Chapter 4

The Molecular Basis of ABA-Mediated Plant Response to Drought

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Additional information is available at the end of the chapter

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1. Introduction

'Drought stress is as complicated and difficult to plant biology as cancer is to mammalian biology' said Jian-Kang Zhu, a molecular geneticist at the University of California, Riverside. The capacity of a plant to turn on or turn off a series of genes that further alter plant physiology and morphology allows a plant to tolerate, escape or avoid drought stress. Many countries around the world experience drought stress in different ways but it always leads to a decreased annual yield of crops. Deciphering the basis of the molecular response to stress and the mechanism for the adaptation and acquisition of tolerance can facilitate the creation of cultivars with increased drought tolerance. Drought response is a complex mechanism that has been investigated using a broad spectrum of 'omics' techniques, such as molecular genetics, functional genomics, transcriptomics, proteomics and metabolomics combined with advanced phenotyping techniques. The response of plants to dehydration stress has been extensively studied in a wide range of species with particular emphasis on model plants such as Arabidopsis. Taking advantage of the knowledge already obtained from Arabidopsis and other model species, it is possible to gain insight into the stress response in crops such as barley or wheat.

The best known trigger of the cascade of drought signaling is abscisic acid (ABA). Knowledge about the complexity of ABA signaling in regards to stress response is still full of gaps but the recent identification of ABA receptors and the key factors of the first step of ABA signal transduction in Arabidopsis provided an important insight into this mechanism ([1-4]. The actions of the other ABA signaling components, such as phosphatases, kinases, transcription factors and their roles in abiotic stress response during different developmental stages is also documented in crops [5]. Under drought conditions, ABA induces the expression of many genes whose products are involved in the response

to drought, among which are positive and negative regulators of ABA signaling, transcription factors and genes encode enzymes that are involved in the synthesis of osmoprotectants. It is important to mention that ABA is not the only phytohormone involved in stress response. There is much evidence of cross-talk between ABA and other phytohormones, such as jasmonates and ethylene [6].

Recent advances in functional genomics have revealed the importance of posttranscriptional regulation of gene expression performed by microRNA. Deep sequencing methods have enabled the identification of the miRNA involved in drought response in barley and rice. Further analysis also showed their potential roles in stress signaling by identifying their targets [7-8].

The molecular basis of drought response and the interaction between genes and proteins involved in this mechanism can be studied using of advanced molecular techniques only when a good drought assay that mimics natural drought conditions can be applied in the laboratory. Many protocols for drought assays have been developed that can be implemented in the study of different species ranging from Arabidopsis to crops. Another important issue is the method of phenotyping and the spectrum of physiological parameters that are measured [9]. The techniques used most often are: chlorophyll fluorescence, stomatal conductance and relative water content (RWC) [10-12]. Combining these molecular techniques with advanced methods of phenotyping would enable drought tolerant forms to be produced. This would contribute to beginning the Blue Revolution advocated by Kofi Annan in his April 2000 Millennium Address: "We need a Blue Revolution in agriculture that focuses on increasing productivity per unit of water – more crop per drop". This chapter reviews the newest aspects of the molecular and physiological mechanisms of drought stress response in crops.

2. Abscisic acid – The best known stress messenger

Since its isolation from cotton in the 1960s [13], the role of abscisic acid (ABA) in plant development and in the response of plants to environmental signals has been extensively studied. Analysis of Arabidopsis under salt and drought stress has revealed the important role ABA plays in response to these stresses [14-16]. Endogenous ABA concentrations increase under drought stress due to induction of ABA biosynthesis genes [14]. The increase in ABA reprograms the gene expression pattern to regulate water relations through adjustment of cellular osmotic pressure, the closure of stomata, a reduced leaf canopy, deeper root growth and changes in root system architecture [17-19].

Biosynthesis of ABA has been relatively well characterized in Arabidopsis and some data is available for other species, such as maize, tomato, potato and barley [20-24]. Knowledge about ABA biosynthesis derived from studies in Arabidopsis is highly applicable to other plant species, because the pathway and the respective genes are conserved in angiosperms. ABA is synthesized through the cleavage of a C40 carotenoid precursor, followed by a two-step conversion of the intermediate xanthoxin to ABA via ABA-aldehyde [25-27]. The path-

way begins with isopentyl pyrophosphate (IPP) which is the biological isoprene unit and the precursor of all terpenoids, as well as many plant hormones. The next step is the epoxidation of zeaxanthin and antheraxanthin to violaxanthin which is catalyzed by zeaxanthin epoxidase (ZEP), which was first identified in tobacco [28]. After a series of violaxanthin modifications which are controlled by the enzyme ABA4, violaxanthin is converted into 9-cis-epoxycarotenoid [29]. Oxidative cleavage of the major epoxycarotenoid 9-cis-neoxanthin by the 9-cis-epoxycarotenoid dioxygenase (NCED) yields a C15 intermediate - xanthoxin [30]. This step is the last one that occurs in the plastid. Xanthoxin is exported to the cytoplasm where two-step reaction via ABA-aldehyde takes place. The first step is catalyzed by a short-chain alcohol dehydrogenase/reductase (SDR) that is encoded by the *AtABA2* (*ABA deficient 2*) gene [31-33] and generates ABA aldehyde. Then the ABA aldehyde oxidase (AAO) with the molybdenum cofactor (MoCo) catalyzes the last step in the biosynthesis pathway - the conversion of ABA-aldehyde into ABA [34].

Drought stress has been shown to up-regulate *NCED3* expression in Arabidopsis [14], maize [21], tomato [35], bean [15] and avocado [36]. A significant increase in NCED transcript levels can be detected within 15 to 30 min after leaf detachment or dehydration treatment [15; 37], indicating activation of NCED genes can be fairly quick. Cheng et al. [32] reported that the *AtNCED3* gene (and *AtZEP* (*Zeaxanthin Epoxidase*) and *AtAAO3* (*ABA aldehyde oxidase*)) could be induced in the Landsberg erecta background by ABA and studies in rice showed that *OsNCED3* expression was induced by dehydration [38]. Immunohistochemical analysis, using antibodies raised against AtNCED3, revealed that the protein is accumulated in the leaf vascular parenchyma cells in response to drought stress. it was not detected under non-stressed conditions. These data indicate that the drought induction of ABA biosynthesis occurs primarily in vascular tissues and that vascular-derived ABA might trigger stomatal closure via transport to guard cells [39]. *AtNCED3* expression is up-regulated by drought conditions across observed species and decreases after rehydration. At the same time, the expression level of *AtCYP707A1*, *2*, *3* and *4* (*CYTOCHROME P450, FAMILY 707, SUBFAMILY A, POLYPEPTIDE 1*, *2*, *3*, *4*) were induced by rehydration [40-41]. These genes, which encode the hydroxylases that are responsible mostly for ABA catabolism, were identified in Arabidopsis, rice [42], barley [43], wheat [44] and soybean [45]. *OsABA8ox1* (*ABA-8-hydroxylase 1*) expression is induced dramatically by rehydration, which can lead to a decrease in the ABA content in rice leaves [42].

The balance between active and inactive ABA is very important for plant stress response and is achieved not only by biosynthesis and catabolism reactions, but also by conjugation and deconjugation. ABA can be inactivated at the C-1 hydroxyl group by different chemical compounds that form various conjugates and accumulate in vacuoles or in the apoplastic space [46]. The most widespread conjugate is ABA glucosyl ester (ABA-GE) which is catalyzed by ABA glucosyltransferase [47-48]. Lee et al [49] identified the AtBG1 (BETA-1,3-GLUCANASE 1) protein which is responsible for the release of ABA from ABA-GE. Their findings showed that ABA de-conjugation plays a significant role in providing an ABA pool for plants that allows them to adjust to changing physiological and environmental conditions.

The ability of ABA to move long distances allows it to serve as a critical stress messenger. ABA transport was long assumed to be a diffusive process, mainly due to the ability of ABA to diffuse passively across biological membranes when it is in a protonated state [50]. The last step of ABA biosynthesis occurs in the cytosol where pH is estimated to be 7.2-7.4. In the apoplastic space, where ABA is meant to be transported before reaching the target cell, the pH is estimated to be around 5.0-6.0. Although ABA can be passively transported from a low pH to a higher one with a pH gradient, there is a need for the transporter to allow ABA to get into the target cell and to be exported from the cell to the apoplast. During stress response, the strong alkalization of apoplastic pH would slow ABA diffusive transport from the apoplastic space to the target cells. Because of the predominance of a non-protonated ABA state, there is a need for the existence of ABA transporters. The identification of ABA transporters in target cell membranes, such as the cell membranes of guard cells, has resolved the problem of how ABA gets into the cells when passive transport is decreased under stress conditions. One of the identified ABA importers is ABCG40 (ARABIDOPSIS THALIANA ATP-BINDING CASSETTE G40) described by Kang et al [51]. The expression of *ABCG40* is not tissue specific and its product localizes in cell membranes [51]. Kuromori et al [52] identified another ABA importer - ABCG22 (ARABIDOPSIS THALIANA ATP-BINDING CASSETTE G22). The gene encoding this transporter is mainly expressed in guard cells. Also, the expulsion of ABA into the intercellular space is mediated by transporters such as ABCG25 (ARABIDOPSIS THALIANA ATP-BINDING CASSETTE G25). *ABCG25* is expressed mainly in vacuolar tissue, where ABA is synthesized [53].

A breakthrough in understanding ABA signaling occurred recently when several groups identified key ABA receptors. Chemical genetics emerged as the solution for 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, among them PYR1 (PYRABACTIN-RESISTANCE 1) have been identified [3]. PYR1 is one of the 14 homologs (PYL – PYRABACTIN RESISTANCE LIKE) present in the Arabidopsis genome [1-4]. After receiving ABA from ABC transporters, the PYR/PYL/RCAR-ABA (PYRABACTIN-RESISTANCE 1/ PYRABACTIN RESISTANCE LIKE/ REGULATORY COMPONENT OF ABA RECEPTOR) complex perceives ABA intracellularly and forms ternary complexes inhibiting clade A of PP2Cs (PROTEIN PHOSPHATASE 2C), the negative regulators of ABA signaling, such as ABI1 (ABA INSENSITIVE 1), ABI2 (ABA INSENSITIVE 2), HAB1 (HYPERSENSITIVE TO ABA1) [1-2; Table 1].

This allows the activation of down-stream targets of PP2Cs – the Sucrose nonfermenting 1-related subfamily 2 protein kinases (SnRK2), such as SnRK2.2/D, SnRK2.3/E and SnRK2.6/OST1/E which are the key players in the regulation of ABA signaling [54-57; Figure 1].

The last enzyme, OST1 (OPEN STOMATA1), 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 [58]. Mutants for the other two ABA-activated kinases, *SnRK2.2* and *SnRK2.3*, did not show a drought-sensitive phenotype

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[59]. The triple mutant *snrk2.2/d snrk2.3/I snrk2.6/e* displayed an extremely sensitive phenotype under water deficit conditions. Transcriptomic studies of the triple mutant showed a down-regulation of genes encoding PP2Cs, which suggested a feedback loop in the transcription regulation of PP2Cs by SnRKs [54].

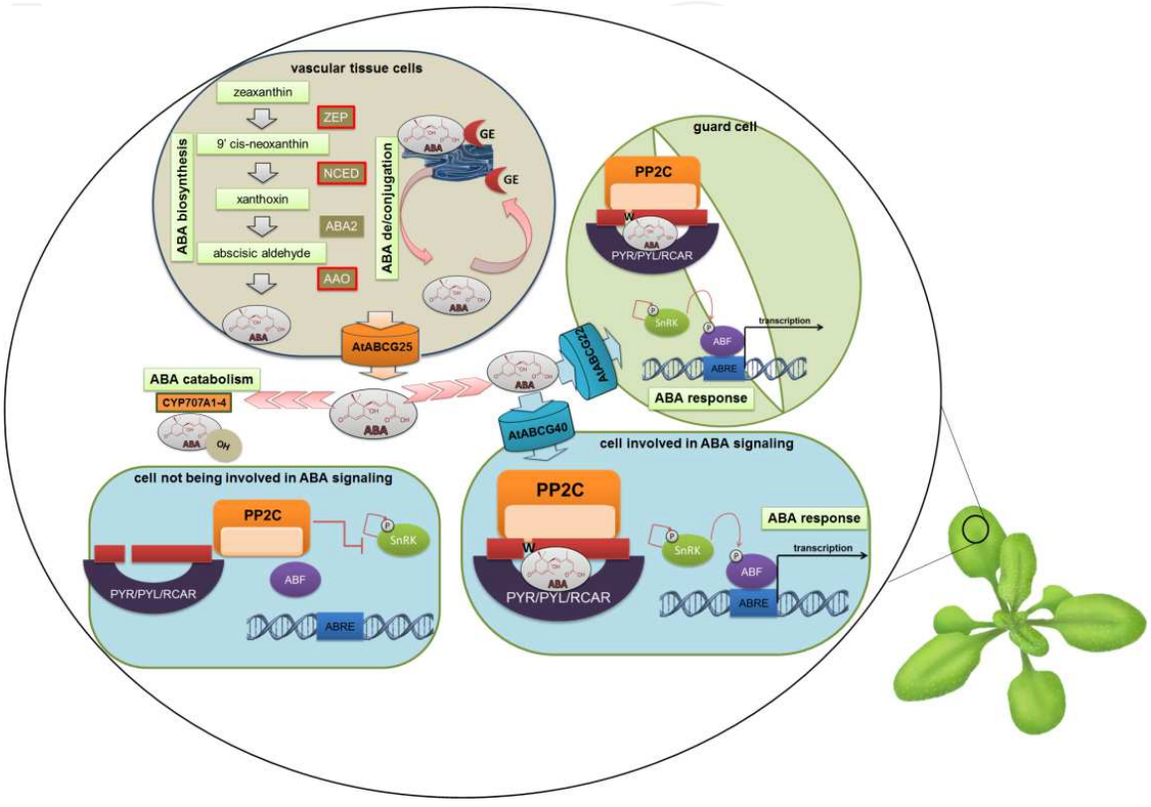

Figure 1. ABA synthesis, catabolism, conjugation and response in a scheme.

One of the earliest plant responses to water deficit condition, and one regulated mainly in an ABA-dependent manner, is the closure of stomata. The closing or opening of the pore is a result of the osmotic shrinking or swelling, of the two surrounding stoma guard cells. ABA acts directly on the guard cells and induces stomata closure via an efflux of potassium and anions from the guard cells [60]. ABA regulation of the membrane ion channels is mediated by increased cytosolic Ca^{2+} resulting from the release of Ca^{2+} from intracellular stores and a Ca^{2+} influx from the extracellular space. It is worth noting that a number of mutations that affect ABA signaling in regards to stomatal action during drought have been characterized. Dominant mutations have been described in genes that encode type-2C phosphatases - ABI1 (ABA INSENSITIVE 1) and ABI2 (ABA INSENSITIVE 2) [61-62], whereas recessive mutations that lead to supersensitivity to ABA in regards to stomata closure are found in genes that encode farnesyltransferase β-subunit - ERA1 (ENHANCED RESPONSIVE TO ABA1) [63-64], a larger subunit of cap binding complex CBP80 (CAP BINDING PROTEIN 80) [65] and the Sm-like snRNP protein SAD1 (SUPERSENSITIVE TO ABA AND DROUGHT 1) [66].

RCAR	PYR/PYL	PP2C interactors
RCAR1	PYL9	ABI1[1],[4], ABI2[1], HAB1[1]
RCAR2	PYL7	ABI1[4]
RCAR3	PYL8	HAB1[3], ABI1[4]
RCAR4	PYL10	ABI1[4]
RCAR5	PYL11	HAB1[3], ABI1[4]
RCAR6	PYL12	PP2CA/AHG3[2]
RCAR7	PYL13	
RCAR8	PYL5	HAB1[3], ABI1[4]
RCAR9	PYL6	ABI1[1],[4], ABI2[1], HAB1[1]
RCAR10	PYL4	HAB1[2], ABI1[4]
RCAR11	PYR1	HAB1[2], ABI1[4]
RCAR12	PYL1	HAB1[2], ABI1[4]
RCAR13	PYL3	HAB1[2]
RCAR14	PYL2	HAB1[2]

Table 1. The nomenclature of the different soluble receptors and their PP2Cs interactors

3. Abscisic acid is not the only phytohormone in stress response

The effectiveness of ABA is regulated not only by the length of a drought or the previous stress history of a given plant, but also by other phytohormones such as jasmonates, cytokinins and ethylene. The role of jasmonic acid (JA) has been well established in regards to plant development and defense responses [67]. Recently, it was also shown that jasmonic acid (JA) and methyl jasmonate (MeJA) are involved in the regulation of drought response. When JA or MeJA are applied exogenously to plants they are converted into a biologically active form (+)-7-iso-Jasmonoyl-L-isoleucine (JA-Ile). JA-Ile is then bound by the receptor SCF^{COI} complex that contains the CORONATINE INSENSITIVE1 (COI1) F-box protein [68-69]. This interaction leads to the degradation of the repressor protein – JAZ (Jasmonate ZIM-domain) by the 26S proteasome, it allows MYC2 (MYC DOMAIN TRANSCRIPTION FACTOR 2) activation of a distinct JA response genes [70-72]. In the absence of JA, JAZ inhibits MYC2 in order to activate the transcription of JA-inducible genes. It was showed that *MYC2* is up-regulated not only by JA, but also by ABA and drought. The described interaction between the protein specific to jasmonates - JAZ and both jasmonates and also ABA and drought-inducible MYC2 suggest the important regulatory role of JA in an ABA-dependent response to drought. A similar mechanism has been described in rice [73]. It was shown that, in addition to ABA, jasmonates also trigger stomatal closure in response to drought in

various species, including Arabidopsis and barley [74-76]. Low endogenous ABA content in the ABA-deficient mutant *aba2* impairs MeJA (methyl-jasmonate)-stimulated Ca^{2+} elevation, which is, in turn, important metal closure. Furthermore, MeJA stimulates the expression of the ABA biosynthetic gene, *NCED3*. MeJA signaling in guard cells requires the presence of endogenous ABA [77]. Another example of cross talk between ABA and jasmonates during stress response is the up-regulation by JA of *AtPYL4* (*PYRABACTINE LIKE 4*), *AtPYL5* (*PYRABACTINE LIKE 5*) and *AtPYL6* (*PYRABACTINE LIKE 6*), which are members of the *PYR/PYL/RCAR* ABA receptor family [78]. These studies showed the importance and conservation across the species of the role of JA in ABA-dependent response to drought.

Cytokinins (CKs) are another group of hormones involved in stress responses [79-80]. Cytokinins regulate cell proliferation and differentiation [81]. Abiotic stresses, such as drought, decrease the biosynthesis and transport of CKs from roots to shoots [82]. An increased concentration of CKs in xylem has been shown to decrease stomatal sensitivity to ABA [83]. The same effect was observed when exogenous CKs were applied [84-85]. When a plant encounters mild drought conditions, it is not necessary to close the stomata and further limit its photosynthetic rate. Since the decline in CK content increases the stomatal sensitivity to ABA, avoidance of this phenomenon might help in obtaining a better yield from plants that experience mild drought. CK up-regulation can be achieved by reduced expression of a gene that encodes cytokinin oxidase, an enzyme that degrades CKs. In addition to maintaining a better photosynthetic rate, increased levels of CKs lead to enhanced activity of the cell-cycle genes, and the consequent, increase in cell number may result in improved grain filling [86]. The process of grain filling is actually an increase in cell number and cell filling in the endosperm [87]. There is a generally positive relationship between endosperm cell number and grain weight in wheat [88], barley [89], maize [90] and rice [91]. Thus, endosperm cell number is one important factor determining grain weight [87]. Taking into account that endosperm cell number in cereal crops is established during an early phase of development, it is assumed that this step can be regulated by cytokinins [87]. Another manipulation of the CK level in plant tissues was achieved by seed inoculation with CK-producing bacteria, gradually releasing CKs within the physiological concentration range [92]. Wheat plants in which seeds were treated with such bacteria and grown under mild drought condition gave a 30-60% higher yield than non-treated controls. Since a high level of CKs improves grain quality and photosynthesis rate, and a high level of ABA increases root extension rate, osmoprotectant activity, and solute biosynthesis, another aim of breeders is to obtain a high content of both ABA and CKs under mild drought conditions Wilkinson et al. [6].

Ethylene, a gaseous plant hormone that inhibits root growth and development, is involved in stress-induced leaf senescence and can contribute to reducing the rate of photosynthesis [93-95]. ABA can modulate the influence of ethylene on stomatal conductance. Contradictory results have been published regarding the role of ethylene in stomatal action. Desikan et al. [96] showed that ethylene induces stomatal closure, whereas Tanaka et al. [97] and Wilkinson and Davies [98] proved that ethylene can antagonize ABA action in the stomata. This

is probably due to the fact that the concentration of neither hormone is important for the final effect but rather the ratio of ABA to ethylene [99; 18].

4. With a little help from arabidopsis – Transferring knowledge from weeds to crops

A small genome, short life cycle, small stature, prolific seed production, ease of transformation, a completely sequenced genome, a near saturation insertion mutant collection, a genome array that contains the entire transcriptome – these are the major advantages of using the model plant Arabidopsis in studies on the molecular basis of responses to environmental stresses including drought. The identification of stress-related genes, their functions and the pathways they are involved in, has been facilitated by an increasing number of molecular tools, genetic resources and the large number of web-based databases available for Arabidopsis (Table 2).

Genomic resources and results obtained of Arabidopsis provide a resource for exploitation in crops. Using sequence homology, EST (Expressed Sequence Tag) libraries, and the full-length cDNA repositories available for crop species, there is a possibility of a simple transfer of data revealed in Arabidopsis to identify a gene of interest in a crop species (Figure 2).

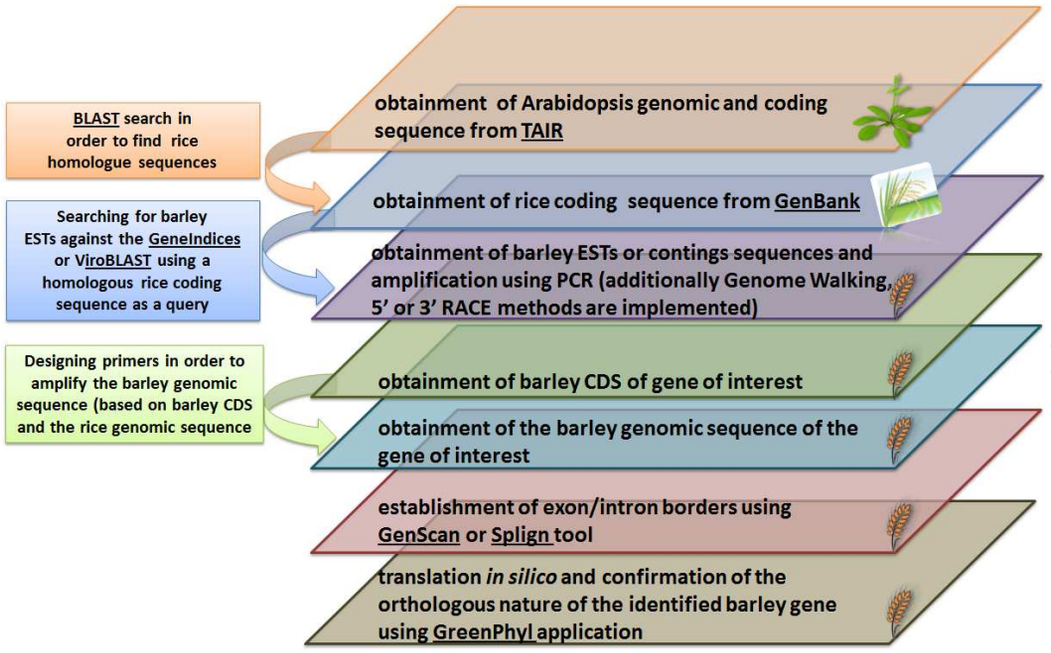

Figure 2. The pipeline of identification of barley homologous gene based on Arabidopsis and rice information. GenBank: http://www.ncbi.nlm.nih.gov/genbank/; TAIR: www.arabidopsis.org; BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi; GeneIndices: http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=barley; GenScan: http://genes.mit.edu/GENSCAN.html; Splign: http://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi; GreenPhyl: http://greenphyl.cirad.fr/v2/cgi-bin/index.cgi.

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Type	Resource/ database	URL
Integrative databases	TAIR	*http://www.arabidopsis.org/*
	MIPS	*http://mips.helholtz-muenchen.de*
	PlantGDB	*http://www.plantgdb.org/*
	EnsEMBLPlants	*http://plants.ensembl.org/index.html*
	Brachypodiumdb	*http://db.brachypodium.org/*
	EBI	*http://www.ebi.ac.uk/embl/*
	GenBank	*http://www.ncbi.nlm.nih.gov/genbank/*
	Gramene	*http://www.gramene.org/*
	Oryzabase	*http://www.shigen.nig.ac.jp/rice/oryzabase/*
	GrainGenes	*http://wheat.pw.usda.gov/*
Large-scale collections of full-length cDNA clones	flcDNA *A. thaliana*	*http://rarge.psc.riken.jp/*
	flcDNA *O. sativa*	http://cdna01.dna.affrc.go.jp/cDNA/ http://www.ncgr.ac.cn/ricd
	flcDNA *H. vulgare*	*http://www.shigen.nig.ac.jp/barley/*
	flcDNA *T. aestivum*	*http://trifldb.psc.riken.jp/index.pl*
	flcDNA *Z. mays*	*http://www.maizecdna.org/*
	ViroBLAST	*http://indra.mullins.microbiol.washington.edu/viroblast/viroblast.php*
TF databases	AGRIS	*http://arabidopsis.med.ohio-state.edu/*
	PlantTFDB	*http://planttfdb.cbi.edu.cn/*
	GRASSIUS	*http://grassius.org/*
Microarray bulk data retrieval	NASCarrays	*http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl*
	ArrayExpress	*http://www.ebi.ac.uk/arrayexpress/*
	AtGenExpress	*http://www.weigelworld.org/resources/microarray/AtGenExpress/*
	GEO	*http://www.ncbi.nlm.nih.gov/geo/*
	PlexDB	*http://www.plexdb.org/*
Gene expression analysis resources	Genevestigator	*https://www.genevestigator.com/gv/*
	BAR	*http://esc4037-shemp.csb.utoronto.ca/welcome.htm*
	eFP Browser	*http://esc4037-shemp.csb.utoronto.ca/efp/cgi-bin/efpWeb.cgi*
Functional information	GeneMANIA	*http://www.genemania.org/*
	IntACT	*http://www.ebi.ac.uk/intact/*
	BioGRID	*http://thebiogrid.org/*

Table 2. Web-based resources for gene expression analysis for Arabidopsis and other species, including crops.

In many cases, not only structural proteins, such as ion channels are conserved between Arabidopsis and other plant species, but also regulatory proteins, such as transcription factors. In addition, it is worth adding that entire transcriptional regulons can also be conserved, as in case of the ABA signalosome PYR/PYL/RCAR-PP2Cs-SNRKs. 'Only after we understand how plants respond to stress — in many cases first in Arabidopsis and then applying the Arabidopsis model to crop plants — will we be able to begin engineering stress tolerance' [100].

During the last decade, microarrays have become a routine tool for the analysis of transcripts, not only in model Arabidopsis but also in crops, such as barley and rice. Interestingly, interspecies comparisons between distantly related species, Arabidopsis and rice or barley revealed conserved patterns of expression in the case of many orthologs genes [101-103]. Comparative analyses showed that orthologous of specific genes in rice or barley are also responsive to stress similar to Arabidoposis [103; 102]. Mochida et al. [104] used publicly available transcriptome data to investigate regulatory networks of the genes involved in various developmental aspects including drought in barley. On the basis of a comparative analysis between barley and model species, such as Arabidopsis or Brachypodium, modules of genes putatively involved in drought response have been identified. In addition to these computational approaches, Moumeni et al. [105] have undertaken a comparative analysis of the rice root transcriptome under drought stress. They used two pairs each of drought-tolerant and susceptible rice NILs (Near Isogenic Lines). Global gene expression analysis revealed that about 55% of the genes differentially expressed were in rice roots under drought stress. The drought-tolerant lines showed an up-regulation of the genes involved in secondary metabolism, amino acid metabolism, response to stimulus, defense response, transcription and signal transduction. Proteomic analysis of drought-sensitive and drought-tolerant barley lines performed by Kausar et al. [106] revealed an increased level of metabolism, photosynthesis and amino acid synthesis-related proteins in tolerant genotypes, whereas a decreased level was observed in sensitive forms. The data confirmed the results described previously in other species and should that similar processes play a significant role in barley's adaptation to stress conditions.

5. The huge role of tiny molecules (microRNA) in drought response

Small non-coding RNAs – miRNAs, which were first reported in the nematode *Ceanorhabditis elegans* in 1993 [107] and which are responsible for the phenomenon of RNA interference, have become recognized as very important regulatory components of the cell signaling. miRNAs have been shown to be highly conserved gene expression regulators across species [108-109]. The first plant miRNA was isolated from Arabidopsis [110]. To date, approximately 5000 plant miRNAs have been identified and deposited in miRbase (19.0 release) including 299 miRNA from Arabidopsis, 135 from Brachypodium, 206 from sorghum, 42 from wheat, 591 from rice, 172 from maize and 67 from barley [111]. miRNAs are small regulatory RNAs of a 20-22 nucleotide length that are encoded by endogenous *MIR* genes. Their primary transcripts are partially double-stranded stem-loop structures. Pri-miRNAs in plants

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are processed by DCL1 (DICER-LIKE 1) HYL1 (HYPONASTIC LEAVES 1), SE (SERRATED) proteins into pre-miRNA hairpin precursors which are finally converted into short duplexes – mature miRNAs. The duplexes are then methylated at the 3′ terminus and exported to the cytoplasm. In the cytoplasm, single-stranded miRNAs are incorporated in the AGO (ARGONAUTE) protein, the catalytic compound of the RISC (RNA-INDUCED SILENCING COMPLEX) complex, and guide the RISC to the target mRNAs by sequence complementarity to negatively regulate their expression [112].

Plant microRNAs are involved in various developmental processes including flowering, and leaf, stem and root development [113-115]. Jones-Rhoades and Bartel [116] drew the attention of plant biologists to the miRNA engagement in stress response for the first time. To gain an insight into the role of miRNAs in the regulation of transcripts in response to drought, several projects on the identification of the miRNAs related to stress response in crops were undertaken. Using deep sequencing techniques, Zhou et al [117] identified nineteen new miRNAs that are induced by drought in rice, among them eleven down-regulated and eight up-regulated miRNAs. In addition, they identified nine miRNAs that showed an opposite expression to that observed in drought-stressed Arabidopsis (Table 3). A similar approach was used by Kulcheski et al. [118] in soybean, which revealed 11 miRNAs that are related to drought stress (Table 3). Based on bioinformatic prediction and then verification of the obtained results using RT-qPCR, Xu et al. [119] identified 21 miRNAs differently expressed during water stress in maize (Table 3). A similar approach using bioinformatic prediction of miRNAs on dehydration stress was undertaken by Kantar et al. [7], who found four miRNAs that are related to drought stress in barley (Table 3). Deep sequencing of a small RNA library in the case of barley was performed by Lv et al. [8]. They showed that six miRNAs specific for stress response. hvu-MIRn026a, hvu-MIRn029, hvu-MIR035, hvu-MIR156d exhibited higher expression in response to salt and drought stress, whereas hvu-MIR396d and hvu-MIR399b showed a higher expression only in drought-stressed plants. Additionally, the authors observed that hvu-mir029 was highly expressed after drought treatment and at a very low level under non-stressed conditions, which suggests the important role of this molecule in water deficit response (Table 3).

To understand the function of newly identified miRNAs, the putative target transcripts have to be predicted. In order to identify microRNAs target transcripts, Kantar et al [7] performed computational studies and a modified 5′ RLM-RACE (RNA ligase-mediated 5′ rapid amplification of cDNA ends) in barley. Seven cleaved miRNA transcripts were retrieved from drought-stressed leaf samples as targets for hvu-MIR165, hvu-MIR166, hvu-MIR156, hvu-MIR2055, hvu-MIR171, hvu-MIR172, hvu-MIR397 and hvu-MIR159. The identified targets are mainly transcription factors that play a role in plant development, morphology and determination of the flowering time. *SCRL6* (*SCARECROW LIKE 6*) encodes a transcription factor that is involved in diverse plant developmental processes such as leaf or root growth and is the target of hvu-MIR171, *ARF10* (*AUXIN RESPONSIVE FACTOR 10*) encodes a transcription factor that negatively regulates auxin signaling and is the target of hvu-MIR160, *SBP* (*SQUAMOSA PROMOTER BINDING PROTEIN*) is a transcription factor that is mainly important for leaf development and is the target of hvu-MIR156a, and *MYB33* (*MYB DOMAIN*

PROTEIN 33) is a transcription factor that is involved in ABA and GA signaling and is the target of hvu-MIR159a [7].

Species	Identified miRNA related to drought	References
rice	osa-MIR170, osa-MIR172, osa-MIR397, osa-MIR408, osa-MIR529, osa-MIR896, osa-MIR1030, osa-MIR1035, osa-MIR1050, osa-MIR1088, osa-MIR1126, osa-MIR395, osa-MIR474, osa-MIR845, osa-MIR851, osa-MIR854, osa-MIR901, osa-MIR903 and osa-MIR1125, osa-MIR156, osa-MIR168, osa-MIR170, osa-MIR171, osa-MIR172, osa-MIR319, osa-MIR396, osa-MIR397, osa-MIR408	[117]
soybean	gma-MIR166-5p, gma-MIR169f-3p, gma-MIR1513c, gma-MIR397ab, gma-MIR-Seq13, gma-MIR-Seq11, gma-MIRSeq15, gma-MIR166f, gma-MIR-482bd-3p, gma-MIR4415b, gma-MIR-Seq07	[118]
maize	zma-MIR161, zma-MIR397, zma-MIR446, zma-MIR479, zma-MIR530, zma-MIR776, zma-MIR782, zma-MIR815a, zma-MIR818a, zma-MIR820, zma-MIR828, zma-MIR834, zmaMIR1, zma-MIR2, zma-MIR3, zma-MIR4, zma-MIR5, zma-MIR6, zma-MIR7, zma-MIR8, zma-MIR9	[119]
barley	hvu-MIR156, hvu-MIR166, hvu-MIR171, hvu-MIR408	[7]
	hvu-MIRn026a, hvu-MIRn029, hvu-MIR035, hvu-MIR156d, hvu-MIR396d, hvu-MIR399b	[8]

* red indicates down-regulation by drought, green indicates up-regulation by drought, blue indicates regulation opposite to that observed in Arabidopsis, black indicates no information about regulation by drought

Table 3. miRNA related to drought in different crop species.

6. From the cell to the organism level – Phenotyping of drought-treated crops

In order to understand gene-to-phenotype relationships in the plant response to drought stress, it is vital to decipher the physiological and genetic bases of this process. Recent advances in crop physiology, genomics and plant phenotyping have provided a broader knowledge and better tools for crop improvement under stress conditions [120]. Maintaining a high yield under drought conditions has become a priority for breeders. However, the physiological basis of yield maintenance under drought is not yet fully understood, of the complexity of the mechanisms that plants can use to maintain growth in conditions due to water deficit [120]. Quantitative trait loci (QTL) for genes conferring a yield benefit under drought conditions first need to be identified in phenotypic screens and then incorporated into crops using marker-assisted selection [121]. Direct selection for yield in drought-prone environments, however, has proven to be difficult. Drought stress is a dynamic process and

can occur at different periods of the crop cycle and with different intensities. Consequently, plants have developed various strategies in response to drought: tolerance, escape and avoidance. Ludlow [122] defined three strategies plants use to cope with drought stress: drought tolerance is the ability of a plant to cope with water deficit through low tissue water potential, drought escape is defined as completion of the life cycle just before a severe drought starts, and drought avoidance is plant maintenance of high tissue water potential by minimizing water loss or maximizing water uptake. The final mechanism conveys the ability to survive and recover rapidly after a severe stress through protective mechanisms, such as cell wall folding, membrane protection, and the accumulation of antioxidants [123-124].

In order to incorporate traits that confer drought tolerance into molecular breeding programs, phenotyping protocols are extremely important [125]. With the wide availability of genetic resources, such as mutant populations (TILLING) or mapping populations, high-throughput phenotyping will become an essential asset in closing the gap between plant physiology and genetics [126- 127]. It is worth noting that a complex set of both abiotic and biotic stresses shapes the natural environment during plant development drought stress is just one of many factors. It is hard to exclude one of the stress pathways and to analyze it in isolation from others because the cascade of stress response is a complicated web of overlapping pathways. When studying drought tolerance in plants, it is very difficult to control and monitor the level and onset of water deficit, since it is a dynamic process and a combination of the available water in the soil and the plant water status. Continuous measurements are needed in order to link the level of drought experienced by the plant with the physiological changes occurring in response to it [125]. Under greenhouse conditions, water use can be monitored by weighing the pots or using TDR (Time Domain Reflectometry) soil moisture meters [128]. The water supply can be regulated at high-throughput automated screening facilities by using the classical water withdrawal approach [14] and maintaining a constant soil water status [129].

Another difficult issue is how to describe plant response to drought at the physiological level using properly chosen physiological, but also morphological, traits. In breeding programs for improved drought tolerance, crop traits associated with the conceptual framework for yield drought adaptation have been proposed by Passioura [130]. This framework has three important drivers: (1) water uptake (WU), (2) water-use efficiency (WUE) and (3) harvest index (HI). Several traits are highly associated with these three aspects of Passioura model. With regard to WU, the best method would be direct selection for variation in root architecture but since this is hard to perform, stomatal conductance, mainly the canopy temperature, is measured. This provides indirect indicators of water uptake by roots [131]. To estimate WUE, carbon isotope discrimination is used. A high affinity of Rubisco for the more common ^{12}C isotope over the ^{13}C indicates a lower WUE, whereas a lower discrimination value indicates a higher WUE [131]. In the case of HI, the extreme sensitivity of reproductive processes to drought may result in reproductive failure, which is associated with a low HI value [132].

Water stress reduces photosynthesis in the leaves of higher plants. It is linked with a decreased diffusion of CO_2 from the atmosphere to the site of carboxylation [133-134]. Underlying this process is the stomatal closure during short-term drought and photoinhibition damage, and the inactivation of RuBisCO under long-term stress [135].

Stomatal closure is one of the first responses to drought conditions which might result in cell dehydration or runaway xylem cavitation [136]. A good illustration of this process is stomatal behavior in the midday, when either stomatal closure or decreased stomatal conductance can be observed. Both responses are mediated by ABA synthesized in response to dehydration conditions [18]. When decreased stomatal conductance is combined with sustained high irradiance, leaves are subjected to excess energy relative to the available CO_2 and the rate of reducing power can overcome the rate of its use in the Calvin cycle. These processes lead to the down-regulation of photosynthetic and even photoinhibition. Plants have evolved mechanisms of defense to protect photosynthesis. Such protection can be achieved by the regulated thermal dissipation that occurs in the light-harvesting complexes [137].

Processes associated with the photosynthetic apparatus can be measured using chlorophyll fluorescence. Experiments with chlorophyll fluorescence were first carried out by Kautsky and Hirsch [138]. Since then, this technique has progressed quickly and chlorophyll fluorescence can be easily measured using commercially available chlorophyll fluorimeters which enable the measurements of the photochemical and non-photochemical processes involved in the fluorescence quenching that occurs in the presence of light [139]. The Fv/Fm ratio representing the maximum quantum yield of the primary photochemical reaction of photosystem II (PSII) is the most often used parameter. Environmental stresses that affect PSII efficiency lead to the characteristic decrease in the value of this parameter [140]. Fluorescence kinetics of chlorophyll a, the 'OJIP/JIP-test' named after the basic steps of the transient by which parameters quantifying PSII behavior are calculated (O is the fluorescence intensity F0 (at 50 μs); J is the fluorescence intensities FJ (at 2 ms); I is FI (at 30 ms) and P is the maximal fluorescence intensity, FP = FM) is an informative tool for studying the effects of different environmental stresses on photosynthesis [141-142;10;143]. This analysis offers simple equations to express the equilibrium between the inflow and outflow of the entire energy flux within PSII; it also provides information about the fate of absorbed energy. Some of the parameters calculated using the JIP-test are related to energy fluxes for light absorption (ABS), the trapping of excitation energy (TR) and electron transport (ETR) per reaction center (RC) or per sample area called cross-section (CS). Their estimates are based on the analysis of several groups of measured and calculated parameters. Analyses performed using these parameters are quick and the measurements are non-invasive [10].

In addition to the photosynthesis process, it was observed that the alteration of leaf angle caused by dehydration, towards smaller angles, would diminish intercepted radiation and carbon assimilation, and also have an important protective role against excess solar energy [144]. There is also a correlation between the rate of photosynthesis and the age of the leaf. Younger leaves tend to be more resistant to drought than older ones. When a severe reduction in the size of the leaf canopy occurs, as a result of shedding older

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leaves, it allows a plant to recover faster following rehydration [145]. Photosynthetic recovery following rehydration plays a pivotal role in drought-tolerance mechanisms and prevents a dramatic decline in crop yields [146]. It was shown that recovery from a severe stress is a two-step process. The first phase occurs during the first hours or days after rewatering and corresponds to an improvement of leaf water status and the reopening of stomata [147]. The second stage lasts a few days and requires the *de novo* synthesis of photosynthetic proteins [148-149].

It is also worth noting that other phenotype analyses should be performed in order to obtain a complete picture of the stress response of a given plant. Relative Water Content (RWC), which was proposed by Sinclair and Ludlow [12], is the most often used assay to assess plant response to a water deficit. This simple test allows the establishment of relative water content in a leaf of control and drought-treated plants. Detached leaves are weighed and saturated with water for 24 h, then again weighed and dried for 48 h and weighed again. RWC is calculated from the following formula: RWC (%) = [(FM - DM)/(TM - DM)] * 100, where, FM, DM, and TM are the fresh, dry and turgid masses of the tissue weighted, respectively.

The degree of cell membrane stability (CMS) is considered to be one of the best physiological indicators of drought-stress tolerance. It can be evaluated using measurements of solute leakage from plant tissue [150-151].

In response to drought stress, plants are able to adjust osmotic pressure by synthesizing osmoprotectants such as proline, the water soluble carbohydrates that behave like a molecular weapon against dehydration within the cell. There are several methods used in order to estimate the accumulation of endogenous proline or sugars in drought-treated plants [152].

Several morphological traits that have an impact on drought tolerance have been observed. Growth inhibition resulting from drought-induced ABA biosynthesis was observed in plants exposed to stress [153]. A number of studies have shown that wax deposition on the leaf surface increased in response to drought and an associated improvement in drought tolerance was observed in oat, rice, sorghum, wheat and barley plants that had an increased wax layer [154 -157]. Enhanced drought tolerance was also gained by plants having a reduced number of stomata, which was probably dependent on the accumulation of waxes [158]. Yang et al [158] performed analysis on an ox-*win1/shn1* (overexpressor *wax inducer 1/shine 1*) mutant. *WIN1/SHN1* encodes a transcription factor that regulates the expression of genes that control the accumulation of cuticular wax. Analyses performed by Yang et al [158] showed that induction of *WIN1/SHN1* expression by drought is correlated with an increased expression of the genes involved in wax accumulation, and on the other hand, a decreased expression of the genes involved in stomatal development. These results suggest that the drought-tolerant phenotype of analyzed by Yang et al [158] forms caused by induction of *WIN1/SHN1* may be due to a reduced number of stomata as well as wax accumulation.

There are now several high-throughput phenotyping techniques available for the measurement of some of the traits described above. One of these is thermal infrared imaging, or infrared thermography (IRT), which is used to measure the leaf or canopy temperature.

Evaporation is a main determinant of leaf temperature. There is a direct relationship between leaf temperature, transpiration rate and stomatal conductance [159-161]. Drought-tolerant genotypes can maintain a higher stomatal conductance and also a higher rate of photosynthesis, as was mentioned above, thus these genotypes could be identified as having a lower canopy temperature than the sensitive genotypes [162-163].

7. GM crops – are they a solution?

Genetic modification of crops is a controversial issue. Some aspects of genetic modification that have potential to improve drought tolerance in crops are presented here. Biotechnological approaches may involve the overexpression of genes related to osmotic adjustment, chaperones and antioxidants [reviewed in 164-165]. Also, ectopic expression or suppression of regulatory genes, such as genes that encode transcription factors, is widely used [166]. Recent studies on rice led to the identification of genes involved in three pathways that can be manipulated in order to improve drought tolerance in crops: the gene that encodes β-carotene hydroxylase, which confers drought resistance by increasing xanthophylls and ABA synthesis [167], the *DST1* (*DROUGHT AND SALT TOLERANT 1*) gene that regulates stomatal closure and density under drought stress [168] and the *TLD1*/ *OsGH3.13* (*INCREASED NUMBER OF TILLERS, ENLARGED LEAF ANGLES, AND DWARFISM*) gene whose down-regulation enhanced drought tolerance in rice [169]. Although several genes that can improve the drought tolerance of crops have already been identified, progress in the commercialization of the traits controlled by these genes has been slow [165]. One of the genes that has been successfully introduced into a crop plant and that gave improved drought tolerance in field trials was 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 [170]. Results from field experiments showed that a maize line expressing the *CspB* gene had a higher yield under water deficit conditions than the control and expressed a yield equivalent to the control under non-stressed conditions. Tests are in progress in 2012 on commercial farms, [171; http://www.monsanto.com/products/Pages/corn-pipeline.aspx#firstgendroughttolerantcorn]. The value of a biotechnological approach to improving crop yields under drought stress conditions is becoming evident with the first demonstrations of improved drought tolerance in crops in the field (reviewed in [171]).

8. Conclusions and perspectives

In order to achieve a full understanding of drought-response mechanisms in plants and to make use of this understanding to produce crops with improved drought tolerance, there is a need to combine the data derived from different studies. Detailed analyses of the networks of protein interactions, the co-expression of genes, metabolic factors, etc. should provide insights into the key regulators of drought response [172-173]. Biotechnological approaches

can also be promising in improving drought tolerance in crops based on previously obtained and integrated knowledge [171].

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