. Several studies have reported an enhanced drought or osmotic stress (PEG) tolerance in transgenic plants overexpressing some ROS scavenging-related gene such as Mn-SOD [86], specific channel protein such as water-transporting aquaporin TaAQP7 [89], osmolyte biosynthesis-related gene such as P5CS involved in proline biosynthesis [40], or dehydration-protective protein such as durum wheat dehydrin DHN5 [14]. However, drought tolerance

represents a multigenic trait in which several diverse proteins are involved. Therefore, it seems that transgenic plants overexpressing a single gene (protein) involved in drought response usually exhibit rather a delayed stress onset than an enhanced stress tolerance [51]. However, some examples of genetic manipulation leading to an altered phenotype with differential drought response can be given.

Overexpression of NADP-ME can significantly affect stomatal closure and water use efficiency in transgenic plants due to an altered metabolism of malate resulting in decreased free K^+ levels in stomatal guard cells and thus decreased stomatal conductance and higher WUE [49].

Wheat and barley have C3 photosynthesis type which is limited by stomatal conductance and CO_2 availability in intercellular space due to a dual enzymatic activity of RubisCO. It is known that nonphotosynthesising isoforms of the major enzymes of Hatch–Slack cycle phosphoenolpyruvate carboxylase (PEPC), NADP malic enzyme (NADP-ME) and pyruvate phosphate dikinase (PPDK) which are present also in C3 plants play important roles in plant stress responses including pH homeostasis, processing of Krebs cycle intermediates, and the like [23]. It is also known that a modification of C3 photosynthesis type to C4 photosynthesis type significantly enhances WUE in hot and dry climates while increasing CO_2 assimilation due to CO_2 prefixation by PEPC. In C3 plants, enzymes of the Hatch–Slack cycle active in CO_2 prefixation are already present in their genomes; however, the

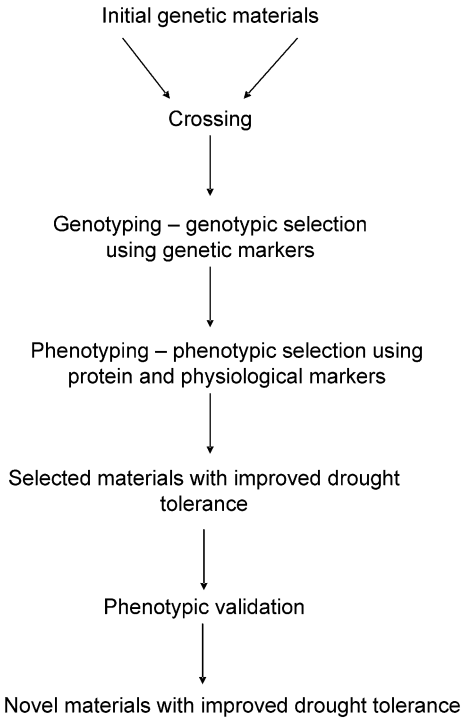

Fig. 11 2 A generalised schematic workflow of breeding process utilising genotyping and phenotyping approaches to obtain novel genetic materials with improved drought tolerance

Hatch–Slack pathway is not active. A possible way to increase wheat water use efficiency and its final yield in hot and arid climates represents wheat transformation from C3 to C4 photosynthesis type, that is, modification of leaf anatomy to C4-specific Kranz anatomy and activation of the Hatch–Slack C4 pathway. Currently, a large research project aimed at development of C4 rice supported by the Bill and Melinda Gates Foundation has already been launched at the International Rice Research Institute (IRRI) in Manila, Phillippines (The C4 Rice Project: http://c4rice.irri.org/) [78].

11.6 Conclusions and Future Perspectives

Wheat and barley belong to the most grown crops in the world, common wheat (*T. aestivum*) being the third and barley being the fourth most produced cereal crops in the world, respectively. Therefore, wheat and barley are grown in diverse environments differing in the severity and timing of drought periods with respect to the plant life cycle. Moreover, under field conditions, plants are usually exposed to combined effects of multiple abiotic and also biotic stresses [54, 55]. It also has to be kept in mind that the same ambient conditions reveal different effects on different genotypes and plant growth stages indicating that plant-associated parameters such as tissue relative water content (RWC) are more relevant parameters of stress than just soil water content [67]. Therefore, no universal breeding strategy to enhance drought tolerance in wheat and barley can be outlined. However, it can be summarised that the crucial factors affecting final grain yield in drought-prone environments include mechanisms associated with plant water uptake from soil (architecture of plant root system), leaf photoprotection (photosynthetic pigments, leaf rolling, waxy cuticle), stomatal openness and transpirational efficiency, delayed leaf senescense (*stay-green* phenotype), and assimilate partitioning between grains and other parts of the plant, that is, characteristics associated with plant harvest index. The crucial parameters determining crop yield under drought include water use, WUE, and HI, a so-called Passioura's equation (reviewed in [64, 67]. Tardieu [73] has suggested that there are multiple ways to achieve enhanced drought tolerance and improved crop yield in wheat and barley which are dependent on the given genetic material, environmental constraints, and management practices (G × E × M interactions). A generalised schematic workflow of approaches utilised during breeding of wheat and barley for improved drought tolerance in a given environment is given in Fig. 11.2.

In areas where drought occurs in the plant vegetative stage and irrigation is applied, plant fast growth leading to formation of a sufficiently deep root system and sufficient leaf area (LAI) to shade soil surface and minimise water evaporation is crucial. In contrast, when drought occurs regularly during a grain-filling period (terminal drought), a conservative, water-saving strategy associated with stomata closure, delayed leaf senescence, high TE and WUE (low $\Delta^{13}C$), seems to represent the most efficient strategy inasmuch as it spares water in soil and the water can be

utilised for grain filling and yield formation. It has been shown that every extra millilitre of water extracted from soil during the grain-filling period can increase the final yield in wheat by 55–59 kg per ha [39, 53]. Australian wheat cultivars Drysdale and Rees were bred for low $\Delta^{13}C$, that is, a water-saving strategy which seems to lead to the highest and most stable final grain yield in areas suffering from terminal drought. However, in areas with more variable patterns of rainy and drought periods and annual rainfall higher than 400 mm, wheat cultivars with high $\Delta^{13}C$ (i.e., cultivars whose stomata remain open under drought) seem to provide higher yield than low $\Delta^{13}C$ cultivars even upon terminal drought, mainly due to higher plant biomass at anthesis and subsequent assimilate reallocation from stem to grain. Moreover, high $\Delta^{13}C$ cultivars tend to have a higher harvest index [21, 62]. Furthermore, terminal drought effects can be avoided by early vigour [21]. It can thus be concluded that there is no universal breeding strategy to achieve the highest as well as most stable yield upon drought and that a selection of the most efficient strategy depends on a given environment, especially on timing and severity of drought stress with respect to the plant life cycle (reviewed in [6, 73]).

Recently, a boom of whole genome sequencing in major crops including common wheat [80] and barley [79] together with a boom of high-throughput omics techniques (transcriptome, proteome, and metabolome analyses coupled with functional studies of protein subcellular localisation, protein–protein interactions, and PTMs) have provided a more complex insight into molecular mechanisms underlying an active plant stress response. In addition, the data obtained from whole genome sequencing projects together with the results of quantitative transcriptomic and proteomic studies have enabled researchers to identify candidate genes underlying several QTLs associated with important morphological, physiological, biochemical, and agronomical traits underlying an enhanced drought tolerance and to design functional markers revealing 100 % linkage with a given QTL. Moreover, due to a direct involvement of proteins in the resulting plant phenotype, quantitative proteomic studies can lead to an identification of protein quantitative loci (PQLs), which, according to Riccardi et al. [65], represent those proteins that reveal reproducible quantitative differences in relative abundance in genotypes with differential stress tolerance, and which were mapped to a reproducible QTL for a given trait. The results of genome sequencing projects together with quantitative transcriptomic and proteomic studies will thus help the researchers and the breeders to identify promising (significant quantitative effect, stability over a wide range of environments) QTLs and to match these QTLs to functional genetic markers (candidate genes) thus facilitating transfer of these QTLs into novel genetic materials.

Last, but not least, phenotyping of novel genetic materials will remain the most important step for evaluation of novel genetic materials for their tolerance to drought. Recently, high-throughput phenotyping platforms have enabled the researchers and the breeders to obtain large datasets on several morphological, physiological, and yield-related characteristics for large sets of plant materials (thousands of different genetic lines, sets of DHs, NILs, RILs, etc.) practically at a given time point [4]. This will surely facilitate selection of suitable genetic materials

during the breeding process aimed at improvement of desired phenotypic traits underlying enhanced drought tolerance and final yield.

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Chapter 12
Transcription Factors Involved in Plant Drought Tolerance Regulation

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12.1 Introduction

The complex genetic basis related to drought tolerance requires a deeper understanding of molecular and physiological mechanisms, also concerning regulation of networks involved in related responses such as water deficit, osmotic, oxidative, and heat stress. The recognition of these mechanisms is fundamental for the development of adapted cultivars to each type of abiotic stress [180, 227]. When exposed to drought, plants induce a series of changes at the physiological, biochemical, and molecular levels. Such modifications may affect cell viability due to the production of reactive oxygen species (ROS) responsible for the oxidation of multicellular components, such as proteins, lipids, and nucleic acids [90, 258].

Drought is also responsible for inhibition of respiration, stomatal closure, stimulation of root growth, changes in photosynthesis, and assimilation of nutrients, among others [123, 175, 201]. In addition to the mentioned effects, the refered stress stimulates the production of abscisic acid (ABA), a central regulator of the stress response, which confers tolerance, increasing expression of many stress-responsive genes by inducing signaling cascades [37, 112].

In addition to regulatory events mediated by ABA, other groups of genes activated by drought confer tolerance to the plant, including transcription factors (TFs) that play a role as regulators and central molecular switches in the control of

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gene expression. Thus, any change in TFs activity would result in modulation of a genetic network of the plant, making them potential targets for induction of adaptive responses to abiotic stresses as studied in the present chapter [221].

12.2 Plant abiotic stresses: signalling pathways, phytormones and transcription factors activation

Major abiotic stresses—including high salinity, drought, cold, and heat—influence the entire plant metabolism and adversely affect growth, development, fertility, and productivity of crops [120]. After detection of stress, plants stimulate signal transduction by various signaling pathways, inducing a range of physiological and biochemical responses at the cellular and whole-organism levels to enable them to maintain homeostasis against these conditions [74, 120]. Many protein kinases participate in abiotic stress signal transduction, including mitogen-activated protein kinases (MAPKs), calmodulin-dependent protein kinases (CDPKs), receptor protein kinases (RPKs), and ribosomal protein kinases [200].

Membrane disorganization, protein denaturation, metabolic toxicity, repression of cell growth, and photosynthesis are some of the consequences of abiotic stresses on plants. Also, plants alter their metabolism by the accumulation of osmolytes and generation of second messengers such as calcium and ROS [46, 175]. Modulation of intracellular Ca^{2+} levels is sensed by calcium-binding proteins, initiating a protein phosphorylation cascade that targets TFs, which convey these signaling cues and activate or repress expression of stress-inducible genes and enhance stress tolerance [69, 244]. Stress-induced changes may participate in the generation of phytohormones including abscisic acid, ethylene (ET), jasmonic acid (JA), and salicylic acid (SA). Various interactions can take place between the phytohormone and TFs to amplify the initial signal and induce a second round of signaling that can follow the same pathway or use altogether different signaling pathway components [9].

The ultimate goal of signal transduction pathways is to elicit a specific well-timed biological response and to modify gene transcription. Many TF-families have been suggested to play a major role for transcriptional reprogramming associated with plant stress response [164]. These TF genes are capable of controlling the expression of a broad range of elements through sequence-specific interactions with *cis*-regulatory DNA elements in the promoters of target stress-related genes containing TF binding sites, specifically activating or repressing the expression of these target genes, directing the expression in a synchronized manner [62].

Among all described phytohormones, ABA is an important player in the regulation of responses during abiotic stresses, especially regarding drought and salt stresses. It promotes rapid changes in rates of transcription, transcript processing, and stability, also modifying conformational states of regulatory molecules that control RNA processing such as TFs [37].

Molecular analysis of promoters of ABA-responsive genes led to the identification of a conserved *cis*-acting element, designated ABRE (ABA-responsive element; [31, 263]). ABREs can be recognized by ABRE-binding proteins (AREBs) or ABRE-binding factors (ABFs), which belong to group A of the basic leucine zipper (bZIP) TFs [223]. In *Arabidopsis*, approximately 75 distinct bZIP-type TFs have been identified [89]. Recent genomewide analysis based on RNA-Seq, EST (expressed sequence tag), and microarray analyses have revealed different numbers of bZIP superfamily members in plants, with 125 representatives in maize [238], 45 in castor bean [100], 55 in grapevine [134], and 64 in cucumber [15]. Hence, TFs are important regulators that control gene expression under abiotic stress. Among them, NAC (no apical meristem, ATAF1/2), AP2/ERF (Apetala2/ethylene response factor), MYB, C2H2 zinc finger, and WRKY superfamilies [74, 119, 159, 169] deserve mentioning. This section exemplarily describes functional aspects of some important TF families with a functional role in plant response to abiotic stress (Table 12.1).

12.2.1 bZIP-Type Transcription Factors Family

bZIP proteins can be identified based on the presence of a bZIP domain, characterized by 60–80 amino acids in length, a basic region (BR), and a leucine zipper (LZ) that is functionally distinct [85]. In the model plant *A. thaliana*, the bZIP family is divided into 10 groups (A, B, C, D, E, F, G, H, I, and S), considering amino acid sequence similarities of bZIP domains and protein structure. For *Arabidopsis,* it has been proposed that members of the A, B, and S groups play crucial roles in ABA-signaling and abiotic stress response [89, 130].

AREB/ABFs belongs to the A-group bZIP comprising nine homologues in *Arabidopsis*, and can bind to ABRE, a *cis*-regulatory element found in promoters of many ABA- and stress-responsive genes. This element plays a pivotal role in the regulation of abiotic stress response including drought, salt, and cold [53, 54]. Among the nine members of AREB/ABF identified in *Arabidopsis,* AREB1/ABF2, AREB2/ABF4, and ABF3 are induced by ABA treatment during vegetative growth, dehydration, and high salinity [32, 223]. In *Arabidopsis* overexpression of ABF3 or ABF4 resulted in ABA hypersensitivity and enhanced drought tolerance with changes in expression levels of ABA- and other stress-regulated genes.

Fujita et al. [51] have reported that overexpression of AREB1 in transgenic *Arabidopsis* plants leads to ABA hypersensitivity and enhanced drought tolerance whereas overexpression of ABF2 increased plant tolerance to high salinity, drought, heat, and oxidative stresses [109, 128]. AtbZIP37 (ABF3) and AtbZIP38 (ABF4/AREB2) were upregulated in response to ABA signal, dehydration, and salinity. Overexpression of ABF3 and ABF4 also resulted in ABA hypersensitivity and several other ABA/stress-associated phenotypes, including enhanced drought tolerance [106, 165]. Similarly, overexpression of the *PtABF* gene of *Poncirus trifoliata* (Rutaceae) enhanced dehydration and drought tolerance in tobacco [81].

Table 12.1 Transcription factors (TFs) described in this chapter

TF Family	Organisms	Gene name	Inducers	Function in abiotic stress	Reference
bZIP	*Arabidopsis thaliana*	AREB1/ABF2	ABA, drought, salt stress	Regulator of ABA signaling under drought stress	Fujita et al. [51]
bZIP	*Arabidopsis thaliana*	AtbZIP37 (ABF3)	ABA, drought, salt stress	Regulator of ABA signaling under drought stress	Kang et al. [106]
bZIP	*Arabidopsis thaliana*	AtbZIP38 (ABF4/AREB2)	ABA, drought, cold, salt stress	Mediates drought stress response at the molecular level	Kang et al. [106]
bZIP	*Poncirus trifoliata*	PtABF	ABA, drought, cold	Regulation of expression of genes involved in stress response and tolerance	Huang et al. [81]
bZIP	*Oryza sativa*	TRAB1	ABA, drought, salt stress	Regulation of gene expression via phosphorylation in response to ABA	Hobo et al. [75], Kagaya et al. [104]
bZIP	*Oryza sativa*	OsABI5	ABA, salt stress	Regulation of adaptive stress response and plant fertility	Zou et al. [262]
bZIP	*Oryza sativa*	OsbZIP23	Drought, salt	Regulation of expression of genes involved in stress response and tolerance	Xiang et al. [243]
bZIP	*Oryza sativa*	OsbZIP72	Drought, ABA	Regulation of expression of ABA-stress-related genes and enhanced drought tolerance	Lu et al. [137]
bZIP	*Oryza sativa*	OsbZIP46	Drought, ABA, heat	Regulation of expression of ABA-stress-related genes	Tang et al. [214]
bZIP	*Tamarix hispida*	ThbZIP1	ABA, drought, salt stress	Mediates ABA and salt stress response at the molecular level	Ji et al. [97]
bZIP	*Capsicum annuum*	CaBZ1	Drought, salt, and ABA	Increased the expression of ABA, regulation of stress-related genes	Moon et al. [153]
bZIP	*Glycine max*	GmbZIP1	Drought, salt, cold, ABA, low temperature	Regulation of expression of ABA-stress-related genes	Gao et al. [57]
bZIP	*Vitis vinifera*	VvbZIP23	Drought, salt, cold, ABA	Regulation of genes involved in abiotic response	Tak and Mhatre [211]
bZIP	*Vitis vinifera*	VvbZIP45	Drought, ABA, heat	Not reported	Liu et al. [135], Nicolas et al. [161]
bZIP	*Solanum lycopersicum*	SIAREB1	Drought, salt	Regulation of genes involved in abiotic response	Orellana et al. [168]
bZIP	*Solanum lycopersicum*	SIAREB2	Drought, salt	Regulation of genes involved in abiotic response	Orellana et al. [168]

(continued)

Table 12.1 (continued)

TF Family	Organisms	Gene name	Inducers	Function in abiotic stress	Reference
WRKY	*Arabidopsis thaliana*	AtWRKY33	Salt, cold, oxidative stress, UV radiation	Control of cellular ROS levels in abiotic stress signaling	Jiang and Deyholos [98]
WRKY	*Arabidopsis thaliana*	AtWRKY25	Cold, heat	Regulation of heat inducible genes	Jiang and Deyholos [98]
WRKY	*Arabidopsis thaliana*	AtWRKY39	Heat	Regulator of SA-dependent heat stress defense pathways	Li et al. [126]
WRKY	*Arabidopsis thaliana*	AtWRKY36	ABA, drought	Regulation of expression of ABA-stress-related genes	Ren et al. [186]
WRKY	*Oryza sativa*	OsWRKY45	ABA, drought, salt	Regulation of genes involved in abiotic response	Ren et al. [186]
WRKY	*Oryza sativa*	OsWRKY11	Heat, drought	Regulation of genes involved in desiccation tolerance	Wu et al. [242]
WRKY	*Glycine max*	GmWRKY20	ABA, salt, drought, cold	Mediates ABA signaling and regulates the expression of wax biosynthetic genes	Luo et al. [140]
WRKY	*Glycine max*	GmWRKY54	Salt, drought	Regulation of genes involved in abiotic response	Zhou et al. [262]
WRKY	*Gossypium hirsutum*	GhWRKY25	Salt, drought, cold, ABA, SA	Regulation of the expression of ROS-related genes	Liu et al. [136]
WRKY	*Gossypium hirsutum*	GhWRKY17	Salt, drought, H_2O_2	Regulation of expression of ABA-stress-related genes	Yan et al. [250]
WRKY	*Triticum aestivum*	TaWRKY10	Salt, cold, H_2O_2	Regulation of osmotic balance, ROS scavenging and transcription of stress-related genes	Wang et al. [232]
WRKY	*Triticum aestivum*	TaWRKY44	Salt, cold, drought, H_2O_2	ROS elimination and activation of stressed-associated genes	Wang et al. [235, 236]
WRKY	*Dendrathema grandiflorum*	DgWRKY3	Salt, drought	Regulation of genes involved in osmotic adjustment, membrane protection, and oxidative stress response	Liu et al. [133]
WRKY	*Tamarix hispida*	ThWRKY14	ABA, salt, drought	Regulation of genes involved in abiotic response	Zheng et al. [260]
NAC	*Arabidopsis thaliana*	ANAC019, ANAC055, ANAC072	ABA, drought, salt	Regulation of genes involved in abiotic response	Tran et al. [218]
NAC	*Oryza sativa*	OsNAC6/SNAC2	Salt, cold, drought	Regulation of abiotic stress-responsive gene expression	Nakashima et al. [160], Hu et al. [77]

(continued)

Table 12.1 (continued)

TF Family	Organisms	Gene name	Inducers	Function in abiotic stress	Reference
NAC	*Oryza sativa*	OsNAC5	Salt, cold, drought	Regulation of OsLEA3 expression	Takasaki et al. [212]
NAC	*Oryza sativa*	OsNAC10	Salt, cold, drought	Enhancement of grain yield under drought conditions	Jeong et al. [95]
NAC	*Oryza sativa*	ONAC063	Salt, cold	Regulation of abiotic stress-responsive gene expression	Yokotani et al. [252]
NAC	*Oryza sativa*	SNAC1	Salt, cold, drought, ABA	Increase of stomatal closure and expression of genes involved in stress tolerance	Hu et al. [76], Liu et al. [134, 135]
NAC	*Glycine max*	GmNAC2	Salt, cold, drought	Downregulation of the expression of ROS-related genes	Pinheiro et al. [176], Tran et al. [220]

Several members of rice bZIP TFs were able to withstand various abiotic stresses with the ability to improve drought and salt tolerance in transgenic rice. The first member described in rice was TRAB1, a TF-induced by ABA under drought and salt stress. TRAB1 can be activated via ABA-dependent phosphorylation [75]. The second member identified was OsABI5, a homologue of TRAB1 that encodes a protein that can bind to ABRE and was induced by ABA and high salinity, being downregulated in seedlings grown under drought and cold conditions. Overexpression of OsABI5 in rice provided high sensitivity to salinity stress [261, 262]. Expression of OsbZIP23 also was induced by drought, salinity, and ABA treatment. Transgenic rice overexpression OsbZIP23 exhibited ABA hypersensitivity and improved tolerance to drought and salinity [243]. Overexpression of another member of bZIP, OsbZIP72, also exhibited hypersensitivity to ABA and led to drought tolerance in transgenic rice plants [137], whereas OsbZIP46 increased ABA sensitivity but had no positive effect on drought resistance [214]. Recently, Liu et al. [135] showed that OsbZIP71 was strongly induced by drought and ABA treatments and repressed under salt stress. Quantitative real-time PCR revealed that abiotic stress-related genes were upregulated in overexpressing plants. Thus, these reports indicate that OsbZIP factors have significant roles in the control of abiotic stress tolerance, with potential biotechnological applications for improving abiotic stress tolerance of plants.

Similar results were obtained with transgenic *Arabidopsis* plants overexpressing ThbZIP1 from *Tamarix hispida* (Tamaricaceae). The overexpression led to significant tolerance to drought and salt stress, but plants were sensitive to ABA treatment. Microarray analysis showed that many ROS scavenging genes were upregulated by ThbZIP1 under salt stress conditions [97]. Also, Zhang et al. [257] showed that ABP9, a maize bZIP gene, conferred drought, salt, and cold tolerance in transgenic plants, but plants overexpressing ABP9 also displayed significant sensitivity to ABA. Transgenic plants overexpressing ABP9 presented improved salt stress tolerance by the modulation of ROS levels. In addition, both ABP9 and ThbZIP1 transformed plants showed improved water-retention capacity [97, 257].

A novel bZIP TF from hot pepper, CaBZ1, presented homology to other bZIP genes of *Arabidopsis* group S, and its expression was strongly induced by multiple stress stimuli, such as ABA, cold, and salinity [153]. Overexpression of CaBZ1 reduced water loss, increased ABA-induced stomatal closure, and altered expression levels of stress-inducible and TF genes, such as NACs and CBFs [153]. Consistently, a novel bZIP from soybean named GmbIZP1 also conferred stress tolerance not only to drought but also to salt and cold stresses [57].

In grape (*Vitis vinifera*, Vitaceae), a bZIP member named VvbZIP23 was isolated from cultivar "Mangoo" and was identified as an important plant regulator of abiotic stress responses. Its expression was found to be strongly induced by a wide spectrum of abiotic stresses, including drought, salt, cold, and application of abscisic, salicylic, and jasmonic acid [211]. Some evidences indicated that VvbZIP45 transcripts were also involved in abiotic stress response with upregulation by ABA, including drought stress [134, 161].

In cultivated tomato (*Solanum lycopersicum,* Solanaceae) the expression of two AREB/ABFs proteins (SlAREB1 and SlAREB2) was induced by both drought and

salinity, although expression of SlAREB1 was strongly affected. Tomato mutants overexpressing SlAREB1 showed increased tolerance to salinity and drought stresses compared with wild-type plants. Notably, microarray analyses revealed that many defense genes associated with abiotic and biotic stress were upregulated [168]. More importantly, the results suggested that SlAREB1 TF was involved in ABA response to abiotic stress and possibly also in response to pathogens during plant defense, mediating crosstalk between abiotic and biotic responses in tomato plants, similar to CAbZIP1 from pepper expressed in *Arabidopsis* [124].

Considering the multiple functions of bZIP TFs in abiotic stress response, these potential candidate genes have received far less attention for application in the improvement of drought tolerance in crops. Such an approach would be interesting towards a better understanding of the network regulation associated with bZIP TFs.

12.2.2 WRKY TFs

The WRKY family is defined by the presence of highly conserved WRKY-DBD of 60 amino acids in length, which contains the almost invariant WRKYGQK sequence motif at the N-terminal and a zinc-binding motif with features of C–C–H–H ($C–X_{4–5}–C–X_{22–23}–H–X–H$) or C–C–H–C ($C–X_7–C–X_{23}–H–X–C$) at the C-terminal region [44]. WRKY proteins have been found to modulate gene expression in plants under biotic and abiotic stresses. In addition to roles in response to such stresses, WRKY proteins are involved in a whole range of physiological processes that have profound effects on plant growth and development, such as senescence, dormancy, morphogenesis of trichomes and embryos, and metabolism [190]. WRKY acts via protein–protein interaction and even cross- and autoregulation [170].

Specific WRKY TFs, which help in the expression of a cluster of stress-responsive genes, are being targeted [16]. For example, in *Arabidopsis* AtWRKY33 downstream targets genes with functions in detoxification of ROS (such as glutathione *S*-transferase *GSTU11*, peroxidases, and lipoxygenase *LOX1*), increasing salinity tolerance [98]. AtWRKY33 also affected tolerance to heat through modulation of transcriptional reprogramming [127], indicating a crossover of regulatory roles within various stress responses. Crossregulation among AtWRKY25, AtWRKY26, and AtWRKY33 is essential in promoting tolerance against high-temperature stress by positive regulation of the cooperation between the heat-shock protein and ethylene-activated signaling pathways [127]. Furthermore, AtWRKY39 is heat stress induced and acts as a positive regulator of SA-dependent heat stress defense pathways [126]. Another good example in rice is the OsWRKY11 gene, whose overexpression under the control of heat shock protein HSP101 promoter led to enhanced heat and drought tolerance in transgenic rice [242].

WRKY genes may mediate crosstalk between plant abiotic tolerance and ABA-related signaling. For instance, overexpression of GhWRKY17 from cotton (*Gossypium hirsutum*, Malvaceae) in *Nicotiana benthamiana* (Solanaceae)

responded to drought and salt stress through ABA signaling [250]. Transcription levels of ABA-inducible genes, including AREB, DREB, and LEA, were repressed under drought and salt stress conditions [250]. Similar results were obtained for *Tamarix hispida* after overexpression of ThWRKY4, conferring tolerance to salt stress. Expression of ThWRKY4 alone was not sufficient to activate some stress-related genes (bZIP, DOF, bHLH), but needed to be activated by ABA signals [260]. Results showed that OsWRKY45 also plays a role in ABA signaling and drought tolerance in rice [215]. Moreover, overexpression of OsWRKY45 resulted in constitutive expression of ABA-induced responses and abiotic-related stress factors, also markedly enhancing drought resistance [179]. Interestingly, AtWRKY63 (ABO3) mutants exhibited ABA-induced stomatal closure resulting in lower drought tolerance and higher ABA sensitivity [186]. Consistent with these findings, expression of the wild soybean GmWRKY20 gene in *Arabidopsis* enhanced drought tolerance and also regulated ABA signaling. GmWRKY20 overexpression lines were more sensitive to ABA during stomatal closure when compared with the wild type [140]. On the other hand, transgenic plants overexpressing GmWRKY13 exhibited decreased sensitivity to ABA, and exhibited less tolerance towards high salt and mannitol treatment in comparison to the wild types [262]. Taken together, these data demonstrate that WRKY TFs play key roles in the ABA-dependent pathway and drought-responsive signaling networks [140].

Involvement of some soybean WRKY genes with salt and cold tolerance was experimentally demonstrated. For example, GmWRKY21 transgenic plants were tolerant to cold stress, whereas overexpression of GmWRKY54 in *Arabidopsis* conferred drought and salt tolerance [26, 262]. High levels of GmWRKY54 are thought to induce expression of salt tolerance Zn finger (STZ/Zat10) and DREB2A [262]. In Arabidopsis, AtWRKY8 was implicated in the modulation of salinity stress and showed high upregulation upon NaCl treatment [79], whereas overexpression of AtWRKY18 and AtWRKY60 increased plant sensitivity to salt and osmotic stresses [27]. GhWRKY25 from cotton also led to improved tolerance to salt stress, but reduced plant tolerance to drought stress [136], whereas a reverse phenomenon was reported for DgWRKY3 from chrysanthemum (*Dendranthema grandiflorum*, Asteraceae). Heterologous expression of DgWRKY3 in tobacco revealed that transgene expression was lower under drought when compared to salinity stress [133].

In wheat, WRKY confers multiple abiotic stress tolerance. Exposure to salt, cold, drought, and H_2O_2 induced the expression of TaWRKY10, TaWRKY2, TaWRKY19, and TaWRKY44. Overexpression of TaWRKY10 and TaWRKY44 enhanced drought and salt tolerance by direct or indirect activation of stress-related genes, ROS scavenging, and osmotic balance [232]. TaWRKY19 and TaWRKY2 overexpression conferred higher tolerance to salinity, drought, and low temperature in *Arabidopsis* [162]. Together, these examples illustrate how stress-responsive WRKY TFs may represent convergence points between pathways with different functions, also indicating them as a promising target for applied studies in crop species.

12.2.3 The Role of NAC TFs in Plants under Abiotic Stress

The NAC TF gene family is one of the largest and most diversified in plants with about 110 genes in *Arabidopsis* [92], 150 genes in rice [163], and 101 genes in soybean [176]. Transcriptomic studies indicate that a large number of NAC TFs are associated with abiotic stress, such as drought, salinity, and cold, as well as the ABA phytohormone. Involvement of NAC TFs in abiotic stress is regulated through binding to regulatory promoter regions at the transcriptional level, alternative splicing at the posttranscriptional level, association with ubiquitins, and dimerization and/or interaction with other non-NAC proteins at the posttranslational level [178].

Some stress-responsive NAC genes are grouped into the SNAC category which includes three subgroups [163]. In *Arabidopsis* members of subgroup III-3 (ANAC019, ANAC055, and RD26 or ANAC072) were induced by high salinity, drought, JA, and under the control of a central ABA perception and signaling network [92, 218]. Overexpressing of these three NAC TFs conferred drought tolerance in transgenic *Arabidopsis* by upregulation of several stress-inducible genes [218]. These TFs bind to ERD1 (early responsive to dehydration stress 1) promoter, which is induced by dehydration [156, 218]. Interestingly, ERD1 upregulation depends on co-overexpression of two *cis*-elements (ZF homeodomain transcriptional activator ZFHD1 and NAC TFs), suggesting cooperative regulation of stress responses via members of different TF families [130]. ANAC019 and RD26 also conferred ABA-hypersensitivity, suggesting positive regulation of ABA signaling [92, 218].

Several members of the NAC family in rice have been identified with potential association with abiotic stress response or signaling. For example, SNAC1 overexpression improved drought tolerance under field conditions, also conferring strong tolerance to salinity [76]. OsNAC6/SNAC2 was also induced by drought, cold, and high salinity. OsNAC6 overexpression in rice transgenic plants improved drought and high-salt stress through modification of expression of a significant number of stress-responsive genes with several functions such as detoxification, redox homeostasis, and proteolytic degradation [33]. Overexpression of OsNAC5 also affected plant tolerance to salinity by upregulating the expression of stress-inducible genes, such as OsLEA3 [212]. Song et al. [204] showed that transgenic rice plants with reduced OsNAC5 expression by RNA interference (RNAi) resulted in less tolerant individuals to abiotic stresses than control plants. Recent results demonstrated that overexpression of OsNAC5 enhanced drought tolerance and grain yield under field conditions [96]. Furthermore, overexpression of OsNAC10, OSNAC45, and ONAC063 also enhanced abiotic stress tolerance of rice playing an important role in inducing responses to high-salinity stress [95, 252].

NAC genes also improved stress tolerance in other crops. In *Brassica napus* (Brassicaceae) nine BnNAC were induced in response to cold, ABA treatment, and drought [72]. In soybean, NAC was shown to be induced by abiotic stress, participating in stress tolerance. GmNAC2 acts as a negative stress regulator and its overexpression led to hypersensitivity to drought, salinity, and cold [99].

12.3 Epigenetic Control of TF Response under Abiotic Stresses

The tridimensional structure of the DNA in eukaryotes is dynamic, directly influencing gene expression. During the cell cycle, due to the association of DNA with proteins called histones, given regions may have a lower or a higher level of folding. These topological alterations reflect, respectively, permissive or inhibiting states regarding the access of the transcriptional machinery (TFs, effector proteins, etc.). Certain genomic regions have constitutively lower (called euchormatin) or higher levels of folding (constitutive heterochromatin). Additionally, there are those regions whose topology/accessibility may be variable (facultative heterochromatin) during the life cycle of the organism. This occurs due to histone modifications in N-terminal tails, which act as a substrate to chemical associations that change the chromatin structure and/or signaling for the recruitment of inhibitors/activators of gene expression [94]. There are a number of these alterations in such proteins (for a review see [17, 30]. The set of modifications orchestrated by combinations involving these proteins is named "histone code", being recognized by other proteins and resulting in downstream events [94, 208].

Another type of chemical alteration of chromatin, also associated with transcriptional regulation, is represented by the methylation of cytosines in target genes. In plants, methylation of promoter sequences is generally associated with inhibition of transcription. Otherwise, methylation on coding regions usually has discrete effects during the referred process [206, 265]. As exposed, it is clear that both chemical histone modifications and methylation of cytosines alter expression of genes without causing changes in their nucleotide sequences. Additionally, such modifications represent no mutations and may be transferred (inherited) from one generation to the next [70, 152]. Thus, such processes are representative of so-called epigenetic phenomena. Russo [192] conceptualizes this term as "any inheritable modification of gene expression coming from the alteration on the accessibility of the transcriptional machinery to target genes, and not of mutation on its sequences." Additionally, there are recent adjustments of this concept, suggesting that the requirement of heritability is falling into disuse and that epigenetic may be defined as "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" [22]. Thus, there is no consensus or concrete definition of the term epigenetic, a condition also highlighted by Richards et al. [187].

Once the DNA is on a permissive configuration for the access of the transcriptional machinery, TFs interact with specific promoter regions and execute their function, helping the RNA polymerase II to initiate the transcription. However, recent findings revealed that expression of TFs may also be directly regulated by epigenetic modifications. This was initially observed in *A. thaliana* for the gene ATX1 (*Arabidopsis* homologue of trithorax). ATX1 carries a SET [Su(var)3-9, E(z) and Trithorax] domain that is highly conserved and presents histone methylase activity [183]. ATX1 is an epigenetic regulator of histone H3K4 methyltransferase activity (i.e., it methylates lysine four of histone H3; [3]), a modification associated

with gene activation [195]. It is also known that ATX1 does not act on global methylation of K4 residues in H3, acting only in part of them [2]. However, an analysis revealed that a high amount of genes (around 1600) exhibited expression changes on *atx1* mutants, with approximately half of the genes being inducted and the other half suppressed [4]. Aiming to explain the occurrence of such drastic and opposite alterations (induction versus suppression) in the *Arabidopsis* transcriptome after knocking out of a single gene, target genes with altered expression have been prospected. As an outcome, 60 TF coding genes were identified, 42 of them activated and 18 repressed [4]. Once there was a direct correlation between epigenetic modification and expression of TFs, Alvarez-Venegas et al. [5] conducted a series of analyses to determine how the action of ATX1 influenced the activity of TFs and also of a high number of additional genes. Because TF WRKY70 was suppressed 7.2 times in control samples during the previous test, this gene was chosen as a study model [4]. This particular TF is important because it represents a convergence point between the jasmonate signaling pathway (acting as a suppressor) and salicylic acid pathway (acting as an activator), being an important component of the biotic stress response (Fig. 12.1a, b). The authors observed that WRKY70 is the primary target of ATX1, which acts on the nucleosomes of this TF, methylating [specifically, trimethylating (me3)] K4 residues of H3 (H3K4me3) histones. Such epigenetic modification was responsible for the activation of WRKY70. An alteration of the expression of ATX1 led, consequently, to a change in expression of WRKY70 (Fig. 12.1a, b) and all genes regulated by this specific TF (induced of and suppressed). This explained, at least partially, how the alteration of one single gene caused an abrupt change in the expression of myriad other genes.

In relation to TFs associated with abiotic stress response, Alvarez-Venegas et al. [4] also observed that an *atx1* mutant influenced the activity of ZAT10 (suppression of 2.7 times) and R26 (suppression of 3.72 times), among other genes. The first gene encodes a TF that induces the expression of genes associated with ROS response. Some evidence indicates that ZAT10 enhances tolerance of plants to stresses such as high salinity, heat, and osmotic stresses [146]. The second gene (R26) encodes an NAM TF, which mediates regulation between ABA and JA signaling pathways during responses to drought or wounding stresses [50]. These data suggested that epigenetic mechanisms may regulate TFs associated with drought stress, a presumption confirmed by recent experiments carried out by Verkest et al. [226]. These authors analyzed a population of isogenic lines (identic genotypes) of rapeseed (*B. napus*) to select individuals with best indicators regarding drought tolerance [high NAD(P)H content and low respiration] when compared to other lines. Inasmuch as the population used consisted of identic genotypes, any variation in this index could be attributed to epigenetic factors. After several selection steps, two lines were selected: PEG1 and PEG2. The first presented significant tolerance to drought when compared to its control and PEG2. ChIP-seq (chromatin immunoprecipitation sequencing) data revealed that many genomic regions of PEG1 were enriched for H3K4, being absent in its control. The contrast of these regions enriched with epigenetic markers when compared to

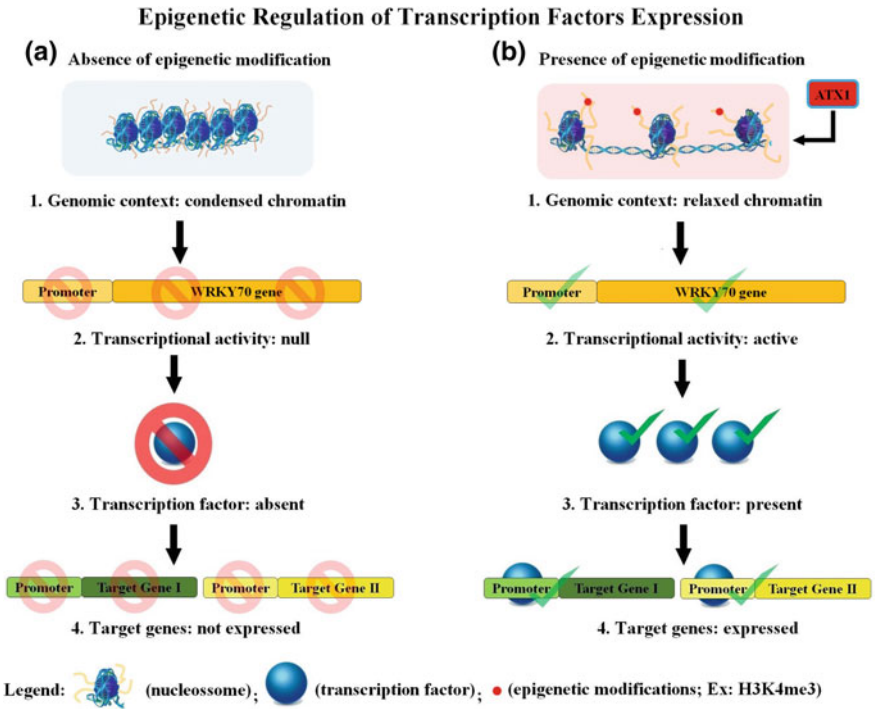

Fig. 12.1 Scheme involving the epigenetic regulation of the expression of WRKY70 transcription factor, by the methylase of histones ATX1 and the developments in the activity of target genes composing the SA (salicylic acid) signaling pathway: **a** situation highlighting the absence of expression of WRKY70 due to the high level of chromatin condensation (absence of ATX1 activity) and, as a result, the absence of expression of its target genes; **b** situation highlighting the induction of WRKY expression due to the occurrence of epigenetic modification (performed by ATX1)

transcriptome data has shown that a significant portion of genes associated with H3K4 was differentially expressed, including many TFs as AP2/EREBP, WRKY, NAC, GRAS, C3H, C2H2, HSF, bHLH, and C2C2.

In addition to acting directly in the regulation of TF expression, epigenetic phenomena act indirectly in the regulation of responses during periods of unfavorable conditions for plant development. Every epigenetic modification may influence the access of TFs [94] or their recruitment [237], and several reports indicate on alteration in plant epigenomes submitted to drought and associated stresses, including osmotic and heat stress, among others (Table 12.2). Despite advances in gene regulation towards developmental processes and stress response, further studies are mandatory for a better understanding of epigenetic mechanisms, also considering the application of this knowledge for plant breeding purposes.

Table 12.2 Selection of reports concerning epigenetic alterations in response to drought or associated stresses (osmotic stress, heat, etc.) in plans

Analyzed epigenetic markers	Species	Target gene	Analyzed Tissue	Epigenetic state during stress	Tolerance to studied stress	Reference
H3K27me3 and and H3K27me2 (2)	*Solanum lycopersicum*	*Asr2*	Root	Decrease in the content of H3K27me2	not analyzed	Gonzales et al. [61]
H3T3ph	*Arabidopsis thaliana*	Genome wide	Leaf	Decrease in the phosphorylation	hypersensitivity	Wang et al. [234]
H3K4me3, H3K9Ac and H3K27Ac	*Physcomitrella patens*	Genome wide	Gametophores	Increase in the content of H3K4me3, H3K9Ac and H3K27Ac	not analyzed	Widiez et al. [240]
Cytosine methylation	*Oryza sativa*	Genome wide	Leaf	hypomethylation in tolerant acession and hypermetylation in sensible accession	not analyzed	Gayacharan and Joel [58]

12.4 Bioinformatic Studies of TFs Involved in Abiotic Stresses

The advent of experimental high-performance platforms, in particular, next-generation sequencing (NGS), optimized assay systems, and advanced bioinformatics approaches have enabled comprehensive and maximized studies of plant genomes in a quick and economically viable manner. Transcriptome studies may be used for quantitative analysis of thousands of expressed genes related to germination, growth and development, flowering, and conditions of biotic and abiotic stresses, allowing us to understand plant mechanisms of the stress response [166]. The identification of the expression of regulatory elements in the huge amount of new data has been facilitated by bioinformatic methods and by the availability of several online repositories. Algorithms designed for data interpretation allow robust biological discoveries, such as prediction of possible TFs, their location in the genome and evaluation of their regulation in stress conditions, as compared to nonstressed controls. TF identification can be divided into two simplified approaches; the first one consists in detecting these elements in databases, by performing an analysis of these repositories, and the second approach is based on pattern recognition in sequences, allowing their grouping in different plant TF families previously described.

Stress-response mechanisms involve complex regulation of multiple genes and TFs. The first step involves the identification of these factors to unravel mechanisms

associated with their regulation [10, 13, 40]. The main computational methods developed for identifying TFs on a genomewide scale involved in silico approaches to evaluate the presence or absence of TFs and definition of their characteristics (DNA binding domains, auxiliary areas, and lost domains; [68]). The first TF analysis at the genomic level was performed by Riechmann et al. [188] for *A. thaliana*.

Several available databases focused initially on identification and annotation of TFs, such as PlnTFDB [173], PlantTFDB [99], LegumeTFDB [150], and TreeTFDB [151]. Databases for specific organisms were also created, including SoyDB [230], DATF [65], and DPTF [264], all for plants such as soybean (*Glycine max*), *Arabidopsis*, and *Populus trichocarpa* (Salicaceae), which may be used for evolutionary studies of TF in plants, as for prediction of TFs in new sequenced genomes. Public databases such as PlantTFDB (http://planttfdb.cbi.pku.edu.cn) and PlnTFDB (http://plntfdb.bio.uni-potsdam.de/v3.0) have catalogued and predicted TFs for more than 50 plant species of different taxonomic categories, including algae, bryophytes, gymnosperms, and angiosperms. Additionally, in 2009, the STIFDB (Stress-Responsive Transcription Factor Database) was created to summarize information from abiotic stress-responsive genes and TFs associated with these genes in *A. thaliana* [198]. Its assembly is the result of data clustering from analytical profiles of stress responses in plants [12, 194] and computational studies of stress-induced gene regulation [59]. The new version (STIFDB2 database; http://caps.ncbs.res.in/stifdb2) added data regarding two rice varieties (*O. sativa* subsp. *japonica* and *O. sativa* subsp. *indica*). The data were compiled from an analysis of 15 different stress types, including cold, osmotic, dehydration, heat, salinity, radiation, and oxidative stress, among others. Different families and subfamilies of TFs, their DNA binding domains and reference data from the literature related to his prediction/description are also listed in the new version. Its construction was based on mining genomic data and identification of 5984 unique genes related to stress, with more than 38,500 associations signaling and/or responding to stress. Briefly, the database can be used to identify potential stress-induced TFs and their gene regulation and may also be extrapolated to determine protein–protein interactions between TFs. With the inclusion of orthologues from other species, the database can be used to study evolutionary conservation and carry out comparative analyses between TFs responsive to abiotic stress in different plant species [156].

PlnTFDB is another useful database, consisting of a public repository, that aims to identify and catalogue all genes involved in transcriptional control in plants, currently containing 28,190 protein models and 26,180 sequences of different proteins, organized into 84 families, with classification based on domain structure. The classification of TFs is based on domain structures previously built in the Pfam database, with prediction via hmmpfam, an alignment tool included in the HMMER package, which is based on the identification of protein motifs via HMM (hidden Markov model; [42, 173]). Despite its functionality, few tools are available in PlnTFDB, allowing analysis of sequences just like a TF repository of information. Many domains are included in other databases/repositories such as PROSITE (http://prosite.expasy.org), a database that includes protein domains, families, and

functional sites; and SMART (http://smart.embl-heidelberg.de) allowing gene identification, annotation of domains, and sequence architecture.

In addition, the DBD (http://www.transcriptionfactor.org) database deserves mentioning with tools for TF prediction in complete sequenced genomes, using resources provided by Pfam (http://pfam.xfam.org) as well as information on domain organization, also systematizing information for gene families and genomes [121]. DBD provides for users the possibility to search DNA binding domains in a given protein sequence. Despite its functionality, this database only focuses on TFs, not including transcription regulators (TRs).

Regarding the identification of TFs and TRs by comparing functional domains, some tools and scripts are available, including the unpublished tool ITAK (http://bioinfo.bti.cornell.edu/cgi-bin/itak/index.cgi). This tool adopts the same domain classification of PlnTFDB, based on data catalogued in Pfam to predict genes coding for TFs and TRs. This tool is available in two versions (online and standalone). The online version, even being friendly to the user, analyzes only 50 protein sequences at a time, making its use impractical to evaluate sequence analysis on a genomic scale. In contrast, the standalone version is capable of realizing more robust analyses, but may be a challenge for most biologists, because the implementation is based on Linux command lines, often requiring optimization on a cluster computer system.

Another tool that provides a functional protein analysis, classifying families by prediction of domains and important target sites in sequences, is InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan5) which allows the alignment of sequences against a database of protein signatures. InterProScan comprises 14 search programs for domain signature or domain pattern, being useful for prediction of TFs and TRs [84]. This tool has been widely used for large-scale analysis, as in the case of *Medicago truncatula* (Fabaceae), which had its factors and transcription regulators identified in different gene families by the presence of known binding DNA domains [105]. For soybean, Wang et al. [230] developed a protocol for TF and TR prediction using InterProScan and a previous mapping in a species database. More recently, a group of online tools called MEME (http://meme-suite.org) was developed, which includes 13 tools for discovery and enrichment of motifs as well as motif pattern comparison (motif–motif comparisons). Motifs are responsible for many biological functions, therefore their detection and characterization comprise necessary steps in the analysis of molecular cell interactions, including regulation of gene expression [14].

In recent years, probabilistic models have been used for performing sequence alignments, especially using HMM-based software. One of the most widely known software, HMMER, implements in its algorithm a probabilistic inference to build complex models of specific positions in a given sequence by HMM. This software is a powerful tool for homology search, based on probabilistic inference methods [43, 48, 102]. Today HMMER is a major tool for domain search and TF classification, implemented in most plant TF databases, being widely used to find TFs related to abiotic stress.

In microalgae, a basal group of Viridiplantae, computational identification of TFs has been carried out for *Chlamydomonas reinhardtii*, *Volvox carteri, Galdieria sulfuraria*, and *Nannochloropsis oceanica* (Eustigmatophyceae; [173, 228]). Relationships and differences between TFs of this ancient group with higher plants remain uncertain. Hu et al. [80] analyzed three different genomes [*N. oceanica* (IMET1 strain), *N. oceanica* (CCMP1779 strain), and *N. gaditana* (CCMP526 strain)] by comparison of conserved domains, using the PlantTFDB and its implemented pipeline [99]. The analysis uncovered 125, 119, and 85 TFs in the three genomes, respectively, corresponding to 1.26, 0.99, and 0.94 % of their proteomes. The predicted TFs were then classified into different families based on the DNA binding domain. Considering the three analyses, 26 families were shared, with 19 TFs common for all three species, whereas MYB, bZIP, MYB-related, and NF-YC were the most abundant TF families, representing more than 40 % of the factors found.

In higher plants, a total of 1533 TF-genes was identified for *Arabidopsis*, being classified in 34 families [188], whereas 1611 TFs were predicted for rice, classified in 37 families of genes [245]. In turn, for soybean 51 HMM models deposited in Pfam and 11 models created via the HMMER tool were used to predict existing TFs in the soybean genome. It was possible to identify 5035 protein sequences corresponding to 4342 different loci from 61 TF families. From these sequences a database for specific soybean TFs was created: the SoybeanTFDB (http://soybeantfdb.psc.riken.jp), grouping basic and relevant information about the motifs found, promoter regions, and genome distribution, as well as the alignment of members of the 61 TF families found. Focusing on abiotic stress response, promoter regions of TFs were analyzed to identify *cis* elements in the PLACE database (http://www.dna.affrc.go.jp/PLACE), a repository of identified *cis-motifs*, including those that respond to biotic stresses [73, 150].

This type of analysis focused on specific families is available for several plant species, but only some important TF groups for specific stresses and growth regulation had been studied. This often happens because most TF families are vast and complex in higher plants. This is the case of WRKY, NAC, and bZIP families, which are highly diverse and active in plant expression reprogramming under stress conditions [158, 189, 197]. For example, Ooka et al. [167] identified in their work 75 isoforms of NAC in rice and 105 for *Arabidopsis*, whereas at least 157 orthologues in rice were identified by Lu et al. [139]. In soybean at least 101 members of NAC were predicted [176], and 163 complete sequences were predicted to *P. trichocarpa*, being grouped into 18 different subfamilies [78]. For WRKY at least 74 members have been characterized in *Arabidopsis*, and more than 100 members have been reported for rice [241, 256]. Similarly high numbers were found in grape (*Vitis vinifera*, Vitaceae) and tomato (*S. lycopersicum*) with 98 and 84 members, respectively [82, 233]. Members of bZIP TFs have also been predicted in several organisms, such as *Cucumis sativus* (Curcubitaceae), with 64 isoforms identified [15], and 75 members were predicted for *Arabidopsis* [89], 92 for *Sorghum bicolor* (Poaceae, [231]), and 96 for *Brachypodium distachyon* (Poaceae, [131]).

The identification based on computational methods is faster when compared to traditional molecular biological methods. Its efficiency is directly related to data integration from public databases, associated with sensitive prediction algorithms, providing a new approach for understanding main plant molecular responses related to stress response, adaptation, and tolerance [99, 156, 173]. Until now, there has been no universal pipeline for identification and classification of plant TFs that can be used by the scientific community in a comprehensive way. However, prediction of TFs in plant genomes can be made without great difficulty when appropriate tools and repositories are integrated into a workflow. Here, we present two workflows for TFs prediction, using tools and resources available in the previously mentioned databases (Fig. 12.2).

Predictions based on sequence alignment/motifs (HMM; Fig. 12.2a), use TF databases currently available. Initially, it is necessary to select protein families of sequences of interest in the respective database (e.g., PlantTFDB, PlnTFDB, etc.) to be used as seed sequences to generate the alignment and build the motif pattern using the hmmbuild tool implemented in the HMMER 3.0 package. The motif generated for a given TF family is then confronted via the hmmsearch tool (also implemented in the package) against the protein or translated nucleotide sequences for the chosen species, using a cut-off value of e^{-05} (or lower). This step will select candidates for TFs in a selected organism that show similarity with predicted TFs available. Sequences obtained in this step should be annotated against GenBank to confirm their function, as well as the similarity level with homologous sequences previously characterized. This analysis is simple and can be applied to all TF families available in repositories. The disadvantage of this workflow resides in the fact that this is not a comprehensive analysis, requiring a longer time to increase the number of TF families analyzed.

In contrast, the workflow outlined in Fig. 12.2b presents a pipeline for a global analysis of TFs. Prediction, in this case, is based on HMM alignment of motifs of known families via the ITAK tool. The script implemented in the program will identify all TF candidates, generating files for alignments, classification, and translation of nucleotide sequences for the chosen genome, using a recommended cut-off of e^{-05} (or lower). Also here, these candidates can then be aligned via BLAST against public TF databases and GenBank to identify their function and confirm similarity to orthologues from other species. The disadvantage of this method is the requirement of the command line to install packages and run scripts, a limitation for many biologists, in addition to the necessity of computing power, often associated with routine implementation on computer clusters, paralleling the process to accelerate the analysis.

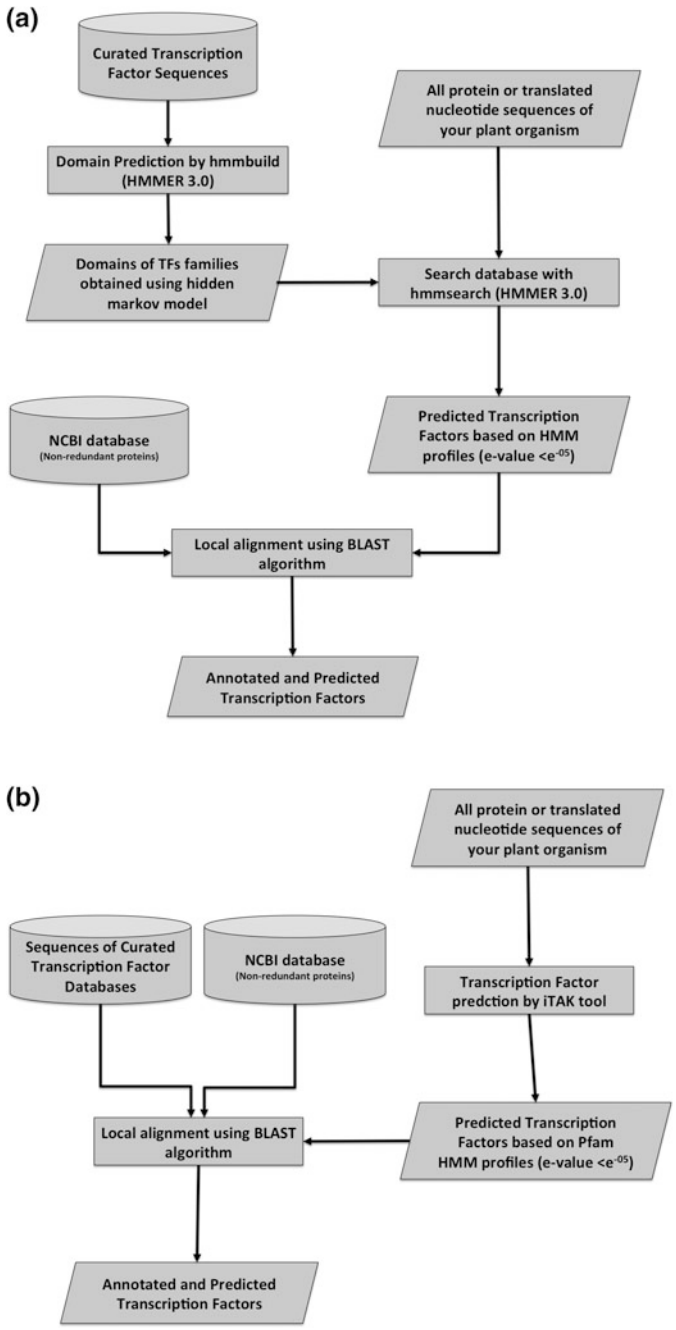

Fig. 12.2 Exemplary workflows for in silico transcription factor prediction, using available TF databases: **a** scheme for TF prediction using HMMER 3.0 via alignment of sequences against HMM models obtained via hmmbuild (aligning sequences obtained in public databases); **b** predicting transcription factors in the genome, based on ITAK tool (offline version) and annotation against NCBI GenBank

12.5 Interaction Network

Modeling metabolic pathways is fundamental to a system biological analysis of complex processes, facilitating studies about signaling mechanisms including abiotic and biotic stresses and allowing identification of molecular targets for biotechnological applications in crop plants. Generation of interaction networks also contributes to the understanding of metabolic, physiologic, and cellular mechanisms involved in such processes. Among regulators, many TFs figure as hubs, acting in the expression control of several stress-inducible genes, forming gene networks and/or signaling cascades. Regulation of gene expression is complex and controlled by an interaction network between different regulatory protein and *cis*-regulatory sequences present in promoters of their target genes [67]. Promoter regions act as molecular "switches" in gene expression, but also as signal transduction termination points in signaling processes [157, 219, 248, 249]. The interaction between TFs and their DNA binding sites are an integral part of regulatory networks [24].

Changes in expression patterns occur after perception of a given environmental perturbation by the plant, with the molecular machinery reprogramming TFs activity, allowing the creation of an effective defensive state. Many of these genes are multifunctional, able to induce tolerance to more than one stress situation [32, 36, 147, 184, 244]. The interaction between abiotic and biotic stresses, for example, induces complex responses to different stressors. Under unfavorable condictions, accumulation of certain metabolites may modify plant response to both biotic/abiotic stresses, favoring crosstalking response, protecting the organism from more than one type of stress pressure [182]. Thus, changes in ion flux eventually increase ROS and hormone synthesis, as the first responses after stress perception, whereas the resulting signal transduction triggers metabolic defense reprogramming [19]. For example, Tsutsui et al. [222] reported that DREB TF may regulate stress response between abiotic and biotic stress in *A. thaliana*, increasing plant resistance to cold and pathogens simultaneously.

Available sequences of reference genomes revolutionized the knowledge of plant genetics and its interactome networks, especially considering protein–protein interaction (PPIs) networks, allowing a comprehensive identification of genes involved in the response to abiotic and biotic stresses. Plant protein–protein interactome networks can be generated either using in vivo or in silico methods. The most reliable experimental methods for plant interactome mapping are yeast two-hybrid (Y2H, Y3H), bimolecular fluorescence complementation (BiFC) with affinity purification and mass spectrometry (AP-MS; [255]). Considering in silico methods in PPI detection, there are sequence-based approaches, structure-based approaches, chromosome proximity, gene fusion, orthologue-based sequence approach, phylogenetic profile analysis, gene coexpression profiles, sequence coevolution, synthetic lethality data, in silico two-hybrid systems, gene cluster versus gene neighbor analysis, protein domain information, protein interface analysis, and protein docking methods, among others [11, 181, 202, 203]. Another

in silico method used to understand main mechanisms associated with signaling and gene response regard pathway model representations, built using computational tools. Software that allows description of molecular interactions is based on System Biology Markup Language (SBML; http://www.sbml.org), that allows consistent and intuitive graphical construction of front pathways related, for example, to evaluate plant environmental stress responses. The maps cited here are traditionally created via CellDesigner v. 4.4 (http://www.celldesigner.org/), a modeling tool for the design of biochemical networks with graphical user interface [55, 83, 113].

In recent years, several approaches were designed to predict plant interactomes in species of agronomic importance, such as soybean, *Citrus sinensis* (Rutaceae), and rice, providing insights into mechanisms and pathways involved mainly with disease resistance [155]. Currently, some plant repositories and databases for whole genome scale, for transcriptomic and proteomic data are available and some act as repositories of plant protein–protein interaction platforms. Many of these repositories are available online for searches and free downloads, such as STRING, IntAct, MINT, TAIR, and BioGRID [8, 23, 207, 209].

For *Arabidopsis* and some other plants, stresses including drought, osmotic, salinity, oxidative, wounding, and heavy metals were found to induce expression of TF proteins that modulate specific genes with defined binding regions (Fig. 12.3; [213]). Several reviews focus on specific TF roles under abiotic stress, with the main emphasis on families that present key roles in stresses such as drought, salinity, high temperature, and hormones, being responsible for the modulation of several genes. Due to the fine-tuned relationship between TFs and other genes, it is clear that the understanding of such processes is directly related to the deciphering of regulatory networks, especially in interactions between members and the dynamic interaction among these, as well as analysis of each member that acts in more than one signaling pathway. This type of analysis has been generated for several TF families, with emphasis on the basic leucine zipper, AP2/ERF, NAM/ATAF1/CUC2 (NAC), WRKY, MYB, and basic helix–loop–helix (bHLH). Many members of these TF families were characterized with respect to their role in abiotic stress pathways, resulting in an increase of plant tolerance/resistance against stresses [28, 35, 38, 40, 53, 149, 164, 178, 191].

TFs function through interaction with a broad range of proteins, including other TFs, cofactors, and chromatin modifiers [29, 63]. Tolerance to abiotic stresses occurs in plants when TFs interact with multiple proteins. For many TFs, the formation of dimers is necessary prior to DNA binding, including homo- or heterodimers [56]. Dimerization can be a mechanism of either positive or negative transcription control [196]. Examples of TFs that require dimerization for binding include bZIP and bHLH [6]. The group-A subfamily of bZIP-type includes ABA response elements factors (ABF1-ABF4) and ABA-responsive element binding proteins (AREB1-AREB3), which interact with ABA-responsive elements (ABREs) under water deficit condition in *Arabidopsis* [52]. AREB proteins bind to ABA-responsive elements, which are major *cis*-acting elements in ABA-dependent pathways [253]. Overexpression of AREB1 in rice and soybean enhances drought tolerance [18, 165]. Overexpression of AREB2/ABF3 and ABF4 in *Arabidopsis*

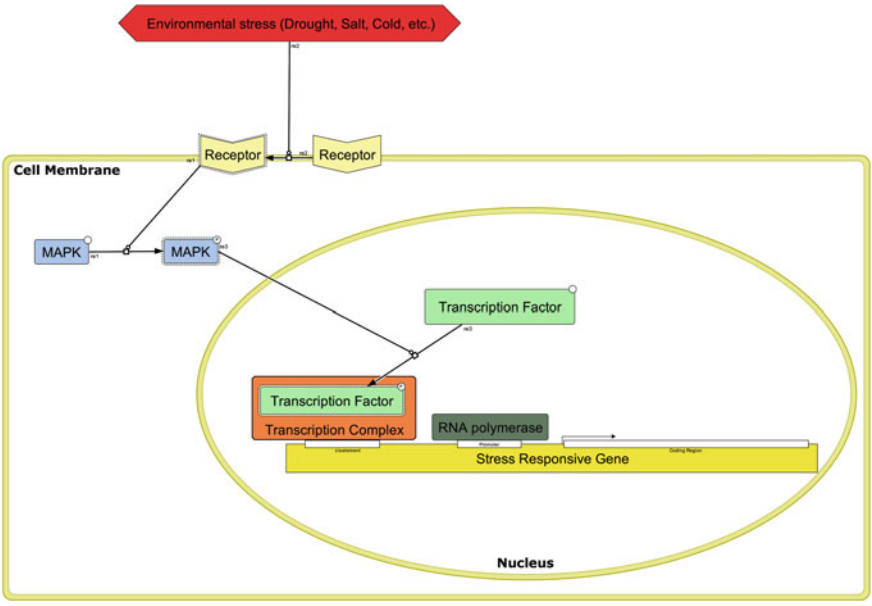

Fig. 12.3 A simplified model of abiotic transcriptional activation and gene expression regulation created using CellDesigner ver. 4.4. The graphical representation is based on system biology graphical notation (SBGN). The model shows proteins (transcription factors, general MAPK), general receptors, genes, and protein complex. The active state of the molecules is indicated by a dashed line surrounding the molecule. The phosphorylated state is indicated by letter "P" inside the protein residue symbol. The frame in *yellow* represents the cellular membrane and compartments

also confers drought tolerance [109], whereas overexpression of an active form of AREB1/ABF2 exhibits enhanced drought tolerance [51]. These three AREB/ABF TFs (from both rice and *Arabidopsis*) need ABA for full target gene activation. In turn, *areb1 areb2 abf3* triple mutant presented reduced tolerance to drought stress as compared to single and double *AREB/ABF* knockout mutants, indicating cooperative action between these three TFs [223, 253].

Analysis of the promoter region of other dehydration-induced genes led to the discovery of a group within the AP2 TF subfamily named DREB/CBF (dehydration responsive element binding/C-repeat binding factor), which is unique to plant species and implicated in ABA-independent regulation (Fig. 12.4).

Proteins of the DREB subfamily activate the C-repeat or dehydration response element (DRE/CRT) located in the promoters of drought-inducible genes and regulate expression of genes under water deficit [132]. In turn, members of the DREB1/CBF subgroup (DREB1A/CBF3, DREB1B/CBF1, and DREB1/CBF2) are cold inducible and are known to be involved in cold stress response, whereas

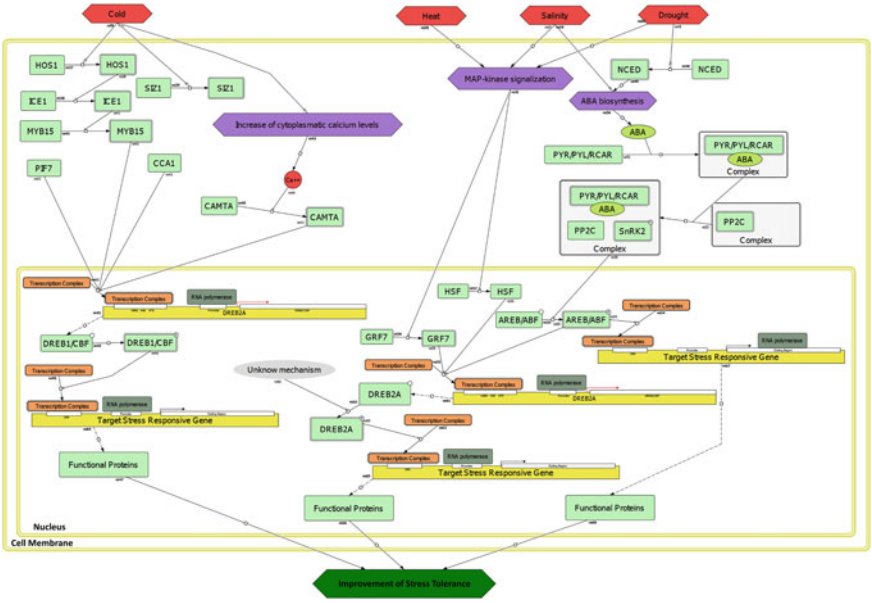

Fig. 12.4 Representation of abiotic stress signaling networks mediated by AREB, DREB1, and DREB2-type transcription factors. Model created using CellDesigner ver. 4.4, with a graphical representation of plant abiotic stress responses in abscisic acid (ABA)-dependent and ABA-independent gene expression. Drought, salt, high and low temperature model the level and activity of the TF group and their target genes. The *upper part* of the figure shows transcription cascades involved in rapid responses to abiotic stress, driven by kinases (MAPK) or not. *Lower parts* of the figure show transcription cascades involved in gene activation. The pathways suggest crosstalk between the stress types. The active state of the molecules is indicated by a *dashed line* surrounding the molecule. The phosphorylated state is indicated by the letter "P" inside the protein residue symbol. The frame in *yellow* represents the cellular membrane and compartments

the DREB2 group (DREB2A and DREB2B) plays a role in dehydration and heat stress response [149]. The physical interaction between AREB/ABFs and DREB1A/CBF3, DREB2A, and DREB2C has been described [125]. Lee et al. [125] showed that the domains of AP2 proteins (DREB1A and DREB2A) interact with ABF2 and that additional ABF family members (ABF3 and ABF4) interact with DREB2C. The interaction between DREB2C, ABF2, and ABF4 can be observed in the *Arabidopsis* predicted network (Fig. 12.5a), indicating crosstalk between elements of ABA-dependent and ABA-independent response pathways. Subsequently, Kim et al. [110] showed that an ABRE promoter sequence and AREB/ABF were involved in DREB2A expression, suggesting a complex interaction between AREB and DREB regulons at gene expression and also at the protein levels.

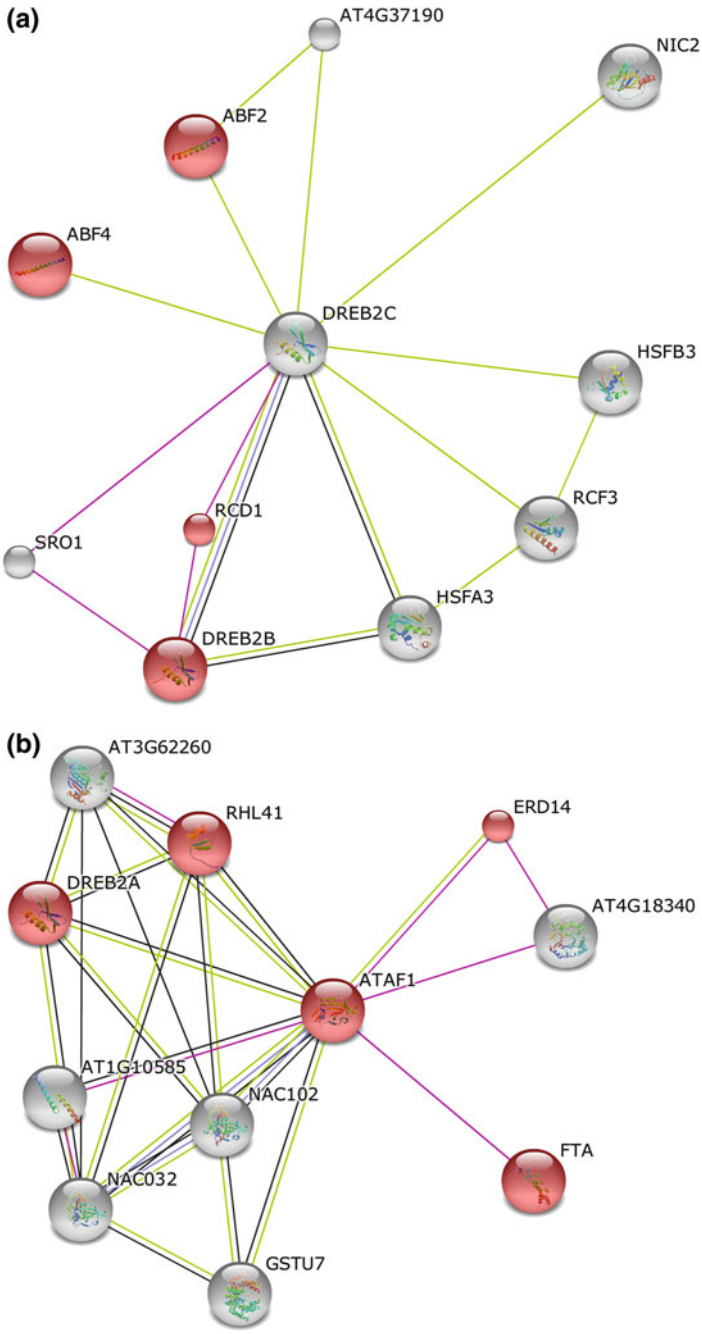

Fig. 12.5 Schematic representation of the transcription factor. **a** DREB2C and **b** AtATAF1 from *Arabidopsis thaliana* interaction with other proteins predicted in silico by STRING database program version 9.0 [210]. *Red colors* indicate GO Biological Process response to water deprivation

Yeast-two–hybrid assays involving DREB2A, DREB2B, and DREB2C showed their interaction with the regulatory hub protein RCD1 (radical induced cell death 1) and its closest paralogue SRO1 (similar to RCD one; [91]), which is also observed in *Arabidopsis* DREB2C predicted interaction (Fig. 12.5a). RCD1 is degraded during heat shock stress and RCD1-interaction–deficient *dreb2a* splice variant (DREB1a.2), which lacks the RCD1-interacting region and is accumulated during heat shock. This observation suggests that removal of RCD1 protein or loss of interaction with DREB2A is necessary for DREB2A function under abiotic stress and that RCD1 may mediate REB2A degradation [224]. Also, predicted interaction analysis has connected DREB2C to RCF3, which encodes a KH-domain containing putative RNA-binding protein. RCF3 is an important upstream regulator of heat-stress–responsive gene expression and thermotolerance in *Arabidopsis*, indicating that interaction between DREB2 and RCF3 cooperates in regulating heat tolerance in *Arabidopsis* [64].

Recently expression of an NAC member, ATAF1, was also induced by different abiotic stresses. Jensen et al. [93] reported that *Arabidopsis* ATF1 may regulate ABA-dependent gene expression of ABRE regulons. Interestingly, cooperative action of ANAC096 and AREB/ABF factors (ABF2/AREB1 and ABF4/AREB2) was necessary for dehydration and osmotic stress response [247]. In soybean, GmNAC20 may participate in the regulation of stress tolerance through activation of the DREB/CBF-COR pathway [66]. The *Arabidopsis* ATAF1 predicted interaction allowed the identification of an interaction between ATAF1 and DREB2A, probably playing a significant role in drought response (Fig. 12.5b). ATAF1 also interacts with the ERD14 protein, which is ABA-regulated. EDR14 is a member of the dehydrin family that accumulates in response to abiotic stresses in *Arabidopsis*, including drought, low temperature, and salinity [1]. Functional analyses of ABA-regulated ERD14 showed prevention of heat-induced aggregation and/or inactivation of distinct enzyme substrates [118]. Thus, ERD proteins act protecting the cell metabolism during stress and the induction of these genes by ABA during heat shock may help to prevent denaturing effects.

Other examples of TFs that form dimers are WRKY proteins (AtWRKY18, AtWRKY40, and AtWRKY60) from *Arabidopsis*. They can interact with themselves and with each other to form homo- and heterocomplexes through the leucine zipper present at the N-termini of WRKY proteins [246]. For instance, yeast-two–hybrid assays revealed that AtWRKY40 and AtWRKY60 interact with AtWRKY36 and AtWRKY38 [7]. Subsequently, the yeast-two–hybrid system also revealed a significant interaction of AtWRKY30 with AtWRKY53, AtWRKY54, and AtWRKY70 [21]. Diverse WRKY genes are under direct positive or negative control by WRKY TF-factors via a specific feedback mechanism (auto/crossregulation; [171]).

The WRKY family is one of the best-studied TFs regarding plant protein interactome networks. AtWRKY33 has been shown to be induced by oxidative stress, and its overexpression in *Arabidopsis* improved oxidative and drought stresses [98]. To illustrate the strong interaction between WRKY33 and other proteins, we exemplarily used the database view of STRING (Fig. 12.6).

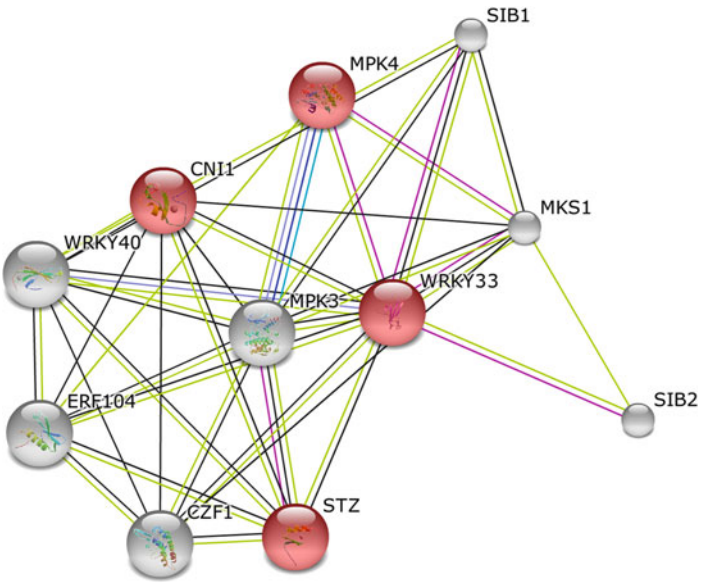

Fig. 12.6 Schematic representation of the AtWRKY33 interaction with other proteins predicted in silico by STRING database program version 9.0 [210]

The output shows that *At*WRKY33 interacted with the VG protein that contained a conserved FXXVQXLTG region, named VQ domain, such as SIB1 (sigma factor-interacting protein 1). These proteins play diverse roles in plant defense response, drought and salt tolerance, and also growth and development [101]. Based on recent reports, Kim et al. [111] suggested that the interaction of WRKY TF with the VQ domain proteins regulates the DNA binding activity of the TF either as positive or negative cofactors of WRKY TFs. Lai et al. [122] revealed that the VQ motif of SIB1 is necessary for its interaction with WRKY33 in plant defense against necrotrophic pathogens. In turn, Hu et al. [79] showed that the VQ9 protein interacted with WRKY8 in an experiment using the yeast-two–hybrid system. Mutation of VQ9 increased tolerance to salt stress. Therefore, VQ9 protein probably acts as a transcriptional repressor that antagonizes WRKY8 to maintain a proper balance of WRKY8-mediated signaling pathways to allow salinity stress tolerance [79]. In rice, proteins bearing the VQ domain are coregulators of WRKY TFs in response to disease resistance and response to environmental stress [111]. Also in grapevines, VQ14 and V17 may cooperate with VvWRKY25 and VvWRKY27 to mediate abiotic stress responses [235, 236].

WRKY proteins contain clustered proline-directed serines (SP clusters) as potential phosphorylation sites of MPKs [86]. Furthermore, phosphorylation of WRKY by MPKs could be an important means to transduce the signal to the nucleus, a fact also pointed out by previous evaluations with MPKs that implicated an abiotic tolerance. In *Arabidopsis*, MPK4 and MPK6 are activated by cold, salt,

and drought stress. During salt and cold stresses, MPK6 (and MPK4) was activated by MKK2 [216], whereas MPK3/6 activated WRKY33 expression. In vivo, WRKY33 protein autoregulates their expressions via a positive feedback loop by binding to their own promoter [142]. Upon drought stress, rice OsWRKY30 can be phosphorylated by MPK3 and MPK6, which then lead to an enhanced drought tolerance [199]. According to Banerjee and Roychoudhury [16], the MAPK cascades involved in phosphorylating WRKYs associated with abiotic stress are less studied in comparison to the biotic counterparts. Another significant interaction of WRKY33 was observed regarding STZ, a zinc-finger protein that exhibits a repressor domain activated under severe salt stress to control survival mechanisms, leading to increased salt stress tolerance [146, 193]. Transgenic lines of soybean overexpressing GmWRKY54 showed enhanced salt and drought tolerance, maybe through regulation of STZ/Zat10 [262].

12.6 Molecular Modification of TFs

As a consequence of external stimuli and intracellular signals, TFs can be targets of covalent modifications (posttranslational modifications, PTM), usually of enzymatic nature, during or after their respective biosynthesis [107]. Such PTMs add functional groups to TFs, such as ethyl, methyl groups, causing changes in their features, including protein stability, binding specificity, transcriptional activity, subcellular distribution, and even regarding interactions with other proteins [47] as well as epigenetic modifications [20].

So, every functional aspect of TF may be influenced by these changes, which can act in isolated forms or interconnected [47]. Thus, cross or associated answers from various PTMs have been reported [251]. For example, acetylation of some H3 lysine (K) residues in a particular position can mask the occurrence of ubiquitination (another PTM, which also occurs in K residues), possibly signaling for protein degradation via proteasome 26S [49, 217]. Considering TFs as regulators of multiple processes, even a potential PTM code, such as the proposed histone code, has been proposed [20], highlighting the importance of these modifications.

Among several databases currently available on the Web, a bank that covers different PTM proteins is available (69 types; http://ptmcode.embl.de; [145]), including phosphorylation, acetylation, methylation, adenylation, glycosylation, ubiquitination, hydroxylation, ADP-ribosylation, amidation, carboxylation, sulfation, and prenylation [145]. Some of these PTM deserve mentioning, as most frequently cited in relation to TFs, such as phosphorylation, acetylation, ubiquitination, and sumoylation.

Protein phosphorylations are the most frequent PTMs, being also the better studied [34, 108]. They regard reversible modifications that result in conformational changes in protein structure, generally associated with biological activation of proteins, turning them on or off. In turn, dephosphorylation, promoted by a

phosphatase enzyme, reverses the situation and may lead to inactivation of the protein. The addition of one or more phosphate groups by a kinase at an amino acid of a target protein chain—usually serine (S), threonine (T), or, to a lesser extent, tyrosine (Y)—increases the negative charge around these modifications. Such additions expose other amino acids located more centrally in the protein, in a way that a nonpolar and hydrophobic region of a protein can become polar and hydrophilic, leading to a conformational change in the protein structure and interaction with other residues [114].

TFs are generally phosphorylated at multiple sites, and this phosphorylation process may regulate the function of a given TF [115]. Many studies aiming at the understanding of how phosphorylation/dephosphorylation regulates TFs function are available and the process is well documented (e.g., [87, 172, 239]).

An important initiative generated a database covering triplets of genes, including the ability of a TF to regulate a second gene (the target gene) which is dependent on one or more PTMs, that are catalyzed by a third gene, usually a modifying enzyme [45]. Given that TFs are targets of different kinases and phosphatases, phosphorylation can integrate a set of information from extracellular stimuli that induce signal transduction pathways which provide a prompt response with the required flexibility in gene regulation [87].

Another PTM regards protein acetylation, in which an acetyl group is incorporated by replacing a hydrogen atom in a given amino acid. This modification is reversible (deacetylation) and can neutralize the positive charge (generally of a K) that may change the function of a given protein [185]. These changes can involve features such as DNA recognition, protein–protein interaction, and protein stability, among others. Acetylase enzymes can act on DNA-binding proteins, such as histones and TFs, or even on extranuclear proteins such as tubulin [117]. Histone acetylation is closely related to chromatin remodeling. In this process, certain regulatory elements (which are complexed with histones) are important for the expression of certain genes in eukaryotes.

On the other hand, acetylation of nonhistone proteins has been associated with the modulation of cell signaling in multiple levels and this participation would be analogous to that involving phosphorylation and dephosphorylation. In the excellent review of Spange et al. [205] examples of proteins affected by acetylation are compiled, including TFs, highlighting biological implications brought about by each modification. Exemplary cases of TF are also presented by Polevoda and Sherman [177], among a diversity of acetylated proteins.

In a study with human cells, Martínez-Balbás et al. [143] demonstrated that acetylation of the DNA binding domain E2F1 TF influenced the ability of this TF to bind to DNA, also influencing its transcriptional activation capacity and the half-life of the respective protein. E2F is an important TF family active in the cell cycle, coordinating the transcription of genes necessary for the progression of the cell into the S phase. Expression of some genes of this family has been also studied in other plants including rice and tobacco [116]. In *A. thaliana*, E2F1 was described by Jager et al. [88] as a member of a multigene family with different activities.

It should be kept in mind that changes in the ability to bind to DNA depend on where acetylation occurred in the protein chain and that acetylation will not always stimulate transcription [177]. According to Khidekel and Hsieh-Wilson [107], acetylation may decrease TF binding to promoters, mitigate transcription, stop TF-coactivator ligation, or still cause nuclear kidnapping of TFs. A Web resource called AceK, which aims to locate acetylated sites in histones and nonhistone proteins, is available at http://csb.cse.yzu.edu.tw/AceK/ [138].

Another PTM type that involves TFs regards ubiquitination. In this process an ubiquitin (small eukaryotic regulatory protein with 8.5 kDa) binds to the last amino acid of the chain (Glycine 76) with a K amino acid in the target protein. This binding may signalize for degradation of the target via proteasome (ubiquitin 26S proteasome system, UPS) or may change its cellular localization, its activity, or even prevent or promote other protein interactions [25]. This PTM may involve a single ubiquitin (monoubiquitination) or a chain of ubiquitin (polyubiquitination), but only the polyubiquitination of specific lysine residues (K48 and K29) signalize the protein degradation via UPS. The UPS pathway influences some processes associated with plant development, including response to biotic and abiotic stresses [141]. Other types of polyubiquitination have been related to diverse events, such as endocytosis, intracellular trafficking, and protein activation [174]. In turn, monoubiquitination has been implicated in events such as DNA repair and gene silencing [154]. The process related to this PTM involves different steps: activation, conjugation, and connection that are commonly performed by enzymes as a ubiquitin-activating enzyme (UBA; E1), a ubiquitin-conjugating enzyme (UBC; E2), and ubiquitin ligase (E3). The completion of these steps culminates by the conjugation of ubiquitin to a K residue of the substrate protein [25].

The relation of E3 ubiquitin ligases with their target proteins and plant innate immunity has also been studied. E3 ubiquitins (Ub)-ligase proteins—whose importance is reflected by the presence of over 1400 coding genes in *A. thaliana* [144]—are classified in different families, according to their structural and functional characteristics, reflecting their specificity to given substrates. Duplan and Rivas [41] reported a positive action of an RING-type Ub-ligase in the basal resistance activation (PTI-PAMP–triggered immunity; [103]) of *Vitis pseudoreticulata* after fungal infection, by inducing proteolysis of the target-TF (*WRKY11*) via UPS. In this case, the target TF has been considered to be a negative regulator of plant defense response [254].

Another PTM regards sumoylation, characterized by a labile covalent bond of a c-terminal glycine residue of SUMO (small ubiquitin-like modifier protein) to a K acceptor of a target protein [71]. Thus, SUMO also competes with other PTM by K substrate. This reversible association may change the protein activity and its nuclear localization, also affecting protein–protein interaction [225]. One explanation for these changes would be that the presence of multiple sites of monosumoylation or formation of polysumoylation of a protein greatly increases its mass and surface area, affecting protein interaction with other proteins and also its activity. The importance of this conformational change in the regulation of transcription is not entirely understood. However, TF sumoylation has been associated with repression

of the activity of certain targets. In this regard, some mechanisms have been proposed. One is that sumoylation promotes interaction of TFs with corepressors (such as histone deacetylase corepressors), suggesting a complex crosstalk between acetylation and sumoylation, affecting gene regulation [60].

In *A. thaliana*, TF MYB30 is sumoylated in K283, and this has been associated with ABA (abscisic acid phytohormone) signaling, in response to abiotic stress [259]. The replacement of K283R blocks sumoylation in *A. thaliana* protoplasts. In the same way, bZIP TF ABI5 also suffers sumoylation (mainly in K391) resulting in a negative regulation of the ABA signaling pathway [148]. In both cases, sumoylation serves to balance expression of genes that are responsive to ABA [259]. A list of these genes with variations in their expression can be accessed in Zheng et al. [259].

It is evident that TFs and their amino acid residues are subject to various PTMs. Furthermore, some residues of target proteins, such as K, are subject to different PTMs such as acetylation, sumoylation, and ubiquitination, among others, allowing various combinations and influencing general and specific responses involving many different pathways.

12.7 Concluding Remarks and Perspectives

Many studies showed that TFs are key regulators of both ABA-dependent and ABA-independent abiotic stress response. Several examples demonstrate how TFs can be coregulated under different abiotic stress and might be part of the same signal transduction networks.

In the course of the last 15 years, significant efforts have focused on the generation and improvement of high-throughput analytical technologies for investigating the roles and mechanisms associated with TFs. Many actual findings considering responses to drought and related abiotic stresses rely on the development of omics inferences, especially genomics, transcriptomics, and proteomics, helping to uncover the complex roles of TF networks.

Next-generation sequencing is getting more reliable and cheaper, and shall bring additional evidence to a better understanding of TF function in nonmodel plants and groups scarcely studied, such as most woody species. The availability of a higher sequencing coverage in transcriptomes is necessary, considering that TFs present discrete expression but can induce drastic changes under stress, as exemplified in this review.

Here we selected some key TFs with proposed similar functions in distinct plant species, uncovering their critical role in the induction of signaling cascades, leading to cell reprogramming after stress perception. However, many others deserve attention and possibly the combination of different TFs will be advisable in biotechnological inferences. Additional databanks and tools for bioinformatics and system biology analysis are being developed and will allow the recognition of new interactions in different species that can be compared for identification of new

mechanisms considering these important molecules. Furthermore, chromatin modeling and mechanisms of posttranscriptional regulation of TFs regard frontiers to be unveiled, whereas the possible manipulation of these processes for breeding purposes is still in its infancy.

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Chapter 13
Mutation Breeding and Drought Stress Tolerance in Plants

Mohammad Taher Hallajian

13.1 Introduction

Mutagenesis is a process by which the genetic information of an organism is changed in a stable manner, resulting in a mutation. It may occur spontaneously in nature, or as a result of exposure to mutagens. Mutagenesis in the laboratory is an important technique whereby DNA mutations are deliberately engineered to produce mutant genes, proteins, strains of bacteria, or other genetically modified organisms. Various constituents of a gene, such as its control elements and its gene product, may be mutated so that the function of a gene or protein can be examined in detail. The mutation may also produce mutant proteins with interesting properties, or enhanced or novel functions that may be of commercial use. Plant mutagenesis is rapidly coming of age in the aftermath of recent developments in high-resolution molecular and biochemical techniques. By combining the high variation of mutagenized populations with novel screening methods, traits that are almost impossible to identify by conventional breeding are now being developed and characterized at the molecular level [1]. Mutation induction continues to contribute to crop improvement, using physical mutagens such as gamma ray, X-ray, fast neutron, and chemical mutagens such as ethyl-methane-sulfonate (EMS) and sodium azide (IAEA 2009). Ionizing radiation includes ultraviolet (UV) light, X-ray, gamma rays, and neutrons. These high-energy forms of radiation cause double-strand breaks of the DNA double helix. Radiation causes deletions of nucleotides from the DNA sequence. These deletions can cause reading-frame

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shifts, inactive protein products, or faulty transcripts. Chemical mutagens affect the DNA molecule through chemical reactions within the genome. Base analogues are chemicals with similar properties to the DNA bases. They can be incorporated by the cell into the genome, replacing the proper base such as alkylating agents, nitrous acid, and intercalating agents [2]. Transposable elements are a special class of mutagen. They are self-replicating segments of DNA that excise and/or insert themselves within the genome. Also known as transposons, these strange sequences were first proposed by the pioneering Barbara McClintock working on maize [3]. Transposable elements, unlike other forms of mutagenesis, do not act upon the genome in a completely random fashion [4].

Among physical mutagens, gamma radiation has been widely used for mutation induction of both seed and vegetative propagated crops. Recently ion energy technology—heavy ion beam (HIB) and low energy ion beam (LIB)—is being utilized for mutation induction in wide-ranging crops. HIB is predominantly used for inducing mutations in plants. The spontaneous mutation rate is pretty low and cannot be exploited for breeding and that is why artificial mutations are induced with physical and chemical mutagen treatment. Many useful genetic changes have been induced by mutagen treatment including high yield, flower color, disease resistance, early maturation, and so on in crops, vegetables, medicinal herbs, fruit, and ornamental plants. However, the major problem with fruit-breeding work is the long life cycle of many fruit crops, which varies from 3 to 25 years or even more. In fruit crops, mutagenesis has been quite useful in isolation of useful mutants such as plant size, blooming time, fruit ripening, fruit color, and resistance to pathogens [5].

13.2 History of Mutation Breeding

Historically the use of mutagenesis inbreeding has involved forward genetic screens and the selection of individual mutants with improved traits and their incorporation into breeding programs. DeVries [6] suggested the use of radiation to induce mutations. The discovery that X-ray induced mutations in *Drosophila melanogaster* [7] and *Hordeum vulgare* [8] led to the use of radiation-induced mutations for changing plant traits by plant breeders and geneticists [9]. Since the discovery of X-rays about 100 years ago, the use of ionizing radiation, such as X-rays, gamma rays, and neutrons for inducing variation, has become an established technology [10]. Mutagenesis has been used in plant breeding since Muller's discovery of the mutagenic effects of X-rays on Drosophila flies [7]. Induced mutations have been used in the improvement of major crops such as wheat, rice, barley, cotton, peanuts, and beans, which are seed propagated [10].

Mutation breeding became more widely used in the 1950s, after the US atomic bombing of Japan at the end of World War II in 1945. In the wake of the

devastation, there was a desire to find uses for the "peaceful atom" that were helpful to humanity. Atomic gardens were set up in the United States and Europe, and even in Japan, with the aim of creating high-yielding and disease-resistant crops.

Hermann J. Muller, founder of mutation genetics and winner of the Nobel Prize in Physiology or Medicine in 1946, summed up the broad range of aspects and implications of mutation research in his Nobel Lecture on "The Production of Mutations."

Auerbach and Robson (1946) reported the use of chemicals such as mustard gas to be highly mutagenic. Since then a number of agents have been discovered that can increase the frequency of artificially induced mutations [9].

Atomic gardens, built around gamma-ray emitters, were popular among breeders in the 1960s and Japan still operates one. China began launching seeds into space in 1987 to take advantage of cosmic radiation and low gravity, developing more than 40 mutant crops with higher yields and better disease resistance, including varieties of rice, wheat, and pepper [11].

Bhabha Atomic Research Centre (BARC) developed 41 new crop varieties (Trombay varieties) by radiation-induced mutation and crossbreeding; these have been released and officially notified by the Ministry of Agriculture, Government of India for commercial cultivation. It started in 1973 with Trombay Groundnut (TG-1) cultivated mainly in Gujarat and Maharashtra [12].

Ion beams have been widely used in the research on material surface modification since the 1970s. Their application for mutation induction was started with low-energy ions in China in the late 1980s and with heavy ions in Japan in the early 1990s. Although ion beam technology has been used for food crop improvement in China, it has been more extensively used for floriculture plants in Japan. With the aid of IAEA and UNDP, China has been able to develop new, higher-yielding rice cultivars and extend them to farmers across the country as they strive to produce more food from the 33 million ha under cultivation with rice (https://www.iaea.org).

Thus far, over 3000 mutant varieties have been officially released over 60 countries including rice, wheat, barley, sorghum, legumes, cotton, edible oil, ornamental plants, and fruits (www.mvd.iaea.org) [13]. China and India are the major producers of mutant varieties to feed their ever-growing human population. Among all crops, the highest number of mutant varieties released is in rice. In 2005, The International Atomic Energy Agency (IAEA), Vienna, Austria was conferred the Nobel Peace Prize for its contributions to the peaceful applications of nuclear energy in various fields including food and agriculture. The year 2008 marked the eightieth anniversary of mutation induction in crop plants, when an international symposium on induced mutations in plants was organized in Vienna, Austria [14]. The role of mutation breeding in increasing the genetic variability for desired traits in various crop plants have been proved beyond doubt by a number of scientists [15–20].

13.3 GMOs Versus Mutation Breeding Versus Conventional Breeding

When countries reject or ban genetically modified crops over safety concerns, agricultural companies often turn to developing new strains using mutagenesis, wherein plants are subjected to radiation treatments or doused in toxic chemicals that randomly scramble genes to produce new traits.

Is mutagenesis really safer than genetic modification? BioChica, a scientist in molecular genetics addresses the safety differences of GMOs, mutagenesis, and conventional breeding by looking at peer-reviewed academic papers and other publications. A paper from the Proceedings of the National Academy of Sciences, which compared GM rice to "mutant" rice, concluded that, although there are unintended genetic changes in the GM rice, there were far fewer than in rice bred through mutagenesis, although the potential for harm in both cases is trivial. Food safety should be regulated, but the regulations should be on the food product itself, not on the method used to grow a particular crop (www.geneticliteracyproject.org).

GM proponents often compare GM with mutation breeding (or mutagenesis), which they argue that mutation breeding is used by conventional plant breeders and that mutation-bred plants have a history of safe use and do not cause ill health. GM proponents also say that genetic modification is more precise than mutation breeding, and imply that therefore, GM plants should not be regulated any more strictly than those produced by mutation breeding. Once plants carrying radiation-induced mutations have been created, they are crossed with other crop varieties using conventional breeding (the same process is used with GM crop varieties). However, mutation breeding is not in itself conventional breeding (http://earthopensource.org/gmomythsandtruths).

Foreign gene transferring to organisms and producing new traits can cause serious safety problems. For example, in Iran, scientists developed stem borer resistant rice plants via transferring the BT gene to rice but this foreign gene produces toxin in the plant, especially in part "spikes" and this issue endangers human food security. Even, in some cases, transferring a foreign gene can produce allergic interactions, genetic disorders, and different diseases, whereas in mutagenesis, the organism's DNA changes after irradiation and no foreign DNA is entered. In mutation breeding, in order to produce enough and favorable mutant populations, radio sensitivity and postradiation recovery studies must be carried out. LD50 and the optimum dose of irradiation for any explant are determined. Moreover, in this method, many harmful traits are deleted morphologically from the mutant population during the selection process in consecutive years. Also, the nutritional quality of selective samples must be analyzed in different generations of mutation in terms of safety.

Mutation induction techniques can greatly increase the gene mutation frequency and create new germplasm, new materials, and new varieties in a relatively short period of time. The genetic variability increases considerably, by hundreds or a thousand times higher than the natural mutation frequency. The variation spectrum

is wide and various, among them useful variation increases significantly, including some rare mutations that are not easily observed in nature or by the crossing method [13].

13.4 Mutation Breeding Strategy

Plant breeding categorized into three subtypes as mutation breeding, recombination breeding, and transgenic breeding has the potential of generating variation and selection of target lines [9].

Mutation breeding is the process of exposing plant explants to mutagens, physical or chemical agents for inducing genetic variation. It offers good prospects for the domestication of promising underutilized wild species, for agricultural or horticultural uses, as well as for improving adaptation of recently introduced crops to unsuitable environments. One of the most crucial requirements for a successful breeding program is the selection of an effective and efficient dose of a mutagen for mutagenizing the starting material and producing a high frequency of the desirable mutation.

In the case of mutation breeding, the basic fundamental and unique feature is the generation of new mutated alleles. The key steps include analysis of difference in the sensitivity of different genotypes and plant tissues to different mutations often measured using lethal doses (LD), generation of genetic chimeras after mutagenic treatment, and analysis of their effect on transmission of mutated alleles and segregation in the subsequent generation, and also often the recessive nature of induced mutations [9].

The ability to handle large mutagenized populations in a confined space, faster progeny turnover in vegetatively propagated species, and the ability to screen for several biotic and abiotic stress factors in the culture environment make in vitro approaches very efficient. Mutant screening has developed revolutionary changes in the past decade with reverse genetic approaches taking precedence. Therefore, integration of mutation techniques with molecular approaches is providing exciting opportunities for modern plant breeding. Mutation breeding can be enhanced by genetic selection for novel alleles. Through targeted mutation breeding, genotypes with induced or natural mutations in candidate genes are identified for cultivar development. For most horticultural plants, targeted mutation breeding may be a more economically feasible approach to trait development than through transgenic technology.

Like any other scientific innovative technology, mutation breeding has its advantages and limitations. One of the biggest advantages is the creation of new genetic alleles that are not in germplasm pools and the induction of new gene alleles for a commercial variety such that new varieties carrying the desired mutation alleles can be directly used as a commercial variety. Also, the limited genetic changes of any single plant of a mutated population and the often recessive nature enable breeders to develop a new variety in a short breeding cycle. A limitation is

its limited power in generating the dominant alleles that might be desired; its less effectiveness than cross-breeding for a trait needs a combination of multiple alleles, such as tolerance to abiotic stresses. The low mutation frequency requires growing and screening a large population for selection of desired mutants with reasonable confidence [21].

The prime strategy in mutation-based plant breeding has been to upgrade the well-adapted varieties by altering one or two major traits. These include characters such as plant height, maturity, seed shattering, and disease resistance, which contributed to increased yield and quality traits, such as oil profile and content, malting quality, and size and quality of starch granules. For example, short height genotypes in rice, wheat, barley, and maize have contributed significantly to increasing grain yield because of their resistance to lodging and high planting density [22].

A sequential strategy is essential for any mutation breeding steps where mutagenic induction and its mutagenesis are more helpful for autogamous crops than the cross-pollinating one. This is due to several problems regarding the incorporation, selection, and maintenance of recessive mutations in crop plants, many plant breeding problems in the cross-pollinating species, and sometimes many handling-based problems in existing variability. Where the lack of variability exists for specific and simply inherited traits, the basis of choosing between induced mutations and hybridization is essentially the same in self- and cross-fertilizing species. However, the genetic consequences of the failure of recessive imitations to express in crossfertilizing systems without forced selling or sib-mating must be taken into consideration in assessing the cost of such ventures. The efficiency of mutation breeding, more than any other breeding method, is dependent on the effectiveness; useful variants can be recognized in the M2 or M3 generation [9].

In crops where diversity for a given trait is low or nonexistent, induced mutagenesis provides an avenue of possibility.

13.4.1 Mutation Breeding in Seed-Propagated Species

Seeds treated with mutagenic agents give rise to chimeric plants. Chimeric plants produce both mutant and nonmutant seed. This can be problematic; however, one just needs to plant more seeds to find the desired mutants. As long as an efficient screening method is in place, this should produce no significant pitfalls. Mutagenic treatment of seed is by far the most popular method in mutation-breeding programs [2].

13.4.1.1 Mutation Breeding in Self-Fertilizing Species

Breeding mutant traits is fairly straightforward in crops that are capable of self-fertilization. Because many mutations are recessive, after mutagenic treatment, the material should be self-fertilized and advanced to at least the M2 before

phenotypic screening. At this point plants will be segregating for the recessive mutant trait.

Because mutagens act randomly upon the genome, it is important to collect as many positive mutants as possible. This allows the breeder to have a series of lines from which to select for performance in addition to the presence of the mutant trait [2].

After mutagenic treatment, plant materials (seeds, tissues, organs, etc.) and plants grown from them are in the M1 generation; the seeds harvested from M1 plants and the plants grown from these seed are the M2 generation. M1 and M2 populations are populations that are composed of M1 and M2 plants. Their genetic structure is quite different from those of a traditional crossbreeding program, that is, F1 and F2 populations. In diploid plants, the mutation rate per cell is double of the mutation rate per gene. The chance of a simultaneous occurrence of two or more mutations at the same locus of the two homologous chromosomes in diploid plants is rare and so is the chance of segregation of homozygous mutants in the M1 population.

In seed-propagated crops, only germline cells can transmit their genotype into subsequent progenies. Therefore in materials treated with a mutagen, only cells that develop into inflorescences ("initial cells" or "genetic effective cells" in a seed), can transmit mutated alleles into the M2 generation. When a mutation (A→a′) is induced at one of the genes of a homozygous AA locus, the phenotype becomes heterozygous (Aa′). Typically, the mutant allele is completely recessive to the original allele and the Aa′ phenotype cannot be discriminated from the original AA phenotype. Hence, the vast majority of induced mutants cannot be screened in the M1 generation, although there are a few examples of dominant mutations. Mutants that have homozygous mutant alleles (a′a′) will appear in the M2 population as a result of self-pollination of M1 plants. For example, early flowering, semi-dwarf, and male sterile mutants can be visually recognized. There are basically two methods of establishing an M2 population in self-pollinated crops such as barley and wheat. In such monocots the inflorescence is known as a spike [13].

M1-Spike Progeny Method

This is a method in which M2 seeds are harvested separately from each spike of M1 plants. The method was developed by Stadler [8] and has been used effectively by Swedish research groups guided by Gustafsson [23]. Usually 10–20 plants are grown out from seed of individual M1-spikes.

One-Plant-One-Grain Method

The method of constructing the M2 population by planting only one grain (seed) from each plant was proposed as the "one-plant-one-grain method." In practice, the seeds are harvested not from each M1 plant, but from each spike, hence this method

is also known as the "one-spike-one-grain method." If a few seeds per spike are used for the establishment of the M2 population, this approach is called the "one-spike-few-grain method." In mutation breeding, a single target mutant from the entire M2 population is sufficient for utilization and multiplication of the mutants for further selection and testing [13].

13.4.1.2 Mutation Breeding in Cross-Fertilizing Species

Cross-fertilizing species raise some difficulties. Because species that are predominantly cross-fertilizing typically exhibit significant inbreeding depression, the necessary self-fertilizations to identify mutants in the population result in reduced plant vigor due to the genetic background and not necessarily the mutations. This compounds the difficulty of successfully identifying mutations. Crop species with self-infertility mechanisms are especially hard to use mutation breeding methods without elaborate crossing schemes. Because the genetic structure of segregating generations in cross-pollinated crops is quite different from those in self-pollinated ones, the methods for selecting mutants in self-fertilizing species are not applicable for cross-fertilizing species [2].

In some cross-fertilizing (allogamous) crop species (e.g., maize, melon, cucumber, oil palm), male and female flowers are spatially separated. In such species, it is possible to produce M2 populations by artificial selfing of M1 plants by pollinating the female flowers with pollen from male flowers of the same plant. However, homozygous mutants would not appear in such M2 populations, inasmuch as male and female gametes usually derive from different cells in the seed embryo. Because M2 plants are free from chimerism, and therefore homozygous M3 seeds are produced through selfing of heterozygous plants (Aa′) in M2, subsequently homozygous mutant plants are segregated out in M3 populations. In many cross-fertilizing species, however, selfing is not successful due to self-incompatibility systems, and in other cases artificial selfing on a large scale is impractical due to the very small size of flowers [13].

Crossing-Within Spike Progeny Method

The crossing-within spike progeny (CSP) method is composed of: (1) harvesting seeds separately from each spike of the M1 plants or plants in a recurrently treated population; (2) sowing the seeds derived from each spike in a small hill-plot (plot of a small area, e.g., 0.5×0.5 m) in the next generation; (3) isolating each hill from the others by bagging all the plants of each hill-plot just prior to the start of flowering; and (4) harvesting seeds from each hill-plot and sowing them as a hill-plot progeny for the selection of mutants in the next generation. This method is based on half-sib mating, a type of inbreeding that is achieved within each hill-plot from which homozygous mutants will segregate out in the following generation (M3). Under open pollination, the eggs are fertilized with pollen from the other plants. Seed

harvested from the mutated spike are sown in a hill-plot and the plants are bagged just before flowering to avoid fertilization with the pollen from the other hills. Fertilization is performed within each hill-plot. Avoiding chimeric structure within the M1-spike is important in mutant selection in cross-pollinating crops. Because the bagging of each hill-plot just before flowering time in M2 is a laborious task, it is desirable to increase the frequency of the mutated gene in the population in the generation in which bagging is performed. Recurrent mutagenic treatments for successive generations meet these two requirements [13].

Unlike a self-pollinated species, seed sterility does not increase drastically after recurrent treatment in a cross-pollinated species, which was shown in Italian ryegrass after gamma-ray and chemical treatments [24].

In a cross-breeding program, most important traits to be selected are quantitative and, in general, controlled by polygenes. Selections for a specific trait are made in the later generations when most of the loci governing trait are fixed. In a mutation-breeding program, the targeted traits are those usually governed by a single major gene and the selection of mutants is performed primarily in the M2 generation. Seed sterility may be observed and is often caused by the mutagenic treatment. The treatments with radiation often induce chromosome translocations and inversions that lead to pollen and seed sterility. Because either type of induced sterility is more or less inherited by the subsequent generations, hence, if the objective is not to develop mutants for fertility, inflorescences with normal seed fertility should be selected from the M1 plants. In the selection of mutants in self-pollinating crops, the most important point in M3 screening is to evaluate the mutations that have been selected at the M2 generation. The mutation must be highly heritable and fixed in the following generation by selfing the M2-spike progeny in the field. If the undesirable characteristics are associated with a mutant and cannot be removed by ordinary selection, it may be necessary to backcross (BC) the mutant to the original variety and select a promising progeny from the BC population. Generation of doubled haploids from M1, M2, M3, and other mutation generations is a valuable means of producing homozygous mutants and is particularly valuable in species that have a long generation time, for example, perennial trees [13].

13.4.2 Mutation Breeding in Vegetative Propagated Species

Mutation breeding is the only straightforward alternative for improving seedless crops. In other words, mutation breeding is the most suitable method for the breeding of vegetatively propagated crops (VPCs), because the new mutant varieties and the original ones have the same genetic background except for the mutated genes. In VPCs that do not produce any seed (e.g., banana), mutation breeding becomes one of the few available options (other than transformation), inasmuch as cross-breeding is not possible.

All cells exposed to the mutagen will not necessarily incur mutations, but those that do incur mutations will give rise to cells exhibiting the mutation. Identification and propagation of the necessarily large numbers of plants to identify successful mutants is difficult for many vegetatively propagated plants, however, once one is identified, the mutation is fixed in the cloned progeny. Crop species where in vitro techniques exist and can be used to mutate plant material, allow for the regeneration of large numbers of plantlets. This system is highly amenable to both vegetative and seed propagation. A large number of plant species are asexually or vegetatively propagated and are known as vegetatively propagated crops. They include many ornamentals, root and tuber crops, woody perennial and forest trees, fruit crops, and other crops such as peppermint, sugarcane, tea, and many grasses. Cross-breeding of VPCs is often difficult due to various biological limitations, for example, their long vegetative phase, high heterozygosity and polyploidy, incompatibility and other cross barriers, apomixis, and sterility. Mutation techniques can overcome many of these barriers and can be used for the improvement of many VPCs [13].

The possibilities of mutation breeding in vegetatively propagated crops depend on many factors, such as the genetics of the characters involved, the mutagen to be used, the handling of the material after treatment, the availability of a selective screening method, and so on.

The decision as to which breeding strategy (mutation vs. cross-breeding) is appropriate for a specific situation is usually economic: which method is the easiest, the fastest, and the least costly. Effective methods to develop mutants that express phenotypic variation on an individual plant level include: adventitious bud techniques, continuous pruning, grafting and cutting-back techniques, and in vitro culture techniques. Generally, stable mutants are not produced until after several vegetative generations.

In VPCs, meristematic buds are usually used as target material for mutation induction. Many plants possess natural systems of vegetative propagation, especially by tubers, bulbs, rhizomes, stolons, apomictic seed, and so on. In addition, many new in vivo methods, such as stem or leaf cuttings or grafts, and in vitro methods such as cell or tissue culture are also used in commercial production. In practice, the ease of dissociating chimeras after mutagenic treatment plays an important role in the choice of target material. The use of heterozygous starting material (Aa) will be more practical for mutation work than homozygous material (AA or aa), because induced mutations are mostly recessive hence they can be expressed only in plants with the original genotype Aa where the dominant allele is mutated [13].

General considerations for initiating a mutation breeding program of VPCs are:

- The trait for which the variation is sought, and its commercial value/potential, end-user demands
- Genetics of the trait (dominance, recessiveness, pleiotropy, linkage)
- The crop, variety, mode of propagation, degree of heterozygosity, and ploidy level

- Need for mutation induction as an alternative to existing conventional or modern methods
- Plant material to be used for treatment and methods to handle chimerism
- Available information on mutation breeding limitations, if any [25].

Mutation Breeding Steps in VPCs.

In the first year, explants (e.g., shoot meristems or axillary buds) are treated with mutagens with optimal doses. Shoot growth (M1V1 generation) is initiated and assessed for the occurrence of chimera. In the second year, after vegetative propagation of M1V1, M1V2 shoots are assessed for possible occurrence of periclinal or homohistont mutated parts and vegetative propagation of M1V2 shoots is carried out for the isolation of induced mutations. In the third year, genetic uniformity is checked through growth and preliminary evaluation of the mutants (M1V3). In the following years, growth assessment of the mutants is done throughout the vegetative and reproductive stages and evaluation of the mutant's performance for agronomic traits will be fulfilled. The exact number of years needed for assessment varies from plant species to species. Final assessment is carried out in the last MV generation for release as a mutant variety. Inheritance in VPCs is often complex owing to high levels of heterozygosity and ploidy, which make genetic analysis difficult [13].

13.4.3 Ploidy and Mutation Breeding

Mutagenesis of polyploid plant species is difficult. Because most mutations are recessive, plants must be homozygous to display the trait. Polyploidy conditions can further complicate the process of reaching homozygosity for the mutation, so selfing must be carried out in additional generations to ensure presence of the mutation [2]. They can more efficiently repair damage to their DNA. The ploidy level of the target species also influences mutation response. Ploidy deficiencies include a reduction in genome number, for example, from diploid barley to haploid barley. Haploid production and thereby doubled haploid production by inducing embryogenesis in haploid (gametic) cell cultures to produce homozygous lines is a valuable technology in plant breeding and genetics of many species.

Polyploidy either by genome duplication (autoploids) or genome addition (alloploids) has occurred naturally in the evolution of many species and has also been induced for crop improvement. One effect of polyploidy is to increase the volume of the nucleus; this in turn increases cell size and tissue, organ, and plant size [13].

13.5 Molecular Mutation Breeding

The term "molecular mutation breeding" is defined as mutation breeding in which molecular biological knowledge, techniques, and tools are used. Experimental mutagenesis of the early twentieth century led to unprecedented breakthroughs in plant breeding; this, however, was based largely on phenotypic selection and without much knowledge of the genetic controls of target traits. Techniques in molecular genetics are rapidly evolving and methods in handling the large datasets produced (bioinformatics) are expected to have a massive impact on molecular mutation breeding. High-throughput DNA technologies for mutation screening such as targeting induced local lesions in genomes (TILLING), high-resolution melt analysis (HRM), EcoTILLING, and so on are the key techniques and resources in molecular mutation breeding. Molecular mutation breeding will significantly increase both the efficiency and efficacy of mutation techniques in crop breeding [9].

Induced mutagenesis was almost irrelevant to reverse genetics before the development of TILLING and similar generic reverse genetics strategies. This approach can be used both for functional genomics and practical breeding. In plant genomics research, in addition to identifying the function of a particular gene in a given plant species, the effect of various mutant alleles can be assessed using TILLING technologies. Starting with a homozygous population is desirable. From a technical point of view, the TILLING protocol includes four main phases: (1) generation of a mutant population, (2) selection of target genes (DNA preparation and pooling), (3) molecular screening, and (4) recovery of mutants [13].

13.5.1 De-TILLING

Fast neutron mutagenesis often results in kilobase-scale DNA deletions. As a new knockout technique to obtain deletion mutants for target genes, a strategy to screen for rare deletion mutants in large fast neutron mutagenized populations was first developed by Li et al. [26, 27] and demonstrated in *Arabidopsis* and rice. It combines fast neutron mutagenesis and high-throughput PCR screening, named "Deleteagene" (delete-a-gene). This strategy has been further developed and named deletion-TILLING, or de-TILLING [28].

The de-TILLING method includes three key technological aspects:

1. Fast neutron mutagenesis, which generates DNA deletions in different sizes
2. A DNA pooling strategy to reduce the number of PCRs needed
3. Technologies that allow a mutant allele, possessing an internal deletion, to be amplified in pools with excessive genomic target sequence [13].

13.5.2 New Platforms for TILLING

Typical or simplified TILLING systems are based on the hetero-duplex cleavage by endonucleases such as Cel1. There are instruments that can be used to differentiate hetero-duplex from homo-duplex, hence no cleavage is needed. Using CSCE or HRM, the only step required is a simple PCR before either capillary electrophoresis or DNA melting curve analysis [13].

13.5.2.1 CSCE-Based TILLING

CSCE is a nonenzymatic differential DNA conformation technique for SNP discovery. After PCR amplification, and the denaturing and reannealing of amplicons, several duplex species are formed, for example, homo-duplex of wild type (WT-WT) and mutant (M-M) and hetero-duplex of WT-M. Because of the mismatch formed in the hetero-duplex, it migrates at a different speed from the homo-duplex during electrophoresis in capillaries filled with CAP, a semi-denaturing polymer, thus allowing the identification of pools containing a mutation within the target fragment [13].

13.5.2.2 HRM-Based TILLING

Similar to CSCE, HRM is also a nonenzymatic mutation screening technique; it reveals sequence variants due to distinct patterns in DNA melting curve shape. It has been used recently in many ways as a novel approach to study genetic variation in many fields with applications ranging from qualitative SNP detection to semi-quantitative analysis of methylation. When using equipment and reagents such as the Light Cycler® 480 System and its accompanying High Resolution Melting Master mix, which contains a saturating fluorescent dye, realtime PCR is carried out using a touchdown protocol, with annealing temperatures ranging from 70 to 60 °C [13].

13.5.3 EcoTILLING

The genomes of individuals within a single species contain significant genetic variation that has arisen from spontaneous mutation. The vast majority of this diversity is in the form of single nucleotide changes commonly referred to as simple nucleotide polymorphisms (SNPs). Such naturally occurring SNPs are of great interest to scientists because they are useful as genetic markers in mapping, breeding, and genotyping and can provide information concerning gene structure, linkage disequilibrium, population structure, or adaptation [9]. Both nucleotide

changes and small insertions and deletions are identified, including at least some repeat number polymorphisms. This method is called EcoTILLING. The technology is applicable to any organism even including those that are heterozygous and polyploid [29].

13.6 Application of Mutation Breeding in Improvement of Quantitative Traits of Plants

Mutagenesis for resistance to abiotic stresses is a well-known effective and efficient breeding approach in order to create new desirable genetic variability, as the use of the traditional breeding methods have narrowed genetic variability in the cultivated crop species over a long period. A quantitative trait mutant cannot be detected with a high level of confidence owing to its interaction with environmental factors, therefore a different procedure is recommended for selecting such mutants. If the heritability of the targeted quantitative trait is low and is much influenced by environmental effects, mutants should be screened from the M3 population based on the mean phenotypic value of progeny derived from M2 spikes or plants. For a quantitative trait, normally M3 families and subsequent generations are used for phenotypic screening. When screening for a quantitative trait, such as yield, disease resistance, abiotic stress tolerance, or quality, mutant selection should be postponed until the M3 generation [13].

In India, breeding work for salinity resistance is mainly carried out at the Central Soil Salinity Research Institute, Karnal (http://www.agriinfo.in). Also, in Bangladesh, breeding for crop quality and abiotic and biotic stress tolerance is mainly carried out by the Bangladesh Institute of Nuclear Agriculture with the collaboration of IRRI (http://www.bina.gov.bd). Thus far, two salt-tolerant rice varieties (BINA dhan 8 and BINA dhan 10) and two submergence-tolerant rice varieties (BINA dhan 11 and BINA dhan 12) have been developed by BINA.

In the United States, there are firms such as Arcadia Biosciences that specialize in TILLING, and a number of crops are currently being developed around the world, including salt-resistant tomatoes, drought-resistant soya beans, and strawberries with a longer shelf life, gluten-free cereals, fungus-resistant barley, and yellow tomatoes (http://www.gmo-safety.eu/News).

13.6.1 Mutagenesis for Tolerance to Drought Stress in Plants

In recent years, drought has occurred more and more commonly as a result of global warming and climate change. The plant traits improved by mutation breeding include: yield, flowering and ripening time, adaptability, plant type and growth

habit, resistance to lodging and stem breakage, shattering and shedding resistance, tolerance to temperature, drought, heat, and salinity [30].

13.6.1.1 Probability of Obtaining Mutants

Often a few loci possess significantly higher genetic effects. Mutations at a gene at such loci with a large genetic effect can be selected after mutagenic treatment. The occurrence of a grain carrying a mutant gene with the low probability of only 10^{-12} per gene per generation due to a spontaneous mutation will still amount to several thousands of independent occurrences (and thus grains carrying the mutant gene) worldwide [31].

It is important to determine the most efficient sizes of M1 and M2 populations to ensure a reasonable probability of identifying a desired mutant. Factors such as chimerical nature, harvesting and growing practices, and the genetic nature of traits of interest can all significantly affect the population size and hence the efficiency in mutant development. A quantitative trait such as yield or quality of grains is generally controlled by many genes and, in addition, influenced by environmental factors. The mutation rate of a gene is very low, therefore the chance of simultaneous occurrence of mutations at two or more genes is negligible. Hence the selection of mutants in mutation breeding is usually unsuccessful for a quantitative trait, particularly when the number of loci controlling the trait is many and the effect of each locus is small as compared with environmental variation. But many quantitative trait locus (QTL) studies revealed that the genetic effect among the contributing loci is not equal [13].

As mentioned above, determination of the target population size in M1 is the most crucial component of mutation breeding. It is obvious that the population size will depend on the inheritance pattern of the gene. If the mutation is monogenic recessive, the probability of recovering a mutant phenotype will be higher than for a trait controlled by more than one gene. In practice, 10 times of the size has to be considered, because the mutation produced may be useful or undesirable. Mutation breeding is an input-intensive process. It is therefore advisable to select mutagens with high mutation frequency, so that Ml generation size can be reduced. It is to be remembered that germline mutations take place only in the initial cells of the embryo, therefore depending on the nature of the species, products of initial divisions should be screened. For example, cereals such as rice, wheat, barley, oat, and the like produce multiple tillers. Those tillers that generate first (primary tillers) have the maximum chance to carry a mutation. In the case of tuber crops such as potato, the mutation may be present in any of the stems arising from different discs of a tuber, therefore each of them has an equal chance to give rise to a mutation. Genetically, a mutant plant in Ml should be heterozygous, because during treatment only one allele is affected by one mutation. Only dominant mutations can be identified [9].

13.6.1.2 Determining an Optimal Treatment Dose

Mutation frequency usually increases linearly with an increasing dose of mutagenic treatment, but survival and regeneration capacity decrease with increasing dose. The radiosensitive curve should be determined to calculate LD50 dose (lethal dose) for each experimental plant to avoid either very high or very low dosage. Moreover, plants and even varieties differ in radiosensitivity [32]. In order to obtain the highest of favorable mutations, it is necessary to determine LD50 and optimum dose. This dose is a dose that causes the maximum favorable mutations with minimum damage to the plant.

In seed-propagative plants, seed is the main material for mutagenesis in most cases. In vegetative-propagative crops, when determining an optimal dose for treatment, these two aspects should be considered. In VPCs no meiosis is experienced throughout the process of mutant development, therefore, the genetic constitution of a mutated cell is inherited by its lineage cell lines and plants unchanged. Therefore, it is impossible to separate useful mutations from unwanted ones that occurred in the same cells. This is in sharp contrast to seed-propagated crops, where unwanted mutant genes can be separated from desired ones through self-crossing or backcrossing. Therefore, a dose lower than LD50 is preferred in mutation breeding of VPCs.

13.6.1.3 Management of Early Generations of Mutation

The treated seeds need to be handled with care. The seeds treated with physical mutagens can be stored before sowing. However, the seeds treated with chemical mutagens should be washed thoroughly and be planted as soon as possible. The time of sowing should be slightly later (2 or 3 weeks) than normal so as to reduce excessive vegetative growth. The purpose of isolation of the M1 is to avoid the introduction of genetic variability other than that induced with the mutagenic treatment. Mechanically isolation can be achieved by bagging spikes in cereals using plastic or paper bags to prevent cross-pollination and bird damage. Even if the objective of a mutation project is to select mutants with enhanced tolerance to drought or salinity, the M1 plants should be grown in a nonstressed condition; otherwise, there will be insufficient number of plants generated in the M2. M1 plants should be grown either at a reasonable distance from other varieties (physical isolation) or in a time period when no other plants would flower simultaneously (biological isolation). In the case where the seed yield from each branch is reasonably adequate, it is suggested that each primary branch may be harvested separately. In the case of cereals, the individual plant or spike can be harvested [33, 34].

Many mutants with desired traits are selected in the second or third generation after mutagenic treatment and subsequently released as new cultivars after agronomic evaluation in regional and national trials.

In seed-propagated crops, sowing of the M2 generation depends upon the method of harvesting of the M1 generation. Two methods of sowing the M2 generation can be followed. First, M1 plant to row, where all seeds produced from a single plant are grown in a row. The success of its use will depend, to a large extent, on how well the branching has been controlled because it tends to dilute the yield of M2 mutants. The second method is of M1 spike or branch to row, which offers the greatest precision with regard to the origin of a mutant when the material treated is genetically homogeneous as regards the nonmutant allele and when outcrossing is controlled.

From M1 to M3 through from germination to harvest, the mutated plants are carefully observed for all viable mutations. The plants with different morphological traits are isolated for chimeric and dominant mutations through their life period in M1, and they are isolated for recessive mutations in M2 and M3. All kinds of morphological mutants isolated in M1 and M2 are confirmed for true mutations in M2 and M3, respectively. Individual plant selections based upon phenotypic variations are started in the segregating M2 population, focusing on agronomic and yield characters [9].

In vegetative-propagated crops, selection for interesting mutants can take place three to four years after irradiation. In this way, some commercial mutants have been obtained [35].

Visual selection of the mutant phenotypic variation is the most effective method of selection in VPCs. It can be used effectively for identifying common traits and characteristics such as color changes, plant morphology, earliness, resistance to pests and diseases, and so on. Selection usually starts from M1V2 and continues for confirmation in M1V3 or M1V4 generation. Because the probability of identifying desired mutants in a homogeneous M1V2 population is quite low, selection is necessary in the M1V3 generation or in some cases in the M1V4 generation [13].

Mainly three types of screening/selection techniques can be employed for the selection of mutants in M2 and subsequent generation via visual, mechanical/physical, and other methods [36].

Visual screening is the most effective and efficient method for identifying mutant phenotypes. Visual selection often is the prime basis for selecting for disease resistance, earliness, plant height, color changes, ion-shattering, adaptation to soil, climate, growing period, and so on. Mechanical or physical selection can be used very efficiently for seed size, shape, weight, density, and the like, using appropriate sieving machinery. In other categories, chemical, biochemical, physiological, or physiochemical-like screening procedures may be needed for selecting certain types of mutants [9].

13.6.1.4 Screening and Selection of Mutants for Resistance to Drought

A quantitative trait mutant cannot be detected with a high level of confidence owing to its interaction with environmental factors, therefore, a different procedure is recommended for selecting such mutants. Selection of mutants on the basis of the

mean value of M2-plant–derived progeny in the M3 instead of the value of the M2 plant is a possible solution. If the heritability of the targeted quantitative trait is low and is much influenced by environmental effects, mutants should be screened from the M3 population based on the mean phenotypic value of progeny derived from M2 spikes or plants [13]. The mutants confirmed in M3 are screened for resistance to drought and heat in field and greenhouse experiments. In the drought-resistant experiments, the mutants are selected for the background of their parents. In other words, the mutants are used according to the reaction of parents to abiotic stresses, and compared with their parents and international standard cultivars (ICARDA).

In these experiments, chlorophyll content (CC), drought resistance score (DR) using a 1–9 visual scale, root (RL) and shoot lengths (SL), plant height (PH), canopy width (CW), biological yield (BY) per plant and seed yield (SY) per plant, 100-seed weight (SW), and harvest index (HI) are studied. CC is recorded using a chlorophyll meter. Of course, the evaluation scale of drought resistance (tolerance) in vegetative stages is different and depends on the kind of plant species and sensitive stage of plant growth. For example, in rice, the drought tolerance scale is on leaf rolling score (0–9) at the end of the vegetative stage. Yield is another screening scale that is estimated in all plants after finishing of drought stress, usually at the end of the reproductive stage. In rice, the most important factor of yield is spikelet fertility. Therefore the drought tolerance level of mutant rice plants is estimated on a 1–9 scale of spikelet fertility.

13.6.1.5 Evaluation of Preliminary Yield of Promising Drought-Tolerant Mutant Lines

Yield traits are the most important criteria for water-deficit tolerance screening [37–40]. In rain-fed paddy fields, water shortage has been well known as being a seri-ous issue, especially in the reproductive stage, during which plants are particularly sensitive, leading to low crop yield [37, 39]. The flowering stage is the most drought-sensitive stage as drought stress during this stage results in loss of yield due to low spikelet fertility and low full seeds. In vegetative-propagated species such as sweet potato, bulk irradiation is done at respective optimum dosages and explants are propagated in vitro up to M1V4 and M1V5 stages to dissolve chimeras and obtain stable mutations.

Promising mutant lines are further identified and evaluated in a replicated preliminary yield evaluation trial. Also, in seed-propagated species such rice, selective mutant lines are grown up to M4 and M5 to achieve genetic stability and enrich germplasm by selecting genotypes with superior qualitative and quantitative traits. Preliminary yield of promising drought-tolerant mutant lines is evaluated in completely random design in three replications [41].

13.6.1.6 Assessment of Adaptability and Stability of Promising Drought-Tolerant Mutant Lines in Advancing Generations

Usually, this evaluation is carried out in different ecological regions for at least two years. The assessment method of promising mutant lines is approximately similar to other native genotypes and cultivars.

A randomized complete block design with three replications is applied. In seed-propagated species such as cereal, promising mutant lines with control and commercial cultivars are sown on plots randomly. With attention to lack of purity and homogeneity of genotypes and in result, different propagative and reproductive specifications especially in open-pollinated species, spaces between mutant plants in plots are variable on studied mutant genotypes and cultivars. All agronomic practices recommended for production are applied equally for each plot. Plot base and individual plant base data are collected. Common traits that are evaluated include percentage of fertility, plant height, flowering date, and yield, especially in plot base. All the data are collected when the crop reaches physiological maturity. These data are subjected to analysis of variance (ANOVA) using statistical software. The data are combined over locations after carrying out analysis of variance for each location separately, and homogeneity tested as suggested by Gomez and Gomez [42]. Means are separated using Duncan's multiple range (Duncan) test. Environment interaction of the genotypes in biplot analysis is conducted using GenStat 15.1 computer software.

13.7 IAEA/FAO Activities on Developing Drought-Tolerant Plants

The International Rice Functional Genomics Consortium announced the public availability of more than 200,000 rice mutant lines, which represent mutations in about half of the known functional genes mapped for rice to date [43].

The database maintained by the UN Food and Agriculture Organization and the International Atomic Energy Agency contains only around 3000 such plant varieties, and this number includes not only food crop plants but also ornamental plants. It also includes not only the primary mutant varieties generated through mutagenesis, but also any varieties that have been created by crossing the primary mutant varieties with other varieties by conventional breeding. Thus the actual number of primary mutant varieties is a fraction of the 3000 varieties listed in the database. These varieties have been officially released in over 60 countries including rice, wheat, barley, sorghum, legumes, cotton, edible oil, ornamental plants, and fruits (www.mvd.iaea.org). Drought-resistant varieties have been developed in several crops in many countries throughout the world. In India, drought- and salinity-resistant or tolerant varieties have been developed in several crops such as cotton, rice, and sugarcane. In cotton, drought-resistant varieties have been

developed through induced mutations. In wheat, drought-resistant varieties have been developed in China and the USSR through induced mutations. In *Luthyrus saliva*, a drought-resistant variety has been developed by induced mutations in USSR (http://www.agriinfo.in). The group in Vienna, according to program head Pierre Lagoda promotes developing more "sustainable" crops by irradiating them to resist threats such as drought, insects, disease, and salinity [11].

The Mutation Breeding Project focused on the improvement of drought tolerance in soybean and sorghum represents one project developed by the Forum for Nuclear Cooperation in Asia (FNCA) in 2002. Ten drought-tolerant mutant lines of sorghum were obtained in Indonesia. In dry seasons, these lines have biomass production and grain yields significantly higher than original variety Durra and the national check variety [44]. Eight drought-tolerant pure soybean lines were selected in Malaysia. Four mutant lines (GH-7, I-209, M-220, and 60-MBB) were already distributed to field test in Malaysia and the Philippines. In the Philippines, five promising drought-tolerant soybean lines were developed. In Vietnam, the results of a local adaptability test at five stations showed that the line D.96 adopted as a national variety in 2004 and named DT96 was tolerant to drought and high yielding [44].

In Iran, Hallajian et al. [45] produced several new promising drought- and salt-tolerant lines in rice. Also, these promising lines had superior qualitative and quantitative traits such as early flowering, dwarfing, high aroma, resistance to herbicide and lodging, high yield, and so on. Now these promising lines are being evaluated in different geographical sites of Iran in order to introduce, register, and release new elite rice cultivars (www.aeoi.org.ir; Fig. 13.1).

In Australia, there have been some reports in improving some rice varieties for their tolerance to some adverse environmental conditions and higher grain yields (IAEA 1980). Characterization of ethyl methane sulfonate (EMS) induced mutants of N22 for water stress and heat tolerance was reported by Panigrahy et al. [46]. Nagina 22 (N22) is a deep-rooted, drought- and heat-tolerant aus rice cultivar.

Scientists from the University of Agricultural Science and BARC produced a large seed variety of groundnut. Hundreds of farmers are producing even up to 7 tons/ha of some varieties of groundnuts in some states. A drought-tolerant early maturing variety and an early maturing large seed variety of groundnuts are being cultivated in large desert areas in Rajasthan [12].

In West Africa, sorghum is also undergoing irradiation treatment and, in field trials, some of the new mutant varieties produced have demonstrated increases in yield of 30–50 %, higher protein content, and earlier maturation compared to local cultivars. Some varieties demonstrated an improved tolerance to drought and the new plants also maintained the important characteristics favored by farmers (www.new-ag.info).

Fig. 13.1 Drought stress in the field and promising drought-tolerant line (2013)

13.8 Conclusion and Future Perspectives

With the imminent threats posed by global climate change to crop production and the ever-increasing and more sophisticated demands of agricultural products, crop improvement efforts have to be more powerful and precise in developing new crop varieties. Breeders therefore require tools that permit achieving subtle changes to the genetic make-up of otherwise superior crop varieties, for example, high yielding but lacking in specific quality traits, and yet leaving the genome largely intact in order not to disturb already stacked alleles of genes. The spectrum of available mutation techniques has significantly increased; as a result, following the recent trend in the release of crop mutant varieties in some countries, the number of officially released mutant varieties listed in the FAO/IAEA Mutant Varieties Database will expand exponentially year after year. The conventional breeding method takes several years to develop a new cultivar/variety from wild species. Induced mutagenesis and its breeding approaches are potential tools and are being highly used in crops to improve their quality and quantitative yield traits.

The availability of genomics information in the public domain coupled with recent advances in molecular and cellular biology techniques have paved the way for transforming old mutation techniques into state-of-the-art technology for both crop improvement and basic genomics research. Some technologies are already in place and when integrated into mutation research, they will greatly increase the efficiency and application of mutation techniques in plant research. For example, the next-generation sequencing technologies, such as the Roche 454 Genome Sequencer-FLXFe Sequencer-FLX Biosystems SOLiDF™ instruments, have the potential to reduce the cost of genome sequencing by several magnitudes, and simplify the process of mutation detection, the key point in mutation research and application programs. In particular, they will enable the identification of mutant genes underlying important quantitative traits such as drought tolerance and yield, something that is still very difficult if not impossible with traditional means (www.iaea.org).

Most of the mutant lines were found to be more resistant to the concerned stresses than their parents and the best international checks. These mutants will be used either (1) directly in the target environments as commercial varieties, or (2) indirectly in breeding programs as useful parents. Thus, mutation-assisted plant breeding will play a crucial role in the generation of “designer crop varieties” to address the uncertainties of global climate variability and change, and the challenges of global food insecurity [9]. Our present knowledge about mutation breeding and drought tolerance in plants indicated that mutation breeding can be applied as a useful tool in developing new drought-tolerant genotypes and cultivars with superior traits and can help to develop cultivation of different plant species in drought periods or low falling regions of the world.

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Chapter 14
Identification of Candidate Genes for Drought Stress Tolerance

Amal Harb

14.1 Introduction

A water deficit can be natural or man-made. The naturally occurring water deficit is known as drought, which is mainly shortage in rainfall [97]. Different specialists define drought in different ways depending on their interest and the level at which they are studying drought [128]. For a plant physiologist, drought means water deficit for days, whereas for the molecular biologist and biochemist it is dehydration for hours [128]. Drought is one of the major abiotic stresses that impede the growth and productivity of plants. Moreover, drought is predicted to persist for decades to come given the global climate change and the various consequences related to it.

At the morphological level, plants under drought stress suffer from growth retardation at the vegetative stage, which is more pronounced in the shoot growth. At the flowering stage, drought is detrimental and results in drastic decrease in the yield of plants. These morphological effects of drought are manifestations of the physiological, biochemical, and molecular changes in drought-stressed plants. Physiologically, plants under drought will lose water and this will impede normal photosynthesis through mainly the closure of stomata and the consequent low availability of CO_2 [24, 126, 137]. In addition, nutrients uptake will be hindered, which will result in both structural and developmental defects as well as physiological and biochemical abnormalities [64].

One of the major biochemical changes in drought-stressed plants is the accumulation of reactive oxygen species (ROS) and the alteration of ROS homeostasis [30, 79, 117, 137]. Under these conditions and depending on the plant resistance to drought, plants usually activate their antioxidant systems [47, 137]. Another biochemical response to drought stress is the accumulation of osmolytes such as

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proline and glycine betaine [201]. These osmolytes help the plant to tolerate water deficit by increasing the osmotic potential inside its cells.

Drought tolerance is one type of drought resistance by which plants can survive the low water availability in their environment [97]. A plethora of studies help in deciphering the molecular responses of plants to drought stress. Starting from the pregenomic era, many drought-responsive genes were identified in Arabidopsis and many crop plants. It was not until the sequencing of the Arabidopsis genome that a quantum leap in our understanding of the molecular basis of drought responses has been witnessed. The complete genome of *Arabidopsis thaliana* was sequenced in the year 2000. The second flowering plant of the sequenced genome was rice, which is considered as a model plant for monocots. For decades, the analysis of different types of microarrays of Arabidopsis plants under drought revealed thousands of drought-responsive genes. Transcriptome analyses of rice plants under drought also revealed thousands of drought-responsive genes. With the substantial progress in the sequencing technologies, the genome sequence of a large number of plants is now complete. Hence, drought-responsive genes were identified in many important crop plants. Through this chapter, drought-responsive and candidate genes for drought tolerance are used synonymously. In this chapter, drought-responsive genes in the model plant Arabidopsis are discussed in detail. Then, drought-responsive genes in many major crops are reviewed.

14.2 Drought Tolerance

The ultimate consequence of drought stress is the loss of water from plants, which results in severe cases in wilting and eventually the death of plants. Drought tolerance is one way that plants resist drought stress [97]. In noncrop plants survival under drought stress is considered as drought tolerance, whereas, in crops drought tolerance is normal or even high productivity under drought stress [127]. Drought-tolerant plants have the ability to survive the low internal water content utilizing different mechanisms. These mechanisms include the increase in ABA concentration, the increase in antioxidants and the accumulation of osmolytes and protective proteins such as late embryogenesis abundant (LEA) proteins [11, 68].

Different groups studied the response of plants to drought utilizing different treatments. In many studies the exposure of plants to osmoticum such as mannitol or high molecular weight polyethylene glycol (PEG) for a few days is considered drought [89, 92]. Yet in other studies plants were left to dehydrate for hours to study the biochemical and molecular changes under dehydration or desiccation [145, 198]. Several studies tested the effect of soil water deficit in pots on the growth, development, physiology, biochemistry, and molecular processes in different plants [2, 60, 80, 121, 141]. Transcriptome analysis of plants under different drought treatments revealed differential gene expression, which resulted from the differences in drought treatment and the developmental stage at which drought was imposed. With the absence of a controlled experiment that compares the differences

in gene expression in plants at the same developmental stage under different drought treatments, it is difficult to assign specific genes exactly to each type of drought treatment. In Arabidopsis, plants were exposed to moderate controlled and progressive soil water deficit in pots. The transcriptome analysis of plants under the two treatments revealed a higher number of genes were differentially expressed in response to progressive drought compared to moderate controlled drought [60]. Moreover, only 178 differentially expressed genes (91 upregulated and 87 downregulated) were shared by both treatments. About 1083 genes (545 upregulated and 538 downregulated) were responsive specifically to moderate controlled drought. The functional analysis of the differentially expressed genes in both treatments showed that response to water deprivation, ABA, osmotic, and oxidative stresses were the major functional groups in the common upregulated genes between progressive drought and early stage of moderate drought [60]. In a comparative study in barley plants under dehydration and soil water deficit, the expression profiling results showed that the majority of the differentially expressed genes (57 %) were in plants under dehydration stress for 6 h [159]. In addition, soil water deficit resulted in the differential expression of 6 and 14 % of the genes after 7 and 11 days of drought treatment, respectively. The results highlight both the quantitative and qualitative differences in the molecular responses of plants under fast dehydration shock compared with slow soil water deficit. In rice, differential gene expression was revealed between moderate and severe progressive drought [21].

With the substantial advances in the sequencing technologies, the detailed dissection of the molecular responses of plants to drought becomes more feasible [148]. In the model plant Arabidopsis, the molecular responses to osmotic, dehydration, and soil water deficit were dissected thoroughly [60, 68, 113, 114, 150, 151, 167, 194]. In rice, the molecular responses of rice plants to drought were dissected by different transcriptomic methods [21, 66, 96, 135, 136, 166]. Moreover, drought-responsive genes were identified in many crop and noncrop plants [49, 101, 140, 196].

Drought-responsive genes were classified according to their function into six major groups [48, 122, 167]. The first group is the transcription factors (TFs), which are considered key players in response to drought. The highly drought-responsive TFs are members of the bZIP, AP2/ERF, MYB, NAC, and WRKY gene family [114, 167]. The second group is protein kinases. Hence, this indicates the crucial role of protein phosphorylation in response to drought. The third group is LEA proteins, which protect plants under drought stress. The fourth group is phytohormones. The major drought-responsive plant hormone is ABA, which usually increases under drought stress [18, 31, 70]. Other plant hormones were revealed to have important roles in response to drought such as auxin and jasmonic acid [3, 60, 65]. The fifth group is osmoprotectants such as proline, trehalose, and glycine betaine [178]. Other gene groups were also shown to have roles in response to drought such as genes for lipid transfer proteins, antioxidant enzymes, ligases, protein ubiquitination, and metabolic enzymes [167].

The expression profiling of plants under drought stress revealed a large number of drought-responsive genes. Nonetheless, not all drought-responsive genes result

in drought tolerance [35]. To test which of the drought-responsive genes are drought-tolerance genes, overexpression of the candidate genes are usually generated. Many genes proved to be drought tolerant in Arabidposis and other plants utilizing this technology [174]. The overexpression of *DREB1A* in Arabidopsis resulted in enhanced drought tolerance, but it causes growth retardation [100]. Moreover, the overexpression of the orthologous gene of rice *OsDREB1A* also enhanced drought tolerance in transgenic Arabidopsis plants [39]. The overexpression of *D*1-pyrroline-5-carboxylate synthetase genes (*AtP5CS* from *A. thaliana* or *OsP5CS* from rice) in petunia plants showed a higher accumulation of proline and improved drought tolerance compared to nontransgenic control plants [197]. Another example is the overexpression of *SHINE* (*SHN*) and *HARDY* (*HRD*), which resulted in improved drought tolerance without growth abnormalities [2, 80]; respectively. In maize, the overexpression of *ZmNF-YB2* resulted in drought tolerance in transgenic maize plants [115]. Indeed, there are a plethora of studies showing enhanced drought tolerance upon the overexpression of different drought-responsive genes [49, 167].

14.3 Methods for Identification of Candidate Genes for Drought Tolerance

The most widely used method to identify candidate genes for drought tolerance is microarrays with its different forms: cDNA or oligonucleotide microarray (gene chip or tiling arrays; [19, 190, 151]). The cDNA microarray does not require the previous knowledge of the genome sequence of the plant under test. Indeed, it was proven to be efficient in the identification of drought-responsive genes in many plants [125, 135, 144, 145, 159, 183]. The revolution in genome sequencing led to the improvement of expression profiling and the development of oligonucleotide microarrays. This type of microarray allows the expression profiling of thousands of genes at the same time. In addition, it allows the differentiation between members of gene families [190]. The oligonucleotide microarray was used to identify drought-responsive genes in Arabidopsis, rice, and other plants. The tiling microarray is used to identify differentially expressed DNA sequences at the level of the whole genome [108]. Unlike the gene chip microarray, the probes used in the tiling array represent sequences that are contiguous in the genome region. Hence, this makes this type of microarray more efficient and with higher resolution for the identification of drought-responsive DNA sequences compared with the gene chip microarray. Other techniques such as differential display, cDNA amplified fragment length polymorphism (cDNA-AFLP), cDNA-SSH, and serial analysis of gene expression (SAGE) were also used to identify drought-responsive genes [32, 34, 36, 40, 54, 71, 109, 111, 133, 154, 169]. The most advanced transcriptomic method is the RNA sequencing (RNA-Seq), which was developed to sequence directly the expressed part of the genome under different environmental conditions [185].

Recently, a significant number of studies used this method to reveal the transcription profile of plants under different abiotic stresses including drought [15, 67, 78, 179].

Another approach for the identification of drought-responsive genes is the screening of gain and loss-of-function mutant population under drought stress [46, 62, 73, 91]. In Arabidopsis, activation tagging mutants were generated using T-DNA vectors that contain several copies of 35S promoter from the cauliflower mosaic virus and *En-I* Maize Transposon system [104, 187]. Moreover, a population of loss-of-function mutants was generated in Arabidopsis by T-DNA insertion [5]. About 129,000 T-DNA insertion mutants were generated in rice [210]. A more recent survey study revealed 200,000 mutants available for functional genomics in rice [90]. These mutants were generated by insertional, physical, and chemical mutagenesis methods. In a recent review, a collection of 246,566 insertion mutants in rice was analyzed. The results showed that about 68 % (211,470) of the inserts are in the genic region of the rice genome [184]. Moreover, there is at least one insertion in 60.5 % of the nuclear genes of rice. Mutant resources were also generated in many important crop plants such as barley, tomato, and soybean [10, 16, 22, 110, 158].

14.4 Candidate Genes for Drought Tolerance in the Model Plant *Arabidopsis Thaliana*

A. thaliana is a diploid (2n) flowering plant with 10 chromosomes. Its small size, short generation time, small genome size, and high seed production per generation made it the model plant for genetic and molecular studies [6]. Arabidopsis research was first established by Friedrich Laibach in 1907 [88], but it was not until the mid-seventies of the twentieth century that Arabidopsis was adopted as a model plant [88]. Since then, many biological processes were thoroughly studied in Arabidopsis at different levels. *A. thaliana* was a good model plant for the understanding and the dissection of developmental, physiological, biochemical, and molecular processes in plants. Indeed, the understanding of many biological processes has been largely enriched using this model plant.

Plant interactions with the surrounding environment and its responses to the different biotic and abiotic stresses were also dissected using *A. thaliana* as a model plant [63, 88, 116]. This was largely facilitated by the complete genome sequence of Arabidopsis, the availability of a large number of mutants, and generally the strong genetic and molecular infrastructure.

Many drought-responsive genes were identified in Arabidopsis before the genomic era. *RD22* (responsive to dehydration 22) was induced by drought and salt stress [199]. *RD29* was shown to be induced by drought, salt, and cold stress [200]. *DREB1A* and *DREB2A* that encode DRE (dehydration-responsive elements) binding protein were isolated from Arabidopsis using the yeast-one-hybrid system [100, 155]. The functional analysis of *DREB2A* revealed the important role of this gene in drought tolerance [145]. *RD22BP1* (MYC) and *ATMYB2* were found to activate the

RD22 gene under drought and ABA [1]. Using the yeast-one-hybrid system, two basic leucine zipper (bZIP) transcription factors were shown to bind to the ABA-responsive element. These two factors were called *AREB1* and *AREB2* and they were induced by drought, salt stress, and ABA [176]. *CBF4* was identified in Arabidopsis as a homologue of *CBF/DREB1*, which was only induced by drought. Moreover, transgenic plants overexpressing *CBF4* were drought tolerant [56]. Three drought-responsive NAC transcription factors were identified in Arabidopsis by the yeast-one-hybrid system [170]. *ANAC019*, *ANAC055*, and *ANAC072* were induced by drought stress and the overexpression transgenic plants were drought tolerant [170].

The full-length cDNA microarray for monitoring the expression of 1300 genes under different abiotic stress revealed 44 drought-responsive genes with 30 novel genes [144]. Among these genes LEA, catalases, aquaporins, and *DREB1A* were identified as drought-responsive genes. In another study, a full-length cDNA microarray of 7000 genes showed the upregulation of 277 genes under drought stress [145]. These genes were grouped into genes for regulatory proteins such as transcription factors and kinases and genes for functional proteins such as heat shock, osmoprotectants, and late embryogenesis proteins.

Gene chip microarrays were used to identify drought-responsive genes. The expression profiles of Arabidopsis plants grown in 200 mM mannitol were revealed using a gene chip of probes for 8100 genes [89]. After 3 h of osmotic stress, 279 and 85 genes were differentially expressed in the leaf and root tissue, respectively. Longer exposure to osmotic stress for 27 h resulted in the differential expression of 82 and 63 genes in the leaf and root tissue, respectively. Moreover, the results highlight the differential regulation of gene expression between different tissues and different durations of osmotic stress. Using an oligonucleotide microarray of 26,090 probes to identify differentially expressed genes under progressive drought, 1969 drought-responsive genes were revealed. Among these 923 genes were upregulated and 728 genes were downregulated [65]. In addition, the expression profile of plants after rewatering showed that the expression of most drought-responsive genes returns to its normal level. Using the Affymetrix AHT1 gene chip, the expression profile of Arabidopsis plants under dehydration stress was revealed [85]. The highest number of upregulated genes in the root was after half an hour of dehydration (325), whereas the highest number of downregulated genes was after one hour of dehydration [154]. In the shoot of dehydrated Arabidopsis plants, the highest number of upregulated genes was after 1 and 3 h of treatment, whereas the highest number of downregulated genes was after half an hour of treatment [85]. Among the upregulated genes in the shoot and root of dehydrated plants for half an hour, 68 and 267 genes were drought specific, respectively. In addition, the time course expression profile of the commonly induced genes by drought, salt, and UV stress showed a unique profile of each gene under the different abiotic stresses. The Affymetrix AHT1 gene chip of 21,180 probe sets for 21,019 genes was used to profile the expression of Arabidopsis plants under moderate controlled and progressive drought [60]. The two drought treatments showed different expression profiles. Moreover, the time course expression profile of Arabidopsis plants under

moderate drought showed differential gene expression among the different stages of drought treatment [60].

A tiling microarray was applied to reveal drought-responsive genes at the whole genome level [107, 209]. In Arabidopsis, dehydration shock induced 1188 AGI (Arabidopsis genome initiative) code genes and it suppressed 217 AGI code genes [107]. Moreover, 115 and 497 non-AGI code genes were induced after 2 and 10 h of dehydration, respectively. Dehydration for 2 and 10 h resulted in suppression of 7 and 78 non-AGI code genes [107]. A tiling array of more than 9000 genes not present in the AHT1 gene chip was used to profile the expression of Arabidopsis plants under various abiotic stresses [209]. The expression profiling of Arabidopsis plants exposed to osmotic stress (300 mM mannitol) revealed new, not annotated stress-responsive genes [209]. A total of 376 genes were upregulated after 1 h of osmotic stress. Among these 120 genes were identified as responsive to osmotic stress for the first time.

The screening of 2000 transposon activation tag lines revealed AP2 transcription factor *SHINE* (*SHN*), which was identified as a drought-tolerance gene [2]. The overexpression of this gene resulted in the decrease in stomatal density, which was proposed as a possible mechanism for the drought tolerance shown by the overexpression lines. Another AP2/ERF-like transcription factor *HARDY* (*HRD*) was identified by gain-of-function as a drought-tolerance gene [80]. The gain-of-function mutant of *HRD* was drought tolerant. Moreover, the overexpression of the *HRD* gene in Arabidopsis and rice resulted in a significant improvement of drought tolerance of the transgenic lines [80]. Indeed, the overexpression of many Arabidopsis drought-responsive genes functioning by different mechanisms resulted in improved drought tolerance. Drought-tolerant plants were resulted from the overexpression of *AtWRKR57*, heat shock transcription factor (*HSFA1b*), the voltage-dependent anion channel 2 gene (*AtVDAC2*), and Arabidopsis ARGOS homologue ORGAN SIZE RELATED1 (*AtOSR1*) and *AtOSR2* [13, 74, 149, 188].

Previous studies showed that phosphorylation is crucial in drought signaling [44, 122, 172, 204]. Indeed, many protein kinases were shown to be induced by drought stress. Two genes of Ca^{2+}-dependent protein kinases *ATCDPK1* and *ATCDPK2* were identified from a cDNA library [177]. The two genes were significantly induced by drought stress. The knockout mutant of ABA-Activated SnRK2 Protein Kinase (*SRK2E*) *srk2e* was hypersensitive to dehydration [204]. In addition, *srk2e* plants were not able to close their stomata under dehydration. Hence, they were wilting under dehydration. In addition, the expression of *RD22* and *RD29B* was suppressed in the *srk2e* plants. SNF1-related protein kinase 2 (SnRK2), *SRK2C* is a protein kinase that was shown to play a crucial role in drought tolerance. The knockout mutant of this gene was hypersensitive to drought stress [175]. Moreover, the overexpression of this gene enhanced drought tolerance of Arabidopsis transgenic plants and resulted in the induction of major drought-responsive genes such as *RD29A*, *COR15A*, and *DREB1A_CBF3* [175]. The nonethylene receptor histidine kinases *AHK1*/*ATHK1*, *AHK2*, *AHK3*, and *CRE1* were induced by drought stress [172]. The gain and loss-of-function mutants showed that *AHK1* is a positive regulator of drought response acting upstream of

the drought-responsive genes *AREB1*, *ANAC*, and *DREB2A*. In another study, the loss-of-function and expression profiling analyses of three cytokinin (CK) receptor histidine kinases *AHK2*, *AHK3*, and *AHK4*/*CRE1* showed that they are negative regulators of drought responses [171]. The genetic analysis of the *AtCPK23* gene that codes for Ca^{2+}-dependent protein kinase showed the loss-of function *cpk23* was drought tolerant [103]. This mutant was shown to have a reduced stomatal opening compared with the wild type. The mitogen-activated protein kinase pathways play a crucial role in the signaling process under various environmental stresses [31, 48, 161]. In a very recent study, Arabidopsis Raf-like MAPKKK gene *Raf43* was induced by multiple abiotic and biotic stresses [180]. Moreover, the knockout mutant of this gene was drought sensitive, whereas the *Raf43* complemented mutant showed improved drought tolerance. A newly identified ABA-activated MAPK signaling module (*MAP3K17*/*18-MKK3-MPK1*/*2*/*7*/*14*) was shown to have slow activation under drought stress compared to the rapid activation of the previously identified MAPK modules [17]. This suggests the involvement of this module in the long-term specific responses to drought.

Drought-tolerant Arabidopsis plants were generated by the transformation of drought-responsive genes from different plant species. The overexpression of the vacuolar pyrophosphatase 1 (*EVP1*) from *Eucalyptus globules* in Arabidopsis plants resulted in drought-tolerant transgenic plants and enhanced the formation of root hairs [45]. Drought-tolerant Arabidopsis plants were also generated by the overexpression of the *Capsicum annuum* drought stress-responsive 6 (*CaDSR6*; [86]). The overexpression of cold-acclimation specific protein 31 from *Medicago Truncatula* (*MtCAS31*) in Arabidopsis resulted in reduced stomatal density and drought tolerance [191]. Transgenic Arabidopsis plants overexpressing MAP kinase 1 from maize (*ZmMAPK1*) were drought tolerant [189]. Transgenic Arabidopsis plants overexpressing the drought-induced NAC transcription factor from *Arachis hypogaea* (*AhNAC2*) were drought tolerant and showed increased hypersensitivity to ABA [98]. More than 70 % of the transgenic plants survived severe drought compared with 25 % of the wild-type plants. The overexpression of NAC transcription factor from *Miscanthus lutarioriparius* (*MlNAC5*) in Arabidopsis plants showed enhanced drought tolerance [203]. Transgenic Arabidposis plants overexpressing rice protein phosphatase 2C (*OsPP108*) and maize ARGOS genes (*ZmARGOS1* and *ZmARGOS8*) showed improved drought tolerance ([149, 153], respectively).

14.5 Candidate Genes for Drought Tolerance in Crop Plants

14.5.1 Rice

Rice is the main food for more than half of the world's population [118]. The rice grains contain carbohydrates, lipids, proteins, and minerals [118]. The widely cultivated species of rice is *Oryza sativa*. The water demands of this species are

very high compared with other major crops. Indeed, it is grown under flood irrigation. Drought is threatening all forms of life on earth, and agriculture is the most harmed sector by water scarcity. Indeed, the growth and productivity of a large number of crops were negatively affected by the unpredicted episodes of drought in different areas of the world. Because of its high water demands, rice will be highly affected by water deficit.

Rice (*O. sativa)* species has a total of 24 (2n) chromosomes. Rice was the first sequenced genome crop because of its relatively simple genome compared with the genome of other crop plants. Indeed, rice is considered as a model for monocot plants. Using whole-genome sequencing, a draft sequence of rice genome *O. sativa* L. ssp. *indica* was released in 2002 [206]. The draft showed a genome size of 466 Mb with 46,022–55,615 genes. In 2005, the International Rice Genome Sequencing Project (IRGSP) published a high-quality map-based genome sequence of rice *O. sativa ssp. japonica* cv. Nipponbare (International Rice Genome Sequencing Project [69]). The estimated genome size of the subspecies *japonica* was 389 Mbp and 37,544 genes were identified. About 71 % of these genes showed a putative orthologue in the model plant Arabidopsis. In an updated release of annotation (Release 4.0; January 12, 2006), 24,799 genes were annotated [124]. Using the Roche 454 pyrosequencing platform, the genome sequence of cultivar Nipponbare was updated and revised [82]. The reference genome enabled the identification of 35,681 protein-coding genes. The first genome sequence of the cultivar Nipponbare covers 95 % of the 389 Mbp, whereas the revised genome sequence covers 96.6–97.1 % of the entire Nipponbare rice genome that was estimated to be 384.2–386.5 Mbp. Hence, the revised genome sequence of rice paved the way for better understanding of the molecular basis of many biological processes including responses to abiotic stresses.

A cDNA microarray of about 1700 independent cDNAs was used to identify drought-responsive genes [135]. The results showed 62 genes were induced by drought stress. Moreover, the results revealed a significant similarity in the differentially expressed genes between rice and Arabidopsis, the model plants for monocots and dicots, respectively. Hence, this suggests some similarities in the response mechanisms to drought stress between monocots and dicots [113]. Two rice genotypes (drought tolerant and drought sensitive) were exposed to polyethyleneglycol (PEG 6000)-induced osmotic stress [183]. Then, the expression profile of these two genotypes was analyzed by cDNA microarray. The results showed that 64 and 79 unique ESTs were induced in the drought-tolerant and the drought-sensitive genotypes, respectively. The induced ESTs in the two genotypes were functionally different. The induced genes in the drought-tolerant genotype code for transcription factors and antioxidant enzymes, whereas those in the drought-sensitive genotype code mainly for proteins for cellular degradation. In another study, a 70-mer oligonucleotide microarray covering 36,926 unique genes was used to profile the expression of different organs of rice plants under drought stress [214]. Samples of shoots at the vegetative stage and flag leaves and panicles at the reproductive stage were taken at three time points of dehydration (1, 3, and 5 h). The global expression analysis revealed a higher number of genes were

induced at the three stages of dehydration in the shoot compared with the flag leaves and the panicle. A total number of 1257, 582, and 614 genes were induced in the shoot, flag leaf, and panicle, respectively. The functional analysis of the differentially expressed genes showed that some of them belonged to the common drought-responsive genes such as late embryogenesis abundant proteins, aquaporins, and transcription factors. In addition, new drought-responsive genes were identified. The annotation of these genes revealed genes for transcription factors, heat shock proteins, protein kinases, and photosynthesis enzymes. Drought-tolerant and -sensitive genotypes of upland rice (*O. sativa* L. var. *japonica*) were exposed to controlled soil water deficit [136]. After that, root samples from the control and the treated plants were taken. A total of 127 transcripts was induced in both genotypes. Eighty-four (84) transcripts were only expressed in the drought-tolerant genotype and 71 transcripts were only expressed in the drought-sensitive genotype. Among the genes that were induced only in the drought-tolerant genotype were genes for signaling, metabolic regulation, protection against oxidative stress, and maintenance of cell turgor. This indicates that maintenance of cell turgor and integrity is a mechanism utilized by the drought-tolerant genotype to resist drought. In another study, cDNA-SSH was used to identify candidate genes for drought tolerance in three lines of rice under osmotic stress [43]. A total of 300 subtracted sequences was revealed, which represent a wide range of metabolic activities and functions such as transcription factors, genes for cell communication, and genes for hormonal signaling.

The expression profile of drought-tolerant and -sensitive rice genotypes under long-term severe drought revealed the induction of a total 413 genes and the repression of a total 245 genes [33]. Moreover, many gene groups were differentially expressed in the drought-tolerant and -sensitive genotypes. A higher number of photosynthetic genes were downregulated in the drought-tolerant genotype than the drought-sensitive genotype. In addition, two *CYP86A2* genes were upregulated in the drought-tolerant genotype, which plays a role in the metabolism of wax and cutin. Expression profile under drought stress of two indica rice genotypes of contrasting drought response (drought-tolerant and drought-sensitive) was revealed using the Affymetrix GeneChip [96]. The expression profile of the drought-tolerant genotype showed the induction of many important enzymes and the activation of α-linolenic acid metabolism. Most of the downregulated genes in the drought-sensitive genotype were genes that result in drought tolerance. The expression analysis of the five DREB2-type rice genes under drought stress showed that *OsDREB2B* has the highest induction compared with the other four genes [106]. The global expression profile of rice plants under drought stress was revealed using the Affymetrix rice genome array containing 48,564 japonica and 1260 indica sequences [181]. Drought was imposed at three developmental stages: 4-tiller stage, panicle elongation stage, and booting stage. Samples from leaf, root, and panicle were used in the expression analysis. A total of 5284 drought-responsive genes was identified, which makes only 10 % of the total transcripts on the microarray. Moreover, quantitative and qualitative differences in the differentially expressed genes were shown among the different tissues and developmental stages. For

example, photosynthetic-related genes were downregulated in the leaf tissue. Most of the induced transcription factors were shown at the panicle elongation stage, which indicates the importance of the regulation of gene expression at this stage. The global transcription profile was analyzed in the elongation zone of leaves of drought-tolerant and -sensitive genotypes using the Affymetrix rice genome array (GEO #GPL2025; [21]). Under mild drought, genes for secondary wall formation were upregulated in the drought-sensitive genotype and they were downregulated in the drought-tolerant genotype. In general, drought responses in rice include genes for regulatory proteins such as transcription factors and kinases and genes for functional proteins and chemicals such as osmoprotectants synthesizing enzymes and aquaporins [58]. Moreover, an important role of phytohormones in the drought response of rice plants was shown [58].

A large number of candidate genes for drought tolerance were used to modify rice plants genetically [167]. In most cases the results were very promising regarding the generation of drought-tolerant rice lines. A few studies tested drought tolerance of transgenic rice plants under drought conditions in the field and showed increased yield and productivity of these plants [72, 138, 192, 193, 205, 207]. Rice transformation with drought-responsive transcription factors such as *OsbZIP71*, *AtABF3*, *OsERF4a*, *AtCBF3/DREB1A*, *OsMYB2*, *OsNAC5*, and *Cdt-NF-YC1* resulted in the improvement of drought tolerance of the transgenic lines [27, 72, 75, 99, 119, 192, 202]. Moreover, rice plants were transformed with different kinases such as *OsSIK1*, *OsSIK2*, *AtNPK1*, *OsCPK4*, and *OsCPK9* [23, 26, 123, 186, 192]. The kinase transgenic plants showed enhanced drought tolerance. Genes for phytohormone synthesis and signaling, LEA proteins, heat shock proteins, antioxidant enzymes, and different metabolic enzymes were successfully used in rice transformation for drought tolerance [38, 76, 86, 130, 139, 182, 193, 211, 217]. Three DREB genes, *OsDREB1E*, *OsDREB1G*, and *OsDREB2B*, were isolated from rice plants [25]. These genes were shown to bind to the C-repeat/DRE element by the yeast-one-hybrid assay. The overexpression of *OsDREB1G* and *OsDREB2B* significantly improved drought tolerance of the transgenic rice plants. After 15 days of progressive drought and 10 days of rewatering, more than 80 % of overexpression plants of *OsDREB1G* and *OsDREB2B* survived.

Some genes were found to regulate rice responses to drought negatively. The loss-of-function of cytochrome P450, *Osdss1* showed delayed germination and early growth. In addition, *Osdss1* plants were drought tolerant with a high accumulation of ABA and reduction in gibberellins [160]. Another example is the MADS box transcription factor *OsMADS26*, which was shown to regulate the response of rice plants to drought stress negatively [83].

Recently, the role of nitric oxide in response to abiotic stresses has been studied [152]. Indeed, a rat nitric oxide synthase (*nNOS*) was overexpressed in rice plants [20]. The transgenic lines had a higher concentration of nitric oxide (NO), accumulated less reactive oxygen species, and had higher activities of antioxidant enzymes compared with the wild type. A number of stress marker genes such as *OsDREB2A*, *OsDREB2B*, *OsSNAC1*, *OsSNAC2*, *OsLEA3*, and *OsRD29A* were expressed at a higher level in the *nNOS* overexpression rice plants compared with

the wild type. The transgenic plants significantly tolerate osmotic stress and soil water deficit.

14.5.2 Maize

Maize (*Zea mays*) is an important C_4 crop plant, which is negatively affected by drought stress especially at the reproductive stage. Maize is a segmental allotetraploid (2n = 2x = 20) of a genome size of 2.4 GB [57, 84, 105]. An improved draft sequence of the maize genome was published in 2009 [143]. Since then, maize had a good genetic and molecular infrastructure, which significantly facilitated the genetic and molecular dissection of maize responses to different abiotic stresses including drought.

Differentially expressed genes in maize seedlings under osmotic stress (20 % PEG 6000) were identified by cDNA-SSH followed by cDNA macroarray [213]. The results showed differential gene expression between the leaves and roots of maize plants under osmotic stress. The differentially expressed genes in maize kernels 15 days postpollination under drought stress were identified using a macroarray of 2500 cDNAs [8]. A total of 25 transcripts was upregulated and 80 transcripts were downregulated. The expression profile of different regions of maize root was revealed by the construction of cDNA libraries from the well-watered and drought-treated plants [132]. Differences in the spatial gene expression of maize root were shown. Using an oligonucleotide microarray of 57,452 transcripts representing more than 30,000 maize genes, the expression profile of the immature ear and tassel under drought stress was revealed [216]. The expression profile was organ-specific, meaning more genes (1513) were differentially expressed in the tassel compared with the ear [193]. Moreover, most of the sugar metabolism genes were only upregulated in the tassel. This highlights one of the mechanisms of drought response in the reproductive tissues of maize. The expression profile of maize plants exposed to controlled drought was identified after 1 and 7 days of treatment using a microarray of 57,452 maize 70-mer oligonucleotides representing about 30,000 genes [208]. The results revealed a lower number of genes (102) were induced at the early stage of drought compared with the late stage of drought (332 genes). In addition, the functional analysis of the induced genes showed that most of the early induced genes were mainly signaling genes, whereas the late induced genes were involved in osmolyte metabolism. The expression profile of the ears of a fungal-resistant maize line exposed to drought stress at day 18 after pollination was identified using a maize microarray with 58 K oligonucleotide probes [102]. The results showed the highest number of differentially expressed genes was at day 35 after pollination. A total of 294 genes was upregulated and 341 genes were downregulated at day 35 after pollination. About 30 % of the upregulated genes and 26 % of the downregulated genes were of known function. Genes for antioxidant enzymes, biosynthesis, and signaling of plant hormones, heat shock proteins, and dehydrins were among the upregulated genes. Among the downregulated genes

were genes for metabolism and defense. Drought-responsive genes were identified in three-leaf seedlings of drought-tolerant and drought-sensitive inbred lines of maize using Affymetrix *Zea mays* genome GeneChip with 17,555 probe sets representing 14,850 genes [212]. The results showed differential responses of the two lines at the transcriptional level. More genes were differentially expressed in the drought-sensitive line than the drought-tolerant line. Moreover, many of the ABA signaling pathways were upregulated in both maize lines. A number of cell-wall—related genes were highly downregulated in the drought-sensitive line. This suggests that normal biosynthesis of the cell wall is one mechanism of drought tolerance.

The expression profile of newly pollinated ovary and basal leaf meristem tissue of maize plants exposed to progressive drought was revealed using RNA-Seq technology [78]. The study showed differential expression of genes between the two tissues. For example, cell division and cell cycle genes were downregulated in the ovary, but not in the leaf meristem. Moreover, ABA-related genes were upregulated to a higher level in the ovary compared with the leaf meristem. The study highlights the increased drought sensitivity of the reproductive tissue compared with that of the vegetative tissue. A deep Solexa/Illumina sequencing was used to identify osmotic responsive genes in maize seedlings grown in 20 % PEG 6000 [147]. A total of 558 and 462 genes was upregulated and downregulated, respectively. Among the upregulated genes were genes for different groups of transcription factors such as NAC, DREB, MYB, and bZIP, late embryogenesis proteins (LEA), and heat shock proteins. Genes for the biosynthesis of gibberellic acid (GA) was downregulated. This indicates that reduction of GA in drought-treated plants is one mechanism to tolerate drought stress.

Drought-responsive genes in the vegetative and reproductive tissues from two developmental stages of maize plants under progressive drought were identified by 454 GS FLX pyro sequencing [12]. More genes were responsive to drought stress in the reproductive tissue (1284) than in the vegetative tissue (915). In addition, the drought-induced genes at the vegetative tissue were mainly for cellular homeostasis, whereas those induced at the reproductive stage were for sugar metabolism. Candidate genes for drought tolerance in maize lines with contrasting drought tolerance were identified by a data mining approach [195]. The genome of the reference maize line B73 and 15 inbred lines were resequenced to detect SNPs, and then common variant analysis was applied to detect SNPs and their associated genes for drought tolerance. After that, the involvement of the predicted genes in drought tolerance was validated by RNA-sequencing. The results showed 262 out of 271 identified genes had differential expression in response to drought stress. Both quantitative and qualitative differential expression was shown among the different maize lines and among the different maize tissues (root, leaf, and ovary).

In a recent study, the expression profile of an insect-resistant genetically modified maize variety (Bt corn) under drought stress was compared with that of a nontransgenic variety [52]. Quantitatively, the number of genes that were either induced or repressed by drought stress was higher in the nontransgenic variety than in the transgenic one. About 153 and 49 genes were induced in the nontransgenic

and transgenic variety, respectively. Transcription factors were among the highly represented induced genes in the nontransgenic variety, whereas most of the induced genes in the transgenic variety were of unknown function. In addition, in the transgenic variety there were no induced genes for carbohydrate metabolism, which was represented by a large number of the induced genes in the nontransgenic variety.

14.5.3 Soybean

Soybean (*Glycine max*) is a legume crop that is important as a protein and oil source. In addition, as do other legumes it fixes nitrogen through the symbiotic interaction with soil nitrogen-fixing bacteria. The genome size of soybean is 1.1 GB. The genome was sequenced and about 46,430 protein-coding genes were predicted [142]. Since then, it became the model for legumes to dissect the molecular and genetic basis of the symbiotic interactions with nitrogen-fixing bacteria.

A DREB transcription factor (*GmDREB1*) was identified in soybean and its expression profile under dehydration stress was studied in drought-tolerant and drought-sensitive genotypes [156]. In general, the gene was induced in both genotypes, but it was highly induced in the roots of the drought-tolerant genotype. The expression profile of putative 83 two-component systems (TCS) was analyzed by quantitative realtime PCR in soybean plants under dehydration stress for different time periods [93]. More dehydration-responsive TCS genes were in the shoot than in the root. Moreover, the number of repressed TCS genes was higher than the induced genes. The results point to the crucial role of the two-component system in the signaling and response to drought stress. In a recent study, the expression of 26 drought-responsive TCS genes was quantified in the shoot and root of drought-tolerant and drought-sensitive genotypes [165]. *GmHK07*, *16*, *GmHP08*, *GmRR04*, *16*, *32*, *34*, *GmPRR39*, and *44* showed high induction in the drought-tolerant genotype. Therefore, these genes are promising candidate genes for drought tolerance. Using the 66 K Affymetrix Soybean Array GeneChip, the genomewide expression profile of soybean plants under progressive drought at the vegetative and reproductive stage was revealed [95]. At the vegetative stage, 846 genes were induced, and 1206 genes were induced at the reproductive stage. Among the induced genes, genes for signaling were more at the reproductive stage than the vegetative stage. At both developmental stages, the repressed genes were enriched for photosynthesis-related genes. Using high-throughput Illumina sequencing, a total of 535 and 1690 genes were upregulated in the leaves and roots of soybean plants exposed to osmotic stress (2 % PEG 8000), respectively [41]. Dehydration-responsive genes in the roots of soybean plants were identified by the generation of DeepSuperSAGE expression analysis [42]. The change in the expression of 38 *GmNAC* genes in soybean seedlings under dehydration was

quantified [94]. Twenty-five (25) *GmNAC* genes were induced in the shoot and root of soybean plants. Among these genes *GmNAC085* showed the highest induction of about 390-fold in the shoot and 20-fold in the root. Drought-tolerant and drought-sensitive soybean plants were exposed to dehydration stress at the vegetative stage and then their expression profiles were revealed by the construction of cDNA from leaves and roots followed by Illumina/Solexa sequencing [26]. Quantitative and qualitative differences in the differentially expressed genes were shown between the two soybean genotypes. In addition, differences were also shown between the expression profile of the leaf and the root. The functions of the differentially expressed genes in the drought-tolerant genotype were enriched for transcription factors, protein kinases, hormone-related genes, and others. Hence, these genes need further dissection for the optimum utilization in the improvement of drought-tolerant soybean plants.

The expression of 23 *GmNAC* in the roots of soybean genotypes of different drought tolerance exposed to progressive drought was quantified by realtime PCR [162]. *GmNAC085*, *092*, *095*, *101*, and *109* showed higher drought induction in the tolerant soybean genotype than the sensitive genotype. Genomewide expression of HD-Zip genes in soybean plants under dehydration stress was determined from the microarray data from the Gene Expression Omnibus (GEO) database at the National Center for Biotechnology Information [29]. Fifty-nine (59) genes out of 88 HD-Zip genes were responsive to dehydration stress. Then, 4-week old soybean plants (genotype Williams 82) were exposed to dehydration and the expression of 12 HD-Zip genes was quantified in the leaves by realtime PCR. Of these genes, 10 were significantly induced after different time periods of dehydration. These genes are *GmHDZ 2*, *9*, *19*, *27*, *32*, *38*, *51*, *72*, *75*, and *83* and they belong to HD-Zip I. In another study, soybean plants (genotype Williams 82) were exposed to dehydration at the first trifoliate stage (V1) and after 0, 1, 6, and 12 h root samples were collected [14]. After that, the expression profiles of members of the homeodomain leucine zipper (HD-Zip) transcription factor family were revealed by RNA-Seq. *GmHDZ 2* and *75* were induced as was found in the leaf tissue by Chen et al. [29]. *GmHDZ 20*, *28*, and *72* were repressed by dehydration stress. The results of the two studies revealed candidate genes from the HD-Zip transcription factor gene family, which could be promising candidate genes for drought tolerance. Using the 66 K Affymetrix Soybean Array GeneChip, the expression profile of the roots of drought-sensitive and drought-tolerant soybean genotypes under short-term and long-term dehydration stress was identified [55]. The results showed the induction of more genes in the drought-tolerant genotype in response to the short-term dehydration than the drought-sensitive genotype. Signaling genes for kinases, phosphatases, and transcription factors and genes for functional proteins such as proteins for the biosynthesis of osmoprotectants were differentially expressed in the two soybean genotypes.

14.5.4 Tomato

Tomato (*Solanum lycopersicum*) is considered a model plant for fruit development. The tomato plant is from the Solanaceae family, which has high water demands. Indeed, drought stress resulted in the reduction in growth and yield of tomato plants. It has a genome of 950 MB and 24 chromosomes (2n) [129]. A draft sequence of 760 MB of tomato genome was published in 2012 [164]. Moreover, the genome sequencing of the closest wild relative of tomato *Solanum pimpinellifolium* (739 MB sequenced) showed a divergence percentage of 0.6 % between the genome of the two tomato species [164]. A comparative study of the transcriptomes of five wild tomato species and one domesticated species, *S. lycopersicum*, revealed differential gene expression between the wild and domesticated tomato species due to both natural and artificial selection [87]. Indeed, the wild species are considered good reservoirs of genes for adaptation to many environmental stresses including drought.

Drought and ABA-induced cDNA clones were identified in wild tomato *Solanum chilense* (*Lycopersicon chilense*) exposed to drought for four days [28]. Two drought-tolerant *Solanum pimpinellifolium* genotypes and one drought-sensitive *S. lycopersicum* genotype were exposed to progressive drought at the 6-leaf stage [49]. Then, the expression profile of the three genotypes was determined using TOM2 microarrays. In general, more genes were differentially expressed in the drought-sensitive genotype. A number of 386 and 388 genes were only up- and down-regulated in the drought-sensitive genotype, respectively. In the three genotypes, 82 genes for transcription factors and 50 signaling-related genes were responsive to drought stress. Genes that were specifically responsive to drought stress in the drought-tolerant genotypes were players in the biochemical pathways of energy conservation and protection against the detrimental effects of drought stress such as the accumulation of reactive oxygen species. These genes are good candidate genes for drought tolerance in tomato. Hence, extensive functional dissection of these genes will help in the enhancement of drought tolerance in tomato. Important genes in ABA signaling of *SlPYL* (ABA receptor), *SlPP2C* (type 2C protein phosphatase), and *SlSnRK2* groups were identified and their expression in tomato leaves under progressive drought was tested [157]. Six *SlPYL* genes were downregulated and *SlPYL1* and *3* were upregulated in dehydrated leaf tissue. All the tested *SlPP2C* genes were upregulated except *SlPP2C6*. Five *SlSnRK2* genes were upregulated: *SlSnRK2.2, SlSnRK2.3*, *SlSnRK2.4, SlSnRK2.6*, and *SlSnRK2C*. Differences in the proteomes of drought-sensitive (*S. lycopersicum*) and drought-tolerant (*S. chilense*) tomato species under dehydration stress were shown [215]. The induced proteins in the drought-tolerant tomato species were highly represented by proteins for posttranscriptional regulation. The genes of these proteins might be of high importance to harness the key players in drought tolerance of wild species of tomato. A set of eight highly drought-responsive genes was selected from previous microarray data, and their expression in drought-tolerant and drought-sensitive tomato genotypes under progressive drought was determined by

semi-quantitative and quantitative realtime PCR [51]. The drought-tolerant genotype was from *Solanum habrochaites* and the drought-sensitive genotype was from *S. lycopersicum*. *SlEFH12* and *SlSNF4-15* were only induced in the drought-tolerant genotype. The functional analysis of *SlEFH12* and *SlSNF4-15* showed that they code for calcium-binding EF hand protein and SNF4-kinase, respectively. Moreover, a transcription factor from the WRKY family *SlWRKY4* showed higher repression in the drought-tolerant genotype. This indicates the highly active signaling processes in the drought-tolerant tomato genotype under drought stress.

Le16 (*Lycopersicon esculentum protein 16*; *Solyc10g075090*) that codes for phospholipid transfer protein was identified in tomato plants [131]. This gene was highly induced by dehydration shock and osmotic stress. Since then, this gene has been considered as a marker for drought stress. The expression of four previously identified drought-responsive genes in *S. lycopersicum Le4*, *Le16*, *Le20*, and *Le25* was determined in the drought-tolerant species *Lycopersicon pennellii* under progressive drought and dehydration shock [77]. *Le16*, *Le20*, and *Le25* were induced. Two AREB/ABF family genes *SlAREB1* and *SlAREB2* were functionally analyzed under drought stress [120]. Both genes were induced by dehydration stress, but *SlAREB1* showed higher induction than *SlAREB2*. Therefore, overexpression and loss-of-function lines were generated. The physiological, biochemical, and molecular responses of the two transgenic lines and the wild type to progressive drought were analyzed. The overexpression lines of *SlAREB1* showed enhanced physiological and biochemical changes under drought stress. Moreover, the transcriptome analysis of the overexpression line under drought stress revealed the upregulation of genes for lipid transfer, antioxidants, enzymes, transcription factors, and late abundant embryogenesis proteins. A cDNA clone coding for an orthologous gene to *Arabidopsis WIN/SHN1* was isolated and named *SlSHN1* [4]. The gene was induced by drought stress and its overexpression resulted in enhanced drought tolerance. Moreover, genes of wax and cutin biosynthesis were upregulated in the *SlSHN1* overexpression lines. Tomato PYR/PYL/RCAR abscisic acid receptors were identified and three of them, Sl06g050500, Sl03g007310, and Sl08g076960, were overexpressed in Arabidopsis plants [50]. The overexpression lines of Sl06g050500 and Sl03g007310 showed enhanced drought tolerance in terms of higher sensitivity to ABA and survival after 20 days of progressive drought. Hence, these genes could be used to improve drought tolerance in tomato and other crop plants. A semi-quantitative expression analysis showed the induction of *SlAREB*, *SlNCED3*, and *SlERF024* in drought-sensitive and drought-tolerant tomato genotypes of *S. lycopersicum* under osmotic stress [9].

14.5.5 Barley

Barley (*Hordeum vulgare* L.) is an important crop, and is the fourth crop produced among the cereal plants [37]. It has high adaptation to different environments, and it

is cultivated in relatively dry areas. Barley is a diploid plant with 14 chromosomes (2n = 2x = 14) and a haploid genome size of about 5.1 GB [163]. About 1.7 Gb of the genomic sequence containing 17,386 annotated barley genes was generated by the sequencing of 15,622 BACs [112].

A cDNA microarray was used to identify the expression profile of the leaves and roots of barley plants under dehydration shock [125]. The results showed differential expression between the leaf and shoot tissues. Moreover, the upregulated transcripts were for ABA-responsive proteins, antioxidants, enzymes, and late embryogeneis proteins. The most represented functional category for the downregulated genes was photosynthesis-related proteins. The expression profile of barley plants under osmotic stress (20 % PEG 6000) was revealed using a cDNA microarray [173]. Twenty-two (22) expressed sequence tags (ESTs) were upregulated and 30 were downregulated. The upregulated ESTs were for genes for carbon and nitrogen metabolism, whereas the downregulated ESTs were for sugar transporters, heat shock proteins, and kinases. A comparative expression profiling was done on barley plants under dehydration shock and progressive drought using a cDNA microarray [159]. A higher number of genes (57 %) were differentially expressed in the dehydrated barley plants. Only 10 % of the differentially expressed genes were common in the two drought treatments. The common induced genes showed a higher transcription level under dehydration compared to progressive drought. This suggests acclimation occurs in response to progressive drought, but not in response to dehydration shock. Therefore, more attention should be paid to which drought treatment simulates drought in the field, which normally leads to acclimation. The Affymetrix Barley1 microarray with probe sets that represents 21,439 genes was used to identify the expression profile in barley plants under gradual progressive drought [168]. A total of 2207 genes was upregulated and 1497 were downregulated by progressive drought. Moreover, the results revealed an increase in the number of differentially expressed genes as the soil water content decreased. The functions of drought-responsive genes were signaling, regulation of transcription, protection of membranes and proteins, ABA biosynthesis, and water and ion uptake.

Transcriptome analysis of drought-tolerant and drought-sensitive barley genotypes under progressive drought at the reproductive stage was done using the 22 K Affymetrix Barley 1 microarray [53]. A total of 17 genes was upregulated specifically in the drought-tolerant genotypes. The functional analysis of these genes revealed the induction of genes for stomatal closure via carbon metabolism, the biosynthesis of osmoprotectants, antioxidant machinery, and genes for protein and membrane stability. This indicates the mechanism(s) for drought tolerance used by the drought-tolerant genotypes. RNA-seq was used to identify the expression profile of genotypes of wild barley collected from different habitats in the southwestern part of the Fertile Crescent [67]. These plants were exposed to drought at the reproductive stage, and samples of spikelets were collected at the end of drought treatment. The results showed similar functional processes resulted in drought tolerance and reproductive success in the drought-tolerant barley genotypes collected from different habitats in the Fertile Crescent.

The expression of three dehydrin genes *Dhn6*, *Dhn11*, and *Dhn13* were quantified by realtime PCR in drought-tolerant and drought-sensitive barley genotypes under dehydration shock [134]. The three genes showed specific expression profiles after different time periods of dehydration. For example, *Dnh6* was highly induced in the drought-tolerant genotypes after 8 h of dehydration, then after 12 h a similar expression level was shown in two drought-tolerant genotypes and one drought-sensitive genotype. In a recent study, the expression of 13 dehydrin genes was quantified in drought-tolerant and drought-sensitive barley genotypes under terminal drought (40 % field capacity (FC); [81]). Five dehdyrin genes (*Dhn1*, *Dhn3*, *Dhn5*, *Dhn7*, and *Dhn9*) were specifically upregulated in the drought-tolerant genotype. In another study, the expression of *Dhn1* and *Dhn9* was semi-quantified in drought-tolerant and drought-sensitive barley genotypes under controlled severe drought (25 % FC; [61]). *Dhn1* was induced in the drought-tolerant genotype after 2 days of drought treatment.

The expression of genes for antioxidant enzymes *CAT2*, *SOD*, and *APX* was semi-quantified in drought-tolerant and drought-sensitive barley genotypes under controlled severe drought (25 % FC) [59]. In general, the three genes were induced after 2 days of drought treatment in the drought-sensitive genotype. *CAT2* was induced after 9 days of drought treatment in the drought-tolerant genotype, whereas *SOD* and *APX* were induced after 16 days of drought treatment.

14.6 Conclusion

The persistence of drought in many areas of the world makes the improvement of drought tolerance in many crops a critical need, which becomes more feasible in the postgenomic era. The quantum leap in biotechnologies for sequencing and molecular analyses facilitates the dissection of the molecular responses to abiotic stresses including drought. Indeed, a plethora of genomic, transcriptomic, proteomic, and metabolomic data about the responses to drought stress are accumulating. These overwhelming data need integrated efforts for the optimum utilization of such data in the improvement of drought-tolerant crops. Recently, an integrated drought database (DroughtDB) was generated to show confirmed drought-tolerance genes from different plant species [7]. The database contains drought-tolerance genes from Arabidopsis, rice, sorghum, maize, brachypodium, tomato, barley, *Aegilops tauschii* (wild relative of wheat), and rye. A cross-species meta-analysis (CSA) using microarray data of progressive drought stress at the reproductive stage of different plant species (Arabidopsis, rice, wheat, and barley) was developed [146]. The analysis revealed 225 differentially expressed genes were common in all analyzed data from the different plants. The functional analysis of these genes showed different conserved functional groups such as carbohydrate metabolism, protein degradation, transcription, and response to stimulus. These functional groups underlie conserved adaptation mechanisms to drought stress in flowering plants. The results of these two studies are valuable because of the comprehensive

and integrated methods of analysis of the scattered drought transcriptome data. Indeed, the results of these studies are highly informative and will significantly help in the improvement of drought-tolerant crops.

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Chapter 15
Analyses of Drought-Tolerance Mechanism of Rice Based on the Transcriptome and Gene Ontology Data

Ali Moumeni and Shoshi Kikuchi

15.1 Introduction

Rice (*Oryza sativa* L.) is one of the most staple food crops for about 50 % of the world's growing population [25]. It provides more than 20 % of the calories consumed by this population worldwide and it is estimated that about 800 million tons of rice will be required to feed the people in 2025 [37]. On the other hand, climate change and water availability are two important factors affecting rice production in rice-growing areas in the world [79]. Global warming and climate change intensify the occurrence and severity of abiotic stresses that seriously affect the growth and development of plants, including the rice crop [48]. Rice is grown in a very diverse condition that ranges from flooded wetland to rainfed dryland [17]. Drought is one of the major environmental constraints, negatively affecting growth and productivity of rice (*O. sativa* L.) and its grain yield potential [25], especially lowland-adapted rice genotypes which are known to be very sensitive to the soil water deficit (SWD) and evaporative demand [49]. Hence, improvement of rice for drought tolerance (DT) requires an understanding of the underlying physiological

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mechanisms and the genetic controls of contributing traits to drought [7]. The mechanisms of response to water-deficit (WD) stress can be studied at the molecular level and at the whole-plant level.

Efforts to identify genes and mechanisms related to drought stress tolerance in rice plants has a long history of employing diverse strategies from quantitative methods to the molecular level, such as microarray analysis and next-generation RNA sequencing, at the field and controlled conditions [46]. To achieve the targets, the employed strategies must be linked with suitable phenotyping protocols at all stages, such as the screening of germplasm collections, mutant libraries, mapping populations, transgenic lines and breeding materials, and the design of omics and quantitative trait loci (QTLs) experiments [54]. The traditional methods of assessment of gene expression levels induced by drought stress rely on measuring a small number of genes, whereas microarray technology is a high-throughput tool that can display changes in expression profiles of a large number of genes at the same time [34].

Genomewide expression technology such as a DNA microarray generates vast amounts of biological data that involve a huge range of biology-oriented information. Biologists currently spend a lot of time searching for all of the available information about each small area of research [3]. This has been made more difficult by the wide variations in terminology that may be common usage at any given time, which inhibit effective searching by both computers and people. Therefore, these huge sets of information need to be managed in the best ways that could be useful to molecular biologists as well as those who can use the information in their studies [13]. To make these biological data useful, the Gene Ontology (GO) tool provides ontologies that explain gene products in three distinct domains of molecular biology. The three nonoverlapping domains are: molecular function (MF) which describes activities, such as catalytic or binding activities, that occur at the molecular level; cellular component (CC) which explains locations, at the levels of subcellular structures and macromolecular complexes; and finally biological process (BP) which depicts biological goals performed by one or more ordered assemblies of MFs [22]. Therefore, different aspects of drought-tolerance analysis in rice through microarray technology and GO tool are reviewed in this chapter.

15.2 Principles of Microarray Technology, Gene Expression Profiling, and Drought Stress

As plants have developed a varied range of cellular adaptive mechanisms, it is difficult or even may not be possible to study microchanges at the molecular level and determine the expression profile of a large number of genes through traditional methods [34]. The term microarray was first introduced in 1995 [58]. In the past decades, development of microarray technology caused an enormous revolution in

biological research [50]. The microarray is one of the suitable technologies that enable researchers to identify the expression of thousands of genes which were thought to be nontraceable. One can analyze the expression of many genes in a single reaction quickly and in an effective way. This technology also enabled biologists to uncover the major aspects underlining the growth and development of plants [15].

15.2.1 Principles of Microarray Technology

A DNA microarray contains thousands of nucleic acid probes that are chemically bound to a substrate, which can be a microchip, a glass slide, or a microsphere-sized bead. Many DNA samples are used to construct an array. Microarrays provide the scanning of the expression of thousands of genes at the same time in a single experiment based on two main principles of nucleic acid hybridization: DNA and RNA specifically interact with the related complementary sequence attached to the array surface; and later, this hybridization happens in proportion to the amount of mRNA in the mixture [4]. All the data are collected and a profile is generated for gene expression in the cell.

There are several types of array and the broadest distinction is whether it is spatially arranged on a surface or on coded beads: The traditional solid-phase array is a collection of orderly microscopic "spots", called features, each with a specific probe attached to a solid surface, such as glass, plastic, or a silicon biochip commonly known as a gene chip, genome chip, DNA chip, or gene array. Thousands of them can be placed in known locations on a single DNA microarray. The alternative bead array is a collection of microscopic polystyrene beads, each with a specific probe and a ratio of two or more dyes, which do not interfere with the fluorescent dyes used on the target sequence [50]. The range of applications for microarray technology is huge. However, there are two typical applications of microarrays that are in widespread use: gene expression profiling to measure the expression of genes between different cell populations, and comparative genomics to analyze genomic alterations such as sequence and single nucleotide polymorphisms.

15.2.2 Gene Expression Profiling and Drought Stress

DNA microarrays can be used to detect DNA, or detect RNA that may or may not be translated into proteins. The process of measuring gene expression via cDNA is called expression analysis or expression profiling. In a gene expression profiling

experiment, the expression levels of the vast array of genes are simultaneously monitored to investigate the effects of specific treatments, diseases, and developmental stages on gene expression. Because any modifications in growth and development are often connected with fundamental changes in gene expression, it is possible to estimate changes at the molecular level [34]. For example, microarray-based gene expression profiling can be used to identify genes whose expression is changed in response to drought stress by comparing gene expression in stressed plants to that in nonstressed plants [2]. Abiotic stresses such as drought trigger a wide range of responses in the plant, from changes in patterns of gene expression and cellular metabolism to changes in growth and yield [52]. The genomewide identification of the genes regulated by drought allows for a more detailed understanding of the transcriptional response to stress and provides a starting point for the further elucidation of the role of individual genes in the stress response.

There are several major components that are fundamental to detect changes in gene expression profiles of rice plant in response to drought stress, such as: (a) plant materials and developmental stages, (b) experimental conditions, and (c) gene expression technologies.

15.2.2.1 Plant Materials and Developmental Stages

To detect gene expression changes in rice plant under drought stress, most researchers focused on using the heterogeneous genetic backgrounds of tolerant and susceptible germplasm at the seedling stage. Using these germplasms obscures the relationship between genetic variation and drought-tolerance phenotypes [29, 46]. A more desirable approach is to use genetic stocks with a common genetic background, but contrasting levels of tolerance to drought stress. One promising approach is to use near-isogenic lines (NILs) with similar genetic backgrounds, but contrasting levels of tolerance to WDs. NILs are invaluable for testing hypotheses in physiological and genetic studies without any interference from variation in other traits [38]. The reproductive stage is very important because (1) rice is highly sensitive to water deficit stress at the reproductive stage, (2) floral fertility is extremely sensitive to water stress, (3) the occurrence of drought at the reproductive stage is more frequent in rainfed drought-prone areas, and (4) the yield loss at the reproductive stage drought is more severe than drought at the seedling or vegetative stage [47]. Recently, several studies were reported in the case of using pairs of NILs in the background of IR64 with contrasting DT at the reproductive stage, including: (a) the IR77298-14-1-2-B family: IR77298-14-1-2-B-10 that is highly drought tolerant versus IR77298-14-1-2-B-13 a susceptible line, and (b) the IR77298-5-6-B family including IR77298-5-6-B-18, which is moderately drought tolerant; and IR77298-5-6-B-11, which is highly susceptible [46, 65, 67]. Despite their common genetic background ($\sim$97 %) as backcross progeny from Aday Sel $\times$ IR64, the two pairs of NILs show distinctive differences in their gene expression profiles in response to drought stress [67].

15.2.2.2 Experimental Conditions

Designing a suitable experimental condition is critical in collecting reliable data for testing of rice genotypes against drought stress. Various experimental procedures from field tests [1, 9, 19, 24, 67], to fully controlled conditions in the growth chamber can be applied in this regard. In the majority of experiments, a rapid drought stress will be imposed through growing plant materials in polyethylene glycol (PEG) solution [31, 33, 42], whereas, in an experimental condition that is similar to a field stress situation, the environmental factors that affect experimental conditions are being controlled in a greenhouse under controlled conditions. Plant materials are grown in polyvinyl chloride (PVC) pipe columns measuring 1.05 m and diameter 18 cm filled with soil mix (2 soil: 1 sand) that is adequately fertilized (plants initially grown in the greenhouse but shifted to phytotron before imposing the stress) as described [61]. In this method of evaluation saturated soils in the pots were covered with white plastic covers, with an opening in the middle to facilitate planting. A feeder pipe was inserted for watering the pots. Few pregerminated seeds were transplanted per pot and later thinned to 2 plants at the three-leaf stage. The experimental design was carried out in a randomized complete block design (RCBD) with four replications [46, 67]. Due to the biological complexity of gene expression, the considerations of experimental design are of critical importance if statistically and biologically valid conclusions are to be drawn from the data. The WD regimes were imposed through a drydown method [61] starting 35 DAS to provide long-term drought stress, approximating the field conditions at the reproductive phase wherein grain yield was drastically decreased, until the pot reached the targeted fraction of transpirable soil water (FTSW). The pots were weighed daily during the drydown to estimate the transpiration. Pots were maintained at targeted FTSW until harvest.

15.2.2.3 Gene Expression Analysis and Data Management

The two recent complementary advances such as one in knowledge and another in technology, are largely speeding up the study of gene expression and the discovery of the roles played by specific genes in the stress tolerance including WD [4]. There are three basic types of samples that can be used to construct DNA microarrays: two are genomic and the other is transcriptomic; that is, it measures mRNA levels. What makes them different from each other is the kind of immobilized DNA used to generate the array and, ultimately, the kind of information that is derived from the chip. The target DNA used will also determine the type of control and sample DNA that is used in the hybridization solution. To determine gene expression profiling many reports are available [16, 24, 29]. Recently a two-dye method was used to compare the expression profiles directly of two samples on the Agilent oligoarray [46, 55, 56]. The total RNA was extracted from different tissues and probes

(4 × 44 K microarray formats) were blotted onto a glass slide (25 × 75 mm) in three biological replicates. Figure 15.1 shows details of different steps in a two-color microarray experiment from RNA sampling to data analysis in an organism exposed to environmental stress.

The high-throughput techniques such as microarray normally generate a huge number of datasets of gene expression values in plants that are exposed to different treatments. Hence, there are many challenges in the analysis and interpretation of these huge datasets through a proper way to solve such a problem [18]. To provide the perfect application of biological databases and the knowledge they include, different styles of information from different origins must be combined in ways that make an impression to biologists [22]. One way is summarizing the data into the identified molecular and cellular pathways so the important genes and pathways that might be responsible for the applied treatment will be identified. Another important way is the gene ontology enrichment analysis (GOEA) that provides ontologies to describe attributes of gene products in three nonoverlapping domains of molecular biology [13]. There are various software, Web tools, and databases that can be used for rice transcriptome analyses and each group of DEGs could be annotated and classified according to the functional categories [32, 53, 59]. There are different rice microarray platforms available in the rice oligonucleotide array database (ROAD); 1867 publicly available rice microarray hybridizations are available to analyze gene expression [11].

Understanding a cellular metabolic situation is crucial for the biologically meaningful interpretation of the final consequences of gene expression to physiological adjustments necessary for whole-plant level stress tolerance [45]. To this end, results of differentiation of expression patterns of different tissues such as root and leaf in different rice genotypes through testing of NILs and the susceptible parent IR64 under a severe water stress (0.2 FTSW) indicated that the number of differentially expressed genes (DEGs) under severe WD treatment was higher than mild WD [46], and under high-osmotic treatment than low-osmotic treatment [43]. These DEGs were involved in various functional categories, which play important roles in drought stress tolerance. These functional categories such as cell growth, hormone biosynthesis, cellular transport, amino acid metabolism, reactive oxygen species (ROS), signaling, transcription factors (TFs), and carbohydrate metabolism were selected from metabolic and signaling pathways available in different databases and in the literature [5, 71]; then changes in expression profiles of the genes involved in these main functional categories were analyzed. For instance, cell-wall–related genes were mostly downregulated in roots of different rice genotypes under severe WD, whereas the number of upregulated genes at mild WD was higher in the same tissue and genotypes [47, 63, 67]. These results indicate that severe drought stress seriously affected cell expansion in the roots of almost all rice genotypes at the reproductive stage, whereas activation of xyloglucan genes in the root tips of tolerant rice under drought stress resulted in enhanced root growth and elongation. In these reports, results indicated that many genes involved in hormone

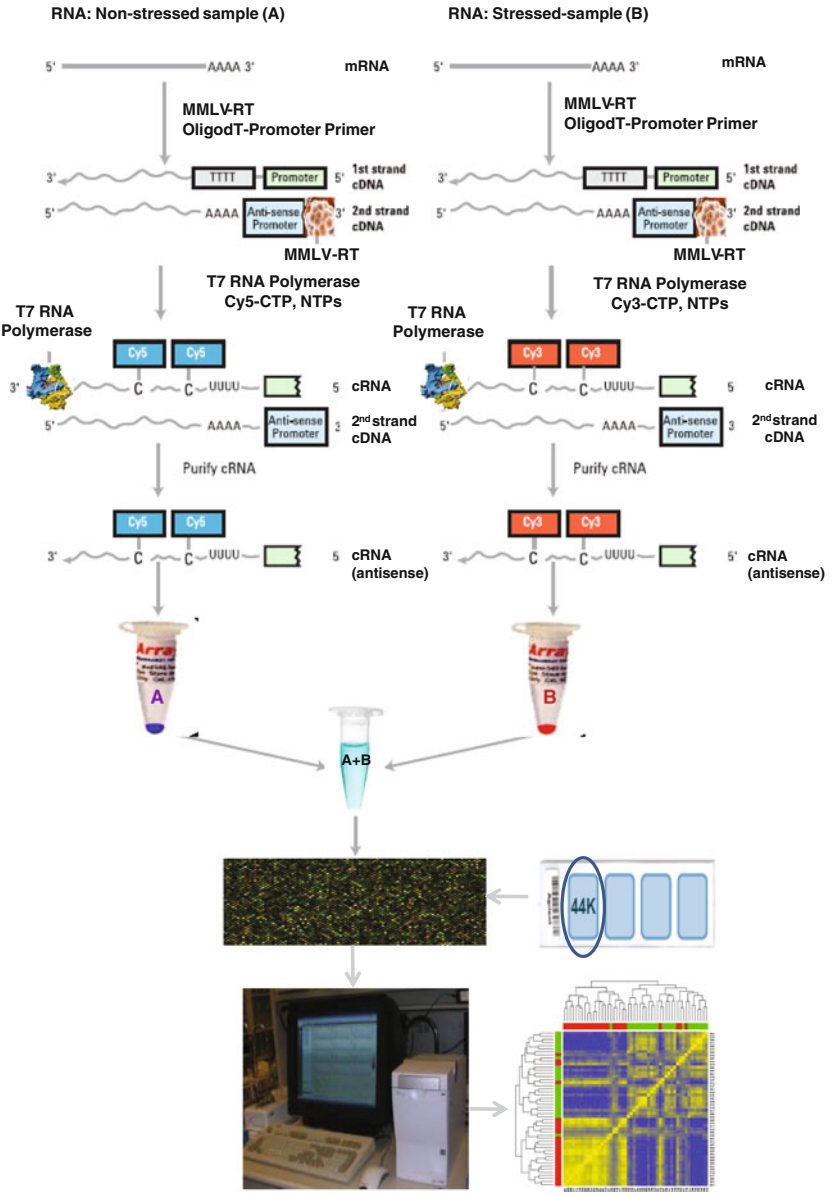

Fig. 15.1 Details of different steps in a two-color microarray experiment from RNA sampling to data analysis in an organism exposed to environmental stress. Generation of cRNA for a two-color microarray experiment and extraction of signal intensities of each microarray from scanner, data analysis, and visualization are shown

biosynthesis, such as those related to abscisic acid (ABA), auxins, gibberellins, and ethylene were found to be differentially expressed under severe drought stress at the reproductive stage. ABA biosynthesis, which plays a central role in many aspects of response to various stress signals, drought, and high salinity, were constitutively activated [72]. As for cellular transport, genes related to ion transport, pumps, and secondary transporters were mostly activated and/or repressed in tolerant rice genotypes [43]. The expression patterns of genes encoding enzymes involved in amino acid metabolism, such as proline/arginine metabolism were reported to be up- and downregulated in tolerant genotypes as compared with the susceptible lines [24, 36, 45, 46]. In the case of signaling systems, different studies indicated that expression profiles of important genes involved in stress signaling systems and other stress-regulated genes such as chaperons, including dehydrins and late embryogenesis abundant (LEA), and some other important families mostly activated in response to drought stress treatments [20, 70, 74]. Those genes related to signal transduction such as ABA responsive, calcium-dependent protein kinases (CDPKs), calcineurin B-like protein-interacting protein kinases (CIPKs), calmodulin (CML) and calmodulin-related calcium sensor proteins, and receptor-like cytoplasmic kinases (RLCKs) were reported as both up- and downregulated in rice genotypes in response to drought stress treatments [40, 46, 69]. In nature, when environmental stress happens, plants should be able to "sense" the stress signals prior to responding to the abiotic stress. Because of the complex nature of stress, multiple sensors, instead of a single sensor, are most probably responsible for perception of the stress [21]. A large number of TF genes, which regulate gene expression in response to environmental and physiological signals, from different families such as AP2-EREBP, bHLH, C2H2, GRAS, HB, LOB, MYB-related, ARF, BBR/BPC, C2C2-CO-like, C2C2-Dof, C3H, G2-like, GeBP, NAC, SET, WRKY, and OFP families were also differentially expressed (both up- and downregulated) under drought stress treatments [35, 45, 66, 73]. Using Agilent Rice arrays for investigation of change in expression of the dehydration-responsive element binding protein1 (DREB1), a TF that controls the expression of many stress-inducible genes, in transgenic rice plants overexpressing DREB1 orthologues showed improvement in tolerance to drought, high salt, and low-temperature stress conditions [28]. Table 15.1 summarizes a number of genes and DEGs in main functional pathways that are important in tolerance in response to WD stress in rice genotypes. According to this review, by comparing the expression patterns of rice genotypes in response to different WD treatments through microarray analyses, the important functional categories of genes could be identified, and the microarray technology could clearly differentiate the tolerance and susceptible genotypes of rice.

Table 15.1 Number of genes and DEGs that are involved in main functional classifications that could be important in response to WD treatments in rice near-isogenic lines, and IR64 (susceptible parent)

Category	No. of genes	0.2 FTSW					
		IR64		10		13	
		Up	Down	Up	Down	Up	Down
1 Cell growth							
Cell all							
Expansins	52	15	47	14	55	15	45
Extensins	10	1	6	1	6	1	4
Cellulose synthase	45	11	14	12	21	13	18
Xyluglucans	47	6	26	4	29	8	21
Sub-total	**154**	**33**	**93**	**31**	**111**	**37**	**88**
Cytoskeleton							
Actin filaments	21	7	3	10	1	8	3
Microtubules	18	7	2	6	4	8	1
Sub-total	**39**	**14**	**5**	**16**	**5**	**16**	**4**
2 Hormone biosynthesis							
Abscisic acid	11	7	1	6	2	7	0
Gibberellin	16	4	5	3	4	1	4
Auxin	20	5	6	10	5	7	4
Ethylene	32	16	9	16	9	13	10
Sub-total	**79**	**32**	**21**	**35**	**20**	**28**	**18**
3 Cellular transport							
Ion channels	153	40	42	43	40	40	45
Pump	228	70	48	72	51	70	57
Secondary transporters	682	202	188	217	190	186	195
Unclassified	11	1	7	0	6	0	5
Sub-total	**1074**	**313**	**285**	**332**	**287**	**296**	**302**
4 Amino acid metabolism							
Proline and arginine	42	13	12	16	12	15	12
Glutamate, Asparatate, Alanine	40	11	18	12	18	10	20
Sub-total	**82**	**24**	**30**	**28**	**30**	**25**	**32**
5 Reactive oxygene species							
Flavonoid	20	4	3	7	3	5	5
Superoxide desmutase	8	6	1	6	1	4	1
Ascorbate	12	8	0	8	0	8	0
Thioredoxin	3	1	1	2	1	1	1
Peroxiredoxin	9	6	1	6	1	5	1
Ferritin	3	3	0	3	0	3	0
Glutathione	47	11	17	12	13	10	15
Glutaredoxin	36	11	4	11	4	10	8

(continued)

Table 15.1 (continued)

Category	No. of genes	0.2 FTSW					
		IR64		10		13	
		Up	Down	Up	Down	Up	Down
Sub-total	**138**	**50**	**27**	**55**	**23**	**46**	**31**
6 Signalling and abiotic stress related							
Chaperons	106	44	9	49	9	50	11
LEA	18	15	0	15	1	15	0
Dehydrins	8	8	0	8	0	8	0
ABA responsive	60	22	14	20	15	19	14
CIPK	30	11	6	12	9	9	6
CDPK	30	8	7	8	5	8	8
Calmodulins	37	7	15	8	17	8	16
RLCK	287	46	73	61	73	49	78
Sub-total	**576**	**161**	**124**	**181**	**129**	**166**	**133**
7 Transcription factors	**2336**	**493**	**618**	**487**	**648**	**483**	**622**
8 Carbohydrate metabolism							
Glycolysis	87	30	18	33	21	28	22
Citrate cycle	48	19	4	16	8	18	6
Fructose and Mannose	41	13	6	15	8	12	11
Starch and sucrose	78	24	16	23	17	21	16
Ascorbate and Aldarate	24	9	1	11	2	7	2
Inositol_Phospahe	28	6	5	6	6	8	7
Sub-total	**278**	**95**	**45**	**98**	**56**	**86**	**57**
Grand total	**4756**	**1215**	**1248**	**1263**	**1309**	**1183**	**1287**

DEG Differentially expressed genes; *Up* Up-regulated; and *Down* Down-regulated, Tolerant NIL (10):IR77298-14-1-2-B-10; susceptible NIL(13): IR77298-14-1-2-B-13; 0.2 FTSW: severe WD treatment. Moumeni et al. [46]

15.3 Gene Ontology Enrichment Analysis and Drought Stress

15.3.1 Importance and Application

Extracting the important biological information from the data of microarray experiments is very important, however, this task proved to be difficult for biologists. Such high-throughput experiments mostly generate a large number of candidate genes or proteins, sometimes with noisy results [77]. To overcome this challenge, a systemic annotation vocabulary describing biological information and tools to show masked knowledge using such a vocabulary are required [78]. Because of the challenges in the analyses and interpretation of huge datasets that are generated by microarray technology through a proper way to solve such a problem, other than summarizing the data into the identified molecular and cellular pathways,

the Bioinformatics Society developed several enrichment tools that employ GO as the annotation resource [18]. The GO is normally a controlled vocabulary system that includes a wide range of information for gene function description at a molecular level [22].

Currently, GOEA is widely used to identify the main functional classifications of environmental stress-treated plants, including drought-responsive genes and genes involved in the drought-tolerance mechanism, which generated from microarray experiments [25, 26, 39, 46, 76]. Evolving the main biological information from the microarray data on drought stress in rice through GO analysis is being increased. The effect of WD on the leaf elongation zone in rice through GOEA showed that the GO category "rhythmic process" was found to be significantly enriched in the case of downregulated differentially expressed common genes [10]. Functional classification of 24,027 DEGs in different rice genotypes contrasting with DT at the reproductive stage in root tissue through GO analysis indicated that activated genes in the drought-tolerant genotypes were mostly involved in secondary metabolism, amino acid metabolism, response to stimulus, defense response, transcription, and signal transduction, and downregulated genes were involved in photosynthesis and cell wall growth [46], whereas in leaf tissue, the differentially expressed specific gene's DT in the highly drought-tolerant rice genotype was attributed to the upregulation of genes with calcium ion binding, transferase, hydrolase, and TF activities and in the moderate drought-tolerant rice genotype genes with transporter, catalytic and structural molecule activities were upregulated under WD [47]. Figure 15.2 indicates GO classifications, MF, of up- and downregulated genes in a drought-tolerant near-isogenic line of rice when exposed to severe WD a treatments at the reproductive stage.

Genes related to stress, external stimuli, embryonic development, and the carbohydrate metabolic process were upregulated, whereas genes related to photosynthesis, cell differentiation, the phosphorus metabolic process, and the reproductive process were downregulated by drought in rice genotypes that were classified through GO analysis [17]. The DEGs in the functional category include OsGigantea (OsGI) and Os11g34460, the rice orthologue of FKF1, which together are important to promote flowering in Arabidopsis under drought stress [57]. Identification of a conserved drought stress responsive gene network across tissues and developmental stages in rice through GO analysis showed relatedness of dehydrin gene to the "response to stress" GO term with high significance [62]. Genes involved in photosynthesis and carbohydrate/glucan metabolism classifications were developmentally upregulated under normal conditions but not under drought [30]. GO analysis indicated that those genes involved in three categories of BPes were significantly overrepresented in GO terms of "response to stimulus" including abiotic, endogenous, and biotic stimulus, "metabolism" such as lipid metabolism, amino acid and derivative metabolism, and carbohydrate metabolism, and "signal transduction" [75]. GO analysis is also a proper way in network-based gene clustering to identify the relationships between drought-responsive genes from large microarray datasets and to detect functional modules in rice under drought stress [76]. Therefore, GO analysis is a suitable method to give hints to understand the various regulatory mechanisms following the drought response.

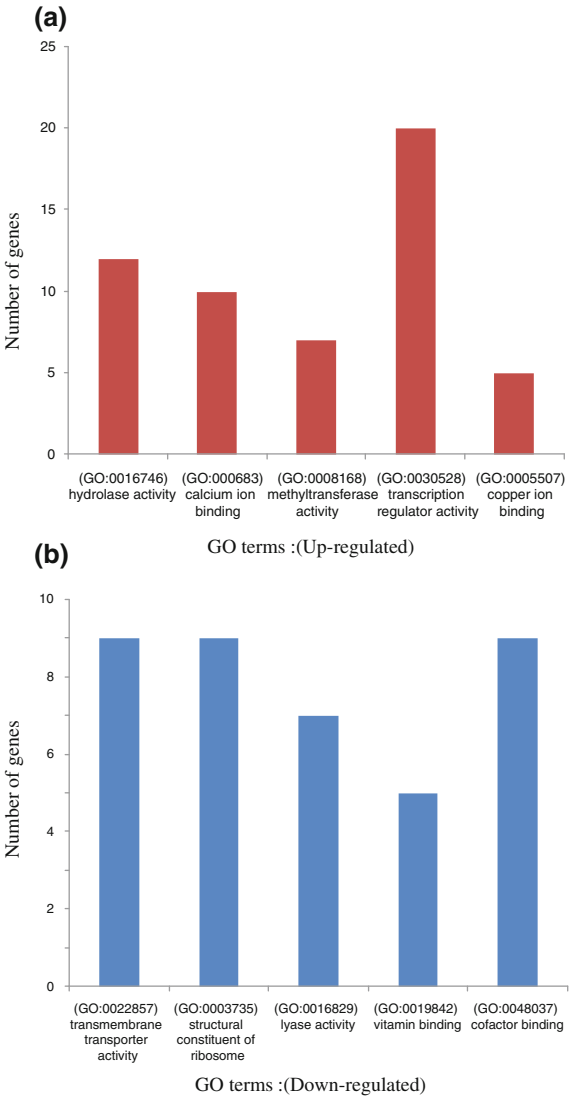

Fig. 15.2 Gene ontolgy classifications, MF, of up- and downregulated genes in a drought-tolerant near-isogenic line (NIL) of rice when exposed to severe WD treatments at the reproductive stage: **a** upregulated and **b** downregulated overrepresented GO terms with number of genes in each GO category

15.3.2 Gene Expression Data Management/Tools

The GO and related annotations to gene products are becoming routine for researchers to include when publishing functional information [27]. Since the first paper was published in 2000 [3], hundreds of peer-reviewed papers have cited the GO. Researchers apply different GOEA tools, among the tens of GO-focused applications and tools available, to identify significant enriched GO categories for all sets of DEGs when exposed to different environmental stresses such as WD

stress. Herein, several important GO tools are introduced. Blast2GO is a GO analysis tool that optimizes function transfer from homologous sequences through an elaborate algorithm that considers similarity, the extension of the homology, the database of choice, the GO hierarchy, and the quality of the original annotations. The tool includes numerous functions for the visualization, management, and statistical analysis of annotation results, including gene set enrichment analysis [14]. AmiGO is a Web application that allows users to query, browse, and visualize ontologies and related gene product annotation (association) data [12]. Another program is BiNGO, a tool to determine which GO categories are statistically overrepresented in a set of genes or a subgraph of a biological network. BiNGO maps the predominant functional themes of a given gene set on the GO hierarchy, and outputs this mapping as a Cytoscape graph [44]. Finally, agriGO, a Web-based tool and database for GO analysis, supports special focus on agricultural species and is user-friendly. The agriGO is designed to provide deep support for the agricultural community in the realm of ontology analysis [18]. Table 15.2 shows different GO tools and programs to analyze gene expression data originated through microarray experiment.

More information about different programs, databases, and literature about GO tools can be found at: http://omictools.com/gene-ontologies-c25-p1.html.

Table 15.2 Different gene ontology (GO) tools and programs to analyze gene expression data originated from high-throughput technology

No	Gene ontology tools	Type of tool	URL	References
1	Blast2GO	Program	http://www.blast2go.com	[14]
2	Genes2GO	Program	http://norstore-trd-bio0.hpc.ntnu.no:8080/Genes2GO/	–
3	agriGO	Program	http://bioinfo.cau.edu.cn/agriGO/	[18]
4	AmiGO	Program	http://amigo.geneontology.org/amigo	[12]
5	Annotare	Program	http://code.google.com/p/annotare/	[60]
6	BiNGO	Program	http://apps.cytoscape.org/apps/bingo	[44]
7	CompGO	Program	http://www.bioconductor.org/packages/release/bioc/html/CompGO.html	[68]
8	FuncAssociate	Program	http://llama.mshri.on.ca/funcassociate/	[8]
9	GoMapMan	Program	http://www.gomapman.org/	[51]
10	GOstat	Program	http://gostat.wehi.edu.au/	[6]
11	PiNGO	Program	http://www.psb.ugent.be/esb/PiNGO/Home.html	[63]
12	REViGO	Program	http://revigo.irb.hr/	[64]

15.4 Conclusion and Future Perspectives

Water scarcity is one of the most pressing issues facing agriculture, especially rice production, today. It changes the expression level of a large number of genes in rice. Through microarray experiments, gene expression profiles of rice plant can be detected under WD. However, testing suitable plant materials such as NILs with similar genetic backgrounds, but contrasting drought-tolerance levels, in long-term drought-stress treatments (i.e., drydown method similar to field condition) at the reproductive stage, which is critical in grain yield loss will help to determine different pathways/mechanisms involved in drought-stress tolerance. Application of a comprehensive 4 × 44 K oligoarray platform will ease the detecting expression level of thousands of transcripts. But, an analysis of such a huge number of genes generated by microarray experiments without summarizing the data is rather impossible. There are different ways to extract the information from these datasets including: (1) functional pathway analysis, and (2) GOEA of the DEGs. Through these methods of functional analysis, most important functional pathways and GO terms with related DEGs were identified.

This work will help breeders improve the DT of rice cultivars. In the meantime, further work on the details of gene regulation, transformation of susceptible genotypes with candidate genes responsible for DT, will provide useful information.

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Chapter 16
Systems Biology Approaches to Improve Drought Stress Tolerance in Plants: State of the Art and Future Challenges

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16.1 Introduction

Abiotic stresses, such as drought or water deficit (WD), play a major role in determining crop productivity [1]. Photosynthesis, together with cell growth, are among the primary processes that are affected by drought [2]. On carbon metabolism, the impact of water deprivation results in changes in the sugar pool used for signaling cellular processes or substrates for biopolymers such as cellulose, starch,

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and proteins [3]. In this context, it is tempting to claim that the yield and quality of the harvested plant parts (e.g., grains, biomass, and stalks) under drought may rely on the crosstalk of regulatory processes at the whole plant level [4]. Research on drought tolerance has generally been conducted using discipline-specific approaches. However, the multiple levels of complexity and crosstalk seen in drought responses can benefit from the use of global system biology to deeply understand the mechanisms involved [5, 6].

During the last decade, the "reductionist" molecular biology and functional biology approaches have been progressively replaced by the "holistic" systems biology approach [7]. The development of postgenome methodologies, such as global analysis of coding and noncoding transcriptomes, proteomes, and metabolomes integrated in solid bioinformatics platforms, has noticeably improved our knowledge and holistic understanding of various plant functions, including the response to abiotic stresses [8]. Systems biology-based analysis can involve multiple levels of complexity, ranging from single organelles, cells, tissues, and organs to whole organisms and even populations. These variables can still be combined with multiple developmental stages and environmental interactions, suggesting an infinite number of permutations to this complexity [5].

Despite the major progress observed in postgenome technologies and knowledge, we are still far from having an integrative description of all the molecular events and players underlying the complex response of a plant to drought stress, which is the distinguishing feature of systems biology. Nevertheless, this is still a challenge for all plant sciences and agricultural researchers. In this chapter, we describe some achievements in the fields of transcriptomics, proteomics, and metabolomics and provide some approaches for data integration to accomplish the plant systems biology goals.

16.2 Studying the Plant Transcriptome Reprogramming Under Drought

Transcriptomics refers to the study of the transcriptome, the complete set of coding (mRNAs) and noncoding (e.g., miRNAs) transcripts produced by the genome under a specific condition. Typically, the comparison of transcriptomes allows the identification of transcripts differentially expressed in a specific tissue or cell, developmental process, or in response to environmental changes. Recent transcriptomic approaches rely on the use of high-throughput methods, such as microarray analysis or next-generation sequencing (NGS) of RNA. A considerable number of reviews have been published gathering knowledge on plant responses to several abiotic stresses obtained from transcriptomic studies [7, 9, 10, 11].

16.2.1 Microarray-Based Approaches

Microarrays are a high-throughput technology based on the affinity of single-stranded DNA sequences to bind to complementary sequences of nucleotides (probes) deposited in an array, also known as a chip or slide. Signal intensity values from hybridization of transcripts with the probes provide information about the expression level of transcripts [12]. The first *Arabidopsis* microarrays, made available in 2000 by Affymetrix, opened the possibility to interrogate large genomes in plants [13]. Since then, microarrays have been customized for several model and crop plants to understand the complex dynamics of the transcriptome, namely under environmental stresses [14].

Studies conducted in the model plant *Arabidopsis thaliana* (L.) Heynh. provided important insights into the molecular mechanisms underlying the reproductive responses and acclimation under drought [15]. In this work, the transcriptome of inflorescences was studied under moderate and severe drought using an Affymetrix GeneChip Expression microarray. More than 4000 genes showed differential expression under severe drought and less than 2000 changed under moderate drought condition. Although these results suggest a putative adaptation to dehydration, they also provide evidence for the existence of distinct sets of genes responsive to different levels of water availability. For instance, the Nuclear Factor Y transcription factor subunits (NY-Fs, CCAAT-binding factors) were specifically induced by moderate drought and might have a specific function under this condition.

Rice (*Oryza sativa* L.) is one of the most important cereal crops in the world but its production is strongly affected by drought. A transgenic rice line constitutively expressing a A20/AN1 zinc-finger protein gene (*OsiSAP1*) showed improved yield under WD [16]. To understand the molecular basis of this response, the authors conducted a transcriptome analysis using an Affymetrix GeneChip Rice Genome array. The results revealed an altered expression of several endogenous genes that includes transcription factors, membrane transporters, signaling components, and genes involved in metabolism, growth, and development. One of the most interesting results is that the majority of the transcripts upregulated were related with stress response, leading to the conclusion that *OsiSAP1* might be a positive regulator of WD responses.

Wheat (*Triticum aestivum* L.) is another cereal crop whose productivity is strongly affected by drought. Two wheat cultivars TAM 111 and TAM 112, with distinct WD adaption mechanisms, were compared at the transcriptomic and physiological levels [17]. Using an Affymetrix GeneChip Wheat Genome array, the authors found that transcripts associated with photosynthesis, carbohydrate metabolism, phytohormone metabolism, and other dehydration responses were uniquely regulated between cultivars. These results suggested a differential role for ABA in regulating physiological and transcriptomic changes associated with WD stress and its potential involvement in the superior adaptation and yield of TAM 112 cultivar.

The maize (*Zea mays* L.) leaf growth zone offers unique possibilities for studying the spatio-temporal and environmental regulation of developmental processes. Using an Agilent 44K maize array, Avramova et al. [18] observed the downregulation of 32 of the 54 cell cycle genes under drought, providing a molecular basis for the inhibited cell division in this condition. Also, they found evidence of upregulation of the photosynthetic machinery and the antioxidant and redox systems, which was corroborated by biochemical assays.

Sorghum (*Sorghum bicolor* (L.) Moench) is an important cereal crop used for food, feed, and ethanol production. Naturally growing in arid and semi-arid regions, it is well adapted to the hot and dry conditions, being a unique model to investigate the molecular mechanisms underlying such resistance. Johnson et al. [19] used microarrays to study the transcriptional response of sorghum plants subjected to heat and drought stresses. Several potential genes involved in the response to stresses were identified, such as the transcription factors myb domain protein (MYB78) or NAC proteins (ATAF1), unique heat shock proteins (HSPs), and genes involved in the polyamine biosynthesis pathways. Interestingly, the authors also found that when combined stresses were applied, the response was different compared to individually applied stresses. In another study, Pasini et al. [20] analyzed the transcriptome of young sorghum leaves from seedlings under a dry-down condition. Most of the differentially expressed genes under stress encode proteins involved in regulation of transcription, such as basic leucine zippers (bZIPs), MYBs, homeobox (HOXs), signal transduction (phosphoesterases, kinases, and phosphatases), carbon metabolism (NAD-dependent malic enzyme, NADP-ME), detoxification (cytochrome family, CYPs), glutathione S-transferase (GST), aldo/keto reductase (AKR) family proteins, and osmoprotection mechanisms (pyrroline-5-carboxylate synthetase family P5CS). Other proteins involved in the stability of protein membranes, such as dehydrin (DHN1), late embryogenesis abundant (LEA), and HSPs were also described.

WD is a major factor limiting soybean (*Glycine max* L.) production in semi-arid and subhumid regions of the world [21]. The economically important DT2008 and the Williams 82 (W82) cultivars have been reported to have a differential drought-tolerance degree to drought, which was associated with their respective root traits [22]. A 66K Soybean Affymetrix GeneChip array was used to compare the root transcriptomes of DT2008 and W82 seedlings under normal, mild, and severe dehydration conditions [23]. Among differentially expressed genes, 822 and 632 genes showed altered expression by dehydration in W82 and DT2008 roots, respectively. The higher transcriptome reprogramming observed in the drought-sensitive W82 cultivar suggests that a larger molecular machinery is activated in this cultivar to cope with the stress. The differences between genotypes were attributed to differential expression of genes involved in osmoprotectant biosynthesis, detoxification, or cell wall-related proteins, kinases, transcription factors, and phosphatase 2C proteins.

16.2.2 Next-Generation Sequencing-Based Approaches

A revolution in transcriptomics studies occurred with the introduction of NGS, opening new opportunities in life sciences [24]. Microarray drawbacks, such as hybridization background or probe cross-hybridization issues, the restriction on transcript detection imposed by the array design, as well as, the need for a sequenced genome were largely overcome by RNA-sequencing [25, 12]. RNA sequencing (RNA-Seq) brings several advantages in plant research: significant increase in coverage depth [26], detection of novel and/or low abundance transcripts and splice variations [27], and detection of noncoding RNAs [28] without requiring prior knowledge of the genome sequence of the target organism. Additionally, RNA–Seq is also well suited for detecting single nucleotide polymorphisms (SNPs, [29]), small insertions and deletions (INDELs, [30]), and allelic specific differences in gene expression in genome-wide analysis studies (GWAS; [31]).

The major commercially available high-throughput sequencing platforms are the Roche 454 Genome Sequencer FLX+System (Roche Applied, http://www.454.com), the Ilumina/HiSeqGenome Analyzer (Solexa, http://www.illumina.com), and the ABI SOLIDsystem (Applied Biosystems, http://www.appliedbiosystems.com). However, the pace of change in this area is rapid and new sequencing platforms were released in 2011: the Ion Torrent's Personal Genome Machine, the Pacific Biosciences' RS, and the Illumina MiSeq [32]. Comprehensive method description, as well as a comparison of their applications, advantages, and limitation may be found in Ansorge [24], Marguerat and Bähler [33], and Quail et al. [32]. The crucial role of bioinformatics to store, manage, and extract valuable information from transcriptome datasets effectively needs to be highlighted [8]. Some dedicated tools and databases are briefly described in Sect. 16.6 of this chapter.

RNA-seq approaches are opening new opportunities to understand crop responses to water limited environments. Drought stress can seriously affect potato (*Solanum tuberosum* L.) tuberization, yield, and quality [34]. A recent transcriptomic study was conducted to unveil the molecular mechanisms governing the potato stolon's response to drought stress and recovery [35]. In this work, the potato plants of Ningshu 4 variant were subjected to severe drought stress and rewatering treatments, at the tuber bulking stage. In comparison to untreated control plants, 3189 and 1797 genes were differentially expressed in drought-treated and recovered plants, respectively. Of these, 263 genes showed opposite expression patterns between drought-treated and recovered plants. Among them, genes homologous to protein phosphatase 2C (PP2C), aspartic protease in guard cell 1 (ASPG1), GA-stimulated transcripts in Arabidopsis 6 (GASA6), and calmodulin-like protein 19 (CML19) were described.

Cotton (*Gossypium hirsutum* L.) is one of the world's most valuable crops, providing much of the planet's natural fiber for the global textile industry. Bowman et al. [36] investigated the impact of drought stress on the root transcriptome. Among the 1530 transcripts differentially expressed, the results indicate the involvement of important biochemical pathways needed for cellular osmotic

balance, abscisic acid, and cellular water uptake. A similar study conducted in cotton leaves demonstrated the activation of abscisic acid, ethylene, and jasmonic acid signaling pathways in response to drought stress [37]. These results highlight drought-induced specific transcriptomic signatures in plants, which are dependent on the organ studied. Recent evidence showed that the expression profile of genes triggered by WD stress can also be dynamically modulated across the day cycle [38]. Such findings suggest the importance of analyzing different time periods to characterize plant responses to stress deeply.

Plant breeders are interested in identifying genes that confer drought tolerance and can then be used for marker-assisted selection for drought improvement. RNA–Seq is also well suited for detecting SNPs in grain legumes. A transcriptomic approach was undertaken to identify genes with a well-defined genotype × environment (G × E) response in two soybean genotypes (PI 416937 vs. Benning cultivar) with contrasting wilting responses [39]. Because many of these genes are located within slow-wilting QTLs, they are strong candidates for genes underlying PI 416937's unique drought avoidance strategy. Common bean (*Phaseolus vulgaris* L.) is one of the most consumed food legumes worldwide but its productivity is strongly hampered by drought [4]. A large number of genes involved in drought stress responses were identified in common bean using RNA-seq approaches [40]. Additionally, 10,482 simple sequence repeats (SSRs) and 4099 SNPs were identified in transcript sequences, providing new resources for gene discovery and development of new functional molecular markers.

RNA-Seq has become a powerful tool for gene expression analysis in plant systems without a reference genome sequence. High-yielding biomass crops of the genus *Miscanthus* are widely recognized as one of the most promising lignocellulosic feedstocks for the production of bioenergy and bioproducts [41]. RNA-Seq contributed to understanding the molecular basis of the higher water use efficiency (WUE) of *Miscanthus lutarioriparius* (L. Liou ex S.L. Chen and Renvoize), native of the semiarid location, when compared to the same species grown in wet central China [42]. Forty-eight candidate genes were assigned to photosynthesis, stomatal regulation, protein metabolism, and abiotic stress responses. Of these genes, nearly 73 % were upregulated in *M. lutarioriparius* from the semi-arid site, which showed enhanced WUE. Importantly, this study demonstrates that candidate genes involved in the adaptation to environmental constraints could be identified by integrating physiological data with molecular/gene expression data.

Microarrays and NGS-based approaches are revolutionizing transcriptomics research in plants, providing an integrative and comprehensive view of the biological phenomenon under study. The identification of noncoding small RNAs, another promissory application of RNA-Seq technologies, is addressed in another section of this review. The decreasing cost of sequencing coupled to the increasing number of international multidisciplinary consortia, such as the 1000 plants (one KP or 1 KP, https://sites.google.com/a/ualberta.ca/onekp/) initiative are generating large-scale gene-sequencing data for over 1000 species of plants. In due time they will constitute important functional resources to be explored to decipher the molecular mechanisms underlying drought resistance in plants.

16.3 The Proteomics Insight

The advances in knowledge on the responses of plants to abiotic stress owe a lot to proteomics technologies. These have been increasing since the 1970s [43]. A number of reviews were published putting together the most important information on plant responses to several abiotic stresses obtained from proteomic studies [44–48]. Readers are advised to refer to them for specific information.

16.3.1 Two-Dimensional Electrophoresis (2DE): The Workhorse of Drought Proteomics?

The alterations of proteome patterns in response to external conditions may be assessed through gel-based or gel-free techniques. Gel-based techniques were the very beginning of proteomics and are still widely used for profiling complex plant proteomes. This classical technology, known as two-dimensional electrophoresis (2DE), allows for the resolution of proteins according to their isoelectric point (pI) and molecular mass (Mr), in the first and second dimensions, respectively [49]. More recently, gel-free bottom-up (shotgun) proteomic techniques had been developed to fill in the gaps of gel-based techniques (for a detailed review, see [50]).

A good part of the knowledge acquired using proteomic technologies on the subject of crop responses to drought was based on classical gel techniques applied in cereals, legumes, forage, and horticultural plants, but also in woody species [51–56]. Roy et al. [57] published a detailed review with more information about these proteomic studies. In this section, we focus on studies done after 2012.

In *Gramineae*, Vítámvás et al. [58] studied the response of barley crowns to different drought conditions using 2DE coupled with matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). After 10 days of WD, 105 differentially abundant spots were detected and 76 were successfully identified. The majority of identified proteins were involved in stress response, amino acid metabolism, carbohydrate metabolism, DNA and RNA regulation and processing. In *S. bicolor*, Jedmowski et al. [59] studied the leaf proteomic response to drought and recovery using a comparative approach between a drought-tolerant (11,434) and a drought-sensitive cultivar (11,431). The results showed that chaperones and proteins related to energy balance and metabolism were the most evident features explaining the differences between the tolerant and sensitive genotypes.

Faghani et al. [60] studied proteomic changes caused by drought in wheat genotypes with contrasting drought tolerance. The authors used the 2DE approach combined with nano-liquid chromatography–mass spectrometry (nanoLC-MS/MS) to identify drought-responsive proteins in roots and leaves of sensitive (SW) and tolerant (SE) genotypes after water withholding (20 % field capacity). Forty root

and 73 leaf differentially accumulated proteins were found. The results showed changes in the abundance of proteins related to defense and oxidative stress and protein processing in roots of both genotypes. Endo-1,3-beta-glucosidase, peroxidase, S-adenosylmethionine synthase (SAMS) and malate dehydrogenase (MDH) were proteins differentially accumulated between genotypes. These proteins were increased in the roots and leaves of the SE genotype and decreased in the SW. The authors highlighted the increased abundance of ascorbate peroxidase (APX) in the SE genotype and the decreased abundance of 14-3-3 and ribosomal proteins in the SW genotype upon drought. These differential protein abundances between genotypes might constitute part of the molecular basis of their contrasting tolerance to drought. Paul et al. [61] performed a comparative proteomic analysis using drought-tolerant rice plants overexpressing the *Arabidopsis* dehydration-responsive element-binding protein 1A (DREB1A). After 7 days of WD and 24 h of recovery, the proteomes of transgenic and wild-type roots were studied using 2DE coupled with MALDI-TOF-TOF. The proteomes of both genotypes were compared resulting in the identification 30, 27, and 20 differentially accumulated proteins in the three environmental conditions studied. Carbohydrate and energy metabolism were the functional categories most represented. In both plants, stress- and defense-related proteins were especially up-accumulated under WD. The putative R40C1 protein accumulated under drought only in transgenic plants is suggested to play a crucial role in the generation of drought-tolerant plants.

In legumes, Zadražnik et al. [62] studied the proteome of two common bean (*P. vulgaris L.*) cultivars differing in drought-tolerance using two-dimensional difference in gel electrophoresis (2D-DIGE) combined with LC-MS/MS. The leaf proteome changes in plants submitted to two stages of drought stress (12 and 17 days) were analyzed. Fifty-eight proteins whose abundance changed significantly under drought were identified. The majority of proteins belonged to photosynthesis and energy metabolism functional categories. The increased abundance of oxygen evolving enhancer proteins in the tolerant cultivar was noticed, contrasting with its decreased abundance in the sensitive one.

16.3.2 Shotgun Proteomics: Making a Blast in Drought Stress Research?

As seen previously, the use of proteomics techniques has led to major breakthroughs in the study of the physiological responses of plants to WD. Such accomplishments were achieved chiefly through the use of gel-based techniques. These are still very important in the context of proteomics research and show a considerable number of advantages: reduced cost of equipment, equipment availability and means in numerous life-sciences laboratories and it is a very visual technique where expression changes can easily be pinpointed. It has, however, an important disadvantage: studies are limited to the proteins that are effectively

visualized and quantified in the gel. Proteomics research can also be conducted using gel-free proteomics or shotgun proteomics. Conceptually, if in 2DE, proteins are separated in a gel and individual proteins are excised from the gel, digested with trypsin, and identified using mass spectrometry (MS), in shotgun proteomics, total protein extracts, that is, the whole proteomes are digested with trypsin, and proteins are identified and quantified resorting to MS [63]. Shotgun proteomics has numerous advantages, the most important of which are the broadness and the comprehensiveness of the results that are achieved, and the high-throughput nature of the research. It is likely that 2DE-based proteomics will still be the workhorse of plant proteomics research for many years. Nevertheless over the last 15 years, proteomics in plants has slowly been evolving and gel-free methods are growing in importance. For further information, readers are directed to a recently published review on this subject [64]. Herein, we focus on the use of shotgun proteomics to study WD plant physiology. We provide specific examples and case studies on the fast growing importance of this technique and its potential to upgrade proteomics research, essentially in crop plants.

In *Gramineae*, Mirzaei et al. [65] used label-free shotgun proteomics to identify proteins associated with drought stress signaling in the rice shoot proteome. The approach, consisting of the comparison of rice plants watered every day and plants in which water was withheld, allowed the identification of almost 1400 proteins that could be divided into 17 major functional groups. The results showed that the majority of the proteins affected belonged to the protein and oxidation-reduction metabolism. Interestingly, transport proteins (essentially plasma membranes and vacuolar transport proteins) were up-accumulated in plants subjected to drought. Given the known difficulties in studying membrane proteins using a 2DE-based approach, this study clearly points out important advantages of shotgun proteomics other than the sheer number of proteins identified.

In legumes, shotgun proteomics has been used to study the effects of flooding and drought stresses in two-day-old soybean roots [66]. Authors detected a total of 48 proteins with changed abundance levels as a consequence of drought stress. Proteins involved in protein synthesis had an increased abundance level as a consequence of WD (and decreased accumulation under flooding stress). The same was also registered for cell organization and redox related proteins. The authors propose SAMS as a key protein in the regulation of stress response and consequently a marker of exposure to WD stress. Kottapalli et al. [67], studied the effect of WD on mid-mature peanut (*Arachis hypogea* L.) using a label-free quantitative proteomics approach. These authors not only studied differences in protein abundance but also established a pod-specific proteome database for this species. This component, not frequently found in proteomics literature, is of the utmost importance given the lack of annotated databases for this non-model species. Regarding the differential protein accumulation, the authors suggested three candidate biological pathways affected by WD that could be related to agronomical traits: glycolysis, sucrose/starch, and fatty acid metabolism.

In species outside the *Gramineae* and *Fabaceae*, the use of shotgun proteomics to study drought stress is very limited. In fact, we could only find two examples,

one with rapeseed (*Brassica napus* L.) and another one with apple (*Malus domestica* Borkh.). Koh et al. [68] used isobaric tags for relative and absolute quantitation (iTRAQ) LC-MS/MS to study the effect of water withdrawal in the rapeseed leaf proteome. The authors identified 417 proteins with changes in abundance as a consequence of drought. Proteins involved in protein folding and degradation, as well as signaling seem to have a decreased abundance, whereas photosynthesis, protein synthesis, and stress-related proteins increased their accumulation as response to drought. Also using iTRAQ-based proteomics analysis, Zhou et al. [69] studied differences in leaf proteome in two cultivars of apple trees with different WUE. The authors identified a total of 4078 proteins, with 600 showing differential abundance. Most of the proteins were related to photosynthesis regulation, indicating that the cultivar with higher WUE maintained the Calvin cycle function by keeping reactive oxygen species (ROS) at normal levels [69].

Nevertheless, the biggest limitation to the use of shotgun proteomics is undoubtedly the access to dedicated (preferably well annotated) databases, a situation in common, for instance, with farm animals [70]. This is a key factor in the determination of the success of the shotgun proteomics approach.

In fact, plants with a limited number of sequences in the databases, which are the vast majority of plants of agronomical interest (see Fig. 16.1), are not likely to achieve protein identification success. To solve this problem researchers have either to establish their own databases, an expensive and know-how demanding process, not available to the majority of plant scientists; or alternatively use databases from other

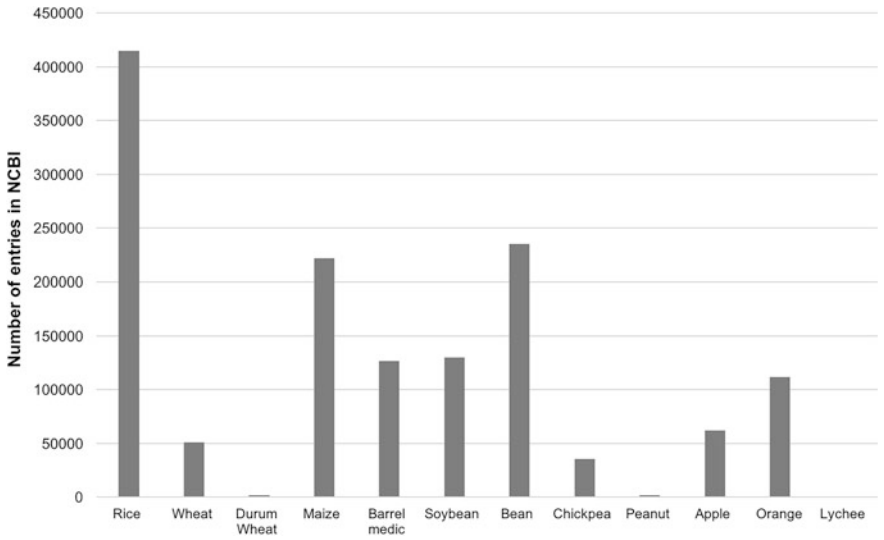

Fig. 16.1 Number of entries in the NCBI protein database (July 2015) for some important crop plants (rice, wheat, maize, soybean, bean, chickpea, peanut, apple, orange, and lychee) and the model legume barrel medic (*Medicago truncatula*). Notice the low representation of important crop plants such as wheat, chickpea, or apple, with strong implications for gel-free shotgun proteomics approaches

species, ideally with high level of synteny among them. Finally, it is noteworthy to mention that shotgun proteomics and 2DE are two complementary techniques and that both approaches are recommended to study the effects of WD in plants.

16.4 The Key Role of Metabolomics

Metabolomics, in addition to genomics, transcriptomics, and proteomics, is another essential approach in systems biology. The plant metabolome is a result of the preceding processes occurring in cells such as gene expression. This is followed by the biosynthesis of enzymes that catalyze biochemical reactions leading to the production of specific metabolites. It is estimated that 0.2–1 million of metabolites occur in the plant kingdom [71]. Many metabolites play an important role in the adaptation to changing environmental conditions and determine responses to biotic and abiotic stresses. Under drought conditions, plants accumulate osmoprotectants that protect tissues from the loss of water. Antioxidants and stress signals (e.g., sugars) are other metabolites essential in the drought response [72]. Both primary and secondary metabolites are involved in the plant's response to WD. Among primary metabolites, amino acids (proline, tryptophane, phenyloalanine, histidine), polyamines (spermine, spermidine, putrescine), carbohydrates (sucrose, raffinose, glucose, fructose, maltose), polyhydrol alcohols, and quaternary ammonium compounds (especially glycine betaine) are reported to contribute to plant protection [73]. The impact of metabolomics on drought stress improvement is covered in Chap. 7 of this book series, therefore a general overview of key metabolites and some case studies are provided, describing the main techniques used to explore the metabolite influence on plant growth, development, and on crop quality.

16.4.1 Key Metabolites Underlying Plants Response to Drought

Numerous reports indicate an increased concentration of polyamines in plant tissues under drought conditions. Polyamines at the physiological pH form cations and interact with negatively charged macromolecules, stabilizing their structures, namely under stress when this damaging situation occurs. They also protect cellular membranes from denaturation maintaining membrane permeability because they bind to the phospholipid head groups [74, 73]. Moreover, polyamines are considered to be signaling compounds that influence the regulation of basic cellular processes. They promote protein synthesis and modulate nucleic acid structures and enzyme activity and functions. Putrescine (Put) regulates the level of abscisic acid (ABA) but the reverse regulation is also known [74]. Cvikrova et al. [75] reported the decrease of Put and spermidine (Spd) content in tobacco plants during 10 days

of drought. Inasmuch as they are intermediates for the production of other polyamines, simultaneously the concentration of spermine (Spm) increased as well as of diaminopropane, which is, in turn, produced by oxidative deamination of Spd and Spm. In contrast, in the case of pakchoi (*Brassica rapa* L. ssp chinensis), the drought stress coincided with an increase of Put content and a decrease in Spd and Spm level [76]. Therefore these studies highlighted that each plant species responds to WD in diverse ways.

Sugars (e.g., sucrose and hexoses), aside from playing an important role as carbon and energy source, also act as osmoprotectants that improve tolerance to drought conditions. By decreasing osmotic stress, they hamper the reduction of cell turgor, also stabilizing membranes and subcellular structures. Moreover, sugars can act as signaling molecules [73]. The relation between enhanced drought tolerance and elevated sugar content in peanut (*Arachis hypogaea* L.) was established by Padmavathi and Rao [77]. The authors tested four cultivars that varied in their WD tolerance. They found that the most tolerant one had the highest increase of soluble sugars in leaves of plants exposed to water stress. Simultaneously, the tolerant cultivar accumulated the largest amount of proline and other amino acids. The significantly elevated levels of sucrose and proline were also reported for water-stressed wheat [78]. In this work, the authors found that the exogenous addition of glycine betaine significantly increased the proline content but not sucrose concentration. The increase of proline content was also observed in tobacco plants exposed to WD [75]. In soybean, the level of proline increased gradually up to the fifth day when plants were maintained under WD conditions but it declined in the recovered plants [79]. In the leaves of the water-stressed oak plants, proline was accumulated in a 2.4-fold higher amount in comparison to control individuals [80]. Glycine betaine also acts as an osmoprotectant, in addition to stabilizing complex proteins, enzymes, and membranes [78]. Although glycine betaine naturally accumulates in significant concentration in spinach (*Spinacia oleracea* L.) and sugar beet (*Beta vulgaris* L.) in response to drought, rice does not accumulate this compound at all [81].

Other compounds involved in drought response are polyols, such as mannitol or sorbitol. They are synthetized from glucose-6-phosphate in a relatively short metabolic pathway. In addition, they are chemically stable and not readily metabolized, therefore their concentration can rise rapidly and does not undergo considerable fluctuations. Polyols are thought to stabilize cellular structures [82]. The mannitol content increased in the leaves of *Coffea arabica* L. plants exposed to WD, and decreased after rewatering [83]. The authors explained that mannitol production was replaced by alternative pathways of carbohydrate synthesis, which is desirable in the recovery from environmental stresses.

The increase of numerous primary metabolites was noticed in the study on *Vitis vinifera* L. exposed to WD lasting for a few days [84]. The authors reported the elevated concentration of citric, palmitic, succinic, and tartaric acids as well as D(-) ribose, sucrose, and myo-inositol. However, no significant changes in the content of monosaccharides were observed.

Apart from the compounds involved in the plant's primary metabolism, many secondary metabolites mitigate the adverse results of abiotic stresses, including water deprivation. Stress conditions alter the metabolism mainly by decreasing primary and increasing secondary metabolite production. The increase of the content of many secondary metabolites such as phenolic compounds, terpenes, essential oils, alkaloids, and others was recorded for numerous species grown under WD conditions [85, 86].

Due to drought, plants limit the loss of water by closing stomata [2]. However, this reduces the uptake of carbon dioxide which, in turn, decreases the efficiency of the Calvin cycle. Therefore, the energy accumulated in light-dependent reactions is consumed in an alternative way: the production of highly reduced products [86]. As examples, phenolic acids, lignins, flavonoids, isoflavonoids, anthocyanins, and coumarins are produced under stress through the phenylpropanoid pathway in which phenylalanine is the main substrate [73]. The content of phenylpropanoids changes upon different stresses including WD. Increased production of polyphenols was reported in the leaves of *V. vinifera* L. under drought conditions which lasted for over five days [84]. The authors explained that plants need these compounds for scavenging ROS resulting from photoinhibition caused by drought and exposure to high irradiation. Ahmed et al. [87] pointed to the raised level of phenols and flavonoids in Tibetan wild barley (*Hordeum vulgare* L. ssp. Spontaneum) leaves in two of the three drought-treated genotypes relative to control ones. Flavonoids play a significant role in stress response due to their antioxidant properties [73]. However, the synthesis of flavonoids in grams (*Vigna mungo* (L.) Hepper and *Vigna radiata* (L.) R. Wilczek) was reduced during 21-day water shortage conditions but their content increased again during the recovery period [88]. On the contrary, a several-fold increase in anthocyanin content under drought and a decline upon rewatering was observed in these species. In the aforementioned studies of Akitha Devi and Giridhar [79], only a minor increase of content of isoflavones (daidzein, genistein, glycitein) was observed in soybean seeds coming from plants subjected to WD for five days during their last vegetative stage. However, WD imposition at the beginning of seed development caused the reduction of flavone concentration, probably as result of seed transport inhibition. Noteworthy, in both grams and soy, an elevated concentration of proline was also reported.

Abiotic stress may be advantageous in the production of aromatic or medicinal herbs because it enhances their quality by raising the synthesis of desirable secondary metabolites. Moderate drought during the cultivation of *Thymus vulgaris* L., *Chelidonium majus* L. and *Petroselinum crispum* L. caused the increase of the concentration of terpenes, alkaloids, and essential oils, respectively [85]. Generally, in water-stressed plants, an increased concentration of glucosinolates is observed and the production of alkaloids is more pronounced [73]. The content of different groups of primary and secondary metabolites is significantly influenced by severe drought stress in grapevine leaves [84]. These authors identified 17 volatile metabolites with potential to discriminate between control and stressed plants. Several aldehydes were among the drought-affected substances, suggesting that they may act as ROS scavengers that can be easily oxidized.

Terpenoids are other metabolites involved in stress response, which act as antioxidants and are responsible for the stabilization of lipid membranes [73]. As an example, a higher content of monoterpenes was reported in *Salvia officinalis* L. grown under drought stress conditions compared to well-watered plants [86].

Lignin is another secondary metabolite involved in drought tolerance. It stiffens cell walls, thus inhibiting cell growth and limiting loss of water [89, 73]. The intensive production of lignin was noticed in white clover (*Trifolium repens* L.) subjected to WD for 12 days [89]. Although a rapid increase of this compound content was observed in the case of roots, in leaves a slower accumulation was seen. The authors associated lignin accumulation with increased phenol oxidase and cinnamyl alcohol dehydrogenase enzyme activity, under drought conditions.

16.4.2 Tools to Assess the Metabolome Changes

Plant metabolites important for WD tolerance are diverse not only in their function, but also in their chemical structures and properties. Therefore, the metabolome complexity requires the use of combined modern analytical platforms including nuclear magnetic resonance (NMR) spectroscopy and MS in combination with different separation techniques, namely gas chromatography (GC), liquid chromatography (LC), and related advances: ultra-high performance liquid chromatography (UPLC) as well as capillary electrophoresis (CE) [90–93]. Recent advances in analytical technology allow for the separation, detection, identification, and quantification of thousands of metabolites. According to recent scientific reports, about 50,000 compounds from the plant kingdom have been detected, and new metabolites are continuously reported in the literature. It is predicted that more than 200,000 of them will be described in the near future [93].

GC coupled with mass spectrometry (GC-MS) is widely used in metabolomics studies because of its high resolution, selectivity, and sensitivity. GC-MS is used as a platform in nontargeted analysis, especially for volatile compounds such as low-molecular weight alcohols, monoterpenes, esters, and nonvolatile compounds after derivatization [90, 91, 93]. LC-MS, which is one of the most resourceful techniques for studying plant metabolomics, allows the analysis of a large group of secondary metabolites such as alkaloids, flavonoids, phenolic acids, fatty acids, and sterols [94, 93]. The LC-MS technique is dedicated to the analysis of labile and nonvolatile polar and nonpolar compounds in native form, as well as their characterization and structure [95]. NMR spectroscopy is used for metabolite fingerprinting and profiling. NMR analysis provides structural information for the identification of compounds of interest and direct quantitative data [96]. Metabolite analysis by NMR platform is rapid, nondestructive, and requires minimal sample preparation methods, although the sensitivity of NMR is much lower compared to MS. However, NMR is more effective in providing information about the structure than MS [94, 96, 95].

Another method applied in biochemical profiling is capillary electrophoresis MS (CE-MS), a sensitive technique useful for quantification of charged metabolites [91]. The significant advantages of CE-MS are the short time and small amounts of samples required for analysis, which makes this method promising for high-throughput metabolomics [95]. Furthermore, this technique has the potential to be applied for single-cell analyses.

Each metabolome is composed of a large number of molecules exhibiting high diversity of chemical structures and physical properties. Each technique has both advantages and limitations, thus a combined use of multiple analysis platforms is required. Some examples of aforementioned techniques' application in metabolomics studies of drought-treated plants are presented in Table 16.1.

Metabolome analyses of numerous organisms have provided an enormous amount of data stored in databases, which gather information about diverse metabolites, biochemical reactions, and pathways (please refer to Sect. 16.6.5 for some examples). The use of collected data eases the identification of studied compounds, resulting in fast and accurate metabolome analysis but they need to be continuously updated [92, 93]. In turn, available statistical approaches for a fast conversion of raw data into biologically reliable information also need to be built and improved [94, 93].

In spite of the fact that many plant metabolites have been described, the knowledge covering metabolomics still remains incomplete. However, the advance in analytical technologies provides constant progress in the detection and identification of unknown compounds. Due to global climatic changes, abiotic stresses such as drought acquire significance because of their influence on growth and

Table 16.1 Some metabolite profiling approaches applied to study the impact of drought in plants

Metabolite	Organism	Organ	Experimental approach	Reference
Sugars, proline	*Zea Mays*	Leaves	GC-MS; UPLC	[97]
Sugars, amino acids	*Glycine max*	Leaves, nodules	NMR	[98]
Sugars, sugar alcohols, amino acids	*Medicago x varia*	leaves, flower buds	GC-MS	[99]
Mannitol	*Coffea arabica*	Leaves	HPLC	[83]
Isoflavones, proline	*Glycine max*	Leaves	HPLC, spectrophotometry	[79]
Polyamines	*Nicotiana tabacum*	Leaves, roots	HPLC	[75]
	Brassica rapa L. subsp. *chinensis*	Leaves	HPLC	[76]
Polyphenols, volatiles	*Vitis vinifera*	Leaves	LC-MS/MS, GC-MS	[84]
Essential oils, phenolics	*Eucalyptus* spp.	Leaves	GC-MS, HPLC, spectrophotometry	[100]

development of plants and crop quality. Therefore, metabolomics plays a major role in bringing new knowledge of the changes in biochemical profiles caused by drought.

16.5 Deciphering the Role of Noncoding Genome in Plant Drought Resistance

Epigenome remodeling by environmental stimuli for transcriptional and genomic stability is an emerging and growing field, which can provide new targets for plant breeding. Noncoding RNAs, and in particular microRNAs (miRNAs) have been subjected to intensive studies in crop plants, namely because their expression could be modulated by epigenome modifications [101–103].

miRNAs are a class of small noncoding (20–24 nucleotides) RNAs that act at the posttranscriptional level, leading to gene silencing through mRNA cleavage and translation repression based on sequence complementation [104, 105]. miRNAs are coded by *MIR* genes that are often located in intergenic regions, and independently transcribed by the RNA polymerase II (RNA POLII) into primary miRNAs (Pri-miRNAs; [106]). Pri-miRNAs are then processed by different members of the Dicer-like (DCL) family. In Arabidopsis, DCL1 (an RNAse III enzyme) is the main enzyme responsible for the cleavage of pri-miRNAs into the stem loop precursors of miRNAs, and also for the processing of the miRNA/miRNA* duplex in the nucleus [107]. *MIR* genes can also be found in exonic and intronic sequences of their coding genes (mirtrons). Their transcription is dependent on their host genes and miRNA processing can bypass the canonical DLC1 precursor-miRNAs processing step [107]. In addition, it was also documented that some plant miRNAs were derived from transposable elements (TE; [108]). Then hua enhancer1 (HEN1) methylates each strand of the miRNA/miRNA* duplex to avoid degradation, and finally the duplex is transported to the cytoplasm with the assistance of the hasty protein (HST). In the cytoplasm, the duplex is separated and the guide miRNA is incorporated into the protein complex known as RNA-induced silencing complex (RISC) through binding with Argonaute1 (AGO1). Then, the RISC assembly binds to its target through sequence complementarity of their mature miRNA, leading to suppression of the target gene's expression, both by cleavage or translation inhibition of the transcript [103].

The expression of miRNA has been demonstrated to be altered in response to drought stress, suggesting that *MIR* genes may be involved in the regulation of the expression of drought-responsive genes [109, 102, 110, 111, 112, 113] (Table 16.2). miRNAs that target genes underlying drought responses may be used to improve crop drought tolerance [110]. Therefore, the identification/characterization of drought-responsive miRNAs is an important goal to prospective new approaches to engineer plants better adapted to cope with drought stress conditions.

Table 16.2 Identification of drought-responsive miRNAS in crop plants

Species	Type of stress	Source
Apium graveolens	Osmotic stress	[114]
Brachypodium distachyon	Water deficit	[115]
Brassica juncea	Osmotic stress	[116]
Cicer arietinum	Water deficit	[117]
Gossypium hirsutum	Osmotic stress	[118, 119]
Hordeum vulgar	Water deficit	[120, 121]
Leymus chinensis	Osmotic stress	[122]
Manihot esculetum	Water deficit	[123]
Nicotiana benthamiana	Osmotic stress	[124]
Nicotiana tabacum	Osmotic stress	[125]
Oenanthe javanica	Osmotic stress	[126]
Oryza sativa	Water deficit	[112, 127, 128]
Panicum virgatum	Osmotic stress	[129]
Saccharum spp	Water deficit	[111, 130, 131]
Solanum tuberosum	Water deficit Osmotic stress	[132–134]
Triticum aestivum	Osmotic stress	[135, 136]
Vigna unguiculata	Water deficit	[137]
Zea mays	Osmotic stress Water deficit	[138–141]

16.5.1 Identification of miRNAs and Their Target Genes

Plant miRNAs were first reported in 2002 [142–144], but the number of miRNAs identified in different plants was boosted due to the use of new generation sequencing technologies and to the development of predictive computational approaches (reviewed by Liu et al. [145] and Kang and Friedländer [146]).

Sequencing and analysis of smallRNA (sRNA) libraries has been the main strategy for the efficient discovery and quantification of conserved, novel, and rare miRNAs in crop plants. A general framework of sRNA data analysis for identification of miRNAs and miRNA targets was discussed in Sun et al. [147], Budak and Akpinar [101], and Kang and Friedländer [146]. Once the sRNA libraries have been sequenced by NGS, the NGS reads are filtered for their quality and mapped against different databases in order to remove noncoding RNAs other than miRNAs (e.g., snoRNA, lncRNA, rRNA) and repetitive sequences. Then, to identify their miRNA precursor sequences candidates, NGS reads are mapped against public/private reference genome sequences available (e.g., the phytozome) and/or blasted against the miRNA databases (e.g., miRBase). Sequences of miRNA precursor candidates are then filtered under different structural feature criteria [148]. Several computational approaches have been used for the discovery of miRNAs associated with the response of a crop to WD and osmotic stress. For example, the suites MirDeep-P

[149], mirDeepFinder (http://www.leonxie.com/deepfinder.php, [150]), UEA sRNA toolkit-Plant version filter pipeline (http://srna-tools.cmp.uea.ac.uk), and miRPlant [151] have been used for miRNA discovery in switchgrass (*Panicum virgatum* L.; [129]), mustard (*Brassica juncea* L. Czern.; [116]), *Saccharum* spp. [130], and rice [151], respectively.

Several miRNA target prediction software packages have been developed based on the high level of complementarity required in plants for miRNA action [152, 153]. Some of them have been used to predict drought-responsive miRNA target transcripts in crop species, such as psRNATarget (www.plantgrn.org/psRNATarget , [154]), TargetFinder (https://github.com/carringtonlab/TargetFinder, [155, 156]), miRNAassist (http://www.borland.com, [157]), or WMD3 (http://wmd3.weigelworld.org [158, 159]). Among the different experimental approaches to validate miRNA target predictions, degradome sequencing (Degradome-Seq) analysis [160] has been used to confirm plant miRNA–mRNA target sites in cotton [119] and barley [121] in the response to osmotic and WD stress, respectively.

Deep sequencing is a robust strategy to identify new and rare miRNAs, however, other platforms based on microarray assays such as the μParaflo microfluidic microarray (LC Sciences, http://www.lcsciences.com/) have been used. The μParaflo microfluidic microarray allows an expeditious identification of differentially expressed miRNAs, which can be used as a high-throughput platform to study the expression of hundred miRNAs and validate results from deep sequencing small libraries. It is important to note that this platform could be easily customized for new species-specific miRNAs. This platform had been used to identify differentially expressed during osmotic stress responses in maize [138, 141]. Quantitative PCR-based methods are also widely used to profile and validate the expression of miRNAs and their targets. Loop-PCR [161, 162] is a reliable method to quantify the expression distinguishing a single-nucleotide difference of miRNAs. Indeed, loop-PCR has been used to quantify the expression of miRNAs, induced or repressed during drought, in different crop species such as *Cicer arietinum* L. [117], *G. hirsutum* L. [119], *H. vulgare* L. [120], *Manihot esculeta* Crantz [123], *Nicotiana tobacum* L. [125], *Oenanthe javanica* (Blume), DC [126], *P. virgatum* L. [129], *Saccharum spp* [111, 130], *Vigna unguiculata* (L.), Walp [137], and *Z. mays* L. [139, 140]. Another strategy to quantify miRNAs involves a first step of adenylation of the 3′-end of miRNA, and then reverse transcription of the poly-adenylated miRNA, followed by a second step for qPCR amplification. This strategy was used to profile the differentially expressed miRNAs in *Brachypodium distachyon* (L.) Beauv [115] and in *B. juncea* (L.) Czern [116].

16.5.2 miRNA-Mediated Regulation of Drought Stress Responses

Drought-responsive miRNAs are expected to regulate negatively the expression of drought-responsive genes. A growing number of publications on identification of

crop miRNAs and associated targets implicated in drought stress resistance are available. For a comprehensive example, please refer to the work of Ferdous et al. [110] and Table 16.2.

Chickpea (*C. arietinum* L.) plants with enhanced miR408 expression showed plantacyanin transcript repression, subsequent regulation of dehydration-responsive element-binding (DREB), and other drought-responsive genes, resulting in enhanced tolerance to WD [117]. In another study, in maize, Luan et al. [139] reported that the regulation of the transcription factor NF-YA by miR169 plays a critical role during plant development and plant responses to abiotic stress, namely in drought stress. In potato, miR172, miR396a, miR396c, and miR4233 may regulate the pyrroline-5-carboxylate synthetase (P5CS) gene, whereas miR2673 and miR6461 may regulate the pyrroline-5-carboxylate reductase (P5CR) and proline dehydrogenase (ProDH) genes, which are three genes involved in proline metabolism [132]. The expression of stress-responsive miRNAs seems to be highly dependent on the genotype, developmental stage, and tissue, but also on the dose and time of drought stress. The miRNA expression analysis in leaves and roots of two cultivars of cowpea (*V. unguiculata* (L.) Walp), with different responses to drought stress, revealed that the same miRNA had a cultivar and tissue-dependent response to drought stress [137]. A similar feature was described for barley, in which hvu-miR159b, hvumiR166a, ath-miR172a, osa-miR393a, and hvu-miR5048 were differentially expressed between irrigated and drought conditions [121]. Interestingly, hvu-miR159b and hvu-miR166a were upregulated in leaf tissues, whereas in root tissues they were downregulated under drought. Seven drought-responsive *T. aestivum* MIR precursors (TaMIR1136, TaMIR156, TaMIR408, TaMIR1119, TaMIR1129, TaMIR1133, and TaMIR1139) showed dose-dependent and typical temporal expression patterns during drought induction [136]. Also for cotton, the changes in miRNAs and their target expressions were dose-dependent and tissue-dependent under drought conditions [118]. Kansal et al. [127] analyzed the dynamic of spikelet miRNA population in the anthesis stage during drought stress in rice. These authors found specific patterns associated with drought-tolerant or -susceptible cultivars suggesting an evolution of distinct and variety-specific regulatory mechanisms. Importantly, a significant proportion of these drought-responsive genes co-located within QTLs related to drought tolerance and associated traits.

Despite the large amount of studies on the identification and characterization of miRNAs in crops, there is a need to perform more system-level research to better understand their role in the gene–gene interaction networks [110, 163]. Indeed, several questions still need to be addressed: (a) the interaction between the miRNA and their potential target genes is not well known; (b) the pleiotropic effects associated with overexpression of miRNA must be considered; (c) the presence of isomiRs–miRNAs variants that originate from the same miRNA precursor and shared similar sequences. This adds another level of complexity to gene regulation, as they can display targets and function, contributing for a precise temporal and spatial gene regulation [109]; (d) miRNAs can also inhibit expression of MIRs genes and targets genes by directing DNA methylation [164]; and (e) primary

transcripts of microRNAs encode regulatory peptides (miPEP) that were found to enhance the transcription of the pri-miRNA [165]. Moreover, translation inhibition by miRNA in plants and the balance between cleavage and translation repression are still unclear regulatory mechanisms of gene expression. These challenging questions should be addressed in order to optimize miRNA-based technology to improve agricultural crop resistance to drought stress in a climate-changing world.

16.6 The Crucial Role of Bioinformatics

Computational analysis of biological data has played a crucial role in every aspect of omics-based research. The advent of high-throughput techniques to screen the changes in transcriptomes, proteomes, or metabolomes at a global scale resulted in a large amount of genome-scale datasets. As an example, the European Bioinformatics Institute (EBI) hosts a database that currently has more than 629 million sequence entries (release 125, European Nucleotide Archive, accessed 10 October, 2015). The availability of fully annotated genome sequences facilitates DNA-based comparative genomics studies to understand evolutionary or phylogenetic relationships between species. Large-scale gene-expression data are crucial to unveil gene expression patterns, transcriptional modules, identify co-expressed genes, transcriptional regulators, and conserved *cis*-element motifs. MS data provide valuable information about abundance patterns of proteins and metabolites, allowing the development of detailed protein–protein interaction and metabolic network maps. In this context, bioinformatics tools are needed to manage the various types of genome-scale datasets effectively and to integrate them and extract a biologically meaningful interpretation of the plant system under study [166].

Bioinformaticians use computational and statistical skills to analyze biological data and extract new knowledge. This is done using both new experimentally generated datasets and publicly available data. To accomplish this goal, omics datasets need to be carefully recorded and stored in databases, in which data can be queried, compared, and analyzed [167]. The use of common formats to enable data exchange and reproducible methods for data annotation is a prerequisite to allow dataset comparison. The integration of the observed changes into a pathway analysis or data modeling approaches may further extend bioinformatics analysis. In this section, we provide an overview of several omics dataset´s repositories and bioinformatics tools available for noncomputational plant science researchers that can be freely accessed online.

16.6.1 General Sequence Repository Databases

Several repository databases are used to share nucleotide sequencing information, covering raw sequencing data, sequence assembly information and functional

annotation. In this section, we focus on the International Nucleotide Sequence Database Collaboration (INSDC; http://www.insdc.org). The INSDC initiative gathers three partners: the DNA Databank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/) at the National Institute for Genetics in Mishima (Japan), the European Molecular Biology Laboratory's European Bioinformatics Institute (EMBL-EBI; http://www.ebi.ac.uk/ena) in Hinxton (United Kingdom), and the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/genbanK) in Bethesda (Maryland, United States). INSDC partners work closely together to provide data formats and conventions that enable consistent data submission to their databases and support regular data exchange around the globe [168]. In addition to the permanent scientific record for nucleic acid sequencing, INSDC provides information relating to samples and experimental configurations [169]. Within INSDC-associated databases, data access is provided though browser/search tools and allows large-scale file downloads.

16.6.2 Plant Genome Repositories

The number of plant genomes sequenced and annotated is growing rapidly, as the result of genome sequencing projects launched by consortia gathering worldwide plant genomics and bioinformatics groups. Access to the raw genome sequence alone is not enough for their full use. Online genome browsers provide supplemental resources for data analysis. These can include locations of genes and their transcripts, GC content, links to external databases (for more information of the protein product of a gene, for example) and online BLAST services. Some examples of currently available genome databases and browsers can be seen in Table 16.3.

Not all genome browsers focus on a single species. Phytozome (http://www.phytozome.net, [177]) and EnsemblPlants (http://plants.ensembl.org, [178]) are examples of genome browsers with user-friendly interfaces for accessing and comparing genome-scale data from species of scientific interest across the taxonomy. The last release of Phytozome (version 10.3.1, accessed 10 October, 2015) provides access to 61 sequenced and annotated green plant genomes. Currently,

Table 16.3 Some examples of current available genome databases

Organism/Family	Database	Reference
Arabidopsis	Arabidopsis Information Resource (TAIR)	[170]
Grasses	Gramene	[171]
Solanaceae	Solanaceae Genomics Network (SGN)	[172]
Physic nut	Jatropha Genome Database (JGD)	[173]
Rosaceae	Genome Database for Rosacear (GDR)	[174]
Legumes	Legume Information System (LIS)	[175]
	Legume Integrative Platform (Legume IP)	[176]

Ensembl Plants (release 28, accessed 10 October, 2015) provides access to 39 species. These databases provide comparative genomics information, such as synteny and sequence conservation, genome alignments, phylogenetic trees, and gene orthology relationships.

Several genome browsers have implemented BioMart [179] an open-source standardized interface for data query and download. Users familiar with the BioMart interface in one database will find an identical one when using another browser that also has the service. In addition to a point-and-click interface, BioMart provides an application programming interface (API), making it accessible from within programming languages such as R. This makes it easier to integrate data retrieval in an analysis pipeline and, by specifying the database version that is to be accessed, to increase reproducibility.

16.6.3 Transcriptome Repositories

NGS experiments have provided a long list of coding and noncoding transcripts for a wide range of model and crop plants [8]. By browsing Sequence Read Archive (SRA, [180]) in NCBI (http://www.ncbi.nlm.nih.gov/sra) for the taxa *Viridiplantae* (NCBI Taxonomy ID: 33090), we presently (on 10 October, 2015) retrieved 95,749 entries, 93,630 out of which refer to "seed plants". The rate of deposition of new sequenced transcriptome data is increasing, driven mainly by the increasing number of running NGS projects and the increasing availability and decreasing cost of sequencing. SRA stores raw sequence data from all available NGS technologies and alignment information in the form of read placements on a reference sequence. Coupled to these molecular data, a comprehensive description of the samples (Biosample, http://www.ncbi.nlm.nih.gov/biosample) and experiments (Bioproject, http://www.ncbi.nlm.nih.gov/bioproject) undertaken can also be queried.

Two repositories specialized in gene expression data: ArrayExpress from EBI and Gene Expression Omnibus (GEO) from NCBI. Like other EBI and NCBI resources, the datasets are mirrored between the two. Processed gene expression data are provided in addition to raw sequence information. When submitting data to these repositories, researchers are requested to provide information regarding the samples, experimental design, protocols used, and analyses performed. This information makes it easier to find, use, and compare data from published experiments. Most journals require that the authors of any publication that has created a transcriptomics dataset make it available to the community through one of these repositories.

miRBase is the public repository for all published microRNA sequences and associated annotation [181, 182]. All miRBase microRNA sequence data and annotation are accessible through the website (http://www.mirbase.org/). Presently, miRBase gathers 28,645 miRNA entry sequences and respective annotations. Similarly to coding transcriptomes, the rate of deposition of new miRNA is increasing, especially in crops that are not commonly used as model organisms. By

browsing miRBase for *S. tuberosum*, for example, we could retrieve 224 miRNA precursors (stem-loop) as well as the sequences of resulting mature miRNAs. No less relevant is the emergence of validated miRNA-target databases, to support the development of miRNA research further. miRTarBase (http://mirtarbase.mbc.nctu.edu.tw, [183]) and DIANA-TarBase (http://www.microrna.gr/tarbase, [184]) are the most updated and comprehensive databases with information of experimentally validated miRNA-target interactions. PASmiR (http://pcsb.ahau.edu.cn:8080/PASmiR) is another comprehensive literature-curated and Web-accessible repository for miRNAs involved in plant response to abiotic stresses [185].

16.6.4 Protein Data Repositories

The Universal Protein Resource (UniProt, http://www.uniprot.org) is the most comprehensive data repository on protein sequence and functional annotation. The UniProt database has doubled in size to 80 million sequences during the past year [186]. Currently, UniProt is constituted of several components: (a) UniProtKB/Swiss-Prot contains manually curated and reviewed entries; (b) UniProtKB/TrEMBL contains automatically annotated but not manually curated entries; (c) UniRef provides clustered entries sets based on the percentage of identity of protein sequences; and (d) UniParc database is a comprehensive set of all known sequences indexed by their unique sequence checksums. Additionally, in UniProt reference proteomes it is possible to retrieve annotated proteomes from a particular taxonomic group. Presently, 48 proteomes of *Magnoliophyta* division could be browsed.

Concomitantly, several repositories have been established to store protein and peptide identifications derived from MS, with the proteomics identifications being (PRIDE) database (http://www.ebi.ac.uk/pride, [187]) the most comprehensive one.

16.6.5 Metabolome Repositories

Metabolomics, the comprehensive analysis of metabolites in a biological specimen, is a promising technology for research plant sciences. In recent years, several metabolomics reference databases devoted to a specific species or analytical approach have been made available to the public. MetaboLights (http://www.ebi.ac.uk/metabolights; [188]) appeared recently as an open-access repository for metabolomics studies. MetaboLights has the advantage of being cross-species and cross-techniques which is an added value for comparative or systems biology approaches. Using this database, the metabolite structures and their reference spectra can be retrieved, as well as their biological roles, locations, concentrations, and raw data from metabolomics experiments.

16.6.6 How to Give Biological Relevance to Omics Datasets?

The vast amounts of data produced by genome-wide techniques require careful data processing. The specifics vary with the type of experiment performed, but all analysis pipelines should include stringent data quality control, appropriate normalization techniques, and rigorous statistical testing. It is important to consult with bioinformaticians and statisticians when designing the experiment, as the design affects the kind of analysis that can be done. Once the data are available, with the high number of elements assayed at one time (e.g., transcripts, proteins), it is often helpful to do some exploratory data analysis. Clustering has become an integral step in exploratory data analysis in order to group similar elements into classes [189]. Several computational algorithms were developed to order and visualize the underlying patterns in large-scale expression datasets showing similar patterns that can, therefore, be grouped according to their co-regulation or co-expression [167]. Table 16.4 summarizes some free and user-friendly clustering software available on the Web. Dimensionality reduction techniques such as principal component analysis (PCA) can also help to deal with the high number of dimensions in a typical genomewide analysis.

Clustering based on a gene product with known/predicted function, based on Gene Ontology Resources [193], is also an alternative. Gene Ontology provides a nested controlled vocabulary to annotate genes based on the biological function, molecular process, and cellular location for their products. Different software packages (see Table 16.5, for some freely available examples) were developed for grouping annotation sets by category (GO-slims) or perform statistical evaluation of gene sets based on GO terms enrichment (Enrichment Tools).

Table 16.4 Software for clustering transcriptomic and proteomic data

Software	Link	Reference
MultiExperiment Viewer (MeV)	http://www.tm4.org/mev.html	[190]
EXpression Analyzer and DisplayER (EXPANDER)	http://www.cs.tau.ac.il/~rshamir/expander/expander.html	[191]
Statistical Utility for Microarray and Omics data (SUMO)	http://angiogenesis.dkfz.de/oncoexpress/software/sumo/	–
Bioconductor/R	http://www.bioconductor.org/packages/release/BiocViews.html#___Clustering	[192]

Table 16.5 Software for GO enrichment in plant datasets

Software	Link	Reference
GoMiner	http://discover.nci.nih.gov/gomine	[194]
AgriGO	http://bioinfo.cau.edu.cn/agriGO/	[195]
MERCATOR	http://mapman.gabipd.org/web/guest/mercator	[196]
PlantGSEA	http://structuralbiology.cau.edu.cn/PlantGSEA	[197]

More information about how to use and interpret information extracted from GO resources is available at Blake [198].

16.6.6.1 Future Challenges for Bioinformatic Data Analysis

The increasing amount of sequencing throughput and its greater availability poses both serious challenges and opportunities for computational analysis. Bioinformatics does not only have to provide the structures in which to store the information, but also store it in a such way that is retrievable, and comparable to similar data or other types of information [167]. The development of guidelines (also known as "Minimum Information About") for transcriptomics/proteomics experiments may encourage a standardization of procedures favoring the use of similar data formats by the majority of the scientific community. Centralized data repositories play a central role in enforcing these guidelines upon dataset submission, as do journals in requiring that new publications have their data submitted to these repositories.

Sequencing brings genomewide techniques to nonmodel organisms. As long as the nucleic acids have been extracted efficiently, the sequencer will work well for a newly discovered species as it does for yeast, and there is no need to create bespoke microarrays. However, researchers embarking on these projects should not underestimate the difficulties involved. Measuring the levels of gene expression in RNA-seq experiments is much easier if a well-annotated genome is available. For example, having to do *de novo* transcriptome assembly increases the experiment's complexity and in some cases can be a whole project on its own. Nevertheless, the expanding popularity and increasing standardization of genome-wide techniques bring great opportunities for the study of non-model plants and crops.

16.7 Integrating Data—The Quest for Systems Biology Approaches to Understand Plant Drought Adaptation

Systems biology comprises the iterative cycling between experimental (wet-lab) and computational (dry-lab) approaches with the aim of generating a comprehensive understanding of biological systems [199].

One of the major challenges of systems biology is the integration of the different large omics datasets originated from the same biological sample to attain a holistic overview of all regulatory processes and reactions. This is relevant to identify the complex gene regulatory networks composed of genes, noncoding RNAs, proteins, metabolites, and signaling components [200] and their relevance for the plant's development and response to environmental stimuli [201]. Effective data integration depends not only on experimental design but also on data quality, the availability of statistical methods and algorithms that can address the data complexity extracting

Table 16.6 Omics integrative and visualization tools for networks and biological pathways

Software	Link	Reference
VANTED	https://immersive-analytics.infotech.monash.edu/vanted	[199]
Cytoscape	http://www.cytoscape.org	[206]
MapMan	http://mapman.gabipd.org/web/guest/mapman	[208]
WikiPathways	http://www.wikipathways.org	[209]
GenMAPP	http://www.genmapp.org	[210]

usable information. Sussman et al. [202] provide additional considerations for researchers aiming to develop approaches envisaging data integration.

Much effort has gone into developing methods and applications (see Table 16.6) to integrate various genome-scale data types. These software platforms are used to identify and decompose cellular regulatory networks, and if desired, to perform modeling studies and assess their relevance for the plant's development and response environmental stimuli [201, 203]. Many of these tools are tightly integrated with public databases (e.g., UniProtKB/SwissProt), thus allowing users to visualize and interpret their own data in the context of previous knowledge [204]. Among the relevant tools available, the VANTED [199] framework seems to be one of the most promising for plant systems biology, because it integrates the MetaCrop database [205] as a data source. Nevertheless, it is worth highlighting that the Cytoscape platform [206] is one of the most used (for an example see the work of Verdier et al. [207]).

The integrative analysis of transcriptome changes coupled with proteomic and metabolomics data would lead to a characterization of the changes observed from a gene-to-metabolite perspective, providing groundbreaking knowledge on the molecular response of plants to drought. However, this objective is still far from being completely accomplished. Although several studies on animal cells take full advantage of omics data integration (e.g., see Wilmes et al. [211] and Villar et al. [212]), very few reports on the literature are available for plants (either cells or tissues) indicating that this is still a huge challenge for plant researchers. However, we herein refer to some successful case studies in which data integration made significant advances on the global molecular understanding of plant drought responses.

In a very recent study, Rabara et al. [213] identified major changes in physiology, metabolites, mRNA levels, and promoter activities during the tobacco (*Nicotiana tabacum* L.) response to drought. The integration of all datasets allowed the identification of features unique to tobacco or members of the *Solanaceae* family reponse to drought, such as increased production of glutathione and tocopherol, ammonia detoxification accumulation of amino acids acting as osmolytes, and activation of the raffinose pathway. Data also pointed to extensive regulation of nitrogen metabolism, activation of gamma-aminobutyric acid (GABA) shunt to control cytoplasmic pH, maintain C/N balance, and oxidative stress protection.

Global transcriptional and metabolic responses to drought and rewatering were investigated in *Medicago truncatula* Gaertn., a model legume species [214].

Integration of metabolomic and transcriptomic data highlighted a remarkable regulatory role of myo-inositol and proline pathways on drought-stress adaptation in this species.

The metabolomic and transcriptomic changes in anthers, pistils before pollination, and pollinated pistils were studied in a heat-tolerant rice (N22) and a heat-sensitive (Moroberekan) rice cultivar under heat and drought stress [215]. The carbon-starved anthers (CSA) gene was highly expressed in the susceptible line, whereas the tolerant line responded with high expression of genes encoding a sugar transporter (MST8) and a cell wall invertase (INV4), markers for high sink strength. Overall results highlighted that sugar metabolism is a crucial metabolic and transcriptional component that defines the reproductive success of rice under stress.

Data integration provides a comprehensive overview of all regulatory processes driving the response to environmental/developmental perturbation studied. By linking these network models with phenotypic traits, a number of new targets (e.g., genes) will be made available for molecular breeding approaches aiming at the improvement of plant responses to drought stress. Although the potentialities of omics data integration are enormous, its application is still mostly restricted to model plants [6] and focused on the integration of two types of omics datasets (e.g., transcriptomic and metabolomic or transcriptomic and proteomic). Inasmuch as any systems-level response is a result of complex interplay between gene regulation, posttranslational modifications, and metabolic fluxes, these studies might have missed responses visible only by investigating all three omics levels simultaneously [216]. Nevertheless, the data generated with model systems can still be extrapolated to crops using translational genomics approaches.

16.8 Conclusions and Future Prospects

The threatening scenario imposed by climate change will severely affect agriculture, especially in drought-prone regions of the developing world [217]. Consequently, there is a need for concerted crop improvement approaches to mitigate crop failure under marginal environments. In a context of food security, plant improvement should develop crops able to cope with environmental injuries, such as drought, but still capable of achieving yield with substantial quality thus contributing to the implementation of a sustainable agricultural system.

Drought stress is one of the major environmental stresses affecting plant productivity and numerous studies have addressed the different mechanisms underlying a plant's adaptation to water deprivation (for a review see Chaves and Oliveira [218]). In this review, we focused on the recent developments of plant genomic resources by the use of available high-throughput technologies. Several genes, molecular markers, proteins, or metabolites with a relevant role on drought stress tolerance have been unveiled. This knowledge can be used to generate stress-tolerant plants via marker-assisted breeding or new molecular breeding techniques. Although pioneer works have been conducted on model systems such

as Arabidopsis and *M. truncatula*, numerous studies are being presently conducted on economically important crops, with considerable success. The main conclusion of our review is that systems biology approaches are boosting the translation of knowledge to generate new crop genomic resources.

The application of high-throughput techniques as described for transcriptomics, proteomics, and metabolomics approaches, has revolutionized drought stress research. Nevertheless, important setbacks need to be overcome, such as cost and access to cutting-edge analytic platforms including access to advanced integrative data analysis platforms and know-how. Several initiatives to make the availability of these techniques widespread to multiple end-users were developed. The past European initiatives PRIME-XS (7th Framework Program, http://www.primexs.eu) or Cooperation in Science and Technology (COST) actions such as Plant Proteomics in Europe (http://www.cost.eu/COST_Actions/fa/Actions/FA0603) and Next Generation Sequencing Data Analysis Network (http://www.cost.eu/COST_Actions/bmbs/BM1006) have contributed importantly to this goal. Presently, the running COST action, "The quest for tolerant varieties—Phenotyping at plant and cellular level" (http://www.cost.eu/COST_Actions/fa/FA1306) gathers a critical forum on phenotyping and aims to exploit various -omics areas and/or physiology to (re)discover tolerant varieties. This initiative also contributes to grant access to high-throughput facilities and develop know-how on data analysis to support all plant sciences researchers willing to implement systems biology approaches to understand drought stress tolerance in plants.

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Chapter 17
Transgenic Plants for Higher Antioxidant Content and Drought Stress Tolerance

Chandrama Prakash Upadhyaya and Mohammad Anwar Hossain

17.1 Introduction

Stress, by definition, is any external factor that exerts a detrimental impact on the plant growth and development. Therefore, it is vital to understand the basics of plant responses to various abiotic stresses associated with climate change. Abiotic stresses are the extreme restriction for crop production worldwide and account for yield reductions of as much as 50–60 % [2, 164]. Crop plants, as sessile organisms, encounter unavoidable abiotic stresses during their life cycles, including salinity, drought, extreme temperatures, metal toxicity, flooding, UV-B radiation, ozone, and so on, which all pose a serious threat to plant growth and development, metabolism, and productivity [83, 84, 86, 88]. Among these abiotic stresses, drought is supposed to be the most complex and devastating on a global scale [156], and its frequency is expected to increase as a consequence of climate change [33, 109]. According to the *Water Initiative* report of The World Economic Forum at Davos [99], water shortages are expected to deplete global crop production losses of up to 30 % by 2025, compared to current yields. Therefore, drought stress represents a major threat for sustaining food security under current conditions and will be more of a danger in the future, as climate change is projected to induce more frequent and more intense higher temperatures and drier conditions in many regions of the world [59, 129, 171, 177].

Drought is one of the most important limiting factors for agricultural crop production all around the world. Drought stress both during vegetative and early

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reproductive growth reduces yield. The assimilation of vegetative parts contributes to yield and that is why vegetative-stage drought is important; more emphatically, drought in the reproductive stage diminishes the yield directly by hampering the reproductive parts or developmental stages, which are very susceptible to all kinds of stress, including drought. Drought hampers the yield by obstructing the panicle, peduncle, rachis, tiller growth, and development; reducing the number of seeds, seed size, and seed quality. The complex nature of the morphological, physiological, and phonological traits of plant genotypes are influenced by the soil moisture, which determines the yield. Effects of drought on crop plants also depend on their developmental stages. Although the pattern of damage may differ, the result is the same (i.e., reduction of yield). The primary effect of drought stress is largely a reduction in plant growth, which depends on cell division, cell enlargement, and differentiation, and involves genetic, physiological, ecological, and morphological events, and their complex interactions. These events are seriously inhibited by drought stress, which adversely affects a variety of vital physiological and biochemical processes in plants, including stomatal conductance, membrane electron transport, carbon dioxide (CO_2) diffusion, carboxylation efficiency, water-use efficiency (WUE), respiration, transpiration, water loss, photosynthesis, and membrane functions (Fig. 17.1). Disruption of these key functions limits growth and developmental processes, and leads to reductions in final crop yield [161].

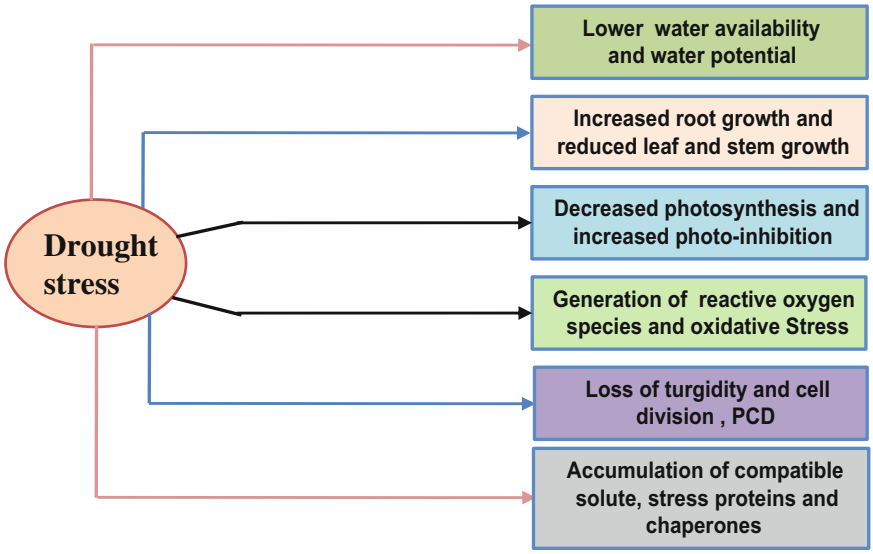

Fig. 17.1 Possible effects of drought stress in plants. Reduced water uptake results in a decrease in tissue water content and reduction in turgidity due to drought. Under drought stress conditions, cell elongation in higher plants is inhibited by reduced turgor pressure. Drought stress also inhibit cell elongation, and expansion result in growth reduction. Severe drought conditions limit photosynthesis due to a decrease in the enzyme activities required for photosynthesis. Drought stress disturbs the balance between the production of ROS and antioxidant defense, causing oxidative stress

Drought stress, like other kinds of abiotic stresses, aggravates the production of ROS such as $O_2^{\bullet -}$, $^1O_2$, H_2O_2, and $OH^{\bullet}$ to levels that are often beyond the plant's scavenging capacity. This causes oxidative stress that damages cells and cellular components, disrupts the physiological and biochemical life processes, and even leads to cell death [58, 85, 109, 120, 182]. Improvements in tolerance and resistance to drought stress will require intensive studies, and researchers are now focusing on oxidative stress as one of the basic damage responses in almost all kinds of stress. In this chapter, we review the generation of ROS under drought stress. We also review recent reports on development of drought stress tolerance transgenic plants via the antioxidant mechanism or removal of ROS from different cellular organelles.

17.2 Oxidative Stress and Reactive Oxygen Species

The O_2 molecule is a free radical, contain two impaired electrons with the same spin quantum number. This spin makes O_2 prefer to accept its electrons one at a time, leading to the generation of the so-called ROS. Reactive oxygen intermediates (ROIs) are partially reduced forms of atmospheric oxygen (O_2). They typically result from the excitation of O_2 to form $^1O_2$ or from the transfer of one, two, or three electrons to O_2 to form a $O_2^{\bullet -}$, H_2O_2, or a $OH^{\bullet}$. In contrast to atmospheric oxygen, ROIs are capable of unrestricted oxidation of various cellular components which in turn lead to the oxidative damage of the cell [12, 14, 42, 80]. It has been estimated that 2 % of O_2 consumption leads to the formation of ROS in plant tissues [26]. Increase of ROS as a result of numerous environmental stresses is the key to the loss of crop productivity worldwide [135]. ROS affects several cellular functions via damaging nucleic acids, oxidizing proteins, and causing lipid peroxidation [66]. Gratao et al. [75] established that whether ROS will act as protective, damaging, or signaling factors depends on the subtle equilibrium between ROS production and scavenging at the proper cellular site and time. ROS are also produced continuously as by-products of several metabolic pathways that are localized in different cellular compartments such as chloroplast, mitochondria, and peroxisomes [46, 149]. In higher plants and algae, photosynthesis takes place in chloroplasts, which contain a highly organized thylakoid membrane system that contains components of the light-capturing photosynthetic apparatus and provides all structural properties for optimal light harvesting. Oxygen generated in the chloroplasts during photosynthesis can accept electrons passing through the photosystems, thus forming $O_2^{\bullet -}$. Under steady-state conditions, the ROS molecules are scavenged by various antioxidative defense mechanisms [66, 77]. The equilibrium between the production and the scavenging of ROS may be perturbed by various biotic and abiotic stress factors such as salinity, UV radiation, drought, heavy metals, extreme temperature, nutrient deficiency, air pollution, herbicides, and pathogen attacks. These disturbances in equilibrium lead to a sudden increase in intracellular levels of

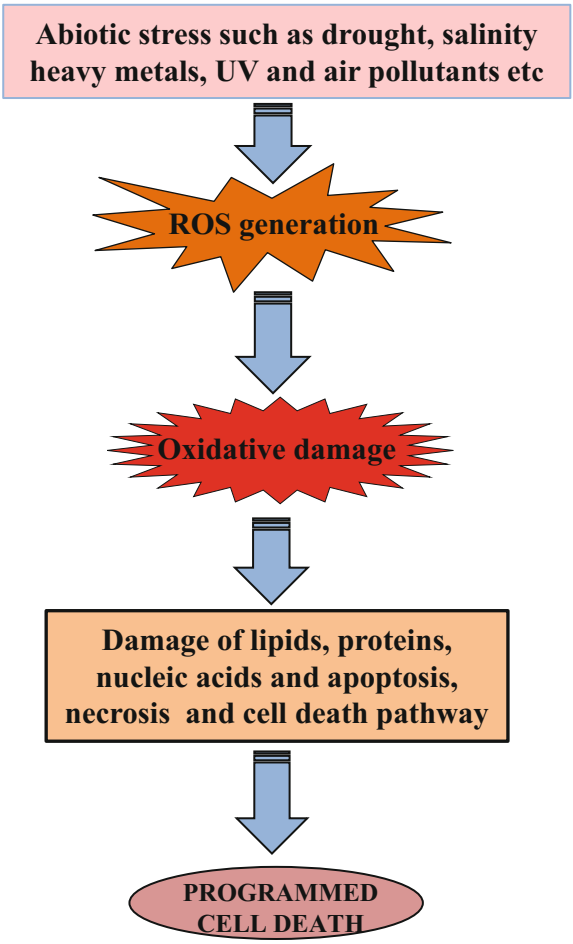

Fig. 17.2 Reactive oxygen species induced oxidative damage to proteins, lipids, and nucleic acids. Oxidative stress induced by the accumulation of ROS can bring out a range of stress responses. Exposure of cells to severe oxidative stress can elicit lethal response pathways such as apoptosis and possibly other forms of cell death pathways which can ultimately lead to programmed cell death

ROS that can cause significant damage to cell structures (Fig. 17.2). Exposure of cells to severe oxidative stress can elicit a lethal response pathway such as apoptosis and possibly other forms of cell death pathway that can ultimately lead to programmed cell death.

Plants, being sedentary, are inept to escape from stress and often display exceptional adaptive responses to overcome these stresses. A rapid transient production of huge amounts of reactive oxygen species called an oxidative burst is supposed to be the most common feature associated with plant responses to stress. Leshem and Kuiper [118] proposed a hypothesis termed the "General Adaptation Syndrome (GAS) response" according to which different types of stress evoke a similar adaptive response and implicating the role of ROS in the underlying

adaptive response mechanism. The sites of oxidative burst are the cell wall and the plasma membrane, involving the enzymes NADH peroxidase (NADH-PX) and NADPH oxidase (NADPH-OX or RBOH (respiratory burst oxidase homologue), respectively [1, 28, 152]. Interesting facts were also reported by some researchers including Gapper and Dolan [70] and Moller et al. [138]. Their experiments proved that ROS generated in response to environmental stress are necessary for cell function, regulation, and development. For instance, plant cells use ROS for polymerization of lignin during cell wall formation. Several other enzymes associated with the cell wall also contribute to the generation of ROS including lipoxygenase, oxalate oxidase, xanthine oxidase, amine oxidases, and peroxidases [3, 24, 197]. Plant cells generate H_2O_2 during normal metabolism via the Mehler reaction in chloroplasts, electron transport in mitochondria, and photorespiration in peroxisomes. ROS generated by chloroplasts as by-products of photosynthesis include $^1O_2$ and the $O_2^{\bullet -}$, whereas the main species generated by peroxisomes are $O_2^{\bullet -}$ and H_2O_2 [15, 46]. In the dark, most ROS are generated in mitochondria, which mainly form $O_2^{\bullet -}$, by over reduction of the electron transport chain [188]. Superoxide can be converted into H_2O_2 in a reaction catalyzed by superoxide dismutase (SOD) and H_2O_2 serves as an inert diffusible species that can give rise to reactive $OH^{\bullet}$ through the catalysis by free transition metal ions [78]. H_2O_2 has a property to diffuse freely, and this movement is facilitated through peroxiporin membrane channels. Although H_2O_2 is relatively stable and found in the plant cell at µmolar concentration range, the residual ROS have short half-lives [36]. It has now been a well-established fact that out of several primary ROS, $OH^{\bullet}$ is the most reactive and is capable of oxidizing all known biomolecules at diffusion-limited rates of reaction. According to estimates, the average diffusion distance before $OH^{\bullet}$ reaction with a cellular component is only 3 nm (i.e., approximately the average diameter of a typical protein) [98]. The degree of cytotoxic damage induced by ROS ultimately depends on the redox homeostasis or the balance between ROS detoxification and ROS production mechanisms in the cell.

An antioxidative system consisting of antioxidant molecules and enzymes is in place in different compartments of plant cells that scavenges or detoxifies the ROS and keeps cellular redox homeostasis. Under physiological steady-state conditions, ROS are scavenged by different antioxidants. However, the balance between production and scavenging of ROS is disturbed by a number of adversative environmental stress factors, resulting in a rapid increase of intracellular ROS levels. Although high concentrations of ROS can cause irreversible damage to the macromolecules leading to cell death, at the same time they can also influence signaling and gene expression, indicating that cells have evolved strategies to utilize ROS to their advantage in various cellular programs and functions. The role of ROS is increasingly implicated in cell signaling processes involving the induction of stress-related genes regulated by a network of transcription factors. Transcription factors are proteins that act together with other transcriptional regulators, including chromatin-modifying proteins, for binding or obstruction of RNA polymerases with

the DNA template [10, 114]. The antioxidant enzymes include SOD, catalase (CAT), glutathione peroxidase (GPX), and the ascorbate–glutathione cycle (Halliwell-Asada pathway) enzymes: ascorbate peroxidase (APX), glutathione reductase, monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR) [74, 135] (Table 17.1). In the case of animals, the GPXs function as key enzymes that scavenge H_2O_2; in plants this function mainly belongs to CAT and the enzymes of the ascorbate–glutathione cycle. On the other hand, studies have indicated that GPXs or GST/GPXs become the main H_2O_2-scavenging enzymes under extreme/persistent stress conditions [79]. In addition to these antioxidant enzymes that scavenge ROS, plants synthesize antioxidant molecules that include L-ascorbic acid (vitamin C), glutathione, α-tocopherol (vitamin E), and carotenoids [4, 7].

17.3 ROI Signal Transduction Pathway

Recent studies have identified several components involved in the signal transduction pathway of plants that senses ROIs. These include the mitogen-activated protein (MAP) kinase kinase kinases AtANP1 and NtNPK1, and the MAP kinases AtMPK3/6 and Ntp46MAPK [109, 169]. In addition, calmodulin has been implicated in ROI signaling [48, 82]. A hypothetical model depicting some of the players involved in this pathway is shown in Fig. 17.3. H_2O_2 is sensed by a sensor that might be a two-component histidine kinase, as in yeast [42]. Calmodulin and a MAP-kinase cascade are then activated, resulting in the activation or suppression of several transcription factors. These regulate the response of plants to oxidative stress [48, 127]. Crosstalk with the pathogen-response signal transduction pathway also occurs and might involve interactions between different MAP-kinase pathways, feedback loops, and the action of NO and SA as key hormonal regulators. This model (Fig. 17.3) is simplified and is likely to change as research advances our understanding of this pathway.

ROIs act as signals that mediate the systemic activation of gene expression in response to pathogen attack [9], wounding [153], and high light [145]. They were suggested to act in conjunction with a compound that travels systemically and activates their production in distal parts of the plant, where they mediate the induction of gene expression. The involvement of ROIs in the regulation of stomatal closure [155] and in other cellular responses involving auxin [213] might suggest that more signaling pathways involving ROIs as inducers of systemic signals await discovery. It is unlikely that ROIs can travel systemically because they are highly reactive and would be scavenged along the way by the many antioxidative mechanisms and antioxidants present in the apoplast. However, it is possible that a wave of activity similar to the “oxidative burst” is activated in cells

Table 17.1 Important antioxidants and their localization in plant cells

Antioxidant	Localization of antioxidant*	Respective ROS	References
Enzymatic			
Superoxide dismutase (SOD) (EC 1.15.1.1)	Chl, Mit, Per, Cyt, Apo	$O_2^{\bullet-}$	[23, 124]
Catalase (CAT) (EC 1.11.1.6)	Per, Gly	H_2O_2	[135, 202]
Ascorbate peroxidase (APX) (EC 1.11.1.11)	Chl, Mit, Per, Cyt, Apo, Gly	H_2O_2	[14, 32]
Peroxidase (POX) (EC 1.11.1.7)	Vac, Cyt, CW	H_2O_2,	[12, 135]
Glutathione reductase (GR) (EC 1.6.4.2)	Chl, Cyt, Mit	Reduction of glutathione	[39, 53]
Glutathione peroxidase (GPX) (EC 111.1.9)	Cyt	H_2O_2, Lipid peroxyl radicals (ROO), organic hydroperoxide (ROOH)	[51, 94, 135]
Glutatjione S-transferase (GST) (EC 2.5.1.18)	Cyt, Mit, ER	Organic hydroperoxide (ROOH)	[68, 166]
Dehydroascorbate reductase (DHAR) (EC 1.8.5.1)	Chl, Mit, Per	Regeneration of ascorbate from dehydroascorbate (DHA)	[13, 133]
Monodehydroascorbate reductase (MDHAR) (EC 1.6.5.4)	Chl, Cyt, Mit, Per	Reduction of monodehydroascorbate (MDA) to give rise to ascorbate	[13, 20, 100, 133]
Nonenzymatic			
Glutathione	Chl, Mit, Per, Cyt, Apo	H_2O_2,$^{\bullet}OH$, $^1O_2$, $O_2^{\bullet-}$	[14, 101, 135, 149]
Ascorbic acid	Chl, Mit, Per, Cyt, Apo	H_2O_2,$^{\bullet}OH$, $^1O_2$, $O_2^{\bullet-}$	[14, 135, 149, 180]
α-tocopherol	Membrane	•OH, $^1O_2$, ROO, ROOH	[12, 93, 135, 144]
Carotenoids	Chloroplast	$^1O_2$	[12, 135]
Flavonoids	Vac	$^{\bullet}OH$, $^1O_2$, $O_2^{\bullet-}$, ROO and peroxinitrice	[30, 198]

*Chl-chloroplast; Mit-mitochondrion; Per-peroxisome; Gly-glyoxisome; Cyt-cytosol; Apo-apoplast; CW-cell wall; Vac-vacuole; ER-endoplasmic reticulum

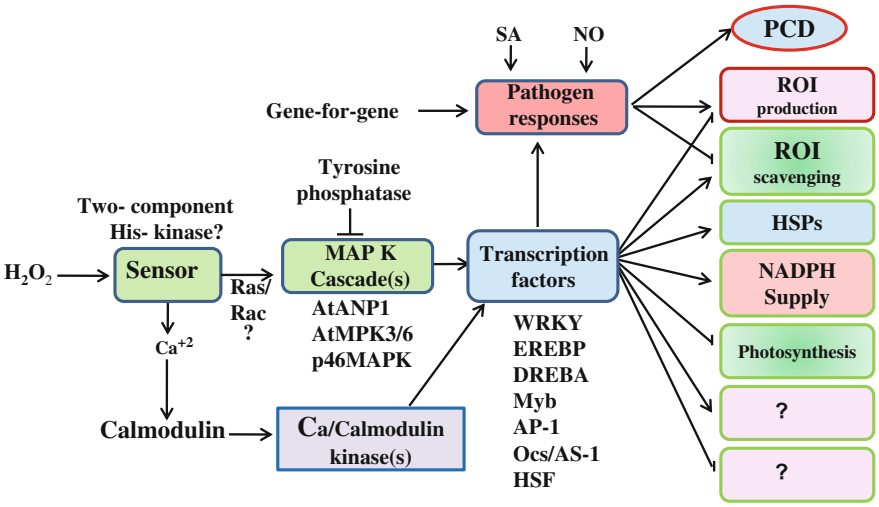

Fig. 17.3 A suggested model for the activation of signal transduction events during oxidative stress. Adopted from Mittler [135] H_2O_2 is detected by a cellular receptor or sensor. Its detection results in the activation of a mitogenactivated-protein kinase (MAPK) cascade and a group of transcription factors that control different cellular pathways. H_2O_2 sensing is also linked to changes in the levels of Ca_2^+ and calmodulin, and to the activation or induction of a Ca_2^+–calmodulin kinase that can also activate or suppress the activity of transcription factors. The regulation of gene expression by the different transcription factors results in the induction of various defense pathways, such as reactive oxygen intermediate (ROI) scavenging and heat-shock proteins (HSPs), and in the suppression of some ROI-producing mechanisms and photosynthesis. There is also crosstalk with the plant–pathogen signal transduction pathway, which might depend on pathogen recognition by the gene-for-gene mechanism and can result in an inverse effect on the regulation of ROI-production and ROI-scavenging mechanisms, as well as on the activation of programmed cell death (PCD). The plant hormones nitric oxide (NO) and salicylic acid (SA) are key regulators of this response [135]

along the systemic path and in distal tissues, resulting in the accumulation of ROIs. Future studies using plants with altered levels of ROI-scavenging and/or ROI-producing mechanisms might resolve this question.

17.4 Drought-Induced ROS and Effect on Plant Growth and Metabolism

Almost all the abiotic stresses result in increased ROS production in the plant cell [166]. Among all the abiotic stresses, drought-induced ROS production has been documented in several plant species growing under different environments [21, 89, 104, 172]. Under normal conditions, most cellular compartments of plants keep a

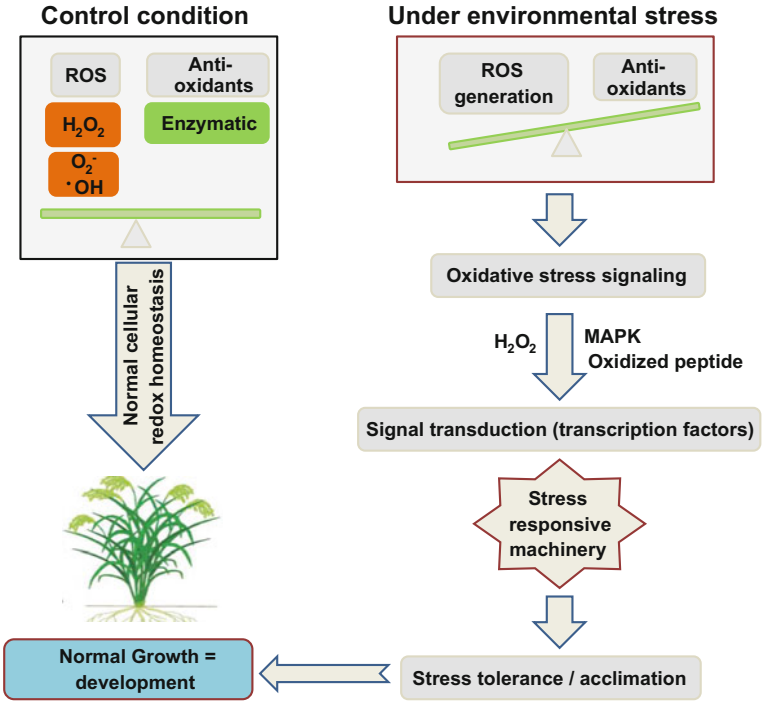

Fig. 17.4 Plant growth and development under normal and drought stress conditions. ROS production beyond the plant's quenching capability is often defined as a disruption of redox signaling and redox control. During the drought stress, an increased ROS level is prominent which can cause oxidative stress by damaging membrane proteins, lipids, photosynthetic pigments, and nucleic acids through the oxidation process and these are significantly augmented under drought stress. However ROS at optimum level orchestrate plant adaptive responses to drought stress

reducing environment and maintain a steady homeostatic state. However, during the stress, an increased ROS level is prominent. ROS production beyond the plant's quenching capability is often defined as a disruption of redox signaling and redox control [103], which can cause oxidative stress by damaging membrane proteins, lipids, photosynthetic pigments, and nucleic acids through the oxidation process and these are significantly augmented under drought stress [85, 109, 120, 182] (Fig. 17.4).

Drought stresses damage the photosynthetic pigments and photosynthetic apparatus and other enzymatic processes, resulting in the production of ROIs dangerous beyond the plant's scavenging capacity and imposing oxidative stress [148]. Water deficit makes the cellular content more viscous, causing the denaturation of proteins, thus the membrane of the photosynthetic apparatus as well as the cell membrane denatures, and at the same time the enzymes of the Calvin cycle are inactivated, the efficiency of carboxylation reaction and CO_2 fixation by RuBisCO

is reduced, and this results in increased photorespiration, which is one of the key reasons of ROS production [141].

Drought-induced photorespiration is reported to cause more than 70 % of total H_2O_2 generation in the plant cell [150, 151]. The rate of regeneration of NADP is also reduced under drought, therefore, the electrons of the electron transport chain cannot be accepted properly and finally surplus reduction of the electron transport chain causes leakage of electrons to O_2 and the production of ROS ($O_2^{\bullet -}$, $^1O_2$, H_2O_2, and $^{\bullet}OH$). Drought-induced stomatal closure is another common phenomenon that reduces the CO_2 availability in the fixation site of the Calvin cycle [193, 194]. In addition, stressful conditions also cause the chloroplasts to receive excessive excitation energy beyond their capability to combine it and thus ferredoxin remains in the over reduced condition during photosynthetic electron transfer; the electrons having a high state of energy are transferred from Photosystem I to molecular oxygen. During this transfer, the $O_2^{\bullet -}$ is generated through the Mehler reaction and this superoxide radical leads to the production of more harmful oxygen radicals such as $^{\bullet}OH$ [43, 135]. They also proposed that the drought stress response occurs in three successive phases. Normal ROS steady-state level is disturbed by drought stress where initially the enhancement of ROS production due to stomatal closure shifts the equilibrium upwards and this triggers defense signal transduction pathways. Prolonged drought stress results in aggravated ROS production that cannot be counterbalanced by the antioxidant system, leading to toxic oxidative events that ultimately result in cell death. Several other studies have shown that drought stress causes imbalance between light capture and its utilization in Photosystem II changes the photochemistry of chloroplasts, which causes excess production of highly reactive ROS species [64, 157]. Lipid peroxidation is thought to be the most common and obvious damaging process for all living organisms, including animals and plants [74]. Lipid peroxidation affects normal cellular functioning and the lipid-derived radicals accelerate oxidative stress [139]. There are several reports of enhanced electrolyte leakage under drought stress due to oxidative stress, and subsequent lipid peroxidation and plasmalemma injury such as in maize [47], rice [76], and rapeseed [85]. Drought stress produces ROS that subsequently impair the photosystem and reaction center of the photosynthetic apparatus. Singlet oxygen damages light-harvesting complexes [121]. The highly reactive $^1O_2$ can react with proteins, lipids, and pigments [190]. Lu and Zhang [124] reported that Photosystem II is more susceptible to drought as compared to Photosystem I, and a decline in D1 and D2 proteins causes the destruction of Photosystem II. Again, due to differences in the antioxidative capacity, oxidative damage was not found to be uniform in the different cells or tissues in C4 plants [52, 65]. Interestingly, the oxidative stress causes additional damage to bundle sheath tissue as compared to the mesophyll tissue [106]. Higher leakage of electrons to O_2 occurred during photosynthesis under drought stress and as compared to unstressed wheat seedlings the drought stress caused approximately 50 % higher leakage of photosynthetic electrons through the Mehler reaction [27, 179]. Sgherri et al. [173] also reported similar results in the case of sunflower. The production of hydroxyl radicals in

thylakoids under drought was considered as an additional menace because of their oxidizing potential to react with almost all biological molecules [42]. Their accumulation leads to a chain of deleterious reactions, and exerts harmful effects by damaging thylakoidal membranes and the photosynthetic apparatus; several reports state that no enzymatic reactions were found to remove or reduce the highly reactive $^{\cdot}OH$ [207]. Reactive nitrogenous species were also found to increase under drought. An increased nitric oxide (NO) level of water-stressed grapevine leaves was observed by Patakas et al. [154]. Arasimowicz-Jelonek et al. [11] also reported a higher level of NO in cucumber roots during water stress. The physiology and cellular status of root and leaf tissues of *Medicago truncatula* were observed under 11 days of drought stress [61]. Cellular damage, membrane damage, enhanced levels of reactive oxygen and nitrogen species, and reduced stomatal conductance were key features of that stress in *Medicago* and rewatering resulted in partial or complete alleviation of the stress-induced damage.

Drought stress responses towards the production of H_2O_2 and lipid peroxidation differ by genotype, and sometimes with the variety of genotypes, duration of water stress and the age of the plant are also important factors. Uzilday et al. [193, 194] compared the effects of drought among two C3 (*Cleome spinosa*) and C4 (*Cleome gynandra*) plants. These plants were exposed to drought stress for 5 and 10 days. Lipid peroxidation as represented by MDA and H_2O_2 contents remarkably increased in *C. spinosa* as compared to *C. gynandra* under drought stress, which proved the higher sensitivity of the C3 plant towards drought stress. Selote and Khanna-Chopra [172] also reported that increased H_2O_2 and lipid peroxidation *T. aestivum* (20 days old and 9–11 days water stress), *Populus przewalskii* (water stress at 2 months old) [117], and *Pinus densata*, *Pinus tabulaformis*, and *Pinus yunnanensis* (water stress for 1 month in 1-year-old plants) [69]. It is well established that as a result of drought stress the generated harmful ROS damage the cell structures, proteins, lipids, carbohydrates, and nucleic acids, and disrupt cellular homeostasis, in severe cases leading to cell death. However, in spite of the detrimental effects of ROS, they also play major physiological roles in intracellular signaling, cellular regulation, and as secondary messengers, which have been studied in several reports. In addition, the role of ROS as signals for gene expression has also been confirmed by several authors [96, 74, 132].

17.5 Antioxidant Defense System in Plants Under Drought Stress: Transgenics Approach

Water is the most important to complete the plant life cycle with most plant cells consisting of at least 70 % water on the basis of fresh weight. When there is scarcity of water, plant water status is disturbed causing imbalance in osmotic and ionic homeostasis, loss of cell turgidity, and damage to functional and structural cellular

membranes and proteins. Therefore, water-stressed plants show wilting, loss of photosynthetic capacity, and are unable to sequester assimilates into the appropriate plant organs. Severe drought conditions result in reduced yield and ultimately the plant death. The overall aim of genetically improving crops for drought resistance is to develop plants able to obtain water and use it to produce sufficient yields for human needs under drought conditions. Although advances have been made in developing crops that are genetically improved with traits such as herbicide and pesticide resistance, attempts to improve plant drought resistance have been hindered by the complexity of plant drought resistance mechanisms at the whole plant, cellular, metabolic, and genetic levels. Interaction between these mechanisms and the complex nature of drought itself adds another layer of difficulty to this problem. Plants acquire well-organized enzymatic and nonenzymatic defense systems that function together to control the flow of uncontrolled oxidation under various stress condition and protect plant cells from oxidative damage by scavenging ROS. The well-documented antioxidant enzymes in plants are SOD, catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX), guaicol peroxidase (GPOX), glutathione-*S*-transferase (GST), and so on. Among the nonenzymatic antioxidant components, ascorbic acid, glutathione (GSH), phenolic compounds, alkaloids, nonprotein amino acids, and α-tocopherols are commonly found in plants [74] (Fig. 17.5). The improvement of the antioxidant defense system via the transgenic approach is considered to be effective in the development of resistance and adaptive features in plants against drought stress. It has been supported by many research findings that the enhanced activities of components of the antioxidant system such as antioxidant enzymes and nonenzymatic compounds via the transgenics approach decrease oxidative damage, and develop and improve the drought tolerance and resistance of plants [43, 174]. Enzymatic antioxidants include SOD, CAT, APX, MDHAR, DHAR, and GR and nonenzymatic antioxidants are GSH, ascorbic acid, tocopherols, and proline.

17.5.1 Enzymatic Components

Plants have different antioxidant enzymes that are compartment-specific and present in various cell organelles, including chloroplasts, mitochondria, peroxisomes, cytosol, and stroma, by which ROS production remains under control via highly efficient scavenging mechanisms. The major oxidative enzymes and nonenzymatic components, their respective ROS, cellular localization, are presented in Table 17.1.

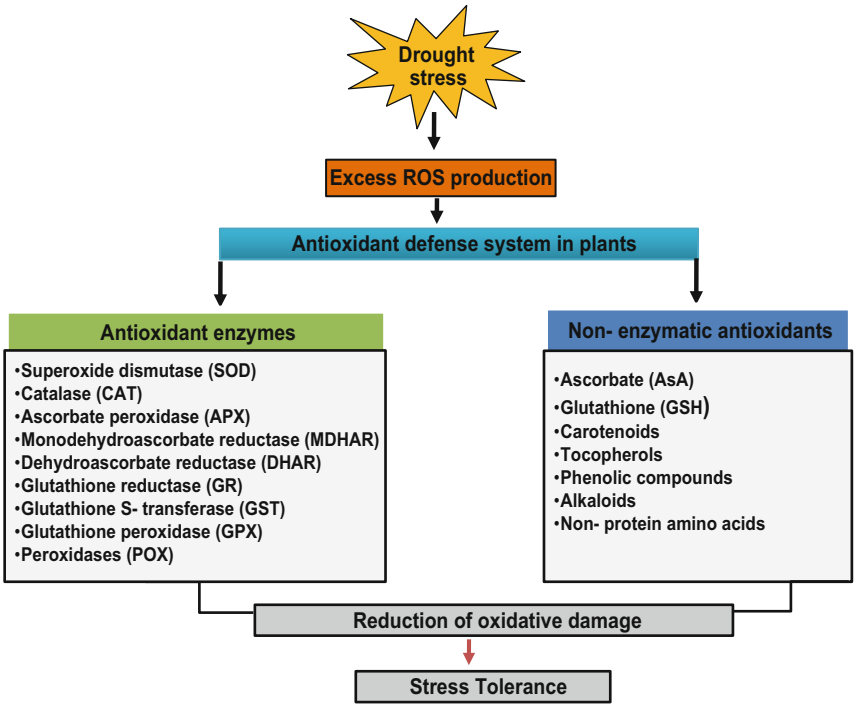

Fig. 17.5 Antioxidant defense system in plants under drought stress-induced oxidative stress. Plants acquire well-organized enzymatic and nonenzymatic defense systems that function together to control the flow of uncontrolled oxidation under various stress conditions and protect plant cells from oxidative damage by scavenging ROS. Antioxidant enzymes and nonenzymatic antioxidant components, commonly found in plants protect the cellular homeostasis level and the ROS is scavenged

17.5.1.1 Superoxide Dismutase (SOD)

SOD is a kind of metalloenzyme and is the most effective intracellular enzymatic antioxidant that is ubiquitous in all aerobic organisms and in all subcellular compartments prone to ROS-mediated oxidative stress. It is well established that various environmental stresses often lead to the increased generation of ROS where SOD has been proposed to provide the first line of defense against the toxic effects of elevated levels of ROS, important for plant stress tolerance. The SODs remove $O_2^{\bullet -}$ by catalyzing its dismutation: one $O_2^{\bullet -}$ being reduced to H_2O_2 and another oxidized to O_2. It removes $O_2^{\bullet -}$ and hence decreases the risk of $OH^{\bullet}$ ion formation via the metal catalyzed Habere Weiss-type reaction. This reaction has a 10,000-fold faster rate than spontaneous dismutation. SODs are classified on the basis of their metal cofactors into three known types: the copper/zinc (Cu/Zn-SOD), the manganese (Mn-SOD), and the iron (Fe-SOD), which are localized in different cellular compartments [135]. In the *A. thaliana* genome, three Fe-SOD genes (FSD1, FSD2, and

FSD3), three Cu/ZnSOD genes (CSD1, CSD2, and CSD3), and one MnSOD gene (MSD1) have been reported [108]. The Mn-SOD is found in the mitochondria of eukaryotic cells and in peroxisomes [45]; some Cu/Zn-SOD isozymes are found in the cytosolic fractions, and also in chloroplasts of higher plants [44]. The Fe-SOD isozymes, often not detected in plants [60] are usually associated with the chloroplast compartment when present [8]. All forms of SOD are nuclear-encoded and targeted to their respective subcellular compartments by an amino terminal targeting sequence. Several forms of SOD have been cloned from a variety of plants [170].

There have been many reports of the production of abiotic stress-tolerant transgenic plants overexpressing different SODs (Table 17.2). *Cu/Zn-SOD* overexpressing transgenic tobacco plants showed multiple stress tolerance [22]. The transgenics plants were able to show tolerance to salt and polyethylene glycol (PEG)-induced drought stresses and enhancement in the chloroplast antioxidant system. They showed that overexpression of rice cytosolic *Cu/Zn-SOD* in chloroplasts of tobacco plant improved their photosynthetic performance during photooxidative stresses such as high salt, drought, and PEG treatment when compared to untransformed plants. Although in both plants, net photosynthesis was steadily decreased, the rate of reduction was lower in the transgenic plants. The constitutively expressed *Cu/Zn-SOD* acts as an early scavenger, protecting the transgenic plants from the initial damage before their endogenous defense system is activated, and the higher SOD activity in the transgenic plants during the stress keeps the superoxide radical at a lower level leading to reduced oxidative damage and higher photosynthetic rate. When they match the result of net photosynthesis and antioxidant enzyme activities during salt stress, water deficiency, and PEG-induced stress, it was interpreted that the enhanced tolerance is caused by the overexpressed *Cu/Zn-SOD* and increased cellular antioxidant enzymes. Prashanth et al. [159] also showed the overexpression of *Avicennia marina* Cu/Zn SOD in transgenic rice and reported that the transgenic plants were more tolerant to MV-mediated oxidative stress, salinity stress, and drought stresses. Interestingly, Faize et al. [58] aimed to test whether overexpression of cytosolic SOD or cytosolic APX, alone or in combination, could enhance the tolerance of tobacco to drought stress and if the expression of these transgenes could affect the antioxidant metabolism in the soluble and chloroplastic fractions. To accomplish this goal, transgenic tobacco overexpressing each or both (SOD/APX) transgenes was generated and tolerance of the plants to a mild water stress was evaluated by analyzing the antioxidant metabolism in the soluble and chloroplastic fractions. The effect of the transgenes on the photosynthesis rate and on chlorophyll fluorescence parameters was also studied. The results showed that the overexpression of at least cytosolic APX protects tobacco plants from water stress and affects the antioxidant metabolism in the cytosol as well as in the chloroplast. High levels of cyt SOD and cyt APX gene transcripts as well of their respective activities suggested that the transgenes were constitutively and functionally expressed. Interestingly, some of the plant lines harboring only the cyt SOD transgene also had high APX activity. This was the case for at least six transgenic lines. This result was not surprising inasmuch as the reaction product of the SOD activity (H_2O_2) is the substrate for APX activity.

Table 17.2 ROS scavenging enzymatic antioxidants and their role in transgenic plants for drought stress tolerance

Gene	Target transgenic	Gene source	Response in transgenic plants	References
Superoxide dismutase (SOD)				
Cu/Zn SOD	*Nicotiana tabacum*	*Oryza sativa* L.	Enhanced tolerance to salt, water, PEG-induced drought stresses and enhancement in chloroplast antioxidant system	[22]
Cu/Zn SOD	*Oryza sativa* cv. Pusa Basmati 1	*Avicennia marina*	Transgenic plants were more tolerant to MV-mediated oxidative stress, salinity stress, and drought stress	[159]
Mn SOD	*Triticum aestivum cv* Oasis	*Nicotiana plumbaginifolia*	Photooxidative stress tolerance, lower oxidative damage, higher H_2O_2, and significant increase in SOD and GR activity	[130, 136]
Cu/Zn SOD + APX	*Nicotiana tabacum*	*Pisum sativum*	Increase in SOD and APX activity, drought stress tolerance	[58]
MnSOD	*Oryza sativa*	*Pisum sativum*	Enhanced tolerance to drought, and enhancement in cellular antioxidant system	[199]
Mn SOD + Fe SOD	*Medicago sativa* L.	*Nicotiana plumbaginifolia* and *Arabidopsis thaliana*	Mild water stress tolerance with high photosynthetic activity	[168]
Catalase (CAT)				
CAT-2	*Lycopersicon esculentum*	*Escherichia coli*	Transgenic plants have higher CAT activity as compared to wild-type plants. The transgenic plants showed increased tolerance to the oxidative damage caused by drought stress or chilling stress under high light intensity (1000 µmol m^{-2} s^{-1})	[206]
Ascorbate peroxidase (APX)				
c APX	*Lycopersicon esculentum cv.* Zhongshu No.5	*Pisum sativum*	Enhanced tolerance to drought, UV-B heat, and chilling stresses, increase in APX activity	[200, 201]
APX3	*Nicotiana tabacum*	*Arabidopsis thaliana*	Water deficit tolerance with higher photosynthesis	[204]
APX 2	*Nicotiana tabacum*	*Cucumber*	Increased drought tolerance due to reduced stomatal conductance	[62]

(continued)

Table 17.2 (continued)

Gene	Target transgenic	Gene source	Response in transgenic plants	References
POD	*Nicotiana tabacum*	*Ipomoea batatas*	Resistance to various abiotic (salt, mannitol, MV and H_2O_2) and biotic stress (*Phytophthora parasitica*)	[107]
Glutathione reductase (GR)				
GR	*Triticum Aestivum cv. Oasis*	*Escherichia coli*	Higher GSH content and GSH/GSH + GSSG ratio than control; no increase in SOD and GR activity	[130, 136]
GR	*Nicotiana tabacum*	*Arabidopsis thaliana*	Several oxidative stress tolerances including MV, water, and chilling stress tolerance.	[50]
GR + DHAR + GSH	*Nicotiana tabacum*	Brassica	Increased tolerance of the transgenic plants to a variety of abiotic stresses like MV-induced oxidative stress, salt, cold, and drought stresses	[115, 116]
GR	*Gossypium hirsutum* L.	*Nicotiana tabacum*	No oxidative stress tolerance	[125]
Monodehydroascorbate reductase (MDHAR)				
MDHAR1	*Nicotiana tabacum*	*Arabidopsis thaliana*	Ozone, salt, and PEG-induced drought stress tolerance due to higher MDAR activity and higher level of reduced AsA	[55]
Dehydroascorbate reductase (DHAR)				
DHAR	*Nicotiana tabacum*	*Arabidopsis thaliana*	Ozone and drought tolerance with higher DHAR activity and reduced AsA content	[54]
DHAR	*Solanum tuberosum*	*Arabidopsis thaliana*	Enhanced DHAR activity with faster growth, even under drought and salt stress conditions	[56]
Glutathione S-Transferase (GST)				
GST	*Nicotiana tabacum*	*Prosopis juliflora*	Survived better than control plants fewer than 15 % PEG-induced water stress	[72]
GST	*Nicotiana tabacum*	*Pyrus pyrifolia*	Transgenic tobacco lines showed relatively normal growth under drought, NaCl, and cadmium (Cd) stresses activity	[123]

(continued)

Table 17.2 (continued)

Gene	Target transgenic	Gene source	Response in transgenic plants	References
GST + GPX	*Nicotiana tabacum*	*Nicotiana tabacum*	Transgenic tobacco seedlings provide increased GSH-dependent peroxide scavenging that leads to reduced oxidative damage	[167]
GST	*Nicotiana tabacum*	Cotton	Increased activities of GST and GPX which strengthen the antioxidant defense of transgenic plants to resist the oxidative stress	[209]
GST	*Arabidopsis*	*Lycopersicon esculentum*	Enhanced resistance to salt and drought stress	[102]
Glutathione peroxidase (GPX)				
GPX2	*Arabidopsis thaliana*	*Synechcystis PCC 6803*	Tolerance to H_2O_2, Fe^{+2}, MV, chilling, high salinity or drought stresses	[67]

In conclusion, they found that coexpression of cytosolic antioxidant genes (SOD and APX) has only minor effects during drought, similar to those exhibited when expressing cytosolic APX alone. However, a partial protection of photosynthesis and membrane integrity was observed.

17.5.1.2 Catalases (CAT)

CATs are a tetrameric heme-containing enzyme which plays a pivotal role in directly dismutating H_2O_2 into H_2O and O_2 and is critical for ROS detoxification during stressed conditions [71]. CAT has one of the highest turnover rates of all enzymes: one molecule of CAT can convert 26 million molecules of H_2O_2 to H_2O and O_2 per minute. CAT is important in the removal of H_2O_2 generated in peroxisomes by oxidases involved in beta-oxidation of fatty acids, photorespiration, and purine catabolism. The CAT isozymes have been studied extensively in higher plants [158]. It has also been reported that apart from reaction with H_2O_2, CAT also react with some hydroperoxides such as methyl hydrogen peroxide [6]. Azpilicueta et al. [19] reported that incubation of *H. annuus* leaf discs with 300 and 500 mM $CdCl_2$ under light conditions increased the CATA3 transcript level but this transcript was not induced by Cd in etiolated plants. Moreover, in roots of the transgenic CAT-deficient tobacco lines (CAT 1AS), the DNA damage induced by Cd was higher than in wild-type tobacco roots [73]. There are reports of the development of drought-tolerant transgenic plants overexpressing different CAT (Table 17.2). Transgenic tomato plants overexpressing *CAT2* showed an increase in CAT activity and thus enhanced tolerance to drought [206].

17.5.1.3 Ascorbate Peroxidase (APX)

APX plays an essential role in scavenging ROS and protecting cells in higher plants, algae, euglena, and other organisms. APX is involved in scavenging of H_2O_2 in water–water and AsA-GSH cycles and utilizes AsA as the electron donor. The APX enzyme family consists of at least five different isoforms including thylakoid (t-APX) and glyoxisome membrane forms (gm-APX), as well as chloroplast stromal soluble form (s-APX), cytosolic form (c-APX) [149]. APX has a higher affinity for H_2O_2 (mM range) than CAT and peroxidase (POD) and it may have a more crucial role in the management of ROS during stress. It has also been noted that overexpression of APX in *Nicotiana tabacum* chloroplasts enhanced plant tolerance to salt and water deficit [22]. Wang et al. [199–201] demonstrated the effect of increased cytosolic ascorbate peroxidase (cAPX) on drought, water deficit, chilling, and UV-B stress tolerance using transformed tomato (*Lycopersicon esculentum* cv. Zhongshu No. 5) plants. They showed in laboratory or field tests, the potential to enhance tolerance to drought, UV-B, and sunscald stress by gene transfer. Overexpression of *cAPX* in transgenic tomato enhanced resistance to heat (40 °C) and UV-B stress compared to wild-type plants. APX activity in leaves of *cAPX* transgenic plants was several-fold higher than in leaves of wild-type plants when exposed to heat, UV-B, and drought stresses. Tobacco (*Nicotiana tabacum* L.) plants were transformed to constitutively overexpress the *Arabidopsis thaliana* gene for *APX3* [204]. Following repeated water-deficit cycles, fruit number and seed mass of transgenic tobacco were significantly higher than those of control plants. Although these data did not support the idea that overexpression of the gene for APX3 enhances protection of the photosynthetic apparatus during water deficit, overexpression of *APX3* may affect other cellular metabolisms that result in higher CO_2 assimilation under moderate water-deficit conditions and therefore higher seed mass after repeated water-deficit treatments.

Kim et al. [107] demonstrated that transgenic tobacco plants overexpressing *swpa4* (sweet potato POD c-DNA) showed increased H_2O_2 production followed by the upregulation of multiple apoplastic acidic PR genes. The *swpa4* transgenic plants manifested significantly enhanced tolerance to a variety of abiotic and biotic stresses in the H_2O_2-regulated stress-response signaling pathway. In order to assess the effects of *swpa4* expression on drought stress tolerance in soil-grown whole plants, 2-month-old plants were not watered for 8 days, and then watered for 8 days for recovery. More bleaching and a greater loss of PSII photosynthetic efficiency was observed in the control plants compared to the transgenic plants. In addition, the tobacco leaf discs from transgenic lines 1 and 2 exhibited higher levels of tolerance in the presence of mannitol (drought inducing) and NaCl, as seen by assessments of lipid peroxidation and total Chl. On the basis of the above results, it was evident that the *swpa4* transgenic plants are more tolerant to drought H_2O_2, dehydration, and high salinity than the control plants. Overexpression of APX in transgenic plants conferred drought stress tolerance is presented in Table 17.2.

17.5.1.4 Glutathione Reductase (GR)

Glutathione reductase, also known as GSR or GR (EC 1.8.1.7), belongs to the family of NADPH-dependent oxidoreductase and occurs in both prokaryotic and eukaryotic organisms. Although GR is located in chloroplasts, cytosol, and mitochondria, more than 80 % of its activity in photosynthetic tissues was reported to be of chloroplastic isoform [16]. GR plays an essential central role in cell defense against reactive oxygen metabolites by efficiently maintaining the cellular reduced GSH pool through catalyzing the reduction of GSSG to GSH with the accompanying oxidation of NADPH [38]. GR is a flavo-protein oxidoreductase, found in both prokaryotes and eukaryotes [165]. It is a potential enzyme of the AsA–GSH cycle and plays an essential role in the defense system against ROS by sustaining the reduced status of GSH. It is localized predominantly in chloroplasts, but a small amount of this enzyme has also been found in mitochondria and cytosol [39, 53]. GR catalyzes the reduction of GSH (glutathione), a molecule involved in many metabolic regulatory and antioxidative processes in plants where GR catalyzes the NADPH-dependent reaction of the disulphide bond of GSSG and is thus important for maintaining the GSH pool [35, 163]. GSH plays an important role within the cell system, which includes participation in the AsA–GSH cycle, maintenance of the sulfhydryl (-SH) group, and a substrate for GSTs. GR and GSH play a crucial role in determining the tolerance of a plant under various stresses [35].

In addition to reports of differential modulation of GR in metal metalloids, salinity and drought stresses, increased GR activity has been widely observed in many plant species including *T. aestivum* [87], *Z. mays* [110], *Cucumis sativus* [41], *N. tabacum* [186], and *Phaseolus aureus* [111] under high temperature (HT) stress. In *N. tabacum*, coexpression of GR resulted in the increased tolerance of the transgenic plants to a variety of abiotic stresses such as MV-induced oxidative stress, salt, cold, and drought stresses [116]. This was due to the changes in enzyme activities, and levels or redox state of AsA and GSH. Contour-Ansel et al. [38] investigated the variations in GR gene expression in two cowpea (*Vigna unguiculata*) cultivars viz. EPACE-1 (drought-resistant) and 1183 (drought-sensitive) using reverse-transcription where two new cDNAs encoding a putative dual-targeted and a cytosolic GR gene were cloned and sequenced. Drought stress induced an upregulation of the expression of the cytosolic GR gene directly related to the intensity of the stress in both cultivars. Although a noticeable activation of the antioxidant metabolism was observed in both cultivars, the drought-tolerant cultivar responded faster than the sensitive cultivar under a fast desiccation. Moreover, exogenous ABA enhanced significantly the activity and expression levels of GR in both cultivars after treatment for 24 h. In *Proteus vulgaris*, two cDNAs of the enzyme GR encoding a dual targeted isoform (dtGR) and a cytosolic isoform (cGR) were cloned and their expression under drought stress was observed [189]. Moderate drought stress induced an upregulation of the expression of cGR in the susceptible cultivars, whereas dtGR expression decreased. However, in tolerant cultivars the expression remained stable suggesting that that moderate drought stress may lead to a hardening process and tolerance.

Overexpression of a eukaryotic GR from *B. campestris* (*BcGR*) and *E. coli* GR (*EcGR*) was studied in *E. coli* in pET-28a. It was found that BcGR overproducing *E. coli* showed better growth and survival rate than the control but far better growth was noted in *E. coli* strain transformed with the inducible EcGR in the presence of oxidative stress, water stress, and Cd [208]. In an interesting study, transgenic *N. tabacum* with 30–70 % less GR activity were used to find out the possible mechanism of GR against oxidative stress. Transgenic plants with less GR activity showed enhanced sensitivity to oxidative stress. It was suggested that GR plays an important role in the regeneration of GSH and thus protects against oxidative stress also by maintaining the AsA pool [50]. Shu et al. [175] isolated the tomato chloroplast GR gene (*LeGR*) and produced antisense transgenic tomato lines that showed depletion of tomato chloroplast GR. Further investigation revealed that transgenic plants accumulated more H_2O_2, GSSG whereas GSH content decreased in transgenic plants with no changes in total GSH. These seedlings also showed worse performance in terms of physiological parameters. On the other hand, WT plants showed higher activities of enzymes and synthesis of metabolites. Transgenic plants that produce GR have been found to be abiotic stress tolerant (Table 17.2).

17.5.1.5 Monodehydroascorbate Reductase (MDHAR) and Dehydroascorbate Reductase (DHAR)

MDHAR is a flavin adenin dinucleotide (FAD) enzyme that is present as chloroplastic and cytosolic isozymes. MDHAR exhibits a high specificity for monodehydroasorbate (MDHA) as the electron acceptor, preferring NADH rather than NADPH as the electron donor. Asada [14] studied the multistep reduction of FAD in detail. The first step is the reduction of the enzyme-FAD to form a charge transfer complex. The reduced enzyme donates electrons successively to MDHA, producing two molecules of ascorbate via a semiquinone form [E-FAD-NADP(P)]. It is well established that the disproportionation by photoreduced ferrodoxin (redFd) in the thylakoids is of great importance. Because red Fd can reduce MDHA more effectively than NADP, MDHAR cannot participate in the reduction of MDHA in the thylakoidal scavenging system. Therefore, MDHAR only function in the presence of NAD(P)H [14]. Accompanying APX, MDHAR is also located in peroxisomes and mitochondria, where it scavenges H_2O_2 [44]. Similarly, DHAR regenerates ASH from the oxidized state and regulates the cellular ASH redox state (which is crucial for tolerance to various abiotic stresses) leading to the production of ROS. Eltayeb et al. [55] reported the overexpression of monodehydroascorbate reductase in transgenic tobacco confers enhanced tolerance to ozone, salt, and polyethylene glycol-induced drought stresses. To examine whether an overexpressed level of MDAR could minimize the deleterious effects of environmental stresses, they developed transgenic tobacco plants overexpressing the *Arabidopsis thaliana* MDAR gene (*AtMDAR1*) in the cytosol. Incorporation of the transgene in the genome of tobacco plants was confirmed by PCR and Southern-blot analysis and its expression was confirmed by Northern and Western-blot analyses. These transgenic

plants exhibited up to 2.1-fold higher MDAR activity and a 2.2-fold higher level of reduced AsA compared to nontransformed control plants. The transgenic plants showed enhanced stress tolerance in term of significantly higher net photosynthesis rates under ozone, salt, and polyethylene glycol stresses and greater PSII effective quantum yield under ozone and salt stresses. Furthermore, these transgenic plants exhibited a significantly lower hydrogen peroxide level when tested under salt stress. These results demonstrate that an overexpressed level of MDAR properly confers enhanced tolerance against ozone, salt, and PEG stress. In addition, Eltayeb et al. [54] also showed that the transgenic tobacco overexpressing dehydroascorbate reductase in cytosol showed enhanced tolerance to ozone and drought stresses. In order to examine the protective role of DHAR against oxidative stress, we developed transgenic tobacco plants overexpressing the cytosolic *DHAR* gene from *Arabidopsis thaliana*. Incorporation of the transgene in the genome of tobacco plants was confirmed by polymerase chain reaction and Southern blot analysis, and its expression was confirmed by Northern and Western blot analyses. These transgenic plants exhibited 2.3–3.1-fold higher DHAR activity and 1.9–2.1-fold higher level of reduced AsA compared with nontransformed control plants. The transgenic plants showed maintained redox status of AsA and exhibited an enhanced tolerance to ozone, drought, salt, and polyethylene glycol stresses in terms of higher net photosynthesis. In this study, we report for the first time that the elevation of the AsA level by targeting DHAR overexpression in cytosol properly provides a significantly enhanced oxidative stress tolerance imposed by drought and salt. Similarly, transgenic potato plants overexpressing the *Arabidopsis* DHAR gene in the cytosol exhibited enhanced DHAR activity with faster growth, even under drought and salt stress conditions [56]. Overexpression of MDAR and DHAR in transgenic plants has been summarized in Table 17.2.

17.5.1.6 Glutathione S-Transferases (GST)

The plant glutathione transferases, formerly known as glutathione S-transferases (GST, EC 2.5.1.18) are a large and diverse group of enzymes that catalyze the conjugation of electrophilic xenobiotic substrates with the tripeptide glutathione. Glutathione S-transferases (GSTs) are ubiquitous enzymes in animals and plants, and they are multifunctional proteins encoded by a large gene family. GSTs are involved in response to the oxidative stress including drought, salt, heavy metals, and so on. Under oxidative stress, the excessive ROS induce an increase in GST levels, and then the GSTs metabolize the toxic products of lipid peroxidation, damaged DNA, and other molecules [51].

Overexpression of GST in transgenic plants has been summarized in Table 17.2. It has also been found that GST overexpression also enhances plant tolerance to various abiotic stresses. Liu et al. [123] isolated and characterized a full-length cDNA of a novel zeta GST gene, *PpGST*, from fruit of *Pyrus pyrifolia* Nakai cv. Huobali and the same gene was successfully integrated into the genome of the transgenic tobacco lines and expressed. Growth of T1 generation plants of *PpGST*

transgenic lines and WT under nonstressful conditions was similar, however, the transgenic tobacco lines showed relatively normal growth under drought, NaCl, and cadmium (Cd) stresses. Furthermore, the T1 transgenic tobacco lines showed a significantly slower superoxide anion production rate than the WT under abiotic stress. Simultaneously, the MDA content of each T1 transgenic tobacco plant was only slightly increased and significantly less than that of the WT under drought, salt, and Cd stress. Together with the GST activity of the transgenic tobacco lines, which was significantly increased under stressful conditions, as compared with that in WT, overexpression of *PpGST* in tobacco enhanced the tolerance of transgenic tobacco lines to oxidative damage caused by drought, NaCl, and Cd stresses. Transgenic tobacco seedlings overexpressing GST and GPX showed enhanced seedling growth under a stressed environment. Additionally, a significant increase in MDHAR activity, GSH, and ASH content along with GST and GPX has also been noted in transgenic GST/GPX expressing (*GSTþ*) seedlings than WT. These results indicated that overexpression of GST/GPX in transgenic tobacco seedlings provides increased GSH-dependent peroxide scavenging and alterations in GSH and ASH metabolism that lead to reduced oxidative damage [167]. Transgenic tobacco plants overexpressing Gstcr1 showed significant increase in the activities of GST and GPX which strengthen the antioxidant defense of transgenic plants to resist oxidative stress [209]. Transgenic tobacco plants overexpressing *Prosopis juliflora* GST (*PjGSTU1*) survived better than control plants with fewer than 15 % undergoing PEG stress. Furthermore, GFP fusion studies revealed the presence of PjGSTU1 in the chloroplast of transgenic plants that was correlated with its role in ROS removal [72, 64]. Very recently, Jing et al. [102] generated transgenic *Arabidopsis* overexpressing tomato glutathione S-transferase designated *LeGSTU2* that showed enhanced resistance to salt and drought stress. The increased tolerance of transgenic plants was correlated with the changes in proline, malondialdehyde, and antioxidative emzyme activities.

17.5.1.7 Glutathione Peroxidase (GPX)

GPXs (EC 1.11.1.9) are a large family of diverse isozymes that use GSH to reduce H_2O_2 and organic and lipid hydroperoxides, and therefore help keep plant cells from oxidative stress [150, 151]. Millar et al. [131] identified a family of seven related proteins in cytosol, chloroplast, mitochondria, and endoplasmic reticulum, named AtGPX1–AtGPX7 in *Arabidopsis*. Recently, Yang et al. [205] introduced the radish phospholipid hydroperoxide GPX gene (RsPHGPx) into a yeast PHGPx-deletion mutant and found that it significantly rescued the growth of the recombinant cell exposed to linolenic acid, indicating a similar role to the yeast PHGPx3 gene (ScPHGPx3) in protection of membrane. Glutathione peroxidase (GPX)-like proteins (GPX-1 and GPX-2) of Synechocystis PCC 6803 (S. PCC 6803) reduce unsaturated fatty acid hydroperoxides using NADPH, but not reduced glutathione (GSH), as an electron donor. Gaber et al. [67] generated transgenic *Arabidopsis* plants overexpressing S. PCC 6803 GPX- 2 in the cytosol (AcGPX2)

or chloroplasts (ApGPX2). Both transgenic lines (AcGPX2 and ApGPX2) showed enhanced tolerance to oxidative damage caused by treatment with H_2O_2, Fe ions, or methylviologen, and environmental stress conditions, such as chilling with high light intensity, high salinity, or drought. The degree of tolerance of the transgenic plants to all types of stress was correlated with the levels of lipid peroxide suppressed by the overexpression of S.PCC 6803 GPX-2. Under conditions of oxidative stress due to the H_2O_2 treatment, the NADPH/(NADP:NADPH) ratio in the transgenic plants was lower than that in the wild-type plants. This indicated that the expression of S. PCC6803 GPX-2 contributes to the reduction in unsaturated fatty acid hydroperoxides using NADPH in situ under stress conditions in the transgenic plants. Overexpression of GPX has been found to enhance drought stress tolerance in transgenic plants (Table 17.2).

17.5.2 Nonenzymatic Antioxidants

17.5.2.1 Glutathione (GSH)

Tripeptide glutathione (glu-cys-gly; GSH) is one of the crucial metabolites in plants that are considered to be the most important intracellular defense against ROS-induced oxidative damage. It occurs abundantly in reduced form (GSH) in plant tissues and is localized in all cell compartments such as cytosol, endoplasmic reticulum, vacuole, mitochondria, chloroplasts, and peroxisomes, as well as in apoplast [101, 134] and plays a central role in several physiological processes, including regulation of sulfate transport, signal transduction, conjugation of metabolites, detoxification of xenobiotics [146], and the expression of stress-responsive genes. It is well established that GSH also plays an important role in several growth and development related events in plants, including cell differentiation, cell death and senescence, pathogen resistance, and enzymatic regulation [162]. GSH provides a substrate for multiple cellular reactions that yield GSSG (i.e., two glutathione molecules linked by a disulfide bond). The balance between the GSH and GSSG is an indispensable component in maintaining a cellular redox state. GSH is necessary to maintain the normal reduced state of cells so as to counteract the inhibitory effects of ROS-induced oxidative stress [66]. It is a potential scavenger of $^1O_2$, H_2O_2 [31] and the dangerous ROS such as $OH^{\bullet}$ [103]. Moreover, GSH plays a key role in the antioxidative guard system by regenerating another potential water-soluble antioxidant such as AsA, via the ASH–GSH cycle [63]. It has been reported that when the intensity of a stress increases, GSH concentrations usually decline and the redox state becomes more oxidized, leading to deterioration of the system [188]. GSH is particularly important in plant chloroplasts because it helps to protect the photosynthetic apparatus from oxidative damage. Overexpression of a chloroplast-targeted γ-glutamylcysteine synthetase (*γ-ECS*) in transgenic tobacco plants resulted in a threefold increase in GSH level [40].

17.5.2.2 Ascorbic Acid (AsA)

Ascorbic acid is the most abundant, powerful, and water-soluble antioxidant and it acts to prevent or minimize the damage caused by ROS in plants [18, 181]. It occurs in all plant tissues, usually being higher in photosynthetic cells and meristems (and some fruits). Its concentration is reported to be highest in mature leaves with fully developed chloroplast and highest chlorophyll. It has been reported that AsA mostly remain available in reduced form in leaves and chloroplast under normal physiological conditions [180]. About 30–40 % of the total ascorbate is in the chloroplast and stromal concentrations as high as 50 mM have been reported [66]. In plants, mitochondria play a central role in the metabolism of AsA. Plant mitochondria do not only synthesize AsA by L-galactono-g-lactone dehydrogenase but also take part in the regeneration of AsA from its oxidized forms [185]. The regeneration of AsA is extremely important because fully oxidized dehydroascorbic acid has a short half-life and would be lost unless it is reduced back. AsA is considered as a most powerful ROS scavenger because of its ability to donate electrons in a number of enzymatic and nonenzymatic reactions. It can provide protection to membranes by directly scavenging the $O_2^{\cdot-}$ and $OH^{\cdot}$ and by regenerating α-tocopherol from the tocopheroxyl radical. In chloroplast, AsA acts as a cofactor of violaxantin de-epoxidase thus sustaining dissipation of excess excitation energy [169]. In addition to the importance of AsA in the AsA–GSH cycle, it also plays an important role in preserving the activities of enzymes that contain prosthetic transition metal ions [149]. The AsA redox system consists of L-AsA, MDHA, and DHA. Both oxidized forms of AsA are relatively unstable in aqueous environments whereas DHA can be chemically reduced by GSH to AsA [63].

Plants with higher ascorbate content can effectively scavenge the excessive ROS generated during stress conditions, and confer increased tolerance to abiotic stresses. Increased abiotic stress sensitivity of the *Arabidopsis* vtc mutant is attributed to the low intrinsic ascorbate levels and impaired ascorbate–glutathione cycle, which resulted in an enhanced ROS activity and a significant decrease in the CO_2 assimilatory capacity [97]. Moreover, deficiency of ascorbate may limit the recycling of α-tocopheroxyl radicals to α-tocopherol, which may, in turn, increase the oxidation of thylakoid membrane lipids in drought conditions [143]. Several transgenic plants overproducing ascorbate showed an enhanced salt and drought tolerance with reduced membrane lipid peroxidation and chlorophyll content loss. These plants also exhibited a higher survival rate under stress conditions and a significantly higher seed germination rate, fresh weight, and root length (Wang et al. [199, 200]; Sun et al. [184, 211]. Transgenic potato plants expressing the strawberry *GalUR* gene and rat *GLOase* gene with several-fold increased biosynthesis of AsA also exhibited a better survival under salinity and drought stress conditions including a reduction in the level of lipid peroxidation [90, 91, 92, 192].

17.5.2.3 Proline (Pro)

Proline (an osmolyte) is considered to be a potent antioxidant and potential inhibitor of programmed cell death. Hence, Pro are now regarded as nonenzymatic antioxidants that plant microbes and animals, require to moderate the adverse effects of ROS [37]. The synthesis of L-Pro from L-glutamic acid via D1-pyrroline-5-carboxylate (P5C) is catalyzed by the activities of the enzymes D1-pyrroline-5-carboxylate synthetase (P5CS) and D1-pyrroline-5-carboxylate reductase (P5CR) in plants [196]. On the other hand, mitochondrial enzymes Pro dehydrogenase (oxidase; ProDH) and P5C dehydrogenase (P5CDH) metabolize L-Pro into L-Glu via P5C. It has been established that following salt, drought, and metal stress, the enhanced accumulation of Pro may be due to increased synthesis or decreased degradation. Free Pro has been anticipated to play a role as an osmoprotectant, a protein stabilizer, a metal chelator, an inhibitor of lipid peroxidation, and/or $OH^{\bullet}$ and $^{1}O_2$ scavenger [17, 95, 191]. Pro, mannitol, sorbitol, and myo-inositol have been tested for $OH^{\bullet}$ scavenging capacity and out of these, the Pro seemed to be more effective scavenger of $OH^{\bullet}$ [178]. Thus, Pro is not only a vital molecule in redox signaling, but also an operative quencher of ROS formed under salt, metal, and dehydration stress conditions in plants and algae [5]. It was suggested that the ability of Pro to scavenge ROS and ability to inhibit ROS-mediated apoptosis can be an important function in response to cellular stress. Increased accumulation of Pro has been correlated with improved tolerance to various abiotic stresses especially salt and drought. Enhanced synthesis of Pro under drought or salt stress has been implicated as a mechanism to alleviate cytoplasmic acidosis and maintain NADP:NADPH at values compatible with metabolism [81]. An additional advantage of the refilling of NADP supply by Pro synthesis may be to support redox cycling, which is especially important in plant antioxidant defense mechanisms during stress. There is now enough evidence suggesting the important role for Pro synthesis in potentiating pentose–phosphate pathway activity, as this pathway is a most important component of antioxidative defense mechanisms, which need NADPH to maintain GSH and ASH in the reduced state.

It has also been found that overexpression of Pro biosynthetic pathway genes enhance abiotic stress tolerance in transgenic plants. Su and Wu [183] reported that both constitutive expression and stress-inducible expression of the P5CS cDNA in transgenic *O. sativa* have led to the accumulation of P5CS mRNA and Pro which resulted in higher salt and water deficiency stress tolerance. Vendruscolo et al. [195] reported the effects of water deficit on wheat plants transformed with the *Vigna aconitifolia* D1-pyrroline-5-carboxylate synthetase (P5CS) cDNA that encodes the key regulatory enzyme in proline biosynthesis, under the control of a stress-induced promoter complex-AIPC. Transgenic wheat plants submitted to 15 days of water shortage presented a distinct response. They found that drought resulted in the accumulation of proline. The tolerance to water deficit observed in transgenic plants was mainly due to protection mechanisms against oxidative stress and not caused by osmotic adjustment. Molinari et al. [135] reported the evaluation of the stress-inducible production of proline in transgenic sugarcane. After 9 days without

irrigation, proline content in transgenic events was on the average 2.5-fold higher than in controls. However, no osmotic adjustment was observed in plants over-producing proline during the water-deficit period. The photochemical efficiency of PSII observed was higher (65 %) in the transgenic events at the end of the water-deficit experiment. The effects of proline on lipid peroxidation as MDA levels and on the decline of Chl in leaf discs along the drought period suggest that proline protected the plants against the oxidative stress caused by the water deficit. The overall capacity of transgenic plants to tolerate water-deficit stress could be assessed by the significantly higher biomass yields 12 days after withholding water. These results suggested that stress-inducible proline accumulation in transgenic sugarcane plants under water-deficit stress acts as a component of an antioxidative defense system rather than as an osmotic adjustment mediator. Yamada et al. [203] transformed petunia plants with Δ^1-pyrroline-5-carboxylate synthetase genes (*AtP5CS* from *Arabidopsis thaliana* L. or *OsP5CS* from *Oryza sativa* L.). The transgenic plants accumulated Pro and their drought tolerance was tested. The Pro content amounted to 0.57–1.01 % of the total amino acids in the transgenic plants, or 1.5–2.6 times that in wild-type plants grown under normal conditions. The transgenic plant lines tolerated 14 d of drought stress, which confirms that both *P5CS* transgenes had full functionality. Exogenous L-Pro treatment caused the plants to accumulate Pro; plants treated with 5 mM L-Pro accumulated up to 18 times more free Pro than untreated plants. Exogenous L-Pro restricted the growth of wild-type petunias more than that of *Arabidopsis* plants. The capacity for free Pro accumulation might depend on the plant species. The growth of petunia plants was influenced not only by the Pro concentration in the plants, but by the ratio of the Pro content to the total amino acids, because the growth of the transgenic petunia plants appeared normal. Simon-Sarkadi et al. [176] reported stress-induced changes in the free amino acid composition in transgenic soybean plants having increased proline content. Following drought stress at supraoptimal temperature the increase in proline content in transgenic (T) soybean [*Glycine max* (L.) Merr. cv. Ibis] plants overexpressing the gene coding for the last enzyme of Pro biosynthesis, L-Δ^1-pyrroline-5-carboxylate reductase, was much greater than in wild-type (W) plants (105-fold vs. 19-fold after 7 d). The results indicate that manipulating the content of a single amino acid influences the whole free amino acid composition in soybean.

17.5.2.4 α-Tocopherols (Vitamin E)

Tocopherols are lipid-soluble antioxidants and considered as potential scavengers of ROS and lipid radicals [93]. Tocopherols are also considered as a major antioxidant in biomembranes, where they play both antioxidant and nonantioxidant functions. Tocopherols are considered general antioxidants for protection of membrane stability, including quenching or scavenging ROS like $^1O_2$. Tocopherols are confined in plants in the thylakoid membrane of chloroplasts. Out of four isomers of tocopherols (α, β, γ, £) found in plants, α -tocopherol has the highest antioxidative activity due to the presence of three methyl groups in its molecular structure [105]. It is synthesized

from β-tocopherol in chloroplasts by g-tocopherol methyltransferase (*γ-TMT*; VTE4). A high level of α-tocopherol has been found in the leaves of many plant species including Arabidopsis but these are low in γ-tocopherol. It has been found that nitration of γ-tocopherol is considered to be an important mechanism for the regulation and detoxification of NOx in animal tissues. Germinating seeds of *Brassica napus*, *N. tabacum*, and *A. thaliana* also showed the presence of 5-NγT. It can be said that γ-tocopherol or 5-NγT prolongs early development by reducing NOx concentration [49]. Tocopherol has been shown to prevent the chain propagation step in lipid auto-oxidation which makes it an effective free radical trap. Additionally, it has been estimated that one molecule of α-tocopherol can scavenge up to 120 $^1O_2$ molecules by resonance energy transfer [144]. Different plant studies have indicated the positive relationship between tocopherol biosynthesis/accumulation and water stress [142]. Several reports have shown there is a remarkable elevation of α-tocopherol under water-deficit conditions in pea [139, 187], wheat and cereals [25, 160], rosemary [142], and lavender. Liu et al. [122] observed that transgenic tobacco plants overexpressing the vte1 gene enhanced α-tocopherol synthesis resulting in better protection to water deficiency; this was also associated with upregulated antioxidant defense such as decreased lipid peroxidation, electrolyte leakage, and H_2O_2 levels. However, severe drought stress (30 % PEG) resulted in a loss of α-tocopherol in rice chloroplast [29].

Successful efforts to improve plant performance against drought through engineering tocopherol level and composition have been reported in the literature. Liu et al. [122] reported enhanced tolerance to drought stress in transgenic tobacco plants overexpressing VTE1 for increased tocopherol production from *Arabidopsis thaliana.* Yusuf et al. [210] and Kumar et al. [112] observed that α-tocopherol-enriched transgenic *B. juncea* plants constitutively overexpressing the *γ-TMT* gene showed enhanced tolerance to drought stress (200 mM mannitol) compared to wild-type plants. Transgenic plants showed enhanced activities of SOD, CAT, APX, and GR and decreased the levels of MDA, H_2O_2, and electrolyte leakage compared to wild type. Their findings implicated the role of higher α-tocopherol levels in conferring better tolerance against salt, heavy metal, and osmotic stresses and also established the existence of interplay between this lipid-soluble antioxidant and other water-soluble components of plant antioxidant defense. In the investigation with Arabidopsis plants Cela et al. [34] observed a 3.6- and 13.5-fold increase in the level of α- and γ-tocopherol, respectively, under water deficiency. However, some important genes (e.g., VTE2, VTE1, and VTE4) responsible for tocopherol biosynthesis did not change significantly [34]. Espinoza et al. [57] reported that tobacco seedlings overexpressing VTE2.1, which encodes the enzyme HPT, can catalyze the prenylation step in tocopherol biosynthesis under drought stress. The elevated level of α- tocopherol may enhance photosynthetic efficiency and lower lipid peroxidation leading to better oxidative protection.

17.6 Conclusion and Future Perspectives

Drought stress is a complex phenomenon and plants cope with drought stress via several morphological, anatomical, and biochemical adaptations at the cellular and organelle levels. A better understanding of the effects of drought on plants is therefore essential in order to establish improved management practices and breeding efforts in agriculture, and to allow prediction of the fate of natural vegetation under severe climate change. Coordinated approaches involving traditional plant breeding, along with molecular approaches, should be followed to identify and cultivate drought-tolerant varieties. It is well documented that drought stress leads to the overproduction of ROS in plants which is highly reactive and toxic and ultimately results in oxidative stress. Overall, the involvement of ROS in various metabolic processes in plant cells might have general implications. Oxidative stress is a condition in which ROS or free radicals are generated at the cellular level, which can exert their toxic effects on the cells. These species may affect cell membrane properties and cause oxidative damage to nucleic acids, lipids, and proteins that may make them nonfunctional. Different exogenous protectants, such as osmoprotectants (proline, glycine betaine, trehalose, etc.), plant hormones (gibberellic acids, jasmonic acid, brassinosteroids, salicylic acid, etc.), antioxidants (AsA, GSH, tocopherols, etc.), signaling molecules (NO, H_2O_2, etc.), and polyamines (spermidine, spermine, putrescine, etc.), have been found effective in mitigating drought-induced damage in plants. Crop plants that are tailored to have improved capacity for biosynthesis or bioaccumulation of these protectants might show enhanced drought tolerance. It is well known that plant cells and their organelles such as chloroplasts, mitochondria, and peroxisomes employ antioxidant defense systems to protect themselves against ROS-induced oxidative stress. A great deal of research has also established that the induction of the cellular antioxidant machinery is important for protection against ROS and ROS-induced cellular damage. Overexpression of ROS scavenging enzymes including isoforms of SOD (Mn-SOD, Cu/Zn-SOD, Fe-SOD), CAT, APX, GR, DHAR, GST, and GPX resulted in drought stress tolerance in various crop plants due to efficient ROS scavenging capacity. However, pyramiding of ROS scavenging enzymes may also be used to obtain durable resistance to drought stress. Therefore, transgenic plants have tremendous potential in the near future to mitigate drought stress as well as to increase the quality of plant products.

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Chapter 18
Engineering Glycinebetaine Metabolism for Enhanced Drought Stress Tolerance in Plants

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Abbreviations

APX	Ascorbate peroxidase
BADH	Betaine aldehyde dehydrogenase
CAT	Catalase
CDH	Choline dehydrogenase
CMO	Choline monooxygenase
COD/COX	Choline oxidase
DHAR	Dehydroascorbate reductase
GB	Glycinebetaine
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSMT	Glycinesarcosine methyltransferase
MDA	Malonaldehyde
MDHAR	Monodehydroascorbate reductase
POD	Peroxidase
PSII	Photosystem II
ROS	Reactive oxygen species
RWC	Relative water content
SDMT	Sarcosine dimethylglycine methyltransferase
SOD	Superoxide dismutase

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18.1 Introduction

Plants are frequently exposed to various environments due to their sessile growth behavior. Unfavorable environmental stress can disturb cellular structures and impair key physiological functions. Drought represents one of the major environmental factors limiting plant growth, geographic distribution, and productivity [1, 2], and can impose an osmotic stress that leads to turgor loss. Consequently, changes in cellular status occur, including disorganized membranes, proteins undergoing loss of activity or being denatured, and excess levels of ROS secretion that lead to oxidative damage of subcellular organs. Eventually, inhibition of photosynthesis, metabolic dysfunction, and damage to cellular structures contribute to growth perturbances, reduced fertility, and premature senescence in plants [3].

Naturally, plants have developed differential physiological and biochemical strategies to cope with the stress imposed by drought conditions [1]. Accumulation of compatible solutes and the acceleration of reactive oxygen species (ROS)-scavenging systems are considered the most suitable approaches to protect against drought stress [4, 5]. Low molecular-weight, highly soluble compounds, for example, proline, trehalose, and glycinebetaine (GB), are nontoxic at high concentrations and have been detected in a wide variety of organisms [6].

Introduction of new pathways for improved biosynthesis of various compatible solutes in plants has resulted in improved tolerance to various abiotic stress conditions [7–9]. Two basic functions have been attributed to these solutes: osmotic adjustment and cellular compatibility; nevertheless, a molecular mechanism of compatible solutes, including GB under drought stress tolerance is largely unknown. Compared with the natural accumulation of these compatible solutes, their lower accumulation in nonaccumulator plants by transgenesis is a challenging issue. Normally, compatible solutes might not significantly contribute to osmotic adjustment at very low levels. Therefore, these compounds are expected to play major roles in ROS scavenging, protecting the integrity of macromolecules (i.e., nucleic acids, proteins, lipids), and acting as a reservoir of carbon and nitrogen sources [10–14].

As a fully N-methylsubstituted derivative of glycine, GB is present in bacteria, animals, and higher plants [15, 16]. The functions of GB have been characterized by its role in stabilizing the quaternary structure of complex proteins, enzymes, and photosynthetic machinery (e.g., PSII complexes and RuBisCo), protecting transcriptional machinery and maintaining the integrity of membranes [6, 16]. Indeed, many studies of the protective role of GB in plants have been widely accepted [16, 17]. The exogenous application of GB to non- or low-accumulating plants renders tolerance to various abiotic stresses, and does so by reducing the adverse effects of environmental stress and enhancing subsequent plant growth and yield [15]. Externally applied GB is readily absorbed by rapidly penetrating through the leaves, or being taken up via roots, and then being transported to other organs

Table 18.1 Enhanced tolerance to drought stress via exogenously applied GB in plants

Plant species	Experiment environment	Reference
Avena sativa	Greenhouse, field	[79]
Brassica rapa	Greenhouse	[80]
	Greenhouse, field	[79]
Hordeum vulgare	Greenhouse, field	[79]
Lycopersion esculentum	Field	[81]
	Growth chamber	[18]
	Greenhouse	[80]
	Greenhouse	[82]
Phaseolus vulgaris	Greenhouse	[83]
Triticum aestivum	Greenhouse	[82, 84]
	Greenhouse, field	[84, 85]
Carapa guianensis	Greenhouse	[86]
Oryza sativa	Field	[95]
Nicotiana tabacum	Greenhouse	[87]
Helianthus annuus	Field	[88]

where it can play additional roles [18]. Many reports have demonstrated the positive effects of the exogenous application of GB on plant growth and yield under drought conditions, for example, in tobacco, wheat, turnip, tomato, common beans (*Phaseolus vulgaris*), rice, and sunflower (Table 18.1).

GB plays a vital role in imparting drought stress tolerance in plants [19–23]. No report has yet demonstrated any negative effects of GB on plant growth and productivity under normal and stressful conditions. Naturally, some plant species including rice, mustard, Arabidopsis, and tobacco do not produce GB under stressful or nonstressful conditions [6]. In these species, and in some naturally GB-accumulating plants, transgenic plants with overexpressing GB synthesizing genes, including *CDH* (*betA*), *BADH*, *CMO*, *COD*, *ApGSMT*, and *ApDMT*, exhibited increased production of GB and enhanced stress tolerance (Table 18.2). Although those transgenic plants might accumulate GB at different levels, all plants exhibited improved drought tolerance. Therefore, both the exogenous application of GB (Table 18.1) and engineered synthesis via manipulation of the GB-biosynthetic pathway into plants (Table 18.2) have increased their tolerance capacity against drought stress. Such approaches of GB application in plant improvement have provided encouraging results, not only for engineering stress tolerance, but also for investigating the working mechanisms of GB.

Table 18.2 Transgenic plants engineered to synthesize glycinebetaine and their enhanced tolerance to drought stress

Plant species	Gene	Remark	Reference
Arabidopsis thaliana	*MpGSMT + MpSDMT*	Protection of photosynthesis	[21]
Solanum tuberosum	*codA*	Protection of photosynthesis; prevention membrane lipid peroxidation and degradation of chlorophyll; increased ROS detoxification	[19]
	BADH	Protection of membrane integrity and yield loss	[23]
Zea mays	*ApGSMT2 + ApDMT2*	Protection of photosynthesis and membrane integrity	[34]
	betA	Protection of membrane integrity	[89]
	CDH	Protection of membrane integrity, enzyme activity, photosynthesis, and yield loss	[22]
Lycopersicon esculentum	*codA*	Protection chlorophyll content and RWC	[90]
Nicotiana tabacum	*ApGSMT2 + ApDMT2*	Protection of photosynthesis and membrane integrity	(42)
	BvCMO	Protection of photosynthesis	[91]
Triticum aestivum	*BADH*	Protection against damage of membrane; decreased photoinhibition of PSII	[49]
	betA	Protection of photosynthesis, yield loss and membrane integrity; increased ROS detoxification	[53]
	BADH	Protection of photosynthesis; enhanced antioxidant activity	[92]
Medicago sativa	*codA*	Maintained higher RWC; increased proline content	[93]
Gossypium hirsutum	*betA*	Protection against damage of membrane and photosynthesis	[50]
Oryza sativa	*codA*	Protection against damage of membrane, enzyme activity, photosynthesis, and yield loss; regulation of ROS detoxification and transcriptome changes	[20]
Brassica napus	*cox*	Protection of photosynthesis and membrane integrity	[94]

18.2 Pathways of GB Biosynthesis in Plants

The most well-known pathways of GB biosynthesis that exist in plants, animals, and microorganisms start with choline as a precursor molecule (Fig. 18.1). In naturally GB-accumulating plants (Fig. 18.1a), GB is synthesized by a two-step pathway that is initiated by choline monooxygenase (CMO), which converts

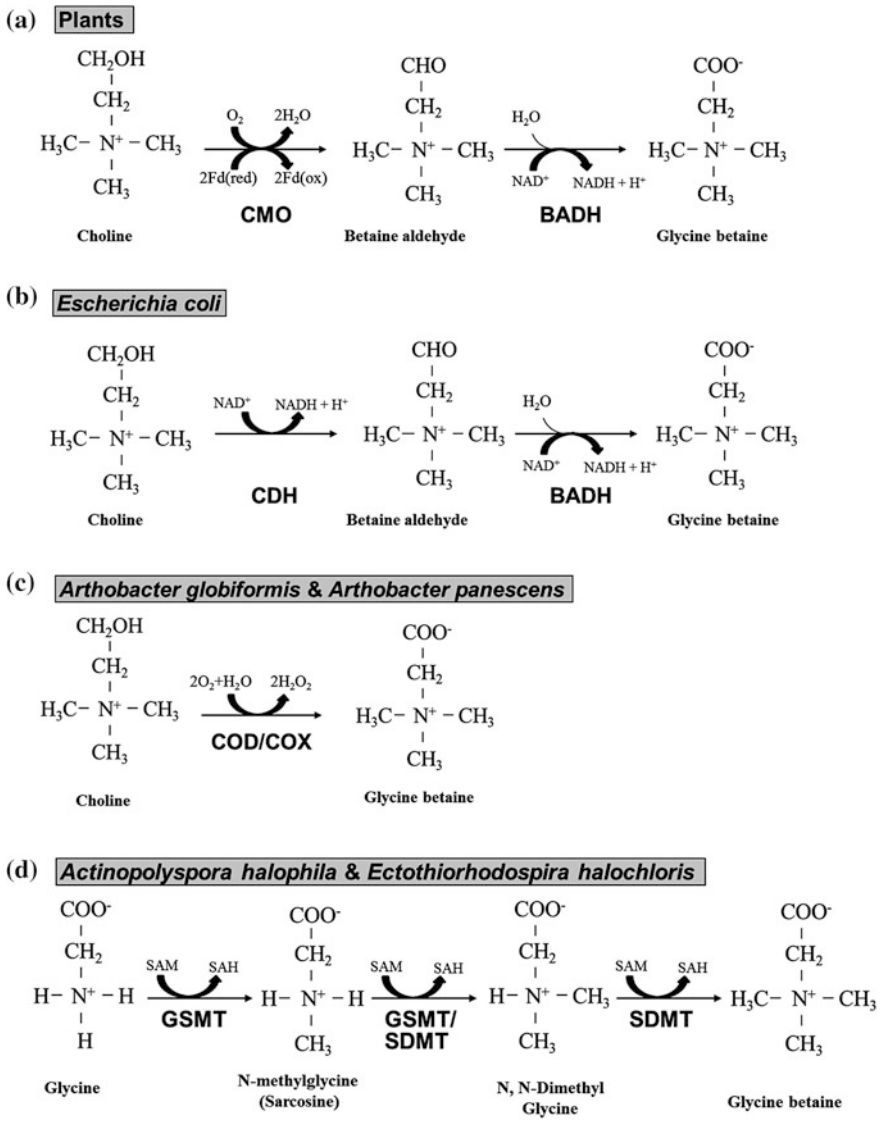

Fig. 18.1 Pathways for GB biosynthesis from different organisms. **a** Two-step dehydrogenated glycinebetaine biosynthetic pathway catalyzed by choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (*BADH*). Fd (*red*) ferredoxin in its reduced form, Fd (ox) ferredoxin in its oxidized form. **b** *E. coli* two-enzyme pathway steered by choline dehydrogenase (*CDH*) and BADH enzymes. **c** One-enzyme pathway in which choline is oxidized by choline oxidase (COD/COX) in *A. globiformis* and *A. pascens*, respectively. **d** A three-step methylation pathway in *Actinopolyspora halophila* and *Ectothiorhodospira halochloris* in which glycine is stepwise methylated to glycine betaine via glycinesarcosine methyltransferase (*GSMT*) and sarcosine dimethylglycine methyltransferase (*SDMT*). SDMT catalyzes the last two steps. *SAM* S-denosylhomocysteine; *SAH* S-adenosylmethionine

choline into betaine aldehyde that is followed by an NAD^+ dependent enzyme called betaine aldehyde dehydrogenase (BADH) that ultimately generates GB. These enzymes are mainly found in the chloroplast stroma [24]. In *Escherichia coli* and animals (Fig. 18.1b), GB is synthesized by the enzyme choline dehydrogenase (CDH) in association with BADH [25], whereas in soil-living bacteria, such as *Arthrobacter globiformis* and *A. panescens* (Fig. 18.1c), choline oxidase (codA) converts choline into GB and hydrogen peroxide (H_2O_2) in a single step reaction [26, 27]. This one-step enzyme pathway seems to be more suitable for GB pathway engineering in as much as only one overexpressed gene is required in transgenic plants rather than that found in two-enzyme pathways (e.g., CMO + BADH, CDH + BADH; [7, 15, 16]).

An alternative pathway that can be utilized to synthesize GB has only been found in extremely halophytic cyanobacteria, for example, in *Actinopolyspora halophila* and *Ectothiorhodospira halochloris* (Fig. 18.1d). In these bacteria, GB is synthesized from glycine instead of choline for GB synthesis by a three-step N-methylation reaction [17, 28, 29] that is catalyzed by two enzymes, that is, glycine sarcosine methyltransferase (GSMT) and sarcosine dimethylglycine methyltransferase (SDMT). The first methylation step is catalyzed by GSMT and the second reaction is catalyzed by both GSMT and SDMT, whereas the third methylation is catalyzed solely by SDMT. Transgenic Arabidopsis plants expressing these genes synthesize more GB than those only expressing CMO [30].

18.3 Genetically Engineered Biosynthesis of GB and Drought Stress Tolerance

The cloning of various genes encoding enzymes that catalyze the biosynthesis of GB from various microorganisms and plants, and their expression in transgenic plants has been extensively reported. Among the different GB biosynthetic genes, *codA* from *A. globiformis* has been widely used for GB production in transgenic plants because of its one-step conversion of choline into GB. Other genes have also been used individually to produce transgenic plants, with the exception of *GSMT* and *SDMT*. These applications have been successfully reported in diverse plant species to enhance tolerance to a variety of abiotic stresses, such as Arabidopsis [21], Brassica [31], persimmon [32], tomato [33], maize [34], rice [35], potato [36], sweet potato [37], and wheat [38, 39].

In many plant species with engineered GB accumulation, GB-biosynthetic enzymes were targeted to the chloroplasts. In some studies, these enzymes were also reported to be targeted to the cytosol [22], the peroxisomes [40], or to both the cytosol and chloroplasts simultaneously [41]. Transgenic rice expressing the chloroplast-targeted choline oxidase encoded by the *codA* gene from *A. globiformis* has been evaluated for tolerance to water stress [20]. During seedling generation, and the vegetative and reproductive stages, transgenic plants have maintained higher

activity of Photosystem II and showed improved physiological performance. For example, enhanced detoxification of ROS as compared to wild-type (WT) plants under conditions of water stress. Using three types of transgenic tomato plants expressing choline oxidase by a *codA* gene that was targeted to chloroplasts, cytosol, or simultaneously to both the chloroplasts and cytosol [41], all three types of transgenic plants exhibited greater chilling and salt stress tolerance as compared to their WT plant counterparts. However, it was determined that GB accumulation in chloroplasts was more effective than that in the cytosol in protecting plants against abiotic stress, even though the level of GB was at its lowest in chloroplasts. There was a significant correlation between the levels of GB in chloroplasts and the extent of tolerance to oxidative stress; whereas no correlation could be built between the levels of GB in the cytosolic and stress tolerance. This suggested that the accumulation of GB in chloroplasts was the most important parameter for protecting the function of GB in plants. Some reports have demonstrated that transgenic plants could accumulate different levels of GB with improved tolerance against drought stress conditions (Table 18.2). In transgenic maize plants generated using the *betA* gene for cytosol-targeted CDH, the total amount of GB in the leaves was much higher than that found in WT plants. However, GB levels in the chloroplasts of both transgenic and WT plants were very low. Nonetheless, these transgenic maize plants exhibited greater drought stress tolerance than WT plants [22].

The enhanced GB synthesis in transgenic maize may exert protection on the activity of Photosystem II, leaf cell membrane integrity, the activity of enzymes, including the enzymes associated with sugar and amino acid metabolism, which led to greater increases in total soluble sugars and free amino acids under dehydration conditions [22]. Meanwhile, it was found that the reproductive development of transgenic maize was less inhibited by drought stress and the grain yields of transgenic plants were greater than those of WT plants [22]. In the field, after drought stress, transgenic lines accumulated threefold more glycinebetaine as compared to WT. These results indicate that there is a positive correlation between glycinebetaine concentration and drought tolerance in the transgenic maize. Therefore, it was suggested that in maize plants, the enhanced tolerance to drought stress resulted from GB being present in the cytosol.

Coexpression of *ApGSMT2* and *ApDMT2* from *Aphanothece halophytica* in tobacco resulted in increased GB levels [42]. The potential functions of *ApGSMT2* and *ApDMT2* in GB synthesis were first studied in transgenic *E. coli* [43], which had increased levels of GB and improved salt tolerance. Compared to transgenic tobacco expressing *betA*, transgenic tobacco coexpressing both *ApGSMT2* and *ApDMT2* accumulated more GB and exhibited enhanced drought resistance with improved seed germination performance, higher relative water content, less cell membrane structural damage, and improved photosynthetic capacity under drought stress. Transgenic maize showing increased expression levels of *ApGSMT2* and *ApDMT2* under drought conditions facilitated GB accumulation in their leaves and conferred improved drought tolerance [34]. Other changes in the transgenic plants included an increased accumulation of sugars and free amino acids, greater chlorophyll content,

a higher photosynthesis rate and enhanced biomass, and lower malonaldehyde (MDA) and electrolyte leakage as compared to the WT counterparts.

18.4 GB Enhances Antioxidative Defenses in Plants

ROS are consistently produced in plants under normal growth conditions but are maintained at a low level through the ROS scavenging system. During stressful conditions, such as drought and low temperature, ROS generation can overwhelm the scavenging mechanisms and become toxic for plant cells. It has been reported in various studies that GB improves the ROS scavenging capacity of plants under abiotic stress [44–46]. It is still an arguable point whether GB has any direct ROS scavenging capability. However, it was previously demonstrated that GB played a vital role in maintaining the activities of ROS scavenging enzymes such as those found in the ascorbate–glutathione cycle [16, 17].

When subjected to water stress, ROS accumulated in plant cells; at the same time the antioxidant system, and especially SOD, which is the most important antioxidant enzyme, was induced to scavenge the newly produced ROS. In transgenic potato plants that expressed the chloroplast-targeted *codA*, the activities of both SOD and CAT increased more significantly than in their WT counterparts during water-stress–rehydration treatment [19]. Meanwhile, WT exhibited a higher MDA content than the transgenic lines under stress conditions. Therefore, after stress, transgenic plants were more capable of eliminating ROS to protect the plant as compared to WT.

Cell membrane stability is often affected by lipid peroxidation that is caused by ROS under stress conditions [47, 48], which results in the production of MDA. [49] reported that transgenic wheat overexpressing a *BADH* gene caused GB overaccumulation, which helps alleviate lipid peroxidation and maintains cell membrane stability, which was consistent with previous in vitro studies [50]. Nevertheless, GB cannot eliminate ROS directly [51]; therefore, the enhanced activities of the antioxidative enzymes and antioxidant content may represent key factors that are involved in GB-mediated decreases in ROS. The antioxidant enzymes are primarily localized in the chloroplast [52], thus it is hypothesized that overaccumulated GB in vivo may be partly transported into the chloroplast, which then stabilized the structures of these enzymes under stress conditions. The increased nonenzyme antioxidants such as AsA and GSH in transgenic plants may indirectly affect the corresponding synthetic enzymes.

Electrolyte leakage and MDA levels have been widely used as indicators to identify cell membrane permeability and lipid peroxidation caused by ROS. [53] reported that drought stress led to lower membrane stability along with much higher activities of SOD and POD in all *betA* transgenic and WT wheat lines. However, the *betA* transgenic lines were less injured and exhibited greater root length and growth as compared with the WT plants. Drought stress caused severe electrolyte leakage in WT plants, whereas the transgenic lines were much less affected. In

addition, the MDA levels in the transgenic wheat lines were significantly lower than the WT plants under drought stress, which may be an important factor in making electrolyte leakage from the transgenic plants lower than that found in the WT plants. The elevated accumulation of GB in transgenic lines may play an important role in maintaining the cell membrane stability by reducing ROS indirectly, and this was partly mediated by the increased activities of some antioxidant enzymes such as SOD and POD.

Similarly, under conditions of transgenic cotton displaying enhanced GB by expressing the *betA* gene [50], a lower percentage of ion leakage was found, and decreased lipid membrane peroxidation was detected in transgenic cotton plants than was found in WT plants. The low ion leakage that occurred in the transgenic plants might have been due to decreased lipid peroxidation under drought stress conditions. Simultaneously, the decreased lipid peroxidation under drought stress in the transgenic plants would reflect lower levels of ROS as compared the WT plants. Cell membrane stability could be affected by lipid peroxidation caused by ROS under stressful conditions [54]. Therefore, elevated accumulation of GB in transgenic cotton might be helpful in maintaining cell membrane stability by reducing ROS secretion, which would be partially achieved following increased activities of some antioxidant enzymes such as SOD, and concordantly with that found by prior work [55, 56].

18.5 Protection of the Photosynthetic Machinery

The photosynthetic activity of the chloroplast is one of the most stress-sensitive physiological processes in plants. Stress might damage the thylakoid membrane, disturb its functions, and ultimately decrease photosynthesis and plant yield [57, 58]. Preservation of the photosynthetic apparatus is an important strategy aimed at enhancing crop yield under stressful conditions. GB is regarded as one of the most effective osmoprotectants due to its many advantages mentioned above, in addition to being a compatible solute [16, 17].

Wang et al. [49] reported that BADH transgenic wheat showed improved protection of the thylakoid membrane structure, composition, and function during drought stress processes. Drought stress resulted in swollen and loosely scattered thylakoid lamellae in WT plants, but this was not obvious in transgenic lines. Moreover, the stress-induced chloroplast and thylakoid damage was more severe in WT plants than in transgenic lines, suggesting that overaccumulation of GB resulted in protection of the photosynthetic apparatus in wheat leaves. GB may stabilize the lipid composition of the thylakoid membrane, and GB appears to enhance xanthophyll cycle-dependent nonradioactive energy dissipation, systems that might be involved in the protection of the thylakoid structure and function.

During drought stress, ROS are continuously produced in the chloroplast and mitochondria as by-products of metabolism. However, their production is enhanced under abiotic stress, which leads to photoinhibition of PSII in the chloroplast. GB

antagonizes inhibition of protein biosynthesis, and thus enhances PSII repair leading to increased stress tolerance. In transgenic potato, overaccumulated with GB, much higher photosynthetic parameters were seen as compared to nontransformed plants [19]. For example, under drought stress, the photosynthetic rate (Pn) and stomatal conductance (Gs) in transgenic plants decreased only slightly as compared with control treatment. In addition, the intercellular carbon dioxide concentration (Ci) and transpiration rate (Tr) were higher in transgenic plants than were found in non-transformed plants, indicating that these parameters were directly related to Pn and Gs. As GB can protect the repair machinery of PS [59, 60], the photosynthetic parameters of transgenic plants could be recovered more efficiently after water stress treatment during the recovery stages. Moreover, the higher leaf water potential in transgenic plants sustained a higher Tr. This demonstrated that GB synthesis that was produced by introduced *codA* gene expression in transgenic plants could prevent membrane lipid peroxidation and degradation of chlorophyll caused by stress.

The performance of the *betA*-transgenic wheat lines was superior to that found in WT plants by maintaining higher rates of photosynthesis and suffering decreased injury after drought stress [53]. To understand the photosynthetic limiting factors under drought stress, the maximal efficiency of PSII photochemistry (Fv/Fm) was measured in dark acclimated leaves. The Fv/Fm value is the most frequently used parameter to indicate the extent of injury to the PSII complex due to stress factors including drought. The Fv/Fm value in the WT declined significantly during drought stress, whereas in the transgenic lines, it decreased more slowly. Drought stress leads to a substantial reduction in net photosynthesis, which occurs as a result of stomatal closure that restricts the diffusion of CO_2 into the leaves, or other factors such as inhibition of Rubisco or adenosine triphosphate synthesis [61]. The higher levels of GB may have effectively stabilized the activity of macromolecules by protecting highly complex proteins, such as RuBP carboxylase or the PSII complex [62–64].

Transgenic rice with chloroplast-targeted codA enzyme from *A. globiformis* showed enhanced tolerance to water stress at different developmental stages, from seed germination to the reproductive stage. Chloroplast-targeted biosynthesis of GB protects photosynthetic machinery more efficiently than cytosol-targeted production [41, 65]. As the production of ROS is enhanced by abiotic stresses and the major site of production of ROS is the chloroplast, the enhanced GB accumulation alleviates the extent of photoinhibition of PSII. GB protects the oxygen-evolving PSII and stabilizes the activity of Rubisco enzyme at high concentrations of NaCl [66]. However, their reports showed that GB enhances the repair of PSII by protecting the repair machinery rather than directly protecting PSII against photo damage [59, 60]. Therefore, enhanced PSII activity in transgenic Indica rice lines may be due to chloroplast accumulation of GB, which protects the PSII repair machinery and thus maintains efficient photosynthesis under drought stress [15, 59, 67]. Higher PSII quantum yield in transgenic plants during drought stress could result in improved vegetative and reproductive growth.

18.6 GB-Induced Expression of Specific Genes and Drought Stress Tolerance

Extensive studies on GB have suggested its variable roles in protecting plants under stressful conditions. As suggested by Fan et al., several mechanisms have been proposed for GB-mediated abiotic stress tolerance in plants, including osmoregulation, stabilization of native structure of proteins and enzymes, membrane integrity, protection of photosynthesis, and ROS detoxification [37]. Increased proline biosynthesis was also noticed, and yet direct evidence indicating their interaction is lacking. Nevertheless, GB, together with proline can destabilize the double-helix structure of DNA, which lowers the melting temperature of DNA in vivo [68], which indicates that these molecules could activate replication and transcription to regulate gene expression under abiotic stress. Indeed, there are many differentially expressing endogenous genes that have been identified in GB-mediated stress tolerance of rice [20]. With all this increasing evidence of GB function in mind, it would be interesting to determine the regulatory mechanism responsible for natural GB accumulators in normal and stressful conditions, and to compare these profiles with the data acquired from GB-transgenic plants. This information might provide new insights into the function of GB.

As mentioned above, the production of ROS is closely associated with various types of abiotic stress. The role of the ROS scavenging systems in GB-mediated tolerance of plant cells to abiotic stress has become increasingly important. In Arabidopsis, it was found that upon exogenous application of GB, seven genes encoding proteins directly involved in scavenging ROS were upregulated. These proteins included glutathione reductase, cytoplasmic Cu/Zn superoxide dismutase, glutathione S-transferase, peroxisomal Cu/Zn superoxide dismutase, catalase 2, monodehydroascorbate reductase, and ascorbate oxidase [69]. These observations provide strong evidence for the involvement of ROS-scavenging systems in GB-mediated stress tolerance. The model of how GB-upregulated genes in the roots of Arabidopsis prevent ROS accumulation in cell walls has been suggested in studies of preventing ROS signaling that was associated with chilling stress [70, 71].

Transgenic Indica rice plants that had been transformed with a *codA* construct for chloroplast-targeted choline oxidase exhibited increased tolerance to water stress and enhanced detoxification of ROS under water stress [20]. Transcriptome analysis showed altered expression of several transcripts in the transgenic rice, even under unstressed conditions in relation to WT plants. Out of 165 upregulated genes, 50 genes are known to be involved in the response of plants to abiotic stress. Among stress-related enhanced genes, some are of particular interest. One such gene encoding the aquaporin protein showed a 5.18-fold upregulation. Aquaporins are water channel proteins, and their protective role during drought and salinity stress is well known [72, 73]. Interestingly, aquaporins were found to regulate the diffusion of H_2O_2 across the plasma membrane [74, 75]. Another such upregulated gene codes for an NAC domain-containing transcription factor. Overexpression of this gene alone conferred tolerance to drought and salinity stress in both laboratory

and field conditions [76–78]. Likewise, other major components of stress-responsive pathways, heat shock proteins, MYB-related proteins, ethylene-responsive proteins, zinc finger proteins, PR proteins, receptor-like kinase, sugar and ion transporters, and cytochrome P450 proteins were also shown to enhance gene expression in *codA* transgenic rice. Upregulation of these genes might be responsible for the observed stress tolerance in transgenic rice. These studies provided strong evidence that enhanced expression of stress-responsive genes in GB-accumulating transgenic plants might be a plausible explanation for GB-mediated stress tolerance.

18.7 Conclusion and Perspectives

GB is an extremely efficient compatible solute that is strongly associated with enhanced tolerance of accumulating plants in drought stress environments. Meanwhile, the biosynthesis of GB, and its mechanisms of augmenting tolerance to drought stress have been extensively studied (Table 18.2). As demonstrated in the model that was suggested by Fan et al. [37], possible mechanisms for the GB-enhanced tolerance in plants to drought stress might include, but are certainly

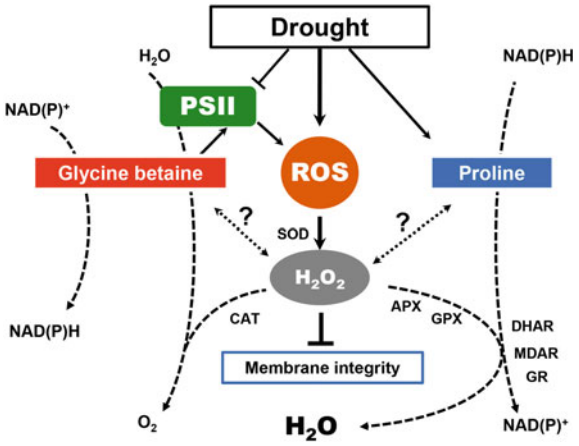

Fig. 18.2 Mechanism of glycine betaine (GB) function in drought stress tolerance. Enhanced accumulation of GB by the expression of GB synthetic genes in plants changes the redox homeostasis, improves PSII, and blocks ROS generation. Additional NADP(H) production also facilitates proline accumulation and ROS scavenging. Under stress conditions, GB promotes ROS scavenging by the systemic upregulation of ROS-scavenging gene expression, which eventually protects cellular functions and membrane integrity. Key: *APX* ascorbate peroxidase; *BADH* betaine aldehyde dehydrogenase; *CAT* catalase; *DHAR* dehydroascorbate reductase; *GPX* glutathione peroxidase; *GR* glutathione reductase; *MDHAR* monodehydroascorbate reductase; *POD* peroxidase; *PSII* photosystem II; *ROS* reactive oxygen species; *SOD* superoxide dismutase. (Modified from [37])

not limited to: [36] alleviating the production of ROS, [44] protection of the photosynthetic machinery, and [45] induction of specific genes whose products are involved in stress tolerance. Nevertheless, further understanding of the molecular mechanisms of GB function in plants is required (see Fig. 18.2), such as how H_2O_2 regulates or affects the biosynthesis of GB at the molecular level, and precisely how GB is incorporated or interacts with proline function in plants.

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Chapter 19
Genetically Modified Crops with Drought Tolerance: Achievements, Challenges, and Perspectives

Chanjuan Liang

19.1 Introduction

Drought is the single largest abiotic stress factor leading to reduced crop yields. Approximately one-third of the Earth's land area is arid or semi-arid. The situation is aggravated by the shortage of water resources because of widespread water pollution and unpredictable climatic change [79, 83]. Water availability is particularly critical for agricultural crops to maintain high yields in variable growing seasons. Thus, agricultural drought, namely water deficiency, adversely affects plant and crop production by reducing leaf size, stem extension and root proliferation, disturbing plant water and nutrient relations, and inhibiting water-use efficiency [16]. For example, the average annual yield loss of maize (one of the most important grains on Earth) attributable to drought is approximately 15 % [3]. Soybean (the most important legume on Earth) is considered the most drought-sensitive plant because drought may reduce soybean yield by approximately 40 % [17, 25]. During periods of severe drought, these losses can be much higher and can potentially result in complete crop failure. Obviously, drought is currently the leading threat to the world's food security. At the same time, it is a big challenge to achieve an average annual increase in cereal production of 44 million metric tons per year for meeting the demand of 9 billion people by 2050 [32]. Developing drought-tolerant crops may become a factor to maintain plant growth and productivity. Drought-tolerant crops can be cultured in areas where other crops cannot easily grow, thereby sustaining and potentially expanding the area for agricultural production.

In recent years, many countries and international organizations have launched research projects on exploring the drought-tolerance and water-saving mechanisms

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of plants to identify key genes or tools for improving plant drought resistance. With the rapid development of the theory and technology of modern biology, researchers have been justified that modern molecular-biological technology is more rapid and efficient than conventional selection and breeding in terms of ability to produce drought-tolerant crops [53]. However, broad adoption of genetically modified crops (GM crops), including crops with drought tolerance, will depend on adequate safety assessment and related public acceptance. So far, conventionally bred varieties with similar drought-tolerant characteristics generally do not need to go through a pre-market safety assessment. To meet safety concerns in the case of GM crop varieties, many countries have formulated specific regulations to assess the safety of these crops for human and animal consumption and for the environment, prior to market approval [27, 29, 33, 62]. In this chapter, we first provide a brief overview of recent developments in drought-tolerant GM crops. Subsequently, we address current food and environmental safety assessment strategies, discuss the challenges, and indicate necessary steps in further developments.

19.2 Genetically Modified (GM) Crops with Drought Tolerance

For more than two decades, scientists have conducted vast amounts of research, including studies on morphological traits, and the structural, physiological, biochemical, and molecular regulation to reveal the mechanisms of drought responses of plants. The response of plants to drought stress is a complex process involving many genes and signaling pathways. Shinozaki and Yamaguchi-Shinozaki [72] indicated that genes involved in these responses can be grouped in two main classes: single function genes and regulatory genes based on their biological function. The single function genes encode enzymes associated with the accumulation of osmolytes, proteins, and enzymes scavenging oxygen radicals (ROS), proteins associated with the uptake and transport of water and ions (ion transporters, channels), and proteins involved in lipid biosynthesis [68]. The regulatory genes are involved in signaling cascades and transcriptional or posttranscriptional regulation of gene expression such as transcription factors, protein kinases, protein phosphatases, and proteinases [72]. Meanwhile, regulatory proteins have been proven to play crucial roles in the responses of plants to drought stress conditions. Consequently, the modification of the expression of a regulatory gene is more efficacious and is probably to be widely used in GM crops with abiotic stress tolerance (the next generation of GM crops) [68]. In addition, transcription factors are a dispensable group of proteins that modulate gene expression to respond to drought stress at the transcriptional level. Many transcriptional regulators involved in plant responses to drought belong to one of the large transcription factor families such as APETALA2/Ethy-lene-responsive element binding protein (AP2/EREBP), basic leucine zipper (bZIP), NAM-ATAF1/2-CUC2 (NAC), MYB, and zinc finger [31]. Recent reviews

Table 19.1 Recent reviews on drought tolerance in plants

Title of review	References
Recent advances in the dissection of drought-stress regulatory networks and strategies for development of drought-tolerant transgenic rice plants	[78]
General mechanisms of drought response and their application in drought-resistance improvement in plants	[31]
Introduction to desiccation biology: from old borders to new frontiers	[54]
Engineering crop plants against abiotic stress: current achievements and prospects	[2]
Tolerance to drought and salt stress in plants: unraveling the signaling networks	[36]
Genetic engineering and breeding of drought-resistant crops	[42]
Recent progress in drought- and salt-tolerance studies in Brassica crops	[86]
Mechanism of ABA signal transduction: agricultural highlights for improving drought tolerance	[47]
Current state of the problem of water relations in plants under water deficit	[51]
Challenges and perspectives to improve crop drought and salinity tolerance	[18]
Drought tolerance in modern and wild wheat	[11]
Potentials toward genetic engineering of drought-tolerant soybean	[77]
Any trait or trait-related allele can confer drought tolerance: just design the right drought scenario	[76]
Targeting, metabolic pathways for genetic engineering abiotic stress tolerance in crops	[68]
Tackling drought stress: receptor-like kinases present new approaches	[58]
Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks	[50]
Transgenic plants for abiotic stress tolerance: current status	[44]
Recent molecular advances on downstream plant responses to abiotic stress	[26]
Drought tolerance through biotechnology: improving translation from the laboratory to farmers' fields	[24]
Progress studies of drought-responsive genes in rice	[38]
Models and tools for studying drought stress responses in peas	[57]
Transcription factors as tools to engineer enhanced drought stress tolerance in plants	[43]

on drought-tolerant crops are summarized in Table 19.1, for those who are interested in this specific topic.

Although many genes have been identified that can improve drought tolerance in plants and some drought-tolerant crops have been developed, progress has been rather limited regarding producing such crops for field condition or commercialization. The first drought-tolerant GM crop plant is MON 87460, a maize (*Zea mays* L.) product developed by Monsanto Company in 2009, and first planted in the United States in 2013, increased 5.5-fold from 50,000 ha in 2013 to 275,000 ha in 2014 (http://www.isaaa.org/resources/publications/briefs/49/toptenfacts/default.asp). And Water Efficient Maize for Africa aimed at delivering biotech drought-tolerant maize to

selected countries in Africa by 2017. MON 87460 expresses cold shock protein B (CSPB) from *Bacillus subtilis* to impart drought tolerance. In bacteria, cold shock proteins are believed to help preserve normal cellular functions by binding cellular RNA and maintaining RNA stability and translation under certain stresses. Similarly as in bacteria, the CSPB protein in MON 87460 maintains normal cellular functions under drought-stress conditions by preserving RNA stability and translation [60]. In addition, the overexpression of CSPB was shown to provide stress tolerance to arabidopsis and rice [13]. A drought-tolerant GM rice containing an osmotin gene is in the advanced R&D pipeline worldwide, and expected to be commercialized possibly in India after 2015 [74].

Demonstration of drought efficacy of GM traits in the field is a critical step for showing commercially relevant drought tolerance. Deikman et al. [24] reviewed some developed drought-tolerant GM crops including rice, cotton, maize, and canola in field testing from 2009 to mid-2011. Many of these recent discoveries have been in rice, which is both an excellent model species for basic research, and one of the world's most important crops. *Stress-responsive NAC1* (*SNAC1*), an NAC-type transcription factor, is specifically induced in guard cells under drought-stress conditions. Overexpression of *SNAC1* in rice resulted in significantly enhanced drought tolerance under severe drought conditions in the field at the reproductive stage (22–34 % higher seed setting) without any phenotypic changes or yield penalty under normal growth conditions [41]. The *SNAC1*-overexpressing rice showed a significant reduction in stomatal aperture, and the enhanced drought tolerance appeared to be due partially to increased water use efficiency and increased abscisic acid (ABA) sensitivity in guard cells [41]. When *SNAC1* was overexpressed in wheat, transgenic wheat also showed improved drought tolerance [69]. In addition, drought tolerance in GM rice plants overexpressing *OsNAC5* [46], *OsNAC9*/*SNAC1* [67], or *OsNAC10* [45] under control of the root-specific promoter has also been examined in the field. Xiao et al. [84] examined drought tolerance of transgenic rice plants overexpressing seven well-documented stress-related genes with an actin promoter under field conditions. The seven genes were *CBF3/DREB1A*, an AP2/ERF-type transcription factor; *SOS2*, aserine/threonine protein kinase; *NCED2* and *LOS5*, enzymes involved in ABA biosynthesis; *NPK1*, a mitogenactivated protein kinase; *ZAT10*, a C2H2-type zinc finger transcription factor; and *NHX1*, a vacuolar Na^+/H^+ antiporter. Although drought stress in the field decreased grain yield in these GM rice, grains yields in *LOS5, ZAT10*, and *NHX1* overexpressors were less affected. Increased grain yield was observed in GM rice plants overexpressing the *AP37* gene, an AP2/ERF-type transcription factor, being subjected to drought stress in the field [64]. Finally, the GM rice plant overexpressing *EDT1/HDG11*, a homeodomain-leucine zipper transcription factor, has been evaluated under field conditions. The drought-treated GM rice plants had higher grain yields than those observed in the drought-treated non-GM rice plants [85].

In addition, isopentenyltransferase (IPT) is a critical enzyme in the cytokinin biosynthetic pathway. The expression of IPT under the control of a maturation- and stress-induced promoter has been shown to delay stress-induced plant senescence that resulted in an enhanced drought tolerance in both monocot and dicot plants.

Qin et al. [66] extended the earlier findings in tobacco and rice to peanut (*Arachis hypogaea* L.). Regulated expression of IPT in peanut significantly improved drought tolerance under both laboratory and field conditions. *LOS5/ABA3* gene encoding molybdenum cofactor sulphurase is involved in aldehyde oxidase (AO) activity in Arabidopsis, which indirectly regulates ABA biosynthesis and increased stress tolerance. Li et al. [55] used a constitutive superpromoter to drive *LOS5/ABA3* overexpression in soybean (*Glycine max* L.) to enhance drought tolerance in growth chamber and under field conditions. They found that the seed yield of transgenic plants is at least 21 % higher than that of wild-type plants under drought stress conditions in the field. The Arabidopsis gene *AVP1* encodes a vacuolar pyrophosphatase that functions as a proton pump on the vacuolar membrane. Overexpression of *AVP1* in Arabidopsis, tomato, and rice enhances plant performance under salt and drought stress conditions [65]. Dehydration responsive element binding (DREB) proteins, belonging to the AP2/ERF family, have been extensively characterized for their role in response to drought stresses [59]. Recently, de Paiva Rolla et al. [23] assessed the performance of soybean plants overexpressing the transcription factor DREB1A under drought conditions in the greenhouse and in the field. In the field, some yield components (the number of seeds, the number of pods with seeds, and the total number of pods) were increased when drought was introduced during the vegetative stage although the DREB protein plants did not outperform the cultivar BR16 in terms of yield. The greenhouse data suggest that the higher survival rates of DREB plants are because of lower water use due to lower transpiration rates under well-watered conditions.

Currently, researchers have made substantial progress in the genetic improvement of drought tolerance. However, before applying these GM varieties with drought tolerance for benefiting agricultural production, an extensive safety assessment must be conducted, especially when it is intended for a food or feed product.

19.3 Food Safety Assessment of GM Crops with Drought Tolerance

19.3.1 General Practice in Food Safety Assessment of GM Crops

At an early stage in the introduction of recombinant-DNA technology in modern plant breeding and biotechnological food production systems, efforts began to define internationally harmonized evaluation strategies for the safety of foods derived from genetically modified organisms (GMO). Global consensus has been reached on the basic scientific strategy to assess GM plants and derived products thereof [33]. This strategy, based on the concept of substantial equivalence, has now been incorporated in regulations in many countries [12, 29, 34, 35], and is basically a comparative safety assessment [49]. The principle of substantial

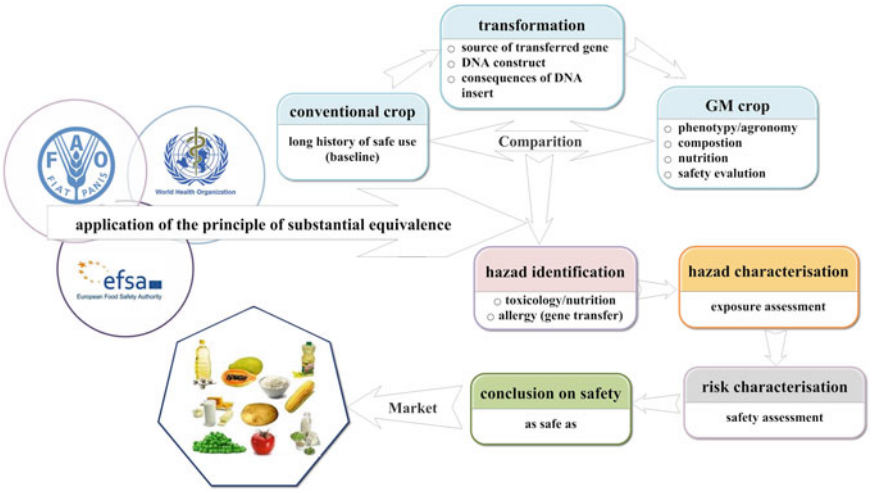

Fig. 19.1 Food safety assessment strategies for genetically modified (GM) crops

equivalence is part of a safety assessment framework, based on the idea of existing foods as a basis for comparing the properties of GMO with the appropriate counterpart [52]. The underlying assumption of this comparative assessment approach is that the comparable conventional varieties which have a long history of safe use are used as a baseline for the safety assessment of the newly developed plant varieties. The application of this comparative approach is considered to identify differences between the GM plant and its non-GM comparator including intended as well as unintended changes. Thus, the outcome of the assessment will not be that the GM plant is safe or not, but the result will be formulated in terms of "as safe as" comparable conventional variety that we consider as safe [27, 29, 33] (Fig. 19.1).

Generally, the safety assessment of a GMO involves the following steps: (i) characterization of the parent crop; (ii) characterization of the donor organism(s) from which any recombinant DNA sequences are derived, the transformation process, and the introduced recombinant DNA sequences; (iii) a safety assessment of the introduced gene products (proteins and metabolites); and (iv) a food safety assessment of the whole food divided from the GM crop or the edible part of the GM crop. According to the EFSA guidance, testing of the whole food derived from a GM plant is only required if the composition has been substantially modified or if there are indications for the potential occurrence of unintended effects. To assess GM crops, scientists need to focus on several aspects [48]: (i) molecular characterization of the introduced genetic fragment and resulting new proteins or metabolites (in addition, an increasing number of European member states routinely ask for characterization of the insertion point of the transgenic fragment); (ii) analysis of the composition of the relevant plant parts with respect to key nutrients and antinutrients, including natural toxins and potential allergens; (iii) potential for gene transfer of specific genes from the GM food to—particularly—microorganisms in

the human and animal gastrointestinal tract; (iv) potential allergenicity of the new gene products, or alteration of the intrinsic allergenicity of the GM food organism; (v) estimated intake levels of the newly introduced proteins as well as of the final product, including any altered constituent; (vi) a toxicological and nutritional evaluation of the resulting data; and (vii) additional toxicity testing (of the whole food) where necessary. The potential hazards of GM plants to human and animal health may be associated with toxicity, allergenicity, intolerance, nutritional quality, and microbiological safety of the food, and the possible side effects due to disruption of the metabolic pathways [20].

Presently, a total of 17 different crops are available to risk assessors in the OECD consensus documents. This first phase aims to identify the hazard [49]. Hazard is defined as the potential of a chemical agent to cause harmful effect(s), and risk as a function of probability that an adverse effect will occur due to the presence of a hazardous compound in food and the severity of the adverse effect (exposure × toxicity). If, on the basis of the above-mentioned basic dataset, there are no indications for adverse effects in the GM plant that may lead to decreased food or feed safety, then in most countries no additional studies will be required. If, on the other hand, differences are observed between the GM plant and comparable conventional varieties, additional toxicological and nutritional studies may be required to characterize this hazard in terms of food and feed safety and nutritional status of the newly developed GM plant [49]. If hazards are identified, additional intake assessment data may also be required to conclude the risk assessment. This will especially be the case if it is likely that the new crop will replace important parts of the diets of humans or animals, or aims at specific groups within the population.

19.3.2 Specific Food Safety Aspects of GM Crops with Drought Tolerance

New GM plant varieties with drought tolerance will also be assessed in this way. Usually, the drought tolerance will be based on the introduction of single gene encoding transcription factors or proteins involved in biochemical pathways. Most of these transcription factor genes and other genes involved are either already known as general drought-related genes, and derived drought-related proteins, or are osmosis- or transporter-related genes that will enable the plant to keep growing under water-deficiency conditions [8]. For instance, compositional changes could result from GM approaches to improve crop tolerance to drought. The environmental effects may lead to greater changes in levels of particular crop components than the apparent difference between the GM crop and its comparator [10]. Drought imposes an osmotic stress and plants use changes in metabolic processes as part of their response, for example, by interconverting starch or fructan with simple sugars and synthesizing compatible solutes such as proline and glycine betaine. Therefore, strategies aimed at manipulating these responses could result in compositional changes [39]. In addition, GM crops with stress-related genes may, in theory, more often have hazardous

characteristics, as similar genes may also be involved in defense systems against, for instance, pests or pathogens in general. On a case-by-case basis it needs to be evaluated if drought-related gene products may also affect the health of the human or animal consumer. This may either be a direct effect, an expression product with potentially toxic characteristics, or an indirect effect, as a result of induced changes in the physiology of the plant that may have adverse effects on the health, or nutritional status, of humans and animals. For GM crops with osmosis- or transporter-related genes and more general transcription factors [71, 73], the same case-by-case approach will be followed; but here it seems less likely that the new product will have direct toxicologically relevant characteristics. The genes used can be derived from plants or bacteria. When derived from plants, the expressed products are often already a part of the human diet. When derived from bacteria, this may lead to more extensive studies to complete the hazard characterization phase because most bacteria are unknown with regard to their safety in the human diet [56].

For the comparative approach another important aspect is that the newly developed GM crop with drought tolerance could also be grown under water deficiency conditions compared to its conventional counterpart. For instance, the compositional analysis is based on the growing of the GM crop and its conventional counterpart under identical circumstances; this may not be representative in the case

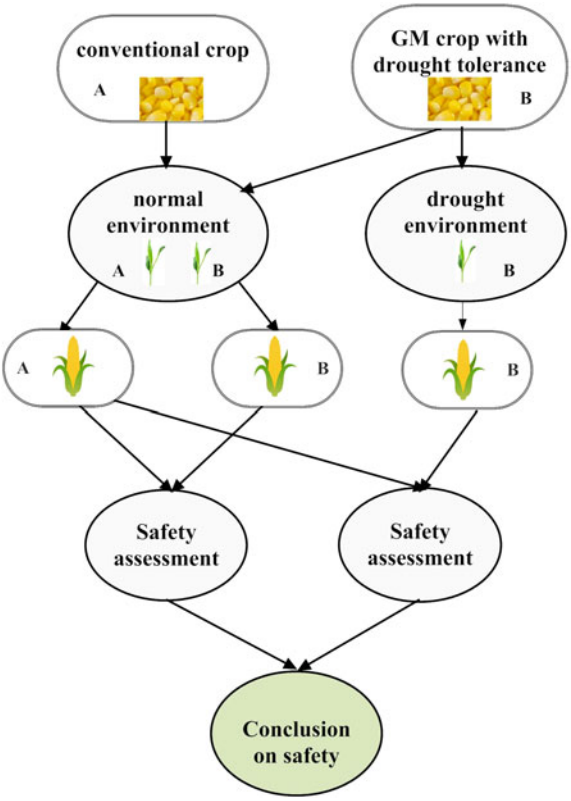

Fig. 19.2 A triangle method proposed for assessing safety of genetically modified (GM) crops with drought tolerance

of abiotic stress-tolerant crops [82]. The GM crop with drought tolerance is aimed to grow under water deficiency conditions that are disadvantageous or even lethal for the conventional counterpart. It may thus be necessary not only to compare the GM crop and its conventional counterpart directly under the "normal" environmental conditions of the conventional crop. In addition, compositional analyses of the GM drought-tolerant crop should be compared when growing under both the "normal" conditions of the conventional crop and the aimed stress conditions for the new GM crop. In this way, by this triangle equation, the physiological consequences of the newly introduced trait can be best estimated and assessed for their potential toxicological and nutritional consequences (Fig. 19.2) [56].

The principle of substantial equivalence has now been incorporated in regulations in many countries, however, implementation of the principle led to controversy and hampers the precision of the actual safety assessment. Risk assessors still face challenges in applying the comparative approach to assess food safety of GM crops with drought tolerance. Moreover, before releasing for human consumption, postmarketing monitoring should be done to monitor the adverse effects or other health outcomes related to the consumption of transgenic-derived foods [52].

19.4 Environmental Safety Assessment of GM Crops with Drought Tolerance

19.4.1 General Practice in Environmental Safety Assessment of GM Crops

To assess the environmental safety of newly developed GM crops is the second step in all countries where GM plants are regulated before commercial use [1, 19]. The basic environmental safety assessment strategy for GMOs is, similar to the food/feed safety assessment, based on a comparison of the GMO of with their non-GM counterparts [30]. The underlying assumption of this comparative assessment approach is that traditionally cultivated crops have a long history of safe use, and then can serve as a baseline for the environmental safety assessment [28]. The safety assessment process follows six steps which are: problem formulation including hazard identification; hazard characterization; exposure characterization that includes levels and likelihood of exposure; risk characterization that integrates hazard, magnitude of the potential consequences and likelihood of occurrence; risk management strategies; and conclusions [28] (Fig. 19.3).

The environmental safety assessment should identify and evaluate potential adverse effects of a GM crop, and elucidate the requirements for risk management [75]. The specific areas to be concerned by applicants and risk assessors include: (i) the potential for increased weediness or invasiveness of the crops, and any changes in ecological fitness; (ii) gene flow by pollination to weeds and wild plants; (iii) gene flow into microorganisms and its stabilization; (iv) potential (chemical)

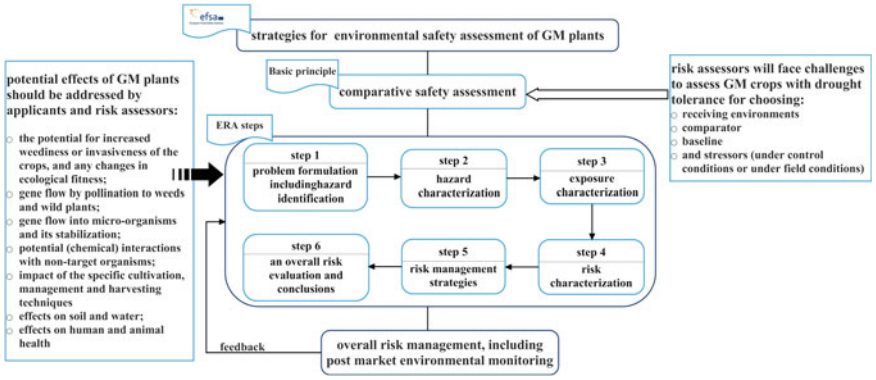

Fig. 19.3 Environmental safety assessment strategies for genetically modified (GM) crops

interactions with nontarget organisms; (v) impact of the specific cultivation, management, and harvesting techniques, such as changes in the weed control regime, and effects on wildlife biodiversity, reduced efficiency of controlling pests, disease, and weeds, and the like; (vi) effects on soil and water; and (vii) safety of foods/feed derived from GM crops for humans and animals [15, 19, 21, 81, 82]. Each specific area of concern is considered in a structured and systematic way following the above-mentioned steps (Fig. 19.3). In addition, each safety assessment procedure for new GM crops that may enter the environment considers the possibility, probability, and consequence of harm on a case-by-case basis, meaning that the required information may vary depending on the type of the GM plants and trait(s) concerned, their intended use(s), and the potential receiving environment(s) [28].

19.4.2 Specific Environmental Safety Aspects of GM Crops with Drought Tolerance

The scientific principles underlying the environmental safety assessments completed for the first generation of GM crops with insect and herbicide resistance commercialized to date are now being applied to crops that are modified for improving tolerance to abiotic stress [61]. The environmental safety of GM crops with abiotic stress tolerance, such as GM crops with drought tolerance, may be more complex as they are likely also to be grown in different environments from the conventional counterparts [5]. EFSA provides detailed indications on how to set up the comparative assessment for GM crops. However, when applying these

indications to drought-tolerant GM crops, risk assessors will face challenges to choose receiving environments, comparator and baseline, and stressors (under control conditions or under field conditions). The possible receiving environments are related to select test sites and conditions whereas a comparative analysis is planning [9]. If GM crops with drought tolerance would only be planted in the same environments as the nonmodified plants, the trait would not extend the range of GM crops, and not beyond the range of previous cultivation. However, such extension needs to be taken into account if drought-tolerant GM crops were to be grown in water-limited conditions where their wild counterparts are less likely to grow. Drought-tolerant GM crops may thus invade new habitats, and consequently, the biodiversity of those habitats could be affected. If the conventional comparator will not be grown in the same environments as the GM crop, comparative analysis may meet problems. This would be a rather extreme case; more likely is growth in areas with occasional water-limited problems, with a possible extension to, for example, more dry areas not used before. On the other hand, nutrient efficiency might just be used in the same environment, but enabling lower use of fertilizer. Both aspects would need to be assessed. The choice of appropriate comparators is essential to indentify hazard, and to test potential differences under both well-watered and water-limited conditions. If conventional comparators cannot tolerate the drought stress, they will be impaired and not be suitable as a comparator (baseline) under drought conditions. To control stressors is possible in growth rooms or greenhouses, but results might not be directly translatable to the field. Under field conditions, it would be hard to ensure crops exposed to a single stressor. That is particularly relevant if crosstalk or multiple phenotypic effects are identified. In addition, tolerance to abiotic stress is likely to be sensitive to the presence and level of stressors. Hence, there is a need to quantify stressors across a wide geographic region with varying environmental conditions. There is a clear need for further guidance in these aspects of the environmental safety assessment.

Environmental safety assessment of GM plants with drought tolerance may be more difficult due to the challenges in choosing receiving environments, comparator (baseline), and stressors (exposure pathways and potential environmental effects). Drought tolerance could be a fitness-enhancing trait that increases the reproductive and vegetative growth and competitive ability of plants under selective pressure (drought condition). Compared to the first-generation insect-resistant and herbicide-tolerant crops that have not been more invasive in natural habitats [6, 7], GM crops with increased fitness advantage may have an invasive potential in both agricultural and natural environments [14, 61]. However, there are limited data available that could evaluate the potential for increased weediness or invasiveness in a GM crop with a fitness-enhancing abiotic stress-tolerance trait(s). Fitness-enhancing traits may not necessarily increase weediness potential because weediness is associated with a complexity of plant characteristics, and also highly depends on the limiting factors of the receiving environment (agricultural conditions). For instance, Sammons et al. [70] provided some information on characterization of drought-tolerant maize MON 87460 for use in environmental safety assessment. Some data are used for the

environmental safety assessment of MON 87460 including evaluation of MON 87460 for agronomic and phenotypic parameters, ecological interactions, reactions to abiotic stressors, root growth, and across-season water use with those of the conventional comparator. The only consistent difference observed between MON 87460 and the comparator was a yield benefit under water-limited conditions, being consistent with the product concept. Beyond that, no differences in weediness or pest potential, in season-long water consumption, or in root growth and development were observed. Transgenic sugarcane lines expressing the *OsDREB1A* and *ZmDof1* genes were developed that showed drought tolerance and improved nitrogen use efficiency. Regarding weediness potential of these transgenic sugarcane lines, the introduction of these genes could not change all of the characteristics that regulate or limit the persistence of sugarcane [63]. Studies on crop-wild recombinant inbred lines (RILs) of lettuce showed that abiotic stress-related potentially fitness-enhancing quantitative trait loci (QTLs) could be inherited from the crop [80], but that there were significant differences between phenotypic, abiotic stress-related characteristics in the greenhouse and the field experiments [40]. Essentially, the key step is to define and quantify weediness and fitness based on a set of scientifically testable characteristics. Optimally, testing transgenic traits for their environmental impact should then follow testable hypotheses based on the trait assessed. However, the quantification of "fitness" is not an easy task, as this clearly depends on the environmental context, for example, the novel GM plant in the receiving environment [4].

19.5 Conclusion and Future Perspectives

Worldwide climate change is an acute threat to agricultural productivity, pushing more than 100 million people back into poverty by 2030, as reported by the World Bank Group [37]. To contribute to global food security to some extent in the years to come, there are currently worldwide efforts to develop new crop varieties with tolerance to drought by conventional breeding, but also by using the tools of modern biotechnology.

GM crops with drought tolerance are under development, but few of them have reached the market. With increasing knowledge to decipher comprehensively the complicated mechanisms of drought tolerance in model plants, it is a formidable challenge to modify genetically such complex, interacting metabolic systems in crops to achieve greater production under drought conditions, probably requiring considerable time. In addition, it is also a big challenge to translate results from models in the greenhouse to crops in the field because field testing to demonstrate improved yields under water-limited conditions is challenging and expensive [24]. Prior to market approval, GM plant varieties, including drought-tolerant plant varieties, require in most countries an extensive food and environmental safety assessment, contrary to similar conventionally bred crops. All aspects mentioned above are facing changes for the contribution of GM crops with drought tolerance to food security.

For the food safety assessment, all aspects of drought-tolerant GM plant varieties seem to be generally covered by current worldwide harmonized scientific safety assessment approaches (application of the principle of substantial equivalence). It may be necessary to culture the new GM drought-tolerant crops both under conventional (well-watered) environmental conditions, together with the conventional counterpart, as well as under the stressed (water-limiting) environmental conditions for this new crop variety to assess for potential effects that may only show under the drought conditions.

Thus far, the drought-tolerant GM crop varieties are assessed by using the same environmental safety assessment procedures as used for the current first-generation GM plants with insect resistance and herbicide tolerance. However, depending on the different nature of drought traits, needs for additional considerations should be examined in the safety assessment process. Data on the response of conventional plants to the stress condition and to the potential receiving environment should both be available. If the conventional comparator has not been under cultivation in the potential receiving environment where GM crops with drought tolerance will be, comparative analysis will be challenged. In addition, in some cases genes with drought tolerance may also confer tolerance to other stresses. Therefore, these stresses should also be considered in that environment. More specifically, to what extent these transgenes may impart increased fitness under actual field conditions and thus change the population ecology of wild relatives. As a result, both the direct and indirect environmental effects of the introduced new GM drought-tolerant crops will be more difficult to estimate. de Jong and Rong [22] indicated that even with advances in basic research and genetic technology, there will remain a considerable margin of uncertainty with predicting introgression from crops to wild relatives. Thus, there is a need to develop globally harmonized protocols on how to best assess these effects of GM crop varieties with drought tolerance in which one is careful with the amount of detail required for environmental risk assessment research.

As drought-tolerant crop varieties developed by conventional breeding methods are actively being introduced as well, knowledge of these could be used for comparisons. We recommend that international scientific platforms take the lead in the development of harmonized protocols and procedures, in the short term, to ascertain that drought-tolerant GM crops can be introduced on the basis of an adequate safety assessment for human and animal consumption, and with regard to the environment.

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Chapter 20
Present Status and Future Prospects of Transgenic Approaches for Drought Tolerance

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20.1 Introduction

A global water shortage brought about by climate change, worldwide population increase, and reduction in fresh water resources represents a severe challenge to crops [39]. Freshwater consumption in agriculture constitutes the largest demand taking up 75 % of supply, with irrigation in developing countries possibly consuming over 90 % of available freshwater [90]. Drought stress is bound to intensify under global climate change, and greenhouse emissions have not only expanded the arid land mass but also elevated temperatures, adversely affecting agriculture [115]. Many crop species including rice, maize, wheat, soybean, pearl millet, and canola are sensitive to water-deficit conditions, and are particularly dependent on water availability during embryo and flower development [13, 15, 16]. The severity on field crops is highly dependent on the onset, duration, and intensity of water loss during critical periods in plant development.

Nonetheless, plants have evolved morphologically and physiologically to adapt to drought stress. Mechanisms developed to survive include modification in root depth, generation of an efficient root system, and reduction of water loss by stomatal closure [8, 143]. Multiple signaling networks regulate the expression of stress-responsive genes to enhance drought tolerance, which has been defined as the ability to thrive and reproduce in water deficit [126, 133]. These quantitative traits are governed by complex genetic processes [40] and this chapter discusses several approaches to achieve drought tolerance, with emphasis on gene functions in signaling pathways (Fig. 20.1).

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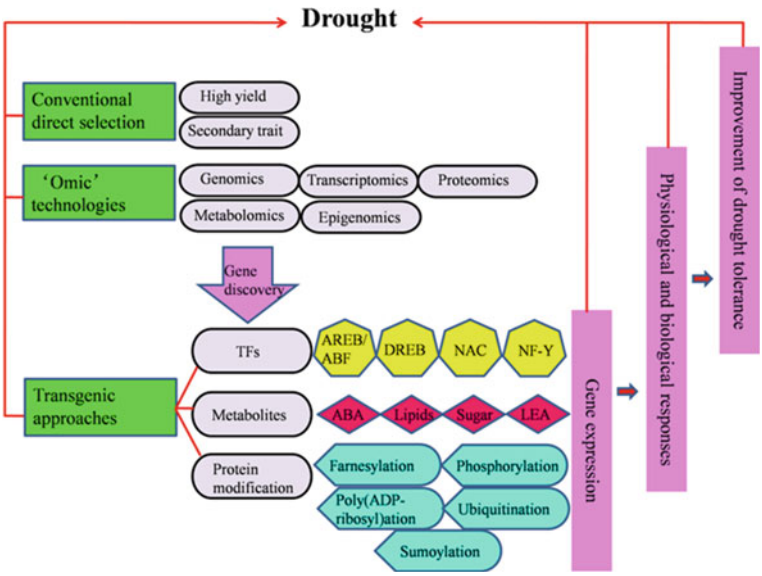

Fig. 20.1 Multiple strategies to achieve drought tolerance in plants. Methods utilized include conventional direct selection, "omic" technologies, and transgenic approaches. These strategies involve modifications in gene expression, as well as in physiological and biological responses to improve drought tolerance. ABA, abscisic acid; ADP, adenosine diphosphate; AREB/ABF, ABA-responsive element binding protein/factors; DREB, dehydration-responsive element binding proteins; LEA, late embryogenesis abundant; NAC, no apical meristem (NAM), *Arabidopsis thaliana* activation factor1/2 (ATAF1/2) and cup-shaped cotyledon2 (CUC2); NF-Y, nuclear factor Y; and TFs, transcription factors

20.2 Strategies to Acquire Drought Tolerance

20.2.1 Conventional Methods

Conventional approaches in plant breeding are usually based on direct selection of high-yielding cultivars. Plant breeders have successfully produced several drought-tolerant varieties in rice through direct selection for grain yield; this has been proven effective and highly heritable [74, 75]. Other efforts in enhancing drought tolerance rely on secondary traits. Root architecture such as thickness, depth of rooting, root penetration ability, and its branching angle and distribution pattern can influence the ability of a plant to withstand drought [24]. Other criteria are related to adjustments in osmosis and water potential of leaves at the panicle [8, 24].

Drought is accompanied by other abiotic stresses such as high temperature which in turn intensifies the water deficit effect by promoting evapotranspiration, adversely affecting photosynthetic kinetics and nutrient uptake [40]. To identify the

desired cultivars, breeders have used time-consuming and labor-intensive methods in screening out large numbers of genotypes [88]. Hence, considerable attempts have already been made to improve crop yield in the twentieth century using conventional direct selection of high-yielding cultivars and the identification of secondary traits to withstand drought (Fig. 20.1).

20.2.2 "Omic" Technologies

"Omic" approaches involve large-scale multidimensional studies with detailed investigations on entire genetic, structural, or functional components. "Omic" technologies such as genomics, transcriptomics, proteomics, metabolomics, and epigenomics can rapidly generate high-throughput "omics" data (Fig. 20.1). Genomics refers to the study of gene structure and function using whole genome sequences. Advanced sequencing methods, especially next-generation sequencing technologies, provide the basis for such investigations. Different sequence-based markers such as simple sequence repeat markers and single nucleotide polymorphism markers are utilized [128, 165]. Quantitative trait locus (QTL) mapping provides an effective way to determine gene combinations or genomic regions related to complex traits in drought tolerance [62]. In pearl millet (*Pennisetum glaucum* (L.) R. Br.), grain production under drought stress conditions has been mapped to several QTLs, one of which related to both grain yield and drought tolerance has been mapped to linkage group 2 [159]. Attempts have been made to identify the candidate genes for this QTL to enable the eventual introduction of desirable alleles into other varieties [159].

In wheat, drought-tolerant related QTLs have also been identified [71, 91], and their specific components and positions in the genome have been mapped [113]. Reproductive organs and roots of wheat have been strategically targeted for protection in drought stress because they tend to be the most adversely affected [11, 109]. However, such drought-associated alleles remain to be identified from wheat. In barley, 38 QTLs associated with water deficit have emerged and mapping of these drought-related QTLs have been achieved [93, 142, 137]. Moreover, detailed positional mapping of several candidate genes have been reported [37, 47].

In rice, the QTLs for drought-related high grain yield have been identified for use in enhancing rice production in lowland and upland cultivars [138, 139]. These QTLs were mapped by analysis of huge populations of genomic components including *qDTY3.2* and *qDTY1.1* [139, 140], which were strongly associated with grain yield under drought. It has been reported that interactions between different loci may further enhance grain productivity [31].

Transcriptomics involving a total set of transcripts from an organism is expected to provide insights that direct investigations on the regulation of plant stress responses. Expressed sequence tags, suppression subtractive hybridization, and spotted microarrays are utilized in transcriptome analysis; high-throughput technologies allow examination of entire gene expression in a genome and microarrays

and RNA-sequencing (RNA-seq) provide whole transcriptome profiling. Microarray technology, by which different genes are probed, requires background information of the whole genome, but RNA-seq does not need such information and has added advantages in the identification of the noncoding RNAs. Fu et al. [41] revealed that RNA-seq provided more accurate data than microarray analysis. These advanced technologies together with the earlier "functional gene" analyses have identified many transcription factors (TFs) in drought stress suitable for applications in transgenic crops [9, 38, 43, 55, 61, 64, 66, 81, 100, 101, 105, 110, 117, 129, 130, 134].

Proteomics represents a large-scale study involving sets of proteins expressed in a specific cell type or within an organism. A single gene upon transcription, translation, and posttranslational modification can give rise to various protein derivations that will contribute to a diverse proteome [6]. Furthermore, the proteome is dynamic. The availability of the Rice Proteome Database makes it convenient for researchers to compare proteomic maps under water deficit conditions and seek out proteins that are upregulated or downregulated under drought stress [72]. Drought-induced proteins in rice leaves and anthers include the late embryogenesis abundant-like protein [69], glyceraldehyde-3-phosphate dehydrogenase [80], actin-binding proteins [80], isoflavone reductase-like protein [120], the r40C1 protein [69], a Rieske Fe-S protein [120], Rubisco large subunit [5], chloroplast ATPase [5], Cu–Zn superoxide dismutase [69, 120], glutathione dehydroascorbate reductase [120], and nucleoside diphosphate kinase [120]. In wheat, proteins altered in expression under water deficit are mostly related to carbon metabolism, photosynthesis, and amino acid metabolism [79].

Metabolomics refers to the identification and quantification of the entire set of primary and secondary metabolites in a given biological process. It is a direct way to explore biochemical pathways and the functional status of an organism. Metabolomics is supported by the development of various platforms for metabolite analysis including gas chromatography mass spectrometry, liquid chromatography mass spectrometry (GC-MS), Fourier transform ion cyclotron resonance mass spectrometry, capillary electrophoresis mass spectrometry, and nuclear magnetic resonance [77]. Semel et al. [123] used GC-MS based metabolite analysis on tomato (*Solanum lycopersicum*) fruits from irrigated and nonirrigated plants to identify amino acids including proline which has been proposed to function in drought stress, as well as fatty and organic acids of the tricarboxylic acid cycle associated with mitochondrial metabolism that can overcome osmotic stress, and sugars with confirmed roles in water stress such as erythritol, fructose, glucose, isomaltose, sucrose, ribose, and trehalose. Castor (*Ricinus communis* L.) accumulated more proline, soluble sugars, free amino acids, and potassium in response to water shortage and the plants became more resistant to drought [7].

Epigenomics focuses on the molecular mechanism of epigenetics including heritable DNA methylation, histone modification, and the effects of small RNAs that can regulate a wide range of processes in response to biotic and abiotic stresses. Gonzalez et al. [46] reported that the tomato Asr2 protein declined under restricted

water availability and the upstream regulatory region of its gene lacked cytosine methylation in simulated drought, thereby linking epigenetics with drought adaptation in plants.

20.2.3 Transgenic Approaches

Transgenic approaches involve the transfer of target genes including those encoding TFs, regulating metabolites, and affecting protein modification to engineer plant traits (Fig. 20.1). The transgene can be derived from the same plant species or from another genus. Transgenic crops generated to enhance grain yield and drought tolerance include rice [60, 61, 114], barley [2], and maize [102]. Investigations are ongoing to better characterize genes related to molecular or biochemical processes including *cis*-acting regulatory elements and signaling pathways involved in the drought response from various plants. The present status and future prospects of this approach are reviewed in the following two sections.

20.3 Present Status of Transgenic Approaches for Drought Tolerance

20.3.1 Regulatory Genes Implicated in Drought Tolerance

In the past two decades ever since the introduction of the first commercialized genetically engineered *Flavr Savr* tomato [73], transgenic plants that harbor desirable traits have been rapidly generated and adopted in many countries [20]. Most of these transgenic plants target simple monogenic traits including herbicide, insect or disease resistance, or have been conferred a trait that is related to a simple molecular pathway [18]. Investigations in bioinformatics, plant physiology, and molecular biology related to genome-wide regulatory networks in different cell types and tissues are ongoing to comprehend better how plants respond to stress treatments. Development of gene technology and the availability of complete genome sequences from different plant species enable rapid identification of regulatory genes and signal transduction networks implicated in drought stress. Such regulatory processes occur at the transcriptional level and *cis*-regulatory elements in the 5′-flanking region of target genes have been mapped to their corresponding DNA-binding TFs [149]. An improved understanding of the genetic mechanisms of plant responses under water deficit conditions has opened up new avenues for applied research [141]. Also, transgenic plants with improved resistance to drought stress generated by the expression of regulatory genes have been produced [9, 43, 55, 61, 64, 66, 81, 100, 101, 105, 110, 117, 129, 130, 134].

TFs generally refer to DNA-binding proteins that regulate the expression of target genes [76]. They may work independently or as part of a protein complex

[20]. TFs and the *cis*-regulatory elements to which they bind are the key points in gene regulatory networks. It has been reported that TFs are master regulators that control the expression of target genes in a whole network model and affect many cellular processes [86]. Activity changes of a single TF could culminate in significant consequences on the response of the plant to abiotic or biotic stresses. TFs have become interesting targets to pursue in the development of future agricultural products and TF-based technologies appear promising in crop genetic engineering. TFs can be classified into various gene families based on the presence of DNA-binding domains in addition to others [147]. In different plant species, similar physiological processes can be linked to one or two common TFs that regulate the expression of genes in the same family [20]. Several TFs implicated in drought stress have been identified using systematic functional genomics strategies; these include the ABA-responsive elements binding protein/factors (AREB/ABFs), NAC (consisting of no apical meristem (NAM), *A. thaliana* activation factor1/2 (ATAF1/2), and cup-shaped cotyledon2 (CUC2), dehydration-responsive element binding proteins/C-repeat binding factor (DREB/CBF), and nuclear factor Y (NF-Y) [3, 55, 70, 102, 126, 133, 154, 166, 169].

The accumulation of abscisic acid (ABA) in osmotic stress arising from water deficit suggested that it plays vital roles in drought responses and tolerance [132]. Upon drought stress, AREB/ABFs are activated and bind to ABA-responsive elements that contain conserved ACGTGT/GC motifs [101]. It has been reported that more than one ABRE is required to control the expression of ABA-responsive genes [23]. During the vegetative growth stage, AREB/ABFs belonging to the basic leucine zipper (bZIP) family regulate ABA-dependent gene expression and in Arabidopsis more than 70 AREB/ABFs homologues have been identified [162]. AREB1/ABF2 has been reported to control ABA signaling and drought stress responses [101]. These TFs can be divided into nine groups [59]. In Arabidopsis, the overexpression of AREB1/ABF2, ABF3, or AREB2/ABF4 promoted drought tolerance and ABA hypersensitivity [43, 64]. Transgenic rice and soybean ectopically expressing AREB1 also showed better drought protection [9, 105]. Knowledge of ABA signal transduction has been advanced following reports on three major ABA signaling participants, namely SNF1-related protein kinase, protein phosphatase 2C, and the pyrabactin resistance/PYR1-like/regulatory component of the ABA receptor [134].

NAC forms a plant-specific TF family, which consists of more than 100 members all containing the conserved NAC domain (CATGTG) [106]. In Arabidopsis, three NAC TFs respond to drought by binding to the NAC recognition sequence (NARS) at the 5′-flanking region of *EARLY RESPONSIVE TO DEHYDRATION1* (*ERD1*) which is induced by drought, high salinity, and ABA [130]. *NACs* in soybean (*GmNACs*) and canola (*BnNAC5-1*) were reported to be drought-inducible [49, 131]. In rice, more than 40 *NAC* genes respond to drought or salt stress [38], and transgenic rice overexpressing stress-responsive *NAC1* (*SNAC1*), *SNAC2*, *OsNAC6*, and *OsNAC5* exhibited improved drought and/or cold protection [55, 56, 61, 100, 129, 130]. The most undesirable effect of transgenic *NAC*-overexpressors was related to growth retardation; however, the utilization of

stress-inducible promoters overcame this disadvantage [60, 61, 98–100, 114, 129]. Nakashima et al. [98] reported that the *LIP9*, *OsNAC6*, *OsLEA14a*, *OsRAB16D*, *OsLEA3-1*, and *OsHOX24* promoters were induced by drought, high salinity, and ABA treatment, and transgenic plants overexpressing *OsNAC6* under the control of the *OsHOX24* promoter did not show any growth inhibitory effects of NAC. A root-specific promoter *RCc3* has been successfully applied in rice under field conditions and rice plants transformed with *RCc3:OsNAC5*, *RCc3:OsNAC9*, and *RCc3:OsNAC10* showed enhanced drought tolerance and grain yield without growth retardation [60, 61, 114]. Studies have also demonstrated that SNACs play a significant role in drought stress and a combination of SNACs with stress-responsive or tissue-specific promoters could represent effective ways to combat water deficit [38, 129, 130].

The DREBs of the plant-specific APETALA2/ethylene-responsive factor (AP2/ERF) TF superfamily, also known as CBF, are associated with ABA-independent dehydration-responsive TFs because they are responsive to dehydration but not ABA [160]. These TFs can be divided into two groups: DREB1/CBF and DREB2. DREBs are reported to participate in the regulation of dehydration and cold responses, as well as in plant development [42, 97, 78]. The transgenic approach by overexpressing DREB1/CBF has been broadly used in various species, including Arabidopsis, potato, rice, soybean, peanut, chrysanthemum, tobacco, wheat, and tomato [14, 51, 54, 58, 66, 67, 81, 105, 110, 117]. However, growth defects have become one obvious side effect of DREB1/CBF in drought improvement [54, 66, 81]. Utilization of stress-responsive promoters such as *RD29A* to drive *DREB1/CBF* has overcome this drawback in Arabidopsis, bread wheat, and tall fescue [66, 110, 167].

DREB2A, inactivated posttranslationally under normal growth conditions, was engineered to a constitutively active (CA) version following deletion of its negative regulatory domain [118]. Transgenic Arabidopsis overexpressing *DREB2A CA* upregulated stress-related genes and improved protection to water deficit albeit with growth retardation [118]. Also, transgenic soybean expressing Arabidopsis DREB2A CA under the control of the stress-inducible *RD29A* promoter exhibited enhanced tolerance to water deficit [35]. Although Arabidopsis DREB2A is involved in dehydration and salt stress, it is also temperature responsive [119]. DREB2 homologues have been identified in different species such as rice, wheat, maize, barley, soybean, and sunflower [30, 34, 92, 95, 158]. Transgenic Arabidopsis expressing maize ZmDREB2A and soybean GmDREB2 under the control of inducible or constitutive promoters provided protection against drought, heat, and salt stress minus growth defects [21, 111].

NF-Ys are ubiquitous CCAAT-binding TFs, which consist of A, B, and C subunits [48]. AtNF-YB1 was the first isolated one following an Arabidopsis systematic genomics screen [20]. The overexpression of ZmNF-YB2 conferred enhanced drought tolerance in maize, indicating a common stress-responsive regulator in both maize and Arabidopsis [102]. More importantly, field-grown maize showed higher grain yield than the control when exposed to drought [102].

Microarray analysis revealed that AtNF-YB1 regulates the drought tolerance pathway differently from CBF or ABA, suggesting that independent molecular pathways do exist [102].

Some other non-TF genes related to epigenetic regulation have also been implicated in the drought response and these include MULTICOPY SUPPRESSOR OF IRA1 (MSI1), a subunit of the Polycomb protein complexes and the CHROMATIN ASSEMBLY FACTOR1 [4, 50]. MSI1 was proposed to be a negative regulator in Arabidopsis water stress, by directly binding to chromatin of a downstream stress-signaling target *RD20* to repress expression of a subset of stress-inducible genes [4, 50]. Furthermore, given that MSI1 targets silencing complexes to the chromatin, during nucleosome assembly and remodeling to maintain epigenetic memory, the regulation of MSI1 in drought stress responses involves epigenetic modification and presents new insights between plant stress and chromatin function [4, 50].

20.3.2 Genes Related to Metabolites and Osmoprotectants

Transgenic plants have been engineered to alter metabolic or physiological pathways to combat drought stress (Fig. 20.1). ABA is a key metabolite of the signaling network that helps plants to adapt to water-deficit conditions [161]. Increasing ABA content by metabolic engineering has successfully protected different plant species against water-deficit conditions. *LOS5/ABA3* encodes an enzyme that regulates the final step in ABA biosynthesis in Arabidopsis [155]. Drought tolerance was enhanced in maize and tobacco transformed with *LOS5/ABA3* [83, 164]. These transgenic plants under water-deficit stress exhibited reduction in leaf wilting and electrolyte leakage, induction in antioxidant enzyme activity, and higher proline and ABA content [83, 164]. Sequential induction of a set of adaptive physiological and biochemical responses culminated in drought tolerance [83, 164]. Other methods that regulate ABA homeostasis in plants focused on 9-*cis*-epoxycarotenoid dioxygenase (NCED), another rate-limiting enzyme in the ABA biosynthesis pathway [84]. In Arabidopsis, drought-inducible AtNCED3 regulated the level of endogenous ABA under water-deficit conditions [84]. Arabidopsis overexpressing *AtNCED3* exhibited a reduction in leaf transpiration rate and increases in endogenous ABA content and expression levels of drought- and ABA-inducible genes culminated in improved drought tolerance [84]. Similarly, the constitutive overexpression of tomato *NCED1* in petunia enhanced drought resistance, albeit with negative pleiotropic effects on growth and development [36]. On the other hand, its overexpression under the control of a stress-inducible promoter from *RD29A*, increased leaf ABA and proline concentrations, and drought resistance with no side effects on plant growth [36].

Exogenous applications of eicosapolyenoic acids can mimic ABA and drought responses [163]. Transgenic Arabidopsis expressing *Isochrysis galbana* $C_{18}\Delta^9$-SPECIFIC POLYUNSATURATED FATTY ACID ELONGASE1 (IgASE1) which

generates precursors of eicosapolyenoic acids, showed elevated ABA content and improved drought tolerance but its leaf area and biomass decreased [163]. The expression of stress- and ABA biosynthesis-related genes such as *NCED3*, *ABA1*, and *AAO3*, were upregulated, suggesting that the accumulation of eicosapolyenoic acids reduces the adversity of water deficit in transgenic Arabidopsis through the action of ABA [163].

The metabolism of membrane phospholipids is adversely affected by drought and dramatic changes in membrane lipid composition [10, 27, 68, 89, 144]. Membrane lipids can function in signal transduction in plant responses to drought stress [150, 151]. Phospholipase C (PLC) and phospholipase D (PLD) have been reported to participate in water deficiency and ABA signaling [10, 27, 68, 89, 144]. Transgenic ZmPLC1-expressing maize lines exhibited better drought protection and elevated relative water content, osmotic adjustment and photosynthesis rates, reduced percentage of ion leakage, and lowered lipid membrane peroxidation [144]. Bargmann et al. [10] reported that the *pldα1* and *pldδ* single and double knockout mutants were hypersensitive to hyperosmotic stress. PLDα1 is known to interact with ACYL-COA-BINDING PROTEIN1 (ACBP1), of which its mRNA expression is ABA-inducible and its recombinant protein binds phosphatidic acid, an important lipid messenger in ABA signaling [32]. In transgenic Arabidopsis overexpressing AtACBP1, seed dormancy and ABA responsiveness during germination and seedling development were affected, but drought tolerance was not [32]. However, transgenic Arabidopsis overexpressing AtACBP2 (a homologue of AtACBP1) exhibited higher ABA-mediated reactive oxygen species (ROS) production in guard cells and acquired drought resistance by effective stomatal closure and reduced water loss [33]. Phosphatidylinositol synthase (PIS) responds to water deficit treatment through phospholipid signaling in plants [82]. Transgenic maize overexpressing *ZmPIS* accumulated more leaf phospholipids and galactolipids and showed elevated expression of phospholipid metabolism- and ABA biosynthesis-related genes, suggesting that the PIS-regulated plant response to drought stress is linked to membrane lipid composition and ABA synthesis [82].

Osmotic adjustment (OA) is an important mechanism that helps plants maintain water absorption, thereby sustaining high photosynthetic rates under drought [162]. Accumulation of osmoprotectants help plants adapt to drought stress, and genes associated with the production of various osmoprotectants including trehalose, mannitol, glycine betaine, and proline, have been applied in several species to increase the osmolyte content under water-deficit conditions [1, 65, 83, 112, 122, 164]. Arabidopsis overexpressing trehalose-6-phosphate synthase (TPS), a key enzyme in trehalose synthesis, displayed enhanced drought tolerance, but exhibited dwarfism and stunted roots arising from the accumulation of the sugar intermediate trehalose-6-phosphate (T6P) [122]. Karim et al. [65] successfully overcame this adverse phenotype by using various strategies; when *Saccharomyces cerevisiae TPS1* and *TPS2* driven from the Arabidopsis *RBCS1A* constitutive promoter were used to transform tobacco, T6P was converted to trehalose and the adverse side effects were not observed [65]. A drought-inducible Arabidopsis *AtRAB18* promoter was also used

to switch on *ScTPS1* and *ScTPS2* so that trehalose synthesis in tobacco occurred only upon water-deficit stress, to avoid any ill effects on plant development [65]. The third strategy confined trehalose production in Arabidopsis chloroplasts by fusing ScTps1 with a chloroplast-specific transit peptide [65]. Expression of mannitol-1-phosphate dehydrogenase to increase mannitol content in wheat resulted in significant improvement to drought stress [1]. Proline is thought to be an important osmolyte that accumulates in drought stress, and genetic engineering of proline biosynthesis in maize, tomato, tobacco, and rice culminated in drought tolerance [83, 164]. Other approaches taken as alternatives to the accumulation of target compounds involve the overexpression of chaperone-like proteins such as the heat and cold shock proteins, late embryogenesis abundant proteins and reactive oxygen species scavenging-related proteins [17, 80, 107, 121, 127, 153, 156].

20.3.3 Genes Associated with Posttranslational Modification

In plants, posttranslational modification is a vital cellular process in signal transduction in response to biotic and abiotic stimuli (Fig. 20.1). Large-scale investigations on posttranslational changes associated with drought have only been carried out in some model plants.

Protein farnesylation, catalyzed by farnesyltransferases (FTs) which conjugate C15-prenyl residues to the *C*-termini of specific substrates, regulates ABA signaling in plants under water-deficit conditions [25]. FTs identified in plants are heterodimers containing α (FTA)- and β (FTB)-subunits encoded by specific gene families [44]. In canola, molecular manipulation of AtFTB to enhance drought tolerance has been successfully attained [146]. Furthermore, the downregulation of *ERA1* encoding Arabidopsis FTB by RNAi technology enhanced ABA sensitivity and improved drought resistance by reducing stomatal conductance and water transpiration [146]. Wang et al. [145] demonstrated that FTA too can be used to improve drought resistance in canola. Canola transformed with a *BnFTA* RNAi construct driven from the tissue-specific and drought-inducible Arabidopsis hydroxypyruvate reductase (*AtHPR1*) promoter inhibited *FTA* expression and sustained crop yield [145]. In rice, the downregulation of FT/squalene synthase (SQS) using RNAi technology enhanced drought resistance and maintained yield [87]. These transgenic RNAi rice lines exhibited better drought tolerance at both vegetative and reproductive stages with a reduction of stomatal conductance resulting in 14–39 % increase in yield over the wild type [87].

In plants, phosphorylation by protein kinase is the most common posttranslational modification that regulates dehydration-responsive gene expression [22]. Ca^{2+} functions as a second messenger in signal transduction under dehydration, and Ca^{2+}-dependent protein kinases (CDPKs) phosphorylate Ca^{2+}-stimulated proteins that may represent key sensors in stress-induced Ca^{2+} fluxes [53]. In Arabidopsis, 34 CDPKs have been identified and many are associated with ABA signaling and drought tolerance including CPK6 and CPK10 [157, 168]. Their overexpression

enhanced resistance under dehydration through regulation of ABA- and Ca^{2+}-mediated stomatal movement [157, 168]. In rice, 31 *CDPK* genes have been identified but only two, *OsCPK7* and *OsCPK9*, were drought-inducible [26, 148]. Using rice overexpressing lines and RNAi technology, a positive correlation of OsCPK7 and OsCPK9 with drought resistance was demonstrated due to a change in osmotic adjustment capability [26, 148].

Another class of Ca^{2+}-sensing protein kinases related to environment-signaling are the calcineurin B-like protein-interacting protein kinases (CIPKs) [26]. Among the 30 *OsCIPKs*, 20 were differentially induced by various stresses such as drought, salinity, cold, polyethylene glycol, and ABA treatment [152]. For example, OsCIPK03, OsCIPK12, and OsCIPK15 conferred tolerance to cold, drought, and salt stress, respectively, in transgenic rice suggesting that rice CIPKs represent good candidates for genomic manipulation [152].

Mitogen-activated protein kinases (MAPKs) consist of MAP kinase (MAPK), MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK), and they function in response to external stimuli including dehydration, cold, and high salt stress [169]. Stimulus at the cell surface initiates signal transduction of sequential protein phosphorylation and activation by MAPKKK, MAPKK, and MAPK, that further phosphorylates TFs to active forms to regulate the expression of downstream genes [96]. Ning et al. [103] reported that the drought-responsive *DSM1*-encoded MAPKKK, a protein kinase from the Raf family which forms an early signaling component, was subcellularly targeted to the rice nucleus and controlled the scavenging of ROS. Two *dsm1* mutants were more sensitive to drought stress during seedling and panicle development and rice *DSM1*-overexpressor seedlings exhibited better protection [103]. The implication of MAPKs in improving drought resistance has also been reported in rice [124]. OsWRKY30 enhanced drought tolerance in rice overexpressing lines following its phosphorylation by MAPKs [124].

Protein poly(ADP-ribosyl)ation refers to protein modification by the attachment of long branching poly(ADP-ribose) polymers synthesized by poly(ADP-ribose) polymerase (PARP) [63]. It has been reported that activation of poly(ADP-ribose) polymers consumes NAD^+ and interferes with cellular energy homeostasis [28]. Confining NAD^+ consumption by reducing PARP activity improved drought tolerance in both Arabidopsis and oilseed rape [28].

Ubiquitination and sumoylation link ubiquitin and SUMOs, respectively, to specific proteins and regulate drought-stress responses [85, 94]. These processes are catalyzed by ubiquitin ligases including ubiquitin-activating enzymes (E1 to E3) and SUMO ligases such as the E1-activating, E2-conjugating, and E3-ligating enzymes, respectively [94, 85]. In transgenic Arabidopsis, rice and cotton, molecular studies of these enzymes including SIZ1, AIRP1, DIS1, and SAP5 demonstrated that they can regulate drought response both positively and negatively [19, 52, 94, 104, 108, 116].

20.4 Conclusions and Future Prospects

Drought represents one of the most important environmental stress factors that threatens agriculture. It induces a wide range of responses as the plant attempts to re-establish homeostasis and repair damaged cellular components. Although conventional breeding favors adaptation and survival, engineered plants that can be generated in a relatively shorter period from the wealth of "omics" data, information on functional genetics and genetic resources pave the way of the future. "Omic" research has greatly promoted applications in transgenic technology by improving protection from various stresses utilizing the identification of gene combinations and genomic regions underpinning complex traits in drought tolerance [62]. Such multidimensional cum detailed investigations are costly, but new technologies in computational instrumentation and statistical tools are expected to bring timely solutions in this rapidly expanding area of research [29].

A better understanding of the molecular mechanisms on drought tolerance in economical crops followed by genetic modification will no doubt address productivity problems in hotter and drier regions arising from global climate change [12]. Research on model plants has been applied to nonmodel crops by means of translational genomics [57]. However, given the differences in drought tolerance among species and genotypes, specific genome studies on drought signaling pathways are deemed to be of greater importance and the current development in high-throughput phenotyping technologies will facilitate investigations [162].

Although it is more straightforward to manipulate a single transgene with respect to expected outcomes, such modified plants may become stunted or less sustainable [20]. Sometimes, the transgenic lines accumulate too many intermediate products and built-in feedback mechanisms posing another barrier, so multiple gene manipulation has usually been employed [65, 66, 110, 167]. A common solution involves the application of a specific promoter to regulate stress-inducible expression of the drought-tolerant gene; alternatively multiple gene coexpression that depletes the toxic intermediates is used [65, 66, 110, 167]. Finally, targeting the functional protein to its natural location may alleviate any harmful effects in genetic manipulation [162].

Although time consuming and costly to generate, commercial production of transgenic crops will outperform its wild type under stress conditions as the effectiveness of drought tolerance by gene modification in rice and maize has been proven [55, 56, 61, 82, 83, 100, 102, 111, 129, 144]. Some of these crops showed drought protection even in field trials [145, 146]. Nevertheless, many of these field trials did not translate to significant advantages in yield [56, 100, 111, 129]. Among the few exceptions, a higher productivity from transgenic crops was achieved in extreme drought [55]. None of the innovations listed have been practiced in agriculture, but a number of biotechnology institutions have plans to commercialize in the coming years [125]. The application of stress-metabolite profiling, functional

genomics, and proteomics will reveal more key regulators in plant drought-stress responses [135], so that more genes can be subsequently targeted for crop transformation to combat drought.

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Chapter 21
Drought Stress and Chromatin: An Epigenetic Perspective

Asif Khan and Gaurav Zinta

21.1 Introduction: Chromatin and Drought Stress

In the current scenario of increased urbanization, burgeoning human population, and impending global climate change, plant scientists are endowed with the responsibility to deal with abiotic stress in general and drought stress in particular as a major factor limiting crop growth and productivity [1–4].

Plants are sessile and sensitive autotrophs that cannot move but avoid unfavorable circumstances through specialized morpho-physiological and developmental transitions accustoming to a dynamic environment. Water is a fundamental requirement for plant growth and survival, and water deficit negatively affects various plant processes including seed germination, photosynthesis, transpiration, metabolite transport and central metabolism including carbon and nitrogen fixation [5–7]. In the field conditions, drought stress is often accompanied by other abiotic stresses such as high temperature and salinity, nutrient deprivation, and so on. The negative impact of drought stress is observed to be aggravated by such stress combinations, leading to oxidative damage to cell membranes and proteins [8–10].

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Over the years, the immediate drought stress response of a plant in the form of regulatory genes (e.g., transcription factors, RNA helicases, protein kinases), stress protectants (e.g., osmoprotectants), hormones (e.g., ABA), small RNAs (miRNAs, siRNAs), as well as a complex interplay of the above factors has tremendously enhanced our knowledge of how plants cope with drought stress during their respective lifetimes [11, 12].

The advent of next generation sequencing (NGS) technologies has fueled the postgenomics era with a road map for direct visualization of expression profiles of thousands of genes, *cis* or *trans* regulatory elements, alternative splice variants, and chromosomal modifications [13]. Moreover, based on the genome sequence information of *Arabidopsis thaliana* and the availability of functional genomic tools such as loss or gain of function T-DNA or transposon insertional mutants, it is now possible to assign fully a visible phenotype to a particular gene (genotype) [14]. Similarly, genomes of various crops such as rice, maize, and chickpea, among others have been sequenced, which has resulted in the development of various genomic tools to understand plant responses to stresses at the molecular level better.

However, a paradox exists in the form of epigenomics wherein the phenotype is embodied by factor(s) other than the underlying DNA sequence called the chromatin [15]. Such an epigenetic or chromatin regulation is heritably transmitted, and is best symbolized by DNA methylation and histone modifications [16–19]. Epigenetics can thus ascribe potent transcriptional status of a cell in response to changing outside stimuli [20–22].

Drought stress once perceived by a plant causes elaborate reprogramming at epigenetic, transcriptional, post-transcriptional, and post-translational levels [23, 24]. Recent research attributes such reprogramming to two factors: synthesis of phytohormone abscisic acid (ABA) during drought stress causing genome-wide transcriptional changes and stress signaling [25–27], and widespread stress-responsive transcriptional changes correlated to chromatin organization and structure [28–31]. It has been envisioned that changes in histone modifications (methylation, acetylation, phosphorylation, sumolyation, and ubiquitination) and DNA methylation (CG, CHG, and CHH, where H represents A, C, or T) can rapidly and reversibly alter gene expression in response to drought stress [32–35]. An additional tier of transcriptional regulation exists in the form of noncoding RNAs (microRNAs, siRNA) with variable effects on stress-induced chromatin release or compaction [36–39]. Excellent reviews describing their role in abiotic and drought stress have been documented [40–42] and their role is not discussed further here.

In this book chapter we enlighten readers with diverse roles of chromatin (both histone and DNA) modifier genes in response to drought stress episode, how these candidate genes need to be engineered for long-term or transgenerational stress memory, and what needs to be done for sustainable drought-free agriculture in near future through study of such chromatin-modifying genes (Fig. 21.1).

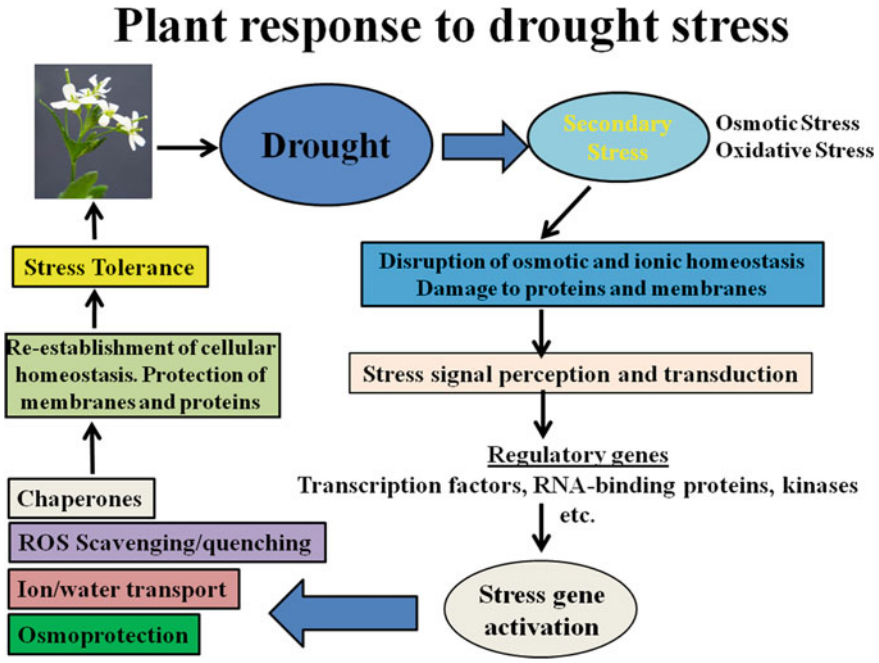

Fig. 21.1 Plant response to drought stress

21.2 Transcriptional Control of Drought Stress via Chromatin-Modifying Genes

21.2.1 The Histone Code

Long before the relationship between the drought signaling cascades and chromatin conformation were deciphered, chromatin has been viewed as an ideal candidate for genome versus environment interaction [43]. The eukaryotic nucleus is the site of DNA transcription characterized by chromatin compacted in the form of a basal unit called nucleosome, 147 bp of DNA sequence wrapped around a histone octamer. The core histones of the octamer constitute H2A, H2B, H3, and H4 which are further condensed and connected via linker histone H1 [44–46]. This physical compaction of the genome is important from a transcription point of view as it restrains accessibility of RNA polymerase. On the basis of compactness, chromatin is classified into heterochromatin and euchromatin. Heterochromatin represents closed conformation and relates to the repressed transcriptional state, whereas euchromatin depicts an open conformation and relates to an active transcriptional state [47]. Such a switch for active transcription is often engendered through covalent modifications of N-terminal regions of histones designated as histone tails. The histone tails are rich in basic amino acids such as lysine and arginine that

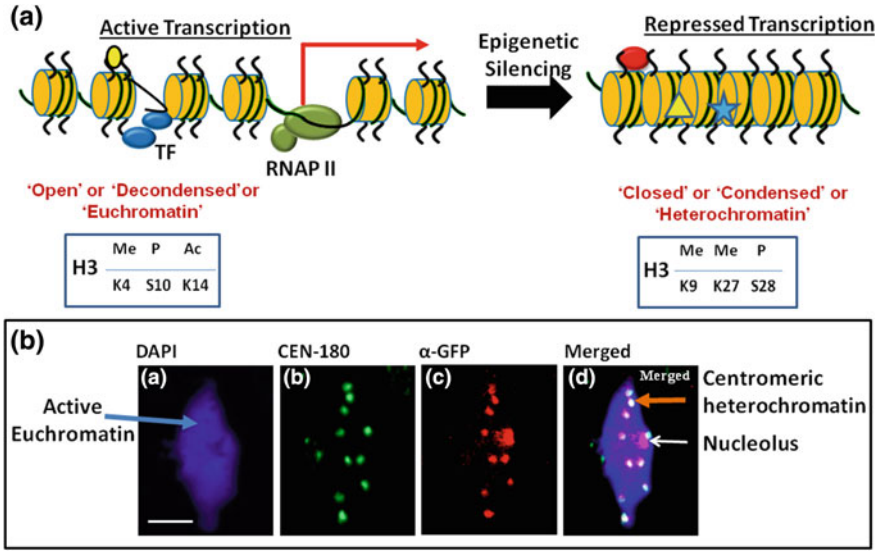

Fig. 21.2 **a** The histone code-The N-terminal region of H3 histone tail is site of covalent modifications that contribute to activation or repression of transcription **b** Heterochromatin and euchromatin represent distinct domains in labelled nuclei [82] RNAP II stands for RNA polymerase II, DAPI-(4',6-diamidino-2-phenylindole) represents fluorescent DNA stain, CEN-180 represents marker for centromeric repeats, α-GFP represents anti-GFP antibody

undergo methylation, acetylation, phosphorylation, ubiquitination, sumoylation, or ADP-ribosylation that dictate corresponding gene expression profile [48–51]. For instance, acetylation of H3 and H4 N-tails nullifies their positive charge, increasing the affinity of RNA polymerase and transcription factors towards the template strand. Thus histone acetylation is a mark for an active transcriptional state [52–54] whereas histone deacetylation confers epigenetic gene silencing [55–57]. Conversely, methylation of lysine residues stands as a more complex phenomenon with varying transcriptional outcomes depending on degree (mono-, di-, or trimethylation) and amino acid modified. For example, actively transcribed genes are found to be enriched in H3K4 and H3K36 trimethylation, whereas methylation of H3K27 and H3K9 signifies repressed chromatin, respectively [49] (Fig. 21.2).

21.2.2 ABA-Induced Chromatin Changes Under Drought Stress

The plant hormone ABA is a key hormone regulating diverse cellular and physiological processes whose synthesis is the prima facie response of a plant undergoing water deficit [25, 58, 59]. The tight connection between ABA signaling and various histone-modifying enzymes has been thoroughly documented and we

dissect this intimacy by first revisiting what we know about ABA and drought stress signaling components followed by case-specific discussion of enhanced drought tolerance and ABA response and vice versa. The de novo response of a plant challenged for water conservation is stomatal closure to prevent transpiration through synthesis and binding of ABA to PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) in a tripartite complex [60, 61] with the clade A protein phosphatase 2C (PP2C) phosphatases [62, 63]. This alters a cascade of downstream signaling components (*abi1*, *abi2*, *abi3*, *abi4*, *and abi5*) preventing deactivation of sucrose nonfermenting related kinase2s (SnRK2s) [27, 64, 65]. The SnRK2s then relay phosphorylation of stress-responsive transcription factors [66] and ion transporters [67–69] that affect ABA-dependent drought stress tolerance. The plant community has recently focused its attention on drought stress and ABA-mediated changes in histone modifications [70]. For example, Sokol et al. found in Arabidopsis and tobacco cell cultures that a brief duration of ABA or salt stress was enough to trigger genomewide H3S10 phosphorylation and H4K14 acetylation [71]. Another brilliant study found a positive correlation between degree of drought stress, euchromatin (H3K4me3 and H3K9ac), and decreased nucleosomal occupancy at drought stress responsive genes, RD20 and RD29A, respectively [33]. Interestingly, increased nucleosomal density and histone deacetylase activity were observed during the recovery period from drought stress for resetting the chromatin status at the same genes [32–34]. Further studies are needed to link other post-translational histone modifications to water-stress–triggered transcriptional reprogramming.

21.2.3 Histone-Modifying Enzymes in Drought-Induced Transcriptional Reprogramming

Three classes of histone-modifying enzymes, namely histone deacetylases, histone methyltransferases, histone arginine methyltransferases, and histone variants (e.g., H1S and H2A.Z) have been implicated to act alone or in conjunction with drought-responsive components of ABA signaling, and we summarized them in Table 21.1 with their role and stress phenotype to give a comprehensive mechanistic view.

Until now we have pictured different post-translational histone modifications in light of drought stress response and also the role of plant hormone ABA supporting the idea for correct histone alterations in the ensuing drought stress process. More studies are needed using mutants of these histone-modifying genes with clearcut drought stress phenotype to pinpoint their direct targets [72]. This can be achieved by using either inducible promoter systems or tissue-specific removal of the desired gene of interest. Also care should be taken while assessing phenotypes of such mutants as it might also account for a pleiotropic effect of hormone ABA. Alternatively ABA-sensitive fluorescence energy resonance transfer (FRET) sensors such as ABACUS can be used to magnify single-cell dynamics under drought stress [73]. That the hormone ABA plays a role in these modifications is clear but

Table 21.1 Histone modifying genes in drought stress tolerance

Gene locus	Abbreviation	Gene product	Biological function	Phenotypic aberrations	References
AT5G03740	AtHD2C/HDT3	HD2-type histone deacetylase	Histone deacetylation, response to drought and salt stress	AtHD2C-ABA hyposensitive *athd2c*-ABA hypersensitive	[34, 35, 118]
AT5G63110/AT4G38130	AtHDA6/HDA19	RPD3-type histone deacetylase	Histone deacetylation,seed dormancy, repression of seed germination, response to drought and salt stress	AtHDA-ABA hyposensitive *athd2c*-ABA hypersensitive	[45, 46, 119]
AT4G16420	ADA2b	Histone acetyltransferase (HAT)	Component of GCN5 HAT complex, response to salt and drought stress, auxin–cytokinin concentrations, cold acclimation	*ada2b*- Pleiotropic developmental effects, ABA hyposensitive, negative regulator of freezing tolerance	[120, 121]
AT2G31650	ATX1/SDG27	Histone-lysine N-methyltransferase	Specification of floral organ identity, positive regulator of NCED3, a key ABA biosynthesis enzyme	Decreased tolerance to drought stress, *atx1*- hypersensitive ATX1- hyposensitive	[122]
AT5G11530	EMF2	Histone lysine K27 trimethylation	Meristem identity, hyperosmotic salinity response, negative regulation of flower development	Positive regulator of ABA response	[123, 124]
AT4G31120	AtPRTM5/CAU/SKB1	Protein arginine methyltransferase	Vernalization, epigenetic silencing of flowering locus c (FLC), mRNA splicing, circadian rhythm	Increased drought tolerance and ABA hypersensitivity	[125, 126]
AT2G18050	HIS1-3	Linker histone H1-3	Response to dehydration, nucleosome asembly	Increased drought tolerance and ABA hypersensitivity	[127]
AT1G52740	H2A.Z	Histone variant	Mutants display disruption of major stress responsive genes that tend to contain increased gene-body H2A.Z	Increased drought tolerance	[128]

whether there are components that are common to multiple or combinations of stress response needs to be further deciphered.

21.2.4 DNA Methylation

In flowering plants, heritable DNA methylation forms another part of the regulatory loop (other than histone modifications) in transcriptional silencing, via deposition of a methyl group at carbon five (C5) of cytosine nitrogen bases of a DNA sequence [74]. In short, cytosine methylation takes place in three sequence contexts as CG, CHG, and CHH (where H can either be A, C, or T but not G). DNA methyltransferase I (MET1), a homologue of the mammalian methyltransferase DNMT1 and a plant-specific methyltransferase, chromomethyltransferase 3 (CMT3), are two enzymes that catalyze symmetric methylation at CG and CHG sites. Asymmetric CHH methylation, however, is maintained *denovo* through domains rearranged methyltransferase 2 (DRM2), a homologue of the mammalian DNMT3A/b and an intricate web of new and emerging players via the RNA-directed DNA methylation (RdDM) pathway [36, 75, 76].

Additionally, repression of transposable elements (TEs) in all sequence (CG, CHG, and CHH) contexts occurs via DECREASE IN DNA METHYLATION (DDM1), SWItch/Sucrose Non-Fermentable (SWI/SNF) superfamily chromatin remodeler [77] that also associates with CMT2 chromo methyltransferase and the canonical RdDM pathway for heterochromatin formation at respective loci [78]. Abiotic stresses may trigger hypo- (gene body or promoters) or hypermethylation (at repressive loci such as transposons) [75, 76] contributing to stress adaptation. A classic example here is of halophytic mangrove trees that are smaller in size and show global hypomethylation than plants growing near riverbanks [79]. Similarly, low relative humidity can trigger transcriptional silencing of SPEECHLESS (SPCH) and FAMA (FMA), two genes functioning in stomatal development, via de novo cytosine methylation and RdDM pathway resulting in a lower stomatal index [80, 81]. Moreover, the RdDM pathway has been implicated in promoting basal thermal tolerance and enhancing multiple abiotic stress tolerance in conjunction with a variety of stress-responsive proteins [82, 83]. Conclusively, single base changes in the methylation landscape can endow tremendous phenotypic plasticity to the plants that should be harnessed in the near future in terms of better drought-resistant plants [84].

21.2.5 Chromatin Remodelers for Drought Stress-Mediated Signaling

Chromatin remodelers are enzymes that noncovalently channel the energy derived through ATP hydrolysis to promote or suppress transcription of a given DNA sequence [85–87]. As such, two different classes of ATP chromatin remodelers

switch/sucrose nonfermentable (SWI/SNF) and chromodomain (CHD) families function to remodel the nucleosome in a drought stimulus driven environment. Of particular relevance are the BRAHMA (BRM) and MINUSCULE (MINU) from the SWI/SNF subfamily of ATPases and CHD subgroup chromatin remodeler PICKLE (PKL) [88, 89]. For example, *brm* mutants displayed ABA hypersensitivity and upregulation of ABI5 suggesting a direct role of BRM in repressing ABI5 gene expression. Recently, a detailed molecular mechanism elucidated the direct link between the BRAHMA ATPase chromatin remodeler and ABA signaling components via reversible phospho-/dephosphorylation has been elucidated [90]. A similar stress phenotype was observed for CHD domain PKL remodeler whose expression also inversely correlates with abi5 expression. More studies are needed for deducing direct targets of PKL due to its dual role as a promoter and repressor of Polycomb group proteins [91, 92].

21.3 Drought Stress Memory: Concept and Key Players

Exposure to mild stress or repeated stress exposure makes plants more tolerant to the subsequent lethal stress [41, 93–96]. This phenomenon is known as "stress memory" in plants, and some good examples of this are seed priming, acclimation to temperature, and systemic acquired resistance. Growing evidence suggests that stress memory is heritable in nature, and transmitted both mitotically as well as meiotically, which results in short- (somatic) and long-term (transgenerational) effects in plants, respectively [97, 98]. Stress memory has been observed in various plants and in response to different abiotic and biotic stresses [30, 32, 98, 99]. Thus, memorizing the previous stress exposure and modifying stress responses accordingly seems to be a fundamental process in plants, and forms the basis of plant stress adaptation. Although care should be taken while assessing the inheritable nature of such stress induced chromatin encoded traits [100].

In the case of *Arabidopsis thaliana* multiple exposure to drought stress trained the transcriptional responses [93], and two stress memory genes, viz. Response to Dehydration (RD29B) and RAB18, were identified whose expression levels were considerably higher at the subsequent stress exposure as compared to the initial stress episode. Similarly in maize, a monocot species, the transcriptional stress memory phenomenon was observed under repeated drought stress exposure [94]. In this study, the comparison of maize and *Arabidopsis* transcriptional responses revealed that related genes showed the memory responses, indicating the conservation of drought stress memory genes in both monocots and dicots. As patterns of gene expression are often correlated with the chromatin changes, Ding et al. [93] observed that non-memory genes (RD29A and COR15A) displayed dynamically changing H3K4me3 patterns, whereas stress memory genes (RD29B and RAB18) maintained increased H3K4me3 during the recovery phase. At the physiological level, pre-stressed plants maintain higher leaf water content during the subsequent drought stress exposure, and ABA plays an important role in regulating guard cell stomatal aperture during

the recovery period [101]. Here, ABA-dependent SnRK2.2 and SnRK2.3 were found be involved in regulating guard cell stomatal memory. Similarly, it was previously shown that ABA plays a crucial role in memorizing the stressful environmental experiences in plants [102]. Although important for plant survival, changes induced by long-term stress memory are difficult to comprehend. Another issue with transgenerational stress inheritance corresponds to the duration of stress memory, which can be attributed to the inherent nature of stress which may or may not subside resulting in residual stress proteins or metabolites (from previous response) still being active [103]. In yeast, increased H3K4 hypermethylation by SET1 methyltransferase has been shown to respond to changing environmental stimuli while safeguarding a molecular memory of the same [104]. Recently, Sani et al. in a series of stress-priming experiments have shown that mild stress leads to retention of long-term stress memory with little morphological differences between primed and control plants [105].

Intriguingly, euchromatic H3K4me3 and heterochromatic H3K27 are regulated by trithorax (TrxG) and Polycomb Repressive Complex (PRC2) or polycomb group proteins (PcG) whose continued presence at the replication fork and on mitotic chromatin depicts the dynamic nature of two opposing chromatin in regulating long-term somatic stress memory [106–111]. Lastly, as important as it is to determine factors that enhance stress memory perception and transmission, equally important is to bring forth factors that suppress stress memory, resetting it to the prestressed state in the next generation. A big leap in this direction has been the discovery of role of decrease in DNA methylation1 (DDM1) and Morpheus' molecule1 (MOM1) genes in erasing epigenetic heat stress memory and preventing transgenerational inheritance [112]. More studies need to be undertaken for similar candidate genes in the case of drought-stressed plants.

21.4 Future Strategies of Chromatin Control for Sustainable Drought Stress Tolerance in Crop Plants

Burgeoning climate change, population explosion, and limited natural resources all pinpoint sustainable agriculture as the last resort for survival of the human race. Combating drought stress is crucial to this strategy and equally relevant is to devise methods wherein the drought stress response is monitored with respect to the changing environment but also the corresponding stress memory is transmitted to the offspring leading to enhanced crop yield and productivity. Major challenges and breakthroughs in the chromatin control of drought stress should be:

I. Thorough understanding and control of dynamic but reciprocal nature of chromatin regulators in isolated as well as combined stresses mimicking near field-like conditions [11, 113]
II. Bypassing the mitotic and meiotic constraints for transgenerational inheritance of somatic stress memory [114, 103]

III. Enhancing the single base pair resolution of N-terminal histone modifier mutants using novel technologies such as clustered regularly interspersed short palindromic repeat (CRISPR)/CRISPR-associated protein9 (Cas9) [115, 116] as well as tissue-specific chromatin profiling under drought stress using isolation of nuclei tagged in specific cell types (INTACT) [117] will further help to enlarge and envision a drought-free near future.

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