Chapter 5
Biotechnology for Drought and Salinity Tolerance of Crops

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Contents

1 Introduction

There is a growing imbalance between supply and demand of the major cereals, viz., wheat, rice and maize, which together provide 70 % of the calorific intake for the world's population. Whilst in recent years, genetic and agronomic developments

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have steadily increased the production of these crops, the rate of increase is still less than that needed to match the requisite demand. This has caused price volatility and fuelled concerns over long-term food security. The demand for cereals is increasing in response to increased population and wealth. However, the loss of land for crop production due to urbanisation, degradation and alternate uses (e.g. for bioenergy crops or leisure) and the projected changes in climate are major obstacles against further increases in production.

The availability of water is a major determinant of plant production, and shortages of water are recognised as major threats to food security (Parry et al. 2005). In areas with low rainfall and high evapotranspiration (semiarid areas), plant growth can also be decreased by soil salinisation, a problem which is exacerbated by irrigation of poorly draining soils with low-quality water (Tardieu 2013). Developing high-yielding crops for water-limited environments is a major challenge. Conventional breeding for yield under any conditions is difficult because of the complex nature of the trait, which is determined by multiple genes, the large size of cereal genomes and the comparatively limited gene pool available for breeders (Malik et al. 2003). There is now genome mapping and sequence data available for some major food crops such as rice (Sakata et al. 2002) and sorghum (Paterson et al. 2009). However, exploitation of genomic data for improved crop performance under drought is limited by the complexity of the underlying traits which are often determined by multiple genes (Parry et al. 2005; Parry and Reynolds 2007) and the seasonal and year-on-year variation of water availability. However, biotechnological tools including plant transformation, random and targeted mutagenesis, transposon/T-DNA tagging and RNA interference (RNAi) permit the linking of genes to their biological function, thereby elucidating their contribution to traits, in ways not previously possible (closing the genotype to phenotype gap) (Pérez-Clémente et al. 2013). With this information, biotechnology has the potential to deliver higher and more stable yields for saline and water-limited environments (Fig. 5.1).

Genetic engineering has been used successfully to improve agronomically important traits in cereals (Vasil 2007). Herbicide-tolerant and insect-resistant genetically modified (GM) maize varieties have been in use since the mid-1990s, and insect-resistant GM rice varieties have been approved for commercialisation in China. The market for 'biotech maize' is now well established worldwide, with significant cultivation even in Europe (Halford 2006). The market for wheat biotechnology has proved more difficult to establish, but experimental GM lines have been produced with improved end-use quality traits (Shewry 2007; Tamás et al. 2009). Here we review the biotechnological methodologies that are available and the prospects for their successful application for improving drought and salt tolerance in cereals.

2 Genetic Approaches

Genetic analysis has played a role in wheat breeding for more than a century, and by the 1970s, the chromosomal locations had been established for major genes controlling dwarfing, spike morphology, grain colour and hardness, the major

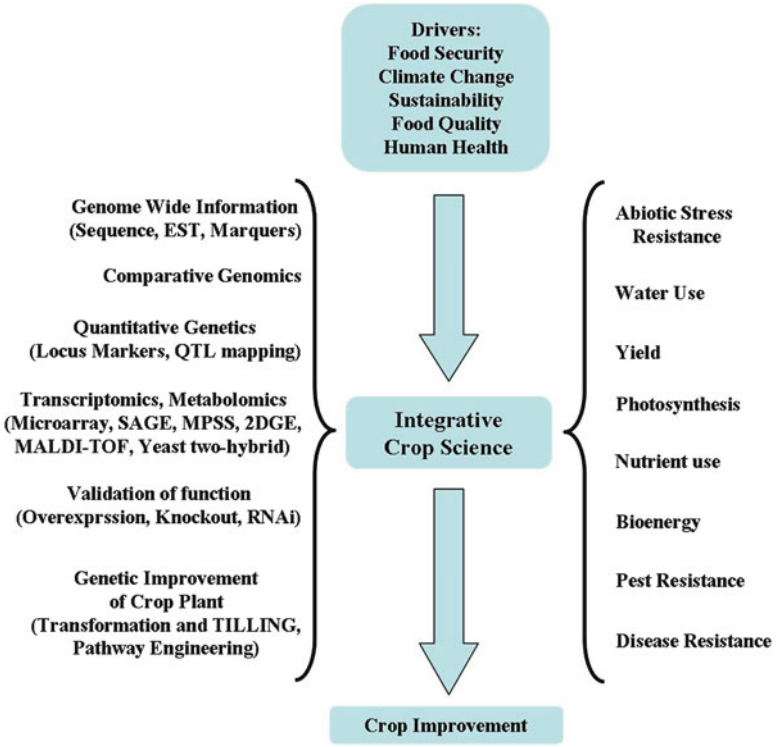

Fig. 5.1 Targets and approaches for improving crop performance under stress conditions. *2DGE* two-dimensional gel electrophoresis; *EST* expression sequence tag; *MALDI-TOF* matrix-assisted laser desorption/ionisation time of flight; *MPSS* massively parallel signature sequencing; *QTL* quantitative trait locus; *SAGE* serial analysis of gene expression

classes of storage proteins, vernalisation and photoperiod response (Snape 1998). Traits that are controlled by multiple genes and loci (quantitative traits), including yield and drought tolerance, have been more intractable, and it was not until the last decade of the twentieth century that progress began to accelerate. This was brought about by the development of genetic maps based on markers; initially these markers were based on restriction fragment length polymorphisms (RFLP), but subsequently a range of markers have been developed, including amplified fragment length polymorphisms (AFLP), random amplification of polymorphic DNA (RAPD), variable number tandem repeats (VNTR), microsatellite polymorphisms based on simple sequence repeats (SSR), single-nucleotide polymorphism (SNP), single feature polymorphism (SFP) and restriction site-associated DNA markers (RAD). Researchers and breeders have been able to construct genetic maps using these markers that enable a trait that segregates in a cross, to be attributed to a specific location in the genome, even if the exact gene responsible is not known. The locus that is identified is known as a quantitative trait locus, or QTL.

2.1 Targeting QTL for Tolerance to Drought and Salinity

Marker-assisted selections of target QTLs are powerful support for improving productivity under drought and/or saline conditions which will assist selection in the breeding process. One of the major difficulties in drought QTLs identification in crops in general and wheat in particular is the identification of the key physiological and morphological determinants of drought tolerance. Most QTLs for drought tolerance in wheat have been identified through yield and yield measurement under water-limited conditions (Maccaferri et al. 2008). Considerable progress has already been made in deconvoluting traits related to water use and in identifying variation in component traits (e.g. root traits—Clark et al. 2008; Courtois et al. 2009; leaf traits—Khowaja and Price 2008; Khowaja et al. 2009). The component traits may have direct impacts on the uptake and use of water or affect these processes indirectly, for example, in some water-limited environments, a shorter life cycle may enable a crop to escape from water limitation. Yields can be increased if such traits are strategically targeted and effectively selected for drought stress tolerance (Richards et al. 2010).

2.2 Mutagenesis and TILLING

Genetic mutation is a powerful tool that establishes a direct link between the biochemical function of a gene product and its role in vivo. Chemical mutagens have been used for forward genetic screens in a variety of organisms. Compounds, such as EMS (ethyl methanesulfonate) and DMS (dimethyl sulphate), are used to generate mutants. This class of mutagens causes a large number of random point mutations in the genome, thus theoretically multiple allele of any gene can be obtained in the population (Greene et al. 2003). Despite the clear advantages of EMS mutagenesis, until recently, it has been useful as a tool for reverse genetics because of the lack of high-throughput techniques for detecting point mutations.

Modern genomics makes reverse genetics possible as large amounts of genomic and expressed sequence information become available. In the last few years, the TILLING method (for Targeting Induced Local Lesions in Genomes; McCallum et al. 2000) have been developed. TILLING has been used successfully as a functional genomics discovery platform in model organisms such as *Arabidopsis* (McCallum et al. 2000; Till et al. 2006) and in plant systems including rice, barley, maize, wheat and soybean (Caldwell et al. 2004; Till et al. 2004; Slade et al. 2005; Anai 2012; Chen et al. 2012). TILLING has several advantages over other techniques used to detect single-bp polymorphism. Alleles generated by TILLING can be readily used in traditional breeding programmes, since the technology is non-transgenic and the mutations are stably inherited. This makes TILLING an attractive strategy not only for functional genomics but also for agricultural applications. It can be predicted that more and more direct or indirect benefits will be revealed through continuous applications of TILLING in the near future.

3 RNA Interference and Its Application in Cereals

RNAi is a potent and highly specific gene-silencing phenomenon that is based on sequence-specific RNA degradation following by the formation of double-stranded (dsRNA) homologous in sequence to the targeted gene (Marx 2000; Baulcombe 2004).

The natural function of RNAi and its related processes seem to be protection of the genome against invasion by mobile genetic elements such as transposons and viruses. Given the gene-specific feature of RNAi, it is conceivable that this method will play an important role in therapeutic application. RNAi has proven to be very efficient in interfering with gene expression in various plant systems such as *Arabidopsis thaliana* and rice (Chuang and Meyerowitz 2000; Miki et al. 2005).

Functional genomics using RNAi is particularly an attractive technique for genomic mapping and annotation in plants. RNAi has been successfully used for functional genomics studies in bread wheat (Travella et al. 2006) as well as plant model systems such as *Arabidopsis* and maize (McGinnis et al. 2005). To develop RNAi technology for functional genomics, there is a need to characterise, in molecular detail, the silencing of homologous genes as well as the inheritance of RNAi-induced phenotype (Travella et al. 2006).

4 Transcriptome Analyses of Plant Drought and Salt Stress Response

The transcriptomics approach deals with comprehensive analysis of gene expression in a cell. Understanding the transcriptome is essential for analysing the genomic function and the molecular constituents of cells and tissues. Different technologies have been developed to study the transcriptome, including northern hybridisation and quantitative real-time PCR (Q-RT-PCR). The above low-throughput techniques are still used for validating the results obtained from global approaches. Advances in genomics technologies allow measurement of transcript levels of thousands of genes at the same time. The DNA microarray, using the principle of nucleic acid hybridisation of mRNA or cDNA fragments, is among these techniques.

4.1 DNA Microarrays

Microarray technology is a powerful tool for analysing the expression profiles of many genes (Richmond and Somerville 2000; Seki et al. 2004). Basically, there are two types of microarray formats: cDNA arrays and oligoarrays. Despite its power and usefulness, microarray technology is both expensive and time intensive. Besides several technical problems such as contamination of DNA in spots on arrays, uneven hybridisation and spurious hybridisation, it requires multiple biological and technical replications for generating reliable data. Microarray technology has been applied to

the analysis of expression profiles in response to abiotic stresses, such as drought, high salinity and cold (Kawasaki et al. 2001; Seki et al. 2001, 2002; Chen et al. 2002; Fowler and Thomashow 2002; Kreps et al. 2002; Lee et al. 2005). Stress-responsive genes have been identified in many plant species, such as *Arabidopsis* (Fowler and Thomashow 2002; Lee et al. 2005), *Arabidopsis*-related halophyte, *Thellungiella halophila* (Inan et al. 2004; Taji et al. 2004; Gong et al. 2005; Wong et al. 2006), rice (Kawasaki et al. 2001; Rabbani et al. 2003; Lan et al. 2005), barley (Oztur et al. 2002), wheat (Gulick et al. 2005), maize (Wang et al. 2003; Yu and Setter 2003), pine (Watkinson et al. 2003), hot pepper (Hwang et al. 2005), potato (Rensink et al. 2005), poplar (Gu et al. 2004; Brosche et al. 2005) and sorghum (Buchanan et al. 2005).

4.2 *High-Throughput Approaches for the Identification of Drought and Salt-Tolerance Genes in Plants*

The development of automated sequencing technologies has led to the production of sequencing machines with dramatically lower costs and higher throughput than the technology of just 2 years ago. The high-throughput sequencing technologies opened new view into the fields, thus allowing scientists to decode the genomes of many organisms (Soon et al. 2013). Various methods have been developed previously to directly determine cDNA sequences, based mostly around traditional (and more expensive) Sanger sequencing, whilst others include methodologies such as serial analysis of gene expression (SAGE) (Velculescu et al. 1995; Harbers and Carninci 2005), cap analysis gene expression (CAGE) (Nakamura and Carninci 2004; Shiraki et al. 2003; Kodzius et al. 2006) and massively parallel signature sequencing (MPSS) (Peiffer et al. 2008; Reinartz et al. 2002; Brenner et al. 2000). Recently, mapping and quantifying of transcriptomes can be easily done with the development of novel high-throughput DNA sequencing methods. This method, termed as RNA-Seq (RNA sequencing), has clear advantages over existing approaches and is expected to revolutionise the manner in which transcriptomes are analysed. It has already been applied to *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Arabidopsis thaliana* and mouse and human cells (Wilhelm et al. 2008; Nagalakshmi et al. 2008; Lister et al. 2008; Mortazavi et al. 2008; Cloonan et al. 2008; Marioni et al. 2008). The unbiased information on transcript sequence abundance and unparalleled ability of HTS to quantitative yield has afforded some remarkable new insights into transcriptome complexity and regulation. RNA-Seq provides quantitative readout and extremely reproducible transcript abundance (e.g. Li et al. 2008; Marioni et al. 2008; Pan et al. 2008; Wang et al. 2008a). RNA-Seq offers a large dynamic range of expression levels and a high-level reproducibility and less RNA sample than either large-scale Sanger expressed sequenced tag (EST) sequencing or tiling arrays (Soneson and Delorenzi 2013). Transcriptome Sequencing (RNA-Seq) can be done with a variety of platforms to test many ideas and hypotheses such as HiSeq (Illumina, formerly Solexa), 5500xl SOLiD System (Live Technologies) and 454 Genome Analyzer FLX (Roche).

The various technologies differ in the procedures used to array the DNA fragments. There are two key features that determine which sequencing platform is best suited for each experiment: the length of sequenced reads and the total number of sequenced reads output. In general, the 454 Genome Analyzer FLX sequencer generates reads of up to 200–300 bp and is currently best suited for applications involving de novo genome and transcriptome assemblies. In contrast, HiSeq and SOLiD generate approximately 35 bp reads and are best suited for resequencing or applications such as gene profiling where the short length of the microread is not a concern.

4.3 Gene Expression Profiling for Abiotic Stress Tolerance in Crops

Several new stress-related pathways, in addition to the previously well-described stress-related genes, have been related to abiotic stress transcriptome profiling in model species such as *Arabidopsis* and rice (Desikan et al. 2001; Kreps et al. 2002; Chen et al. 2002; Seki et al. 2002; Oh et al. 2005; Wang et al. 2011). ESTs are currently used as an efficient and fast method for profiling genes expressed in various tissues, cell types or stages of development (Andrews et al. 2001). Based on the research results, estimates of gene number in the cereals are very similar to other complex organisms; for example, a total of approximately 13,000 abiotic stress-related ESTs were reported in barley and rice (Zhang et al. 2004) and approximately 21,000 ESTs in wheat (Mochida et al. 2004). The clustering of ESTs sequence generated from abiotic stress-treated cDNA libraries provides information on gene number and gene families involved in stress responses. Gene expression profiling using cDNA macroarrays or microarrays will provide an opportunity for the discovery of higher number of transcripts and pathways related to stress tolerance mechanisms. There are few published reports on the use of barley or wheat chips for studying altered gene expression in response to abiotic stress.

5 Proteomic Approaches for Abiotic Stress Response

The importance of protein profiling has long been acknowledged in plant abiotic stress studies. Proteomics not only involves large-scale identification of proteins but also deals with analysis of all protein isoforms and post-translational modifications, protein-protein interactions, enzymatic assays for the functional determination, localisation studies of gene products and promoter activity and structural information of protein complexes (Wilkins et al. 1996; Brosche et al. 2005). The advancement in MS techniques (O'Farrell 1975) coupled with database searching have played a crucial role in proteomics for proteins identification. Databases have been constructed containing all expressed proteins from plant organs and cell organelles of various species (Table 5.1).

Table 5.1 Websites for plant omics research

Transcriptomics-related websites	
Genevestigator	http://www.genevestigator.com/gv/index.jsp
Gene expression omnibus	http://www.ncbi.nlm.nih.gov/projects/geo
Stanford Microarray Database	http://smd.stanford.edu/index.shtml
ArrayExpress	http://www.ebi.ac.uk/arrayexpress
PLEXdb	http://www.barleybase.org/plexdb/html/index.php
TIGR *Arabidopsis* arrays	http://www.jcvi.org/arabidopsis/qpcr/
Rice transcriptional database	http://microarray.rice.dna.affrc.go.jp
Rice Expression Database (RED)	http://red.dna.affrc.go.jp/RED/
BarleyBase	http://www.barleybase.org
Zeamage	www.maizearray.org
TIGR Solanaceae Genomics Resource	http://www.jcvi.org/potato/
Soybean Genomics and Microarray Database	http://psi081.ba.ars.usda.gov/SGMD/default.htm
Tomato Expression Database	http://ted.bti.cornell.edu
Genomics-related websites	
EMBL nucleotide sequence database	http://www.ebi.ac.uk/embl
National Center for Biotechnology Information	http://www.ncbi.nlm.nih.gov
Gramene	http://www.gramene.org
GrainGenes	http://wheat.pw.usda.gov
Gene Ontology	www.geneontology.org
The Arabidopsis Information Resource (TAIR)	http://arabidopsis.org/index.jsp
Rice Genome Project (RGP)	http://rgp.dna.affrc.go.jp/
RiceGE	http://signal.salk.edu/cgi-bin/RiceGE
Oryzabase	http://www.shigen.nig.ac.jp/rice/oryzabase/top/top.jsp
Maize Sequence	http://maizesequence.org/index.html
Maize genome resources	http://www.maizegenome.org/
Maize Genetics and Genomics Database	http://www.maizegdp.org/genome/
Sorghum Genomics	http://sorghoblast3.tamu.edu
Proteomics-related websites	
Proteome analysis at EBI	http://www.ebi.ac.uk/proteome/
Swiss-Prot	http://us.expasy.org/sprot/
Arabidopsis Membrane Protein Library	http://www.cbs.umn.edu/arabidopsis/
Database for *A. thaliana* annotation	http://luggagefast.Stanford.EDU/group/arabprotein/
ExPASy *A. thaliana* 2D-proteome database	http://expasy.ch/cgi-bin/map2/def?ARABIDOPSIS
PlantsP: Functional Genomics of Plant Phosphorylation	http://PlantsP.sdsc.edu/

A major limitation of the current technology is the reduced coverage and inability to detect low abundance proteins. High-resolution 2D gels can resolve about 1,000 proteins that are highly abundant in a crude mixture. Even under optimal conditions, approximately 25 % of the proteome may be observed (Zivy and deVienne 2000). However, development in direct mass spectrometric analysis is increasing

sensitivity, robustness and data handling (Wilkins et al. 1996). A number of proteome-wide platforms have been developed to complement mass spectrometric platforms. Yeast two-hybrid systems (Unlu et al. 1997) can detect weak interactions between low abundance proteins. Analogous to DNA microarrays, protein microarrays (Bayer et al. 2005) allow rapid interrogation of protein activity. The intensity or identity of resulting protein-protein interactions may be determined by fluorescence imaging or mass spectrometry.

New insights have been obtained on plant adaptation to abiotic stresses through application of proteomics approach to organelles and tissues in several plant species (Eldakak et al. 2013). Proteomics provided excellent opportunities to study the response of plants to stresses caused by heat, drought, salinity, ozone, heavy metals, UV light, nutrient deficiencies and elevated CO_2 conditions (Majoul et al. 2000). Proteome of poplar leaves (MacBeath 2002), rice anthers and leaves (Taylor et al. 2005; Renaut et al. 2006) and mitochondria of *Pisum sativum* (Fields and Song 1989) have been analysed to study plant response to cold stress. The effect of salinity stress, especially in crops plants, was investigated by comparative proteome studies in various tissue types in rice (Imin et al. 2004; Cui et al. 2005; Parker et al. 2006; Chitteti and Peng 2006), wheat (Yan et al. 2005; Zhang et al. 2009) and barley (Wang et al. 2008b).

Although proteomics has been exploited in abiotic stress tolerance studies in plants, large-scale proteomics studies are still limited. Application of proteomic approach particularly the comparative proteomics studies provided essential information about stress-induced alterations in protein quantity and quality and specific modifications of proteome (Abbasi and komatsu 2004).

6 Genetic Transformations of Cereals

Cereal improvement by genetic engineering requires the delivery, integration and expression of defined foreign genes into suitable regenerable explants. The available technologies and approaches used for production of transgenic cereal crops are complicated, and their efficiency is low. Moreover, different varieties of the same cereal crop and even different explants of the same variety would often require different methods for transformation. Two main methods are widely used for cereal transformation: (1) DNA transfer via particle bombardment developed by Sanford (1988) based on the use of the helium-driven PDS-1000/He particle gun and (2) *Agrobacterium*-based systems which exploit the ability to transfer a particular T-DNA on the tumour-inducing (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome. Both of these methods involve delivery of the transgene to callus tissue, followed by selection of transformed cells and regeneration of plantlets carrying the gene of interest. Each method has its advantages and limitations: biolistic transformation facilitates a broad variety of transformation strategies with a wide range of gene expression, has no host limitations or biological constraints, and diverse cell types can be targeted efficiently for

foreign DNA delivery (Altpeter et al. 2005). The main limitations of the bombardment approach include the insertion of backbone vector DNA and the insertion of multiple copies and fragmentation of the DNA during bombardment (Hu et al. 2003; Janakiraman et al. 2002). Numerous downstream breeding cycles are needed to select out those transgenic plants with good insertions and then to regenerate the homozygous lines used in breeding programmes for the development of a commercial product. Whereas *Agrobacterium*-mediated method is a simple, low cost alternative to particle bombardment. In addition, the *Agrobacterium*-mediated transformation system facilitates the precise integration of a small number of gene copies into the plant genome and shows a greater degree of stability for the transgene. Unlike microprojectile bombardment, *Agrobacterium* method seems to induce less transgene silencing, since introduced genes remain transcriptional active and has higher transformation efficiency than the microprojectile bombardment method. For all these reasons, *Agrobacterium*-mediated transformation has been adopted as the method of choice for most cereals. Therefore, main focus in this review will be on this transformation method.

The first success in cereal transformation using *Agrobacterium* was reported by Hiei et al. (1994) for stable transformation of rice. *Agrobacterium*-mediated transformation of other agronomically important cereal crop species, such as barley (Tingay et al. 1997), maize (Ishida et al. 1996) and wheat (Cheng et al. 1997), has now succeeded. For wheat, most of the research effort has focused on the model spring genotype 'Bobwhite' (Cheng et al. 1997, 2003; Haliloglu and Baenziger 2003; Hu et al. 2003), but reports of *Agrobacterium* transformation have been made using other spring varieties, Verry5, Cadenza, Fielder (Jones et al. 2005; Weir et al. 2001; Khanna and Daggard 2003; Wu et al. 2003), and the winter-type Florida (Wu et al. 2003; Jones et al. 2005).

Most of the protocols, efficiently used for cereal transformation, generally rely on the use of hypervirulent *Agrobacterium* strains such as AGL-0 and AGL-1 in wheat and barley (Tingay et al. 1997; Wu et al. 2003; Hensel et al. 2008), EHA101 and EHA105 in maize (Hood et al. 1986) as well as hypervirulent derivatives of LBA4404 in barley, maize and wheat (Khanna and Daggard 2003; Kumlehn et al. 2006; Hensel et al. 2008).

The recovery of stable plant cells after *Agrobacterium*-mediated transformation remains however influenced by many factors such as *Agrobacterium* strain, *Agrobacterium* density and surfactants, genotype, explant, binary vector, selectable marker gene and promoter, inoculation and coculture medium, inoculation and coculture conditions, regeneration medium, desiccation, osmotic treatment and tissue culture (Shrawat and Lorz 2006; Cheng et al. 2004). Some of these factors represent a drawback in extending the *Agrobacterium*-mediated transformation system to elite cultivars of economically important cereals. These limitations inspired some investigators to search for new alternative transformation procedures such as in planta transformation which involves no in vitro culture of plant cells or tissue (Supartana et al. 2005; Lin et al. 2009) or the flower dipping method originally developed for *Arabidopsis thaliana* transformation (Zale et al. 2009). Although these alternative methods seem simple and straightforward, yet they are technically challenging, and the results are not always convincing.

7 Conclusions and Future Perspective

Plants are often exposed to multiple abiotic stresses. Considerable advances have been made in understanding the plant's adaptation in stress environments and complex genetics involving multitude of gene and stress tolerance mechanisms. There is a great potential of genetic breeding for drought and salinity tolerance through the contribution of wild relatives to the identification of drought and salinity QTLs and functional markers. Gene expressing profiling has been widely used to understand mechanisms involved in the response of plants to abiotic stresses. Its application will determine a new revolution in crop research as technologies with lower costs. Future research effort should be directed using the omics approaches to elucidate plant's response to abiotic stresses. High-throughput omics technologies coupled with easily accessible integrated databases should now facilitate the elucidation of the complex stress regulatory network and their components to understand the mechanism of stress tolerance. The real benefits of these technologies, however, will only be realised when the knowledge and the tools resulting from the advances in omics field are translated into a product with improved abiotic stress tolerance in field environment.

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