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Vijay Rani Rajpal · Deepmala Sehgal
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Genomics Assisted Breeding of Crops for Abiotic Stress Tolerance, Vol. II

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Preface: Volume II

Breeding climate change-resilient varieties, capable of withstanding broad-spectrum stresses such as drought, heat, cold, salinity, flood, submergence, has become a major goal in plant breeding programs worldwide. The impetus for this common objective has arisen from severe negative effects of the climate change on crop production in the past two decades. Particularly in less-developed countries where the consequences of changing climate can have a devastating socioeconomic impact due to the burgeoning population, increasing the resilience of crops to climate change is the need of the hour for ensuring food and nutritional security.

Further, the objective of reaching a level of production, which is sufficient to sustain an adequate level of global food security, needs to be accomplished in a short span of time. Hence, scientists and breeders all over the world have adopted and integrated genomics-based tools in their breeding pipelines. Genomics-based approaches have been extensively deployed to dissect the genetic makeup of abiotic stress adaptation. Given the quantitative nature of abiotic stress tolerance, identification of quantitative trait loci, genome-wide association mapping, and/or application of transcriptomics have been the main target of research to identify the genetic loci or even candidate genes regulating the adaptive response of crops to abiotic stresses.

Genomics-assisted breeding is benefiting from the recent upsurge in high-throughput sequencing and phenotyping platforms, allowing rapid identification of genes underpinning abiotic stress tolerance. Even in minor and/or orphan crops, the number of available high-quality reference genomes has been constantly growing due to the widespread application of genome sequencing technology. This will not only expedite the dissection and cloning of the loci controlling abiotic stress tolerance but also will expand opportunities to tap into wild relatives of crops, hence increasing the reservoir of genetic diversity available to breeders.

This book elaborates the progress and prospects of genomics-assisted breeding for improving abiotic stress resilience in various crops in a simple but comprehensive mode using suitable examples. This compilation will prove useful to not

only scientists and Ph.D. students who are working on a specific crop or tacking a particular abiotic stress tolerance but to a broad community of readers including graduates and postgraduates who wants to be updated with pros and cons of various genomics-assisted approaches that has been utilized for genetic improvement of crop plants.

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We are very sure that this book will interest scientists, graduates, undergraduates, and postdocs who are investigating abiotic stress tolerance and are actively involved in crop improvement through genomics-based approaches.

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Chapter 1
Genetics and Genomics of Stomatal Traits for Improvement of Abiotic Stress Tolerance in Cereals

Fahimeh Shahinnia, Penny J. Tricker, Mohammad-Reza Hajirezaei and Zhonghua Chen

Abstract In traditional breeding programmes for improving abiotic stress tolerance of cereals, direct selection for grain yield is slow and costly, requiring many years and sites of field trials. Grain yield largely depends on the flag leaf characteristics and functions and is correlated to the ability of the plant to regulate its water content and to synthesize, store and relocate carbohydrates from leaves to grains. Despite the recognition of the importance of the flag leaf in cereals, little is known about genetic control of its cellular structure and development under stress. The leaf stomata cells regulate water loss by transpiration and photosynthetic CO_2 uptake in plants. In order to maintain a high photosynthetic rate for higher yield under drought and salinity conditions, it is critical to explore the mechanisms of control of stomata. A major crucial challenge in breeding for abiotic stress tolerance is the knowledge about the physiological and genetic mechanisms that regulate stomatal morphology and development connected to grain yield. Quantitative trait loci (QTL) mapping has been used to identify the genes that are subject to natural variation of stomatal traits in wheat, barley and rice. Over the last decade, several studies have demonstrated the importance of stomatal density and size and their positive association with physiological processes in grain yield. Further, considerable genetic variation exists for stomatal and epidermal cell traits that could be exploited for marker-assisted breeding and used for creation of new effective traits in cereals.

Keywords Epigenetic control · Indirect selection · QTL · Stomatal features Stomatal regulation · Stress response

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1.1 Introduction

The most important food, feed, and bioenergy crops are produced from the grass family Poaceae which includes cereal grains, pasture grasses, sugar cane, and bamboos (Chase 2004). Humans gain more than 70% of essential calories from the grasses. The economic value of grasses is difficult to estimate, but the yield of wheat in 2014 alone was valued at over $200 billion globally (http://www.fao.org/statistics/en/). Poaceae are now the most persistent plants populating mountains, rainforests, deserts, and even intertidal coastal regions (Kellogg 2001; Prasad et al. 2005; Dai et al. 2012, 2014). The spread and diversification of grasses began in the understoreys of tropical forests around 65 million years ago (Mya). The adaptability and evolution of faster and exceeding transpiration-efficient stomata in grasses have enhanced during global acidification 30–45 Mya (Kellogg 2001; Franks and Farquhar 2007).

Abiotic stresses, mainly drought and salinity are among the main causes of yield losses in crops worldwide (Vinocur and Altman 2005). In contrast to control of plant resistance to biotic stresses by single genes, the multigenic response to abiotic stresses is complex and thus more difficult to control and manipulate. Hence, both selections for yield and for less complex characters such as stomatal traits should be considered to enhance crop tolerance. Stomata as a barrier for gaseous exchange between the environment and plant cells are subjected to different regulations involving internal (morphological features, genetic factors, epigenetic and hormonal regulation and ion channels) and external (light, CO_2, and humidity) factors (Fig. 1.1). Stomata exposed to different environmental adversities have altered size and density and induce an endogenously triggered signaling pathway, which involves various genes, gene modification and concomitant activation of the related metabolism such as hormone biosynthesis and ion transporters.

Light, CO_2 concentration and humidity play a crucial role in determining how morphological features are established within the leaves and how the internal factors such as specific genes and/or hormones can be triggered. During the past decades, physiological aspects of stomatal characteristics have been widely investigated, mainly in the model plant *Arabidopsis*. Pillitteri and Torii (2012) reported that at least 40 known genes in *Arabidopsis* control stomatal regulation and development. However, the genetic control of stomatal size, density and index that includes the assembly and modification of new leaves under changing environmental conditions in crops is less understood (Hetherington and Woodward 2003; Doheny-Adams et al. 2012). Stomatal traits contribute to the physiological reactions of plants to climate changes and accessibility of water (Gailing et al. 2008). Decreasing stomatal density is correlated with increasing CO_2 over the last century (Woodward and Kelly 1995; Ferris et al. 2002). Discrepancies in photosynthetic demand, surface properties, light penetration and the internal architecture of leaves most likely influence stomatal initiation, allocation and features (Ferris et al. 2002). Application of genetic and genomics-based approaches would identify agronomical desirable alleles present at quantitative trait loci (QTL) that affect stress responses in plants. Therefore, a better understanding of the genetic bases underlying stomatal traits and development in response to harsh

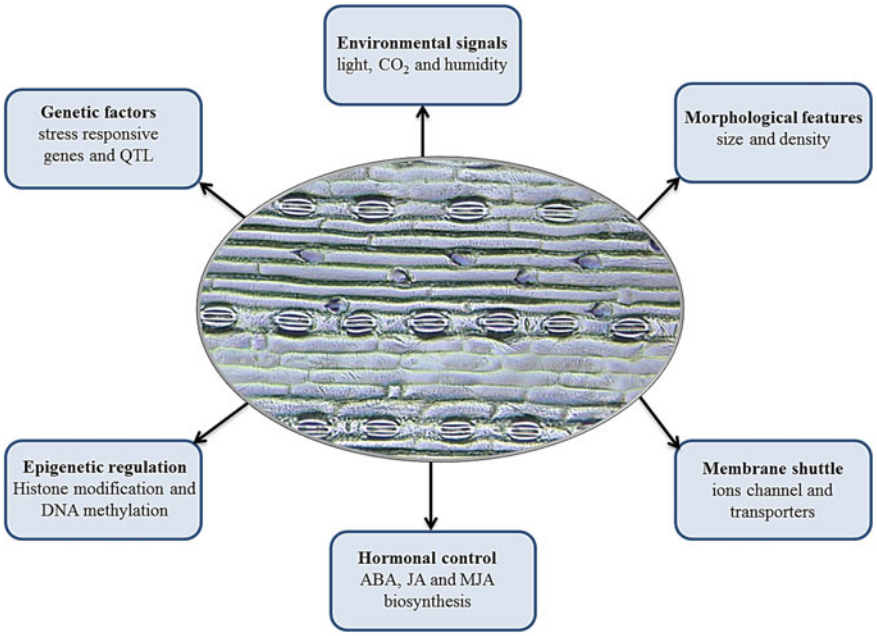

Fig. 1.1 Schematic model for processes regulating stomata during development and under stress. Leaf imprint was taken from the adaxial leaf surface of the RAC875 bread wheat cultivar (Shahinnia et al. 2016)

environmental conditions enable breeders to develop resilient crops more effectively. In this chapter, we addresses the influence of internal processes regulating stomatal functions under abiotic stress conditions and review the progress made in molecular mapping of important stomatal traits and in comparative genomics.

1.2 Stomatal Responses for Stress Tolerance

Grain yield in cereals is determined by the procedure of grain filling and is strictly associated with flag leaf characteristics (Slewinski 2012; Biswal and Kohli 2013). Drought stress predominantly affects flag leaf structure during its development. To select drought tolerance plants, morphological and physiological characteristics of the flag leaf such as superior area, leaf rolling, relative dry weight, delayed senescence, weight loss, carbon and chlorophyll contents, residual transpiration and higher carbon isotope discrimination (CID) have been suggested (Nezhadahmadi et al. 2013). Leaf structural features such as silica and trichomes, stomatal traits, epidermal and bulliform cells are considered to have an important role in controlling water loss and gas exchange damages (Chen et al. 2011; Khazaie et al. 2011; Xu

and Zhou 2008). Water loss through the stomatal pores contributes to 70% of total water usage in plants (Hetherington and Woodward 2003). Stomata regulate 95% of gaseous fluxes between the leaf surface and the environment (Lawson and Blatt 2014).

Both, plant and environment influence the operation of the stomatal aperture and, therefore, both internal and external factors affect stomatal regulation of transpiration. To better cope with temporary dry conditions, stomata must open to allow CO_2 uptake and close during water-stress periods to minimize water loss by leaves (Ainsworth and Rogers 2007). In case of the prolongation of drought period, plants have to complete the growth cycle with a limited amount of water stored in the soil. Under this circumstance, stomata are able to adjust stomatal conductance to enhance CO_2 uptake and transpiration rates for a greater water use efficiency (Kim et al. 2004). Morphological and physiological characteristics such as stomatal size and frequency, stomatal conductance, photosynthesis rate, transpiration, and water use efficiency were suggested to affect grain yields of crops in stressed and non-stressed conditions (Khazaei et al. 2010; Aminian et al. 2011). Venora and Calcagno (1991) demonstrated that stomatal size negatively correlate with water loss in durum wheat, grown under normal conditions. In contrast, in bread wheat, Wang and Clarke (1993) demonstrated a positive correlation between stomatal frequency and the rate of water loss. Higher stomatal frequency has been suggested to be linked with higher water use efficiency and photosynthetic pathways in C_4 plants in comparison to C_3 plants (Hardy et al. 1995b). Leaf stomatal conductance is positively correlated with stomatal density and leaf net CO_2 assimilation rate and increases with temperate drought stress in the grass, *Leymus chinensi* (Xu and Zhou 2008). Water-use efficiency is thoroughly associated with stomatal frequency, through its influence on photosynthesis rate and stomatal conductance. These are among the traits that have been studied in order to use them either for indirect selection for yield or their relationships with other physiological characters. Significant genetic variation for stomatal conductance and photosynthesis rate was found in wheat cultivars, which showed positive correlation with grain yield (Richards 2000). Despite the recognition of the importance of such traits for selecting tolerant plants, little is known about genetic and genomic resources related to stomatal traits, genes and genetic networks that alter the biochemical and physiological pathways, signalling, synthesis, accumulation, transport and efficient use of initial resources in cereals (Biswal and Kohli 2013).

1.3 Evaluation of Stomatal Features

Stomatal guard cells regulate stomatal closing and opening in response to environmental changes. The dumb-bell shape and the kidney shape are two broad types of morphology for guard cells (Hetherington and Woodward 2003). Several stomatal traits such as stomatal pore size, stomatal density, stomatal index and stomatal aperture area can be easily measured. The precision and quickness of evaluating stomatal traits are major obstacles to use those traits in breeding selection (Liu et al.

2014). Two groups of procedures are usually used to visualize stomata, monitoring of replicas, castings of epidermal features or imprints and controlling the fresh or prepared material (Gitz and Baker 2009). Each method has its own unique strengths and weaknesses that must be taken into account depending on the species and the experimental goals. Direct observation techniques include sectioning and fixing fresh leaf materials or teasing the epidermis from the leaf and mounting in buffer solution to view under bright field or fluorescence microscopy. While in impression methods, peels are made by applying a low viscosity plastic or resin such as fingernail polish, silicon rubber, nitrocellulose, vinyl film and cyanoacrylate glue to the leaf surface and letting the liquid to stabilize (Hardy et al. 1995a). The thin film is gently peeled from the leaf surface using a transparent tape, or fine forceps and mounted on a glass slide in order to visualize via bright field microscopy, followed by image analysis using an appropriate image analyser program. The outcome is a stable impression of the epidermis surface for long-term storage (Meister and Bolhàr Nordenkampf 2001). As an alternative, other leaf preparation methods such as air drying, tetramethylsilane air drying, critical point drying and freeze substitution have been proposed for stomatal traits evaluation and proceed further by scanning electron microscopy (SEM) (Hardy et al. 1995a).

Apart from the morphological traits, more recently, chlorophyll fluorescence and thermal imaging have been proposed as techniques to assess stomatal responsiveness and speed, concurrently with photosynthesis. It is ideal for phenotyping plants with no damage in carbon assimilation (McAusland et al. 2013).

Plant phenotyping methods can be complemented with the molecular and genetic technical advances, for quick and applied screening of plants with desired stomatal characteristics.

1.4 Mapping of QTL for Stomatal Traits

A QTL is a location on the genome, genetically associated with variation in the phenotypes of a quantitative trait. Chromosomal location, closely linked markers, estimated additive allelic effects and percentage of phenotypic variance for stomatal traits can be explored through QTL mapping in a bi-parental mapping population (Pinto et al. 2010; Shahinnia et al. 2009). The advent and development of molecular markers in quantitative genetics significantly eases exploration of complex quantitatively inherited traits. Construction of high density genetic linkage maps for cereals has made it possible to detect the poly genes for such traits into individual Mendelian factors (Shahinnia et al. 2006). Dissected regions can be used in marker-assisted selection through fine mapping of the identified QTL controlling favourite traits (Pinto et al. 2010). Genetic and phenotypic variation in stomatal traits has been identified (Gailing et al. 2008; Khazaei et al. 2010; Laza et al. 2010); however; the genetic mechanisms for these traits remain unknown. In poplar, genetic variation and QTL were found for stomatal size, initiation, density and epidermal cell number which delivered initial

evidence that leaf stomatal and cell traits can be detected by QTL analysis (Ferris et al. 2002).

In cereals, three QTLs for stomatal density were identified on chromosomes 1, 3 and 7 using 100 lines of F_2 population from the cross between two *Hordeum chilense* accessions. Two QTLs on chromosome 3 overlapped with a QTL that was assigned for avoidance of leaf rust. Further, 101 recombinant inbred lines (RILs) have been developed through a cross between Indica rice and a tropical Japonica varieties (Laza et al. (2010). Under normal field conditions, they identified ten QTLs for stomatal density and four QTLs for size on chromosomes 1, 2, 3, 4, 6 and 10 across vegetative stage, heading time and leaf adaxial and abaxial surfaces. Each QTL explained 9.7–14.3% of total phenotypic variation for stomatal size and 9.3–15.2% for density. Different allelic effects of parental lines were detected dependent on growth stage in lowland rice. A crucial aspect of adaptation to salinity stress in barely is dedicated to genetic control of stomata regulation (Chen et al. 2005, 2007a, b; Munns and Tester 2008; Munns et al. 2010). Genotypic variation for stomatal behaviour were studuied in barley cultivars using four experimental trials (Liu et al. 2014, 2017). Treating salt-tolerant CM72 and salt-sensitive Gairdner with 200 mM Sodium chloride revealed significant differences for stomatal characteristics like stomatal aperture width and aperture width/length as well as guard cell volume. Genotyping of 108 double haploid (DH) lines obtained from a cross between the parental lines was done with Diversity Array Technology (DArT) and Simple Sequence Repeats (SSR) markers. The QTL QSA-T.CmGa.1H for stomatal area was located in the interval of DArT markers bPb-9081 on chromosome 1H (Liu et al. 2017). The association between grain yield, stomatal traits and slow anion channel genes for improving salinity tolerance was investigated in barley by Liu et al. (2014). They found one QTL for relative stomatal aperture width/length on chromosome 3H. This QTL overlapped with the QTL for salinity tolerance. This trait exhibited significant correlation with relative biomass in a DH population of barley. Panio et al. (2013) using 161 F8-F9 RILs, obtained from a cross between two durum wheat cultivars, detected one QTL for stomatal-conductance on chromosome 7A, explaining 12.8% of phenotypic variation under irrigated conditions in the field. Using 144 DH lines derived from a cross between RAC875 (drought tolerant) and Kukri (drought sensitive) Australian bread wheat cultivars, 21 important QTLs were identified for stomatal traits and yield in low rainfall environments (Shahinnia et al. 2016). The QTLs for stomatal density and size-related traits were found to be located on chromosomes 1A, 1B, 2B, and 7A in both field and controlled conditions. Remarkably, some of these loci overlapped with QTL on chromosome 7A that controlled kernel number per spike, normalized difference vegetation index, harvest index and yield in the same population (Bennett et al. 2012a, b). The RAC875 drought tolerant parental line showed numerous and smaller stomata in comparison to Kukri, under field- and controlled-conditions (Shahinnia et al. 2016).

1.5 Hormonal Signalling Pathway and the Effect of ABA on Stomatal Closure

Stomatal complexes, as a regulatory site of atmospheric CO_2 uptake and of transpiration, contain important specialized cells that are controlled by external CO_2, hormonal stimulant and environmental conditions. Recently, the interaction and role of the hormones in response to abiotic and biotic stress has been summarized in the model plant *Arabidopsis* and a few other crop plants (Acharya and Assmann 2009; Raghavendra et al. 2010; Araújo et al. 2011; Zhu et al. 2012a; Misra et al. 2015). Abscisic acid (ABA), a terpenoid derived from carotinoid, serves as a unique stomatal regulator that causes stomatal closure and opening through a complex regulatory network (Umezawa et al. 2010). Further, ABA receptor was supposed to associate with Mg-chelatase-H-subunit and act as a positive regulator in seed germination, post-germination growth and stomatal movement in *Arabidopsis* (Shen et al. 2006). ABA signalling includes the activation of ion channels via SLAC1 (a guard cell anion transporter) in conjunction with OST1 (a protein kinase, Open Stomata1) as positive regulator of stomatal closure and the type 2C protein phosphatases (PP2C) ABI1 and ABI2 as negative regulators (Geiger et al. 2009; Raghavendra et al. 2010; Araújo et al. 2011), the involvement of reactive oxygen species (ROS), cytosolic calcium concentration and pH changes (Zhu et al. 2012b). Further regulatory components were found through the studies with a synthetic growth inhibitor pyrabactin, which is functioning through PYrabactin Resistance1 (PYR1) and Pyr1-Like proteins (PYL) and is required for ABA signaling in vivo. ABA binds to PYR1, which in turn inactivates PP2C proteins indicating that the PYR/PYL/RCAR proteins are in charge of the inhibition of the PP2C proteins (Kim et al. 2010). PP2C proteins in turn inactivate SnRK2s kinases through dephosphorylation. In general, in the presence and absence of ABA, PYLs modifies the conformation of PP2C proteins and inhibit their activity and bring SnRK2s into action (Zhang et al. 2015).

Besides ABA, additional hormones showed distinct functions in stomatal regulation including auxin, cytokinins, ethylene, gibberellins, jasmonates, salicylic acid, strigolactones and brassinosteroids (Acharya and Assmann 2009; Misra et al. 2015). Interestingly, in *Vicia faba*, cytokinins appear to exert their function through the reduction of hydrogen peroxide, which has been shown repeatedly to act as a stress indicator, whereas auxin prevents hydrogen peroxide generation and thus induces stomatal opening in darkness (Song et al. 2006). Using genetic studies with *Arabidopsis thaliana* mutants, jasmonate (JA) and methyljasmonate (MJA) have been shown to share several characteristic signalling components with ABA and induce stomata closure in various species (Munemasa et al. 2011). Although several signalling components for ABA and JA such as calcium involvement, ROS production, protein phosphorylation and modulation of ion channels are similar, JA and/or MeJA cannot prevent or replace the ABA signalling mechanisms, for instance under drought stress (Murata et al. 2015). Salicylic acid (SA), a known pathogen-related hormone appears to also play a role in stomatal closure in which SA induces the production of intercellular ROS and inactivates the plasma membrane potassium channels. Further-

more, ethylene as a gaseous plant hormone was supposed to induce stomatal closure in *Arabidopsis* in a ROS-dependent way mediated by the NAD(P)H oxidase AtRBOHF (Desikan et al. 2006). However, due to contrasting published data in which ethylene acted in different ways on stomatal regulation by promotion of stomatal closure in *Arachis hypogea* (Pallas and Kays 1982) and *Arabidopsis thaliana* (Desikan et al. 2006) or induction of stomatal opening in *Vicia faba* (Levitt et al. 1987) and *Dianthus caryophyllus* and S*olanum lycopersicum* (Madhavan et al. 1983), the function of ethylene in stomatal regulation appears to be dependent on environmental conditions. A direct function of other hormones including gibberellin, strigolactones and brassinosteroids in stomatal regulation has not been implicated yet.

However, these hormones may have an indirect regulatory function in stomatal movement (Acharya and Assmann 2009; Daszkowska-Golec and Szarejko 2013). To date, most studies on stomatal movement were carried out with the model plant *Arabidopsis* or a few crop plants such as *V. faba*. Similar mechanisms are expected in cereals, however, recent studies emphasized that regulatory responses can be influenced by various environmental adversities (Mori and Murata 2011; Merilo et al. 2014). Chen et al. (2013) demonstrated a partial recovery of ABA- or soil drying-induced stomatal closure of older leaves in wheat initiated by the ethylene receptor antagonist, 1-methylcyclopropane, or by inoculation with the rhizobacterium *Variovoray paradoxus* 5C-2. This study showed clearly that the relative sensitivity of stomatal closure to ABA and dry soil is likely due to modified stomatal sensitivity to ethylene and not to increased ethylene synthesis. In addition, Shen et al. (2015) used epidermal peel assays from wheat, barley and *Brachypodium* and showed that stomatal closure in response to ABA and CO_2 was similar to that reported for non-graminacious model plants. Recently, foliar application of different barley genotypes with MeJA under limited water regimes was reported to result in an additional increase of ABA concentration but without any effect on auxin concentration (Pazirandeh et al. 2015).

Altogether, the signalling network in the guard cells of graminaceous species might share some similarities to that of model species. However, whether the signalling components and the interaction for different hormones during stress, for instance drought, are homogenously distributed among graminaceous and non-graminaceous plants is a matter of further investigations. Indeed, this would lead to the identification of genetic determinants and open future strategies to improve water use efficiency and pathogen invasion of cereal plants and thus enhance yield capacity influenced by climate change.

1.6 Complex Cereal Stomata Are Better Designed for Abiotic Stress Response

Stomata of cereals are complex structure formed by two dumb-bell shaped guard cells and by two subsidiary cells (Pallaghy 1971; Raschke and Fellows 1971). Subsidiary

cells are specialised to provide the guard cells with K^+ and anions during stomatal opening and removal of these ions during stomatal closure. The closure of wheat (*Triticum aestivum*) stomata is magnificently faster than other species (Franks and Farquhar 2007). Light-induced stomatal opening occurred within 30 min in barley (Koers et al. 2011) as compared to tobacco, wherein it took more than 2 h (Kollist et al. 2014). In grasses, large and fast modifications in stomatal conductance and aperture is linked to the "Shuttle Ion Transport" between guard and subsidiary cells within the stomatal complex and the existence of a concerted membrane transport system (Mumm et al. 2011; Raschke and Fellows 1971).

1.7 Membrane Transporters for Cereal Stomatal Function

Several studies have been already performed to investigate the stomatal membrane transporters in *Vicia faba* and *Arabidopsis*, but they are less understood in cereals such as maize, rice or barley (Chen et al. 2012; Hills et al. 2012; Wang et al. 2012). Most of these ion transporters in stomata are potential targets of candidate genes for improving abiotic stress tolerance in cereals (Schroeder 2013). Furthermore, potassium channels activated by hyperpolarization or depolarisation have been characterized in both guard cells and subsidiary cells of maize (Majore et al. 2002; Mumm et al. 2011; Wolf et al. 2006). Interestingly, Buchsenschutz et al. (2005) showed that transcripts for *ZORK*, responsible for potassium release, was present in subsidiary and guard cells of maize that are regulated differently by the cytosolic pH.

Membrane potential and calcium play a crucial role in regulation of maize potassium channels in both cell types (Majore et al. 2002; Philippar et al. 2003; Wolf et al. 2005; Buchsenschutz et al. 2005). Still, a non-selective maize cation channel type, called MgC, is activated rapidly upon membrane depolarization in subsidiary and guard cells. It was shown that abscisic acid had no influence on the MgC channels but differentially regulated the time-dependent K^+ release via *ZORK*. Thus, an antiparallel-directed potassium transport between subsidiary and guard cells is suggested to drive stomatal movements in maize and potentially many other cereals (Wolf et al. 2005, 2006).

Voltage-independent slow anion channels (SLAC/SLAH) and aluminium activated malate transporter (ALMT) are known in guard cells and subsidiary cell of cereals. ZmSLACs were identified in both cell types and were shown to be dependent on cytosolic Ca^{2+} and pH. Stomatal closure was initiated by hyperpolarisation and cytosolic acidification of subsidiary cells, which; however, resulted in reverse responses during stomatal opening (Mumm et al. 2011). Furthermore, *ZmALMT12* is expressed in guard cells that transport malate in an aluminum-insensitive and highly voltage-dependent manner. In addition, powdery mildew (*Blumeria graminis*) stimulates S-type anion channels in barley (*Hordeum vulgare*) whereas stomatal guard cells mediate anions efflux for stomatal closure (Koers et al. 2011). *HvSLAC1* and *HvSLAH3* are the responsible genes coding for mentioned channels (Liu et al. 2014). The kinetic properties of pumps and co-transporters are less studied in grass stomata.

One of the few examples is the H^+-ATPase of maize that is localised on the plasma-membrane of stomatal guard cells. The H^+-ATPase enrichment in guard cells is relevant to active ion transport during stomata opening (Villalba et al. 1991). In addition, proteins designated as ATP-binding cassette (ABC), were supposed to be involved in the membrane transport of various molecules (Verrier et al. 2008; Kang et al. 2010; Kuromori et al. 2010). In maize, ABC transporters ZmMRP3 and ZmMRP4 are targeted to the tonoplast, co-regulating the anthocyanin pathway (Goodman et al. 2004). However, there is limited evidence for a role of ABC transporters in stomatal regulation in grasses.

1.8 Comparative Genomics for Stomatal Traits in Cereals

The genome sequencing has revolutionized plant breeding techniques for global sustainable agriculture. The availability of complete genome assemblies of major cereal crops and their wild relatives has led to the discovery of genes for key agronomy and stress tolerance traits (Schroeder 2013). Stomatal membrane transporter genes are candidates for bioinformatics probing across plant species. Based on the known *Arabidopsis* genes regulating stomatal guard cell response to ABA, we obtained over ten thousand gene sequences and their predicted protein sequences from the sequenced genomes of 26 plant and algae species. Among those, 5,126 are potential transporters belonging to 24 protein families (Chen et al. 2017). In five major cereal crops, *Triticum aestivum*, *Oryza sativa*, *Zea mays*, *Sorghum bicolor*, and *Hordeum vulgare*, there were, on an average, 236 predicted stomatal transporter proteins as compared to 174 in *Arabidopsis* (Cai et al. 2017; Chen et al. 2017). This demonstrated that the key stomatal membrane transporters are highly conserved and are present in large numbers in cereals. Comparative genomics provides an exciting way to evaluate the membrane transporters governing stomatal opening and closure in cereals. Along with the maker assisted selection, the genomic analysis will assist the identification of key genes encoding stomatal traits for abiotic stress tolerance such as salinity tolerance (Liu et al. 2017) in cereals. Further research is required to compare the function of these transporters and their roles in abiotic stress tolerance.

1.9 Epigenetic Control of Stomata

Genetic control of stomatal traits, mediated by transporter and hormonal control of function, is not the whole story of regulation in the genome. Recent evidence has shown that an additional layer of regulation, the epigenome, is involved in both stomatal development and functioning. This is especially important when considering the interaction between genotype and environment as there is evidence that the environment and abiotic stress in particular, may influence stomata through epigenetic regulation. Abiotic stress leads to transcriptional reprogramming during guard

cell development (reviewed in Simmons and Bergmann 2016) and stomatal closure (Ma et al. 2009). Relaxed or repressed transcriptional states are defined by the 'open-ness' of chromatin, the matrix in which the genome is packaged, which may be regulated epigenetically by modifications to histones or by methylation of DNA (Bell et al. 2011). These epigenetic modifications may also persist to provide an epigenetic memory of previously experienced stress, and may therefore be responsible for priming plants to alter their responses to stress (reviewed in Bruce et al. 2007; Conrath 2011).

The fundamental unit of organized chromatin is the nucleosome where DNA is wrapped around a histone octamer consisting of two copies each of the histones H2A, H2B, H3 and H4 and further organized into arrays associated with the linker histone H1. Histone tails are subject to non-covalent modification by epigenetic marks such as acetylation, phosphorylation, dimethylation and ubiquitination that activate transcription, and biotinylation, sumoylation and trimethylation that repress transcription (Berger 2007). Together, these modifications combine to create four chromatin states that are the signatures of, respectively, active genes, repressed genes, silent repeat elements and intergenic regions (Roudier et al. 2011).

ABA production in response to abiotic stresses induces chromatin remodelling by the modification of histone tails and by altering the balance of histone linker H1 variants (Scippa et al. 2004; Sridha and Wu 2006; Rutowicz et al. 2015). Rutowicz et al. (2015) demonstrated that the linker variant H1.3 is found in a guard cell-specific pool and is required for stomatal functioning in *Arabidopsis thaliana*. Increased extracellular calcium (Ca^{2+}) mediates stomatal closure through the calcium signalling gene *CAS* and is epigenetically regulated by the histone methylase CAU1, thus altering stomatal closure and drought tolerance (Fu et al. 2013). Additional histone modifications have been observed in response to ABA, water and salt stress and in the phenotypic and developmental responses to these stresses (reviewed in Han and Wagner 2014). To unravel epigenetic cause from effect and determine the influence of the histone code at genetic loci is not trivial. Quantitative genetic approaches that rely on identifiable DNA polymorphisms may need to be combined with the use of inducible loss-of-function mutants, fine-scale analysis of chromatin dynamics and the separation of different histone: chromatin states (Han and Wagner 2014).

Epigenetic modifications also affect stomatal development and thus regulate stomatal density and index (the proportion of epidermal cells forming stomatal guard cells). In addition to its role in stomatal functioning, histone H1.3 variant affects the expression of guard cell-specific genes including the master regulators of the guard cell lineage *SPEECHLESS* (*SPCH*), *MUTE*, *ERECTA*-family/*TMM* genes and the mitogen-activated protein kinase MKK9 (Rutowicz et al. 2015) correlated with the decrease in stomatal density in the *h1.3* mutant. Disruption of trimethylation of lysine 27 on H3 causes the Stoma-in-Stoma (SIS) phenotype where new stomata are formed within the shells of the old (Lee et al. 2014). Remarkably, Lee et al. (2014) demonstrated that stomatal cell fate was stabilized by epigenetic repression of stem cell genes by the chromatin-modifying Polycomb Repressive Complex 2 and that differentiation could be reprogrammed. H3K27 trimethylation and the SIS phenotype were also induced in transgenic *FOUR LIPS* when a transgene of the final, differen-

tiating gene in the guard cell lineage *FAMA* was expressed under its own promoter, *FAMA*^{trans}. The connections between the beginning and end of the stomatal lineage and how epigenetic regulation is involved in programming and differentiation are now being unravelled (Torii 2015).

Environmental signals regulate stomatal development through the transcriptional and post-transcriptional control of *SPCH*, the master transcription factor that determines entry into, and perpetuation within, the stomatal lineage (reviewed in Simmons and Bergmann 2016). The expression of both *SPCH* and *FAMA* is inversely correlated with increased DNA methylation around the loci in response to a low humidity environment, controlled by short-interfering, non-coding RNAs (Tricker et al. 2012). In the *ros1* demethylase mutant, where the promoter of the peptide ligand *EPF2* gene is not actively demethylated, stomatal lineage cells proliferate so that active DNA demethylation combats the action of RNA-directed DNA methylation controlling *SPCH* (Yamamuro et al. 2014).

Epigenetic modifications may persist and have a role in priming plants for renewed exposure to stress (reviewed in Kinoshita and Seki 2014). Ding et al. (2012) showed that the transcription of *Arabidopsis* stress-responsive genes was altered during multiple exposures to dehydration stress, and recovery was correlated with H3K4 methylation so that plants were effectively 'trained' by previous exposure. More recently, Virlouvet and Fromm (2015) demonstrated ABA-dependent, guard cell-specific transcriptional memory. DNA methylation and the low stomatal index phenotypes induced by low relative humidity persist at the *SPCH* locus and prime plants for increased tolerance to subsequent drought (Tricker et al. 2013a). Remarkably, both DNA methylation and the phenotype persist through at least one generation, but are reversed by exposure to the same stress (Tricker et al. 2013b) suggesting an adaptive, epigenetic 'memory' passed from parent to progeny that escapes reprogramming.

Although regulation by the epigenome in response to abiotic stress is complex, it may provide us with an additional opportunity to select for quantitative traits using quantitative (epi) genetics. In epigenetic recombinant inbred line populations (epiRILs), the control of stress tolerance by DNA methylation is demonstrably heritable and amenable to selection at epiQTL (Cortijo et al. 2014; Kooke et al. 2015; Zhang et al. 2013). The epigenetic regulation of stomatal traits, in particular via DNA de/methylation, with measurable phenotypes, suggests that selection at epiQTL will increase the pool of variation beyond DNA sequence-based variation and may have the additional benefit of pump-priming adaptation (Tricker 2015).

1.10 Genetic Manipulation of Stomatal Traits

Genetic engineering of stomatal size, density and patterning are among the approaches for improving water use efficiency in cereals. The major challenge to achieve this goal is preventing concession of carbon gain when stomata regulate CO_2 access to the photosynthetic tissues of the leaf (Lawson et al. 2012).

Interestingly, smaller stomata show a faster response than larger stomata (Hetherington and Woodward 2003). It was shown that larger stomata often display slower responses to stress conditions, since the guard cell size and geometry affect the speed of stomatal movements. Engineering of stomatal mechanics and guard cell characteristics can lead to fine-tuning of the stomatal response or sensitivity to environmental changes. Also, gaseous conductance of stomata per unit of leaf area can be modified by altering stomatal densities (Lawson and Blatt 2014).

Engineering stomatal signalling and metabolism will affect stomatal function in response to stress as well as manipulating stomatal anatomy, patterning and speed. For example, overexpression of maize (*Zea mays*) NAD-malic enzyme in tobacco resulted in plants with a decreased stomatal conductance but increases in biomass per unit of water used, suggesting that modification of both stomata and mesophyll processes could enhance plant water use efficiency (Laporte et al. 2002).

Although it is possible to engineer stomatal characteristics, it is essential to recognise possible interactions between other traits in this chain. Reprogramming of stomatal function should not make the plants more susceptible to environmental limitations. Such approaches may be dependent on the type of stress and differences in stomatal behaviour in different species, plant water status and leaf age. Progress to these ends can be achieved from combinations of physiological and molecular genetic methods together with quantitative systems analysis. This will also benefit from supplementary evidence about the quantitative kinetics and signal transduction pathways in plants (reviewed in Lawson and Blatt 2014).

1.11 Evaluation of Stomatal Traits for Indirect Selection of Abiotic Stress Tolerant Crops

The enhancement of abiotic stress resilience in cereals by traditional breeding is challenging due to the complex inheritance and multigenic control of this trait (Vinocur and Altman 2005). Direct selection for grain yield and biomass under abiotic stress is often ineffective because of the low heritability especially in early segregating generations. In addition, grain yield and biomass are complex traits for which gene × gene and gene × environment interactions create major restrictions for molecular breeding and identification of QTL with major and stable effects (Panio et al. 2013). One way to elevate the efficiency of selection for abiotic stress tolerance is by indirect selection for other traits that are genetically correlated and give early yield prediction in breeding programmes (Dillen et al. 2008).

Stomatal traits reflect micro-morphology and cell physiology and are very promising traits for identification of genetic variation and improvement of biomass and yield under abiotic stresses (Marron et al. 2007; Panio et al. 2013). Assessment of the degree of genetic variation and mapping of chromosomal regions controlling these traits are essential for the development of breeding strategies to increase stress tolerance in cereals. Dissecting common QTL controlling stomatal traits in association

with yield indicates that stomatal traits can be an underlying mechanism increasing yield at specific loci and used as an alternative to elucidate a target QTL (Shahinnia et al. 2016). This could eventually facilitate the understanding of the function of these loci, identifying candidate genes and accelerating positional cloning of yield QTL.

1.12 Conclusions

Improving water use efficiency and resilience of crops to the contrary effects of climate change is an important topic in research and the scientific agenda. Stomata play a significant role in reducing water loss and increasing photosynthesis rate that can be subjected for manipulation, aimed at increasing stress tolerance. In the post-genomic era, identification of genes responsible for stomatal behaviour in response to fluctuating environmental conditions will be feasible using quantitative genetic approaches in combination with next generation sequencing (NGS), RNA-sequencing data and high-throughput non-invasive phenotyping platforms. In conjunction with other 'omics' approaches such as transcriptomics, metabolomics and proteomics, the knowledge-base of stomatal characteristics and behaviour is growing with the goal of improving crop productivity and yield in the ever changing climate regimes.

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Chapter 2
Quantitative Trait Loci Mapping of Heavy Metal Accumulation and Resistance in Crop Plants

Meetu Gupta and Afsana Praveen

Abstract Industrial revolution and anthropogenic activities have enhanced the spread of many heavy metals to different environmental sites from the earths' crust. Environmental pollution caused by toxic heavy metals is a major threat to human health. There are different sources by which plants get exposure to heavy metals, such as fertilizer and pesticides application in fields, mining industries and groundwater. Heavy metals induce damages in plants at physiological, biochemical and molecular level, either directly or indirectly by generation of reactive oxygen species or free radicals. In order to reduce the toxicity of the these heavy metals in plants different strategies can be used, either by application of specific fertilizer, selection of heavy metals tolerant plants or through genetic engineering. Analyzing the genetic and molecular mechanisms that are involved in heavy metals tolerance is expected to enhance the development of heavy metals tolerant plants, and mapping of quantitative trait locus (QTL) associated with their accumulation and resistance is helpful to improve the heavy metal resistance in plants. A QTL that is responsible for controlling heavy metals resistance in plants can be used for marker-assisted selection in selecting low heavy metal content plants or tolerant plants in a breeding program. In this chapter, we focus on mapping QTL for the selection of agronomic traits for improving the heavy metals resistance in breeding program.

Keywords Heavy metals · QTL mapping · Marker assisted selection · Molecular marker · Resistance

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2.1 Introduction

In nature, plants are exposed to different environmental stresses, including both biotic and abiotic. Heavy metals, which include some essential (e.g. Fe, Mn, Zn, Cu, Mg, Mo, Ni, etc.) and non-essential (e.g. As, Hg, Cr, Pb, Ag, etc.) metals, are toxic substances, which are released into the environment and contribute to a variety of toxic effects on living organisms in food chain by its accumulation and biomagnifications. These metals adversely affect crop productivity and growth by affecting physiological, biochemical and molecular responses (Gill 2014). Heavy metals are defined as metals or metalloid having higher density than that of water and are toxic even at low concentration. They eventually accumulate to levels that could harm physiochemical properties of soils and lead to loss of soil fertility and crop yield. Various anthropogenic and geological activities such as addition of pesticides and fertilizers in agricultural field, fossil fuels burning, mining and smelting of metals, industrial production of batteries and other products of metal, sludge and disposal of municipal and sewage waste etc. increase the concentration of heavy metals to a level that are harmful to both animal and plants (Alloway 1990; Raskin et al. 1994; Shen et al. 2002). Accumulation and bioavailability of heavy metal in plants depends on the biology of plant species. Certain pollutants such as arsenic (As) remain in the environment for an extensive period. Arsenic poisoning is mainly found in the regions of South America, Asia, and densely populated flood plains and deltas of South and Southeast Asia, due to consumption of As contaminated drinking water (Brammer and Ravenscroft 2009). In India, groundwater As contamination was first reported in West Bengal in 1983. A number of other states have been exposed to As contaminated water above permissive limit of 50 μg/L from the use of hand tube well. These states are Bihar, Jharkhand, Uttar Pradesh adjacent to the Ganga River, Rajnandgaon village in Chhattisgarh state, Assam and Manipur near Brahamaputra and Imphal rivers (Ghosh and Singh 2001). Lead (Pb) is also found along with other heavy metals, such as Cd, Zn, and it is a major concern due to its extensive distribution which affects human health and causes environmental pollution (Hernandez-Allica et al. 2007). Cadmium (Cd), a highly toxic heavy metal is readily taken up and accumulated by plants due to its high mobility and water solubility properties (Gallego et al. 2012). Copper (Cu) ion is an essential element and is a structural component of many proteins and enzymes that play an important role in growth processes, such as mitochondrial respiration, photosynthesis, cell wall metabolism, mineral nutrition and hormone signaling pathway (Costa et al. 1994; Muccifora 2007). Zinc (Zn) plays a vital role in plant metabolic processes such as metabolism of carbohydrates, protein synthesis and enzyme activation (Cakmak 2000).

The immobile nature of plants enables them to develop unique mechanisms to cope with different stress factors, for example, via altering their physiology and metabolic mechanisms, changes in gene expressions and/or developmental activities. However, variations do exist in tolerance mechanisms in plants. Various specific mechanisms that exist in plants for avoiding or tolerating heavy metal stress include development of mycorrhizas, which restricts metals movement into the root cells, binding of

metals to cell-wall and root exudates, reduced influx of metal ions through plasma membrane, active efflux of metal ions that have entered into the cell, chelation of metals in the cytosol by various ligands like organic acids, amino-acids or peptides, repair and protection of plasma membrane by different proteins and metallothioneins and compartmentalization of chelated products through transporting them into the vacuole (Hossain et al. 2012). Assessment of environmental condition on ecology at molecular and population levels are important in risk quantification and remediation studies. Genetic ecotoxicology is a multifaceted discipline that examines the effects of toxic compounds on the structure and function of DNA. It also includes study of somatic and population genetic effects. Integration of these two approaches i.e. somatic and population genetics would be advantageous to see the changes in population genetic structure and DNA damage, the frequencies of alleles and other genetic markers that differ between genotoxicant-contaminated and reference populations and genetic analysis of gene flow, which may provide insight into patterns of dispersal in contaminated and reference populations. Only a limited number of DNA-based genetic marker studies have been reported in plants in the literature regarding this.

Many quantitative traits of economic value are under polygenic control and are selected for, directly. Such a selection is often ineffective, since the effect of each gene is small, which is also influenced by the environment. Therefore, there should be a procedure for indirect selection, which is not influenced by the environment. In this regard, identification of linkage between genes for quantitative traits and marker loci can lead to significant improvement. Quantitative trait loci (QTL) are regions of DNA that contain genes affecting a particular quantitative trait. Identification of QTL is done by correlating the trait variation with that of genetic variation, and a significant correlation between phenotype and genotype identified at a QTL suggests that particular QTL can be helpful for determining trait expression (Frerot et al. 2010). QTL mapping based on high-density molecular linkage map is used for understanding the genetic mechanism behind phenotypic complexity. QTL mapping is a powerful genetic tool, that is used for identification of number, position and effects of genetic factors involved in phenotypic variation (An et al. 2006). In this chapter, we focus on different mechanism of heavy metals toxicity, uptake, transport and finally different strategies for heavy metals resistance, and QTL mapping for the selection of agronomic traits for improving the heavy metals resistance in breeding program.

2.2 Heavy Metal Toxicity in Plants

Many physiological and metabolic changes occur in plants upon exposure to high concentration of heavy metals with varying degree of toxicity (Dubey 2011; Villiers et al. 2011). Reduction in plant growth occurs due to heavy metal toxicity, which is identified by symptoms such as leaf chlorosis, necrosis, decrease in seed germination, loss of turgidity, damage of photosynthetic apparatus leading to progressing senescence, and cell death (Carrier et al. 2003; Dalcorso et al. 2010). Heavy metal

exposure causes changes at ultra-structural, biochemical and molecular level in plant cell and tissues (Gamalero et al. 2009). Heavy metals contamination in agricultural soil has become an environmental concern, as they are widely distributed in soil and having adverse toxic effects in plants (Gill 2014). At high concentration of heavy metals, toxicity symptoms become apparent in plants, which are mainly due to cellular level interactions (Hall 2002) such as binding of metals to sulfydryl group of proteins, disrupting the structure and leading to inhibition of its activity (Van Assche and Clitjers 1990). Plants that grow in Cd rich soil have shown cells or tissue injury symptoms in the form of inhibition in growth, chlorosis of leaves, root tips browning and ultimately leading to death (Guo et al. 2008). Previous reports showed that Cd interferes with other metals (e.g. Mg, P, K, Ca) uptake, transport and use by plants. It also reduces the absorption and transport of nitrate from roots to shoots by inhibiting the activity of nitrate reductase in the shoots (Hernandez et al. 1996; Das et al. 1997). In roots of sunflower and wheat, Cd toxicity caused reduction in ATPase activity (present in plasma membrane fraction) and lipid peroxidation (Fodor et al. 1995). Inhibition of chlorophyll biosynthesis and reduction in enzymes activity involved in CO_2 fixation have also been reported due to Cd toxicity (Raziuddin et al. 2011). As toxicity leads to disruption of various physiological and biochemical processes (Li et al. 2006; Talukdar 2011). For example, it leads to the generation of reactive oxygen species (ROS) through conversion of arsenate to arsenite, and induces oxidative stress resulting from cellular damages in terms of enhancement of lipid peroxidation, membrane leakage and ROS accumulation (Mascher et al. 2002). As toxicity in plants is also noticeable by low biomass production (Singh et al. 2007). Pb, a toxic heavy metal alters the overall plant growth, reduces or inhibits photosynthesis (Tian et al. 2014). Other toxicity symptoms include decrease in leaf biomass, leaf size, blade thickness, disintegration of chloroplast, alteration in protein, lipids and nucleic acid (Islam et al. 2008; Pena et al. 2008; Ali et al. 2014). Zn is an essential element but excess amount of it causes nutrient imbalance, leaf chlorosis and photosynthesis inhibition resulting in plant growth damage (Street et al. 2007). Toxicity of Al is a major constraint to crops productivity in acidic soils, and it is solubilized into the soil solution in the highly phytotoxic form as Al^{3+}. This form of Al causes root growth inhibition leading to reduction in root system thus affecting the plant's ability to uptake nutrients and water (Uexkull and Mutert 1995; Adam et al. 2011). In trace amount, Cu is an essential element for plants, but at a higher concentration, it can be toxic. It is found in protein bound form in cells, because in free form it generates oxidative stress causing damage to organic molecules. Cu ion in free form readily oxidizes the thiol bond of proteins and causes disruption of their secondary structure (Brian and Lebrun 1999). Mercury (Hg), can exist in different forms such as HgS, $Hg^{2+,}$ Hg and methyl-Hg, but in agricultural soil Hg^{2+} form is found more frequently (Han et al. 2006). It has been reported that Hg^{2+}readily accumulates in aquatic and higher plants, and its high level is phytotoxic to plant cells (Israr et al. 2006). Toxicity symptoms of Hg^{2+}include visible injury and physiological disorders in plants such as closing of leaf stomata, generation of oxidative stress and disruption of lipids (Zhang and Tyerman 1999; Cargnelutti et al. 2006; Zhou et al. 2007). Figure 2.1 shows the mechanisms of heavy metal toxicity in higher plants.

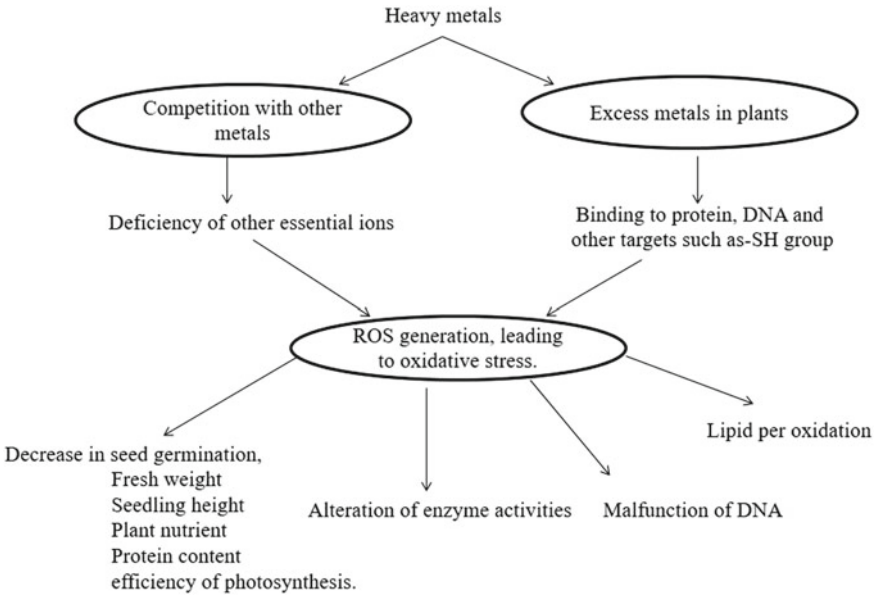

Fig. 2.1 Mechanism of heavy metal toxicity in higher plants

2.3 Heavy Metal Uptake and Transport in Plants

Plants uptake some heavy metals and transport to different tissues through various specific mechanisms, while other heavy metals uptake occurs as a passive process. For example, Cd is a non-essential metal ion, and there is no particular mechanism of uptake for it. Cd and other non-essential metal ions enter into plant cells using a system, which is for essential cations. It has been reported that member of ZIP family metal transporters (ZRT1/IRT 1 like proteins) represent main Fe uptake system in the roots of *Arabidopsis thaliana* (Vert et al. 2002). Previous work on *Saccaharomyces cerevisiae* expressing IRT 1, showed that this gene also contributes in uptake of Zn^{2+}, Mn^{2+}, Co^{2+} and Cd^{2+} (Korshunova et al. 1999). Cu is transported in plant cells through P-type ATPase Cu transporter, a subgroup of the large superfamily of P-type ATPase. Across biological membranes, they consume ATP to pump a range of charged substrates (Palmgren and Axelson 1998). Grotz et al. (1998) cloned four Zn transporter genes (*ZIP1*, *ZIP2*, *ZIP3* and *ZIP4*), expressed in *A. thaliana* and found that in response to Zn deficiency, *ZIP1* and *ZIP3* expressed in roots, which suggested that they were involved in the transport of Zn from the soil into plants. In Zn limited plants, *ZIP 4* expressed in both shoots and roots suggesting that *ZIP4* might be involved in intracellular Zn transport and/or between tissues. Earlier work reported that in higher plants, arsenite is taken up by nodulin2,6-like intrinsic proteins (NIPs) and arsenate by phosphate transporter, whereas other metals such as sulfur, silicon and phosphorus interact with As during its uptake from soil to plants (Zhao et al. 2010).

2.4 QTL Mapping for Improving the Heavy Metals Resistance in Crop Plants

Recently, QTLs have been reported in various crops for heavy metals resistance which can be used for marker-assisted selection (MAS). It has been reported that in soyabean, a cross between AC Hime (high Cd accumulation) and Westag-97 (low Cd accumulation) was done and a recombinant inbred line (RIL) population (F6:8) derived from it, that was used for identification of DNA markers linked to Cda gene/QTLs controlling low Cd accumulation. SSR markers closely linked to Cda 1 genes were identified and that can be used for marker assisted selection for development of cultivars containing low Cd in a breeding program (Jegadeesan et al. 2010). Cai et al. (2008) developed a group of recombinant inbred lines (RILs) by crossing FSW Chinese wheat and ND35 (Al-sensitive Chinese line) to map QTLs for Al resistance. They screened 1,437 SSRs and identified 3 QTLs regulating resistance of Al in FSW. In *Arabidopsis halleri*, QTLs controlling Zn hyperaccumulation were mapped by making an interspecific cross between *A. halleri* and *A. lyratapetraea*. In both low and high pollution treatments, two QTLs were identified on chromosomes 3 and 6, and significant interactions were observed between environment and QTL. The QTL on chromosome 3 was found adjacent to a major QTL that was identified for Zn and Cd tolerance previously, thus suggesting that Zn hyperaccumulation and tolerance have a similar genetic basis, and it might be possible they have simultaneously evolved on soils contaminated with heavy metals (Frerot et al. 2010). Ding et al. (2011) observed a trend in the concentration of As in different parts of maize as leaves > stems > bracts > kernels. They also identified 11 QTLs for As concentration of which three QTLs for leaf As concentration were mapped on chromosomes 1, 5 and 8. In bracts, stems and kernels 2, 3 and 3 QTLs were identified, respectively, for As concentration. These results implied that concentration in different tissues of maize is regulated by different genomic regions and possibly different molecular mechanisms. Maize can be used for phytoremediation in As contaminated paddy soil, and the above identified QTLs can be useful for choosing inbred lines and hybrids containing low As concentration in kernels. Mapping of QTLs controlling Pb content in maize kernels was done by using a RIL population derived from cross of 178 (an inbred line with low accumulation of Pb in the kernels) and 9,782 (a Pb-hyperaccumulator in the kernels). Using the SSR markers, a molecular genetic map was constructed and QTLs were mapped for Pb content in maize kernels on chromsomes 1 and 4. It was found that there was no significant correlation between Pb content in the kernels and other yield related traits such as kernel ear length, row number, ear diameter and weight of per-hundred kernels, indicating that in the process of improving Pb concentration in maize breeding, yield related traits would not change (Zhao et al. 2014). A genome-wide association (GWA) mapping approach has been utilized for mapping grain concentration of As, Cu, Mo and Zn using SNPs in brown rice in five different environments over two years. Majority of loci were significantly associated with variation in grain of these metals. A large number of candidate genes were located near significantly associated SNPs for the uptake

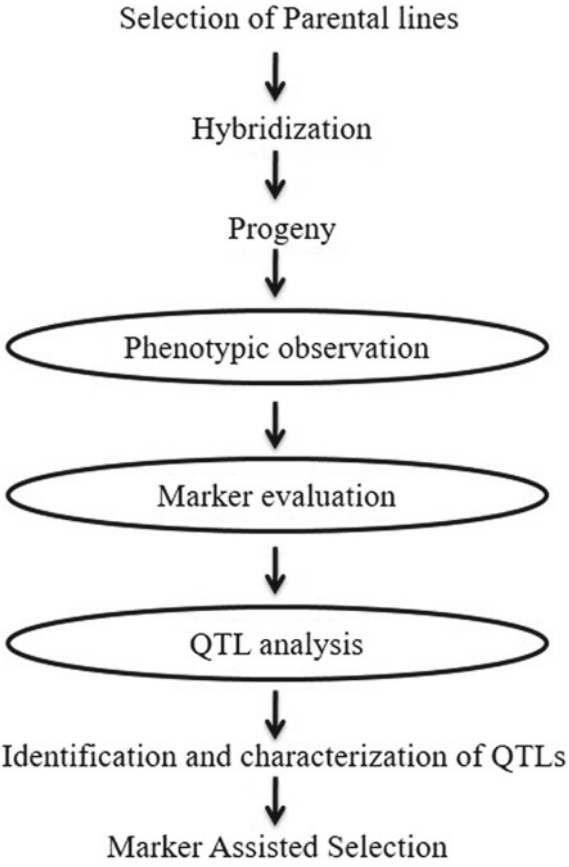

Fig. 2.2 Schematic representation of QTL mapping

or transports of these elements. For further analysis, this study provided a number genome sites and candidate genes (Norton et al. 2014). A Schematic representation of QTL mapping is shown is Fig. 2.2.

2.5 Conclusion

Molecular markers are useful when targeting traits, controlled by several genes. Identification of QTL involved in genetic variation of physiological traits helps in analyzing the genetic effect of each QTL on quantitative traits, and to determine the molecular markers linked to QTLs in order to apply marker-assisted selection for breeding. However, genetic improvement based on selection of markers is still in infancy in many crops. Marker-assisted selection may solve problems associated with genotype × environment interactions and can improve the selection efficiency

to screen metal tolerant plants. Further, the genetic dissection of the quantitative traits controlling the adaptive response of crops to abiotic stress is a prerequisite to allow cost-effective applications of genomics-based approaches to breeding programs aimed at improving the sustainability and stability of yield under adverse conditions.

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Chapter 3
Progress Towards Identification and Validation of Candidate Genes for Abiotic Stress Tolerance in Wheat

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Abstract Identification of candidate gene(s) and its validation in a breeder's germplasm is a prerequisite for any successful marker-assisted selection (MAS) programme for improving abiotic stress tolerance. Once a candidate gene(s) is identified and its effects validated under a stress environment, it becomes a powerful marker resource for developing 'functional markers' to assist genomics-assisted breeding in crops. There are several ways to identify a candidate gene(s) underpinning a specific abiotic stress tolerance mechanism. The most common methods used are various 'omics' approaches targeting transcriptome (transcriptomics), metabolome (metabolomics) or proteome (proteomics), co-location of genes with quantitative trait loci (QTLs) for abiotic stress tolerance traits (called positional candidates), fine mapping of QTLs/QTL cloning, transgenics, RNA interference, mutant screenings and genome wide/candidate gene-based association mapping among others. The advent of next generation sequencing (NGS) technologies has completely revolutionized the identification and characterization of candidate genes underlying various abiotic stress tolerance traits. This review focuses on the approaches taken to identify and validate candidate genes for various abiotic stress tolerances in wheat and the progress made so far in their validation and implementation in wheat breeding programs globally.

Keywords Abiotic stress · Candidate genes · Cloning · Functional markers Omics · Next generation sequencing · Validation

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3.1 Introduction

Wheat is the third most widely cultivated crop after rice and maize. Consumed by more than 40% world population, it is a staple food and the primary source of calories for millions of people worldwide. By 2050, global demand of wheat is projected to increase by 60% to feed the burgeoning world population. Further, with the worsening climate change scenarios, wheat production is anticipated to go down by 29% (Dixon et al. 2009). Abiotic stresses (drought, heat, salinity, metal toxicity, frost etc.) are predicted to be the major factors, which are likely to be responsible for this. Breeding for abiotic stress tolerance, therefore, is a major target in wheat breeding programs globally. Although traditional breeding approaches have pushed the annual genetic gain to up to 1% in grain yield, but to cope with 2% yearly increase in world population under changing climatic conditions, further efforts are required in a short span of time to generate climate resilient high yielding varieties. Hence, with the advent of new sequencing and genotyping platforms such as next generation sequencing (NGS), breeding methodologies have undergone a paradigm shift so much so that the application of 'genomics-assisted breeding' has become common in most breeding programs. Despite the large (~17 Gb) and complex (AABBDD allopolyploid) genome of wheat, these advanced NGS platforms have benefited wheat by generating large number of markers for gene discovery, lack of which had been a limitation in the past.

Wheat scientists are employing NGS platforms regularly to get their populations and germplasm genotyped. As a result, genetic analyses have been conducted in various sets of germplasm and populations and quantitative trait loci (QTL) and/or marker-trait associations (MTAs) using association mapping approaches have been identified for various abiotic stress tolerance traits with an ultimate aim to reach to the candidate gene(s). In addition, other genomics tools such as 'omics' techniques (transcriptomics, proteomics, metabolomics, and ionomics) have also been adopted in contrasting lines/parents, for example differing in tolerance to drought or heat stress or even both, which have not only pinpointed potential candidate genes but have also unveiled the underpinning tolerance mechanisms. Many of the candidate genes have also been cloned and validated using transgenic approach; by creating transgenic wheat, *Arabidopsis* or tobacco lines transformed with the cloned genes in wheat.

Further, a remarkable milestone in wheat genome sequencing has been the sequencing of Chinese Spring (CS42) using the next-generation Roche 454 pyrosequencer (Brenchley et al. 2012). With 5× coverage of wheat genome, ~96,000 genes in bread wheat genome have been estimated. Moreover, genes localized on genomes A, B and D showed conserved orthologous homology with members of grass family (*Brachypodium*, rice, sorghum and barley). This has opened doors for utilizing comparative genomics approach for positional cloning of more candidate genes in wheat by utilizing the genome sequence information of small diploid crop species (Brenchley et al. 2012). Recently, using shotgun-sequencing approach, genome sequence drafts of A and D genome progenitors (*Triticum urartu* and *Aegilops tauschii*) has

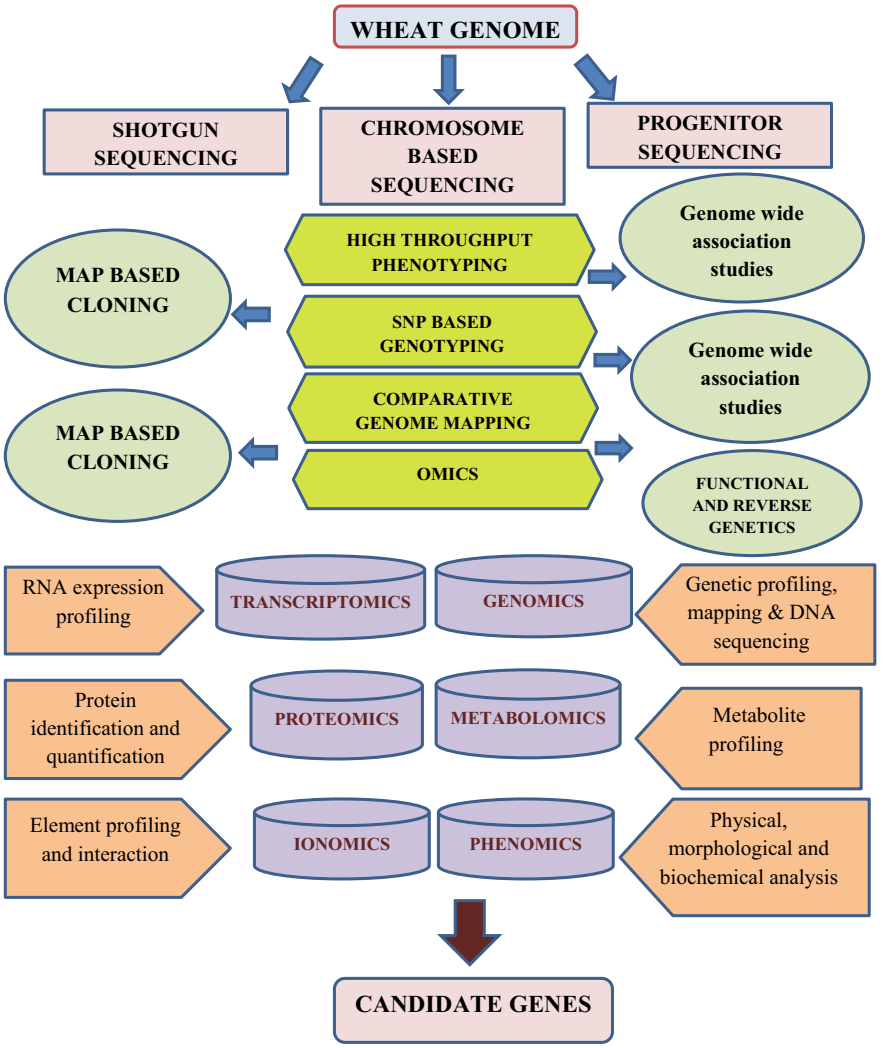

Fig. 3.1 Genomics-based platforms that can facilitate identification of candidate genes for agronomically important traits in wheat

also been acquired, which can further be used in comparative genomics to get an insight into genetics of important traits including abiotic stress tolerance and yield. This chapter reviews these progresses briefly and highlight some examples in detail. Figure 3.1 summarizes how advanced genetics and genomics approaches can be integrated to reach to the candidate genes for abiotic stress tolerance for their deployment in breeding.

3.2 Mapping Approaches Identified Candidate Genes and Quantitative Trait Loci for Abiotic Stress Tolerance in Diverse Elite Germplasm

Biparental QTL mapping and association mapping are the two major strategies followed for genetic dissection of complex and quantitative traits such as abiotic stress tolerance. The former approach has been extensively utilized in wheat, particularly for dissecting drought and heat tolerance (Kirigwi et al. 2007; Mohammadi et al. 2008; McIntyre et al. 2010; Pinto et al. 2010; Vijayalakshmi et al. 2010; Mason et al. 2010, 2011; Alexander et al. 2012; Nezhad et al. 2012; Paliwal et al. 2012; Lopes et al. 2013; Tiwari et al. 2013; Talukder et al. 2014) using low to medium-throughput markers. However, due to the well-known limitations of this approach and the requirement of fine mapping of the identified QTL to reach to the candidate gene (time-taking and very expensive), this approach is no longer attractive among scientists. Nevertheless, Talukder et al. (2014) indicated potential genes for heat tolerance underlying a consistent QTL on 7A by in silico analysis of the QTL flanking markers. They identified stress-responsive gene *srg6*, calcium/calmodulin dependent protein kinase gene and a putative DNA topoisomerase I gene in the QTL region.

With the drastic drop in sequencing and genotyping costs, wheat breeding programs globally have adopted advanced high-density whole-genome genotyping platforms for genotyping their elite germplasm. This has made existing phenotypic data on breeding lines amenable to genome wide association mapping (GWAM), a leading approach for complex trait dissection and identification of novel and superior alleles in the breeder's existing germplasm including wheat (Maccaferi et al. 2011; Ain et al. 2015; Lopes et al. 2015; Mwadzingeni et al. 2017; Sehgal et al. 2017). Using CIMMYT's WAMI (Wheat Association Mapping Initiative) and IBWSN (International Bread Wheat Screening Nursery) association panels, potential novel candidates for drought and heat stress tolerance or both have been identified on chromosomes 2D, 3A, 3B, 4A, 5B and 7B using high-density 9 K SNP and genotyping-by-sequencing (GBS) markers (Edae et al. 2014; Lopes et al. 2015; Sehgal et al. 2017). Two of these candidates included genes belonging to heavy metal transport/detoxification superfamily protein and DNA J heat shock N-terminal domain-containing protein (Sehgal et al. 2017). Ain et al. (2015) conducted GWAM for grain yield in historical wheat cultivars from Pakistan under rainfed conditions. Out of 44 stable MTAs identified for yield and yield-related traits, 14 SNPs showed syntenic relationship to the genes in rice, sorghum and *Brachypodium*. These genes encode proteins, which are important components of pathways triggered in response to stressed environments, for example, aldehyde dehydrogenase, cell number regulator 6 protein, glycosyltransferase-like protein, molybdenum cofactor sulfurase, n-acetyl glucosaminyl transferase III, NADH dehydrogenase, and serine threonine-protein phosphatase 6. The authors suggested these as possible candidate genes for yield improvement as well as for future cloning of these loci.

A modification of association mapping (AM) is candidate gene-based AM, which links phenotypic variation with polymorphic sites in candidate genes to identify

causative polymorphisms. The choice of candidate gene(s) is generally based on the relevant information obtained from genetic, biochemical, physiological or expression studies in both model and non-model plant species (Sehgal and Yadav 2009). Thus, this approach is an alternative to the fine mapping/positional cloning approach and thus a significant shortcut. In wheat, a candidate gene-based association study identified MTAs for drought tolerance traits in known drought stress-induced genes in ABA-dependent (*ERA1*) and ABA-independent (*DREB1A, 1-FEH*) pathways (Edae et al. 2013). In an another candidate gene-based AM in wheat, an SNF-1 type serine-threonine protein kinase *TaSnRK2.8* showed association with plant height, flag leaf width and water-soluble carbohydrates under drought conditions (Zhang et al. 2013). This gene was selected based on previous evidence of its role in enhancing tolerance to drought, salt and low temperature (Zhang et al. 2010). Similarly, Chang et al. (2013) worked on *TaSAP1*, a member of the stress association protein (SAP) gene family in wheat. A high nucleotide diversity was identified in promotor region, which allowed development of three markers T7AM5, T7AM2606 and T7AM39 (InDel5-1810, SNP-2606 and InDel39-1637). Application of these markers in 300 wheat accessions identified six haplotypes and their associations with 1000-grain weight, number of grains per spike, spike length, peduncle length and total number of spikelets per spike under well-watered and drought-stressed conditions.

3.3 Exploitation of 'Omics' Platforms

3.3.1 Transcriptomics

Of all the omics technologies i.e. transcriptomics, metabolomics, proteomics and ionomics; transcriptomics is the most researched for identification of genes and understanding mechanisms for abiotic stress tolerance in various plant species. Transcriptome is the complete set of RNA transcripts in a specific cell type or tissue at a certain developmental stage and/or under a specific physiological condition. By investigating transcriptome of any plant under different treatments, or any developmental stage, a wealth of information can be generated which helps to pinpoint underpinning genes and related mechanisms. There are two key contemporary techniques in this field: microarrays, which quantify a set of predetermined sequences, and RNAseq (a next-generation sequencing technology; see Sect. 3.5, uses high-throughput sequencing to capture all sequences).

A plethora of research reports are available in wheat wherein transcriptome approaches have identified genes involved in abiotic stress response. Related to rice *OsHKT7*, two putative sodium transporter genes i.e., *TmHKT7A1* and *TmHKT7A2* were identified utilizing wheat expressed sequence tag (EST) data, of which later was found to be expressed in roots and leaf sheaths of salt-tolerant durum wheat line (Huang et al. 2006). Monroy et al. (2007) utilized 5,740 feature cDNA amplicon microarray to compare gene expression profile between winter and spring wheat

cultivar in response to cold stress and identified 450 cold-regulated genes, including transcription factors (TFs) and genes involved in signaling and regulatory pathways. *WLIP*19, wheat lip 19 homologue encoding for b-ZIP transcription factor was found to function as transcription regulator of Cor/Lea genes to impart freezing tolerance in wheat (Kobayashi et al. 2008). Higher concentration of WLIP19 under low temperature stress was confirmed using transgenic tobacco and wheat callus. Qin et al. (2008) identified 6,560 probe sets responsive to heat stress in comparison of global expression pattern between Chinese Spring (heat-sensitive) and TAM 107 (heat-tolerant) wheat genotypes. Identified genes were classified in the following categories; heat shock proteins (HSPs), TFs, calcium and sugar signal pathways, RNA metabolism, primary and secondary metabolism, and biotic and abiotic stresses. Kam et al. (2008) identified 37 Q-type zinc-finger protein genes and 30 genes expressing predominantly in roots of *T. aestivum* in response to drought stress. Affymetrix wheat genome array was used for comparison of global expression pattern of drought-sensitive and tolerant genotypes that revealed differential expression of various genes involved in ABA-dependent, ethylene- and IP3-dependent signaling pathways (Ergen et al. 2009). Xu et al. (2008) screened drought-induced cDNA library of wheat and identified three novel homologues of the DBF (DRE binding factor) gene family. Ristic et al. (2009) identified a positive correlation among rubisco activase (RCA) of different wheat genotypes in response to heat stress. Additionally, 5,500 wheat ESTs were identified through suppression subtractive hybridization between heat-stressed and control tissues at 3 stages of development, i.e., seedlings, prepollinated flowers and developing grains (Chauhan et al. 2011) and their expression was confirmed through cDNA macroarray, Northern/RT-PCR as well as real time PCR of the selected genes. Naydenov et al. (2010) conducted microarray analysis of mitochondrial transcriptome profile of wheat under stress and observed 13, 14 and 23 genes responsive to low temperature, high salinity and high osmotic stress, respectively. Expression analysis of 10 MYB TF genes in two wheat RILs was analyzed, and one MYB gene (*TaMYBsdu1*) was identified to be markedly upregulated in leaf and root of long term drought-stressed wheat plants as well as in the salt-tolerant genotypes, thus implying its role as an important regulator involved in adaptation to both salt and drought stresses (Rahaie et al. 2010). Akpinar et al. (2015) identified unique transcripts to function in drought signaling in deep sequencing-based transcriptome analysis of *T. dicoccoides* and *T. durum.*

Recently, microRNAs (miRNA) have emerged as an important regulatory factor in governing plant adaptability to range of experimental conditions. Agharbaoui et al. (2015) characterized de novo miRNAome of hexaploid wheat and identified 199 candidate miRNAs associated with different abiotic stress (cold, salt and Aluminium) response, tolerance and developmental stages. Wang et al. (2015) identified *TaWRKY44* as a positive regulator in drought, salt and osmotic stress that acts through either activation of stress-associated gene expression or ROS elimination via cellular antioxidant system. Zang et al. (2017) identified a novel ferritin gene, *TaFER-5B*, from transcriptome of heat-tolerant wheat cultivar (TAM107). Overexpression of *TaFER-5B* in *Arabidopsis* resulted in enhanced thermotolerance, oxidative and excess ion stress tolerance associated with the ROS scavenging.

3.3.2 Proteomics and Metabolomics

Any external stimuli bring out a change in the plant's constituent proteins and primary or secondary metabolites accumulation. Thus, by assessing the proteome (sum total of all protein constituents of cell) and metabolome (sum total of all metabolites constituents of cell) of plants by proteomics and metabolomics approaches, respectively, plant's response to different stimuli can be investigated and candidate genes can be pinpointed. A proteomic approach, for example, was recently applied to identify protein spots involved in cold responses in wheat (Zhang et al. 2016). Two cultivars contrasting for cold tolerance (UC1110 and PI 610750) as well as their descendants differing in cold-tolerance were investigated by two-dimensional electrophoresis (2DE) method. Sixteen unique proteins were successfully identified, of which 14 had significantly enhanced abundance in the cold-sensitive UC1110 and its 20 descendant lines as compared with the cold-tolerant PI 610750 and its 20 descendant lines. A few of these differentially expressed protein spots were validated by real-time polymerase chain reaction (qRT-PCR) to investigate expression changes at the RNA level. The transcriptional expression patterns of 11 genes was consistent with their protein expression models, thus pointing to their candidacy for cold tolerance. Similarly, Kamal et al. (2010) analyzed abiotic stress-responsive proteins in wheat grain by proteomics technique. Endosperm of wheat grain of four cultivars (two Chinese cvs. China-108, Yennon-78 and two Japanese cvs. Norin-61, Kantou-107) was fractionated and soluble proteins of whole seed were examined by 2DE. Selected protein spots were analyzed, which revealed 124 proteins spots as unique abiotic stress-responsive proteins, induced by heat (31.56%), drought (26.61%), salt (23.38%), cold (21.77%) and other environmental stresses (22.58%). Jiang et al. (2012) characterized wheat proteome of 2 genotypes under drought stress and identified 26, 23 and 17% of differentially expressed proteins involved in carbohydrate metabolism, detoxification and defense, respectively. Budak et al. (2013) characterized proteome of two wild emmer varieties and one durum variety under drought stress and identified 75 differentially expressed proteins. Similarly, Alvarez et al. (2014) identified 1,656 proteins along with 2 unique peptides in root proteome analysis of drought-tolerant (Nesser) and drought-sensitive (Opata) wheat varieties in response to ABA. Of 151 ABA responsive proteins, 100 and 50 proteins displayed an increased and decreased expression level, respectively.

Metabolome changes during abiotic stress are also important to characterize as different metabolites constitutes different developmental changes and thus the characteristic metabolites identified under particular conditions such as specific developmental stage or stress could be used to identify metabolic markers diagnostic for plant stress. Targeted or non-targeted metabolic profiling under stress conditions will enrich our understanding of plant metabolism to develop cultivars with better tolerance and adaptation to stress conditions. Metabolic profiling, however, is limited to analysis of pre-determined metabolites for specific pathways, while metabolic fingerprinting involves a global, high throughput and rapid assay. Targeted GC-MS approach was used to characterize 103 metabolites from leaves of wheat plants i.e.,

Kukri, Excalibur and RAC875 experiencing cyclic drought stress, which revealed cultivar-specific differences indicating different mechanisms adopted by the three tolerant cultivars (Bowne et al. 2012). Two hundred and five metabolites from flag leaves of double haploid (DH) population of Excalibur and Kukri under terminal drought conditions were characterized by Hill et al. (2013) and were correlated with the agronomic data for 29 traits, which identified 95 metabolite QTLs (mQTLs). The study further identified 5 wheat genomic regions that affected both the metabolite and agronomic traits.

A complete understanding of the abiotic stress-responsive metabolome and proteome in wheat will require considerable amount of time and resources, but a methodical and concerted effort will help identify most appropriate genes that could be targeted for wheat improvement.

3.4 Status of Cloned Genes for Abiotic Stress Tolerance and Their Characterization in Wheat

Cloning and gene identification in wheat is a difficult task. However, it is becoming less daunting due to the extensive genomic efforts by the international wheat community. One of the first abiotic stress genes that was cloned in wheat was for aluminum (Al)-tolerance (*ALMT1*; Sasaki et al. 2004). This gene was isolated from root apices of Al-tolerant wheat by subtractive hybridization of cDNAs isolated from near isogenic wheat lines ET8 and ES8. Sequence analysis of *ALMT1* cDNAs from ET8 and ES8 showed differences at six nucleotides resulting in proteins differing at two amino acids. These were designated as *ALMT1-1* and *ALMT1-2* derived from ET8 and ES8, respectively. To establish the association between Al-tolerance and *ALMT1*, populations segregating for Al-tolerance were analyzed for expression of the *ALMT1-1* and *ALMT1-2* alleles. Al-tolerant seedlings showed the expression of either *ALMT1-1* or both *ALMT1-1* and *ALMT1-2* alleles, while Al-sensitive seedlings showed the expression of only *ALMT1-2* allele. Further, at DNA level too *ALMT1-1* allele showed complete co-segregation with Al-tolerance phenotype, which suggested that *ALMT1-1* confers in Al-tolerance. In 2007, another gene for abiotic stress tolerance was fine mapped, Bo1; underlying a major QTL for boron tolerance on 7B (Schnurbusch et al. 2007). Bo1 was fine mapped using the DH population Cranbrook (intolerant) x Halberd (originally used to map the QTL) (Jefferies et al. 2000). To increase the marker density in the Bo1 QTL region as a first step, commonly available RFLP, PCR and SSR markers were used. In addition, intron-based markers were designed from 26 genes identified in the syntenic region of rice genome on chromosome 6L and recombinant lines were selected. One of these markers, AWW5L7, co-segregated with *Bo1* in the 13 recombinant DH lines.

An aquaporin (AQP) gene *TaNIP* (*Triticum asetivum* L. nodulin 26-like intrinsic protein), known to involved in salt tolerance pathway in plants, was cloned and characterized in wheat by Gao et al. (2010). The *TaNIP* gene was identified based

on previous expression gene chip results of a salt-tolerant wheat mutant RH8706-49. This gene was again validated under salt stress in the salt-tolerant and salt-sensitive wheat mutants, which revealed its much higher expression in the salt-tolerant mutant RH8706-49 than in the salt-sensitive mutant H8706-34 after 72 h. Its expression pattern was also tested in the roots and leaves of the same mutants under other abiotic stresses viz. ABA, polyethylene glycol (PEG) and cold treatments. Sequence analysis of the cloned gene revealed that *TaNIP* shares the same conserved structural domains as the AQPs and is a member of the AQP family. The overexpression of *TaNIP* in transgenic Arabidopsis produced higher salt tolerance than wild-type plants. The mechanism behind the salt tolerance was also unraveled i.e. due to accumulation of higher K^+, Ca^{2+} and proline contents and lower Na^+ levels in *TaNIP*-overexpressing *Arabidopsis* plants than the wild-type. In the same year, another group in China cloned wheat gene *TaSnRK2.8* (Zhang et al. 2010), whose role in proving tolerance to multiple stresses (drought, salt and cold stresses) was provided by transferring it to *Arabidopsis.*

The genetics group at IARI (Indian Agricultural Research Institute, India) reported cloning of two heat shock protein (HSP) genes, *HSP 17* and *HSP 90*, in 2012 (Kumar et al. 2012 a, b). Both genes were cloned from thermotolerant wheat cultivar C-306. *HSP 17* belongs to a family of small HSP (sHSP). Its role under heat stress tolerance was provided by qRT-PCR in thermotolerant (C-306) and thermosensitive (HD2329) cultivars. *HSP17* gene showed a 34 fold increase in transcript in C-306 and only 1.5 fold in HD2329 in response to differential treatment of putrescine (1.5–2.5 mM + heat shock of 42°C for 2 h). The *HSP 90* belongs to high molecular weight HSP. *HSP 90* isolated from C-306 was 2.5 kb long and alignment of its sequence with other HSPs in NCBI (National Centre for Biotechnology Information) database showed a large variability in the sequences. Phylogenetic analysis of the diverse sequences have grouped them into four subgroups and *HSP 90* from C-306 belongs to subgroup IV.

Chauhan et al. (2013) cloned heat shock factor (HSF) gene *TaHsfA2d* in bread wheat cv. CPAN1676 and provided evidence of the role of this gene in heat tolerance. Wheat EST showing homology with rice HSF was identified from developing seed tissue EST library and was used for designing the primers for RACE (Reverse Amplification of cDNA Ends)-PCR. The resultant fragments were cloned and sequenced. The sequence analysis revealed highest similarity with HSF of rice *OsHsfA2d* (69%) than with other plants (65% with *Arabidopsis HsfA2*). The evidence of its role in tolerance to heat stress was provided by creating transgenic *Arabidopsis* plants overexpressing *TaHsfA2d*. The transgenic lines possessed higher tolerance towards high temperature and to salinity and drought stresses. Higher yield and biomass accumulation under constant heat stress conditions was also noticed in transgenic *Arabidopsis*. Hu et al. (2013) cloned a transcription factor (TF) gene *TaASR1*, belonging to a family of ASR [Abscisic acid (ABA)-, stress-, and ripening-induced] genes, in cv. Chinese Spring. Although *ASR* genes are known to respond to various stresses in wheat, their exact roles in abiotic stresses tolerance was not known. Cloning of *TaASR1* gene in wheat not only provided evidence of its role under drought stress but also shed light on the exact mechanism. Overexpression of *TaASR1* gene was analyzed in tobacco,

which demonstrated that transgenic lines expressing *TaASR1* gene had lesser malondialdehyde content, ion leakage and ROS, but higher relative water content, and superoxide dismutase and catalase activities than wild type under drought stress. These results suggested strongly that *TaASR1* confers drought stress tolerance by activating the antioxidant system.

In the last four years, many more genes have been cloned and characterized in wheat; conferring pre-harvesting tolerance (*TaSdr*; Zhang et al. 2014), salinity tolerance (*TaAOC1*; Zhao et al. 2014) and multiple abiotic stress tolerance (*TaPP2AbB″- α*; *TaWRKY44*, *TaCRT-D*; Liu et al. 2014; Wang et al. 2015, 2017). *TaSdr* genes (*TaSdr-A1, TaSdr-B1* and *TaSdr-D1*) were cloned by a comparative genomics approach. These are orthologs of rice *OsSdr4*, known to confer seed dormancy in rice. A single nucleotide polymorphism (SNP) (A/G) at the position 11 upstream of the initiation codon was revealed in *TaSdr-B1*, with bases A and G in cultivars with low and high germination indices, respectively. A CAPS (cleaved amplified polymorphism sequence) marker was developed based on this SNP, which was used for validation by linkage and association mapping approaches (Zhang et al. 2014). For validation of *TaAOC1*, transgenic wheat and *Arabidopsis* lines expressing *TaAOC1* were generated. An enhanced level of tolerance to salinity was observed in transgenic lines of both species. Further, transgenic plants accumulated a higher content of jasmonic acid (JA) and developed shorter roots. This study provided first evidence that JA can also be involved in the plant salinity response, in addition to its proven role in defense responses to biotic stresses. The gene *TaPP2AbB"-α* was cloned in wheat cv. Hanxuan 10. The full-length cDNA sequence was obtained using the candidate sequence from rice (NM_001071385.1) and sequence information for a dehydration-inducible cDNA library of wheat D genome progenitor *Aegilops tauschii*. Transgenic *Arabidopsis* plants overexpressing *TaPP2AbB"-α* were generated for its validation, which showed extensive development of lateral roots, especially when treated with mannitol or NaCl. These results suggested that this gene positively regulates lateral root development under osmotic stress. Cloning of *TaWRKY44* revealed a very similar underpinning mechanism for osmotic stress tolerance as shown by *TaASR1* gene i.e. activation of the cellular antioxidant systems. However, *TaWRKY44* conferred drought and salinity tolerance to the transgenic tobacco lines, whereas *TaASR1* conferred mainly drought tolerance. The most recent cloned gene in wheat *TaCRT-D* (Wang et al. 2017) conferred multiple stress tolerance (drought, cold, salt, mannitol) to transgenic *Arabidopsis* plants at multiple stages (seed germination and seedling stages). Most importantly, based on DNA sequence analysis genome-specific and allele-specific markers were developed for the *TaCRT-D* gene for MAS.

3.5 Role of Next Generation Sequencing (NGS) Tools

Genotyping of large breeding populations, especially in huge breeding programs where all germplasm is routinely genotyped, by second or third generation markers

can take several months, and hence can be very expensive. Thus, the need for more efficient technologies that are cost- and time-effective and high-throughput (genotype large populations within a smaller time frame) laid the stone for the advent of NGS or alternatively whole genome sequencing technologies. Availability of NGS has transformed the whole perspective of the identification of DNA markers from fragment-based polymorphism to sequence-based single nucleotide polymorphism (SNP), to expedite the marker identification process and to increase the number of informative markers at a cost as low as 10-40 USD per sample, depending on the type of NGS platform. Of the various NGS technologies, genotyping-by-sequencing (GBS), restriction-site associated DNA-seq (RADseq), sequence-based genotyping (SBG), exome sequencing have already been proved to be effective for next generation plant breeding including wheat (Elshire et al. 2011; Berkman et al. 2012; Poland et al. 2012; Winfield et al. 2012; Kumar et al. 2015). Recent applications include the shotgun sequencing of the wheat chromosomes 7DS, 7BS, and 4A (Berkman et al. 2011 a, b; Hernandez et al. 2012), 5-fold coverage of the wheat cultivar Chinese Spring (http://www.cerealsdb.uk.net/) and deep Illumina sequence data for the D-genome donor *Aegilops tauschii* (http://www.cshl.edu/genome/wheat).

Although, shorter reads are produced by NGS technologies and their error rates are also higher than Sanger sequencing, NGS are popular due to their ability to produce vast quantities of data at a relatively low cost and in a short time. These NGS platforms have particularly benefited wheat, whose large and complex allopolyploid genome had kept it recalcitrant to molecular technologies for a long time. Now generation of genome-wide markers or sequencing of transcriptome by RNAseq or exome sequencing to identify candidate genes is no longer daunting in wheat, and is being carried out routinely in wheat (Poland et al. 2012, van Poecke et al. 2013; Winfield et al. 2012). For instance, Kumar et al. (2015) used Illumina HiSeq 2000 and Roche GS-FLX 454 for high-throughput deep sequencing of whole transcriptome of a heat-sensitive wheat cv. HD2329 under the control (22°C ± 3°C) and heat-stress (42°C, 2 h) conditions. RNAseq expression analysis showed significant differential expression of 1,525 transcripts under heat stress, of which 27 transcripts showed very high (> 10) fold upregulation. Most of the differentially expressed genes (DEGs) were associated with ATP binding, serine threonine kinase activity, zinc ion binding, and metal ion binding. Similarly, Liu et al. (2015) performed deep RNA sequencing of 1-week old wheat seedling leaves subjected to drought, heat and a combination of drought and heat stress (HD) for 1 h and 6 h using the Illumina sequencing platform. Gene ontology enrichment analysis revealed an overlap of drought, heat and HD-responsive genes. Moreover, 4,375 wheat TFs were identified on a whole-genome scale, of which 1,328 were responsive to stress treatments.

Recently, RNAseq analysis in a spaceflight-induced wheat mutant *st1* resulted in identification of candidate genes for salinity tolerance (Xiong et al. 2017). The mutant *st1* was identified in a screen for induced wheat mutants grown in hydroponics with high salinity. Its transcriptome sequence variation analysis revealed that multiple genes involved in Na+transport and genes encoding arginine decarboxylase and polyamine oxidase are contributing to salinity tolerance in *st1*. In addition, 'Butonate metabolism' was identified as a new pathway for salinity tolerance.

3.6 Validation of Candidate Genes

Although comparative genomics is a powerful tool in trait dissection, confirmation of the roles of individual genes is still required through functional validation. In wheat, nearly hundred genes have been reported to be involved in various abiotic stress tolerance via approaches described above and many more are being discovered. A few of them have been cloned and validated using transgenic approach or expression evidences have been generated to validate their role in abiotic stress tolerance in wheat (see above Sect. 3.4). In addition to this approach, two other approaches described below have also been employed for validation.

3.6.1 Allelic Variation in Transcription Factors and Known Target Genes for Abiotic Stress Tolerance

Many families of transcription factors (TFs) have been demonstrated to play a role in stress responses in wheat. Among these, *bZIP, DREB, WRKY, bHLH, MYB* and *NAC* TFs represent the major groups of regulatory genes found to be involved in wheat stress tolerance. The sequence data of few of these genes has been utilized by simple multialignments of their conserved domains to design genome-specific primers, which were then tested on genotypes contrasting for tolerance to different abiotic stresses to identify functional SNPs (Wei et al. 2009; Garg et al. 2012; Mondini et al. 2012). Wei et al. (2009) designed genome-specific and allele-specific markers based on the available sequences of *DREB1* genes in common wheat and related species. Two SNPs (S646 and S770) were detected in *DREB-B1* sequence, which distinguished the Opata 85 and W7984 parents of the ITMI (International Triticeae Mapping Initiative) mapping population. No polymorphism, however, was detected between the orthologous *DREB-A1* and *DREB*-D1 sequences. An allele-specific primer P40 based on SNP S770 in *DREB-B1* sequence was designed and validated using wheat lines differing for drought tolerance. This was subsequently mapped on chromosome 3BL. Mondidni et al. (2012) designed primers from alignment of conserved domains in two TFs; *DREB1*, *WRKY1* and a sodium transporter gene *HKT-1*. These primers were used to test several genotypes of durum wheat that were differentially tolerant to salt and drought stress. By sequencing the polymerase chain reaction (PCR) products from contrasting genotypes, several SNPs were subsequently identified and validated. Garg et al. (2012) chose heat shock protein (*HSP16.9*) as the target gene and designed primers from a partial sequence of *Triticum aestivum* L. *HSP16.9*. These primers were used for amplification of the gene from heat-tolerant (K7903) and heat-susceptible (RAJ4014) genotypes. Sequence analysis of PCR products identified a SNP between these genotypes (A/G) which resulted in a missense mutation from aspartic acid to asparagine residue. An allele-specific

marker was designed based on this SNP and tested on various other heat-tolerant and heat-susceptible genotypes, which revealed 29.89 and 24.14% phenotypic variation for grain weight per spike and thousand grain weight, respectively. This is the first report of HSP-derived SNP marker associated with terminal heat stress in wheat.

3.6.2 *Cosegregation of Fine Mapped QTL/Cloned Genes with Phenotype in the Existing Germplasm or Diverse Accessions*

Only two genes have been validated using this approach; candidate genes for Al and boron tolerance (Soto-Cerda et al. 2015; Schnurbusch and Sutton 2008; Pallotta et al. 2014). Soto-Cerda et al. (2015) not only validated the candidate gene *TaALMT-1* for Al-tolerance but also used it in marker-assisted breeding to introgress the gene in Al-sensitive cultivar Kumpa-INIA. They designed a functional marker $ALMT_{1\text{-}4}$ from upstream of the *TaALMT-1* coding region for screening Al-tolerant Kumpa-INIA lines, which were derived in three backcross generations. Similarly, for boron tolerance Bo1-specific codominant PCR marker AWW5L7 was designed after fine mapping (see Sect. 3.4; Schnurbusch et al. 2007), which was subsequently validated in a range of exotic bread and durum wheat accessions (Schnurbusch et al. 2010) and Australian bread wheat cultivars and breeding lines (Schnurbusch et al. 2010).

3.7 Conclusions

To generate climate resilient and high yielding wheat varieties in a short span of time, scientists globally are utilizing advanced genetics and genomics tools on their current germplasm. As a result, list of potential candidate genes with a probable role in abiotic stress tolerance is increasing in number. Unfortunately, only a handful of these have been validated, and converted into functional markers for MAS. The two best-validated cloned gene-related markers are for boron tolerance QTL *Bo1* (Bo1-specific PCR marker AWW5L7) and Al tolerance QTL ($ALMT_{1\text{-}4}$). Similarly, *HSP16.9*-derived allele-specific SNP marker for screening heat tolerance genotypes, *TaSdr-B1* gene-derived CAPS marker Sdr2B for screening preharvest sprouting tolerance tolerance and the latest *TaCRT-D*-derived PCR-RFLP marker for screening plants for multiple stress tolerance are three another validated functional gene-based markers (for details see above sections). Many other cloned genes have been validated using transgenic approach; most of the time these transgenics have been evaluated under controlled environments. To be able to use these genes for marker-assisted selection, it is important that these are validated directly on breeder's germplasm both under controlled and field conditions. The importance of validating the genes in a breeder's germplasm under field conditions emerged from contrasting results

obtained for *DREB-1A* gene. Greenhouse experiments confirmed the advantages of transgenic *DREB1A*-wheat in recovery after severe water stress. However, under field conditions, the transgenic lines could not outperform the controls in terms of grain yield under water deficit, as was predicted based on greenhouse performance (Saint Pierre et al. 2012). Hence, in future focus should be more on cloning and validating the reported candidate genes rather than discovering new. By increasing the frequency of favorable alleles of the validated genes, robust germplasm can be made ready for developing next generation climate smart varieties.

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Chapter 4
Genomics and Molecular Breeding for Improving Tolerance to Abiotic Stress in Barley (*Hordeum Vulgare* L.)

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Abstract Barley is one of the most important cereal crop in the world, in terms of harvested area, trade value, cattle feed and human nutrition. It is one of the most adapted plant species to marginal environments, where abiotic stresses, such as drought, heat, cold, low fertility and salinity, are prevalent and limit crop productivity. Due to its wide adaptability, barley is often the only crop that can be grown in many countries of West Asia and North Africa, thus representing a very important resource for farmers and the principal feed for livestock in these areas. To cope with these adverse conditions, the selection for barley cultivars with stable and economic yield under variant environments is a primary requirement of any breeding program. Recently, new genomic and molecular tools have increased the number of genes identified in the barley gene pool, involved in abiotic stress tolerance and in the adaptation to unfavorable environments. The complementation of traditional breeding approaches with new analytical selection methodologies is required for future yield gains to meet the global food/feed and industrial demand as well as to cope up with the effects of climate changes. Therefore, exploiting new genomics- and molecular-based breeding strategies to increase barley yield as well as the development of new varieties with improved adaptation to abiotic stresses is crucial. In this chapter, the utilization of genomics- and molecular-based tools and their integration with classical breeding approaches is presented to improve the tolerance to abiotic stresses in barley. Major challenges in breeding for tolerance to major abiotic stresses are described in the beginning, followed by the exploitation and utilization of different genomics

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and genetic resources, and breeding approaches currently used to produce tolerant varieties. The application of marker-assisted selection and markers discovery using quantitative genetics, association mapping and bioinformatics approaches for abiotic stress tolerances in barley are also highlighted. Furthermore, comparative and functional genomics approaches used to understand abiotic stress tolerance mechanisms in plants and their potential application for improving tolerance to abiotic stresses in barley have been discussed. Finally, challenges and future perspectives for the application of genomics- and molecular-based breeding strategies for barley crop improvement under abiotic stress conditions are overviewed.

Keywords Association mapping · Drought tolerance · Frost tolerance · *Hordeum vulgare* · Salinity tolerance

4.1 Introduction

Barley (*Hordeum vulgare* L.) is a cereal plant that belongs to the family Poaceae, and tribe Triticeae. It is a diploid (2n = 14) plant with a complex haploid genome of 5.1 Gb (IBGSC et al. 2012). The first signs of barley domestication were recorded more than 10,000 years ago in the Middle East in a region known as the "Fertile Crescent" (Badr et al. 2000; Pourkheirandish and Komatsuda 2007; Comadran et al. 2012). Archaeo-botanical evidences show that cultivated barley (*H. vulgare ssp. vulgare*) was derived from its wild progenitor (*H. spontaneum*), which is still widely distributed in the Fertile Crescent region particularly in the driest areas (Harlan and Zohary 1966). The domestication process resulted in populations known as "landraces", which were maintained by farmers and were known to have high genetic diversity for tolerance to environmental stresses such as drought, disease and pests (Jarvis et al. 2000; Berthaud et al. 2001). Landraces are considered as a valuable source for sustainable agriculture in the context of future climate change, and their in situ conservation strategies can allow their preservation for future use (Bellucci et al. 2013).

Nowadays, barley is the fourth most important cereal crop in the world after rice, wheat and maize and is one of the most important feed and food crops in dry areas (FAOSTAT 2016). In such areas, barley is considered the crop of choice due to its wide adaptability to different abiotic stresses such as drought and terminal heat (Baum et al. 2007). There are many end-uses of barley including animal feed and forage, human consumption and malting (Horsley et al. 2009). Its productivity varies among years primarily due to the seasonal variability in precipitation and temperatures and associated stresses. Under climate change conditions, the development of new barley varieties is needed to mitigate expected yield reduction associated with several abiotic stresses. To deal with these adverse conditions, breeding for barley varieties with stable and economic yield under prevailing variant environments is needed. Efficient and effective genotypes with tolerance to major abiotic stresses should be utilized to produce new improved cultivars with enhanced productivity to overcome

the adverse effects of climate change (Varshney et al. 2012). In dry environments, breeders are usually establishing their programs based on direct selection for grain yield (Richards et al. 2002). Besides grain yield, many different traits have been targeted by breeders to select for drought tolerance in barley that include growth habit, early growth vigor, flowering time, maturity rate, plant height and grain filling duration (Baum et al. 2007).

Drought stress has always played an important selective role in the evolution of plant growth, development and physiology. The combined effects of drought and temperature and other related stresses on physiology, growth, water relations and yield are significantly higher than their individual effects. Therefore, to cope with these challenges there is an urgent need to identify and utilize new genetic material with high elasticity to climate change (Araus et al. 2008). Plant adaptation is a key factor that will determine the future of crop production systems in response to climate change. Shifting planting dates or switching to short growing-season crop varieties may be the best way to reduce the negative impact of climatic change and associated stresses. Under arid conditions, the selection of drought tolerant genotypes with shorter growing seasons is considered a successful escaping strategy that might enhance crop productivity. Nowadays, the development of new crop varieties with early flowering and maturity and improved stress tolerance is considered a primary objective for many breeders in marginal areas. New varieties that can escape stresses at the most sensitive stages of crop development, such as reproductive and grain filling period, should be considered as the judicial way to alleviate the adverse impact of high temperature and drought. Combining recent advances in genomics with current breeding activities and utilization of modern molecular tools will enable the production of improved lines that are more adapted to dry environments and still highly productive.

4.2 Breeding Challenges for Abiotic Stresses

Abiotic stresses such as drought, salinity, heat and cold have always played an important selective role in the evolution of plant growth, development and physiology and they are always considered as major limiting factors in crop production. Such stresses are considered major constraints on barley production in many areas around the world and the future climate change scenarios predict that the frequency of drought and heat stresses is likely to increase, especially in arid and semi-arid regions (Rizza et al. 2004). Therefore, there is an urgent need to develop new plant varieties with enhanced resistance to abiotic stresses and to cope with the new climate change conditions (Cattivelli et al. 2008).

Barley is considered a drought tolerant cereal crop that can be utilized in rainfed agriculture systems. Among the major cereal crops, barley shows superior drought and heat adaptation and it is considered an excellent model for studying physiological, genetic and breeding aspects of stress tolerance (Ceccarelli and Grando 2002). For instance, in the eastern parts of the Mediterranean basin, barley is generally

grown in arid and semi-arid areas, which are characterized with low rainfall and extreme temperatures. Barley varieties that produce stable and economic yields of both grain and biomass under these inconsistent rainfed conditions are needed. Therefore, improving barley crop yield in dry areas is an important objective for majority of the breeding programs.

In dry environments, breeders are establishing their programs based either on the selection for physiological or developmental traits (analytical breeding) or on direct selection for grain yield (empirical or pragmatic breeding) (Richards et al. 2002). Direct selection for grain yield and biomass under water-limited conditions is difficult due to various factors including the complexity of the traits themselves, complexity of the stress tolerance mechanisms and the annual variability in the amount and timing of rainfall (Lakew et al. 2011). Many different secondary traits have been targeted by breeders to select for stress tolerance in barley that include growth habit, early growth vigor, flowering time, maturity rate, plant height, peduncle length and grain filling duration (Ceccarelli and Grando 2002). The direct selection for grain yield under stress conditions has been hampered by low heritability, epistasis and high genotype by environment (G × E) interactions (Baum et al. 2007). Due to its complexity, the genetic mechanisms underlying the expression of stress tolerance in cereal plants, including barley are poorly understood and these traits are usually difficult to characterize and analyze (Lakew et al. 2011).

Huge efforts were carried out to understand the interactions between genes and environments on yield under rainfed conditions in barley (Ceccarelli and Grando 2002; Richards et al. 2002) and large variations in genotype × season and genotype × location interactions were attributed to its genetic makeup (Richards et al. 2002). Quantitative trait loci (QTL) that contribute to differences in barley adaptation and plant development were found to have a major impact on grain yield in dry environments (Baum et al. 2007). Several candidate genes involved in adaptation to water limitations were identified in drought susceptible and tolerant barley lines (Guo et al. 2009). The expression of these candidate genes was found to be regulated in *cis* indicating that regulatory variation plays a major role in stress tolerance in cereal plants (von Korff et al. 2008).

During the last 50 years, most of the progress in releasing new varieties has been achieved from conventional or traditional breeding methods. In general, such conventional approaches has taken the yield as the main trait for selection, while there was an obvious neglect of other important traits such as tolerance to biotic and abiotic stresses (Ceccarelli and Grando 1996). An example for empirical breeding approach was the gradual replacement of the traditional tall cultivars with the semi-dwarf and fertilizer-responsive varieties with superior harvest indices (Araus et al. 2008). However, to maintain a sustainable increase in cereal yield, the development of location-specific and high-yielding varieties with resistance and/or tolerance to stresses is needed and should be the aim of plant breeders. The complementation of traditional breeding with new analytical selection methodologies, such as molecular tools, is needed for future yield gains to meet the global food demand. Exploiting new breeding strategies to increase barley yield per unit area as well as the development

of new varieties with improved adaptation to drought is considered crucial for crop improvement under rainfed conditions. As drought is unpredictable, the best way to cope with it is to develop tolerant varieties that perform well under such environments (Araus et al. 2008).

4.3 Mechanisms of Abiotic Stress Tolerance in Barley

The advances in understanding of key stress tolerance mechanism in plants, has led to the development of abiotic stress-tolerant plants by the activation of either one or both pathways through the over expression of key regulatory genes (Umezawa et al. 2006). In general, abiotic stress responses are mediated through abscisic acid (ABA)-dependent and -independent signal transduction pathways (Shinozaki and Yamaguchi-Shinozaki 2007). ABA is a phytohormone that regulates various plant growth and development aspects such as seed dormancy, germination, and control of stomata closure as well as in mediating responses to different environmental stresses (Nambara and Marion-Poll 2005). Water deficit condition triggers the accumulation of ABA in plants, which in turn activates signal transduction pathways that are involved in the activation of several genes involved in drought tolerance (Wasilewsk et al. 2008). Under such conditions, ABA promotes the expression of stress-responsive genes that cause growth inhibition, stomatal closure and/or accumulation of osmo-protectants. The role of ABA in stress tolerance was confirmed by ABA-deficient, -insensitive and -hypersensitive mutants (Xiong 2007).

On the other hand, genetical evidences revealed the existence of an ABA-independent stress responsive pathway that plays a major role in tolerance against drought, salinity and cold conditions (Shinozaki and Yamaguchi-Shinozaki 2000). A cross-talk between ABA-dependent and ABA–independent pathways was found to exist, regulating the gene expression of many abiotic stress responsive genes. Such responses are governed by specific genes and their expression is governed by a specialized set of proteins known as transcription factors. The cross-talk between both pathways was found to be regulated via *cis*-element found in many stress-responsive genes indicating that transcriptional regulatory variations play a major role in conferring stress tolerance in plants (Shinozaki and Yamaguchi-Shinozaki 2000). For instance, ABA and different abiotic stresses are known to induce the *H. vulgare abundant protein 1* (*HVA1*) resulting in the accumulation of late embryogenesis abundant (LEA) proteins such as LEA3 (Marttila et al. 1996). The overexpression of *HVA1* in different cereal plants was found to improve tolerance against different stresses (Nguyen and Sticklen 2013). In another study, the overexpression of *HvSNAC1*, a stress-responsive transcription factor, improved drought tolerance in transgenic barley plants at different developmental stages without causing any yield reduction (Al-Abdallat et al. 2014). The drought-inducible expression of *TaDREB2* and *TaDREB3* by using the maize Rab17 promoter in transgenic wheat and barley plants improved tolerance to multiple stresses with less impact on plant growth and development compared to the constitutive over-expression of these genes (Morran

et al. 2011). The overexpression of *TaCBF14* and *TaCBF15* from wheat in transgenic barley plants enhanced frost tolerance and low freezing temperature when compared with wild type spring barley plants (Soltész et al. 2013). Therefore, the identification of new stress tolerance genes using modern genomics and bioinformatics tools and sequence information in combinations with recent advances in genetic transformation and genome editing could have a major impact on barley productivity in dry areas around the globe (Gürel et al. 2016; Lawrenson et al. 2015).

4.4 Bi-Parental and Association Mapping to Dissect Abiotic Stress Response in Barley

Quantitative trait loci (QTL) bi-parental mapping and more recently genome-wide association mapping (GWAM) are used to dissect the genetic architecture of complex traits such as abiotic stress tolerance. Furthermore, second generation mapping resources like multi-parent advanced generation inter-cross (MAGIC) and nested association mapping (NAM) populations are also available to dissect complex traits. MAGIC population allows to: (i) use both linkage and association mapping, without the difficulties of highly structured populations, (ii) sample a greater proportion of genetic variability, (iii) have a population that segregates for multiple QTL/traits, (iv) model cytoplasmic effects (Cavanagh et al. 2008). Despite the privilege of having a MAGIC population, its application in barley is still in infancy. Nevertheless, MAGIC population approach has been successfully used to map major flowering time genes and to confirm its advantages in barley (Sannemann et al. 2015). NAM populations have been recently developed for autogamous species and they offer the advantage of investigating genomic regions with unprecedented genetic resolution by combining the advantages of linkage analysis and association mapping. In barley, NAM populations have been used to study agronomic traits like flowering time and salt tolerance (Maurer et al. 2015; Saade et al. 2016).

QTL mapping is powerful method to identify genes co-segregating with a trait either in F_2 lines, recombinant isogenic lines (RILs) and/or in double haploids (DH). Bi-parental QTL mapping has been intensely used in past years. However, this method suffers from several limitations: (i) only allelic diversity that segregates between the parents of the population can be assayed, (ii) only limited recombination events can be captured in a bi-parental population, and (iii) the usually large pleiotropic and epistatic effects involving major genes segregating within bi-parental crosses, limit our capacity to detect other loci with smaller effects. Relatively low resolution in QTL analysis typically produces large genetic intervals that complicate the determination of the best candidate gene(s) controlling the trait of interest (Balasubramanian et al. 2009). Although bi-parental mapping has been successful in identifying key genetic switches affecting abiotic stress responses, such as frost tolerance in barley (Francia et al. 2004), it can be argued that both the limited size and the genetic origin of the mapping populations capture only a portion of the genetic

diversity of the species (Visioni et al. 2013). Association mapping allows to overcome these limitations. Furthermore, the emergences of high-throughput genotyping platforms have enabled its implementation in crop plants. GWAM became very popular in recent years and is now routinely applied in almost all important crop species including barley. Barley, universally considered as a model crop of the *Triticeae* tribe including rye and wheat (Hayes and Szucs 2006), is a diploid autogamous crop plant where linkage disequilibrium (LD) is predicted to be extensive (Caldwell et al. 2006; Rostoks et al. 2006; Comadran et al. 2011). Therefore, medium resolution GWAM can potentially be used to capture significant genetic effects segregating in the cultivated gene pool. However, there are some complications in performing GWAM studies in barley arising from the inbreeding nature of the crop, in fact, non-random mating and selection cause population stratification (e.g. 2 rows vs. 6 rows or winter vs. spring growth habit) that may produce confounding effects. Population structure increases the chances of both false positives and false negatives, if not properly taken into account using the appropriate structure correction model (Wang et al. 2012; Pasam et al. 2012). The first report on association mapping in barley was on the identification of candidate genes for two simply inherited traits e.g. anthocyanin pigmentation (candidate gene Ant-2; Cockram et al. 2010) and lateral spikelet fertility (candidate gene INT-C; Ramsay et al. 2011). Both studies demonstrated that in the cultivated gene pool there is enough accumulated recombination to identify and functionally validate the candidate genes responsible for the traits. Both GWAM and bi-parental QTL mapping can be utilized as complementary approaches in a breeding program; GWAM can be used to identify the genetic basis of the trait investigated that can facilitate the choice of the parents to develop bi-parental populations for QTL analysis and fine-mapping and for mutagenesis and transgenics (Korte and Fallow 2013).

4.5 Mapping Studies for Frost Tolerance

Low temperature tolerance is induced by cold acclimation, which occurs during the induction of vernalization response, mediated by temperature and photoperiod sensitivity (under short day). Furthermore, cold temperature tolerance is gradually lost once plants switch from the vegetative to the reproductive phase (Galiba et al. 2009). The principal determinants of low temperatures tolerance in barley are the *Frost-Resistance* loci (*Fr-H1* and *Fr-H2*); both located on the long arm of chromosome 5H (Francia et al. 2004; Skinner et al. 2005; Galiba et al. 2009). *Fr-H1* co-segregates with *Vrn-H1* candidate gene *HvBM5A*. Various QTL for frost resistance for winter hardiness (crown fructan content, photoperiod sensitivity and low temperature tolerance) have been mapped on chromosome 5H in the Dicktoo × Morex mapping population (Hayes et al. 1993), the predicted position of *Vrn-H1* (Karsai et al. 1997). The coincidence of low temperature tolerance QTL with *Vrn-H1* has been an interesting focus of research due to the parallelism of *Vrn-H1* expression with both cold tolerance and flowering time. *Fr-H2* co-segregates with a cluster of CBF genes

(C-Repeat Binding Factors), a family of transcription factors involved in low temperature tolerance and in drought stress response (Vágújfalvi et al. 2003; Skinner et al. 2006; Tondelli et al. 2006; Francia et al. 2007). It has been debated in the past, especially in wheat, if the coincident positions of the QTL for frost resistance (Fr-1) and vernalization requirement (*VRN-1*) are due to true pleiotropy of the MADS-box gene, or tight linkage. Recently, Dhillon et al. (2010), using two *Triticum monococcum* mutants (*maintained vegetative phase; mvp*), demonstrated that the allelic variation at *VRN-1* is sufficient to determine differences in freezing tolerance and they suggest that the coincident position of QTL for frost tolerance and vernalization is due to pleiotropy rather than the effect of separate closely linked loci . The *Vrn-H1* mediate the genetic control of flowering time and it may have a role in down regulating the expression of *HvCBF* genes at *Fr-H2*, as suggested by Stockinger et al. (2007). Both vernalization and photoperiod genes play an important role in cold tolerance, the allelic combination of these two loci controls the beginning of reproductive phase that has an important effect on the degree of frost resistance (Turner et al. 2005; Trevaskis et al. 2003; Yan et al. 2003, 2004). More than 13 genes have been identified in the *HvCBFs* cluster on chromosome 5H (Tondelli et al. 2011). These genes encode for *HvCBFs* transcription factors which bind highly conserved regions at promoters of genes involved in drought and cold stress response (Stockinger et al. 1997; Liu et al. 1998; Skinner et al. 2006; Tondelli et al. 2006; Francia et al. 2007). A common problem for both spring and winter barley genotypes is reproductive frost tolerance. Late frost events often overlap with flowering time with negative effects on yield potential and grain quality of barley by damaging reproductive organs in the later stages of their development. QTLs for reproductive frost induced floret sterility and frost induced seed damage have been mapped in a multi-population study performed by Reinheimer et al. (2004). A QTL on chromosome 2HL for frost induced floret sterility was detected in two mapping populations out of three used; while a QTL on chromosome 5HL (position of the *Vrn-H1* gene) was mapped for both frost induced floret sterility and seed damage in all the three bi-parental populations used in the study (Reinheimer et al. 2004). Four association mapping studies have been published to date for frost tolerance in *Triticeae*, three on barley and one in rye. von Zitzewitz et al. (2011) performed a GWAM of winter hardiness traits using a set of accessions consisting of advanced breeding lines from the Oregon barley breeding program. This study identified significant associations with principal determinants of low temperature survival that have been studied for nearly two decades; the *Fr-H1* and *FR-H2* loci. Another study performed using a collection of diverse barley germplasm, representative of barley genetic diversity of the Mediterranean basin over an extended time period, revealed new significant associations for frost tolerance; located in genomic regions never reported before on chromosomes 1H, 2H, 3H and 6H. Two of the significant associations were closely linked to the already known *Fr-H2* and *HvBmy* loci on chromosomes 5H and 4H. A subsequent haplotype analysis revealed that most of the significant SNP loci are fixed in facultative and winter genotypes, while they are freely segregating in the spring barley gene-pool (Visioni et al. 2013). Exploring frost tolerance within unadapted spring gene pool, through association mapping revealed a major role of *Fr-H1/Vrn-H1* and *Fr-H2* loci. This

finding suggests that allele richness at these two loci might exist also in spring barley cultivars (Tondelli et al. 2014a). *Fr-H2* locus overlaps with *HvCBF* transcription factor that plays a major role in response to low-freezing temperature. In particular, a tandem duplication of two genes of the CBF family has been related with the *Fr-H2* QTL effect in the bi-parental mapping population 'Nure' ×'Tremois'mapping population (Knox et al. 2010; Tondelli et al. 2014a). Higher *HvCBF* copy number has been found in both barley and wheat genotypes harboring the recessive winter *vrn-H1* allele (Knox et al. 2010). Recently, variation in copy number of *HvCBFs* co-locating with *Fr-H2* has been investigated through RT-PCR by Francia et al. (2016). Their results are in part in accordance with previous studies and showed that genotypes with increased copy number of *HvCBF2A* and *HvCBF4* showed greater frost resistance. A third Frost-Resistance locus (*Fr-H3*) has been discovered by Fisk et al. (2013) on chromosome 1H, however, further studies are required to confirm the precise position of the locus in the genome and to identify the genetic determinants at this locus. In summary, the three main determinants of low temperature tolerance are *FR-H1*, *FR-H2* and *Fr-H3* in addition to several minor determinants reported by various authors (Fisk et al. 2013; Visioni et al. 2013; Tondelli et al. 2014a). Once the genetic determinants at *Fr-H3* locus will be identified, the variation at the three FR loci can be exploited for MAS in order to fix favorable alleles at those loci (Fisk et al. 2013).

4.6 Mapping Studies for Salinity Tolerance

Soil salinity is a major constraint to crops production because it decreases crop yield and restricts the use of agricultural land; FAO (2008) estimated that approximately 6% of total world land and 20% of total irrigated land is salinized and that poor irrigation management and climate changes are further increasing soil salinity (Athar and Ashraf 2009). Barley is the most salt-tolerant member of the *Triticeae* tribe, its higher level of tolerance depends on its rapid growth and fast phenological development that leads to early maturity under less favorable conditions (Walia et al. 2007; Munns et al. 2006). Salt tolerance is physiologically complex and shows the characteristics of multigenic trait, thus requires changes in many biochemical pathways and in all the major processes like photosynthesis, protein synthesis, energy and lipid metabolism (Parida and Das 2005). Plant responses to salinity are divided into an osmotic phase that inhibits growth of young leaves and an ionic phase, where senescence of mature leaves is accelerated. The level of salinity tolerance at the germination and seedling stages affects the initial plant stand and has been used in the past to screen plants. QTL analysis for salt tolerance, using two different mapping populations, showed that salt tolerance at these two different growing stages is controlled by multiple genes located at different loci (Mano and Takeda 1997). In past years, due to the scarce information about QTL controlling salt stress in literature, physiological traits have been used for screening salinity tolerant genotypes (Munns 2002) such as Na^+ and K^+ concentration in tissues (Chen et al. 2005) and K^+/Na^+

discrimination in ion transport (Chen et al. 2007). Recently, using different genetic approaches, many genes associated with salt tolerance have been identified. These genes have been divided into three groups: (i) genes enhancing osmotic protection and scavenging reactive oxygen species (ROS) radicals (Garg et al. 2002); (ii) genes involved in Na^+, Ca^+ and K^+ transport such as salt overly sensitive (SOS) involved in Na^+/H^+ antiport systems (Apse et al. 1999; Shi et al. 2000); and (iii) transcription factors functioning in signal transduction pathways such as C-Repeat Binding Factors (CBFs) (Wu et al. 2011; Morran et al. 2011). Growth decrease under saline conditions has been attributed to the accumulation of Na^+ in leaves at toxic levels (Mano and Takeda 1997; Shabala et al. 2010). While Cl^- is the principal anion in saline soils, it has detrimental effects on plants, reduces photosynthesis capacity and chlorophyll content (White and Broadley 2001; Tavakkoli et al. 2011). Nguyen et al. (2011) evaluated the effects of several ions and their associations with salt tolerance in 150 double haploids of the Steptoe × Morex mapping population (North American Barley Genome Mapping Project, NABGMP) by comparing shoot and root growth and their ion content between stressed and non-stressed plants. They found significant correlation between salt tolerance and ion contents (Cl^-, Na^+ and K^+) in shoots and roots. QTL analysis revealed significant QTL for both ion contents and salt tolerance on chromosomes 2H and 3H. Using a bi-parental mapping population, derived from the cross between the barley salt tolerant cultivar YYXT and the salinity sensitive cultivar Franklin, Zhou et al. (2012) identified 5 QTLs on chromosomes 1H, 2H, 5H, 6H and 7H accounting for more than 50% of phenotypic variations for salt tolerance at late seedling stage. Some of the QTLs mapped were coincident with the position of previously reported QTL for salt stress tolerance, while others corresponded with QTL for the same traits located in syntenous regions of both rice and wheat genomes. Further, advances on the understanding of salt tolerance in barley comes from association genetics studies. Through GWAS and haplotype analysis, Wu et al. (2011) identified a strong positive association between one haplotype of the gene encoding the transcription factor HvCBF4 and salt tolerance in Tibetan annual wild barley (*Hordeum vulgare* L. spp *spontaneum* and *H. vulgare* L. spp. *agricrithum*). In particular, this haplotype exhibited highly significant shoot dry weight and whole plant dry weight under salt stress, while no significant associations were found between other members of CBFs family and salt tolerance. GWAM for salt tolerance and ion contents performed using a panel of 192 genotypes from a wide geographical range identified two major QTLs for salt tolerance and related traits on chromosome 6H and for ion content on chromosome 4H (Long et al. 2013). The genomic region identified on chromosome 6H was strongly associated with salt tolerance and traits related with plant development and growth vigor under salt stress such as chlorophyll content, plant height, tiller number, and leaf senescence. On chromosome 4H, another strong QTL was detected related with ion content that overlapped with the position of other QTL for salt tolerance and yield related traits under saline conditions, such as number of spikes per plant and tiller numbers (Long et al. 2013). Genes involved in Na^+ exclusion or K^+/Na^+ discrimination have been reported in both bread wheat and durum wheat, respectively known as *Knal* and *Nax2* (Dubcovsky et al. 1996; Byrt et al. 2007). Huang et al. (2008) mapped *HKT1;5* (the candidate gene

for the *Nax2* locus) on chromosome 4H, where a QTL was reported by Long et al. (2013). It has been reported that *HKT1;5* results in salt tolerance and yield increase by 25% in saline soil (Munns et al. 2012). Shavrukov et al. (2010) reported a QTL on chromosome 7H for Na^+ exclusion that can be related with the *HvNax3* locus. Recently, a new QTL for salt tolerance was identified on the long arm chromosome 4H (Fan et al. 2016). Based on in silico analysis, two possible candidate genes were identified: a glutathione-regulated potassium-efflux system protein and respiratory burst oxidase-like protein. Both are related with Na^+ and K^+ homeostasis that plays an important role in salinity tolerance as reported by Munns and Tester (2008). A GWAM study designed to dissect flowering time under salt stress used the NAM population HEB-25 (Schnaithmann et al. 2014), which revealed that the wild alleles of flowering time genes *HvELF3* and *HvCEN* are associated with increased salinity tolerance and with reduced flowering time, resulting in increased thousand kernel weigh and grain yield, respectively (Saade et al. 2016). It is noteworthy that *HvCEN* has already been reported as the most frequently detected QTL associated with reduced flowering time and increased grain yield in a multi environment study performed in eighteen locations in the Mediterranean Basin in the 'Nure' × 'Tremois' bi-parental mapping population (Tondelli et al. 2014b). Furthermore, the same GWAS study reported another QTL located on chromosome 2HL with direct effect on grain yield under salt stress. Lines homozygous for the wild allele at this locus showed 30% more grain yield than the lines homozygous for the allele from the modern cultivar Barke.

4.7 Mapping Studies for Drought Tolerance

Among the different abiotic stresses, drought is by far the most complex and devastating on global scale. Causing the major crop losses worldwide, it continues to be a challenge to breeders. Furthermore, under the scenarios of global climate change, incidences of drought are expected to increase especially in semi-arid and arid regions (Pennisi 2008; Ceccarelli et al. 2010). Attempts have been made to dissect the molecular mechanisms underlying drought tolerance through several approaches such as QTL and association mapping, QTL cloning, functional genomics and transcriptomics, the success has been limited (Mir et al. 2012). This could be explained by the complex genetic basis of the trait, its co-occurrence with other abiotic and biotic stresses and by variability in timing, frequency and severity of drought (von Korff et al. 2008). Plants during their evolution have developed different strategies to cope with drought stress: (i) escape strategy via a short life cycle mostly mediated by photoperiod sensitivity and developmental plasticity; (ii) drought avoidance through enhanced water uptake capacity and reduced water loss; (iii) drought tolerance mediated by osmotic adjustment; and (iv) drought recovery via desiccation tolerance (Chen et al. 2010). Many morphological and physiological traits are found to be linked with drought tolerance, that could be dissected into several components (Karamanos and Papatheohari 1999; Cattivelli et al. 2002). In last twenty years,

many QTLs associated with drought tolerance and related traits have been reported in literature such as QTL for relative water content (RWC), a trait that shows positive correlation with grain yield in the *Triticeae* tribe. QTL for RWC stable across a wide range of environments were detected on chromosome 2H, 4H and 6H, in the population of recombinant isogenic lines (RILs) obtained from a cross between the two barley cultivars Tadmor and Er/Apm (Teulat et al. 2003). Osmotic adjustment (OA) is also considered an important adaptive trait for drought tolerance and, together with RWC contributes in increasing yield and yield stability under drought stress (Blum 1989; Matin et al. 1989). QTL for OA and related traits have been mapped on chromosomes 1H, 2H, 5H, 6H and 7H (Teulat et al. 1998, 2001). Using a RIL population derived from the cross between the cultivar 'Arta' and the genotype of *Hordeum spontaneum* 41-1, Baum et al. (2003) identified several QTLs on seven barley chromosomes for grain yield and other agronomic traits such as biological yield, days to heading and plant height under drought stress in Mediterranean environments. The same population was used by Guo et al. (2008) to map chlorophyll fluorescence parameters (indicators of the photosynthetic capacity during reproductive stage), which are positively correlated with crop yield (Rawson and Constable 1980). No coincident QTLs for fluorescence parameters were detected under well-watered and drought stress conditions, which suggested that the genetic control of traits related to photosynthesis differ under different water conditions. Carbon isotope discrimination (CID) provides a direct measurement of the ratio of dry matter produced to water transpired, also called transpiration efficiency; and it has been associated with drought tolerance in terms of water use efficiency and yield stability in drought prone environments. QTLs for grain CID have been mapped on chromosomes 2H, 3H, 6H and 7H in the RIL population from a cross between the cultivars Tadmor and Er/Apm. Most of these QTLs overlapped with the genomic regions where QTLs for physiological traits related to water status and/or osmotic adjustment were reported (Teulat et al. 2002). Only a few studies have been attempted to dissect drought tolerance using field trials (Baum et al. 2003; Talamè et al. 2004; Forster et al. 2004; von Korff et al. 2006; Comadran et al. 2008; Tondelli et al. 2013). In attempts to identify drought tolerance QTLs suitable for MAS, experiments have been conducted in greenhouses and in field in rainfed and well-irrigated environments, however, in most cases QTL effects were coincident with those detected in environments characterized by moderate to high rainfall. This could be due to the fact that those QTLs are underlying genes related with yield potential. Recently, advanced-backcross lines QTL analysis has been also used to identify novel QTLs related with drought tolerance traits such as proline content and leaf wilting and for yield and seed quality under terminal drought stress (Sayed et al. 2012; Kalladan et al. 2013). Sayed et al. (2012) identified a novel QTL for yield under terminal drought that co-localized with a QTL for drought tolerance index on chromosome 3H. The study of Kalladan et al. (2013) identified several QTLs for proline accumulation, with the most interesting located on chromosome 5H where the drought inducible exotic allele from the wild *H. spontaneum* accession ISR42-8 seems to increase proline accumulation by 53%. Two QTLs associated with leaf wilting decrease were also found in the same DH

population. This remarks the importance of barley wild relatives as potential donors of alleles to increase barley resilience to abiotic stress.

Despite association mapping is considered a powerful approach that is routinely used for quantitative traits dissection in cereal crops, its application on the study of drought stress response has been very scarce. A GWAM study for yield and yield components and developmental and physiological traits under well-watered and drought conditions was performed using a panel composed by cultivated and wild barley (Varshney et al. 2012). A few QTLs explaining low phenotypic variation were detected in only drought sites, and furthermore, these QTLs could not unequivocally related to drought tolerance when compared with QTLs previously mapped by traditional QTL analysis (Varshney et al. 2012). A more recent study indicated that GWAM could be effective for the identification of major QTL for complex traits such as drought tolerance (Wehner et al. 2015). Through GWAM approach, they studied effects of drought stress and drought-induced leaf senescence in barley plants in juvenile phase. Two major QTLs for both biomass yield and SPAD (indicator of leaf senescence) were identified on chromosomes 2H and 5H, the first was located at comparable position in other studies while the second was never reported before. If validated, these two QTLs may represent a starting point for MAS for drought stress at juvenile phase.

4.8 Future Perspectives

Barley is grown in many production systems tailored by different climatic and soil conditions, and also by end user requirements. The wide variability in yield, observed in different growing areas, depends on many factors including the occurrence of abiotic stresses and its combinations. A better understanding of the genetic factors controlling the ability of genotypes to cope with abiotic stresses and their interactions with the environment are of primary importance, especially in the context of predicted climate change scenarios and food security. Despite the continuous efforts of the barley scientific community in the past twenty years, the number of markers for abiotic stress that are currently used in MAS is scarce. MAS has had a limited impact in breeding for multi-genic traits with strong genotype by environment interaction. The limited impact of MAS on barley breeding in past years has been associated with the lack of appropriate type and number of molecular markers, and the lack of effectiveness of how to use markers in breeding programs. Recent advances in next-generation sequencing technologies and bioinformatics together with high-throughput phenotyping methods will increase our ability to identify loci related with abiotic stress tolerance, and in exploiting natural variation at these loci for breeding new varieties through marker assisted selection. Genomic selection (GS) has been implemented especially in animal breeding, and recent research shows its potential for plant breeding although more studies are required to identify the best strategy. Nevertheless, the recent advances in GS looks promising for solving the issue of

selection of multiple genes of small genetic effects for traits for which conventional selection is difficult and phenotyping is time consuming.

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Chapter 5
Innovative Role of DH Breeding in Genomics Assisted-Crop Improvement: Focus on Drought Tolerance in Wheat

H. K. Chaudhary, A. Badiyal, W. Hussain, N. S. Jamwal, N. Kumar, P. Sharma and A. D. Singh

Abstract Wheat production across world is greatly hampered due to huge fluctuations in water availability. There is a dire need to seek more efficient approaches towards genetic tailoring of crops for enhanced drought tolerance in a sustainable way and in less time. Wide hybridization in complementation with genomics-assisted doubled haploidy (DH) breeding, molecular cytogenetic tools and marker-assisted selection can help in quick identification and integration of drought-tolerant genes in wheat. Such approaches result in the genetic up-gradation of elite cultivars with high precision in a very short time span. Development of multiparent advanced generation inter-cross (MAGIC) populations is greatly facilitated by DH breeding for stable incorporation of desirable genes in elite wheat cultivars from a variety of sources. In this chapter, the authors discuss the significance of doubled haploidy breeding in sustainable wheat genome upgradation through its integration with advanced genomic tools for the development of widely adaptable drought resistant high yielding cultivars.

Keywords DH breeding · Genomic · *Imperata cylindrica* · MAGIC population Marker assisted selection · *Triticum aestivum*

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5.1 Introduction

Bread wheat (*Triticum aestivum*) is a globally important crop, accounting for 20% of the calories consumed by humans. It is most widely grown crop, covering more than 200 million hectares of land. The world population is estimated to reach 9.1 billion by the middle of this century, 34% higher than today, comprising of 70% urban population compared to 49% today. Since its domestication about 10,000 years ago, wheat has been continuously grown in the mainstream agriculture and today it comprises the major part of staple human diet and one of the important contributors of the food security mosaic, particularly in the developing nations (Graham and Vance 2003; Bohra et al. 2015). In view of the logarithmically growing demand for food, wheat grain production must increase at the annual rate of two per cent, without an additional land to become available for this crop (Gill et al. 2004).

Systematic wheat breeding began around 100 years ago, but farmers' efforts for the improvement of wheat strains by selective breeding can be traced back to the beginnings of agriculture almost 10,000 years ago. The 'Green Revolution' of the 1960s and thereafter a series of advances in agricultural research, technology and infra-structure triggered a drastic improvement in wheat yields. However, wheat production has struggled to meet global demand and an increasingly variable and unstable climate is adding to the problems of wheat supply. Moreover, in the modern agricultural systems, popular wheat cultivars are sown over wide area for the development of elite cultivars with various desirable agronomic traits such as high yield or superior quality and adaptable to a variety of diverse climates. This practice makes the plants inevitably exposed to a variety of stresses including abiotic and biotic stresses.

Among all abiotic stresses, drought is the most devastating one affecting crop productivity, and is characterized by the complicated interaction of various factors like limiting water availability, lower rainfall, altered precipitation patterns, reducing ground water level and increasing temperature (Toker et al. 2007). Drought stress is considered as the most widespread limitation to wheat productivity and stability in rain-fed production systems and has become an important problem due to increasing water shortages and uneven distribution of rainfall (Rustgi et al. 2013). Consequently, developing wheat cultivars with enhanced drought tolerance and high yield has been the focus of many wheat improvement programs. The limited success in improving drought resistance is primarily due to the difficulty in identifying and accurately measuring the key morpho-physiological determinants of yield under drought conditions (Maccaferri et al. 2009).

The wheat breeders across the world have to join hands to develop drought tolerant wheat cultivars that can sustain crop yield in normal as well as water-stressed conditions. Though the development of such cultivars has always remained the priority of breeders but relatively little breeding work has been carried out on improving crops for drought tolerance. Thus, there is a need to seek more efficient approaches for genetically tailoring crops for enhanced drought tolerance.

Improvement in drought tolerance of a crop through selection and breeding requires a substantial magnitude of heritable variation. Wheat has undergone numerous rigorous selection cycles during the course of domestication due to which the genetic variability has been greatly reduced in current germplasm. To maintain the variability in wheat, wild or close relatives of wheat in primary, secondary and tertiary gene pools have been extensively exploited for sustainable improvement in wheat. Introgression of favorable alleles, gene or gene complexes from wild relatives offers an excellent opportunity to enhance the genetic base of cultivated gene pool for various desirable traits. The wild species of wheat are still a valuable source of useful agronomic traits for the continued improvement of cultivated wheat. Numerous wild relatives of wheat have been evaluated for drought tolerance worldwide through various research agencies. Useful variation for this trait has been identified in *Triticum urartu*, *T. boeticum*, *T. dicoccoides* (Valkoun 2001) and *Aegilops geniculata* (Zaharieva et al. 2001). According to Skovmand et al. (2001), *Ae. tauschii* is the predominant source of variation for drought tolerance. Moreover, genotypes generated from hybridization with *Triticum* wild relatives have been found to produce high grain yield under both favorable and dry conditions. Under drought, the crosses identified to be better performers were those with *Triticum carthlicum, T. dicoccoides, Aegilops species, T. monococcum, T. polonicum*, and *T. dicoccum* (Tadesse et al. 2016). Wide hybridization of wheat with such related grasses coupled with cytogenetic manipulation of the hybrid material can be instrumental in introgressing the genes responsible for drought tolerance from such relatives into wheat. Wheat breeders have followed this approach in addition to the traditional methods across the globe. This has enabled the breeders to identify the genetic variability for drought tolerance among crop cultivars and introduce it into elite cultivars through different mating designs.

In recent years, advances in genetics and genomics have greatly enhanced our understanding of structural and functional aspects of plant genomes and have strengthened our ability to improve crop plants. Genomics approaches offer unprecedented opportunities to dissect complex traits such as drought tolerance and clone the identified genes underlying them. Genomic tools are facilitating the detection of quantitative trait loci (QTL) and the identification of existing favorable alleles of small effect, which have frequently remained unnoticed and have not been included in the gene pool used for breeding. Linkage and association mapping based on high density markers has allowed us to identify QTLs for traits that influence drought resistance and yield in wheat. Once major genes and QTLs that affect yield under drought conditions are identified, their cloning provides a more direct path for mining and manipulating beneficial alleles. While QTL mapping and cloning addressing natural variation will increasingly shed light on mechanisms of adaptation to drought and other adverse conditions, more emphasis on approaches relying on resequencing, candidate gene identification, ‘omics’ platforms and reverse genetics will accelerate the pace of gene/QTL discovery. Genomic selection that estimates marker effects across the whole genome provides a valuable option to improve wheat performance under drought conditions without prior knowledge of the relevant QTLs.

Breeders and molecular geneticists have routinely used populations derived from bi-parental crosses for variety development and mapping QTLs for drought related traits of interest. Typical populations used for QTL mapping include F_2, backcross (BC), doubled haploids (DH) or recombinant inbred (RI) populations derived from two parents. Among these, DH populations are more suitable for QTL mapping studies due to their complete homozygous nature and less time required for their development. One of the major hurdles in harvesting the desired fruits for successful introgressions after wide hybridization of wheat with distantly related species is segregation on advancement of generations. Marker-assisted breeding can help in identifying the segregants containing the introgressed alien chromatin but cannot control the recombination events. The segregation in further generations can lead to the loss of the newly introduced variations or generate novel combinations. To avoid loss of alien chromatin and harness the novelty of recombinants, the introgressed chromatin has to be fixed immediately. Doubled haploidy breeding offers a stable solution to the hurdle. It not only results in the attainment of homozygous population in just one step but also ensures that the manipulation is also integrated stably in the genetic complement of the crop. DH breeding accompanied with marker-assisted selection (MAS) can result in the upgradation of elite cultivars with high precision in a very short time span. These DH lines either can be directly released as varieties for general cultivation or used as parents in breeding programs or even in developing inbred lines for hybrid seed production in case of cross-pollinated species. The use of DHs in the study of quantitative trait loci is indispensable. DH lines are valuable and the most important material for quantitative inheritance studies as these are completely homozygous. This makes them best fit for studying quantitative inheritance as the size of population needed is far less than other types of nearly homozygous inbred lines. Important components of quantitative inheritance like number of genes controlling a quantitative trait, interactions among different genes, gene linkages, additive or additive × additive variances and chromosome locations can be studied using only DH lines in small grain crops.

The commonly used methods of DH production in wheat are anther culture (Ouyang et al. 1973; Chaudhary et al. 2003), wheat × maize (Zenkteler and Nitzsche 1984; Laurie and Bennett 1986; Chaudhary et al. 2002; Dhiman et al. 2012) and wheat × *Imperata cylindrica* (Chaudhary et al. 2005). The androgenesis-mediated haploid induction methods are not generally used in wheat improvement programs due to genotype specificity and poor response of wheat varieties to anther culture. The wheat × maize system, though genotype non-specific and more efficient approach of haploid induction in wheat has failed to produce haploids in wide hybrids like triticale × wheat and wheat × rye (Kishore et al. 2011). Wheat × *Imperata cylindrica*—mediated chromosome elimination approach of doubled haploidy breeding has been identified (Fig. 5.1) as most innovative and efficient alternative for haploid induction in wheat (Chaudhary et al. 2004, 2005, 2013a, b, 2014, 2015, 2016; Chaudhary 2007, 2008a, b, 2009, 2010a, b, 2011, 2012, 2013a, b; Kaila et al. 2012; Tayeng et al. 2012; Chaudhary and Mukai 2004; Rather et al. 2013, 2014; Mayel et al. 2015) as well as in durum wheat (Mahato and Chaudhary 2015). Similar to wheat × maize system, *I. cylindrica*-mediated system is also genotype non-specific and insensitive

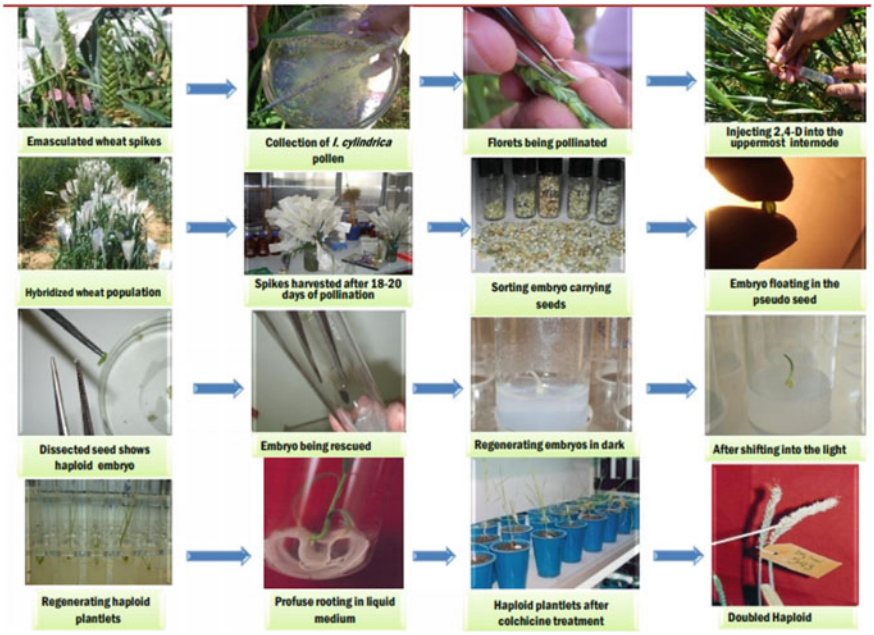

Fig. 5.1 Protocol of *Imperata cylindrica*-mediated wheat doubled haploid production

to crossability inhibitor genes. This novel system is capable of inducing haploids in wheat × rye and triticale x wheat derivatives, where maize was not successful (Pratap et al. 2005; Pratap and Chaudhary 2007a, b, 2012; Kishore et al. 2011; Badiyal et al. 2014, 2016; Jamwal et al. 2016).

Wide hybridization of elite wheat cultivar with a wild relative (carrying genes for drought tolerance) followed by MAS of plants carrying the desired genetic combinations and further development of doubled haploids from such plants in the F_1, advanced segregating generations as well as back crossed generations can lead to instant fixation of introgressed alien chromatin (including some rare recombinants) in the wheat genetic background. This chapter critically analyses how DH breeding can add new dimensions to genomics-assisted crop improvement in wheat with a focus on drought tolerance.

5.2 Doubled Haploids for Development of Bi-parental Mapping Populations

The success of genetic mapping largely depends upon the nature of mapping populations. Several types of mapping populations such as F_2 progenies, F_2 immortal populations, backcross (BC) progenies, recombinant inbred lines (RILs), doubled

haploids (DHs), near isogenic lines (NILs) and nested association mapping (NAM) populations have been used for linkage mapping analysis. Both F_2 and BC populations are simplest and easy to construct, but they are highly heterozygous and cannot be propagated indefinitely through seeds. Due to several advantages associated with DH population as mentioned before, they have been largely used in wheat breeding to map important genes related to drought. Numbers of studies have been conducted on wheat doubled haploids to locate the QTLs for drought tolerance using different molecular markers i.e. restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSR) (see Table 5.1). Wu et al. (2011) located number of QTLs for yield and related characters while working on wheat doubled haploids on all the chromosome of wheat genome except 1A, 4D, 5A, 5B and 6D. Despite their importance in drought tolerance, roots have attracted little attention in genetic studies. Nevertheless, Liu et al. (2013) identified 23 QTLs related to different traits of roots i.e. maximum root length, seminal root number, total root length, root area, root surface area, and seminal root angle under drought conditions.

5.3 Role of Doubled Haploids in Development of Multiparent Advanced Generation Intercross (MAGIC) Populations

The multiparent advanced generation inter-cross (MAGIC) population is one of the new generation of emerging mapping resources within plant genetics (Huang et al. 2015). In case of a MAGIC population, multiple parents (involving 4–20 parents) are used for the development of a mapping population (Cavanagh et al. 2008; Lehmensiek et al. 2009). MAGIC populations have been developed in wheat and are under development in many breeding programs (Gupta et al. 2010).

The development of a MAGIC population starts from the selection of founders based on geographic, genetic and phenotypic diversity. Mixing of parents is done through hybridization in a predefined pattern (see Fig. 5.2). Intercrossing is used for individuals derived from founder population for additional generations. Methods like selfing and double haploidization of individuals are involved either directly on hybrid founder population or after advanced intercrossing to form inbred lines. They are created by several generations of intercrossing among multiple founder lines. Multiple founders allow more allelic diversity to be captured than two parents in a typical bi-parental mapping population. Further, multiple cycles of intercrossing provide greater opportunities for recombination and hence, greater precision in QTL location. During the advanced intercross stages of MAGIC population development, double haploidization of individuals may be preferred to form inbred lines in a faster and easy way (Fig. 5.3).

Table 5.1 List of drought-related QTLs identified in wheat doubled haploid populations

S. no.	Traits	Cross	Method/Crop	Marker system	Chromosomal location	Reference
1.	Grain Yield	Hanxuan 10 (H10)/Lumai 14 (L14)	Anther culture	AFLP/SSR	1B, 4B, 6B	Wu et al. (2011)
		Hanxuan 10/Lumai 14	Anther culture	AFLP/SSR	1A, 5A, 6A	Liu et al. (2013)
2.	Canopy temperature	RAC875/Kukri	Maize	DArT/SSR	3A	Bennett et al. (2012)
3.	Flag leaf waxiness	RAC875/Kukri	Maize	DArT/SSR	4A	Bennett et al. (2012)
4.	Chlorophyll content	Opta/SH233	Maize	SSR	4A, 1B	Ilyas et al. (2014)
5.	Chlorophyll fluorescence kinetics	Opta/SH233	Maize	SSR	7A, 1B, 7D	Ilyas et al. (2014)
6.	Stem water soluble carbohydrate	Hanxuan 10/Lumai14	Anther culture	AFLP/SSR	1A, 1D, 2A, 4A, 4B, 6B, 7A, 7B	Yang et al. (2007)
7.	1000 grain weight	Hanxuan10/Lumai 14	Anther culture	AFLP/SSR	2A, 2D, 3A, 3B, 4A, 5B, 6A, and 7A	Yang et al. (2007)
		CS/SQ1	Maize	RFLP/AFLP/SSR	6B, 5B, 7B, 1D, 3D	Quarrie et al. (2005)
		Hanxuan 10 (H10)/Lumai 14 (L14)	Anther culture	AFLP/SSR	2D, 3A, 3D, 6A,	Wu et al. (2011)
8.	Grain filling efficiency	Hanxuan10/Lumai 14	Anther culture	AFLP/SSR	1B, 2A, 4A, 5A, 6A, 6B, 7B	Yang et al. (2007)
9.	Carbon isotope discrimination	Hanxuan 10 (H10)/Lumai 14 (L14)	Anther culture	AFLP/SSR	1A, 7A, 3B, 7D	Wu et al. (2011)

(continued)

Table 5.1 (continued)

S. no.	Traits	Cross	Method/Crop	Marker system	Chromosomal location	Reference
		Cranbrook/Halberd, Sunco/Tasman & CD87/Katepwa	Maize	DART/SSR/Biochemical	1BL, 2BS, 3BS, 4AS, 4BS, 5AS, 7AS and 7BS	Rebetzke et al. (2008)
10.	Number of spikes per plant	Hanxuan 10 (H10)/Lumai 14 (L14)	Anther culture	AFLP/SSR	1D, 2A, 6B, 7A and 7B	Wu et al. (2011)
		CS/SQ1	Maize	RFLP/AFLP/SSR	1A, 1B, 3D, 4A, 4B, 5A	Quarrie et al. (2005)
11.	Number of grains per spike	Hanxuan 10 (H10)/Lumai 14 (L14)	Anther culture	AFLP/SSR	4A, 3A, 6B, 6A	Wu et al. (2011)
12.	Biomass	Hanxuan10(H10)/Lumai14(L14)	Anther culture	AFLP/SSR	4A, 7A	Wu et al. (2011)
13.	Grain per ear	CS/SQ1	Maize	RFLP/AFLP/SSR	5A, 7A,7B, 4A, 4B, 2B, 1A, 1B	Quarrie et al. (2005)
14.	Heading date	Cranbrook/Halberd,Sunco/Tasman &CD87/Katepwa	Maize	DArT/SSR/Biochemical	2DS, 4AS and 7AL	Rebetzke et al. (2008)
15.	Plant height	Cranbrook/Halberd,Sunco/Tasman & CD87/Katepwa	Maize	DART/SSR/Biochemical	1BL, 4BS and 4DS	Rebetzke et al. (2008)
16.	Maximum Root length	Hanxuan 10/Lumai 14	Anther culture	AFLP/SSR	2D, 3A, 5B	Liu et al. (2013)
17.	Seminal root number	Hanxuan 10/Lumai 14	Anther culture	AFLP/SSR	2B, 3B, 7A	Liu et al. (2013)
18.	Total root length	Hanxuan 10/Lumai 14	Anther culture	AFLP/SSR	1A, 3B, 5B	Liu et al. (2013)
19.	Root area	Hanxuan 10/Lumai 14	Anther culture	AFLP/SSR	2B, 3B, 5B	Liu et al. (2013)
20.	Root surface area	Hanxuan 10/Lumai 14	Anther culture	AFLP/SSR	2B, 3B, 5B	Liu et al. (2013)
21.	Seminal root angle	Hanxuan 10/Lumai 14	Anther culture	AFLP/SSR	2B, 3B	Liu et al. (2013)

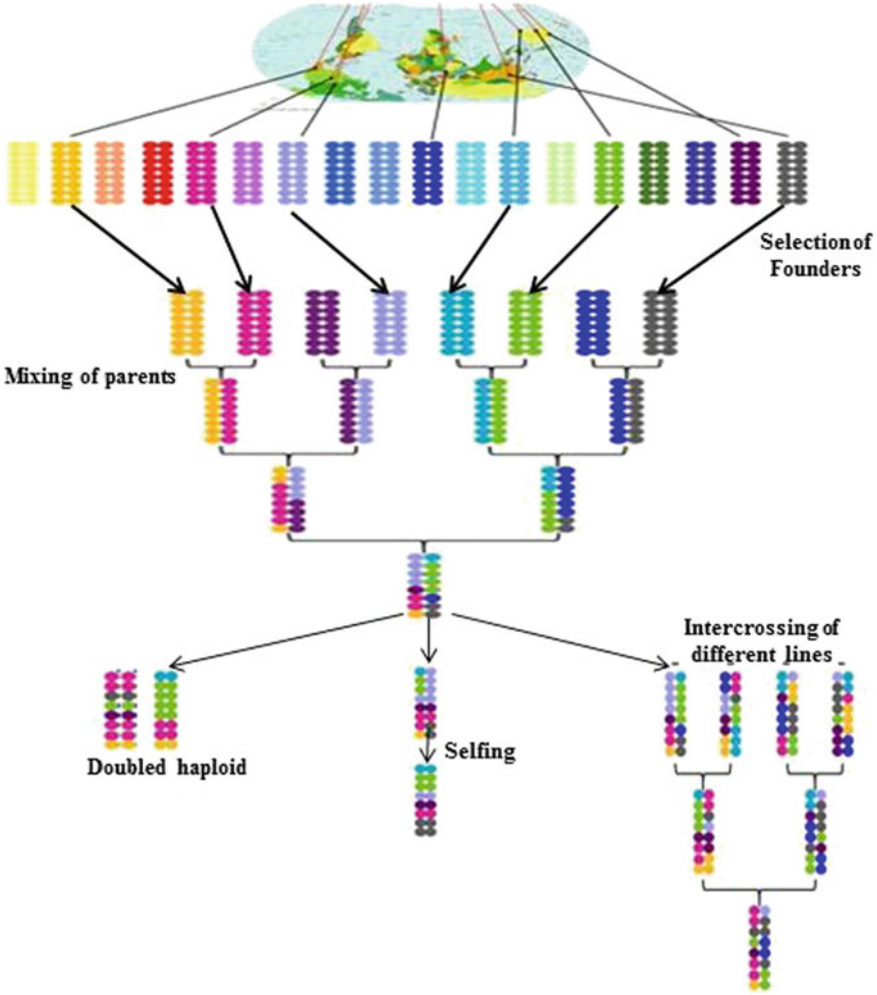

Fig. 5.2 Stages of MAGIC population development design. *Source* Huang et al. (2015)

5.4 Doubled Halpoidy Breeding and Genomic Selection

Traditionally, QTL mapping has been used to identify markers linked to traits. Another approach used to identify markers linked to traits is association mapping in which populations with broad diversity are used. Although these methods are useful in identifying markers linked to traits, their application in breeding programs is limited (Bernardo 2008). This is mainly because the individual marker effects are often small, especially for complex quantitative traits, which are influenced by many genes. In a landmark article, Meuwissen et al. (2001) proposed a new method termed

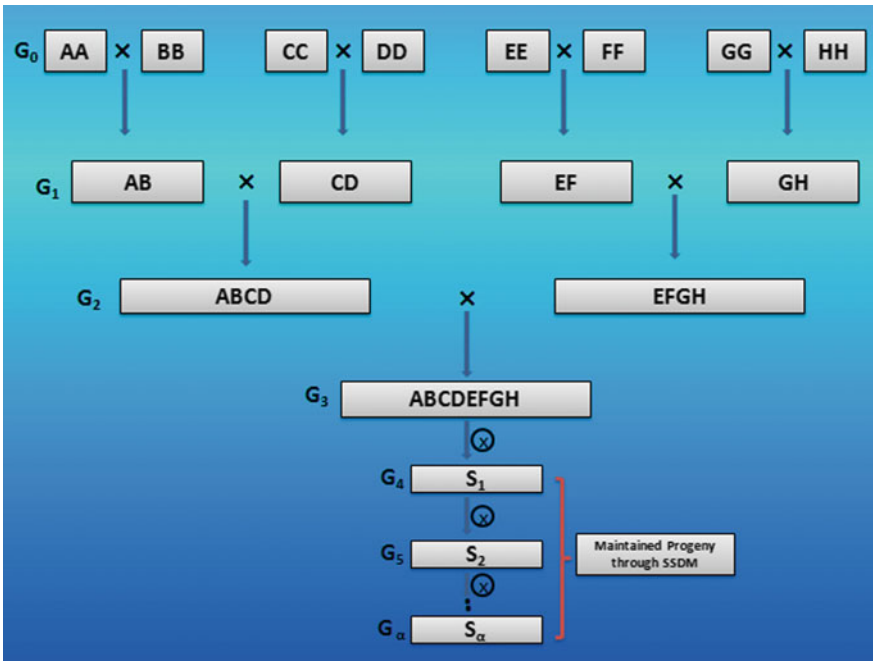

Fig. 5.3 Development of multiparent advanced generation intercross (MAGIC) populations. *Source* Bandillo et al. (2013)

'Genomic Selection' (GS), which uses information from genome-wide markers to predict phenotypes. GS is a revolutionary approach where a breeder's selection is made based on genomic estimated breeding values (GEBVs) obtained from genome-wide DNA marker information. It involves the development of training populations with which to model selection criteria for lines (testing population) within a breeding program. The immortal nature of doubled haploid populations makes them useful for generating data for modeling as is needed for the application of genomic selection. The greater precision in phenotyping attributed to doubled haploid populations should improve the resolution of genomic selection models. Doubled haploid mapping populations in hexaploid wheat have been recently used for comparison of genomic selection models to predict the flowering time and spike grain number under controlled and osmotic stress treatments.

5.5 Genomics-Assisted Breeding to Improve Drought Tolerance in Wheat

After developing adequate mapping population, the next step towards the development of drought tolerant crops is the dissection of drought related traits at molecular as well as phenotypic level. In the recent years, the most imperative science i.e. 'omics' has emerged to play a significant role in crop improvement by facilitating the identification of genes, proteins, and metabolites associated with drought tolerance and also by characterizing their functions (Zargar et al. 2011). Further, advancement in genome mapping and functional genomics technologies has provided new powerful tools for molecular dissection of drought tolerance (Worch et al. 2011). Identification of molecular markers and/or candidate genes associated with drought tolerance/avoidance-related traits provides a better understanding of the molecular basis of drought tolerance and once validated, they can be used in molecular breeding. But for the utmost utilization of molecular markers/candidate genes, precise monitoring of crop at genotypic as well as phenotypic level is required.

The outline of breeding procedure that can be implemented to breed for drought tolerance related traits in wheat in genomics era is given in Fig. 5.4. First and foremost there should be enough variability for these traits in available germplasm. The relevant germplasm can be evaluated and screened for all the traits at multiple locations trials to identify the best and stable lines. These best lines can be utilized to develop the mapping population like bi-parental, association mapping panels etc. After the development of mapping populations, various genetic and genomic tools can be used to identify the key genes underlying these traits and incorporate these genes into desirable cultivars.

5.6 Application of 'Omics' of Drought Tolerance for Precise Genotyping

The established tools of genomics are molecular markers. Since their first use in the study of genetics of agricultural plants, these have enabled discrimination of cultivars and breeding lines and thus have offered the scientific community with most powerful tools to monitor, track and exploit sequence variation in germplasm. Many types of markers have been developed and they are an essential part of structural and functional genomics and for molecular breeding (Varshney et al. 2007). Microsatellite markers or single sequence repeats (SSR) have proved useful in wheat research since they offer reproducibility, multi-allelic nature, co-dominant inheritance, genome specificity, relative abundance and good genome coverage (Varshney et al. 2005; Ganal and Roder 2007). Utilizing them, breeders have localized many stress-related candidate genes on chromosomes (Roder et al. 2004), and have identified various QTLs controlling yield and quality traits (Ganal and Roder 2007) to use them in developing new varieties by MAS or marker-assisted backcrossing (Donini

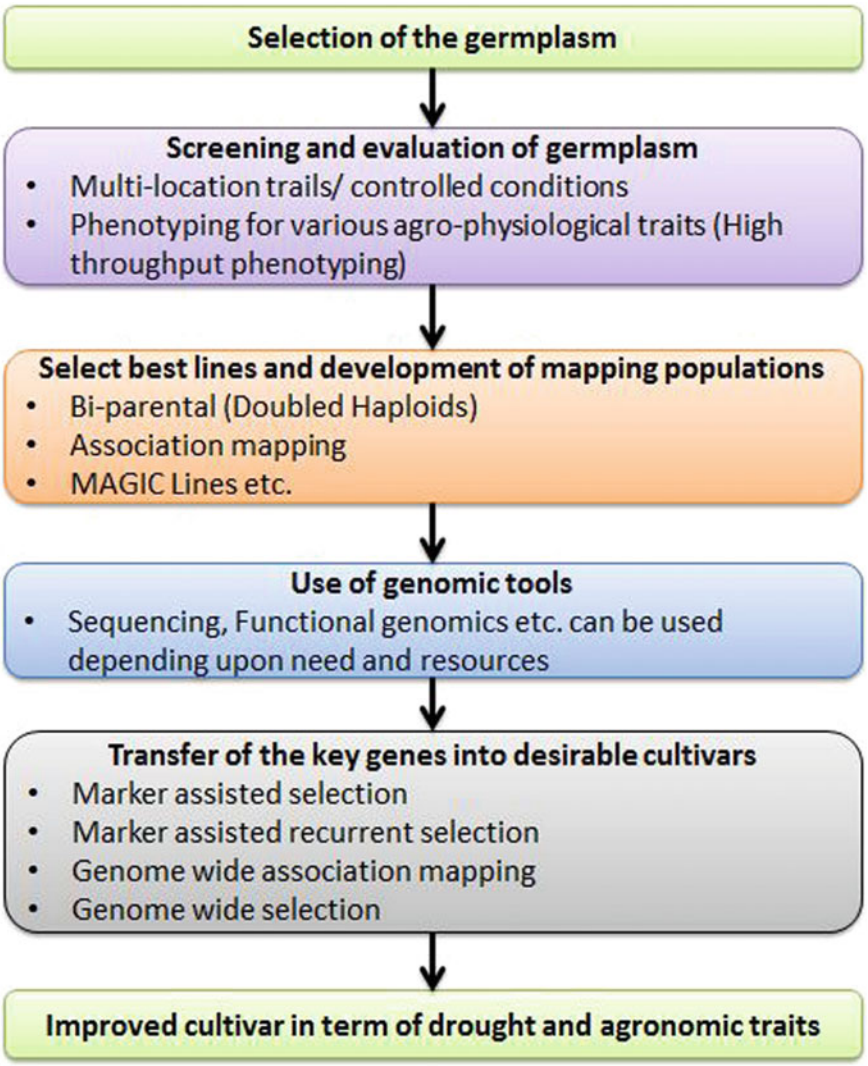

Fig. 5.4 Outline of general overview how to breed and develop improved wheat cultivar for drought tolerance

et al. 2000; Roder et al. 2004). Besides SSRs, another promising type of marker is single-nucleotide polymorphism (SNP), which are widely distributed across the genome and amenable to high multiplex detection systems (Ganal and Roder 2007). The identification of SNP markers depends on comparative sequencing of lines or analysis of expression sequence tags (ESTs).

5.7 Next Generation Sequencing (NGS) Approach

In recent years, the evolution of next generation sequencing (NGS) technologies has enabled many exciting opportunities for crop research in plants with or without a reference genome. Availability of reference genome/transcriptome sequence greatly enhances our ability to decipher the underlying molecular mechanisms of a trait, understand the gene regulatory mechanisms, determine gene expression differences and variations in expressed gene sequences and other structural variations such as copy number variations (CNV) and presence-absence variations (PAV). NGS approach offers an answer to a wide variety of difficult and cumbersome problems such as sequencing of complete genomes and transcriptomes and genome-wide analysis of DNA-protein interactions (Bräutigam and Gowik 2010). NGS along with other accessory technologies can be used for whole genome sequencing, transcriptome sequencing, genome-wide and candidate gene marker development, targeted enrichment of crops and other applications for the sustainable crop improvement. Analysis of NGS data obtained from genome-wide association studies, transcriptomics and epigenomics in combination with data from proteomics, metabolomics and other 'omics' can provide an integrative biology approach to understand the regulation of complex traits like drought. Wheat genome, being hexaploid, large and complex than other crops, could not be explored efficiently using genomic tools but recent advances in new DNA sequencing technologies (454, Solexa, and SOLiD) (Pettersson et al. 2009) will enable low-cost SNP discovery over larger genomic regions in such species.

5.8 Understanding Drought Tolerance Through Precise Phenotyping

After establishing the most suitable target trait for selecting grain yield under drought stress, the next step is to establish the correlation between the candidate gene/genes and their morphological as well as physiological accomplishment as a trait which is tightly connected to yield (Tuberosa 2010). Genetic improvement under drought can be achieved through direct or indirect selection for yield in the target environment (Ceccarelli and Grando 1996; Araus et al. 2008). Though it seems simple to implement but low heritability and high genome × environment (G × E) interactions pose great hurdles in breeding for genetically improved crops under drought-stressed environments. Though, advancement during the recent years in the area of computational biology, bioinformatics and genomics have helped us to understand the genetic basis of drought tolerance yet a direct correlation between the genotype and phenotype could not be established due to a much slow progress in the area of phenomics (Xu and Crouch 2008; Passioura 2010).

Thus, a complementary strategy has been adopted to target traits closely correlated with yield and yield potential and this has been termed as indirect selection, analytical or physiological breeding. Crop physiological studies on cereals under drought environments have identified several indirect traits that can be considered for physiological breeding: radiation and water use efficiency, green leaf duration, harvest index, rate of senescence, grain fill duration, leaf area index, deep roots, vigorous crop establishment, stem-reserve utilization and maintaining cellular hydration (Araus et al. 2008; Reynolds and Tuberosa 2008). Other 'constitutive' type traits have also been selected and have proven very useful in escaping drought such as time to flowering.

It becomes very cumbersome to generate and maintain the data pertaining to aforesaid traits when the population size is large (thousands of plants). Hence, now-a-days the traditional methods of plant phenotyping are being replaced by high-throughput precise and non destructive imaging techniques which include: (i) infrared cameras to scan temperature profiles and transpiration, (ii) fluorescent microscopy and spectroscopy to assess photosynthesis and photosynthetic rates, (iii) three-dimensional cameras to record minute changes in growth responses, (iv) lidar (light detection and ranging) to measure growth rates and (v) magnetic resonance imaging to examine root or leaf physiology (Finkel 2009; Gupta et al. 2012). Digital imaging allows us to monitor, measure and track many aspects of plant development, function and health that were unimaginable using conventional measurement techniques.

Several software programs have been developed for extracting data from digital images taken from roots, shoots, leaves, seeds and grains (Sozzani and Benfey 2011; Cobb et al. 2013). The already developed as well as the futuristic phenomics tools will allow the scanning of thousands of plants in a working day, similar to high-throughput DNA sequencing in the field of genomics (Finkel 2009). The precise and accurate data generated from these facilities is very important and useful for meaningful genetic dissection of complex traits and finds immense applications in various crop improvement programs. One of such high-throughput integrated phenotyping platforms was developed by Lemna Tec, a German company (http://www.lemnatec.com) that includes the pipeline of imaging, image processing automatization and data handling modules. The platform has the capacity to measure almost unlimited sets of parameters easily, allows comprehensive screening and provides statistics on various plant traits in a dynamic way. Depending on the degree of automatization, plants are manually placed on the 3D Scan analyzer or transported via conveyor belts directly from the greenhouses to the imaging chambers. Such chambers provide top and side imaging of both shoot and root systems to quantify plant height/width, biomass and plant architecture.

5.9 Discovery of QTLs Responsive to Drought-Related Traits

After high throughput genotyping and precise phenotyping, data is collected and analyzed for the construction of genetic maps and locate QTLs responsible for drought-related traits. A large number of studies involving linkage mapping have been conducted in several crops to identify QTLs linked to drought tolerance (Cattivelli et al. 2008; Fleury et al. 2010). However, linkage mapping-based QTL mapping does not provide precise information on QTLs because of limitations such as (1) insufficient time for recombination to occur, (2) insufficient phenotypic variation for the trait present in the mapping population and (3) segregation of different QTLs for the same trait in different mapping populations. To overcome some of the above constraints, linkage disequilibrium (LD)-based association mapping has been utilized as an alternative for QTL mapping in crop species (Myles et al. 2009; Rafalski 2010). The technique involves natural population/germplasm collection instead of mapping population for locating the desired QTLs. Using association mapping, several markers linked to drought tolerance traits have been identified in wheat (Sanguineti et al. 2007; Maccaferri et al. 2011), barley (Ivandic et al. 2003; Baum et al. 2007; Varshney et al. 2012) and maize (Lua et al. 2010).

In summary, QTLs for drought tolerance have been identified for several major and important crop species like rice, maize, wheat, barley, sorghum, pearl millet, soybean and chickpea. These QTLs were identified for a variety of important traits including: (1) yield and yield contributing traits under water-deficit conditions (in the case of wheat, maize, rice, soybean and pearl millet), (2) physiological responses including water-soluble carbohydrates, carbon isotope ratio, osmotic potential, chlorophyll content, flag leaf rolling index, grain carbon isotope discrimination, relative water content, leaf osmotic potential, osmotic adjustment, chlorophyll and chlorophyll fluorescence parameters to drought stress (in the case of wheat, maize and rice), (3) flowering time including anthesis to silking interval (in maize), (4) root traits (rice, maize, wheat, soybean and chickpea), (5) stay green (sorghum) and (6) nitrogen fixation (soybean).

However, QTLs identified through linkage mapping-based approaches have been found to be located in 10–20 cM intervals. Such short intervals span several hundred genes and hence identifying the right candidate gene(s) with its significant effect is very difficult and cumbersome. So positional cloning of QTLs have been undertaken in several crop species (Salvi and Tuberosa 2005; Tuberosa and Salvi 2006).

5.10 Marker-Assisted Selection

Several QTLs have been identified for drought tolerance in wheat (Rebetzke et al. 2008; Wu et al. 2011; Liu et al. 2013). Several major loci for yield under different environmental regimes have been mapped which are tightly linked to QTLs for late

senescence of the flag leaf in winter wheat (Verma et al. 2004). However, the major limitation in useful execution of MAS for drought tolerance is the complex nature of drought-associated traits and the segregation of the traits on advancement of generations. Hence, the marker-assisted recurrent selection (MARS) approach, which involves intermating selected individuals in each selection cycle, has been recommended (Eathington et al. 2007; Ribaut and Ragot 2007; Bernardo 2008). It generally involves the use of an F_2 base population and can be used in self-pollinated crops like wheat, barley and chickpea for developing pure lines with superior per se performance (Bernardo 2008). Since MAS is practiced in each cycle following intermating, it overcomes the limitation of inadequate improvement in the frequency of superior alleles in F_2 enrichment (Eathington et al. 2007). MARS for water use efficiency is being exercised in wheat under an Indo-Australian project involving partners from Directorate of Wheat Research, Karnal; Punjab Agricultural University, Ludhiana; Indian Agricultural Research Institute, New Delhi and University of Technology Sydney and Department of Industry, Innovation, Science, Research and Tertiary Education (DIISRTE), Australia. Generation Challenge Programme (GCP) also launched a challenge initiative to improve heat/drought tolerance in wheat through MARS approach involving the IARI, New Delhi, India, Chinese Academy of Agricultural Sciences (CAAS), China and partners from Australia (http://www.generationcp.org/ci_feb_2010_launch_meeting_feature).

Sometimes undesirable or deleterious genes are also accompanied with QTLs from the donor genotypes (linkage drag), which can be harmful to the field performance of the resulting hybrids. This can be minimized through marker-assisted backcrossing (MABC). Following this approach superior lines or cultivars have been developed that contain only the major gene/QTL from the donor parent, while retaining the whole genome of the recurrent parent (Hospital 2003; Varshney and Dubey 2009; Gupta et al. 2010). Although MABC has been used extensively for introgressing resistance to biotic stresses, only a few reports are available on the use of MABC to develop the superior lines/varieties for drought tolerance (Gupta et al. 2017).

5.11 Genome-Wide Selection (GWS)

Genome-wide selection (GWS) or genomic selection (GS) is another important approach to develop superior cultivars with overall excellent performance in a target environment. It utilizes genotyping with genome-wide markers instead of selected markers. In this approach, individuals in a phenotyped population (generally called as the 'training population') are genotyped using genome-wide markers and breeding values of alternative alleles of all the markers are fitted as random effects in a linear model. Individuals in subsequent recurrent selection generations are then selected based purely on the sum of those genomic estimated breeding values (GEBV); Meuwissen et al. 2001). Therefore, GWS reduces the frequency of phenotyping and similarly also increases annual gains from selection by reducing cycle time (Rutkoski et al. 2010). Several scientific groups across the world have recently started

exploring the GWS approach in both self- and cross-pollinated crops with some modifications for both types of crops (Bernardo 2010). The success of the GWS approach is dependent on the availability of a diverse and representative training population. This approach has recently been used to improve durable stem rust resistance in wheat (Rutkoski et al. 2010) and subsequently can be explored to bring together different components of multigenic drought tolerance using the GWS approach.

5.12 Future Strategies

Research in the last three decades has come up with three approaches (viz., plant physiology, molecular genetics and molecular biology) which contributed significantly to the crop improvement programs. The integration of molecular biology and genetics with physiology has led to the identification of the most relevant loci controlling drought tolerance and their respective phenotypic expression. But the challenge in front of breeders is to develop drought tolerant cultivars without compromising their yield potential. Hence, drought tolerance traits should be tested in both stressed and non-stressed environments before being introduced in a MAS breeding program. QTLs for drought-related traits which are closely linked with QTLs for yield potential should be considered as priority targets for MAS. Once the target traits have been identified and introduced in MAS breeding program, these should be integrated stably in the wheat genetic background using advanced breeding approaches like doubled haploidy breeding technique. This integrated approach will lead to the development of stable drought tolerant elite cultivars which will ensure the swift evolution of agriculture in the direction of fulfillment of the food supply of the increasing population.

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Chapter 6
Genomics Assisted Approaches for Improving Abiotic Stress Tolerance in Forage Grasses

Leif Skøt, Rhys Kelly and Mike W. Humphreys

Abstract Ryegrasses, such as Perennial, Italian and Hybrid ryegrass are globally important forage crops in cool season livestock agriculture, and make up most of the acreage used in grassland agriculture. These forages are grown chiefly in Northwest Europe, New Zealand and temperate regions of Japan, Australia, South Africa and South America. Regions dominated by permanent grassland tend to have reasonably high annual rainfall, while lower rainfall regions are dominated by arable crops. However, extreme and unpredictable weather events are likely to occur more often as a result of climate change. This may include dryer hotter summers, and wetter winters. Ryegrass forage crops would thus be exposed to a wide range of abiotic stresses, including drought, cold, flooding and even heat. Generating varieties which can perform well in response to all these diverse stresses is thus an important and difficult challenge for grass breeders. The advent of low cost, high throughput next generation sequencing and genotyping technologies provide new opportunities to increase the speed with which genetic improvement can happen. The availability of high density genotyping platforms makes genomic selection in forages a realistic prospect. They can also be used with great effect in marker-assisted backcrossing strategies to introgress desirable traits from ecotypes or other donor material. The ability of ryegrass and fescue to hybridize, opens up further opportunities for generating new genetic combinations with beneficial characteristics in terms of abiotic stress tolerance from fescue background with the traditional ryegrass properties in terms of forage quality and biomass yield.

Keywords Dehydrins · *Festuca* · *Festulolium* · Forage crops · Fructans · *Lolium perenne* · LEA · Photoinhibition · Ryegrass

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6.1 Introduction

Forage grasses are of key importance providing food and feed for livestock, hay or silage. FAO estimates that around 70% of agricultural land area is used for pasture and fodder crops (http://www.fao.org/ag/agp/agpc/doc/grass_stats/grass-stats.htm). Apart from providing livelihoods for millions of livestock farmers, forage grasses serve many other useful purposes in terms of carbon sequestration, maintenance of biodiversity and provision of wildlife habitats. Given their perennial life cycle, forage crops are highly likely to be exposed to abiotic stresses such as drought, temperature, salinity or flooding at some point. Climate change is likely to exacerbate such events and/or make them more frequent. This can have serious consequences for crop productivity, as well as a big threat to the areas that can be used for forage. Development of varieties that are better at withstanding abiotic stresses is thus an important breeding target.

Forages are predominantly outbreeding and often polyploid, which introduces more complexity to the breeding effort and for elucidating the genetic and molecular mechanisms underlying important complex agronomic traits. Forage crop breeding is generally more resource poor compared to some of the major cereal crops, like rice, maize and wheat. Furthermore, there are many targets to consider in a forage breeding programme, including biomass yield and productivity, forage quality, digestibility, seed yield in addition to biotic and abiotic stresses. This has been suggested as a contributing cause of the generally slower rate of progress in breeding compared to the major cereals (Wilkins and Humphreys 2003; Casler and Brummer 2008; Conaghan and Casler 2011).

Drought, heat, frost and salinity can all lead to water deficit, and this is a major determinant of how plants are distributed geographically. Plants have developed different strategies to deal with water stress (Levitt 1972). Some escape by flowering prior to onset of drought for example. Resistance mechanisms involve avoidance and tolerance. Plants that avoid water deficit do so by either saving or spending water at different stages/time of their life cycle. Water saving plants prevent water loss by minimising stomatal opening during the day, while water spending plants maintain high transpiration rates by accessing water resources usually via large root systems. Tolerance to water deficit can be achieved by maintaining the osmotic potential via synthesis of osmolytes or compatible solutes. Alternatively, repair mechanisms can be used to maintain cell integrity. While the escape strategy is difficult to achieve for perennial crops, the other mechanisms listed above are employed by forage grasses to deal with water deficit. A significant review was published in 2006 dealing with molecular and genomic studies on stress tolerance in forage grasses (Zhang et al. 2006). Here, we will try to review progress since then, with particular emphasis on how the advent of next generation sequencing (NGS) and genotyping is impacting on our understanding of the traits and efforts to breed more resilient forage grass varieties.

6.2 Next Generation Genomic Tools and Resources for Forage Crops

The advent of NGS technology and high throughput genotyping is paving the way for genomics-assisted breeding not only in the major cereals and pulses, but also in a wide array of crop species (Varshney et al. 2014, 2016). Among the forage grasses, genome assemblies are available for perennial ryegrass (*Lolium perenne* L.) (Byrne et al. 2015; Velmurugan et al. 2016). Genome assemblies of two other members of the Pooideae subfamily, barley (*Hordeum vulgare*) (IBGSC 2012) and *Brachypodium distachyon* (IBI 2010) are serving as useful tools for comparative mapping and synteny analysis (Pfeifer et al. 2013). Additionally, a number of transcriptome assemblies have been produced by RNA-seq technology for various purposes (Dinkins et al. 2012; Studer et al. 2012; Bushman et al. 2016; Stočes et al. 2016). High throughput genotyping methods have been developed, including restriction site-associated DNA (RAD) sequencing (Baird et al. 2008), genotyping-by-sequencing (GBS) (Elshire et al. 2011) and diversity arrays technology (DArT) (Jaccoud et al. 2001; Kopecky et al. 2009), all of which have been used in forage grass research and breeding. Together with ryegrass SNP arrays (Blackmore et al. 2015, 2016; Grinberg et al. 2016), these methodologies have almost completely replaced low throughput methods like amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and simple sequence repeats (SSR) methods. Only the latter is still used significantly in plant genetics, mapping and quantitative trait locus (QTL) analysis. High throughput genotyping is providing detailed characterization of germplasm and genetic resources, and for genome-wide association studies (GWAS) of populations for identification of novel allelic variation and mapping of useful traits. Whole genome resequencing is now used in the major crops for germplasm characterization, GWAS and genomics-assisted breeding (Lam et al. 2010; Huang et al. 2012; Mace et al. 2013; Lu et al. 2015). In the near future whole genome resequencing and pan-genomes will be more common even in less resource rich crops such as forages.

6.3 Cold Stress and Freezing Tolerance

6.3.1 Photoinhibition

Freezing temperatures and ice can cause water deficit in plants. When ice forms in the extracellular space, water moves along the water potential gradient from the cytoplasm to the extracellular space causing depletion of water intracellularly. For perennial species growing in climates with cold winters, exposure to low non-freezing temperatures in the autumn is the key to their acclimation to freezing and winter hardiness. This process involves changes in gene expression, morphology and physiology. Recent progress on mechanisms of freezing tolerance, cold acclimation and

de-acclimation in some of the most important temperate forage grasses such as fescue (*Festuca* spp.), ryegrass (*Lolium* spp.) and timothy (*Phleum pratense* L.) has been reviewed (Sandve et al. 2011; Kovi et al. 2016). The complex nature of this trait is highlighted by the fact that plants need to respond not only to water deficit and ice crystal formation, but also to the imbalance between the reduction of enzymatic reactions of the photosynthetic carbon pathway and the rate of electron transfer. This leads to over-excitation of photosystem II (PS II), and triggers photoinhibition of PS II. There is evidence to suggest some link between winter survival and frost tolerance with improved photosynthetic acclimation in androgenic populations derived from hybrids of tetraploid *Festuca pratensis* and *Lolium multiflorum* parents (Rapacz et al. 2004). Subsequent work appear to suggest that in *F. pratensis* a non-photochemical quenching mechanism provides it with tolerance to low temperature photoinhibition, while in *L. multiflorum*, increased photochemical quenching was predominant (Humphreys et al. 2007). Better frost tolerance was observed in a *L. multiflorum* line containing an introgression of a segment of chromosome 4 from *F. pratensis* than in the *L. multiflorum* parent, possibly due to changes in the non-photochemical mechanism providing the photosynthetic acclimation (Humphreys et al. 2007).

6.3.2 Fructans and Frost Tolerance

Fructans are fructose polymers of sucrose, and occur widespread in plants, and they are the most important storage carbohydrate in temperate grasses (Chatterton et al. 1989; Morvan-Bertrand et al. 2001; Hisano et al. 2008). The idea that fructan accumulation might be associated with cold acclimation in temperate grasses has been around for a long time (Eagles 1967; Pollock et al. 1988; Eagles et al. 1993). More recently, transcriptomics work, transgenic experiments, genetic, molecular and biochemical research have provided strong evidence to support a role for fructans in cold acclimation and frost tolerance (Livingston et al. 2009). Fructans are readily polymerised and depolymerised, and the partitioning of solutes was suggested as a mechanism to increase the survival of apices and lateral buds in grasses due to the osmotic effect of the hexoses generated by mobilization and depolymerization (Pontis 1989; Eagles et al. 1993). Expression analyses of perennial ryegrass indicate an important role for genes involved in fructan biosynthesis and metabolism during cold acclimation (Hisano et al. 2008; Abeynayake et al. 2015). Fructans can also interact with cell membranes to stabilize them or minimise damage during freezing (Demel et al. 1998; Hincha et al. 2000; Vereyken et al. 2001). Transgenic ryegrass containing fructosyltransferase genes from wheat provided further support for the role of fructans in protecting cell membranes from damage (Hisano et al. 2004). Genetic transformation of rice with fructosyltransferase genes from wheat, enabled it to make fructans, and this was associated with improved tolerance to low temperature (Kawakami et al. 2008). Recently, transgenic ryegrass with altered patterns of fructan accumulation shows the potential for manipulating the distribution of fruc-

tans between different parts of the plant (Panter et al. 2017). This may have potential for manipulating responses to abiotic stress in grasses.

6.3.3 Antifreeze Proteins

Forage grasses also contain genes encoding antifreeze proteins or ice recrystallization inhibiting (IRI) proteins. It was first reported by Sidebottom et al. (2000), who found an antifreeze protein in perennial ryegrass which seemed to be better at inhibiting ice recrystallization, than acting as an antifreeze. This was consistent with modelling work on the ryegrass antifreeze protein (Kuiper et al. 2001). Phylogenetic analyses of *IRI* genes in grasses demonstrated that the cold tolerant grasses of the Pooideae subfamily, such as perennial ryegrass, wheat and barley, have developed a specific lineage of these genes, probably through expansion of repeat motifs (Sandve et al. 2008). They also identified four *IRI* genes in *L. perenne*. Transgenic plants of *Arabidopsis thaliana* expressing *IRI* genes from *L. perenne* had better tolerance to frost treatment, and lower electrolyte leakage after cold acclimation than control plants (Zhang et al. 2010). The same two genes were also shown to be upregulated in response to cold acclimation treatment in *L. perenne* (Zhang et al. 2017). In *F. pratensis*, an *IRI* gene was mapped to linkage group 5 in close proximity to a QTL for frost tolerance (Alm et al. 2011). This gene was also upregulated in response to cold acclimation treatment (Rudi et al. 2011). It is also interesting to note that the grass *IRI* genes contain a signal peptide targeting the proteins to the apoplast (Sandve et al. 2008; Zhang et al. 2010), consistent with their role in interaction with extracellular ice crystals or in minimising damage to the cell membrane.

6.3.4 Regulation of Cold Tolerance Mechanisms

Plants respond to abiotic stresses such as cold by changing the expression of a range of genes, which in turn elicit various mechanisms by which they cope with these stresses. In *Arabidopsis thaliana*, cold-responsive genes are regulated by a signal transduction mechanism in which transcription factors bind to a cis-acting dehydration-responsive element (DRE)/C-repeat (CRT) to activate gene expression (Yamaguchi-Shinosaki and Shinosaki 2009). The transcription factors are called DREB1/CBF and DREB2. The former regulates cold-responsive gene expression, while the latter is involved in osmotic stress-responsive gene expression, both in an abscisic acid (ABA)-independent manner. Homologues to these genes have been identified in cereals like rice (Dubouzet et al. 2003), wheat (Shen et al. 2003), barley (Skinner et al. 2005) and maize (Qin et al. 2004), and they have all been implicated in cold tolerance. In forage grasses, a homologue of the rice *OsDREB1A*/*CBF3* gene was isolated from perennial ryegrass. Its expression was induced during cold stress, and when transferred to *A. thaliana* it induced the expression of cold-responsive genes

(Xiong and Fei 2006). This would suggest a high degree of functional similarity between these genes in divergent plant species. Expression of ten *CBF* genes identified in perennial ryegrass was increased in response to low temperature (Tamura and Yamada 2007). *CBF* genes from *L. perenne* have also been associated with improved cold tolerance and winter survival (Hulke et al. 2012; Yu et al. 2015). A complex picture emerges from work with *F. pratensis*, from which it is clear that the expression and function of cold-responsive genes and transcription factors involved in the signal transduction pathway, is influenced by vernalization, de-acclimation and re-acclimation (Ergon et al. 2016).

6.4 Drought and Salt Stress

6.4.1 Plant Responses to Osmotic Stress

Drought, heat and salt stress in plants are all likely to lead to water deficit, and plants respond by closing their stomata through ABA production and signalling, in order to minimise water loss by transpiration. Another effect is a reduction in photosynthetic activity due to degradation of the photosynthetic machinery and reduced development of leaf material (Shinozaki and Yamaguchi-Shinozaki 2007; Farooq et al. 2009). Enhanced production of reactive oxygen species (ROS) during water deficit stress can contribute to the degradation of the photosynthetic machinery (Gill and Tuteja 2010). The ability of plants to activate antioxidant pathways is an important element of detoxifying ROS. Gene expression analysis and candidate gene-based association studies in perennial ryegrass have suggested that it is an important element of the defence against drought (Liu and Jiang 2010; Yu et al. 2013). These authors identified two superoxide dismutase genes as being significantly associated with drought tolerance. Expression analysis in Kentucky Bluegrass exposed to salt stress also showed enhanced expression of genes known to be involved in antioxidant metabolism (Bushman et al. 2016).

Other mechanisms used to deal with osmotic stress include heat shock proteins (HSP). They act as molecular chaperones by stabilising protein structure and prevent their denaturation during abiotic stress (Wahid et al. 2007). Recently, three families of heat shock factor genes (*hsf*) (transcription factors regulating the expression of HSPs) were identified in *F. arundinaceae* and *L. perenne* (Wang et al. 2016). The targets for these transcription factors also included ascorbate peroxidase, which is involved in antioxidant metabolism.

Late embryogenesis abundant (LEA) proteins are very hydrophilic, and are believed to confer drought tolerance by increasing the water binding capacity and generating a protective environment for other proteins (Farooq et al. 2009). Genes encoding LEAs from perennial ryegrass have been associated with drought tolerance (Yu et al. 2013).

In a genome-wide association study (Kelly and Skøt, unpublished) found 3 DArT markers associated with biomass retention in drought-treated *L. perenne* varieties. These markers have been mapped onto linkage group 4 close to the *dhn4/5* gene locus, a member of the dehydrin gene family. The dehydrins are another important family of proteins in the ABA-mediated drought response. They are hydrophilic and thus perform a similar role to that of LEAs in retaining water and cellular structure in water stressed cells. Their differential expression in Bermuda grass was shown to confer a drought tolerant phenotype (Hu et al. 2010). Introgression mapping in hybrids between *F. arundinaceae* and *L. multiflorum* revealed that linkage group 3 is another potential source of drought tolerance in these grasses (Humphreys et al. 2005). A QTL mapping study in *F. pratensis* also identified a major QTL on linkage group 3 (Alm et al. 2011), and reviewed in Humphreys et al. (2006).

6.4.2 Other Factors Affecting Tolerance to Abiotic Stress

Neotyphodium/Epichloë is a group of endophytic fungi which can form a symbiotic relationship with temperate grasses such as *L. perenne* and *Festuca* spp. (Schardl et al. 2004). Endophyte-infected grasses can confer a number of advantages in terms of competitiveness, disease resistance and abiotic stress to the grass host (Kane 2011). Exactly how endophyte-infected plants gain better abiotic stress resistance compared to uninfected plants is not yet clear (Malinowski and Belesky 2000).

The potential of *Festulolium* hybrids and lines with introgressions of *Festuca* chromosomal segments in a *Lolium* genome background was alluded to in Sect. 6.3 in terms of cold acclimation and frost tolerance. It has also been shown that such hybrids can have a mitigating effect on run-off during flooding compared to either parental genus (Macleod et al. 2013).

6.5 Conclusions and Future Prospects

Recent advances in genomics technologies have enabled significant progress in our knowledge of the molecular, biochemical and physiological mechanisms behind abiotic stress tolerance in plants, including forage grasses to some extent. The importance of improving abiotic stress tolerance in forage grasses is emphasized by the fact that grassland is often occupying marginal land where other, perhaps more valuable cash crops are impossible to grow profitably, often because of extra pressures from abiotic stresses in terms of temperature and precipitation (Jones et al. 2015). Climate change is likely to impose further stresses, possibly from more erratic and extreme weather patterns in the future. Grasses grown in areas with cold winters would need to be adapted to sudden temperature changes in terms of de-acclimation and re-acclimation (Kovi et al. 2016). In order to counteract the loss of agricultural

land to urban development and desertification, it may also be necessary to breed crops adapted to geographical areas beyond their current limits.

Given the complex multi-genic nature of abiotic stresses they would seem to be good candidates for genomics-assisted breeding methodologies such as genomic selection (GS), as this approach takes into account the small effects of each molecular marker on all chromosomes (Meuwissen et al. 2001). Implementation of GS requires a training population with both phenotypic and genotypic data available. A predictive model is used to obtain a genomic estimated breeding value (GEBV) of the training population. This is then used to predict the phenotypic value in a breeding population which has only got genotypic data. As with plant crops in general, there is great interest in developing GS approaches for forage grasses in order to increase the rate of genetic gain by speeding up the population improvement cycle time. A number of papers have described various scenarios, simulations and methodologies for GS in forage crop breeding (Resende et al. 2014; Lin et al. 2016, 2017a, b; Skot and Grinberg 2017). Apart from prediction accuracy, which is determined primarily by the size of the training population, heritability and to some degree by marker density, the risk of inbreeding and its potential negative effect on breeding programmes with GS, is highlighted (Lin et al. 2016, 2017a, b). Some empirical studies of GS have been carried out in perennial ryegrass, although not with traits relating directly to abiotic stress (Fè et al. 2015, 2016; Grinberg et al. 2016; Byrne et al. 2017). Prediction accuracies vary, but with high heritability traits such as flowering time and disease resistance, the accuracies were high.

GS and related designs for genomics-assisted breeding can in theory be applied to any quantitative trait. It also provides tools and resources for biological discovery (Hickey et al. 2017). Thus, the potential of GS is there, but there is still some way to go before its routine implementation for a wider range of traits, especially abiotic stresses. The desire to implement GS in breeding programmes also highlights the need for accurate phenotype data from field experiments in order to achieve high and reliable prediction (White et al. 2012).

The use of the most appropriate mating designs to minimise inbreeding in outcrossing forage crops is another important factor in breeding programmes. The availability of genomic data provides new opportunities for controlling inbreeding, and this was explored recently in perennial ryegrass where the genomic relationship matrix was used to design the best parent combination to reduce inbreeding while maintaining genetic gain (Lin et al. 2017a). Prior to that, the concept of genomic mating to select complementary parents for the most optimal mating design was proposed (Akdemir and Sanchez 2016). Furthermore, some of the many molecular, genetic and physiological investigations mentioned above have demonstrated that even for complex traits like abiotic stress, key genes involved in signal transduction or other processes can have major effects. This provides opportunities to improve our understanding of the mechanisms controlling these important traits, and to improve stress tolerance through other approaches such as transgenics or genome editing.

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Chapter 7
Molecular Responses to Cold Stress in Temperate Fruit Crops with Focus on *Rosaceae* Family

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Abstract Cold stress is considered as one of the main limiting environmental factors causing a significant loss in the production of fruit crops. Although many fruit crops require chilling during winter to develop fruiting buds, late winter, and early spring frost can severely damage buds, flowers, and fruits and can lead to the reduction of productivity. Among different plant families, the *Rosaceae* family contains several economically pivotal fruit-producing crops, such as *Fragaria* (strawberries), *Malus* (apple), *Rubus* (blueberries) and *Prunus* (stone fruits), which suffer from cold injuries during the blooming period. This chapter provides a general overview of the role of various molecular components involved in sensing and signal transduction processes as well as the regulation of gene expression in response to cold stress in fruit crops. Besides, the impact of next-generation sequencing approaches is highlighted in the molecular studies of the *Rosaceae* family. Also, we have addressed the existing gaps to help researchers identify areas that need more attention.

Keywords Cold stress · Fruit crops · Gene expression · NGS · *Rosaceae* · Signal transduction

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7.1 Introduction

Abiotic stresses impair the growth and development of plants, limit their geographical distribution and reduce their productivity. Cold stress is one of the abiotic stresses that causes tissue injuries, photosynthesis reduction, and delay in the growth of plants. Therefore, enhancing the tolerance of plants in response to cold stresses is one of the primary aims of crop breeding. Plants adapt to adverse environmental conditions by developing various molecular, biochemical and physiological strategies (Cushman and Bohnert 2000; Shinozaki et al. 2003; Yadav 2010). Over the last decades, enormous efforts have been made in deciphering molecular mechanisms of plants in response and adaptation to cold stress (Chinnusamy et al. 2007; Shinozaki et al. 2003).

The *Rosaceae* is one of the important crop families which includes main fruit crops such as apple (*Malus domestica*), pear (*Pyrus communis*), almond (*Prunus dulcis*), apricot (*Prunus armeniaca*), cherry (various *Prunus species*), peach (*Prunus persica*), blackberry (various *Rubus* species), strawberry (genus *Fragaria*) and plum (various *Prunus* species). Although many species of *Rosaceae* require chilling during winter to develop fruiting buds, frost stress during late winter and early spring can significantly damage the reproductive tissues of these plants, consequently leading to the reduction in the productivity (Dai et al. 2013; Stepulaitiene et al. 2013; Szymajda et al. 2013; Alisoltani et al. 2015; Matzneller et al. 2016). In this chapter, we have described different molecular aspects of cold injury in fruit crops with special focus given to the *Rosaceae* family.

7.2 Cold Injury and Cold Acclimation in Fruit Crops

7.2.1 Chilling and Frost Injury

The suite of molecular, biochemical and physiological changes in plants that are induced by low non-zero temperatures, together with the subsequent morphological symptoms is called chilling injury. The rate of chilling injury in crop fruits is primarily associated with both the level of low temperature and the duration of exposure to cold stress. The two most acute chilling injury symptoms observed in the fruit crops are the delay in the ripening and development of fruits. Low temperatures during storage of the fruits can also lead to severe injuries such as flesh browning, reddening, and woolliness, which consequently reduces the fruit's storage life (Crisosto et al. 1999; Lurie and Crisosto 2005). Several other phenotypic symptoms reported in fruit crops under chilling stress are reduction of leaf expansion, loss of rigidity, loss of leaves, chlorosis (loss of the leaf chlorophyll) c) and necrosis (death of most of the plant cells and/or tissues) (Mahajan and Tuteja 2005). Cold stress also affects the development of reproductive tissues of fruit crops. For instance, the risk period of some *Rosaceae*

fruit crops (e.g., almond) starts from late winter which is during tree's blooming period (Rodrigo 2000).

Compared to chilling stress, frost causes the formation of ice crystals in the extracellular spaces in the tissues and damaging plant's cells, and in severe conditions leads to plant's death (Levitt 1980b). The intensity of frost injury is mainly influenced by the type of plant cultivar and severity of cold stress. Tissues drastically injured by frost generally have water-soaked appearance especially after ice thawing (Whiteman 1957; Parsons and Day 1970). The intensity of frost damage also depends on types of plant's organ (e.g., root, trunks, branches, and buds) and developmental stage. As an example, for the most species of *Rosaceae*, the highest lethal temperature 90 (LT90: 90% plant mortality) ranges between −1 and −4°C during post-bloom stage, while the lowest LT90 ranges between −14 and −17°C during silver tip stage (Proebsting and Mills 1978).

7.2.2 Main Injuries Caused by Cold Stress in Fruit Crops

Chilling stress influences fruit crops by affecting their membrane integrity, gene expression, ion leakage, proteins and other biomolecules activities. Although above mentioned changes in plants are considered as the main reasons that help plants to resist low temperatures, the underlying mechanisms of tolerance to cold stress are not fully understood (Dhanapal and Crisosto 2013). The following section discusses the most severe injuries to the crop plants under cold stress in more details.

7.2.2.1 Membrane Damage

The metabolism of chilled cells gets altered due to transition of membrane lipids from crystalline to gel phase, leading to the injury of chilling sensitive plants (Shinozaki and Yamaguchi-Shinozaki 1999). Membrane damage and metabolic dysfunction through stimulation of secondary dehydration stress can also happen due to frost (Mahajan and Tuteja 2005). Murata et al. (1982) and Sun et al. (2015) reported a strong relationship between the degree of sensitivity to cold stress and the proportion of desaturated phosphatidylglycerol across different plant species. Reactive oxygen species (ROS), such as hydrogen peroxide found at higher level in cold stressed plants, can enhance the membrane damage. For example, hydrogen peroxide content has been found to be associated with brownish pistils and fruits in almond and peach during blooming and storage stages at low temperatures (Ding et al. 2009; Alisoltani et al. 2015).

7.2.2.2 Protein Changes and Degradation

The susceptibility of plant tissue to cold injuries depends on the level and balance of certain plant growth hormones (Ismail and Grierson 1977). For instance, abscisic acid (ABA) decreases the ion leakage and prevents the reduction of glutathione and cell membrane phospholipids and can also enhance the stability of microtubular net (Rikin et al. 1979). Similarly, treatment of cold-sensitive plants with methyl jasmonate (JA) induces the synthesis of stress proteins such as heat shock proteins (HSPs), pathogenesis-related proteins (PRPs), and alternative oxidase (Ding et al. 2001, 2002). Proteome analysis in the peach fruits unveiled upregulation of four membrane stability related proteins and repression of three proteins related to phenolic compounds metabolism at 0°C as compared to that of 5°C. It was found that the abundance of enzymes related to sugar metabolism and energy pathways reduces in peach fruit stored at 0°C (Zhang et al. 2010a).

7.2.2.3 Oxidation

Cold stress induces the generation of ROS including singlet oxygen, superoxide radical, hydrogen peroxide and hydroxyl radical, therefore leads to secondary oxidative stress (Mittler 2002). Plants accumulate different types of metabolites during cold stress such as polyamines which are a group of polycationic organic compounds. It was demonstrated that treatment with exogenous polyamines resulted in higher internal polyamine contents and suppressed cold injury (Kramer and Wang 1989). The reduction of cold injury by polyamines is likely associated with their antioxidant and stabilizing activities on the cell membrane.

7.2.2.4 Photosynthesis and Respiration

Photoinhibition of photosynthesis is reported in many cold-sensitive species. As photosystem II (PSII) is more sensitive to light, the slow enzymatic reactions in the thylakoid and decreased carbon metabolism under cold stress can decrease the efficiency of PSII (Allen et al. 2000). Several differentially expressed genes related to dysfunction of the photosystem II in the peach such as degradation of photodamaged D1, D2, CP43, and CP47 has been recognized by Nilo-Poyanco et al. (2018), and they also highlighted the dissimilar abilities of cold-sensitive and non-sensitive varieties to keep the plastids homeostasis under chilling stress.

7.3 Molecular Aspects of Cold Sensing and Signal Transduction in Temperate Fruit Crops

Cold stress could be considered as a physical stress that could be sensed by all molecules and components in plant cells. Different factors are considered as primary sensors of cold stress; cell membrane and cytoskeleton, photosynthesis system and various types of biomolecules. The primary effect of cold on cells is the change in fluidity and rigidity of plant's plasma membranes (Levitt 1980a; Vigh et al. 1993). Several studies have suggested that the cell membrane, through dynamic changes in the physical state of membrane lipids, is one of the primary sensors of cold stress (Mikami et al. 2002). Peroxidation of lipids, which affects membrane integrity, was observed in several *Rosaceae* plants grown under low temperatures, for example, peach (Wang et al. 2006) strawberry (Gülen et al. 2008), almond (Karimi et al. 2013), and apricot (Bayat et al. 2013; Wang et al. 2016b). Wang et al. (2006) demonstrated that treatment of peach fruit with salicylic acid reduces chilling injury, and delays the peroxidation of membrane lipids during cold storage (Wang et al. 2006).

Various proteins such as dehydrin proteins (DHNs), heat-shock proteins (HSPs) and cold-regulated proteins (CORs) are also involved in membrane stabilization in response to cold stress (Janská et al. 2010). Low temperature can also change protein folding, which might be another primary sensor of cold stress (Pastore et al. 2007). Dehydrins are one of the several proteins that have been associated with changes in the cold tolerance of plants. In peach (*Prunus persica* (L.) Batsch) the expression patterns of dehydrin genes (*PpDhn2* and *PpDhn1*) were examined in response to low temperature and water deficit conditions. *PpDhn2* was significantly induced by water-deficit stress but not by low temperatures, whereas *PpDhn1* was induced by both water deficit and low-temperature stresses (Wisniewski et al. 2006). In another study by Zhang et al. (2010a, b), proteome profile of peach fruit stored at low temperature was examined. Differentially expressed (DE) proteins were identified using mass spectrometry in peach fruit stored at 0 and 5°C. Among DE proteins, membrane stability related proteins including enolase, major allergen Prup1, temperature-induced lipocalin, and type II SK2 dehydrin were upregulated. In addition, low temperature of 0°C might regulate the endogenous hydrogen peroxide level, resulting in the activation of genes encoding proteins that stabilizes the membrane (Zhang et al. 2010b). The impacts of the accumulation of dehydrin-like protein in microshoots of pear (*Pyrus communis* L.) was investigated under cold acclimation conditions using immuno-blot, and eight protein bands corresponding to dehydrin-like proteins were characterized in different genotypes (Baniulis et al. 2012). In another study by Li et al. (2012), the expression profile of cell membrane proteins was analyzed in brassinolide-treated and control mango (*Mangifera indica* L.) fruit in response to cold stress. Fourteen DE proteins were identified by mass spectrometry, and among them, remorin type II SK2 dehydrin was upregulated in brassinolide treatment under cold stress. In apple (*Malus domestica* L.), 12 dehydrin genes (*MdDHNs*) were identified with a typical K domain. Expression profiling of nine *MdDHNs* indicated that transcript levels of some *MdDHNs* were significantly upregulated under low temperature,

drought and ABA treatment, which suggested an essential role of the apple dehydrin gene family during stress adaptation (Liang et al. 2012a). In almond, changes in the expression of genes involved in cell membrane structure were observed in both anther and ovary tissues in response to cold stress (Mousavi et al. 2014). The study revealed many upregulated genes involved in lipid metabolic process in both tissues. In pomegranate and loquat, it has been shown that fatty acid compositions correlate with susceptibility of these fruits to chilling injury (Moellering et al. 2010; Cao et al. 2011). Expressed sequence tags (ESTs) analysis *of Prunus campanulata* Maxim. also revealed upregulation of DRP, HSP, MYB, and GPX in plants cultivated at low temperature (Zhang et al. 2015).

In addition to the cell membrane and different types of proteins, metabolic processes are also considered as one of the primary sensors of cold stress. Low temperature causes an imbalance between the source of energy and the sink of metabolites. Photosynthesis acts as the sensor of this imbalance via the redox state of electron-transport components. Photosynthesis also interacts with different processes, such as crosstalk between photosynthetic redox and sugar-signaling pathways, during adaptation to the cold (Ensminger et al. 2006). Cold stress under light leads to the inhibition of the photosystems. It is argued that PSII is more sensitive to cold stress compared to PSI because the increase in the cyclic electron flow around PSI is insufficient to protect PSII (Paredes and Quiles 2015).

After temperature changes are perceived, the signal of cold is transduced to the nucleus where regulation of gene expression and transcription occurs (Zeller et al. 2009). Large numbers of studies have investigated signaling pathways triggered by low temperatures. It is proved that ROS, hormones, and calcium play pivotal roles in signal transduction and regulation of gene expression in response to low temperatures (Liu et al. 2012). Plant hormones such as salicylic acid, abscisic acid, and jasmonic acid are the critical regulators in stress signal transduction and tolerance of the plants to low temperatures (Miura and Tada 2014; Wang et al. 2016a). The increase in ABA content occurs in response to both cold stress and water loss (Wang et al. 2015). The ABA level was found to increase in deeply dormant potato tubers, which was also reported in sweet cherry (Chengguo et al. 2004; Destefano-Beltrán et al. 2006). Several cold-responsive genes are induced through the increase in the level of hormones mentioned above, in particular, ABA. As an example, 19 out of 30 late embryogenesis abundant (LEA) proteins were upregulated by ABA treatment in Chinese plum (*Prunus mume*) (Du et al. 2013).

Protein phosphorylation by protein kinases is crucial for cellular signaling pathways and is essential for the regulation of cold-responsive genes in plants (Chinnusamy et al. 2007). Different types of protein kinases are known to be induced through exposure to cold stress and are involved in perception and transduction of signal under different environmental stresses (Furuya et al. 2013; Jonak et al. 1996). Mousavi et al. (2014) showed that a considerable number of DE genes are involved in signaling processes using high throughput transcriptome sequencing of almond under cold stress. Most of these genes have protein kinase domain and are mainly clustered in two groups; Ca^{2+}/calmodulin-dependent protein kinase (CAMK) and mitogen-activated protein kinase (MAPK). In plant cells, the calmodulin/calcium-

binding protein is a member of the receptor-like kinase family and acts as a primary sensor for changes in free Ca^{2+} levels. The expression level of calmodulin in *Prunus incisa* × *serrula* has been assessed under various abiotic stress conditions, which indicated that calmodulin is differentially regulated in response to multiple stresses (Maghuly et al. 2009). The expression patterns of calmodulin genes were also measured in different tissues of strawberry (Fragaria × ananassa) under various abiotic stresses (Leng et al. 2015). Results revealed a distinct expression profile of this gene in response to heat, cold, and salt stress. In almond, the upregulation of calmodulin was reported in response to cold stress (Alisoltani et al. 2016). The authors also demonstrated a microsatellite variation at calmodulin gene locus in frost-tolerant and frost-sensitive genotypes of almond. They concluded that calmodulin could be used as a functional marker in marker-assisted selection (MAS) of plants for tolerance to cold stress (Alisoltani et al. 2016).

7.4 Regulation of Gene Expression Under Low Temperatures in Temperate Fruit Crops

7.4.1 Transcriptional Regulation of Gene Expression During Cold Conditions

Plants reprogram their genes through regulatory mechanisms (transcriptional, post-transcriptional, and post-translational modifications) in response to cold stress. Therefore, studying the regulatory mechanisms involved in response and adaption to cold stress is pivotal to improve cold tolerance in plants (Alisoltani et al. 2015). A significant finding towards understanding the mechanisms of gene regulation in response to cold stress was the identification of the *Arabidopsis* C-repeat-binding factors (CBFs) which is an AP2/ERF transcription factor, (Gilmour et al. 1998; Medina et al. 1999), also known as DREB1 (Liu et al. 1998). The CRT/DRE motifs are observed in the promoters of different cold-inducible genes (Thomashow 1999), and these motifs activate genes following cold stress (Yamaguchi-Shinozaki and Shinozaki 1994).

Cold-inducible DREB1/CBFs have been identified in numerous members of *Rosaceae*, such as *Prunus avium* (Kitashiba et al. 2004), *Malus* × *domestica* (Yang et al. 2011), *Prunus mume* (Zhang et al. 2013), *Prunus persica* (Artlip et al. 2014), and *Prunus dulcis* (Barros et al. 2012; Alisoltani et al. 2015). The CBF/DREB1 pathway plays a crucial role in the cold response and tolerance of *Rosaceae* plants. Iezzoni et al. (2002) investigated the effect of *CBF1* over-expression on strawberry frost tolerance. Their results showed that, although 50% electrolyte leakage occurred in the two transgenic lines at the −8.2 and −10.3°C respectively, no significant change in the frost tolerance of transgenic lines was detected compared to the wild-type plants. The frost tolerance values were significantly higher than the value for the wild-type plants under −6.4°C (Iezzoni et al. 2002; Owens et al. 2002).

A new gene encoding a DREB1 transcription factor, *MbDREB1,* was cloned and characterized from dwarf apple, *Malus baccata* (Yang et al. 2011). Expression of *MbDREB1* was induced by low temperature. Transgenic plants over-expressing *MbDREB1* showed increased tolerance to cold stress, compared with wild-type *Arabidopsis*. In another study, 68 *MdDREB* genes that were classified into six subgroups were identified in apple. Results from expression analysis revealed that transcript levels of some predicted *MdDREB* genes were significantly upregulated under abiotic stresses (Zhao et al. 2012).

Barros et al. (2012) isolated two almond CBF genes (*PdCBF1* and *PdCBF2*), and they found that low temperatures induce the transcription of these genes. Additionally, this study reported that *PdCBF1* and *PdCBF2* could be induced by ABA and drought treatments. To isolate *CBF* genes in *Prunus mume*, primers were designed based on the CRT/DRE binding factor of peach, sweet cherry and other related *Rosaceae* member sequences and two genes, *PmCBFa* and *PmCBFb*, were isolated from *Prunus mume* (Barros et al. 2012). In this study, phylogenetic analysis indicated differences in monocot and eudicot *CBF* genes. Moreover, sequencing of *CBF* genes from 16 cultivars and wild species of *Prunus mume* revealed intraspecific evolution of these genes (Zhang et al. 2013). *CBF* gene regulation is more complicated in woody plants than in herbaceous plants. Gene expression of five tandem peach *CBF* genes (linkage group at scaffold 5) and one *DREB2* gene revealed the high induction of *CBF* genes by subjective dawn + 4 h (ZT4; where ZT is Zeitgeber time).

In contrast, *CBF* genes were less expressed in leaf, and to a lesser degree in the bark samples, exposed to dawn + 16 h (ZT16) (Artlip et al. 2013). The authors also revealed that the peach *DREB2* ortholog was induced by both low temperature and dehydration (Artlip et al. 2013). In another study on peach fruit, six *CBF* genes (*PpCBF1-6*) were isolated, and their expression profile was assessed in response to low temperature. *PpCBF1/5/6* were all induced at low temperature, whereas no change in expression level of other *CBF* genes was observed (Liang et al. 2013). Comparison with the control plants revealed that the over-expression of a peach *CBF* (*PpCBF1)* in apple enhances the level of frost tolerance. The ectopic expression of *PpCBF1* in apple also affects growth and dormancy of transgenic plants (Wisniewski et al. 2015).

Promoter analysis of *CBF* genes in peach revealed the presence of *cis*-element ICE1 (inducer of *CBF* expression 1) in the upstream sequence of *PpCBF1/5/6* (Liang et al. 2013). ICE1 is a bHLH (MYC-type basic helix–loop–helix) transcription factor, which can bind to the CBF3 promoter and activates the transcription of CBF3 in response to low temperature (Chinnusamy et al. 2003). Feng et al. (2012) found that the *MdCIbHLH1* gene (*Cold-Induced bHLH1*) of apple, which encodes an ICE-like protein, was significantly induced in response to cold stress. The MdCIbHLH1 protein can bind to the promoter of *CBF2* and *CBF3*. The over-expression of the *MdCIbHLH1* gene, as a result, enhanced cold tolerance in transgenic *Arabidopsis*. Authors also suggested that the *MdCIbHLH1* gene, through the CBF pathway, acts in cold stress tolerance of apple. *PuICE1* gene was isolated from *Pyrus ussuriensis*, and its expression level was investigated under different stress conditions (Huang et al. 2015). Results indicated that *PuICE1* could be induced by cold, dehydration and salt, with the greatest induction under cold conditions. Ectopic expression of the *PuICE1*

in tomato improved tolerance to cold stress, and it enhanced the expression levels of six stress-responsive genes in the transgenic plants under cold stress. These findings demonstrated the pivotal roles of *PuICE1* and *PuDREBa* in the cold tolerance of *Pyrus ussuriensis* (Huang et al. 2015). Moreover, there are many other TFs and regulators, for instance, MYB, WRKY, NAC, Dof, SIZ1, and HOS1, which have key roles in cold stress tolerance in the *Rosaceae* members (Alisoltani et al. 2015).

WRKY genes, one of the largest TF families in plants, plays pivotal roles in response and adaptation to various environmental stresses. For instance, the upregulation of *WRKY21* was observed in almond under frost stress (Alisoltani et al. 2015). Authors also reported that the expression of *PdWRKY21* was decreased in the frost-sensitive genotypes of almond compared to other genotypes under frost stress. The induction of *PdWRKY21* gene in the frost-tolerant genotypes of almond under low temperature implies the critical role of *PdWRKY21* in the frost tolerance of almond. In apple, a total of 127 *MdWRKY* genes were identified, some of which were involved in response to waterlogging and drought stress (Meng et al. 2016).

Interaction and inter-relation of different regulatory genes at transcriptional and post-transcriptional, as well as post-translational levels, regulate the gene expression of plants in response to low temperature (Chinnusamy et al. 2007, 2010; Alisoltani et al. 2015). Feng et al. (2012) noted that the degradation of the *MdCIbHLH1* protein in apple can be potentially mediated by ubiquitination and SUMOylation mechanisms under cold stress. The E3 protein ligases participate in the ubiquitination and SUMOylation pathways in plants. The HECT ubiquitin-protein ligases belong to E3 proteins, and are sensitive to cold, drought, and salt stress in apple (Xu et al. 2015). HOS1 is another member of E3s that participates in the ubiquitination process and mediates the degradation of the ICE1 protein under cold stress (Dong et al. 2006). The negative correlation between the expression level of *PdHOS1* gene and *PdMIR7122a-3p* was reported in almond under frost stress (Alisoltani et al. 2015). Based on the findings, the authors suggested that *PdMIR7122a-3p* has a positive role in the frost tolerance of almond. Similar to HOS1, SIZ1 is a member of E3 ligases with a pivotal role in SUMOylation process of plants. However, unlike HOS1, SIZ1 mediates cold tolerance of plants by positive regulation of ICE1 activity. SIZ1 also regulates other proteins, such as MYC2, ANNAt4, and MYB15, under stress conditions (Catala et al. 2007; Miura et al. 2007). In almond, the upregulation of *PdSIZ1*, *PdICE1*, and *PdCBF/DREB1* was observed in frost-tolerant genotypes of almond (Alisoltani et al. 2015).

7.4.2 MicroRNAs as Post-transcriptional Regulators of Genes Under Cold Stress

The main regulatory mechanisms of plants at the post-transcriptional level include alternative splicing (AS) of RNA, and gene silencing through small interfering RNAs (siRNAs) and microRNAs (miRNAs). Post-transcriptional modifications and their

interactions at other regulatory levels can mediate the gene expression in response to various stresses (Guerra et al. 2015). Discovery of small RNAs (sRNAs) and extensive studies on them have produced comprehensive information about post-transcriptional regulatory mechanisms. sRNAs are mainly grouped into two major classes: endogenous siRNAs and miRNAs (Carthew and Sontheimer 2009; Ku et al. 2015). The differences between these two classes of regulatory molecules are related to the structural and biogenesis pathways. siRNA and miRNA are derived from different precursors; the precursors of siRNA are double-stranded, while miRNAs (with self- complementary sequences) have hair-pin-like precursors. The specificity in several factors in synthesis pathway also mediates differences in the biogenesis pathways for each group of sRNAs, including RNA polymerases (Pol), RNA-dependent RNA polymerases (RDR), Dicer-like (DCL) and ARGONAUTE (AGO). The regulatory mechanism for both siRNA and miRNA is post-transcriptional gene silencing (PTGS) through the cleavage of the transcript or translational inhibition (Ku et al. 2015). Different siRNAs are classified into heterochromatic siRNAs (hc-siRNAs), natural antisense siRNAs (nat-siRNAs), trans-acting siRNAs (ta-siRNAs) and repeat-associated siRNAs (ra-siRNAs) (Xia et al. 2012). In different studies, the role of some miRNAs in the biogenesis of ta-siRNAs has been demonstrated. For instance, miR173-ARGONAUTE1 (AGO1) complex and miR390-ARGONAUTE7 (AGO7) complex process the primary transcript of ta-siRNAs by cleavage (Montgomery et al. 2008a, b). The impact of miRNA on ta-siRNAs transcripts have been reported in various members of the *Rosaceae* family. Xia et al. (2012) showed that miR390 and miR828 trigger the production of *MdTAS4* and *MdTAS3* in apple. Interestingly, in this study, an additional miR390-*TAS3-2* pathway was identified that was not previously reported in *Arabidopsis*. Similar results about miR390-*PpTAS3* and miR828-*PpTAS4* pathways have been reported in peach (Zhu et al. 2012). In both apple and peach fruits, a kind of siRNA derived from TAS4, namely *TAS4*-siR81, was found. The homologue of this molecule in *Arabidopsis* targets some members of the transcription factors, such as MYB TFs (Xia et al. 2012; Zhu et al. 2012).

Modulation of gene networks under different abiotic stresses provided an original perspective about the molecular regulatory mechanisms. In the *Rosaceae* family, the gene regulatory network in response to frost stress has been constructed only in almond (Alisoltani et al. 2015). In this network, *PdMIR7122-3p/PdHOS1* was introduced as the only post-transcriptional/translational interaction under cold stress. Under abiotic stresses, miRNAs are known as stress-up regulated/negative regulators and stress-down regulated/positive regulators (Chinnusamy et al. 2010). Different miRNAs related to cold stress are recognized in some species of *Rosaceae*. Several of these miRNAs are specific to some *Rosaceae* family. For instance, miR396, miR414, miR2275, and miR5021 are specific to *Prunus persica* (Barakat et al. 2012), and miR408 and miR1507 are specific to *Pyrus bretschneideri* (Niu et al. 2013). Besides, some miRNAs such as miR156, miR172, and miR535 respond to cold stress in several species (Barakat et al. 2012; Niu et al. 2013; Karimi et al. 2016). The target prediction of these miRNAs revealed that miR156, miR172, and miR396 control the expression levels of squamosa promoter-binding protein-like (*SPL*), APETALA2 protein (*AP2*) and growth-regulating factor (*GRF*), which probably are involved in

the stress response of *Arabidopsis* and rice (Liu et al. 2008; Lv et al. 2010). It was observed that some miRNAs could also act as multiple stress-responsive regulators. For example, miR156, miR159, miR167, miR393, and miR396 in *Prunus persica* were identified as both drought and cold-stress-responsive miRNAs. Furthermore, some miRNAs are involved in developmental processes and stress responses in plants such as miR156 and miR172 (Gao et al. 2012).

Cold-responsive miRNAs have different expression profiles under cold stress based on the type of species. For instance, miR319 and miR393 showed upregulated in *Arabidopsis* (Liu et al. 2008) and almond (Karimi et al. 2016) under cold stress, while miR319 showed down-regulation in poplar (Chen et al. 2012), sugarcane (Thiebaut et al. 2012) and trifoliate orange (Zhang et al. 2014b). Additionally, unlike the *Arabidopsis* and almond, miR393 was down-regulated in wheat under cold stress (Tang et al. 2012). Moreover, the up-regulation of miR169 was observed in several species viz., *Arabidopsis* (Liu et al. 2008), *Brachypodium* (Zhang et al. 2009), rice (Lv et al. 2010) and soybean (Zhang et al. 2014a). The differences in the expression patterns of miRNAs depend on various factors, including regulatory elements in the promoters of miRNAs. Most of the cold-responsive miRNAs have anaerobic induction elements (AREs), which are essential in responses to low temperature, hypoxic and dehydration stresses (Liu et al. 2008). Besides, the existence of ABA-responsive elements (ABREs) and GA-responsive elements (GAREs) in upstream sequence of miR319 in rice could validate the importance of phytohormones in the regulation of cold-responsive miRNAs (Lv et al. 2010). Among cold-responsive miRNAs in *Rosaceae*, miR319 and miR394 have been functionally recognized as the regulators of cold stress response in rice and *Arabidopsis*, respectively (Song et al. 2016; Yang et al. 2013). The over-expression of miR394 suppressed the level of *LCR* transcripts and subsequently induced the expression of *CBF1*, *CBF2*, and *CBF3*. Following these changes, *COR* genes were activated; and *RD29A* and *KIN1* genes were induced afterward. This cascade pathway resulted in cold stress tolerance. Additionally, the over-expression of miR394 increased the amount of proline and soluble surge, which helped improving cold tolerance (Song et al. 2016).

7.5 Impact of High Throughput Technologies in the Study of the Fruit Crops Under Cold Stress

Knowledge in the field of plant science has been revolutionized by whole transcriptome sequencing using next-generation sequencing (NGS) techniques, known as RNA-seq (RNA sequencing). More than 31,500 RNA-seq records can be found in NCBI SRA (Sequence Read Archive), of which about 630 records belong to *Rosaceae* members (Fig. 7.1). The ability to sequence the whole trancriptome under various conditions like abiotic stresses has allowed large-scale comparative analysis of many plants. Polygenic nature of abiotic stresses made them detectable from the entire gene pool including the rare or minor genes.

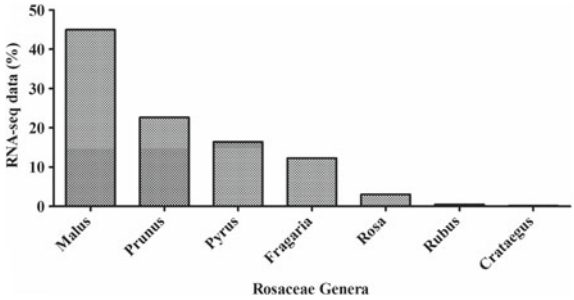

Fig. 7.1 Rate of RNA-seq data deposited on NCBI-SRA for Rosaseae Family. *Malus* sp. and *Prunus* sp. have the highest number of records, respectively

Although there are several ways to characterize differentially expressed genes like (differential display of reverse transcriptase, microarrays, serial analysis of gene expression, selective subtractive hybridization, massive parallel sequence signatures and cDNA-amplified fragment length polymorphisms), each of them have some advantages and disadvantages. Nevertheless, newer techniques are superior to the older ones. The broader range and sensitivity of RNA- seq have contributed dynamically to the rapid application of sequencing for differential expression analysis (Mondal and Sutoh 2013). Genes responsible for tolerance against various biotic and abiotic stresses have been recognized and exploited by many researchers through construction of EST library. Nogueira et al (2003) documented twenty novel cold-inducible genes in sugarcane EST database, which were previously not reported.

High throughput sequencing has also been properly utilized for sequencing of the genome and transcriptome of *Rosaceae* including apricot (Zhong et al. 2013), apple (Ke et al. 2014), peach (Verde et al. 2013; Chen et al. 2014), sweet cherry (Alkio et al. 2014) and almond (Mousavi et al. 2014). The genes responsible for cold acclimatization in blueberry (*Vaccinium corymbosum*) have been found using RNA-seq technology, and their expressions were authenticated through Q-PCR analysis (Rowland et al. 2012). RNA-seq data are indisputably valuable resources to serve as a platform to accelerate the understanding on flower bud development, cold acclimation, chilling unit accumulation/vernalization, flowering, fruit development, and/or nutritional quality traits (Die and Rowland 2013). Consequently, they can boost the identification and functional analysis of potential genes, and transcription factors involved in the metabolism and signaling of hormones under different stresses.

Beginning with the characterization of the plant microRNAs (miRNAs) in *Arabidopsis* (Reinhart et al. 2002), various miRNAs have been predicted and characterized through different approaches in plants; forward genetics, direct cloning and bioinformatics along with experimental confirmation (Jones-Rhoades et al. 2006; Zhang et al. 2009). Advances in sequencing technologies permitted more accurate and efficient identification of miRNAs as they use less time and money compared to the earlier methods (Morozova et al. 2009; Morozova and Marra 2008). Additionally, sRNA sequencing (sRNA-seq) is extensively used to determine the expression levels of known miRNAs, prediction of unknown miRNAs and other small non-coding RNAs (Liu et al. 2011; Lee et al. 2013).

There are numerous software packages available for analyzing sRNA-seq data. Different databases, which are valuable resources for known miRNAs sequences have also been developed; miRBase (Griffiths-Jones et al. 2008), miRNAMap (Hsu et al. 2008), miRGen 2.0 (Alexiou et al. 2009), PMRD (Zhang et al. 2010a, b) and deepBase (Yang and Qu 2012). Among these databases, miRBase (with 821 citations) is the most popular database. Software packages have also been designed for aligning short sequencing reads such as SeqMap (Jiang and Wong 2008), MAQ (Li et al. 2008a), SOAP (Li et al. 2008b), TopHat BWA (Li and Durbin 2009), Bowtie (Langmead 2010) and Bowtie 2 (Langmead and Salzberg 2012). One of the popular tools for the prediction of new miRNAs is mirDeep (Friedländer et al. 2008). RNA folding algorithm from the Vienna Package is used for examining the Hairpin structure (Hofacker et al. 1994, 2003). Moreover, several tools are available for the prediction of miRNAs targets. These tools seek putative binding sites in the 3′ and 5′ UTRs as well as CDS of the candidate mRNA targets, including RNAHybrid (Rehmsmeier et al. 2004), miRU (Zhang 2005) miTarget (Kim et al. 2006), microRNA.org (Betel et al. 2008), DIANAmicroT (Maragkakis et al. 2009) and psRNATarget (Dai and Zhao 2011). According to the citation statistics, a large number of studies have used microRNA.org, RNAHybrid and, psRNATarget for the prediction of miRNA targets.

The impact of miRNAs has been confirmed in different biological and metabolic processes; thus, many studies have focused on the identification and functional analysis of miRNAs. At the post-transcriptional level, miRNAs are pivotal in regulating the gene expression (Sunkar et al. 2007). Some cold-responsive miRNAs have been characterized by microarray technology in *Arabidopsis* and rice (Liu et al. 2008; Lv et al. 2010). Similarly, high-throughput sequencing studies have aimed at scanning miRNAs under cold stress treatments. For instance, cold-responsive miRNAs have been found by Solexa sequencing technology in poplar (Chen et al. 2012), wheat (Tang et al. 2012), tomato (Cao et al. 2014), soybean (Zhang et al. 2014a), tea (Zhang et al. 2014c) and in trifoliate orange (Zhang et al. 2014b). A large number of abiotic stress-responsive miRNAs have been detected by NGS technology. As an example, the sRNA-seq analysis led to identification of stress responsive miRNAs in *Arabidopsis thaliana* under nitrogen starvation (Liang et al. 2012b), in *Glycine max* under cadmium stress (Fang et al. 2013), *in Populus euphratica* under salt stress (Li et al. 2013) and in *Sorghum bicolor* under drought stress (Katiyar et al. 2015).

Moreover, siRNAs have been found under different abiotic stresses, using sRNA-seq approaches. Sunkar and Zhu (2004) and Borsani et al. (2005) were among the first researchers who exhibited the importance of siRNAs in Arabidopsis's response to abiotic stress. The main effects of these non-coding sRNAs have also been exhibited in *Triticum aestivum*. In this study, the expression levels of four siRNAs (siRNA002061_0636_3054.1, siRNA005047_0654_1904.1, siRNA080621_1340_0098.1, and siRNA007927_0100_2975.1) were reported to change in response to heat, cold, drought and salt stresses (Yao et al. 2010). Furthermore, sRNA-seq analyses have been accomplished on various members of the *Rosaceae* family (Table 7.1).

Genome-wide identification of drought-responsive miRNAs in peach under control and drought conditions was conducted by Eldem et al. (2012). In this study, 368

Table 7.1 Details of some of the recent small RNA-seq studies in *Rosaceae* family

Plant species	sRNA library resource	Sequenced raw reads	Conserved miRNAs	Novel/predicted miRNAs	Stress/stimuli-responsive miRNAs	References
Prunus mume	Perfect flower bud	22,561,972	61	61	13 stress-responsive miRNAs	Gao et al. (2012)
	Imperfect flower bud	24,952,690				
Prunus persica	Leaves	10,151,770	157	230	10 cold- responsive miRNAs	Barakat et al. (2012)
	Winter buds	10,899,501				
Prunus persica	Control leaf (LC)	15,499,314	531	197	262 drought-responsive miRNA in leaf	Eldem et al. (2012)
	Drought- stressed leaf (LS)	12,473,137	471	221		
	Control root (RC)	12,703,130	535	238	368 drought-responsive miRNA in root	
	Drought-tressed root (RS)	13,203,304	487	265		
Prunus persica	4 sRNA libraries of root, leaf, flower, mixed fruit	50,000,000	47	47	–	Zhu et al. (2012)

(continued)

Table 7.1 (continued)

Plant species	sRNA library resource	Sequenced raw reads	Conserved miRNAs	Novel/predicted miRNAs	Stress/stimuli-responsive miRNAs	References
Prunus mume	Bud	22,571,296	47	33	2 pathogen responsive miRNAs	Wang et al. (2014)
	Flower	16,407,316				
Prunus persica	Young leaves, young stems and flowers	14,781,274	117	186	–	Luo et al. (2013)
Prunus dulcis	Control anther	50,000,000–58,000,000	131	59	35 cold- responsive miRNAs	Karimi et al. (2016)
	Cold-stressed anther		122			
	Control ovary		123			
	Cold-stressed ovary		119			
Malus domestica	Leaf	59,000,000	33	42	–	Xia et al. (2012)
	Root					
	Flower					
	Fruit					

(continued)

Table 7.1 (continued)

Plant species	sRNA library resource	Sequenced raw reads	Conserved miRNAs	Novel/predicted miRNAs	Stress/stimuli-responsive miRNAs	References
Pyrus bretschneideri	Six small RNA libraries of different stages of fruit development	19,000,000–25,000,000	337	297	6 stress-responsive miRNAs	Wu et al. (2014)
Malus domestica	12 sRNA from Shoot tips of two fire blight-resistant and fire blight-sensitive apple tree	105,646,008	143	116	4 fire blight-responsive miRNAs	Kaja et al. (2015)
Rosa hybrida	Petals of unopened buds (S0)	16,648,213	33	47	50 ethylene-responsive miRNAs	Pei et al. (2013)
	Petals of opened buds (S2)	6,069,761				
	Petals of partially opened flowers exposed to air (C24)	11,579,864				
	Petals of partially opened flowers exposed to ethylene (E24)	15,937,871				

and 465 differentially expressed miRNAs were detected in leaf and root, respectively. In another study on rose petals, miRNA profiling was carried out by Illumina HiSeq-2000, during flowering and in response to ethylene treatment. Here, ethylene treatment functioned as a suppressor for miR164, miR390 and miR396 in rose petals. A total of 28 known miRNAs and 22 novel miRNAs were recognized as ethylene responsive miRNAs (Pei et al. 2013). Wu et al. (2014) applied deep small RNA-seq to find miRNAs involved in the development and quality of the fruit in *Pyrus bretschneideri*. Many miRNAs were identified, of which 297 novel miRNAs were predicted using MIREAP software. KEGG pathway analysis of predicted miRNA targets confirmed that most of these miRNA targets contribute in the development of pear fruit.

Since spring frost is restraining fruit production in the *Rosaceae* and threatening the stone or drupe fruits (such as peach, almond, plum, and apricot) more than the pome fruits (apple, pear, and quince) (Miranda et al. 2005; Folta and Gardiner 2009), some studies have focused on scanning the miRNAs related directly or indirectly in cold stress tolerance. Barakat et al. (2012) characterized ten chilling- stress-responsive miRNAs in leaves and winter buds of *Prunus persica*, revealing that these miRNAs were upregulated in both the leaves and buds of peach. Remarkably, this study demonstrated the co-localization of some conserved new miRNAs and their targets with quantitative trait loci (QTL) for blooming date and chilling requirement. Another study related to *Prunus dulcis* indicated that from 94 miRNA families in reproductive tissues under control and cold stress, 35 miRNAs showed differential expression in response to cold stress. In this study, most predicted targets were categorized into transcription factors group, most of which have a vital role in diverse environmental stresses (Karimi et al. 2016). Besides the studies that seek cold-responsive miRNAs under cold stress, other studies tend to find the miRNAs involved in various processes like flowering and blooming. Identification of these miRNAs and their subsequent engineering could direct researchers to manipulate the flowering process such as flowering date regulation. These approaches could protect fruit trees against cold stress injuries. Gao et al. (2012) used Solexa sequencing technology to characterized miRNAs that participate in the development of *Prunus mume* flowers. In this study, some members of miR319 and miR160a families were found to be influential in the development of the flower. Gene ontology annotation showed that 14% of miRNA targets are classified as stress-responsive genes. In another study on *Prunus mume*, deep sequencing was used to identify miRNAs involved in the process of blooming (Wang et al. 2014). They found that 43% of predicted targets were transcription factors, which are critical in response to environmental changes.

7.6 Conclusion and Perspective

This chapter summarized recent advances made in understanding cold stress responses in the *Rosaceae* family. Impact of molecular components in sensing, signaling and regulating cold stress was also discussed. The general response model to

cold stress has been illustrated in Fig. 7.2. This simplified model includes reception of cold stimulus, signal transduction, gene regulation and the downstream responses of crop plants. To put it briefly, different cellular molecules and organelles can perceive cold signals. The plasma membrane and the receptor-like kinases are crucial in perceiving the signals of cold stress. Several signal transduction pathways, such as hormone signaling pathways, have been described in response to cold stress. In addition to hormonal pathways, *CBF/DREB1* pathway is also pivotal in the induction of cold tolerance in *Rosaceae* members. We also inferred that different TF family members have interrelations with *CBF/DREB1* pathway such as AP2/ERFs, bHLHs, MYBs, MYCs, NACs, and WRKYs. Moreover, varieties of cold-responsive miRNAs and regulatory proteins directly or indirectly interact with the *CBF/DREB1* pathway, which has a central role during cold stress response in the *Rosaceae* crop fruits. However, the molecular mechanism of cold tolerance is complicated, and more studies are needed to elucidate this mechanism completely. Advances in sequencing technology along with the availability of numerous datasets in plants could speed up the survey and understanding of molecular mechanism of cold stress tolerance. The genome and transcriptome of several *Rosaceae* family have been completely sequenced; the assembly and gene annotation are available on the Genome Database for *Rosaceae* and SRA NCBI.

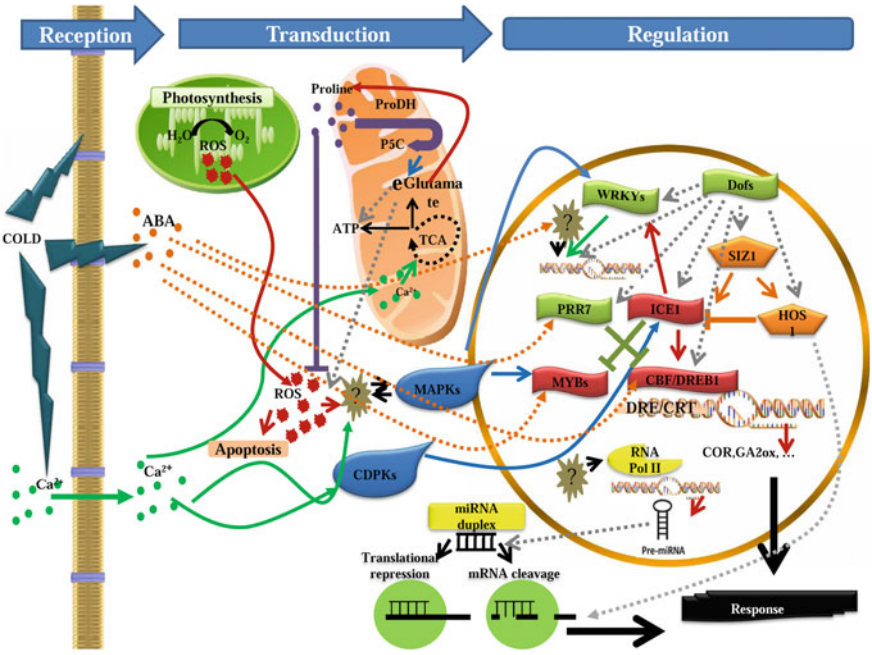

Fig. 7.2 The general response model to cold stress in *Rosaceae* Family. This simplified model includes reception of cold, signal transduction, gene regulation and the downstream responses of crop plants

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Chapter 8
Strategies to Enhance Drought Tolerance in Peanut and Molecular Markers for Crop Improvement

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Abstract The production of peanut (*Arachis hypogaea* L.) in warm environments and on sandy soils makes the crop vulnerable to soil drying in nearly every cropping season. Several traits are being explored to overcome yield decreases resulting from the inevitable water deficits that develop in the soil. In this review, two traits: (1) an early limitation on transpiration rate (TR) as the soil dries, and (2) limitation on maximum TR (TR_{lim}) under high vapor pressure deficit (VPD) in peanut will be discussed. Both of these traits result in water conservation by limiting plant transpiration rates and are potential reasons for genetic variation in Transpiration Efficiency (TE). The basis for transpiration response to soil water deficits and high VPD at the tissue and whole plant levels appears to be leaf and root hydraulic properties. A contributing factor in determining hydraulic limitations is water transport through membranes via aquaporins (AQP). Overall, both of the two traits result in phenotypes with an ability to conserve water especially under late-season drought events. While large genetic variability in these traits has been observed in peanut, breeding efforts are still required to exploit these promising traits in commercial cultivars. This review focuses on the traits in peanut that allow identification of tolerant genotypes with potential yield increase in water-limited environments. A recent progress in molecular marker technology has made it possible to measure polymorphism in peanut and to identify molecular markers or quantitative trait loci (QTL) linked to TE and its surrogate traits despite its low levels of molecular polymorphism and complex polyploid genome. We also reviewed some of these QTLs and their potential application for molecular breeding in peanut under water-limited environments.

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Keywords Aquaporins · Molecular markers · Peanut · VPD · Transpiration efficiency · QTL

8.1 Introduction

Peanut (*Arachis hypogaea* L.) is grown in the warm tropics and in temperate humid regions with sufficiently long warm summers, making it an important oil and food crop in Africa and Asia. In America, it is grown as a food crop. It is often grown on sandy soils with low water-holding capacity and in environments with variable rainfall. Consequently, without irrigation peanut may be frequently subjected to drought stress. Drought is a meteorological event which involves the lack of rainfall for a period to cause moisture diminution in soil resulting in plant water deficit (Kramer 1980). It often affects the crop adversely by drastically lowering plant mass production and seed yield. Peanut is generally considered a drought-tolerant species that has the ability to contend with soil moisture deficit through minimization of plant dehydration, regardless of the fact that water deficit can result in large decreases in pod yield (Clifford et al. 2000). Annual peanut yield losses due to drought have been estimated at US$520 million worldwide (Sharma and Lavanya 2002) and drought leads to approximately 70% of yield loss in peanut (Pandey et al. 2012b).

Several strategies have been proposed to screen the drought tolerance in peanut. In an empirical approach, drought resistant peanut varieties were developed by selecting for high yielding genotypes under imposed water-limited conditions (Branch and Kvien 1992). However, the yield is particularly sensitive to environmental conditions, and there is a considerable influence of G x E on pod yield (Zhang et al. 2013). Therefore, the empirical approach often does not lead to an identification of genotypes that can be used widely. A more targeted approach in developing drought-tolerant cultivars can be dissection of physiological components for specific trait improvement. This trait-based approach allows yield formation under drought to be dissected into a combination of various specific traits, whose importance varies based on the type of environment. This approach has been attempted based on the simple phonological framework of yield (Y) defined as a product of Transpiration (T), Transpiration efficiency (TE) and Harvest Index (HI) (Passioura 1977). Studies on TE (mass produced per unit of water transpired) found extensive genotypic variation for TE (Rao et al. 1993; Rao and Wright 1994; Wright et al. 1994, 1996; Sheshshayee et al. 2003, 2006; Krishnamurthy et al. 2007; Devi et al. 2009; Ratnakumar et al. 2009) in peanut. However, attempts to apply this framework to peanut have not been successful. Challenges such as a negative relationship between TE and HI (Wright et al. 1991, 1996) confounded the simple framework of Passioura (1977).

An alternative to the phenological approach is to develop an understanding of the physiological mechanisms that can contribute directly to drought tolerance. Such understanding opens the possibility for phenotyping traits that alleviate the impact of drought on crops. In this chapter, two plant traits that result in soil water conservation for use during drought periods are reviewed: (i) early limitation on transpiration rate

(TR) as the soil dries, and (ii) limitation on maximum TR under high vapor pressure deficit (VPD) conditions. Both traits allow partial restriction on TR and limit the rate of soil water use, which conserves water in the soil for sustained crop physiological activity when late-season drought is encountered.

To exploit the water conserving traits, it has been proposed that molecular markers and quantitative trait loci (QTL) analysis can be used to identify the genomic locations of genes controlling the two water-conservation traits. Molecular markers are useful in constructing linkage maps, gene mapping, marker-assisted selection (MAS), and gene discovery (Hyten et al. 2010). Combination of trait-based phenotyping strategies with genomic approaches is proposed to expedite breeding efforts. Tremendous progress has been made in peanut to combine trait-based phenotyping strategies with genomic approaches and several molecular markers have been identified for various biotic and abiotic traits (Varshney et al. 2009; Gautami et al. 2012; Pandey et al. 2012a, b, 2014a, b; Varshney et al. 2014; Peng et al. 2016).

8.2 Limited TR with Soil Drying

One of the putative traits to identify drought tolerance is a decreased TR when soils dry to a volumetric water content so that water transfer in the soil is inadequate to match water loss rate of fully open stomata (Ritchie 1981). The fraction of transpirable soil water (FTSW) is used as a covariate for soil moisture available to compare transpiration response and different physiological mechanisms to soil drying. It has been successfully used across a wide range of species and plant processes with threshold of decline generally occurring in the FTSW range of 0.3–0.4 (Sinclair and Ludlow 1986; Weisz et al. 1994; Sadras and Milroy 1996; Ray and Sinclair 1997, 1998; Ray et al. 2002; Bhatnagar-Mathur et al. 2007, 2009; Devi et al. 2009, 2013, 2014; Devi and Sinclair 2011; Shekoofa et al. 2013; Sinclair et al. 2015).

TR response of plants to drying soil has been studied in breeding commercial and transgenic peanut genotypes (Bhatnagar-Mathur et al. 2009; Devi et al. 2009, 2013; Shekoofa et al. 2013). Transgenic peanut genotypes transformed with rd29A:DREB1A showed variation in their TR response when exposed to water-limited conditions with FTSW thresholds ranging from 0.36 to 0.64 (Bhatnagar—Mathur et al. 2009). Devi and Sinclair (2011) and Shekoofa et al. (2013) evaluated transpiration responses in US commercial peanut cultivars exposed to drought stress and noted threshold for the decline in TR was from 0.36 to 0.46. However, in a study of 17 peanut breeding lines from India, a large variation in FTSW threshold for transpiration was observed ranging from 0.22 to 0.71 (Fig. 8.1) (Devi et al. 2009). Therefore, with selection, it seems quite likely that peanut lines with FTSW thresholds for decreased transpiration rate with soil drying at higher than the usual range of 0.3 and 0.4 can be identified.

Decreases in stomatal conductance at high soil water contents allow initiation of water conservation at an earlier stage in soil drying. As a result, the imposition of severe water-deficit stress on the plant is delayed and may increase TE. Simulations

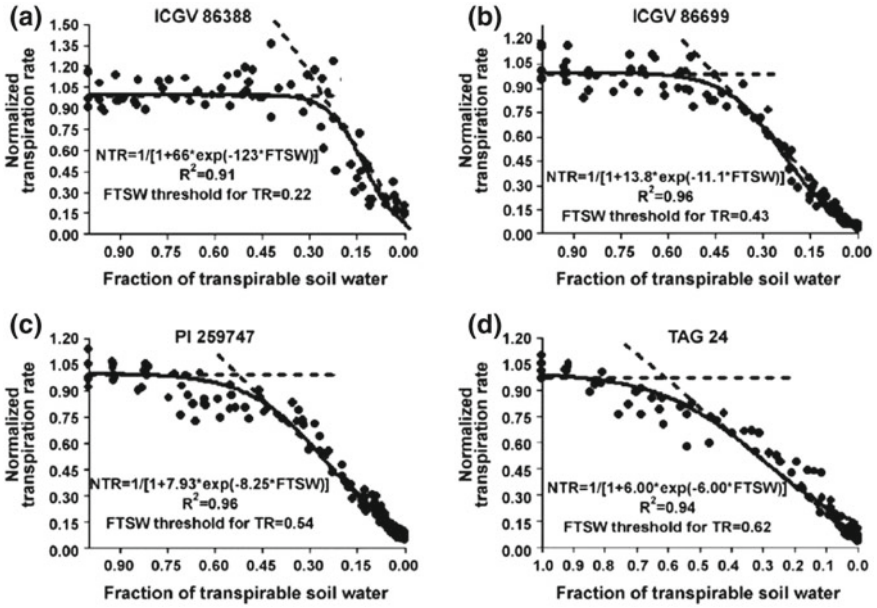

Fig. 8.1 Normalized transpiration rate—Fraction of transpirable soil water of peanut genotypes **a** ICGV 86388 **b** ICGV 86699 **c** PI 259747 and **d** TAG 24 subjected to water deficit conditions. The solid line represents the composite fit of all data using inverse exponential model and dotted lines are the result from two-segmental regression. The figure was adopted from Devi et al. (2009)

models with maize (Sinclair and Muchow 2001) and soybean (Sinclair et al. 2010, 2014) have shown this to be a beneficial trait. Water conservation by declining the stomata conductance early in soil drying in soybean resulted in simulated a yield increase in more than 80% of the growing seasons (Sinclair et al. 2010).

8.3 Limited TR with High VPD

A key in understanding water use, especially as it relates to VPD, is the equation defining the TE ratio.

$$\mathrm{TE} = k_{\mathrm{d}}/(e*_{\mathrm{a}} - e)_{\mathrm{d}} \tag{8.1}$$

where k_{d} is a coefficient reflecting leaf photosynthetic capacity and the biochemical constituents of synthesized plant mass, and $(e^*_{\mathrm{a}} - e)_{\mathrm{d}}$ is VPD (Sinclair et al. 1984; Sinclair 2012). Therefore, VPD has a direct effect on TE and an increasing VPD has an inverse relationship with TE (Bierhuizen and Slatyer 1965; Tanner and Sinclair

1983). TE will clearly be greater under conditions when VPD is low as compared to when VPD is high.

An important outcome of eq. (8.1) is that on a daily basis TE will increase if the daily weighted VPD is decreased. Consequently, during the midday period when VPD is usually at its maximum, decreasing the total contribution of daily water loss during midday will increase TE. Importantly for crop yield, the limitation of TR during the midday results in soil water conservation that will be available to prolong crop physiological activity when late-season drought develops. The potential importance of this response on yield was initially shown in a simulation study of sorghum production in dryland conditions in Australia (Sinclair et al. 2005). Another putative drought-tolerance trait for increasing crop yield is limited-transpiration (TR_{lim}) under high atmospheric VPD (Sinclair et al. 2005). As a standard response, plants have continually increasing TR with increasing VPD (Sinclair et al. 1984). However, TR_{lim} at elevated VPD has been observed in some crops. Maximum TR may occur at VPD of around 2 kPa (Fletcher et al. 2007; Sadok and Sinclair 2009; Devi et al. 2010; Gholipoor et al. 2010; Kholova et al. 2010; Zaman-Allah et al. 2011; Devi et al. 2014). The TR_{lim} trait has the potential for water conservation under high VPD, which, if it occurs early in the growing season, results in increased availability of water during late-season water deficits. Water conservation as a result of the TR_{lim} has been shown to increase yields in soybean and sorghum over a broad range of geographical areas (Sinclair et al. 2005, 2010, 2014).

Genotypic differences of peanut in expression of TR_{lim} has been studied both under controlled environmental conditions and in the field. The sensitivity of TR to VPD in peanut breeding lines and commercial cultivars (Devi et al. 2010; Devi and Sinclair 2011) was noted under controlled environmental conditions. In a study with 17 peanut lines, Devi et al. (2010) found that 9 of the lines had a TR_{lim} with increasing VPD at about 2 kPa. Above ~2 kPa, there was little or no further increase in TR for these 9 genotypes and the remaining 8 lines continued their linear increase in TR with increasing VPD (Fig. 8.2) (Devi et al. 2010). Similarly, 8 commercial peanut varieties exhibited TR_{lim} when tested in the VPDs obtained with low temperature. However, in the high temperature experiment, the uniformly linear increase in transpiration with VPD was displayed by the same peanut lines (Devi and Sinclair 2011).

Recently, the TR_{lim} trait under both growth chamber and field conditions was compared in several peanut genotypes (Shekoofa et al. 2015). In controlled environments, the trait was evaluated on whole plants. There was a difference in expression of the TR_{lim} trait between the two controlled-environment experiments and this was attributed to differences in temperatures conditions. In an experiment conducted in a growth chamber at 31°C, 3 out of 6 peanut genotypes (N05008, Georgia Green, and HTS 02-05) expressed the TR_{lim} trait under high VPD while the other 3 genotypes expressed a linear response in TR to increasing VPD (Table 8.1). In the second growth-chamber experiment at 36°C, all six genotypes expressed a linear response in TR to increasing VPD (Table 8.1). The loss of the TR_{lim} at high temperature is consistent with what was reported by Seversike et al. (2013) for the soybean cultivar Hutcheson in which the expression of TR_{lim} at 30°C was lost at 35°C.

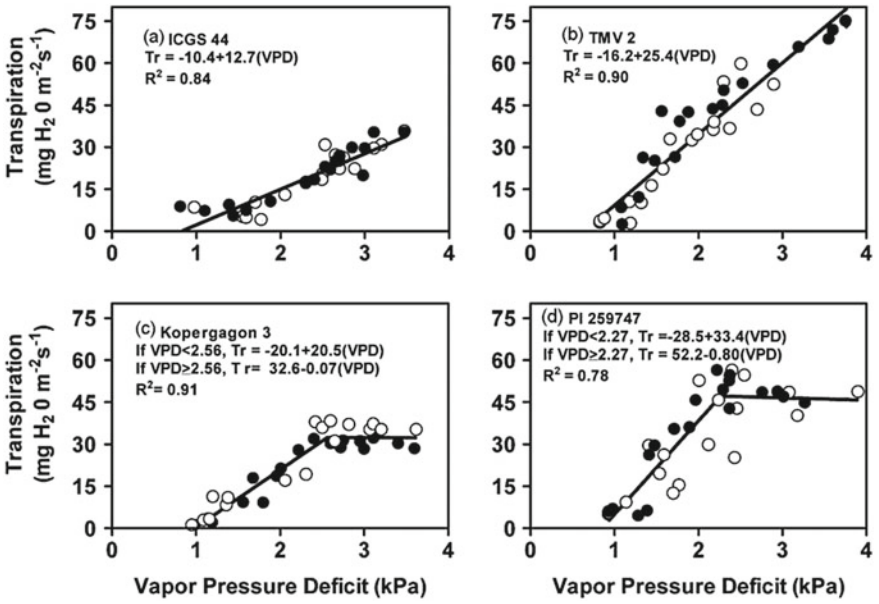

Fig. 8.2 Transpiration rate (mg H_2O m^{-2} S^{-1}) response of peanut genotypes to different levels of vapor pressure deficit (kPa) (Devi et al. 2010). Results from **a** and **b** were fitted with linear regression and **c** and **d** were with two segmental linear regression. Figure redrawn from Devi et al. (2010)

Table 8.1 Regression results of transpiration rate versus vapor pressure deficit for chamber studies (31/26 and 36/26°C day/night temperature) (Shekoofa et al. 2015). Those results represented by the two-segment linear regression include results for the two slopes and the VPD at the breakpoint

32/26°C						36/26°C		
Genotypes	n	Slope1 ± S.E.	Break point (X_0) ± S.E. (kPa)	Slope2 ± S.E.	R^2	Slope1 ± S.E.	Break point (X_0) ± S.E. (kPa)	R^2
SPT06-07	24	13.7 ± 1.26	Linear	–	0.85	30.8 ± 3.45	Linear	0.78
HTS02-05	24	12.2 ± 1.99	2.8 ± 0.22	−10.7 ± 8.30	0.72	28.5 ± 4.77	Linear	0.61
Bailey	24	11.0 ± 1.35	Linear	–	0.75	29.8 ± 4.68	Linear	0.64
Georgia green	24	14.0 ± 2.73	2.3 ± 0.24	−6.3 ± 4.77	0.70	21.81 ± 3.55	Linear	0.63
N05006	24	12.1 ± 1.63	Linear	–	0.72	15.42 ± 3.58	Linear	0.50
N05008	24	16.5 ± 3.19	2.6 ± 0.19	17.1 ± 13.23	0.70	25.98 ± 2.81	Linear	0.79

In the field, none of the 6 peanut genotypes were found to express the TR_{lim} with temperatures 35°C or above (Shekoofa et al. 2015). It appears likely that the lack of expression of the TR_{lim} trait in the field was due to high temperature to which the plants were subjected to in the field. In summary, this study indicated that caution is needed in extrapolating chamber results in identifying the TR_{lim} trait to

the field situation. Temperature differences used in the controlled environment and those experienced in the field may play an important role. Based on the results here, expression of the TR_{lim} trait in peanut appears to be sensitive to a shift in temperature between 31 and 36°C (Shekoofa et al. 2015).

8.4 Plant Hydraulics and Aquaporins

In rhizosphere pressurization experiments, Sinclair et al. (2008) found a congruence in low hydraulic conductance and expression of TR_{lim} at elevated VPD in soybean genotype PI 416937. Low hydraulic conductivity was hypothesized to limit water flow to the guard cells causing a loss of turgor in the guard cell. The loss of turgor in guard cell results in decreased stomatal conductance, which limits water vapor flux through the stomatal pore to the atmosphere under high VPD conditions.

Hydraulic conductivity in leaves appears to be largely facilitated through the activity of aquaporins (AQP), water channeling proteins that allow transmembrane water transport (Heinen et al. 2009). Leaf hydraulic conductance has been found to adjust quickly in response to changes in the environment variables such as atmospheric humidity and soil water status (Nardini and Salleo 2005; Levin et al. 2007; Shatil-Cohen et al. 2011), thus corroborating molecular involvement.

To understand the association of low leaf hydraulic conductance with TR_{lim} at high VPD and its link with AQPs, de-rooted shoots of soybean genotypes were subjected to AQP inhibitors (Sadok and Sinclair 2010). Lack of silver-sensitive AQP in the genotypes with TR_{lim} at high VPD was hypothesized to be responsible for low leaf-hydraulic conductance in soybean (Sadok and Sinclair 2010). Similarly, in peanut, it was demonstrated that treating de-rooted shoots of peanut with AQP inhibitors can result in rapid changes in plant TR (Devi et al. 2012). Clear differences were observed among four peanut genotypes treated with hydrogen tetrachloroaurate ($HAuCl_4$) and silver nitrate ($AgNO_3$). This study indicated that differences in the relative populations of Au and/or Ag-sensitive AQPs might have led to differences in genotype's TR response to VPD.

Shekoofa et al. (2013) examined the response to silver treatment of two peanut RIL (recombinant inbred line) populations derived from parents that diverged in their responses. A large range of response was observed among the RILs with a substantial fraction of RILs expressing more sensitivity to the silver test than either parent, indicating a dominance for a genetic loss in the TR_{lim} trait in this population. Differences in leaf AQP transcript abundance in peanut genotypes differing in the expression of TR_{lim} trait was established in a recent study (Devi et al. 2016). Under exposure to high VPD environment, peanut cultivars with the TR_{lim} trait had decreased AQP transcript abundance for four of the six AQPs tested cultivars (Devi et al. 2016).

8.5 Molecular Markers

Drought is a quantitative trait governed by several genomic regions (genes/QTLs) and to improve such complex trait, several small effect QTLs needs to be selected (Ravi et al. 2011; Gautami et al. 2012). Cultivated peanut is an allotetraploid with a large genome accompanied with a low level of genomic variation (Kochert et al. 1996; Zhao et al. 2012). However, with improved genomic technologies a number of studies have identified polymorphism and molecular markers. Polymorphism in cultivated peanut was first observed by using DNA amplification fingerprinting (DAF) and amplified fragment length polymorphism (AFLP) (He and Prakash 1997). Polymorphism was also noticed using random amplified polymorphic DNA (RAPD) marker in peanut genotypes that are different for various phenotypic traits (Subramanian et al. 2000; Dwivedi et al. 2001). Other work has been done with different molecular markers such as RFLP and isozymes, but very little genetic variation has been detected (Stalker and Mozingo 2001). Hopkins et al. (1999) found 5 simple sequence repeats (SSR) polymorphic markers from 26 primer pairs among 19 peanut accessions tested (Hopkins et al. 1999). Some studies were successful in identifying the polymorphism in peanut utilizing SSR markers (He et al. 2003; Ferguson et al. 2004; Moretzsohn et al. 2005; Cuc et al. 2008).

Krishnamurthy et al. (2007) studied 318 RILs derived from parents ICGV 86031 and TAG 24, which were diverse in their TE and for various other traits related to water use. Phenotypic data on transpiration, TE, SLA (specific leaf area), and SCMR (SPAD chlorophyll meter readings) were collected for the RIL population over two consecutive years. Using the same population, Varshney et al. (2009) first developed the first SSR-based linkage genetic map of cultivated peanut. Out of 1,145 SSR markers screened, 144 (12.6%) showed polymorphism among the studied cultivars. Both phenotypic and genotypic data were analyzed to identify QTLs for drought tolerance in peanut. For each physiological trait studied, 2–5 QTLs were identified, with the phenotypic variation in the range of 3.5–14.1% (Varshney et al. 2009).

Ravi et al. (2011) used the same RIL population derived from ICGV 86031 and TAG 24 in the F8/F9/F10 stage to phenotype for transpiration, TE, SLA, leaf area (LA), SCMR, carbon isotope discrimination, canopy conductance, total dry matter, dry weight, pod weight, seed weight, and stalk weight for 2 to 3 seasons under both water-stressed and well-watered conditions. They used 1,145 SSRs from Varshney et al. (2009) along with an additional 2,070 SSR markers and obtained segregation data for 215 marker loci. This resulted in production of a comprehensive map of cultivated peanut based on a single-mapping population (Ravi et al. 2011).

Gautami et al. (2012) extended the work Varshney et al. (2009) and Ravi et al. (2011) with two new RIL populations based on the crosses ICGS 76 × CSMG 84-1 and ICGS 44 × ICGS 76 along with ICGV 86031 X TAG 24 to identify the genetic basis of drought tolerance and to identify QTL. They used 3,215 SSR markers on the parental genotypes and developed two new genetic maps with 119 SSR loci for ICGS 76 × CSMG 84-1 and 82 SSR loci for ICGS 44 × ICGS 76. These RIL populations segregated for various traits such as transpiration, TE, SLA, SCMR, vegetative

weight/plant at harvest, pod weight/plant and harvest index under both irrigated and intermittent drought stress conditions (Krishnamurthy et al. 2007; Gautami et al. 2012). A total of 153 main effect QTLs and 25 epistatic QTLs for drought-tolerance traits were identified. The major QTLs identified were for transpiration, TE, SLA, SCMR, biomass, shoot dry weight, haulm weight, harvest index, canopy conductance, total dry matter and carbon isotope discrimination.

Fonceka et al. (2012) also mapped 95 QTLs in well-watered and water-limited regimes that differentiated cultivated peanut from a wild relative as well as wild alleles that contributed the positive variation to several traits related to flowering, plant architecture, plant morphology, seed morphology and yield components involved in peanut productivity and adaptation. A comprehensive analysis of marker trait association (MTA) was studied utilizing a reference set of 300 genotypes from 48 countries at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) for several economically important agronomic traits. A total of 68 MTAs were identified under the drought-stress condition for leaf area, leaf dry weight, SCMR, haulm weight, harvest index and seed weight with phenotypic variation ranging from 8.83 to 90.09% (Pandey et al. 2014b).

8.6 Conclusions

In this review, two water conservation traits were considered for improving drought tolerance in peanut. These two traits are: limiting transpiration rate at relative high soil water contents and limiting transpiration rate under high VPD. Both traits result in water conservation during early season of the drought so that plant can utilize the water for the grain development, when the drought occurs later in the growing season. Based on the current research, genetic variation has been identified for these two traits and phenotyping strategies are being developed for drought-stress environments to increase peanut productivity. However, development of the sufficient number of markers and marker technologies are much needed in peanut to understand the association between the molecular markers and phenotypic traits to utilize in breeding programs for the development of drought tolerant varieties. Along with QTLs for drought tolerance there are several other approaches like marker-assisted recurrent selection and genomic selection that need to be explored. These may become the preferred approaches for introgression of a larger number of QTL in order to breed drought-tolerant peanut genotypes.

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Chapter 9
Genetics of Drought Tolerance, Mapping QTLs, Candidate Genes and Their Utilization in Rice Improvement

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Abstract Drought is one of the major challenges in sustaining crop production worldwide with impending risks due to climate change. Rice, a semi-aquatic crop that feeds majority of the global population will be severely stricken by drought stress. Adaptation to drought varies significantly in rice, which is under the regulatory control of several genes involved in several pathways. Most of these genes contribute little to the adaptation process, making it one of the most complex biological mechanisms. Identification of these adaptation genes is the prime target to enable breeding for drought tolerance in rice. Several genes and quantitative trait loci (QTLs) have already been reported in rice associated with traits related to drought tolerance, most of which are identified from landraces as well as in the wild relatives adapted to water limited environments. Recent development in phenotyping and genomic tools has opened up newer vistas of investigation on drought adaptation in rice. This has helped in development and release of improved cultivars with inbuilt tolerance to drought. This chapter summarizes the developments in understanding drought tolerance, its genetics, the underlying mechanisms and efforts for breeding drought tolerant rice cultivars.

Keywords Breeding · Candidate genes · Drought tolerance · Meta QTLs Rice

9.1 Introduction

Drought resistance is one of the most complex and a challenging trait to breed. Drought imposes water stress on plants causing reduced fitness and in severe cases affects the survival of the plants. About 33% of the global population resides in areas prone to water stress and lives below poverty line (Sullivan 2002). Estimates suggest that with worsening climate change scenarios there will be a rise in episodes

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of drought in many parts of the world (Wassmann et al. 2009). Cyclical occurrence of drought approximately in every five years is predominant in eastern Indian states of Jharkhand, Odisha and Chhattisgarh, often resulting in up to 40% loss in total rice production in India (Bhandari et al. 2007; Wassman et al. 2009).

Asia Pacific region tops the world cultivation of rice (*Oryza sativa* L.), the premier staple cereal crop, by accounting for more than 90% of production and consumption. In particular, rice is life for Asia as it supports 1/3rd of calorific requirement of the Asian population. Recent estimates indicate that rice is cultivated in an area of 163.2 million hectares (mha) in more than 117 countries across the world with an annual production of about 751.9 million tonnes (mt) of paddy with an average productivity of 4.60 t/ha (FAO 2017). Estimates show that by 2025, world demand for rough rice would be approximately about 880 mt (Lampe 1995). Average rice yield in India hovers around 3.60 t/ha which is lower than the world average and almost half that is realized in China (6.81 t/ha). However, the Indian scenario of food demand is highly challenging with an estimated grain requirement of 113.3 mt of rice by the year 2021 (Kumar et al. 2009a) to be produced from an area of 43.3 mha, which has produced 104.3 mt during 2014–15 (DES 2016). This comes with an additional challenge of different stresses imposed due to climate change.

9.2 Status of Rice Cultivation in India

The area under rice cultivation has increased from 34.69 to 43.88 mha during 1961–2016, with an increase in production from 35.66 to 104.32 mt. This 192% increase in rice production as against 26% increase in area is solely attributable to green revolution that began in the mid-1960s. However, the annual compounded growth rate of rice production in the country has diminished from 3.55% during 1981–90 to 1.74% during 1991–2000 (Singh and Krishnan 2015) as against annual exacerbated human growth rate of 2.44 and 1.94%, respectively during the corresponding period (www.censusindia.gov.in). This decline in production trends as against the projected population increase is quite alarming, since there is a need to produce146 mt by the year 2030 (Goyal and Singh 2002), with diminishing resources and challenges of climatic vagaries.

In India, rice is cultivated in five diverse regions; northeastern, central, northern, western and southern regions. The north-eastern region comprising of Assam, West Bengal, South Bihar and Odisha has the highest rice cultivation intensity in India, where rice cultivation is primarily confined to the Brahmaputra, Ganga and Mahanadi river basins. This region is characterized by heavy rainfall and therefore supports rice to be grown mainly as a rainfed crop (Ghosh et al. 1960). Currently, the eastern India region includes plains of Assam, Bihar, Chhattisgarh, East Uttar Pradesh, Jharkhand, Odisha and West Bengal which represents 20.9% geographical area of the country, encompassing 61.3% of rice grown area that supports 41.3% of country's rice production (MOSPI 2016). Therefore, eastern India is the key for ensuring national food security. Although the region is rich in natural resources,

the rice productivity remains below the national average primarily because of the challenges of growing under unpredictable water scarcity and frequent droughts. In addition to climatic vagaries, eastern India is dominated by marginal lands rendering rice production uncertain and risky. Eastern states account for 26.9 mha rice area, with different rice ecologies. The largest portion falls under rainfed lowland ecology with nearly 10.6 mha (~39%), followed by irrigated ecology extending about 5.6 mha (~21%) area. Of the remaining, upland rice is spread over about 4.4 mha (~16%), which is prone to frequent drought. Other water rich ecosystems such as rainfed lowland medium deep, deep water and floating rice accounts for about 6.2 mha (~23%) in eastern India (Adhya et al. 2009; Pandey and Bhandari 2009). Eastern India alone accounts to 75% of the total rainfed rice area of 20.7 mha in India (Singh and Singh 2000). Jharkhand is a major state in eastern India, where rice cultivation is traditional. There are three cropping seasons in Jharkhand—*kharif*, *rabi* and summer—of which 75–80% of the net cultivated area in *kharif* season falls under rice crop. Traditionally, the rice is cultivated in an area of 1.48 mha under rainfed condition in Jharkhand with a productivity of 1.0–1.4 t/ha. This low productivity is of high concern in a country wherein 45% of nation's cereal production is contributed by rice and this region alone covers 78% of the total rainfed rice area. Therefore, to ensure food sufficiency it is important to improve rice productivity in the eastern India, where most of the rainfed rice is grown by small-scale farmers for sustenance. In this region, drought is a major detrimental factor, which is either due to low overall rainfall or longer interval between rains (Serraj et al. 2008).

Unpredictable nature of drought in the upland and rainfed lowland ecologies is the primary reason behind poor and unstable rice productivity of the eastern India. Therefore, emphasis should be on intensification of rice production in these areas, by development and adoption of drought tolerant cultivars in conjunction with improved management practices (Yang and Zhang 2010). Several of the popular cultivars in the rainfed areas of eastern India are varieties bred for irrigated ecosystems. They are adopted solely for their high yield potential replacing more resilient local cultivars that are low yielding. Furthermore, farmers in rainfed ecosystems are constrained to choose lowland varieties partly due to unavailability of high yielding and high quality drought resistant varieties (Vikram et al. 2011). These cultivars, such as IR64, IR36, Swarna, BPT5204, Savitri (CR1009), Sarjoo52, Rajendra Sweta and Rajendra Subhashini are highly sensitive to water stress often leading to severe yield loss under drought. Resultantly, there is a compelling need to improve the cultivar adoption using high yielding drought tolerant varieties to enhance rice production in the eastern region (Kumar et al. 2014). In recent years, improved drought tolerant varieties such as CR Dhan 40, Indira Barani Dhan, NDR 97, Sahbhagi Dhan, Shushk Samrat (NDR 1045-2) etc., have been released for cultivation in the drought prone areas. Successful adoption of the drought resistant varieties by farmers imply that future gains for sustaining food security in eastern states can be obtained only through deployment of more drought tolerant cultivars.

9.3 Drought as a Constraint in Rice Production

Rice is inadequately adapted to water limitation (Lafitte et al. 2006). The Asian cultivated rice has been domesticated under diverse agro-ecological systems, including environments with contrasting water availability. This natural adaptation of rice to contrasting and constraining environments, conditioned by the genetic plasticity of rice genome, could conserve a large morphological diversity (Courtois et al. 2000; Robin et al. 2003; Liu et al. 2004). There are two major ecologies for rice, viz., upland and lowland based on the water management. These can either be rainfed or irrigated. Upland ecology is aerobic and normally rainfed with poor water retention, while lowland environments are anaerobic and have flooded soils. Lowland flooding can occur through irrigation or rainfall or both, which can result in either temporary flooding or natural inundation as in the case of flood plains (Tuong and Bouman 2003; Rebolledo et al. 2012). Drought occurs on 50% of the rainfed lowlands and 100% of uplands (Garrity et al. 1986). Rice is versatile in its adaptation and grows in all the conditions, anaerobic, aerobic or both (Wade et al. 1998).

In eastern India, drought is a major challenge for rice production, followed by low light and submergence. Although drought is transient in this region, being predominantly rainfed, the major crop failure in eastern India is attributed to drought-related stresses (Adhya et al. 2009). Unlike lowland rice, upland rainfed farming does not enjoy standing water in rice fields after a rainfall. The risk of rice culture under water deficit and frequent prolonged rainless intervals has been identified as the key factor which affects rice productivity in eastern India, where the largest drought-affected area in the world extending about 13.6 mha prevails (Pandey and Bhandari 2009). The reduction in yield due to annual drought can reach more than 60% in eastern states. Odisha suffered a production loss of 54.6% on account of drought alone during 2002–03 (Adhya et al. 2009). Rainfed lands of erstwhile Bihar state including Jharkhand were recorded to suffer a yield loss of nearly 95–100 thousand tonnes on account of drought and floods, annually. While drought is a major constraint in uplands, flash flood and submergence can also cause serious damage in lowland rainfed conditions (Herdt 1996). The loss due to flood in lowland is about 12–27% per annum (Singh et al. 2012).

9.4 Drought Responses and Adaptation

Rice genotypes vary in their responses to drought situations. To sustain the crop cultivation under drought prone areas it is prudent to genetically improve crops augmenting drought resistance. Rice possesses several traits that contribute to resisting drought situations with tremendous amount of genetic variability for each of these traits. Despite the genetic plasticity, rainfed rice lands of the world extending about 74 mha accounts for only one fourth of global rice grain production (David 1991). This is because, varietal responses to drought rely upon the genetic makeup, degree

of drought and the interaction between the two components (Atlin 2003), all of which together may bring about yield reduction when their effects are modest, or total crop loss when severe. Therefore, degree of genetic response of a rice variety towards drought ranging from susceptibility to resistance, together with physical degree of severity of the stress are main considerations exploited during breeding for improved drought stress resistance (Fukai and Cooper 1995). India has enormous genetic diversity in rice, with several locally adapted landraces that are highly adapted to drought stress (Londo et al. 2006; McNally et al. 2009). Although landraces are relatively low yielders, they possess several genes that can be efficiently deployed for improving drought tolerant mechanisms in future cultivars.

Ontogenetic responses to drought stress in rice vary diversely from seedling to terminal stages. Of the three developmental stages, the most sensitive to drought is the reproductive stage particularly during the microsporogenesis and pollen mother cell differentiation (Sarkarung et al. 1995). Mechanisms of drought adaptation in rice, therefore, depends on the time of onset of the drought as well as the duration, depending upon which responses such as drought escape (reduced duration), drought avoidance (deep roots) and drought tolerance (osmotic balancing) occurs (Price et al. 2002). Levitt (1980) recognizes two forms of drought adaptation, escape and resistance, wherein in escape mechanism, plants accelerates flowering and completes its life cycle before the onset of drought, while in tolerance, physiological alterations are brought in to reduce the detrimental effect of drought on plant development and survival. In drought avoidance, plants maintain leaf water potential and in drought tolerance, either dehydration avoidance or dehydration tolerance are manifested (Price et al. 2002).

Drought resistance has been researched extensively in rice, and several potential contributing traits have been identified (Fukai and Cooper 1995; Nguyen et al. 1997; Price and Courtois 1999). These particularly include traits that help to minimize water starvation such as increased abscisic acid (ABA) production, leaf rolling, reduced respiration, root growth enhancement and stomatal closure. The root system plays a significant role in imparting drought tolerance by sustaining water and mineral uptake to maintain nutrient homeostasis and ionic balance in the plant system. Particularly, a thick and deep root system in an upland rice variety determines its potential to withstand adverse instances of water deficit by acquiring water from deeper soil layers. Possession of thick or thin root system is important when it comes to penetrate deeper into soil, where a hard soil pan may require thicker roots with penetration ability, while a coarse textured soil may require thin roots for penetration (Price et al. 2002). Similarly, there are several shoot oriented traits that are influential in imparting drought resistance in rice. They include osmotic adjustment and dehydration tolerance (Lilley and Ludlow 1996; Lilley et al. 1996; Fukai et al. 1999), high water use efficiency (Dingkuhn et al. 1991), membrane stability (Tripathy et al. 2000), photo-inhibition resistance (Jiao and Ji 2001), rapid leaf rolling and stomatal closure (Dingkuhn et al. 1989, 1999), stay greenness (Hoang and Kobata 2009) and thick epicuticular wax (O'Toole and Cruz 1983). Manifestation of drought resistance indicates a few critical behaviour of the above traits such as sustaining of high ion balance under tissue water deficit (Tripathy et al. 2000), use of maximum avail-

able water (Jones 1993; Condon et al. 2004) and resistance to photo-inhibition and capability for non-photochemical quenching (Horton 2000). Incidence of drought triggers a cascade of signals leading to several biochemical pathways synthesizing osmoprotectants, antioxidants and downstream proteins that aid in activities such as retaining of cell membrane integrity, enzymatic activity associated with carbon fixation and scavenging of reactive oxygen species to evade cell injury under water starved situations (Blum 2009; Farooq et al. 2009). Physiological traits such as leaf water potential, which is an indicator of the whole plant water status and osmotic adjustments are adjudged as a selection criteria for drought tolerance. The maintenance of whole plant water status takes coordination of several mechanisms related to balancing of soil water uptake and restraining of water loss through plant upper parts especially through stomatal apparatus, such as stomatal conductance, internal resistance, transpiration area, leaf rolling, and cellular solute accumulation (Jongdee et al. 2002).

Rice plant suffers significantly from drought stress during flowering stage, culminating in serious yield loss (Kamoshita et al. 2008), the extent of which depends on the degree and duration of water scarcity and the interval between drought spells. It is estimated that yield reduction due to water stress alone surpasses the cumulative loss due to all other abiotic stresses put together. Enhancing yield in water scarce environments would accordingly require drought tolerant rice varieties to sustain notional rice production. On the contrary, most of the popular rice cultivars in use in upland rice areas are lowland varieties developed for irrigated ecosystems. Because of their high yielding potential and preferred grain quality, these varieties are grown on a large scale by rainfed farmers. During normal years of sufficient and distributed rainfall, these varieties do perform well, but fail totally during drought years due to their high susceptibility to drought and incur severe yield loss (Kumar et al. 2008).

9.5 Genetics of Drought Adaptation

Rice is a self-pollinated crop, predominantly inbred and produces homogeneous and homozygous populations. Natural gene flow in rice is limited and any cross breeding will eventually and tends to restore homozygosity. Therefore, ecological and geographic isolations favour development of distinct genetic groups and sub-populations in rice (Garris et al. 2005). Glaszmann et al. (1984) classified world rice germplasm into five distinct groups, *indica*, tropical *japonica*, temperate *japonica*, *aus* and aromatic based on isoenzymatic patterns. These natural sets have enormous admixed genotypes among them, making an array of germplasm with specific adaptation to nearly every rice ecosystem. Further, almost all of the modern high yielding rice varieties have comparatively poor allelic diversity (Fukai and Cooper 1995) having developed from a few founder parents (Javier and Toledo 2004) and hence lack several adaptation specific genes. These adaptation specific genes are however conserved in local germplasm that are fit for the local niche, such as landraces, traditional varieties and wild congeners. For instance, by virtue of

their adaptation to aerobic situations tropical *japonicas* may possess better drought resistance (Lilley et al. 1996; Courtois and Lafitte 1999). Therefore, there is an increased focus on such locally adapted genotypes as sources for genes governing stress tolerance. They possess diverse alleles that confer tolerance through one or the other mechanism, which determines their degree of tolerance, durability and crop stage in which they are active. Since allele diversity are identified by polymorphisms at DNA level, which can be visualized using molecular markers, exploring of variations in the niche adapted germplasm may constitute mining for tolerance genes that can further be used for gene transfer, pyramiding and marker-assisted selection (MAS) (McNally et al. 2009; Jahn et al. 2011; Xu et al. 2011).

By using molecular marker technology and classical genetic linkage analysis several marker loci dispersed on rice genome have been mapped as quantitative trait loci (QTLs) associated with complex traits (Champoux et al. 1995). Tolerance to drought is recognized as a quantitative trait because of its complex nature and myriad of crop responses (morphological, physiological and biochemical) associated with it (Mitra 2001). Evidences indicate that various adaptation mechanisms to cope up with drought have distinct genetic controls, and their interaction may be essential for sustaining plant survival and growth under drought (Yue et al. 2006). Monogenic control of leaf rolling was reported in rice (Singh and MacKill 1991). Tomar and Prasad (1996) reported a gene, *Drt1* with pleiotropic effects on root system, plant height, pigmentation, awning behaviour, etc., in drought tolerant lines challenged with stress. However, because of the difficulty in phenotyping the responses in a breeding population due to extreme influence of environments and poor heritability of such traits, mapping genes governing drought tolerance has been a challenge. Therefore, QTL mapping has been suggested for elucidating the genetics of drought tolerance in rice (Price et al. 2002). These loci are either proximal or centric to genes that control drought resistant traits, hence can be used for improving drought sensitive genotypes using MAS techniques (Subashri et al. 2009; Vikram et al. 2015; Sandhu and Kumar 2017; Menguer et al. 2017). Several QTLs associated with drought resistance and related traits have been mapped in rice (Table 9.1). However, many of these QTLs have been mapped from a few drought tolerant backgrounds and mapping populations involving limited source germplasm. Therefore, consistency and magnitude of phenotypic variation across different genetic background is essential for using the QTLs for MAS-based breeding. Apart from above, QTLs also show inconsistency under different environments rendering them unstable. It is essential to have a stable QTL for successful MAS programme (Podlich et al. 2004; Collins et al. 2008).

Currently, use of several phenotyping facilities, from rain-out shelters to high throughput phenomics platforms (Granier et al. 2006) for drought evaluation in rice, has helped in mapping a large number QTLs associated with drought adaptation for yield and component morphological, physiological and biochemical traits (Table 9.1) (Nguyen et al. 1997; Jongdee et al. 2002; Lafitte et al. 2006; Negin and Moshelion 2017). However, deployment of these QTLs in practical breeding has been a challenge due to enormity of their numbers, poor precision and specificity to certain backgrounds. Notwithstanding, few of these QTLs have been found to be stable

Table 9.1 Quantitative trait loci (QTLs) mapped for drought resistance related traits in rice

Trait	No. of QTLs	Chromosome(s)	R^2 (%)	Population	References
Biomass	8	1, 2, 4, 6	6.3–36.8	IR20/Nootripathu	Prince et al. (2015), Gomez et al. (2010)
	4	3, 6	14.6–24.8	Swarna/WAB450	Saikumar et al. (2014)
	1	12	–	IR74371/Sabitri	Mishra et al. (2013)
	2	1, 3	8.6–20.1	N22/Swarna	Vikram et al. (2011)
	1	1	22.6	N22/IR64	Vikram et al. (2011)
	1	1	30.3	N22/MTU1010	Vikram et al. (2011)
	11	4	14.8–19.8	CT9993/IR62266	Sellamuthu et al. (2011), Kumar et al. (2007), Lanceras et al. (2004)
	3	7, 8, 10	4.6–20.2	IR64/INRC10192	Srividya et al. (2011)
	1	12	18	Way Rarem/Vandana	Bernier et al. (2007)
Canopy temp.	6	2, 4, 7	4.8–32.2	IR20/Nootripathu	Prince et al. (2015), Gomez et al. (2010)
	1	3	16.9	Swarna/WAB450	Saikumar et al. (2014)
	1	1	14.1	Zhenshan 97/IRAT109	Yue et al. (2008)
Drought index	3	2, 4	4.9–18.4	IR20/Nootripathu	Prince et al. (2015), Gomez et al. (2010)
	1	7	22.7	CT9993/IR62266	Sellamuthu et al. (2011)
	1	12	37.0	Way Rarem/Vandana	Bernier et al. (2007)
	1	10	15.5	Zhenshan 97/IRAT109	Yue et al. (2008)
	1	2	2.0	IRAT109/Yuefu	Li et al. (2005)
	1	12	16.1	IR64/Azucena	Hemamalini et al. (2000)
Flowering time	1	6	55.8	IR20/Nootripathu	Prince et al. (2015)
	5	3, 6	7.8–47.9	Swarna/WAB450	Saikumar et al. (2014)
	1	2	4.5	Kali Aus/2*MTU1010	Sandhu et al. (2014)

(continued)

Table 9.1 (continued)

Trait	No. of QTLs	Chromosome(s)	R^2 (%)	Population	References
	1	2	3.0	Kali Aus/2*IR64	Palanog et al. (2014)
Grain weight	2	2, 6	5.9–36.1	IR20/Nootripathu	Prince et al. (2015)
	2	2, 5	6.9–12.3	Guanghui 116/LaGrue	Zhou et al. (2013)
	1	2	2.3	IR64/INRC10192	Srividya et al. (2011)
	1	2	9.7	Zhenshan 97/IRAT109	Zou et al. (2005)
	6	1, 2, 3, 5, 12	5.9–11.3	IR64/Azucena	Thomson et al. (2003)
	1	11	–	Caiapo/*O. rufipogon*	Moncada et al. (2001)
Grain yield	4	1, 6, 8	6.7–20.9	IR20/Nootripathu	Prince et al. (2015)
	4	3, 6	13.0–38.0	Swarna/WAB450	Saikumar et al. (2014)
	5	1, 2	5.0–9.0	Kali Aus/2*IR64	Saikumar et al. (2014), Palanog et al. (2014), Sandhu et al. (2014)
	5	1, 2	6.2–17.0	Kali Aus/2*MTU1010	Palanog et al. (2014), Sandhu et al. (2014)
	24	1, 2, 3, 7, 9, 11, 12	6.3–27.3	Danteshwari/Dagad Deshi	Verma et al. (2014)
	4	2, 9	4.4–10.2	Aday Sel/IR64	Dixit et al. (2012b)
	3	5, 8	6.7–9.7	Guanghui 116/LaGrue	Zhou et al. (2013)
	3	2, 3, 12	3.8–7.5	IR74371-46-1-1/Sabitri	Mishra et al. (2013)
	2	2	2.2–6.9	Apo/Swarna	Dixit et al. (2012b)
	2	1	9.3–32.0	IR64/Azucena	Ghimire et al. (2012)
	6	1, 2, 3, 10	3.2–16.9	N22/Swarna	Vikram et al. (2011)
	9	1, 3, 4, 6, 10, 11	7.3–15.5	CT9993/IR62266	Sellamuthu et al. (2011), Kumar et al. (2007), Lanceras et al. (2004)
	1	8	15.7	IR64/INRC10192	Srividya et al. (2011)
	1	12	33	Way Rarem/Vandana	Bernier et al. (2007)

(continued)

Table 9.1 (continued)

Trait	No. of QTLs	Chromosome(s)	R^2 (%)	Population	References
	1	11	–	Teqing/Lemont	Xu et al. (2005)
Panicle length	2	1, 8	4.3–10.7	IR20/Nootripathu	Prince et al. (2015)
	1	11	33.6	CT9993/IR62266	Sellamuthu et al. (2011)
	1	3	–	Azucena/Bala	Lafitte et al. (2002)
	3	1, 2, 4	7.1–21.2	IR64/Azucena	Thomson et al. (2003)
Panicle number	1	12	–	IR74371/Sabitri	Mishra et al. (2013)
	3	1, 5	12.6–30.2	CT9993/IR62266	Sellamuthu et al. (2011)
	1	4	9.17	Zhenshan 97/IRAT109	Zou et al. (2005)
	1	5	–	Azucena/Bala	Lafitte et al. (2004)
	2	3	7.8	IR64/Azucena	Thomson et al. (2003), Lafitte et al. (2002)
	1	6	–	Caiapo/*O. rufipogin*	Moncada et al. (2001)
Seed-setting rate	6	2, 7, 10	4.8–10.0	IR20/Nootripathu	Prince et al. (2015), Gomez et al. (2010)
	7	3, 4, 5, 6, 9, 12	10.0–18.7	Guanghui 116/La Grue	Zhou et al. (2013)
	1	5	14.7	CT9993/IR62266	Sellamuthu et al. (2011)
	2	6, 8	3.9–15.7	IR64/INRC10192	Srividya et al. (2011)
	5	1, 4, 5, 6, 10	7.1–18.6	IR64/Azucena	Thomson et al. (2003)
Tiller number	1	4	19.8	IR64/Azucena	Hemamalini et al. (2000)
Plant height	10	1, 2, 9, 8	5.0–52.2	IR20/Nootripathu	Prince et al. (2015), Gomez et al. (2010)
	3	3	18.7–27.0	Swarna/WAB450	Saikumar et al. (2014)
	1	1	4.6	Kali Aus/2*MTU1010	Sandhu et al. (2014)
	1	12	1.1	IR74371/Sabitri	Mishra et al. (2013)

(continued)

Table 9.1 (continued)

Trait	No. of QTLs	Chromosome(s)	R^2 (%)	Population	References
	5	1	17.1–52.6	IR64/Azucena	Ghimire et al. (2012), Venuprasad et al. (2002); Lafitte et al. (2002)
	3	1	32.6–53.5	N22/Swarna	Vikram et al. (2011)
	2	1, 11	9.5–15.2	CT9993/IR62266	Sellamuthu et al. (2011)
	5	1, 5, 7, 8	5.4–12.5	IR64/INRC10192	Srividya et al. (2011)
Root thickness	13	1, 2, 3, 4, 6, 7, 8, 9, 12	8.5–31.3	CT9993/IR62266	Zhang et al. (2001)
Root weight	8	1, 2, 4, 6, 9, 10, 12	8.6–20.2	CT9993/IR62266	Zhang et al. (2001)
Root length	5	3, 4, 12	8.3–17.0	CT9993/IR62266	Zhang et al. (2001)

R^2 Phenotypic variation explained

across backgrounds as well as environments. To address this problem, precise identification and positioning of these 'robust' QTLs is needed. Comparative analysis of all the hitherto reported QTLs with necessary information and corresponding map data will be highly relevant in generating consensus maps, and positioning the robust QTLs (Khavkin and Coe 1997; Lin et al. 1995). Earlier attempts used descriptive statistics to attain the congruency of QTLs, while a QTL meta-analysis using mixed models was proposed by Goffinet and Gerber (2000).

9.5.1 Meta QTLs

Meta-analysis is an abstracting method, as indicated by its name in which 'meta' means 'after' or 'beyond' in Greek, which involves a concept of abstraction behind a concept that can be modular in nature and amended by adding new insights into the concept in future. In statistics, meta-analysis involves scrutiny of data from isolated studies to identify and report commonality in the results (Glass 1976; Rosenberg et al. 2004). These consensus QTLs are known as meta-QTLs (mQTLs). In QTL literature, meta-analysis assumes prominence to abstract or to identify meaningful associations between genomic locations and traits by compiling the information from independent QTL mapping experiments (Gyenis et al. 2007), thereby the consensus information can be used directly into marker-assisted breeding programmes (Bernardo and Charcosset 2006). Meta QTL analysis proposed by Goffinet and Gerber (2000) has improved identification of congruent QTLs from the earlier approaches. By this

Table 9.2 List of meta QTLs reported in rice

Trait	No. of studies involved	Total number QTLs	Identified mQTLs	References
Blast resistance	–	435	165	Ballini et al. (2008)
Root traits	24	306	119	Courtois et al. (2009)
Grain yield under drought	15	53	14	Swamy et al. (2011)
Grain yield	11	68	23	Swamy et al. (2011)
Panicle related traits	82	837	87	Wu et al. (2016)
Drought related QTLs	13		–	Khowaja et al. (2009)
Grain size/weight	7	64	6	Daware et al. (2017)
Root development	–	14	1	Coudert et al. (2010)

approach, they demonstrated that consensus information can be elucidated on the QTL positions by using a maize QTL database at University of Missouri. The method involves testing the likelihood of grouping the QTLs in as many as four groups and identification of optimal number of clusters by using a model approach that uses a 'Akaike' information criterion for selection. This is done over a consensus map created by iterative merging of the corresponding linkage maps over which the QTLs are mapped. Arcade et al. (2004) implemented these algorithms into a software suite, 'BioMercator'.

In recent years, several instances of meta-QTL approach are reported from several crops such as rice, maize, wheat, barley, soybean, cocoa, cotton and potato. Meta-QTLs have been mapped for several traits such as yield and related components, abiotic and biotic stress tolerance, fruit and fibre quality, seed quality including micronutrient concentrations, oil content, root system architecture and drought and water stress adaptation in these studies. A list of mQTLs reported in rice for various traits is given in Table 9.2. Reports of marker-assisted transfer of mQTLs for traits such as yield have been published in rice (Sandhu and Kumar 2017).

In rice, drought adaptation is highly complex and much investigated to identify traits and genomic regions associated with it. Meta-QTL analysis has aided tremendously in this effort to consolidate QTLs reported on different chromosomes. By this approach, Khowaja et al. (2009) identified a consistent drought avoidance QTL on chromosome 1, lying proximal to the semi-dwarfing gene, *sd1* that was strongly associated with plant height. Subsequent mQTL studies have revealed that this locus was putatively associated with osmotic balancing mechanisms and leaf rolling behaviour (Trijatmiko et al. 2014). Additionally, Khowaja et al. (2009) located a pleotropic QTL on chromosome 5 influencing leaf and root morphology and another QTL cluster on

chromosome 9 strongly influencing root morphology and behaviour. Subsequently, one mQTL was located on chromosome 12 and three on chromosome 1 (Swamy et al. 2011), identifying them to be ideal candidates for use in marker-assisted selection (MAS). Another meta analyses also revealed mQTLs of similar behaviour, affecting plant height and flowering time on chromosomes 1, 3, 8 and 9 (Sellamuthu et al. 2011). An mQTL on chromosome 8, identified for seed set percentage and grain number under drought, was co-localized with previously reported QTLs for osmotic adjustment (Trijatmiko et al. 2014). While running a meta QTL analysis for panicle traits, a stable mQTL was located on chromosome 10 by Wu et al. (2016), which harboured two strong QTLs for spikelets per panicle and seed setting.

Consistent and robust QTLs are the ones, which often are mapped from several mapping populations across different environments. Isolating information of such QTLs from several independent studies could be a best approach to assemble QTLs quickly and efficiently. This is generally achieved by a meta-QTL (mQTL) approach (Goffinet and Gerber 2000). Genetic variability in Indian rice germplasm, especially in the landraces is enormous and has contributed genes for several abiotic stresses. For instance, QTLs such as *Sub1* for submergence tolerance (Septiningsih et al. 2009), *Saltol* for salt tolerance (Gregorio et al. 1997) and *Pup1* for phosphorus starvation tolerance (Wissuwa et al. 1998, 2002) have been mapped from Indian landraces. Furthermore, several drought tolerance QTLs were also mapped in Indian rice cultivars such as Nootripathu (Gomez et al. 2010), Norungan (Subashri et al. 2009), Nagina 22 (Paterson and Reddy 2005), Dagad Desi (Dixit et al. 2012a, b) and Vandana (Bernier et al. 2007). Meta-QTL analyses have identified promising mQTLs among the drought tolerance QTLs (Table 9.3), such as *qDTY1.1* (Venuprasad et al. 2012), *qDTY2.1* (Venuprasad et al. 2009), *qDTY2.2* (Swamy et al. 2013), *qDTY3.1* (Venuprasad et al. 2009), *qDTY3.2* (Yadawa et al. 2013), *qDTY6.1* (Venuprasad et al. 2012), *qDTY9.1* and *qDTY10.1* (Swamy et al. 2013), and *qDTY12.1* (Bernier et al. 2007). There are several Indian donors for these QTLs, such as Nagina 22 carrying *qDTY1.1* and *qDTY3.2*, Kali *Aus* with *qDTY1.2*, *qDTY1.3*, *qDTY2.2* and *qDTY2.3*, Dagad Desi with *qDTY1.1*, Vandana carrying *qDTY6.1* and MTU1010 with *qDTY10.1* (Kumar et al. 2014). A recent meta-QTL analysis performed using novel QTLs, identified congregation of consistent QTLs on chromosomes 1, 3, 4 and 9. The tolerant alleles of the meta-QTLs were widely distributed among the drought-adapted germplasm of the Eastern India (Thribhuvan 2017).

9.5.2 Candidate Genes

There are several genes reported to impart drought adaptation in rice, most of which have been validated using transgenic approach (Nguyen et al. 1997). The mechanism of drought adaptation is regulated by complex signalling cascades and gene expressions, often involving thousands of genes (Yoo et al. 2017). One of the earliest studies in rice reported transgenic expression of a barley late embryogenesis abundant (LEA) protein, HVA1 (Xu et al. 1996), which improved water stress tolerance in

Table 9.3 Details of yield QTLs under drought being used for marker assisted breeding in rice

QTL	Chr	Markers	Position (Mbp)	Primer sequence	Product (bp)	Donor(s)	Ecology
qDTY1.1	1	RM3285	33.04	aaagcccccaaaagcagtac gtgaaactctggggtgttcg	193	Apo Basmati 334 CT9993 Dhagad Deshi Kali Aus Nagina 22	UL, RL
		RM11943	37.85	cttgttcgaggacgaagataggg ccagtttaccagggtcgaaacc	77		
		RM472	37.88	ccatggcctgagagagagag agctaaatggccatacggtg	296		
		RM431	38.89	tcctgcgaactgaagagttg agagcaaaaccctggttcac	251		
		RM165	40.1	aatcggcgaggtttgctaatgg atacgtggtacgtgacgctttgc	100		
		RM12091	40.25	ctgcaaatgcacaggaatcagg tcctctcgcctttctttctctcc	142		
		RM12146	40.71	agtatgccctgcccactacactagg cagcgaatggcaagagcaacc	100		
qDTY2.1	2	RM12987	10.62	tatcctcacctcacctcaactcacc tcgaaatcgagagagggaaagg	133	Apo Aus 276	RL
		RM5791	10.75	acgacgtcacaaagggttcttgc gaatacgctttcgcctgctacg	100		
		RM521	10.81	ttcccttattcctgctctcc gggatttgcagtgagctagc	260		

(continued)

Table 9.3 (continued)

QTL	Chr	Markers	Position (Mbp)	Primer sequence	Product (bp)	Donor(s)	Ecology
		RM13008	11	aaaggcggaggagaaggtatgg cttggaacgaacgaccaactcc	153		
		RM324	11.39	ctgattccacacacttgtgc gattccacgtcaggatcttc	175		
qDTY2.2	2	RM236	2.11	gcgctggtggaaaatgag ggcatccctctttgattcctc	191	Aday Sel Kali Aus	UL, RL
		RM279	2.88	gcgggagagggatctcct ggctaggagttaacctcgcg	174		
		RM555	4.31	ttggatcagccaaaggagac cagcattgtggcatggatac	223		
		RM5553	4.67	ttcacacgcacatgcacacacc gttcatgtgatcttgctgaccatgc	275		
		RM3294	5.21	atagacggatggcacgagatgc atgtgttcagttcagtggtgagtgg	267		
qDTY2.3	2	RM263	25.89	cccaggctagctcatgaacc gctacgtttgagctaccacg	235	IR74371-46-1-1 Kali Aus Nagina 22 Vandana	UL, RL
		RM573	27.97	ccagcctttgctccaagtac tcttcttccctggaccacac	144		
		RM530	30.56	ttctttattccctcgcactgacc caatgatgccacaaaccgtaacc	189		

(continued)

Table 9.3 (continued)

QTL	Chr	Markers	Position (Mbp)	Primer sequence	Product (bp)	Donor(s)	Ecology
		RM425	32.32	accacagcaggtggaacagg gctagctaagccaacaccaacg	188		
		RM250	32.8	ggttcaaaccaagctgatca gatgaaggccttccacgcag	188		
qDTY3.1	3	RM168	28.09	tgctgcttgcctgcttccttt gaaacgaatcaatccacggc	116	Apo IR55419-04	RL
		RM15791	28.57	agtaagtttgccgcggaggaagc ctccttgtcgatcaccaccatcg	87		
		RM186	28.81	tcctccatctcctccgctcccg gggcgtggtggccttcttcgtc	124		
		RM55	29.05	ccgtcgccgtagtagagaag tcccggttattttaaggcg	226		
		RM520	30.72	aggagcaagaaaagttcccc gccaatgtgtgacgcaatag	114		
		RM293	31.66	tcgttgggaggtatggtacc ctttatctgatccttgggaagg	207		
		RM16030	32.5	gcgaactatgagcatgccaacc ggattacctggtgtgtgcagtgtcc	100		
		RM468	32.67	cccttccttgttgtggctac tgatttctgagagccaaccc	265		

(continued)

Table 9.3 (continued)

QTL	Chr	Markers	Position (Mbp)	Primer sequence	Product (bp)	Donor(s)	Ecology
qDTY3.2	3	RM569	1.89	gacattctcgcttgctcctc tgtcccctctaaaaccctcc	175	Aday Sel IR74371-46-1-1 IR77298-5-6-18 Nagina 22 Moroberekan Vandana	UL, RL
		RM60	0.09	agtcccatgttccacttccg atggctactgcctgtactac	168		
		RM523	1.3	aaggcattgcagctagaagc gcacttgggaggtttgctag	148		
		RM231		ccagattatttcctgaggtc cacttgcatagttctgcattg	182		
		RM517	6.13	ggcttactggcttcgatttg cgtctcctttggttagtgcc	266		
qDTY4.1	4	**RM551**	0.17	agcccagactagcatgattg gaaggcgagaaggatcacag	192	Aday Sel	RL
		RM16368	2.45	tgtccagagaatgacaaagtacgc ggatgtatatctgccaccaaatgc	124		
qDTY6.1	6	RM589	1.39	atcatggtcggtggcttaac caggttccaaccagacactg	186	Apo IR55419-04 Vandana	UL, RL
		RM7639	1.41	ggtaggcacccacccacattgc gtcgttctccgccaccttctcc	156		

(continued)

Table 9.3 (continued)

QTL	Chr	Markers	Position (Mbp)	Primer sequence	Product (bp)	Donor(s)	Ecology
		RM586	1.48	acctcgcgttattaggtaccc gagatacgccaacgagatacc	271		
		RM588	1.61	gttgctctgcctcactcttg aacgagccaacgaagcag	126		
		RM204	3.17	gtgactgacttggtcataggg gctagccatgctctcgtacc	169		
qDTY6.2	6	RM3917	19.73	cgaacgaacgaggacgaacg caggcttgacggacgactcc	199	IR55419-04 Moroberekan	UL, RL
		RM3	20.38	acactgtagcggccactg cctccactgctccacatctt	145		
		RM3187	20.58	tccccacatcgtgtcgtc ttttttccccttctaccctcg	142		
		RM3827	21.95	ggacggattgtaggtaggac cctttcttcaatctgcattc	160		
qDTY9.1	9	RM566	14.65	aatatggtggcgcgtacatcc tgatcgagccaacaacaactgg	143	Aday Sel	RL
		RM24350	15.37	cctttgccgccttctcttgg acgagttcccggttaaccttacg	151		
		RM24390	15.89	gaagctctggatttgcgacatgg gagggcgtgaggtgaagttgg	91		
		RM24421	16.22	atttaaccgcgtttggtgcaatgg gcgggcaaccggagattatgg	93		

(continued)

Table 9.3 (continued)

QTL	Chr	Markers	Position (Mbp)	Primer sequence	Product (bp)	Donor(s)	Ecology
qDTY10.1	10	**RM216**	4.99	gcatggccgatggtaaag tgtataaaaccacacggcca	146	Aday sel Basamti 334 Nagina 22	RL
		RM25089	4.96	gtacgtaagtactcacccttgc ccaacgaactaacacctaacg	168		
qDTY10.2	10	RM258	17.57	tgctgtatgtagctcgcacc tggcctttaaagctgtcgc	148	Aday Sel Moroberekan IR55419-04	RL
		RM304	18.21	tcaaaccggcacatataagac gatagggagctgaaggagatg	160		
		RM171	18.61	aacgcgaggacacgtacttac acgagatacgtacgcctttg	328		
		RM25694	18.96	ccaccctggctactacaacacc cattccaaggtagaggctagaatgg	96		
		RM1146	19.17	tctccctattcccgtgtaaatcg cccgatgatcgattgtacctagc	184		
qDTY11.1	11	RM26243	5.48	tcctatttctctcggaaaccatgc ttgtcctgtgctgtcacgaacc	162		RL
		RM26285	6.48	ccatagctcatagctgccctctcc attccgtccttcagtggctcagg	201		
		RM26334	7.5	gactccctactagtggttctgattcg cctttgacgattgtgatgctacg	193		

(continued)

Table 9.3 (continued)

QTL	Chr	Markers	Position (Mbp)	Primer sequence	Product (bp)	Donor(s)	Ecology
		RM7391	9.84	gatgccacatagcgacttag gtcaatgagttcttcaattcc	193		
		RM6091	13.29	gctgtcctgtccttgaatcc tggtaggctggtgacatgc	126		
qDTY12.1	12	RM28048	14.15	ttcagccgatccattcaattcc gctattggccggaaagtagttagc	93	Dular IR74371-46-1-1 Way Rarem	UL, RL
		RM28099	15.9	tgtgcggatgcgggtaagtcc ccacctgtcaaccaccgaaacc	120		
		RM28130	16.75	cagcagacgttccggttctactcg aggacggtggtggtgatctgg	175		
		RM511	17.44	cttcgatccggtgacgac aacgaaagcgaagctgtctc	130		
		RM28166	17.65	tgcttgcaaacattgcttctgg actgatgtactgaacacgggaagg	195		
		RM28199	18.23	cggcttagggagcgtctgtagg gcatgctagtatggccaccatattcc	179		

Peak markers are highlighted in boldface. For each marker, sequence on the top line is of the forward primer and bottom line is of the reverse primer
Chr chromosome; *UL* rainfed upland; *RL* rainfed lowland

rice. The genes governing drought tolerance can be broadly grouped under three categories; signalling factors, transcription factors and functional proteins. The signal proteins include protein kinases such as mitogen-activated protein kinase (MAPK) namely *OsMAPK5* (Xiong and Yang 2003), *Nicotiana* protein kinase (*NPK1*, Xiao et al. 2009), and drought-hypersensitive mutant 1 (*DSM1*, Ning et al. 2010); calcineurin B-Like protein (CBL)-interacting protein kinase (CIPK) such as *OsCIPK12* (Xiang et al. 2007); calcium-dependent protein kinase (CDPK) such as *OsCDPK7* (Saijo et al. 2000) and other kinases such as rice stress-induced protein kinase gene 1 (*OsSIK1*, Ouyang et al. 2010), salt overly sensitive (*SOS2*, Xiao et al. 2009) and rice glycogen synthase kinase3-like gene1 (*OsGSK1*, Koh et al. 2007). Transcription factors include APETALA2/ethylene response factor (AP2/ERF) such as dehydration-responsive element-binding protein 1 (DREB1)/C-repeat-binding factors (*CBF*, Ito et al. 2006; Ishizaki et al. 2013), *DREB2* (Cui et al. 2011) and *ERF* (Fukao et al. 2011); basic Leucine zipper (bZIP) such as *OsbZIP* (Redillas et al. 2012b; Jeong et al. 2010, 2013) and ABA responsive element binding factor (*ABF*, Oh et al. 2005); no apical meristem (NAM), Arabidopsis transcription activation factor (ATAF), cup-shaped cotyledon (CUC) transcription factors) such as *OsNAC* gene family (Hu et al. 2006; Redillas et al. 2012a); Zinc finger proteins such as C2H2 zinc finger like drought and salt tolerant 1 (*DST1*, Huang et al. 2009), stress associated protein (SAP) like *OsiSAP8* (Kanneganti and Gupta 2008) and cold inducible gene, *OsCOIN* (Liu et al. 2007); and other transcription factors such as basic helix loop helix (bHLH) like *OsbHLH148* (Seo et al. 2011), myeloblastosis (MYB) like *OsMYB2* (Yang et al. 2012), *WRKY* (tryptophan, W; arginine, R; lysine, K; tyrosine, Y, Wu et al. 2009; Rushton et al. 2012), homeodomain-leucine zipper (*HD-Zip*, Zhang et al. 2012b) and jasmonate ZIM-domain protein (*TIFY*, Ye et al. 2009). *OsbZIP66* is a Group-E bZIP TF, which is induced under drought stress, overexpression of which imparts drought tolerance through putative ABA dependent pathway. The promoter region of *OsbZIP66* contains ten ABA responsive *cis*-elements (Yoon et al. 2017). A few other regulatory proteins with proven role in drought response mechanisms are protein degrading E3 ubiquitin ligases, seven in absentia (SINA) protein 1 (*OsDIS1*, Ning et al. 2011), salt-and drought-induced ring finger 1 (*OsSDIR1*, Gao et al. 2011), RING domain-containing E3 ubiquitin ligase (*OsRDCP1*, Bae et al. 2011), and delayed seed germination 1 (*OsDSG1*, Park et al. 2010); protein modifiers like farnesyl transferase/squalene synthase (*SQS1*, Manavalan et al. 2012); phytochrome B (*PHYB*, Liu et al. 2012) and other nuclear proteins such as Ski-interaction protein (*OsSKIPa*, Hou et al. 2009), ribosome-inactivating protein (*OsRIP18*, Jiang et al. 2012) and 14-3-3 proteins (*ZmGF14-6*, Campo et al. 2012). A novel ERF gene, *OsERF109*, is reported to enhance plant survival when repressed in transgenic systems under drought, indicating its negative role in imparting drought tolerance by negatively regulating ethylene biosynthesis (Yu et al. 2017). There are several functional proteins that were reported in rice which includes genes involved in metabolism of ABA and other hormones, osmotic adjustment involving spermine, trehalose, proline, dehydrins/LEA, heat/cold shock proteins (HSP), aquaporins, reactive oxygen species (ROS) scavengers, defence-related proteins, ion channel transporters and genes involved in synthesis of pyramidines, porphyrins,

amino acids, myo-inositol etc. The *DSM2* gene encoding β-carotene hydroxylase is identified to impart resistance to drought and oxidative stresses by enhancing ABA and xanthophyll synthesis (Du et al. 2010). A gold hull and internode 3 (*OsGH3.13*) gene, known as *TLD1* (increased tiller number, wider leaf angle and dwarfism 1), encoding for indole acetic acid (IAA) amido synthetase is reported to impart drought tolerance by downregulating IAA (Zhang et al. 2009). Recently, drought tolerance 11 (*OsDT11*), a putative ABA signalling gene, was reported to enhance drought tolerance when overexpressed, inducing increased ABA accumulation, reduced stomatal density and water loss (Li et al. 2017a). *OsDT11* is located on chromosome 11 (LOC_Os11g10590), and encodes for short chain peptide, a cysteine-rich protein (CRP) that is 88 amino acids long. Another ABA-related gene, abscisic acid stress and ripening 5 (*OsASR5*) showed improved drought tolerance when overexpressed transgenically in *Arabidopsis* and rice, by regulating ABA and hydrogen peroxide-dependent stomatal closure pathway (Li et al. 2017b). Li et al. (2011) demonstrated that overexpression of *OsTPS1* gene enhanced tolerance for abiotic stresses such as cold, salt and drought. In similar fashion, two rice LEA proteins *OsLEA3-1* and *OsLEA3-2* were found to play key roles in drought resistance (Xiao et al. 2007; Duan and Cai 2012). Both *OsLEA3-1* and *OsLEA3-2* were found to safeguard lactate dehydrogenase (LDH) from inactivation on desiccation (Duan and Cai 2012; Hu et al. 2014). Further, HSPs such as *OsHsp17.0*, *OsHsp23.7* and *OsHsp17.7* were established to confer drought resistance in rice on over expression (Murakami et al. 2004; Sato and Yokoya 2008; Zou et al. 2012). The relative water content 3 (*RWC3*) gene from rice was reported to enhance drought tolerance by improving hydraulic conductivity in transgenic plants overexpressing the gene (Lian et al. 2004). *RWC3* is an isoform of rice plasma membrane intrinsic protein (*OsPIP1;3*) encoding for a membrane aquaporin. A recent RNA-seq analysis proposes a negative regulatory pathway of drought tolerance in rice roots mediated by rice phytochrome B (*OsPhyB*) that affects ROS scavenging system by repressing two key enzymes, catalase and ascorbate peroxidase (Yoo et al. 2017). Further, ROS scavenging activity of rice drought stress response 1 (*OsDSR1*), a calmodulin-like (CML) gene is demonstrated to impart enhanced plant survival by preventing the oxidative damage under drought stress (Yin et al. 2017). In transgenic rice seedlings challenged with water stress, a PIN FORMED (*PIN*) gene namely *OsPIN3t*, which is an auxin flux carrier gene, was shown to impart drought resistance by improving polar auxin transport (Zhang et al. 2012a). Other defence-related genes from rice that are reported to enhance drought resilience are type 1 metallothionein (*OsMT1a*, Yang et al. 2009), pathogenesis-related (PR) 4 (*OsPR4a*, Wang et al. 2011) and chymotrypsin inhibitor (*OCPI1*, Huang et al. 2007). Auxiliary improvement of drought resistance was also attributed to metabolism-related proteins from rice such as a putative cytosolic dihydroorotate dehydrogenase (*OsDHODH1*, Liu et al. 2009) involved in pyramidine biosynthesis, ornithine δ-aminotransferase (*OsOAT*) associated with amino acid synthesis (You et al. 2012) and myo-inositol oxygenase (*OsMIOX*) involved in myo-inositol metabolism (Duan et al. 2012). Recently, a novel gene *OsAHL1* (AT-hook content nuclear localized protein) is reported to enhance both drought avoidance and tolerance (Zhou et al. 2016). *OsAHL1* confers drought avoidance by improving root growth on exposure

to drought, and it plays a role in regulating oxidative stress responses thus conferring drought tolerance. In addition to rice genes, a stress-associated protein (SAP) gene *AlSAP* derived from the C4 halophyte grass *Aeluropus littoralis* was found to significantly enhance grain yield in transgenic rice lines subjected to reproductive stage drought stress (Ghneim-Herrera et al. 2017).

9.6 Breeding for Drought Tolerance

Improving crop performance under drought is a worldwide concern under scenarios of increasing recurrence and intensity of dry spell (Mpelasoka et al. 2008; Wassmann et al. 2009). In areas, where rice cultivation depends on rainwater management, such as upland rice in Asia, the repercussions of uncertain dry spells are gruesome. Such macabre situations demand immediate need for replacing the drought sensitive cultivars with better-adapted ones that combine higher yield potential and improved drought resilience (Lafitte et al. 2006; Kumar et al. 2008). However, introducing newer varieties generally face an unpredicted threat from locally adapted flora, which are adapted to perform better under limited water. Consequently, it is prudent to combine efforts to breed varieties that are better weed competitors with ability to access limited water resources quickly and efficiently to garner vegetative phase growth and ultimately a good harvest (Kumar et al. 2009b; Okami et al. 2011).

Although huge variation exists in rice germplasm for drought adaptation, only a handful of varieties are useful in breeding for drought resistance in rice. This may be either due to poor agronomic features tightly linked to drought adaptation or due to incompatibility in using them as source for drought resistant genes. Nevertheless, use of wild rice has long been recognized as an alternate source for drought adaptation genes that are currently absent in cultivated germplasm (Ariyatanakatawong 2015). Widely recognized wild rice for drought research include species such as *O. barthi*, *O. australiensis*, *O. perennis*, *O. longistaminata*, *O. rufipogon* (Brar and Khush 1986), and cultivated African rice, *O. glaberrima* (Sano et al. 1984; Sitch et al. 1989; Jones et al. 1997; Maji et al. 2011; Sarla and Swamy 2005). Seventeen new lines, developed from *O. glaberrima* and *O. sativa* crosses, with superior drought tolerance than the *O. sativa* species, are under production in few countries in Africa (WARDA 2005; Lorieux et al. 2013).

Drought is genetically a complex trait to design a straight-forward breeding solution, because many of the drought-related traits are damages rather than symptoms of stress, which makes them unsuitable for effective genotypic response evaluation and are highly unstable and weighs substantial influence from the environment, thereby exhibiting very low heritability. Therefore, using conventional strategy, drought resistance breeding in rice lags behind when compared to breeding for other stresses. Recent development in genomic tools and technologies has boosted the efforts in breeding by helping to identify genes responsible for drought adaptation and help in deploying these genes in elite cultivars that lack resistance (Roy et al. 2011; Miura et al. 2011).

Earlier breeding efforts towards drought adaptation in rice were primarily devoted to selecting for yield under drought but met limited success due to lack of precision of selection process. Predominantly, efforts were anchored on selection of physiological traits related to turgor and leaf water status and morphological traits such as root system architecture, stomatal apparatus etc. (Kumar et al. 2008). Moreover, most of these studies were conducted under lowland ecology by artificially creating physiological drought. All these earlier attempts for drought tolerance breeding in rice had limited success primarily because of the absence of an effective screening strategy and combined with low heritability of traits (Ouk et al. 2006). Later studies on rice yield under upland situations, however, opened up vistas of exercising selection under such situations (Venuprasad et al. 2007) and combining drought adaptation with high yield potential, a route to develop high yielding drought adapted varieties. This emphasized the importance of precision phenotyping in selection for drought adaptation in rice. Further, the use of modern, high throughput and accurate phenotyping could help in improvement of heritability of selection traits largely, thereby accelerating the breeding progress (Kumar et al. 2008)

Targeted breeding for drought tolerance in rice is now being done by incorporation of QTLs into leading cultivars. This approach focuses primarily on the improvement of mega-varieties using marker-assisted breeding approach, either by transferring single or by pyramiding several QTLs (Singh et al. 2015). QTL introgression into mega-varieties such as IR64 was reported to combine assortment of QTLs such as *qDTY9.1*, *qDTY2.2*, *qDTY10.1*and *qDTY4.1* into several backcross-derived lines. These lines were reported to have a yield advantage of 500 to 1800 kg/ha as against the background cultivar, IR64 under various degrees of stressed conditions (Swamy et al. 2013). Several rice lines have been released for commercial cultivation in different rice growing countries of south and Southeast Asia and Africa in the past ten years (Table 9.4). Most of these are conventionally bred lines that are selected using modern phenotype screens. Recently, products of marker-assisted breeding are also being introduced into cultivar chain (Kumar et al. 2014; Singh et al. 2015). Marker-assisted improvement of popular varieties from major rice growing nations of Asia, such as India (Singh et al. 2015; Dwivedi et al. 2015; Gopala Krishnan et al. 2017), Vietnam (Ha et al. 2016), Philippines (Dixit et al. 2017a), Nepal (Dixit et al. 2017b), and Malaysia (Shamsudin et al. 2016a, b) are underway.

9.7 Conclusions and Future Prospects

In the wake of increasing challenges of water scarcity and global warming, development of drought tolerant varieties to suit the needs of every rice growing nation is a contemporary reality today. Significant research efforts have been made during the past few years for the development of drought tolerant rice cultivars, mostly from the work carried out at International Rice Research Institute, Los Baños (Sandhu and Kumar 2017). These achievements can be attributed to advancement in phenotyping and genotyping technologies. Drought being a complex trait with diverse tolerance

Table 9.4 Recent releases of drought tolerant varieties for commercial cultivation in major rice growing nations of the world

Country	Year	Varietal name	Line	Parentage	Breeding system	Ecosystem
India	2010	Sahbhagi Dhan	IR74371-70-1-1	IR55419-04*2/Way Rarem	Traditional	RL, UL
India	2013	IR64 Sookha 1	IR 87707-445-B-B-B	IR64 + *qDTY2.2* + *qDTY4.1*(Vandana)	MAB	RL
India	2014	DRR Dhan 42	IR87707-445-B-B-B	IR64 + *qDTY2.2* + *qDTY4.1*(Vandana)	MAB	RL
India	2014	Tripura Khara Dhan 1	IR87707-446-B-B-B	IR64 + *qDTY2.2* + *qDTY4.1*(Vandana)	MAB	RL
India	2014	Tripura Khara Dhan 2	IR87707-182-B-B-B	IR64 + *qDTY2.2* + *qDTY4.1*(Vandana)	MAB	RL
India	2014	DRR Dhan 43	IR83876-B-F3	IR03L03/IRRI148	Traditional	RL
India	2014	DRR Dhan 44	IR93376-B-B-130	IR71700-247-1-1-2/IR03L120	Traditional	UL
India	2014	CR Dhan 201	IR83380-B-B-124-1	IR72022-46-2-3-3-2/PSB RC 18	Traditional	UL
India	2014	CR Dhan 202	IR84899-B-154	IR78877-208-B-1-1/IR55423-01	Traditional	UL
India	2014	CR Dhan 203	IR84899-B-185	IR78877-208-B-1-1/IR55423-01	Traditional	UL
India	2014	CR Dhan 204	IR83927-B-B-279	IR78888-208-B-1-2/CT6510-24-1-2	Traditional	UL
India	2014	CR Dhan 205	IR86931-B-578	Nagina22/Swarna	Traditional	UL
India	2014	Tripura Hakuchuk 1	IR83928-B-B-56-4	IR 78877-208-B-1-2/IRRI 148	Traditional	UL

(continued)

Table 9.4 (continued)

Country	Year	Varietal name	Line	Parentage	Breeding system	Ecosystem
India	2014	Tripura Hakuchuk 2	IR82589-B-B-138-2	IR55423-01/IRRI148	Traditional	UL
India	2016	Swarna Shreya	IR84899-B-179-16-1-1-1-1	IR78877-208-B-1-1/IR55423-01	Traditional	RL
Bangladesh	2011	BRRI Dhan 56	IR74371-70-1-1	IR55419-04*2/Way Rarem	Traditional	RL
Bangladesh	2011	BRRI Dhan 57	BR7873-5(NIL)-52-HR6	BR11/CR146-7027-224	Traditional	RL
Bangladesh	2014	BRRI Dhan 66	IR82635-B-B-75-2	IR78875-176-B-2/IR78875-207-B-3	Traditional	RL
Bangladesh	2015	BRRI Dhan 71	IR82589-B-B-84-3	IR55423-01/IRRI148	Traditional	RL
Indonesia	2011	Inpago 7	B12498E-MR-1	IR68886/BP68//Slegreng///Maninjau/Asahan	Traditional	UL
Indonesia	2011	Inpago 8	TB409B-TB-14-3	Cirata/TB 177	Traditional	UL
Indonesia	2011	Inpago LIPI Go 1	IR79971-B-191-B-B	Vandana/Way Rarem	Traditional	UL
Indonesia	2011	Inpago LIPI Go 2	IR79971-B-227-B-B	Vandana/Way Rarem	Traditional	UL
Indonesia	2012	Inpago 9	B12151D-MR-4	UPLRI/IRAT15	Traditional	UL
Indonesia	2013	Inpago Lipi Go 4	IR79971-B-102-B-B	Vandana/Way Rarem	Traditional	UL

(continued)

Table 9.4 (continued)

Country	Year	Varietal name	Line	Parentage	Breeding system	Ecosystem
Mozambique	2013	M'ziva	IR77080-B-B-34-3	IR 70179-1-1-1-1/IR 73885-1-4-3-2-1-6	Traditional	RL
Myanmar	2011	Yeanelo 1	IR54402-01	–	–	RL
Myanmar	2013	Yeanelo 2	UPLRi -7	C22/IR26//C22/OS4	Traditional	UL
Myanmar	2013	Myaungmya May	Pram Bei Kour	Pram Bei Kour	Traditional	RL
Myanmar	2015	Yeanelo 4	IR87707-446-B-B-B	IR64 + *qDTY2.2* + *qDTY4.1*(Vandana)	MAB	RL
Myanmar	2016	Yeanelo 5	IR87705-44-4-B	IR64 + *qDTY4.1* (Aday Sel)	MAB	RL
Myanmar	2016	Yeanelo 6	IR87707-182-B-B-B	IR64 + *qDTY2.2* + *qDTY4.1*(Vandana)	MAB	Rl
Myanmar	2016	Yeanelo 7	IR87705-83-12B	IR64 + *qDTY4.1* (Aday Sel)	MAB	RL
Nepal	2010	Tarahara 1	IR80411-B-49-1-1	IR70181-26-PMI2-9-1-1/IRRI105	Traditional	RL
Nepal	2010	Hardinath 2	IR6144-F-MR-6-0-0	IR1721-11-8-3/KU10	Traditional	RL
Nepal	2011	Sukha Dhan 1	IR74371-46-1-1	IR55419-04*2/Way Rarem	Traditional	RL
Nepal	2011	Sukha Dhan 2	IR74371-54-1-1	IR55419-04*2/Way Rarem	Traditional	RL
Nepal	2011	Sukha Dhan 3	IR74371-70-1-1	IR55419-04*2/Way Rarem	Traditional	RL

(continued)

Table 9.4 (continued)

Country	Year	Varietal name	Line	Parentage	Breeding system	Ecosystem
Nepal	2014	Sukha Dhan 4	IR87707-445-B-B-B	IR64 + *qDTY2.2* + *qDTY4.1*(Vandana)	MAB	RL
Nepal	2014	Sukha Dhan 5	IR83388-B-B-108-3	Swarna/IR72022-46-2-3-3-2	Traditional	RL
Nepal	2014	Sukha Dhan 6	IR 83383-B-B-129-4	IR 72022-46-2-3-3-2/IR 57514-PMI 5-B-1-2	Traditional	RL
Nepal	2017	Hardinath 3	IR93376-B-B-130	IR71700-247-1-1-2/IR03L120	Traditional	UL
Nigeria	2013	Upia 1	IR68	IR 19660-73-4/IR 2415-90-4-3-2//IR 54	Traditional	RL
Nigeria	2013	Upia 2	IR69513-21-Srn2-Ubn1-B	IR57514-SRN-299-3-2-4/IRRI119//IR43524-55-1	Traditional	RL
Nigeria	2013	Upia 3	IR74371-54-1-1	IR55419-04*2/Way Rarem	Traditional	RL
Philippines	2009	Sahod Ulan 1	IR74371-54-1-1	IR55419-04*2/Way Rarem	Traditional	RL, UL
Philippines	2011	Sahod Ulan 2	NSIC Rc272	PR34363-4-Pokkali/AC-45-M5R-19	Traditional	RL
Philippines	2011	Sahod Ulan 3	IR81412-B-B-82-1	IR57514-PMI-5-B-1-2/PSB Rc82	Traditional	RL
Philippines	2011	Sahod Ulan 4	C8108-B-10-2-2-1	C5649-2B-5-2-2-1/IR74627-30-1-1-8	Traditional	RL
Philippines	2011	Sahod Ulan 5	IR81023-B-116-1-2	IR77298-5-6/CT6510-24-1-2	Traditional	RL
Philippines	2011	Sahod Ulan 6	IR72667-16-1-B-B-3	WS91/Abhaya/IR43070-UBN-511-12-1-1-1	Traditional	RL
Philippines	2011	Sahod Ulan 7	C8231-B-1-1	C5649-2B-5-2-2-1/C6518-2B-5-1-1	Traditional	RL

(continued)

Table 9.4 (continued)

Country	Year	Varietal name	Line	Parentage	Breeding system	Ecosystem
Philippines	2011	Sahod Ulan 8	IR74963-262-5-1-3-3	IR43/IR65564-22-2-3/IR68	Traditional	RL
Philippines	2011	Sahod Ulan 9	C6392-2B-3-3-1-2	TOX 4004-36-2-3-2/Katsuri1	Traditional	RL
Philippines	2011	Sahod Ulan 10	PR25769-B-9-1	M9-33B/IR53236-218-3	Traditional	RL
Philippines	2011	Katihan 1	IR79913-B-176-B-4	IR55419-04/Way Rarem	Traditional	UL
Philippines	2013	Sahod Ulan 11	NSIC 2013 Rc346	PR34350-4-Pokkali-24-M5R-10	Traditional	RL
Philippines	2013	Sahod Ulan 12	IR81047-B-106-2-4	IR01A 102/CT6510-24-1-2	Traditional	RL
Philippines	2014	Katihan 2	IR82635-B-B-47-2	IR78875-176-B-2/IR78875-207-B-3	Traditional	UL
Philippines	2014	Katihan 3	IR86857-101-2-1-3	IR80461-B-7-1/IR80508-B-57-3-B	Traditional	UL
Philippines	2014	Katihan 4	IR83140-B-36-B	IR82969-11/IR82870-48	Traditional	UL
Philippines	2015	Sahod Ulan 15	IR83383-B-B-129-4	IR 72022-46-2-3-3-2/IR 57514-PMI 5-B-1-2	Traditional	RL
Philippines	2015	Sahod Ulan 20	IR86781-3-3-1-1	Dhagad Deshi/IR 77080-B-34-3	Traditional	RL
Sri Lanka	2014	–	BG251	BG 34-8*3/IR 20	Traditional	RL

– information not available; *RL* rainfed lowland; *UL* upland; *MAB* marker assisted backcross breeding

mechanisms, there are several loci associated with tolerance that are yet to be discovered. Therefore, integration of technological advancements in genome, proteome and metabolome biology together with computational and statistical advancements can define the future for drought breeding in rice. Methods such as genomic selection is now becoming a reality to be experienced and drive rapid turnover of drought tolerance genotypes, which can be quickly phenotyped and characterised for release as cultivars. Keeping an array of drought adaptable and multiple stress tolerant cultivars is a need of the hour to cope up with the challenges of the future.

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Chapter 10
Genomics-Assisted Breeding for Drought Tolerance in Cowpea

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Abstract The importance of cowpea, *Vigna unguiculata*, in human and animal nutrition and sustainability of soil fertility are recognized globally especially in sub-Saharan Africa (SSA) where the crop is mainly produced in the Savanna and the Sahelian agro ecologies. However, cowpea productivity is adversely affected by both biotic (insect pests, diseases, parasitic weeds, nematodes) and abiotic (drought, heat, low soil fertility) constraints. Appreciable progress has been made in the improvement of cowpea for resistance to some biotic stresses particularly diseases such as bacterial blight, ashy stem blight, marcophomina, parasitic weeds like Striga and Alectra and some insects like aphid, leaf and flower thrips among others. There is need for intensifying research activities with focus on improving cowpea resistance to abiotic stresses. As a crop grown commonly in arid regions, cowpea is subjected to seedling stage, midseason and terminal droughts. In the recent past, the amount of rainfall, during the cropping season in the dry savannah regions of SSA, is getting less. Consequently the cropping season is getting shorter occasioned by late commencement or early cessation of the rain. Farmers in the cowpea producing areas of SSA generally have no access to irrigation hence their crops are grown under rainfed conditions. With the impending higher frequency of drought in the dry savannah region due to climate change, efforts should be made in developing climate resilient cowpea varieties that farmers will grow. Efforts have been made in enhancing tolerance to drought in some improved cowpea varieties using conventional breeding but progress has been slow in this regard. Drought tolerance is a complex trait and many genes are involved in its inheritance. Pyramiding of these genes in improved varieties would therefore, be desirable. Such varieties with pyramided genes are likely to be stable in performance over the years and across several locations in the savannahs. Recent developments in molecular biology could play significant role in the development of such resilient varieties. In a number of crops, molecular markers associated with resistance loci have been identified and are being used in marker assisted breed-

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ing. Marker assisted backcrossing (MABC) is the choice when single traits that are simply inherited are to be moved to varieties with superior performance but lacking in the trait being transferred. Also, marker assisted recurrent selection (MARS) has shown promise in accumulating multiple genes in improved varieties of some crops. Some work has been initiated in cowpea on the use of MARS to pyramid resistance to Striga, yield and drought. Results obtained so far show the potential of this method in pyramiding desirable genes in cowpea. As more resources get committed to cowpea research a solid foundation would be established for the generation of molecular tools that should facilitate their routine application to the improvement of the crop.

Keywords Cowpea · Drought tolerance · MAGIC populations · MARS · Striga

10.1 Introduction

Cowpea (*Vigna unguiculata* L. walp.) belongs to the genus *Vigna* and family *Fabaceae*. It is one of the four cultivated species of the genus with the remaining three being *V. cylindrica, V. sesquipedalis* and *V. textilis*. It is a highly self-pollinating crop, diploid with 2n = 22 and has an estimated genome size of 620 Mb (Chen et al. 2007). Its genome is similar to that of some other warm season legumes, particularly the common bean (*Phaseolus vulgaris* L.) (Vasconcelos et al. 2015). Although, cowpea is one of the most important crops in sub-Saharan Africa where it is considered drought-tolerant as compared to other legumes and cereals cultivated in the semi-arid regions, but it still encounters significant damage and yield losses due to severe and frequent droughts. Its grains and pods play an important role in human nutrition, while biomass provides good nutritious fodder to livestock (Ehlers and Hall 1997; Singh et al. 2003; Boukar et al. 2016). Its grains are rich in carbohydrates, protein and folic acid, and contain respectable amounts of some minerals (Boukar et al. 2011, 2016; Carvalho et al. 2017). The pods are also rich in protein, chlorophyll, carotenoids, phenolics and have high antioxidant activity, low concentrations of nitrates and raffinose family oligosaccharides (Karapanos et al. 2017). It is a great source of income for small holder famers and food vendors. Based on evaluation of 1541 germplasm lines, cowpea grains were estimated to contain on average approximately 25% protein, 53.2 mg/kg iron, while zinc, calcium, magnesium, potassium, and phosphorus content were reported to be 38.1, 826, 1915, 14,890, and 5055 mg/kg, respectively (Boukar et al. 2011). As a leguminous species, cowpea has the ability to fix nitrogen from the atmosphere, some of which is left in the soil for succeeding crops (Sanginga et al. 2000). As a relatively drought tolerant crop, cowpea is excellent for studying the genetic basis of drought tolerance.

Globally, cowpea production is projected at around 6.5 million metric tonnes annually on about 14.5 million hectares. Approximately 83% of the worldwide cowpea production is obtained in Africa, out of which, 80% production is in West-Africa. The world's major producer and consumer of cowpea is Nigeria (45% production), followed by Niger (15%), Brazil (12%), and Burkina Faso (5%). Fatokun et al. (2012a) reported that during 1980–2010, there was an increase of an average rate of 5%, with 3.5% annually in area and 1.5% in yield, implying that 70% of the total growth in cowpea production during this period is accounted for area expansion. Worldwide, the proportion of cowpea in total cultivated area under pulses increased from 10% in 1990 to nearly 20% in 2007 (Boukar et al. 2016). According to Fatokun et al. (2012a) and Boukar et al. (2016), demand for cowpea in West Africa is expected to grow at a faster rate of 2.68% per year than supply (2.55%) over the period 2007–2030. Average cowpea yield in farmer's field is very low due to several biotic and abiotic stresses. Among abiotic stresses, drought is the major constraint in cowpea production in the West African Sahel and dry Savannahs.

Climate change may lead to a higher frequency and severity of drought events as already being experienced in the dry savannah regions of sub-Saharan Africa (SSA). Drought is a major constraint to crop production in SSA where irrigation facilities are grossly inadequate. It has been forecasted that by 2050, water shortages will affect 67% of the world's population (Ceccarelli et al. 2004). Terminal drought is one of the most common environmental stresses that continues to be a challenge to sub-Saharan African farmers and plant breeders. Drought tolerance is a complex trait controlled by polygenes whose expressions are influenced by environmental factors. Therefore, unraveling its genetic basis is crucial for both breeding and basic research. Breeding for drought tolerance with conventional methods could be difficult and time consuming. This calls for concerted efforts by various players such as geneticists, breeders, molecular biologists, physiologists and agronomists among others. Due to rapid population growth particularly in the developing countries where climate change is likely to have greater devastating impact, conventional breeding procedures may not be adequate in developing improved varieties that can ameliorate the challenges. New tools involving molecular breeding have the potential to contribute positively to needed progress in developing appropriate technologies to combat effects of climate change. With the recent progress in genomics, it should be feasible to breed robustly drought tolerant varieties within shorter period. In this chapter, we describe recent developments in genetic and genomic resources, and molecular breeding in cowpea with emphasis on drought tolerance.

10.2 Drought Tolerance Phenotyping and Mechanisms in Cowpea

Breeding for drought tolerance and high yield under drought has not been as successful as for simply inherited traits in cowpea. This is mainly due to the complexity of abiotic factors and plant mechanisms involved in drought tolerance and the lack of simple, accessible and reliable trait-based phenotyping tech-

niques to select drought-tolerant progenies from segregating populations (Singh and Matsui 2002). Drought tolerance in plants is a complex trait since various environmental parameters (air temperature and humidity, soil texture, moisture and fertility) and plant features and strategies (in shoots and roots) operate jointly to enable crop plants cope with drought stress. Therefore, it is important to discriminate and investigate these factors and mechanisms individually, study and understand their interactions and contributions to plant drought tolerance in a given environment or drought scenario, so they will be easy to manipulate by breeders and use in crop improvement (Vadez et al. 2013; Sinclair et al. 2015). Physiological phenotyping for drought tolerance is quite expensive, time consuming, and difficult to use for screening crop germplasm with large number of accessions. However, significant efforts and achievements were made in developing high throughput plant physiology screening methods for improving drought tolerance in cowpea over the past decades.

Singh et al. (1999a) developed a simple "wooden box screening technique" which eliminates the influences of the root system and allows nondestructive identification of plant shoot dehydration tolerance at seedling stage in cowpea. Two types of drought tolerance mechanisms at vegetative stage were identified and described by Mai-Kodomi et al. (1999a). Upon exposure to progressive soil water deficit stress, the Type 1 drought-tolerant lines (TVu-11986 and TVu-11979) stopped growth and conserved water in all the plant tissues, stayed alive for over two weeks without irrigation, and gradually the entire plant parts dried as the drought stress became intense and drastic. The type 2 drought-tolerant lines (Dan'Ila and Kanannado) continued slow growth of the trifoliates but with increased soil moisture deficit stress, their unifoliates senesced early and dropped off with their growing tips remaining turgid and alive relatively longer. Mai-Kodomi et al. (1999b) studied the inheritance of drought tolerance at seedling stage of cowpea. Three cowpea genotypes: TVu-11986 with Type 1 drought tolerance, Dan'Ila with Type 2 drought tolerance, and TVu-7778 as drought sensitive were crossed in multiple combinations, and the segregation pattern revealed that vegetative stage drought tolerance is a dominant trait and both Type 1 and Type 2 reactions are controlled by a single dominant gene but the genes are independent in the two types. The box screening method showed good correlation with drought tolerance at vegetative and reproductive stages, and was also efficient in evaluating and selecting drought-tolerant plants in different crop species (Singh et al. 1999b; Tomar and Kumar 2004; Slabbert et al. 2004; Ewansiha and Singh 2006).

Pot experiments in the screen-house and growth chamber showed that reduced plant leaf area, restricted canopy water loss, transpiration efficiency, delayed leaf senescence, prolonged and sustained stem greenness are important traits for enhancing cowpea growth and grain yield in drought-prone environments (Muchero et al. 2008; Belko et al. 2012, 2013). However, selection based on phenotype has been relatively slow and difficult mainly due to the unpredictable timing, intensity and occurrence of drought and considerable genotype-by-environment interactions and effects on phenotypic expression in the field. Cowpea has a noteworthy capability to survive drought by limiting its water loss or enhancing soil water uptake and use through various anatomical, morphological, biochemical and physiological strategies. Plants "escape" drought by changing phenological development and the

period of a particular growth phase (Agbicodo et al. 2009). Some cowpea varieties evade terminal drought by early flowering (around 12 days), whereas other varieties respond by staying green for weeks and flower later when favorable conditions are re-established (Fatokun et al. 2012b).

Drought "avoidance" strategies are predominantly physiological and morphological alterations to withstand drought stress while maintaining relatively high tissue moisture. A few of the intrinsic and stress-induced mechanisms documented in cowpea include, but not limited to, higher root density or depth (Sicher et al. 2012), decreased leaf area, enlarged leaf waxiness and thickness (Singh and Raja Reddy 2011), reduced stomatal and lenticular conductance and leaf rolling (Fatokun et al. 2012b; Hall 2012). The crop shows very little variations in leaf water content under extreme drought; an isohydric phenomenon, associated with three drought evading mechanisms i.e. stomata closure, paraheliotropism, and high root hydraulic conductivity (Agbicodo et al. 2009). Drought "tolerance" traits are mainly associated with osmotic adjustments, which result from the synthesis and accumulation of compatible solutes in the cytoplasm as well as movement of solutes into the vacuoles (Warren et al. 2011; Khan et al. 2015; Blum 2017). These hydrophilic solutes, by replacing water molecules on membrane and protein surfaces, raise the cellular osmotic pressure and concomitantly the water potential gradient between soil and roots, which allows continued water influx via osmosis. Modifications and/or stabilization of cell walls and membranes also confer tolerance to drought (Lugan et al. 2010; Jin et al. 2016). To lessen the deleterious consequences of water shortage, plants use these mechanisms either independently or jointly.

Belko et al. (2014) evaluated the impacts of reproductive stage drought stress on the growth, development and yields of a diverse set of thirty early and thirty medium maturity cowpea cultivars under post-flowering water stressed (WS) and well-watered (WW) conditions in the field using stress tolerance selection indices e.g. stress tolerance index (STI) and geometric mean productivity (GMP). Overall, lines IT85F3139, IT93K-693-2, IT97K-499-39, IT93K-503-1, IT96D-610, IT97K-207-z15, KVx-61-1, KVx-403, KVx-421-25, and Mouride exhibited the highest grain yield in both WS and WW environments and were therefore identified as the most drought-tolerant lines based on their outstanding STI and GMP values. Vadez et al. (2012a) argued that despite the complexity of the plant's response to drought, simple hypotheses based on soil water availability and plant water-use pattern (water supply vs. demand) can be developed to guide selection of critical plant traits that are capital for adaptation to drought-prone regions. Hence, Belko et al. (2012) tested the hypothesis that water saving shoot traits is important for end-of-cycle drought tolerance and thereby discriminates drought tolerant and susceptible lines. Thus they phenotyped a wide range of cowpea genotypes for their variation in vegetative growth attributes and water-use patterns under different water regimes (WW and WS) and atmospheric vapor pressure deficit (VPD) outdoors, in glasshouse and growth chamber. However, gravimetric measurement of whole plant leaf canopy transpiration rate (TR) involves weighing pots and can be laborious and time consuming. Therefore, Belko et al. (2013) set the conditions (plant age, time of the day) and tested and validated a method in which plant transpiration rate (TR) can be indirectly assessed via

plant canopy temperature (CT) in a high throughput mode using an infrared imaging system. Under WS conditions, canopy transpiration dropped at a lower fraction of transpirable soil water in drought tolerant than in susceptible lines. Tolerant lines also maintained higher transpiration efficiency (TE) and TR, and lower CT under severe water stress (Belko et al. 2012). Under WW conditions, cowpea plants grew and developed larger biomass under low VPD than under high VPD, with a consistent trend of lower leaf area and biomass in drought tolerant lines. Substantial differences existed among cowpea lines in their TR response to natural variation of VPD, with drought tolerant lines having significantly lower TR than sensitive ones, especially at times of highest VPD. Cowpea genotypes also varied in their TR response to progressively increasing VPD, with some tolerant lines displaying a clear VPD breakpoint at about 2.25 kPa, above which there was very little increase in TR whereas sensitive genotypes showed a linear increase in TR as VPD increased. Canopy temperature, estimated with thermal imagery, was highly correlated with TR and could therefore be used as proxy for canopy transpiration (Belko et al. 2013). Plant traits that control canopy water loss when soil water is available at vegetative stage such as low leaf area, low TR by stomata control, and reduced TR in response to high VPD discriminated between drought tolerant and sensitive cowpea lines and, therefore, are reliable indicators of terminal drought stress tolerance. A lower TR could limit plant growth and water use at vegetative stage, and allow drought tolerant genotypes to behave like unstressed plants late in the season when the soil water is progressively depleted. The water saving shoot characteristics of some cowpea genotypes are hypothesized to conserve more water in the soil profile, which is crucial for pod and grain filling and subsequently terminal drought adaptation.

Root-related traits i.e. deep, profuse, dense and extensive root systems are thought to be key in conferring drought tolerance to cultivated crop plants (Lynch 2007; Singh et al. 2010; Gowda et al. 2011). Although variation in root growth and morphology can increase the amount of water uptake under drought and root traits are used as surrogates for soil water extraction (Vadez et al. 2008, 2012; Gowda et al. 2012), the relationships between root traits and water acquisition and their contribution to yield formation under drought remain unclear across crop species (Vadez et al. 2007). Few investigations have been carried out on roots in cowpea and most have focused on the analysis of root growth and morphological differences using limited number of test lines (Matsui and Singh 2003; De Ronde and Spreeth 2007; Onuh and Donald 2009). Moreover, these studies did not address whether root attributes relate to soil water accessibility and use under drought, especially during critical pod and grain filling stages. A “root-box pin-board’ technique was developed to study the two-dimensional root system of large number of cowpea plants and progenies, and permitted characterization of major variations for root system architecture (deep and profuse vs. shallow and dense systems) in cowpea (Singh and Matsui 2002). More recently, Burridge et al. (2016) developed an integrated low-cost and high throughput visual, manual (shovelomics) and image-based (DIRT: digital imaging of root traits, an automated image analysis software) phenotyping technique for in situ field and laboratory evaluation of root phenes in cowpea. The method was used for quantitative evaluation of root architectural traits, and identification and selection of useful root

phenes among 189 lines of cowpea diversity panel. Like in other major crops of economic importance (maize, common bean, soybean), several root phenotypes i.e. adventitious and basal root numbers and growth angle, tap root diameter at different soil layers, secondary or lateral root numbers and branching density, root nodules and diseases scores, significantly varied among tested cowpea genotypes. Genetic analysis was performed to evaluate the relationships between cowpea root traits and agronomic performance and tolerance to parasitic weeds in the field. It was found that plants with steep and profuse root system were better adapted to drought conditions while those with shallow and dense root system were tolerant to low phosphorus and Striga infestation (Burridge et al. 2016). The results therefore suggested the adoption of this integrated root phenotyping platform in the breeding program to improve cowpea adaptation to multiple constraints e.g. vegetative and reproductive stage droughts and Striga infestation.

10.3 Genetic Resources in Cowpea

Genes responsible for resistance/tolerance to several abiotic and biotic stresses have been identified through the germplasm screening, available in different countries. The International Institute of Tropical Agriculture (IITA) is maintaining more than 15,000 accessions of cultivated cowpea and over 2000 wild relatives, in its genetic resources center. Mining these resources has identified several potential donors, which can impart resistance to biotic and abiotic stresses. Several cowpea lines resistant/tolerant to abiotic and biotic stresses have been reported (Ferry and Singh 1997; Singh 2002; Boukar et al. 2013, 2015, 2016). Breeders will continue to rely on these genetic resources as sources of genes for desirable traits in cowpea improvement. They have the potential to provide genes for developing new varieties that will help in combating emerging problems that would arise due to climate change as well as human food and animal feed requirements. It is also worth noting that wild cowpea relatives have hardly been utilized in the development of new improved varieties. A lack of interest in the use of cowpea wild relatives can be attributed to the possibility of linkage drag that may occur from their use as parents. For example, wild cowpea relatives have very small seed size with smooth and unattractive seed coat colors. Several backcrosses to the recurrent parents would be needed in order to recover the cowpea seed size desired by consumers. However, with recent developments in genomics which can facilitate progress in breeding new varieties through marker-assisted selection, more interests may shift in favor of the available crop wild relatives as sources of new genes.

10.4 Genomic Resources in Cowpea

The development of genomic resources for cowpea has lagged behind compared with many other crops. However, because of the advantages associated with the new marker technology, concerted efforts are now being devoted to the development of genomic resources in cowpea. An appreciable amount of progress has already been made from these efforts (Muchero et al. 2009a, 2010, 2013; Amatriain et al. 2017). The molecular markers based genetic linkage maps for cowpea have been published, although not yet aligned with physical maps (Amatriain et al. 2017). These linkage maps have been utilized to identify quantitative trait loci (QTL) associated with morphological as well as stress related traits (Table 10.1) (Boukar et al. 2016). Major recent developments in cowpea genomics include sequence assemblies from 65× coverage whole-genome shotgun (WGS) short reads, a bacterial artificial chromosome (BAC) physical map, minimal tiling path (MTP) BACs, and assembled sequences from 4355 BACs using an improved variety (IT97 K-499-35) which has been released to farmers in several African countries due to its superior yield performance and resistance to *Striga gesnerioides*. Additionally, more than one million SNPs have been discovered from sequences of 36 diverse cowpea accessions supported by the development of a genotyping assay (Illumina Cowpea iSelect Consortium Array) for 51,128 SNPs. Five bi-parental RIL populations (Tvu-14676 × IT84S-2246-4, Sanzi × Vita7, ZN016 × Zhijiang282, CB46 × IT93 K-503-1, and CB27 × IT82E-18) were genotyped with this genotyping platform to produce a consensus genetic map containing 37,372 SNP markers. This genetic map has enabled the anchoring of 100 Mb of WGS and 420 Mb of BAC sequences, an exploration of genetic diversity along each linkage group, and synteny between cowpea and common bean (Amatriain et al. 2017). Updated versions of the cowpea consensus map are accessible via HarvEST:Cowpea (http://harvest.ucr.edu/). With the above listed genomic resources in cowpea, opportunities now abound for the fine mapping of QTLs, map-based cloning, assessment of genetic diversity, association mapping and marker-assisted breeding.

The first DNA marker based genetic linkage map for cowpea was published by Fatokun et al. (1993) followed by Menendez et al. (1997), Ubi et al. (2000) and Ouedraogo et al. (2002a) using RFLP, RAPD, AFLP, cDNA and morphological markers. However, cowpea genome resolution was poor based on these published maps. First attempt to improve these maps was carried out by Muchero et al. (2009a, b), using an Illumina GoldenGate assay having 1,536 EST-derived SNP markers. The authors genotyped a total of 13 recombinant inbred line (RIL) populations, which not only improved the map resolution but also made orthologous gene identification easier by increased synteny with soybean genome (Lucas et al. 2011). The most recent consensus genetic map described above (Amatriain et al. 2017) has a 4-fold increase in marker density and a four-fold increase in resolution (number of bins) over the consensus map of Lucas et al. (2011). This map has dense coverage of all eleven cowpea linkage groups, with 1.85 cM on LG1 being the largest gap.

Table 10.1 List of QTLs for drought and other related traits

Trait	Cross	Population type	Marker system	LG	No. markers/QTLs	PV%	References
Drought traits							
Root architecture traits	Genome wide association study	Diversity panel entries	SNP	All LGs	11 and 21	–	Burridge et al. (2017)
Drought-induced senescence and maturity	CB46 × IT93K503–1	RIL	AFLP	LG1, LG2, LG3, LG5, LG6, LG7, LG8, LG9, LG10	12	5–24; 25–29	Muchero et al. (2009b)
Delayed senescence, biomass, grain yield	Germplasms and IT93 K-503-1 × CB46.	Germplasms and RIL	SNP	–	3	–	Muchero et al. (2013)
Heat tolerance	CB27 × IT82E-18	RIL	SNP	LG2, LG3, LG6, LG7, LG10	5	12–18	Lucas et al. (2013)
Other related traits							
Aphid resistance	CB27 × IT97 K-556-6	RIL	SNP	LG1, LG7	2	4.8–65.7	Huynh et al. (2015)
Macrophomina phaseolina and maturity	IT93 K-503-1 × CB46	RIL	SNP/AFLP	LG2, LG3, LG5, LG6, LG11	9	6.1–40	Muchero et al. (2011)
Fusarium wilt resistance (Fot race 3 & 4)	CB27 × 24-125B-1, IT93 K-503-1 × CB46, CB27 × 24-125B-1, CB27 × IT82E-18.	RIL	SNP	LG1; LG8, LG9, LG3	1; 1	28; 18–47	Pottorff et al. (2012b, P2014)
Bacterial blight resistance	C-152 × V-16; DanIla × TVu7778.	F2:3/RIL	SSR/CISP/SNP	LG3, LG5, LG9; LG8, LG11	3; 3	10.63–30.58; 10–22	Dinesh et al. (2016), Agbicodo et al. (2010)
Golden mosaic virus	IT97 K-499-35 × Canapu T16.	F2	AFLP	–	3	–	Rodrigues et al. (2012)
Striga resistance	IT84S-2246 × Tvu14676; TVx 3236 × IT82D-849; IT93 K-693-2 × IAR1696.	F2	AFLP/SCAR	LG1	2, 4		Ouedraogo et al. (2001), (2002a, b), (2012), Boukar et al. (2004)

(continued)

Table 10.1 (continued)

Trait	Cross	Population type	Marker system	LG	No. markers/QTLs	PV%	References
Seed size	524B × 219-01/Eight different populations.	RIL	SSR/SNP	LG1, LG10/LG2, LG5, LG6, LG7, LG8, LG10	6/10	9–19/47	Andargie et al. (2011), Lucas et al. (2013)
Seed weight	IT2246-4 × TVNu1963/524B × 219-01	F2/RIL	RFLP/SSR	LG2, LG6/LG1, LG2, LG3, LG10	2/6	37–53/8–19	Fatokun et al. (1992), Andargie et al. (2011)
Time of flower opening	524 B × 219-01	RIL	SSR	LG1	5	9–30	Andargie et al. (2013)
Days to flower	524 B × 219-01	RIL	SSR	LG1	3	6–19	Andargie et al. (2013)
Days to first flowering	ZN016 × ZJ282	RIL	SNP	LG11, LG10, LG3	3	10–32	Xu et al. (2013a, b)
Pod number per plant	ZN016 × ZJ282	RIL	SSR	LG3, LG2, LG4	3	11–20	Xu et al. (2013a, b)
Leaf senescence	ZN016 ×ZJ282	RIL	SNP	LG11, LG3, LG7	2	11–29	Xu et al. (2013a, b)

In addition, a core germplasm of landraces collected from across cowpea-growing regions in Africa and other parts of the world has been characterized using SNP markers (Huynh et al. 2013). Using DNA markers, QTLs have been detected by several authors for key traits including drought tolerance (Muchero et al. 2010, 2013), seed quality traits (Lucas et al. 2013), resistance to root-knot nematodes (Huynh et al. 2016), root pathogens including *Macrophomina phaseolina* (Muchero et al. 2011) and fusarium wilt (Pottorff et al. 2012b, 2014), insects (Huynh et al. 2015; Lucas et al. 2012), and the parasitic weed Striga (Boukar et al. 2004; Ouedraogo et al. 2001, 2002a, b, 2012). Fatokun et al. (1993) reported an orthologous QTL for seed size in both cowpea and mungbean using RFLP generated linkage maps of both crops. Same RFLP markers spanned the regions associated with seed weight QTL in the two leguminous crops. In addition, an aphid resistance locus defined by an RFLP marker was reported by Myers et al. (1996). Muchero et al. (2009b) identified 12 QTLs for seedling drought tolerance and maturity using a RIL population based on the cross between IT93 K-503-1 and CB46. A few of these QTLs colocated with QTLs for recovery dry weight (rdw) and stem greenness (stg) under drought stress both under field and greenhouse conditions. A major QTL affecting cowpea leaf shape (associated with drought tolerance) was reported by Pottorff et al. (2012a). Recently, Muchero et al. (2013) utilized phenotypic data from multiple locations and identified seven SNP-trait associations with stay-green trait. Five of these loci also showed pleiotropic effects on biomass, grain yield, and delayed leaf senescence. These QTLs, particularly those identified in two RILs and diverse germplasm can be potential targets for marker-assisted breeding of cowpea varieties with improved drought tolerance. In another study, co-location of three *Macrophomina* resistance QTLs (*Mac-4, Mac-5 & Mac-9*) and three seedling drought response QTLs (*Dro-5, Dro-10 & Dro-7*) were identified from the RIL population IT93 K-503-1 × CB46 (Mucheroet al. 2009b 2011). Burridge et al. (2017) conducted a genome-wide association study and reported 32 significant QTLs for root architecture traits.

10.5 MAGIC Population in Cowpea

QTL mapping using bi-parental populations has limitations because of limited allelic diversity and genomic resolution. A multi-parent advanced generation inter-cross (MAGIC) population strategy has been proposed to integrate multiple alleles and provide increased recombination and mapping resolutions (Bandillo et al. 2013). The increased recombination in MAGIC populations can lead to rearrangements of alleles and greater genotypic diversity.

Cavanagh et al. (2008) proposed that MAGIC populations should provide state-of-the-art approach for developing plant population resources for genetic analysis and increased genetic variability in breeding. In creating a MAGIC population, multiple elite parents are inter-crossed for several cycles followed by single-seed descent, which results in RILs having a mosaic of genome blocks coming from all founders. Development of MAGIC populations have been carried out in a few crops includ-

ing rice, wheat and chickpea (Huang et al. 2015). In cowpea, MAGIC population was established employing eight founder parents (SuVita 2, CB27, IT93K-503-1, IT89KD-288, IT84S-2049, IT82E-18, IT00K-1263, & IT84S-2246-4) that were not only genetically diverse but also carried genes for resistance to several biotic and abiotic stresses, seed quality and agronomic traits relevant to SSA. Phenotyping and genotyping of the MAGIC RILs at F_8 generation showed an average of 99.74% homozygosity and diversity in 38 agronomic traits (Huynh et al. 2017). Due to its wide genetic base, the cowpea MAGIC population has become an important genetic resource for high-resolution genetic mapping and for gene discoveries (Huynh et al. 2017).

10.6 Marker-Assisted Selection in Cowpea

Marker-assisted selection (MAS) offers great prospects for increasing genetic gain per crop cycle, by stacking favorable alleles at target loci and reducing the number of selection cycles. Markers identified in one population need to be validated in other populations or germplasm collections, and closely linked markers flanking the QTL should be used for indirect selection of the trait. Once potential markers, validated QTL are identified, they can be used in breeding. We describe below marker-assisted backcrossing (MABC), marker assisted recurrent selection (MARS) and genome wide association mapping (GWAM) schemes, which are currently being used in our cowpea breeding program to incorporate genes for resistance to some abiotic and biotic stresses (Fig. 10.1).

10.6.1 Marker-Assisted Backcrossing

Marker-assisted backcrossing (MABC) is a fast-track approach to increase the genetic gain of crops and is in use for variety development of several crops (Chamarthi et al. 2011; Varshney et al. 2014; Boukar et al. 2016; Cheng et al. 2017; Ouedraogo et al. 2017). MABC can be used to introgress major genes/QTL from one genetic background (donor parent) to another (recurrent parent) much more precisely than phenotypic selection. The outcome of MABC is a line containing only the major genes/QTL transferred from the donor parent, while retaining a vast proportion of the genome of the recurrent parent. Three types of selection can be done in MABC: foreground, recombinant and background. Foreground selection involves the selection of target genes/QTL on the carrier chromosome with the help of two QTL-linked flanking markers. It can be used to select for laborious or time-consuming traits and it allows selection of heterozygous plants at the seedling stage and therefore identifies plants desirable for backcrossing. Furthermore, identification and selection of recessive alleles can be done, which is otherwise difficult to achieve using conventional methods. Recombination events between the target locus and linked flanking mark-

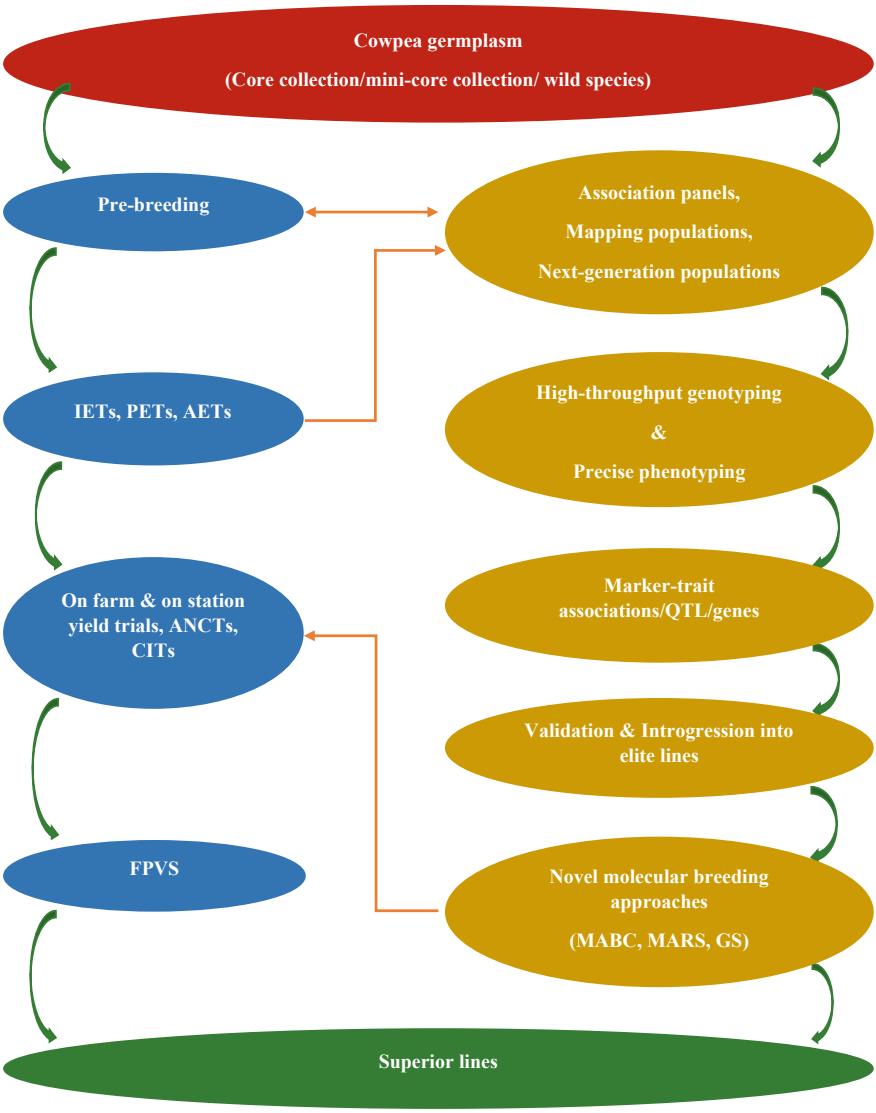

Fig. 10.1 Schematic diagram shows integrated molecular and conventional breeding approaches in cowpea. *MAGIC* Multi-parent Advanced Generation Inter-Cross populations, *QTL* Quantitative Trait Loci, *MABC* Marker Assisted Backcross, *MARS* Markers Assisted Recurrent Selection, *GS* Genomic Selection, *IETs* Initial Evaluation Trials, *PETs* Preliminary evaluation Trials, *AETs* Advance Evaluation Trials, *ANCTs* All Nigeria Co-ordinated Trials, *CITs* Cowpea International Trials, *FPVS* Farmer Preferred Varietal Selection

ers can also be identified in backcross progeny. This can be used to reduce linkage drag, which is difficult to overcome through the use of conventional backcrossing. Background selection involves selection of BC progeny with highest proportion of recurrent parent genome, using unlinked markers present on non-carrier chromosomes (Hospital and Charcosset 1997; Frisch et al. 1999; Chamarthi et al. 2011; Varshney et al. 2014; Batieno et al. 2016). Using tightly linked markers, a target gene can be transferred with minimum linkage drag in two backcross generations, which otherwise would take 8–10 generations by conventional backcrossing (Tanksley et al. 1989).

In cowpea, MABC has been implemented through the CGIAR-GCP-TLI (Consultative Group of International Agricultural Research-Generation Challenge Programme-Tropical Legumes I) project at IITA and NARS centers in collaboration with the University of California, Riverside (UCR). At IITA, using IT97K-499-35 as the donor, two released varieties, IT93K-452-1 and IT89KD-288, have been improved in Nigeria, for Striga resistance. At INERA, efforts are being made to improve Moussa and KVx745-11P for Striga resistance and seed size using IT93K-693-2 and KVx414-22, as donors for Striga resistance and seed size, respectively. At Eduardo Mondlane University (EMU), Mozambique, IT85F-3139 is being improved for grain quality (seed size) using CB27 as a donor.

10.6.2 Marker-Assisted Recurrent Selection

While MABC targets major large effect QTL that has been validated across different genetic backgrounds, MARS aims at accumulating a large number of QTLs in a given population using a subset of markers that are significantly associated with target traits (Bernardo 2008; Ribaut et al. 2010; Chamarthi et al. 2011; Xu et al. 2013a, b; Boukar et al. 2016). In brief, MARS is a modern breeding approach that enables breeders to increase the frequency of several beneficial alleles with small individual but additive effects in recurrent cycles. This involves multiple cycles of marker-based selection that include improvement of F_2 progeny by one cycle of MAS based on marker and phenotypic data, followed by three recombination cycles of the selected progenies based on marker data only and repetition of these cycles to develop the population for multi-location phenotyping (Tester and Langridge 2010). In MARS, a selection index is used that gives weights to markers according to the relative magnitude of their estimated effects on the trait (Lande and Thompson 1990). Several multinational companies, such as Syngenta and Monsanto, are routinely using MARS in their breeding programmes (Ribaut et al. 2010).

In cowpea, MARS has been implemented at IITA and NARS centers in collaboration with UCR by using genomic resources developed during the GCP-TL1 project. At IITA, Nigeria, to develop cowpea varieties with enhanced drought tolerance, two lines (IT84S-2246-4 and IT98K-1111-1) were crossed to develop a MARS population. After QTL mapping with 102 polymorphic SNPs, seven QTLs were identified for yield, drought tolerance and staygreen. One hundred and seventy seven plants

were fixed for favorable alleles at these seven QTLs and advanced breeding lines are being generated. At Eduardo Mondlane University (EMU), Mozambique, the cross CB27 × IT97K-499-35 was used to initiate MARS for large seed, grain quality, and heat tolerance traits. Screening of lines fixed for favorable alleles is going on under drought and irrigated conditions. At ISRA, Senegal, the cross IT93K-503-1 × Mouride has been made for MARS for drought tolerance and resistance to Striga, nematodes and Macrophomina.

10.6.3 Genome-Wide Association Mapping Studies

The genome-wide association mapping (GWAM) approach provides opportunities to explore the tremendous allelic diversity existing in natural germplasm (Deshmukh et al. 2014). A GWAM or whole genome association mapping (WGAM) or linkage disequilibrium mapping (LDM) is used to evaluate associations between markers and trait(s) of interest scored across a large number of individuals. The advancements in genomic technologies have led to a better understanding of the genetic basis of traits using GWAM. This approach results in high-resolution mapping of genetic variability from germplasm sets that have undergone many rounds of recombination (Yu and Buckler 2006). However, to get the associations at a fine mapping resolution, large number of markers are required to screen the genome. Recently GWAM studies have been proven effective by identifying marker-trait associations in several legume crops such as cowpea (Lucas et al. 2013; Burridge et al. 2017; Qin et al. 2017), common bean (Villegas et al. 2017) and soybean (Dhanapal et al. 2015). As mentioned above, a GWAM study in cowpea identified 32 QTLs for root architecture traits. Further, comparisons of results from this study with others revealed QTL co-localizations between root traits and seed weight per plant, pod number and Striga tolerance (Burridge et al. 2017).

10.7 Using Wild Germplasm in Cowpea Breeding

Plant breeders mostly use existing germplasm and landraces to develop new varieties characterized by desirable agronomic traits. In many crops, yields have remained stagnant relatively because sufficient genetic diversity is missing for progress in some of the traits or due to genetic bottlenecks that occurred during the domestication process (Tanksley and McCouch 1997; Gur and Zamir 2004). It is well known that wild relatives provide important sources of genetic variation for crop improvement. However, their exploitation is limited by different sexual incongruity and linkage drag (Wang et al. 2017). Some wild cowpea relatives have been identified as potential sources of genes that confer resistance to a number of pests that have devastating effects on grain yield and stored seeds. *Vigna vexillata* is one such wild cowpea relative with resistance to pod sucking bugs (IITA 1988) and bruchids (Birch

et al. 1986). Strong incompatibility barrier, however, exists between cowpea and *V. vexillata* (Fatokun 2002) which has prevented transferring the desirable genes from the latter to cultivated cowpea through conventional breeding. Some wild cowpea relatives are cross compatible with cultivated types but have hardly been used in developing improved varieties. With the advent of latest genomic tools, it is now feasible to transfer into elite germplasm the favorable alleles left behind by the domestication process more efficiently using genomics-assisted breeding strategies. These tools should facilitate overcoming linkage drag, which is one major reason for the non-interest in the use of wild cowpea lines by breeders. In this context, several methods such as construction of introgression libraries (ILs), advanced backcross-QTL (AB-QTL) analysis, have been suggested for transferring superior alleles from wild species to cultivated lines. AB-QTL analysis has been used in several legume species such as common bean (Blair et al. 2006), and soybean (Chaky et al. 2003) to develop ILs with seed weight, days to flowering and yield traits in common bean and yield traits in soybean. With an initiative of the Global Crop Diversity Trust project in cowpea, we initiated the use of wild cowpea accessions in cowpea breeding programme to introgress genes for drought tolerance into cultivated cowpea lines. In future, AB-QTL approach may be employed to introgress genes for drought tolerance in cowpea improvement efforts.

10.8 Summary

Cowpea [*Vigna unguiculata* (L.) Walp.] is a multipurpose African legume crop, which feeds millions of people and their livestock especially in West and Central Africa. Because of its ability to fix nitrogen, it improves soil fertility, and consequently helps to increase the yields of cereal crops when intercropped or grown in rotation and thus contributes to the sustainability of cropping systems (Singh and Ajeigbe 2007). Cowpea yields in farmer's fields are very low due to several constraints (abiotic and biotic), as well as limited access to quality seeds of improved varieties. Among abiotic constraints, drought is one of the most important factors that could affect all growth stages of the cowpea crop. In the last three decades, efforts of scientists at international and national cowpea research institutions have recorded good progress in variety development through conventional breeding. However, to meet the rising global demand for cowpea to feed the increasing human population, more efforts are required to speed up variety development. With the availability of latest genetic and genomic resources and the establishment of high-throughput SNP genotyping platforms, it is now possible to use modern molecular methods in cowpea to successfully and quickly develop and release improved varieties to farmers, which would help bridge the existing yield gap. However, high throughput plant phenotyping for precise and accurate agronomical, morphological and physiological data in large number of genotypes and segregating populations remains a bottleneck for modern breeding. Further, the shortage of qualified human resources and advanced research equipment and infrastructure in developing countries constitute other chal-

lenges. Although genomic resources for cowpea still lag behind as compared with similar crops, a number of cowpea genetic linkage maps and QTLs associated with desirable traits such as resistance/tolerance to Striga, drought, macrophomina, fusarium wilt, bacterial blight, root-knot nematodes, aphids, and foliar thrips have been reported. Several national and international cowpea breeding programs are exploiting the developed genomics resources to some extent to implement molecular breeding for abiotic and biotic traits, especially by MABC, MARS and GWAM to accelerate cowpea improvement. The recently available MAGIC RIL population and cowpea genome sequence (Amatriain et al. 2017) will further accelerate molecular breeding efficiently in cowpea improvement. The combination of conventional and molecular breeding strategies should result in the development of varieties with genetic gains that would boost cowpea production and productivity in SSA.

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Chapter 11
Hybrid Wheat and Abiotic Stress

Takashi Okada and Ryan Whitford

Abstract Bread wheat (*Triticum aestivum* L.) is one of the major crops for human nutrition and an important one for food security. However, wheat yields are highly dependent upon environmental conditions and are affected by various types of abiotic stresses. One strategy for improving wheat yield stability across environments is to harness hybrid vigour. Estimates of yield improvements associated with hybrid vigour in wheat range from 5 to over 20%, which needs to be further enhanced to meet the future global demand. This yield advantage comes with improved yield stability under both biotic and abiotic stress conditions. This chapter focuses on the current status of hybrid wheat breeding, including hybrid seed production systems, hybrid performance under abiotic stresses and prediction of hybrid performance.

Keywords Abiotic stress · Genomic selection · Hybrid wheat · Heterosis
Male sterility

11.1 History of Hybrid Wheat Breeding and Status

Global food security is of rising concern, and the World Food Summit on Food Security in 2009 set a target of 70% increased food production by 2050, in order to meet future global food demand. This target takes into account population growth and impending changes to climate as well as decreasing availability of arable land (FAO 2009). Capturing heterosis is one of the few high priority strategies towards increasing yield and yield stability (Fig. 11.1a), as has been achieved for allogamous plants such as maize (*Zea mays* L.) and rye (*Secale cereale* L.) (Melchinger and Gumber 1998). For example, average maize yields in the United States have improved by 400% from 1930 to 2002, which can largely be attributed to advances in hybrid breeding (Russell and Sandall 2005). On the other hand, hybrid breeding for autogamous cereals, such

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as rice, barley and wheat, is more challenging due to their self-pollinating nature and reduced expression of heterosis (Coors and Pandey 1999; Longin et al. 2012). Despite this, hybrid breeding of rice in China was successfully established commercially in the mid-70's and China is now the leading country for hybrid rice production. The area within China cultivated to hybrid rice production has increased from 2.1 million ha in 1977 to 15.3 million ha in 1997 (~50% of rice growing area), with growers receiving a yield advantage of between 20 and 30% over traditional line cultivars (Li and Yuan 2000; GRiSP 2013).

In wheat, efforts towards establishing an efficient hybrid seed production system were initiated in the late 1960s and since have had a long history (Curtis and Johnston 1969; Driscoll 1972; Mukai and Tsunewaki 1979; Pickett 1993). Hybrid wheats are expected to provide increased tolerance to a range of abiotic stresses, including drought, heat and frost (Jordaan 1996; Pickett 1993). However, only less than 1% of the current global wheat growing area is planted to hybrids (Longin et al. 2012). France is a leading country in exploiting wheat hybrids, with cultivation also in areas of Germany, China and India. Hybrid wheats remain a small fraction of current global wheat production due to high production costs. This can be attributed to wheat's strong self-pollinating nature, lack of available genetic resources necessary for improving efficient cross-pollination as well as an efficient fertility control system. Overcoming these factors are necessary for stable and economical hybrid seed production (Whitford et al. 2013; Nguyen et al. 2015). Despite such issues, there is strong interest from both public and private sectors to facilitate deployment of wheat hybrids in countries with increasing food security concerns, for example in China and India. An overriding concern is whether the yield benefit from hybrid vigour will be sufficient to offset the substantial costs of restructuring breeding programs for hybrid seed production (Longin et al. 2012; Mette et al. 2015). In the following section, current hybrid wheat seed production systems are explained with discussion on their associated advantages and disadvantages.

11.2 Hybrid Wheat Seed Production Systems

Wheat is a predominantly self-pollinating species, in which natural cross-pollination rates are low, generally less than 5% (de Vries 1974; Tsunewaki 1969). Therefore, most hybrid breeding approaches rely on creating male sterile female inbred to ensure obligate cross pollination with a male fertile inbred (Fig. 11.1a, Whitford et al. 2013). There are three major approaches for ensuring male sterility in wheat: (1) the use of chemical hybridization agents (CHA), (2) cytoplasmic male sterility (CMS) and (3) genic male sterility (GMS) either via genetic modification (GM) or via non-GM methods (Fig. 11.1b–d and Table 11.1). Currently, commercial hybrid wheat seed in Europe is produced by using the CHA Croisor® 100 (Longin et al. 2012; Whitford et al. 2013). Generally, inbred parental lines are grown in alternate female and male strip planting with the CHA applied by spraying the female inbred parental rows at an early stage of floral development (Fig. 11.1b). Timing and dosage

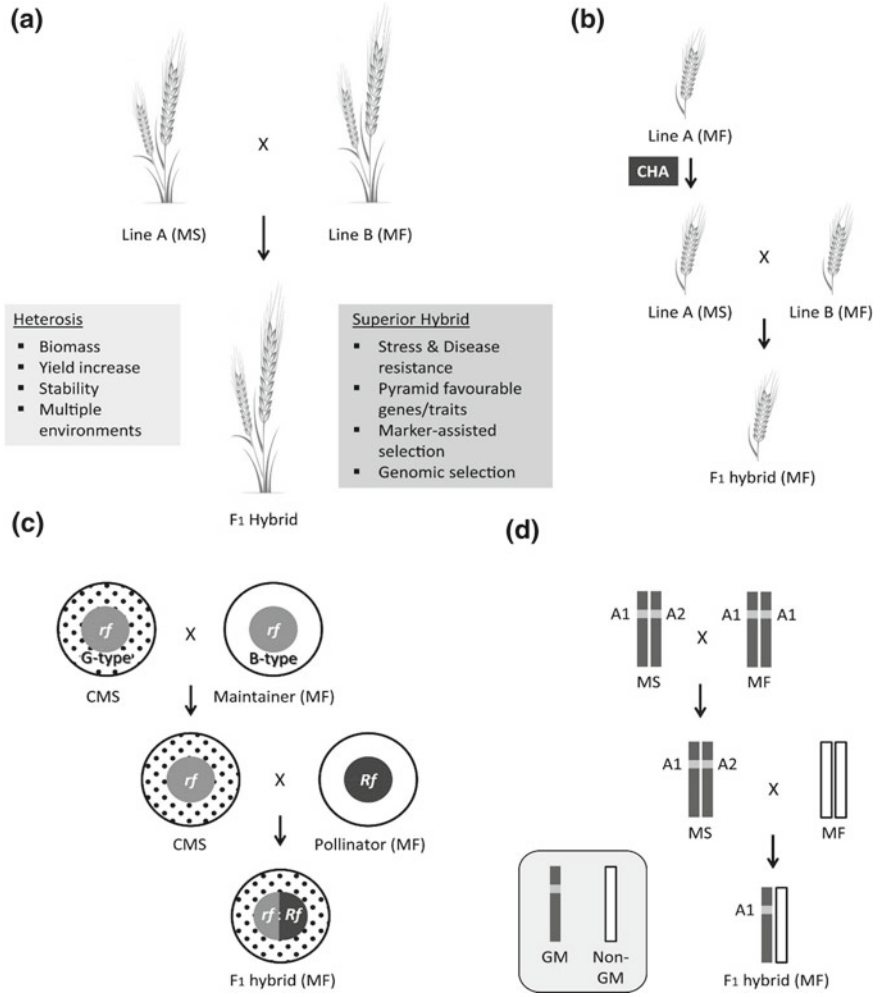

Fig. 11.1 As (**a**) A basic concept of hybrid wheat breeding. Male sterile (MS) line A is crossed with male fertile (MF) pollinator line B to obtain F_1 hybrid seed. F_1 hybrid shows heterosis by increasing biomass, yield and stability under multiple environments. Superior hybrids will be obtained by pyramiding multiple favourable traits with the aid of marker assisted selection and/or genomic selection. (**b**) Schematic representation of hybrid seed production system using chemical hybridizing agent (CHA). (**c**) Hybrid seed production system by using *T. timopheevii* cytoplasmic male sterility (CMS). Large circles represent type of cytoplasm (dotted; G-type from *T. timopheevii* or open; B-type from common wheat) and small filled circles represent nucleus with the allele type of fertility restore gene (*Rf* or *rf*). (**d**) Split-Barnase system for hybrid seed production (Kempe et al. 2013, 2014). The barnase coding information is divided (A1 and A2) and located at two loci on allelic positions of the chromosome. Co-expression of both A1 and A2 fragments induce male sterility, while presence of only one of two components in F_1 hybrid remains fertile. F_1 hybrid is a GM plant in this method. Illustration of wheat in panels (**a**) and (**b**) is designed by Freepik.com

Table 11.1 Advantages and disadvantages of hybrid wheat seed production system

System	Advantage	Disadvantage
Chemical hybridizing agent (CHA)	Simple application/Application to wider varieties/Flexibility of use	Effect of weather conditions/Efficiency and penetrance variation/Toxicity to environment/Adverse effect
Cytoplasmic male sterility (CMS)	Common in hybrid breeding system/High penetrance of MS/Purity of hybrid seed/Environmentally safe/Cost effectiveness	Limited wheat varieties/Incomplete function of fertility restore gene (*Rf*)/Multiple *Rf* genes/Adverse growth effect/Reduced seed set in F_1
Genic male sterility (GMS)	Application to wider varieties/High penetrance of MS/Purity of hybrid seed/Environmentally safe	High cost to establish system/Identification of restorer genes/Public acceptance of GM/Cost of hybrid seed production (GM regulation)

of CHA application is critical for successful induction of male sterility, with modern CHAs being efficacious across genotypes and showing relatively low phytotoxicity (Adugna et al. 2004). However, several limitations exist that hamper the use of CHAs in a large commercial production setting. These include significant effects of weather on the efficiency of sterility induction and therefore hybrid seed purity, environmental phytotoxicity, application cost, and often compromised seed set associated with adverse effects of chemicals (Pickett 1993; Whitford et al. 2013; Mette et al. 2015).

Genetic control of male fertility is more cost-effective, environmentally safe and less dependent upon prevailing weather conditions for sterility induction. CMS has been extensively used as a hybridisation platform for many vegetables and crops (Duvick 1959; Havey 2004). In wheat, cytoplasms from several related species (*Triticum* and *Aegilops* spp.) in combination with various cultivated genotypes have been shown to induce male sterility (Tsunewaki et al. 1976; Mukai and Tsunewaki 1979). To date, the *Triticum timopheevii* cytoplasm is considered the most effective in inducing complete male sterility across many common cultivated wheat genotypes (Tsunewaki et al. 1976), and has therefore been used for commercial production. *T. timopheevii*-based CMS (Wilson and Ross 1962) has found application in commercial production within India, whilst photoperiod sensitive CMS using an *Ae. crassa* cytoplasm (Murai and Tsunewaki 1993) has found commercial application in China (Longin et al. 2012; Mette et al. 2015). Generally, these systems require a three-line crossing strategy to maintain the CMS line and produce the F_1 hybrid seed (Fig. 11.1c). *T. timopheevii* G-type cytoplasm (Tsunewaki et al. 1996, 2002) induces complete male sterility in the absence of a dominant nuclear-encoded fertility restoration gene (*Rf*), while the fertile maintainer line possesses a recessive nuclear *rf* gene and B-type cytoplasm derived from the common wheat. The fertile maintainer, when used as a pollinator on CMS-induced male sterile females, maintains

and multiplies CMS induced male sterile female inbred seed (Fig. 11.1c). Paternal hybrid inbreds contain a dominant *Rf* gene which results in F_1 hybrids of a heterozygous *Rf*:*rf*, yet containing a G-type cytoplasm, therefore enabling pollen fertility restoration and seed set. However, a limitation to the *T. timopheevii* CMS system is that it requires multiple *Rf* genes, each affecting fertility restoration (Bahl and Maan 1973), and therefore F_1 hybrid seed-set is dependent upon dosage of *Rf*s (Adugna et al. 2004). Furthermore, *T. timopheevii* cytoplasm has several unwanted side effects, which include delayed flowering, reduced height and sometimes poor vigour (Jošt et al. 1975; Mukai and Tsunewaki 1979). On the other hand, photoperiod-sensitive CMS (PCMS) utilising the *Ae. crassa* cytoplasm system has potential advantages over *T. timopheevii* system as it simplifies maintenance of CMS female inbred and avoids incomplete fertility restoration (Murai and Tsunewaki 1993; Murai 1997). CMS induction with *Ae. crassa* cytoplasm in the cultivar Norin 26 was shown to require long days ($\geq$15 h) at floral induction stage, while under short days ($\leq$14.5 h) pollen remained fertile (Murai and Tsunewaki 1993). F_1 hybrids carrying the fertility restoring gene (*Rf*) grown under short days did not exhibit full fertility restoration, but revealed up to 40% mid-parent heterosis in grain yield, highlighting its potential application (Murai 1997, 1998; Murai et al. 2008). A major issue in PCMS system is the necessity for growth under long days at floral induction stage. This is a commercial limitation to the application of this system for spring wheat genotypes, as it restricts geographical deployment to high latitudes (Murai and Tsunewaki 1993). Therefore, successful application of CMS systems necessitates stable and high penetrance of CMS in broad germplasm, coupled with the identification of a simple single locus dominant *Rf* that completely restores fertility in the F_1 hybrid.

GMS is advantageous in that it can overcome problems with restricted genotypic combinations and the requirement to track multiple restorer loci, unlike many CMS-based systems. Several dominant and recessive male sterile mutant loci have been identified in wheat with their potential application to hybrid wheat breeding being outlined (Driscoll 1975; Sasakuma et al. 1978; Barlow and Driscoll 1981; Bing-Hua and Jing-yang 1986; Zhou et al. 2008). Driscoll (1972) proposed the XYZ system for hybrid wheat breeding based on the utilisation of a recessive male sterile mutant (*ms*). Pollen fertility is restored by nuclear encoded fertility restoring locus (*Ms*) on the additional rye chromosome 5R, containing the morphological selectable marker locus *hairy peduncle*. The XYZ system utilises these traits in the maintainer line which is used to propagate male sterile female inbred lines for hybrid seed production (Driscoll 1972). A similar system (4E-ms system) uses a blue seed colour marker linked to the fertility restorer (*Ms*) gene present on the 4E chromosome of *Agropyron elongatum* (Zhou et al. 2006, 2008). Both of these systems utilize an alien *Ms* locus and morphological markers on the same chromosome. Poor recombination between alien and cultivated wheat chromatins allow these markers to be tightly linked, therefore ensuring easy separation of male fertile versus male sterile lines, an important component for maintaining hybrid seed purity (Whitford et al. 2013). In each of these systems, however, there is the potential for deleterious effects on plant vigour derived from the alien chromatin.

Alternatively, genetic modification (GM) poses an opportunity to overcome many of these limitations and effectively control fertility. The use of recombinant DNA technology can physically link the necessary gene sequences at a single locus without the requirement of extra unwanted chromatin. An example is the use of the *barnase* gene, encoding a cytotoxic bacterial ribonuclease, for inducing male sterility. Its utility has been demonstrated in wheat transgenics (DeBlock et al. 1997). More recently, Kempe et al. (2013, 2014) has refined the use of *barnase* by developing a split gene system, whereby the *barnase* coding fragment necessary for inducing male sterility works in *trans* through the action of two transgenes (Fig. 11.1d). Although the split-gene system is efficient, the final F_1 hybrid contains a transgenic element, which could be an obstacle for commercialisation considering public concern over GM. An alternate methodology overcoming this issue is the development of Seed Production Technology (SPT), a hybridisation platform whereby the transgene is biologically contained within the maintainer line. This SPT has been successfully developed and implemented for use in the production of maize hybrid seed (Wu et al. 2016). The use of GM plants for hybrid wheat seed production still requires public acceptance in many countries, although we may see the adoption of GM plants in some countries and areas. However, GM regulation fees and associated handling costs will ultimately result in increased hybrid seed price.

In summary, low cost hybrid seed production system and application to broad wheat genotypes are essential for either of the systems (CHA, CMS or GMS) utilized for the induction of male sterility in hybrid wheat breeding. More importantly, improved hybrid performance via increased grain yield and yield stability, are additional critical factors necessary for the successful implementation of a hybrid wheat breeding system.

11.3 Hybrid Performance and Abiotic Stress in Wheat

Hybrid wheat performance has been investigated in many studies using various genotypic combinations, experiment sizes under a range of environmental conditions, measuring mainly yield and yield components (summarized in Longin et al. 2012). More recently, a large-scale field study revealed positive commercial heterosis for grain yield in hybrid winter wheat varieties (Gowda et al. 2012; Longin et al. 2013; Mette et al. 2015). The average yield increase by heterosis was reported to be approximately 10% in hybrids that displayed improved resistance to frost, leaf rust and *Septoria tritici* blotch across multiple field environments (Longin et al. 2013). The higher yield stability across multiple environments was also reported for related autogamous cereals, barley and triticale (Muhleisen et al. 2014). The relative yield advantage of hybrids over pure line cultivars is expected to be even higher under marginal and stress conditions (Oettler et al. 2005). Enhanced yield stability of hybrids compared to inbred lines justifies and facilitates the application of hybrid wheat breeding to cope with increasing pressure from abiotic stressors (Muhleisen et al. 2014).

Assessment of yield stability is done by comparing performance of lines across multiple environmental conditions, including all the potential biotic and abiotic stress factors. Abiotic stresses such as high and low temperatures, drought, salinity and mineral deficiencies or toxicities, severely reduce cereal crop yields. These stresses are becoming more important due to the declining availability of irrigation water and arable land, and increasing stress levels expected in a changing climate (Fleury et al. 2010; Langridge et al. 2006). However, only a limited number of investigations have been reported for hybrid wheat performance and yield stability against specific abiotic stresses. Among these abiotic stresses, low temperature stress was shown to have a significant impact on yield in European, North American and Australian wheat production (Boer et al. 1993; Gu et al. 2008; Worland 1996). Low temperature and frost cause tissue damage, inhibits vegetative growth and affects reproductive development, leading to significant reductions in wheat yield (Galiba et al. 1995; Sutka 1981). Even though the effect of low temperature has been investigated on hybrid wheat more than any other type of abiotic stress, it is limited to only a few studies (Longin et al. 2013; Sutka 1981, 1994). Longin et al. (2013) demonstrated that cereal hybrids were superior to the mean of their parents for resistance to frost. Frost tolerance is a complex trait, shown to be associated with vernalisation sensitive gene *VRN1* (Galiba et al. 1995). QTL analysis has identified a major locus for frost tolerance on chromosome 5A and a minor locus on chromosome 1D (Baga et al. 2007). These genetic loci and associated markers could be used for selection of inbred cross combinations and prediction of frost tolerance in hybrids.

Drought is one of the most critical stresses linked to plant survival, whereby limited water supply and/or periodic conditions of water deficit can affect all stages of the plant life cycle. Crop plants under natural drought conditions are often subject to a combination of stresses including high temperatures, excessive irradiance, soil resistance to root penetration and low water potentials. These conditions affect vegetative growth, tiller number, reproductive development, pollination, and grain filling which lead to significant grain yield reduction (Fleury et al. 2010; Langridge et al. 2006; Mendelsohn et al. 1994; Saini and Westgate 1999). Despite the importance of drought stress, there are only a few studies that have demonstrated hybrid wheat yield stability under drought stress. Hybrid performance under drought condition was investigated in 30 wheat hybrids, which revealed that a few hybrid combinations outperformed line varieties (Riaz and Chowdhry 2003). Because drought tolerance is a complex quantitative trait associated with various phenotypes under complex genetic control, multiple quantitative trait loci (QTLs) have been identified (Bennett et al. 2012; Pinto et al. 2010). Drought stress is quite often accompanied by heat stress and QTLs associated with heat tolerance can be classified as either dependent or independent of drought stress (Mohammadi et al. 2008; Pinto et al. 2010; Bennett et al. 2012). Fokar et al. (1998) demonstrated that F_1 hybrids of spring wheat varieties significantly exceeded the mid-parent value for heat tolerance suggesting that the underlying gene(s) controlling heat tolerance function in a dominant fashion. Moreover, nitrogen use efficiency (NUE) has been investigated using ten combinations of hybrid winter wheat varieties, with heterosis for grain yield being higher at low N levels when compared to high N levels. In this study, an average

of 14.6% mid-parent heterosis was observed at low N conditions relative to 4.0% at high N levels (LeGouis and Pluchard 1996).

As demonstrated by a number of studies, hybrid wheats show both yield increase and stability across multiple environments, especially under low yielding conditions due to abiotic stresses. However, most previous investigations for hybrid wheat performance against various abiotic stresses utilized a small number of hybrid combinations (Sutka 1981; Fokar et al. 1998; LeGouis and Pluchard 1996; Riaz and Chowdhry 2003), except for a recent study (Longin et al. 2013). Further studies are needed to address hybrid performance and yield stability, particularly related to specific abiotic stresses. A thorough understanding of the genetic control for such stresses will be critical for maximizing yield potential and stability, through targeted tracking and pyramiding multiple loci for stress tolerance.

11.4 Superior Hybrid and Prediction of Hybrid Performance

Obtaining superior hybrid wheats and predicting their performance for large numbers of possible inbred combinations requires a modelling system that links genotype to phenotype based on parental selection. Over a century of discussion aimed at defining the genetic basis of heterosis has culminated in three models, namely dominance, overdominance and epistasis (Shull 1908; Bruce 1910; Crow 1948; Birchler et al. 2003; Schnable and Springer 2013). More recently, a role for epigenetics in heterosis has been proposed (Chen 2013; Groszmann et al. 2013), adding another layer of complexity to the genetic basis of heterosis. Therefore, selection of superior hybrids based on genomic and genetic information from a vast number of potential single cross combinations requires a logical and practical selection system. Large-scale field evaluation for yield is costly, therefore breeding programs are limited in the total number of hybrid combinations that can be tested. An ability to accurately predict and select only those elite performing hybrid combinations for multi-site yield trails is a critical component to any hybrid breeding program (Mette et al. 2015; Zhao et al. 2013).

Prediction of hybrid performance is generally based on both general combining ability (GCA) and specific combining ability (SCA), as calculated from pre-existing parental and hybrid performance training sets (Sprague and Loyd 1942; Griffing 1956). The magnitude and the ratio of variance (σ^2) of GCA and SCA ($\sigma^2_{GCA}/\sigma^2_{SCA}$) enables prediction of selection gain in hybrid breeding, whereby GCA effects correlate more with hybrid performance (Longin et al. 2013). A winter wheat experimental study using 940 hybrids revealed that GCA variance is more pronounced than SCA variance with a medium to high ($r = 0.50$–0.92) correlation between predicted GCA and the hybrid performance. This suggested that inbred parental selection predominantly based on GCA effects seems to be a promising strategy towards achieving rapid yield gain (Gowda et al. 2012). The $\sigma^2_{GCA}/\sigma^2_{SCA}$ ratio can vary considerably

depending upon allele frequencies within the genetic pool, therefore increasing the $\sigma^2_{GCA}/\sigma^2_{SCA}$ ratio can be achieved by increasing genetic divergence of the parental groups (Fischer et al. 2008; Reif et al. 2007). The low $\sigma^2_{GCA}/\sigma^2_{SCA}$ ratio observed in wheat could be due to the lack of genetic diversity within the groups (Wurschum et al. 2013). Therefore, breeding genetically divergent heterotic groups for parental genotypes is pivotal for the development of an effective hybrid breeding program. Furthermore, both heterotic group development and the subsequent selection of hybrid cross-combinations can be facilitated by genetic models.

Genomic selection (GS) has great potential to facilitate parental selections so that released hybrid wheat varieties have multiple favourable traits suited to different growing regions. In GS, a training population that is fully phenotyped and genotyped is used to estimate breeding values using statistical models such as RR-BLUP and/or Bayesian methods (Heffner et al. 2009). Development of new genotyping platforms, e.g. high density SNP array and genotyping-by-sequencing (GBS), in combination with more effective computational prediction models improves correlation between true breeding value and the genomic estimated breeding values. Accuracy improvement in breeding value prediction for GS has been demonstrated by increasing marker density via techniques such as GBS (Poland et al. 2012). GS potential for yield improvement has been demonstrated for hybrid wheats on a relatively small scale (90 hybrid combinations), however its efficacy for selection of other agronomic traits has been demonstrated on a larger scale (>1000 hybrid combinations) (Miedaner et al. 2013; Zhao et al. 2013, 2014). These GS studies have successfully shown an increase in the accuracy of prediction for target traits using various prediction models, highlighting its potential for utilization for hybrid wheat breeding.

Recent progress towards sequencing the wheat genome (Brenchley et al. 2012; IWGSC 2014) and the anticipated availability of a high quality reference sequence (IWGSC 2016) will facilitate marker discovery; hence GS for commercial hybrid breeding will become more attractive, practical and realistic. Figure 11.2 provides a schematic overview of a GS-based hybrid wheat breeding program, focusing on abiotic stresses tolerance as target traits. The availability of a wheat genome reference sequence, high-density markers (HDM) combined with the use of GBS for assessing diversity in germplasm collections, can now help in identifying and further developing heterotic groups. These heterotic groups are most effectively developed by recurrent selection using germplasm derived from the broader genetic pool. Hybrid combinations selected by a GS model that incorporates both multi-environment trials and performance prediction, need to be tested and validated in the field. High-throughput phenotyping is an approach for collating data from large parental combination matrices. In combination with HDM or GBS genotypic information, this wealth can be used to train the GS models, which in turn can improve prediction accuracy of breeding values. Furthermore, incorporation of marker-trait associations for biotic and abiotic stress tolerance derived from other studies can improve GS modelling and therefore selection gain in hybrid breeding (Hoffstetter et al. 2016; Spindel et al. 2016). Improved selection of parental combinations will reduce breeding costs and will ensure heterotic yield advantage.

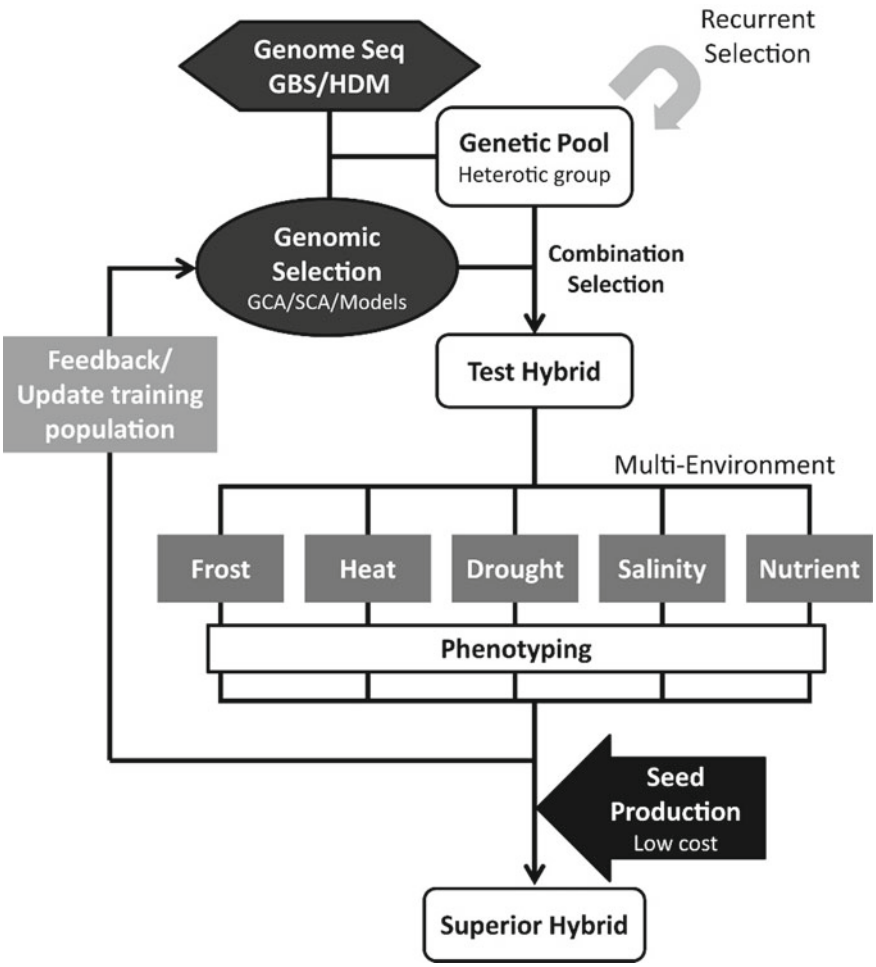

Fig. 11.2 A diagram of hybrid wheat breeding program by using genomic selection (GS) with the focus on abiotic stress tolerance. Abbreviations: GBS, genotyping-by-sequencing; GCA, general combining ability; HDM, high density markers; SCA, specific combining ability

11.5 Conclusion

Hybrid wheat has the potential to significantly increase grain yield and yield stability under marginal, stressed and low-yielding environments. Establishing a system, which reduces hybrid seed production costs, is a key for success. Empirical knowledge and data to support hybrid performance prediction models for specific biotic and abiotic stresses is currently lacking. Research therefore should be focussed on establishing an efficient seed production system, and improving algorithms for genomic

selection. Public-private partnerships for hybrid wheat breeding will be an important component towards achieving these goals in the near future.

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Chapter 12
Genomic Landscapes of Abiotic Stress Responses in Sugarcane

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Abstract Occurrence of abiotic stresses imposes devastating threat to global food security by causing more than 50% loss in crop yield and productivity. Under the scenario of global climate change, these abiotic stresses pose a serious challenge to ensure sustainable food production for the rapidly escalating world population. Plants respond to a wide range of adverse environmental conditions by dynamic regulation of various physiological, developmental, and biochemical pathways in order to tolerate stress and/or to sustain growth. A thorough understanding of such responses to abiotic stresses is, therefore, imperative to design tolerant crop varieties. In sugarcane, genetic advancements have been made by adopting novel crop breeding strategies to obtain improved varieties for abiotic stresses using novel biotechnological approaches, combined with approaches involving genetics, molecular biology, breeding, and physiology. Lately, transgenic approaches have been emerged as versatile tools to combat the adverse impacts of abiotic stresses on crop production and have proven to be one of the prospective ways for the genetic enhancement. Utilization of current molecular biology tools to determine the regulatory mechanisms for abiotic stress tolerance and engineering stress tolerant crops depends on the expression of specific set of stress-related or responsive genes. As a result, several abiotic stress-responsive genes have been identified, isolated, cloned and utilized for building stress tolerance in susceptible genotypes. Transgenic sugarcane lines carrying genes for abiotic stress tolerance have been developed by using *Agrobacterium*-based method besides other methods of gene transfer. Extensive research has been carried out in these areas and several transgenic sugarcane plants with enriched

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abiotic stress tolerance have been advanced for field trials. The present chapter summarizes studies on insights into the molecular responses and genetic manipulation of abiotic stress in sugarcane.

Keywords Abiotic stress tolerance · Transcriptome · Salinity · Genetic engineering · Sugarcane · Transgenic plants

12.1 Introduction

Sugarcane (*Saccharum officinarum* L.) is one of the ten most cultivated crops that contribute more than 80% of the global sugar production (McQualter et al. 2004; Lakshmanan et al. 2005). It is cultivated across the tropical and subtropical regions of the world not only for the production of sugar but also it is considered as a potential bio-fuel resource for ethanol production. However, sugarcane yield is significantly affected by numerous abiotic stresses like salinity, alkalinity, water-logging, drought etc. Amongst these, drought is the major cause of decreased productivity both in terms of magnitude and severity which result in rapid reduction in photosynthesis and growth inhibition (de Andrade et al. 2015). Being a typical glycophyte, sugarcane exhibits modest or no growth along with ≥50% yield reduction when grown under saline conditions (Akhtar et al. 2003; Wiedenfeld 2008). Besides this, in the flood-prone areas, sugarcane crop growth is severely affected by reduced germination and difficulties in root establishment, crop lodging, reduced growth and tillering and consequently yield drop (Solomon 2014). Elucidating tolerance mechanisms would facilitate the development of cultivars tolerant to salinity and drought, allowing cultivation in trivial areas, while assuring the viability and sustainability in such stress-prone areas of the industry (Lakshmanan and Robinson 2014).

Abiotic stresses trigger a series of morphological, physiological, biochemical and molecular changes in plants (Tuteja et al. 2012). Stress-induced gene expression is generally categorized as: (1) genes encoding proteins with yet unknown functions, (2) genes encoding proteins with known structural or enzymatic functions, and (3) genes encoding regulatory proteins (Bhatnagar-Mathur et al. 2008). The defense mechanism has molecular networks including components of hormone signaling, reactive oxygen species (ROS) scavenging system, changes in amino acid profile and lipid peroxidation (Fig. 12.1). Plant genetic engineering offers a powerful mean of over-expression of a broad-spectrum single gene to up or down-regulate specific metabolic step in order to modulate specific abiotic stress response. However, this approach has overlooked the fact that stress tolerance results from the action of several of such stress responsive genes, and single gene tolerance is unlikely to be sustainable. As a result, there has been a continued interest to develop alternative strategies that emerged to transform plants with regulatory genes. Activation of early-responsive genes within minutes of stress signal includes a variety of transcription factors, while late-responsive genes consist of the major stress-responsive genes which modulate and encode the proteins needed for synthesis of membrane stabiliz-

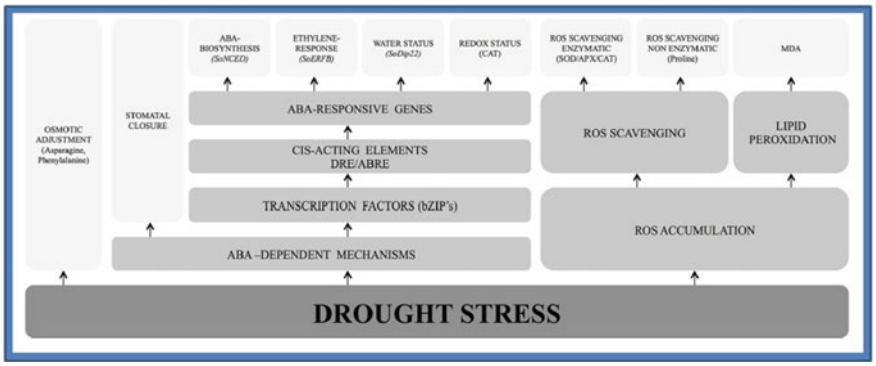

Fig. 12.1 Key components of drought stress-related mechanisms in sugarcane. Impact of drought stress conditions on an array of components those are responsive depending on duration, intensity and genetic makeup of sugarcane cultivars. Pathways for individual components may or may not be influenced by one another and their mutual interactions are always dynamic. (*Source* Ferreira et al. 2017)

ing proteins, antioxidants and osmolytes, for instance, late embryogenesis abundant (LEA) like proteins (Tuteja and Sopory 2008). Recent studies have reported the development of transgenic sugarcane plants comprising of various genes that encode a number of transcription factors (TFs), compatible organic osmolytes, LEA proteins, and heat shock proteins (HSPs). Cis-acting promoter elements and TFs are significant regulators of modulated gene expression and their over-expression has proven to be helpful for promoting stress tolerance in plants (Shinozaki et al. 2003). Genetic engineering allows control on spatio-temporal expression of a particular target gene for optimal function and metabolic efficacy. This is specifically important when the action of a given transcription factor or a gene is desired only at a specific time and/or under specific conditions of stress. This chapter presents various stress-induced biochemical and molecular responses and also summarizes the cloned and characterized transcription factors in sugarcane. The efforts undertaken to impart abiotic stress tolerance in sugarcane by employing several stress-related transgenes are also discussed.

12.2 Stress-Induced Biochemical and Molecular Responses in Sugarcane

Sugarcane possesses a C_4 metabolism and is grown across tropical and subtropical areas (Inman-Bamber and Smith 2005; Zhang et al. 2006). Although acquiescent to high-temperature, sugarcane is prone to freezing temperatures (Zhao and Li 2015). The low temperatures slow down the sugarcane growth, forcing the conversion of reducing sugars into sucrose. Clements (1962) and Du et al. (1999a, b) reported that

the sugarcane cultivars differing in original growth habitat showed sharp differences in cold sensitivity to photosynthesis.

Salinity stress severely brings down sugarcane productivity and quality of the product, due to drastic reduction in photosynthetic efficiency (Akhtar et al. 2003) that continues to accumulate less sucrose in the stalk (Rozeff 1995). In fact this is caused mainly by disruption in the homeostasis of water potential and ion distribution both at cellular and whole plant levels (Munns and Tester 2008). On the other hand, extreme temperatures and drought stress are the two major factors that severely affect agricultural production and cause economic impacts in many regions of the world. Effects of drought on sugarcane growth and development depend on plant growth stage, magnitude and duration of the stress. In general, drought in early and mid growth stages mainly reduces cane yield leading to low sucrose yield. However, moderate or mild drought in late growth stage favors improvements in sucrose content in stalks (Zhao and Li 2015).

Biochemical studies in sugarcane have explored several aspects of cellular metabolism under salinity stress that laid a solid foundation for our understanding of the adaptation mechanisms to high salinity. For instance, in vitro studies found increased leaching of salts from NaCl-treated calli compared to the control calli, which suggested that sugarcane can be considered as a Na^+-excluder plant species. On the other hand, corresponding higher concentration of K^+ ions was found in the control calli than in the NaCl-treated calli. This obvious phenomenon of increased Na^+ and corresponding decline in K^+ ion concentrations ultimately led to growth inhibition and reduced cell viability, demonstrating the typical glycophytic nature of sugarcane (Patade et al. 2009). Wahid and Ghazanfar (2006) reported that a salinity-tolerant clone of sugarcane accumulated less Na^+ and more K^+ compared to a sensitive contrasting clone, and consequently exhibited a higher K^+:Na^+ ratio. Antioxidant properties of flavonoids have been considered important for tolerance, and were found to be greater in tolerant sugarcane clones as compared to the susceptible clones. This confirmed the role of flavonoids to protect sugarcane from ion-induced oxidative stress during salinity stress (Wahid and Ghazanfar 2006). Furthermore, priming of sugarcane setts with NaCl solutions could explain the tolerance behavior and stress memory of sugarcane seedlings to NaCl treatments (Patade et al. 2009). Now, it is well proven that priming treatments modulate numerous pathways in association with induction of an antioxidant pathway that ensures plant growth under stressful conditions (Atreya et al. 2009).

The application of cDNA microarray methodologies in sugarcane has greatly helped to identify candidate genes for water stress tolerance. Rocha et al. (2007) subjected sugarcane plants to water deprived state for 24, 72, and 129 h, and using cDNA microarray approach, changes in gene expression were studied. Their results showed that fifty-two percent of the 179 differentially expressed genes to be drought-responsive. Similarly, Prabu et al. (2010) identified 158 genes in the sugarcane variety Co740 under water-deficit conditions by using PCR-based cDNA suppression subtractive hybridization and dot blot technique. Sequencing and annotation of expressed sequence tags (ESTs) from the public database revealed that most EST-encoded proteins were involved in cellular organization, signal transduction, protein

metabolism, and/or transcription. The WRKY-like transcription factor, abscisic acid (ABA)-inducible gene, drought-induced proteins HVA22, and MIPS were shown to be expressed in the stressed plants (Marone et al. 2001; Jiang et al. 2012). Changes in the generation of ROS alongside changes in superoxide dismutase and ascorbate peroxidase activities were observed in sugarcane leaves when grown under methyl viologen (paraquat)-induced oxidative stress (Chagas et al. 2008). Studies involving drought tolerant and sensitive cultivars resulted in finding a total of 165 genes to be regulated in response to water stress and most of these genes were involved in cellular functions (Rodrigues et al. 2009). On the other hand, short term exposure (up to 24 h) to salinity (200 mM NaCl) and iso-osmotic (20% w/v polyethylene glycol PEG 8000) stresses showed up-regulation of several stress-responsive genes such as shaggy-like kinase (sugarcane shaggy like protein kinase-*SuSk)* and *NHX* genes belonging to the families of Na^+/H^+ and K^+/H^+ antiporters (Patade et al. 2011a, b).

The mechanism of photosynthetic changes in sugarcane leaves upon exposure to chilling temperature (10°C) showed substantial accumulation of aspartate and alanine amino acids (Du et al. 1999a, b). Furthermore, few other experimental evidences concluded that orthophosphate dikinase (PPDK) and NAD-malate dehydrogenase (NADP-MDH) are the key enzymes that regulate the cold sensitivity during photosynthesis process.

Sugarcane transcriptome studies have unraveled modulation of gene expression of several stress-associated genes. The expression profile of 1,536 ESTs was investigated in sugarcane cultivar SP80-3280 exposed to cold stress for 3–48 h (Nogueira et al. 2003). Thirty four cold-inducible ESTs were identified, of which 20 were cold-responsive genes including ESTs for cellulose synthase, an ABI3-interacting protein 2, a phosphate transporter, and a negative transcription regulator. One of the identified transcription factors, *SsNAC23*, belonging to the NAC family was reported to have a role in growth and development of sugarcane and under abiotic and biotic stresses (Olsen et al. 2005). Accumulation of stress-responsive metabolites such as proline, glycine betaine, and soluble sugars was found to be higher in sugarcane sprouts exposed to high temperatures such as 40°C (Wahid 2007). In another study, these authors found that the expression of heat stress-induced dehydrin proteins (DHNs) was shown to improve the integrity of cellular membranes and suggested that expression of DHNs was independent of dehydration stress and DHNs have definitive protective role like other heat stress proteins (Wahid and Close 2007).

The yield and the physiological responses of sugarcane are also affected by toxicity of metals like copper (Cu) and cadmium (Cd), which leads to antioxidant response and increase in metallothioneins (MTs). MTs are low-molecular-weight, cysteine-rich, metal-binding proteins, which play a vital role in detoxification (phytoremediation), metal transport adjustment and metal ion homeostasis (Cobbett and Goldsbrough 2002). DNA gel blot analyses using MT probe found homology of eight fragments with MT Type I, 10 bands with MT Type II and 8 with MT Type III proteins (Sereno et al. 2007). Similar studies conducted with another metal (Zn) revealed that it interfered with the normal mitosis and led to the inhibition of DNA synthesis as well as photosynthetic pigment content within leaf tissue (Jain et al. 2010).

12.3 Genomics of Abiotic Stress Tolerance in Sugarcane

Genomics approaches through transcriptome sequence data are being made available for sugarcane, mainly generated by EST sequencing and also by methodologies such as probe hybridization arrays, or using known genes from other crops. With over 238,000 redundant ESTs derived from 26 diverse cDNA libraries, sugarcane expressed sequence tag (SUCEST) is the biggest database (Vettore et al. 2001). This endeavor brought a broader platform compared to former ESTs produced by other consortia in countries like the United States (Ma et al. 2004) and Australia (Casu et al. 2003, 2004; Bower et al. 2005).

Microarray platforms have also been used to evaluate sugarcane expression profiles. Initially, these were used to examine gene expression variances between maturing and immature stem tissues of sugarcane (Casu et al. 2003, 2004). About 4,715 non-redundant random ESTs obtained from stems (immature and maturing) and roots were studied using glass microarrays. Subsequently, a custom made cDNA microarray (3,598 genes) platform was developed for other purposes such as to profile effects of elevated atmospheric CO_2 level on sugarcane leaves (De Souza et al. 2008). Further attempts were made to characterize sugarcane leaf transcriptome by using SAGE (Serial Analysis of Gene Expression) analysis that showed ~70% transcripts matching to at least one sugarcane assembled sequence (SAS) having pre-assigned putative function (Calsa and Figueira 2007). Gene ontology studies of these transcripts revealed that the gene product was most frequently located in photosystem (PS) I reaction center besides other sites such as PS I, PS II and thylakoid complexes.

In spite of the availability of sufficient data for sugarcane transcriptome representing diverse conditions, only a few reports comprise of transcriptome sequences from drought-stressed libraries. A study involving expression profiling of 1,545 genes concerned in signaling processes showed that about 485 differentially expressed candidate genes were responsive to water deficit stress conditions (Rocha et al. 2007). Another study wherein sequencing of >35,000 ESTs of an Indian subtropical variety was carried out, an attempt to profile the selected EST clusters using real-time PCR resulted in an overall two-fold increase in relative expression of twenty-five stress-related clusters from sugarcane grown under water-deficit stress (Gupta et al. 2010). Iskandar et al. (2011) studied expression profile of genes of proline biosynthesis pathway and found that they were associated with both sucrose accumulation and water deficit in the internode tissue of a high sucrose cultivar under water deficit stress. Except *POX* (down-regulated after 15 days), all other genes showed up-regulation under water-deficit stress. However proline content was negatively correlated with sucrose concentration suggesting that proline has no osmo-protective role in sugarcane culms.

There has also been considerable focus on the transcription factors (TFs) (such as AP2/EREBP, bZIP, WRKY, MYB, and zinc finger proteins), which play a crucial role in stress responses by regulating numerous biochemical and physiological functions of the organism (Grotewold 2008; Du et al. 2013; Ambawat et al. 2013). Accordingly,

development of transgenic plants over-expressing such TFs could improve tolerance to several stresses, particularly to water stress by elevation of water-use efficiency.

Peroxidases, heat shock proteins and water transport proteins are known to play important roles in plant defense under water deficit conditions (Borges et al. 2001; Xiong and Zhu 2002; Wang et al. 2004; Casu et al. 2005). An investigation of 3,575 ESTs of a drought-tolerant sugarcane cultivar yielded total of 165 differentially expressed genes, specifying a huge number of genes related with drought tolerance (Rodrigues et al. 2011). A data mining investigation of SUCEST database yielded enhanced expression of genes encoding co-chaperones, chaperones, and other proteins (Borges et al. 2001). Many of such genes have vital importance for the synthesis of chaperone HSP70 (heat-shock protein) and its co-factors (viz., HSP40), as well as in accumulation to other encoder proteins (viz., HSP100, HSP90), and small HSP chaperones (Wang et al. 2004). The heat stress response regulates chaperone movement of small HSPs in sugarcane (Tiroli and Ramos 2007).

Studies on the expression profiles of twelve genes in the leaves of a drought-tolerant genotype of sugarcane and its comparative analysis with those obtained from other gene expression revealed considerable fluctuations in the patterns of gene expression (De Andrade et al. 2015). This variation was proposed to be due to a high degree of complexity in the response of sugarcane to water stress. Based on semi-quantitative RT-PCR analysis, Prabu et al. (2010) showed increased accumulation of a 22-kDa drought induced protein, along with higher transcript expression of WRKY, MIPS and ornithine-oxo-acid amino transferase during initial stages of stress induction that was followed with a gradual reduction. The study also revealed differential expression of several other sugarcane transcripts in response to water deficit stress using a PCR-based cDNA suppression subtractive hybridization (SSH) technique.

The transcript analyses of sugarcane plants exposed to short-term (up to 24 h) salt (NaCl, 200 mM) or iso-osmotic polyethylene glycol-PEG 8000 (20% w/v) stress conditions was carried out to study differential expression of stress responsive genes (Patade et al. 2011c). The study reported down regulation of a sugarcane homologue of NHX that is a member of Na^+/H^+ and K^+/H^+ antiporter family in response to the salinity stress. Upon long-term exposure to salt or PEG stress, transcript levels of both *PDH* and *P5CS* genes were improved (Patade et al. 2009), which was correlated with increased proline accumulation under stressful conditions (Patade et al. 2011a). Gene expression profiles were compared under water stress in tolerant sugarcane roots (Vantini et al. 2015). Two diverse cultivars, a drought susceptible (SP86-155) and a drought tolerant (RB867515), were estimated at four sampling time points (1, 3, 5, and 10 days) using the cDNA-amplified fragment length polymorphism technique. A transcriptome study of a cold susceptible sugarcane hybrid (CP72-1210) and a cold tolerant *Saccharum spontaneum* (TUS05-05) revealed a total of 35,340 and 34,698 SAS genes, respectively, and were found to be expressed before and after exposure to chilling stress (Park et al. 2015).

Recent studies have also focused on high throughput technologies for unraveling genomics resource under drought stress. Simultaneous application of high-throughput transcriptome profiling by Super-SAGE and the Solexa sequencing tech-

nology has been adopted to study sugarcane transcriptome under drought stress conditions (Kido et al. 2012). The resultant Super-SAGE libraries comprised of 8,787,315 tags (26 bp), and after exclusion of singlets they gave information about 205,975 unitags. Gene Ontology (GO) studies of the ESTtags permitted the in silico identification of 213 upregulated unitags that are responsive to abiotic stresses.

In summary, transcriptomics studies have significantly contributed to the understanding of expression of genes involved in water stress response in sugarcane. Despite the large number of reports, it is necessary to undertake direct correlation of gene expression to higher tolerance level with well-characterized sugarcane genetic lines or mutants (Ferreira et al. 2017). Most studies have been performed at pot level and hence extension of such studies to field conditions needs to be done.

12.4 Sugarcane miRNA and Stress Responses

MicroRNAs (miRNAs) are highly conserved and naturally occurring transcripts generally short in size (20–24 nt), single stranded and, non-coding. Past studies have proved that their expression and a number of miRNAs are either up or down regulated by abiotic stresses, suggesting that they may be involved in regulation of other stress responsive genes during stress and variation (Sunkar and Zhu 2004; Shriram et al. 2016). The contribution of miRNA during drought and salt stress conditions was reported through enhanced expression of miR159 in sugarcane (Patade and Suprasanna 2010). These authors also compared the expression of MYB under salinity and drought stress conditions to study the modification in target gene expression in response to alterations in over or under expression of miR159. Lin et al. (2014) reported a total 57 miRNA families out of which 23 were conserved, and 34 were novel. More than 400 targets genes of 44 miRNA families were identified from drought stress imposed drought tolerant sugarcane cultivar (ROC22) during PEG stress. The study also investigated 11 gene families that were differentially expressed in normal and treated plants. Out of these, nine were up regulated and remaining two gene families were down regulated. The potential targets of these 11 miRNA families were associated with plant growth and stress resistance. With the exceptions of SPBP, NCBP and BCP, the other genes were down-regulated in response to drought stress. Although many miRNAs were recognized, but only few studies have been performed to discover the mature miRNA sequences and investigate their expression in response to drought stress in sugarcane (Ferreira et al. 2012; Thiebaut et al. 2012; Gentile et al. 2013).

Thiebaut et al. (2012) selected eight sugarcane varieties on the basis of drought tolerance. Drought tolerant and sensitive plants were exposed to drought imposition by withholding of irrigation, for every 24 h till 12 weeks, and it was found that higher number of miRNAs was detected only in drought tolerant variety. Similar results were also obtained by Ferreira et al. (2012) using contrasting high and low tolerance sugarcane varieties grown under water limiting conditions under controlled green house conditions for 12 weeks and kept without water for 2–4 days. Few miRNA

like miR397, miR164 were found only in the green house grown (control) plants while some miRNA like miR166, miR160, miR169, miR172 and miR171 were detected in field grown plants. Out of the total microRNAs detected, some were up regulated and some were down regulated, depending on the variety. Prediction of the six precursors and the targets of the differentially expressed miRNA using an in silico approach suggested that majority of the targets played important role in drought tolerance.

Gentile et al. (2013) investigated two drought tolerant sugarcane varieties in the field under irrigation with and without water for 28 weeks. The authors identified 18 miRNA families including 30 established miRNA sequences. Out of these, 13 miRNAs expressed differentially during stress and 7 were commonly distinguished in both the varieties. Only five miRNAs (induced-miR 399 and miR160; repressed-miR166, miR396 and miR171) from irrigated field plants showed a similar profile in both of them. Furthermore involvement of other factors such as type of variety, nature of stressed tissue (leaves, seedlings, root and spikelets) and growth environment (greenhouse, field, hydroponic culture system) on expression pattern of miRNA was also reported (Gentile et al. 2015). These studies contributed greatly to our understanding of the function of miRNAs in the regulation of drought stress under field-grown sugarcane providing important avenues to develop new drought tolerant sugarcane varieties. Thiebaut et al. (2014) performed deep sequencing analysis to identify the small RNAs which are regulated in leaves and roots of sugarcane cultivars with different drought sensitivities (sensitive cultivar SP90-1638, tolerant cultivars SP83-2847 and SP83-5073). The study identified 28 (leaf) and 36 (root) conserved miRNA families which were differentially expressed in leaves and roots, respectively upon exposure to water deficit conditions. Khan et al. (2014) used a subtractive cDNA library from leaf tissue, sequenced the cDNA clones and their putative functions were annotated. The study showed that majority of ESTs were related to stress (15%), catalytic activity (13%), cell growth (10%) and transport related proteins (6%). The authors then conducted an in silico investigation to detect novel microRNAs which have a role in the regulation of plant responses under water stress in sugarcane.

12.5 Genetic Engineering in Sugarcane

Genetic engineering has shown great promise and potential for incorporation of foreign gene resource in sugarcane aimed at genetic enhancement for desirable gene(s) to develop transgenic plants bearing unique traits, such as strengthening of built-in defense mechanism, enhanced nutritional content, yield and agronomic attributes. Initial efforts were made to augment abiotic stress tolerance by modifying the rearrangement of genes through genetic engineering that are either directly linked to encounter adverse environmental conditions; straightly concerned in defense of cells against water scarcity or the genes that encode proteins vital for regulating signal transduction pathways in response to environmental stress. Successful efforts are being made to transfer plant and non-plant genes either individually or in combination, to improve abiotic stress tolerance in sugarcane (Table 12.1). Enhancement in

drought tolerance was achieved by enhancing proline accumulation in transgenic sugarcane (Molinari et al. 2007). Drought tolerant transgenic sugarcane was developed by incorporating *DREB2A* transcription factor (EMBRAPA, Brazil) which is helpful for regulating the genetic machinery for controlling abiotic stresses like drought, salinity, etc. On the other hand, genes for enhanced sucrose accumulation were identified and validated through genetic transformation, and increased sucrose accumulation was achieved in transformed plants compared to control plants (Papini-Terzi et al. 2009). In a first study, transcription activator-like effector nuclease (TALEN) was used to induce mutations in a highly conserved region of the caffeic acid O-methyltransferase (COMT) of sugarcane (Jung and Altpeter 2016) suggesting that such methods can be used for genome editing for other important traits. GM sugarcane has been developed in several countries and is approved in Indonesia for commercial cultivation since it could provide 20–30% higher sugar production under drought (Parisi et al. 2016).

12.6 Conclusions

Abiotic stresses adversely impact sugarcane crop production and productivity by eliciting a series of biochemical, cellular and molecular changes. The genomic endeavors to catalogue these responses to external stimuli are challenging due to the complexity and polyploid genome of sugarcane. The plant tolerance responses are orchestrated by regulatory mechanisms that need to be understood to fine-tune the metabolic pathways to achieve tolerance against different abiotic stresses. Thus, manipulation of metabolic pathways assumes great significance. Genetic engineering has emerged as a novel strategy for sugarcane crop improvement to enable incorporation of genes for novel traits, such as stress tolerance, qualitative and quantitative traits, and plant architecture. Attempts have been made to enhance abiotic stress tolerance through the manipulation of pathways involved in modulating regulator and effector genes involved in protection of plants against salinity, drought, temperature, heavy metals etc. Several candidate genes that produce metabolites, enzymes, osmolytes, osmoprotectants, and chaperones etc. have been used to develop transgenic sugarcane for abiotic stress tolerance. More efforts will be necessary to unravel molecular cross-talk machinery involving signal transduction pathways in plants subjected to combination of stress factors of drought, temperature, and salinity. The recent advancements of genome editing are being attempted in sugarcane. Since most of the traits are polygenic and because of lack of readily available targets, strategies will have to be made available to target a specific gene copy or several homologous copies for successful genetic engineering in sugarcane.

Table 12.1 Examples of transgenic sugarcane developed for abiotic stress tolerance

Gene	Gene origin	Method	Response	Reference
P5CS	*Vigna aconitifolia*	Microprojectile	Overexpression of proline, drought stress tolerance	Molinari et al. (2007)
AVP1	*Arabidopsis thaliana*	*Agrobacterium*	Salt stress tolerance	Kumar et al. (2014)
H+-Ppase AVP1	*Arabidopsis thaliana*	Microprojectile	Drought stress tolerance	Raza et al. (2016)
P5CS	*Vigna aconitifolia*	Microprojectile	Salt stress tolerance	Guerzoni et al. (2014)
RmBetA	*Rhizobium meliloti*	*Agrobacterium*	Drought stress tolerance	Waltz (2014)
Trehalose synthase (TSase)	*Grifola frondosa*	*Agrobacterium*	Osmotic stress tolerance	Wang et al. (2005)
Trehalose synthase (TSase)	*Grifola frondosa*	*Agrobacterium*	Drought stress tolerance	Zhang et al. (2006)
OsglyII	*Oryza sativa*	Microprojectile	Methylglyoxal and salt stress tolerance	Rani et al. (2012)
EaHSP70	*Erianthus arundinaceus*	*Agrobacterium*	Drought stress tolerance	Augustine et al. (2015a)
EaHSP70	*Erianthus arundinaceus*	*Agrobacterium*	Drought and salinity tolerance	Augustine et al. (2015b)
AtDREB2A	*Arabidopsis thaliana*	*Agrobacterium*	Drought stress tolerance	Reis et al. (2014)
EaDREB2	*Erianthus arundinaceus* and *Pisum sativum*	*Agrobacterium*	Drought and salinity tolerance	Augustine et al. (2014)
EaDREB2 + PDH46	*Erianthus arundinaceus* and *Pisum sativum*	*Agrobacterium*	Drought and salinity tolerance	Augustine et al. (2014)
rd29A	*Arabidopsis thaliana*	Microprojectile	Drought stress tolerance	Wu et al. (2008)
PDH45	*Pisum sativum*	*Agrobacterium*	Drought and salinity tolerance	Augustine et al. (2014)
Isopentenyl transferase (IPT)	*Arabidopsis thaliana*	Microprojectile	Cold stress tolerance	Belintani et al. (2012)
AtBI-1	*Arabidopsis thaliana*	Microprojectile	Drought stress tolerance	Ramiro et al. (2016)

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Chapter 13
Genomics Assisted Breeding for Abiotic Stress Tolerance in Millets

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Abstract Large-scale genomic resources have been generated in sorghum, finger millet and pearl millet leading to availability of large number of molecular markers and transcriptome sequences. With the availability of genome sequence in sorghum, pearl millet, and others in progress, integration of genomic technologies in millet breeding has now started in general for most of the stresses. This has raised the status of millets to genome rich crops from resource poor crops. Genomics-assisted breeding is an advanced breeding approach, wherein both the genomic information and the phenotypic selection are considered concurrently for designing phenotypes. Genomics-assisted breeding is strongly supported by third generation DNA sequencing techniques, which have provided enormous nucleotide information. Data mining and allele identification tools have allowed us to generate information for genes of interest and their functional specificity. For genomics-assisted breeding the basic need is to have maximum genomic information, trait specific mapping populations and highly precise phenotyping facilities. In millets, whole genome sequence information of sorghum, pearl millet and foxtail millets are available, which can be utilized efficiently to identify candidate genes for abiotic stress tolerance and for advancing

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breeding strategies such as genomic selection. QTLs conferring stress tolerance have been identified in few of the major millet crops but fine mapping and development of gene specific markers for high throughput selection needs emphasis. This chapter is a brief account of the accomplishments made in field of genomics for important millet crops like sorghum, pearl millet, foxtail millet, proso millet etc. and its application in improving abiotic tolerance.

Keywords Drought · Genomics-assisted breeding · Heat and high temperature Millet

13.1 Introduction

Genomics-assisted breeding is an advanced breeding approach, wherein phenotypic selection (PS) and genomic selection (GS) both are taken into account together. It may also be called as 'Third Generation Plant Breeding'. First generation plant breeding (PB) was dependent entirely on phenotypic selection; in second generation selectable molecular/biochemical information was correlated with traits. In the third generation plant breeding, both the genomic information and the phenotypic selection are considered concurrently for designing phenotypes. The paradigm shift to genomics-assisted breeding happened only due to development of third generation DNA sequencing techniques, which provided enormous nucleotide information. Data mining and allele identification tools have allowed to tag genes of interest and to know its functional specificity. In addition, it has transpired molecular information into designing genetic or genomic architecture of any plant type. Genome wide nucleotide survey and its analysis has become a job of days; locating gene(s) or region of interest on genome using bio-informatics tools has become less daunting. For genomics-assisted breeding to enter into crop improvement domain, the basic needs are to have maximum genomic information, trait specific mapping populations and highly precise phenotyping facilities. These three combinations demand a team approach for designing plants of future. This chapter is a brief account of the accomplishments made in genomics of important millet crops and its application in improving abiotic tolerance.

13.2 Millet Crops: An Overview

Millet is a broad term used for diverse group of small seeded annual C_4 panicoid grasses whose seeds are consumed as food and biomass as fodder. The inherent ability to survive and produce in adverse environments, high genetic adaptability and high nutrition value led to domestication of millet in the Neolitic and Bronze Age period. History of millet cultivation reveals that they are crops of the ancient world (Zhang et al. 2012a, b). Archaeological findings confirm millet production way back to 10,000 years ago (Lua et al. 2009). The ancient vedic Sanskrit text '*Yajurveda*'

Table 13.1 Important millet crops and their common names, center of origins and chromosome numbers

Crop	Scientific name	Origin	Chromosome number
Sorghum	*Sorghum bicolor*	North Eastern Africa	2n = 2x = 20
Pearl Millet	*Pennisetum glaucum*	West Africa	2n = 2x = 14
Finger Millet	*Eleusine coracana*	East Africa, India	2n = 4x = 36
Foxtail Millet	*Setaria italic*	Eastern Asia	2n = 2x = 18
Proso Millet	*Panicum miliaceum*	Egypt and Arabia	2n = 4x = 36
Barnyard Millet	*Echinochloa frumentacea, E. utilis*	India, Japan	2n = 6x = 54
Little Millet	*Panicum sumatrense*	Southeast Asia	2n = 4x = 36

also mentions about foxtail millet (priyangava), barnyard millet (aanava) and black finger millet (shyaamaka). There are nearly 10 genera and 14 species of millets, which belong to Poaceae family. Based on the cultivation area, millets are generally characterized as major or minor; major millets include pearl millet having highest area under cultivation, followed by sorghum, finger millet, proso millet, foxtail millet; whereas millets of minor value are polish millet, Indian barnyard millet, burgu millet, little millet, kodo millet, browntop millet, guinea millet etc. Compared to wheat and rice, millet crops are rich in protein, fiber, minerals, iron and calcium. Millets are natural wealth provided by nature, having huge nutraceutical potential (Table 13.1).

13.2.1 Millets as Potential Abiotic Stress Tolerant Crops

The potential productivity of any crop is affected by biotic and abiotic factors. Important abiotic factors causing yield losses at farmers' field includes water stress, temperature stress, soil related problems like salinity, alkalinity, acidity or elemental toxicity, lodging from wind, rain, snow or hail (Lobell et al. 2009). Abiotic stresses are major constrains to global food security; besides affecting yield, they also affect quality of harvested produce (Wang and Frei 2011). Among the main cultivated crops across globe, millets are considered to be climate resilient due to their minimum vulnerability to environmental stresses, high adaptive potential to vast ecological conditions, high tolerance to abiotic stresses and low input requirement (Bandyopadhyay et al. 2017). There are various morphological, genetical and biochemical factors, which contribute to potential of millets for being abiotic stress tolerant (Table 13.2).

Table 13.2 Important traits in millet crops imparting abiotic stress tolerance

Trait/metabolites/genes	Specificity	Reference
Morphological traits		
Earliness, plasticity to flower as per pattern of rainfall	Early life cycle completion confers escape from water stress, temperature stress at critical growth stages	Bidinger et al. (2007)
Small leaf area, waxy leaves and plant surface, thickened cell wall	Reduced evapo-transpiration rate, high water use efficiency,	Bandyopadhyay et al. (2017)
Early, dense and long root system	Quickly colonizes in soil, avoids early drought and deep roots helps in terminal drought	Li and Brutnell (2011), Passot et al. (2016), Kumar and Panneerselvam (2014)
Physiological/biochemical traits		
Enhanced Photosynthetic rates	Due to C_4 system, millets have high water use efficiency under warm temperature, higher copy number and enhanced expression of MDH (Malate dehydrogenase) and PPDK (pyruvate orthophosphate dikinase) genes	Sage and Zhu (2011)
Enhanced level of metabolites	Increase in antioxidants and reactive oxygen species (ROS) scavenging	Lata et al. (2011)
Molecular/genes traits		
Over-expressed genes in abiotic stress	ASR (Abscisic acid ripening), AGO (Argonaute protein encoding), ATG (Autophagy), LEA (Late embryogenesis abundant protein), ARDP (ABA-responsive DRE binding protein), DREB (dehydration responsive element binding protein), NAC transcription factor, Aldose reductase, Glutamine synthetase, Pyyrroline-5-carboxylate reductase, OPR (12-oxophytodienoic acid reductase), WD-40, PHGPX (Phospholipid hydroperoxide glutathione peroxidase), NAC Transcription Factors, bHLH transcription factor, Dehydrin 7, Heat shock factor, Ascorbate peroxidase, β-carbonic anhydrase, Glutathione reductase, VDAC (Voltage-dependent anion channel), Dehydroascorbate reductase	Bandyopadhyay et al. (2017)

13.2.2 Millet Genomics: An Overview

13.2.2.1 Molecular Markers

Genome characterization using polymerase chain reaction (PCR) and non-PCR based techniques in millets has accounted in identification of numerous markers for appli-

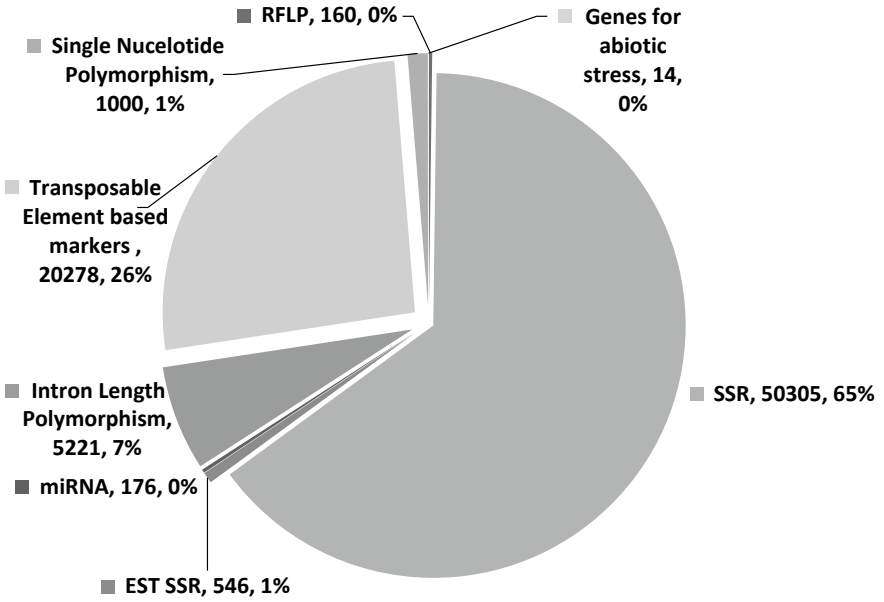

Fig. 13.1 Information on number of molecular markers identified in foxtail millet (Lata 2015; Bandyopadhyay et al. 2017)

cation in genetic improvement (Figs. 13.1, 13.2 and 13.3). In foxtail millet, more than 75,000 markers (Fig. 13.1) are identified [64% (50,305) SSR markers; 26% (20,278) transposable elements-based markers]. In pearl millet, more than 1500 markers (Fig. 13.2) are reported (38% SSR and 38% DArT markers), whereas in finger millet (Fig. 13.3) only 180 markers are reported. In foxtail millet, in the past 20 years four physical maps and three genetic maps (based on single nucleotide polymorphism (SNP), SSR and restriction fragment length polymorphism (RFLP) markers) are reported. In pearl millet, in the past 25 years 7 linkage/genetic maps are reported (based on expressed sequence tags EST-SSR & STS markers; DArT and SSR markers; EST-SSR markers; RFLP and SSR markers and gene-based SNPs).

13.2.2.2 Whole Genome Studies

Whole genomes of millet crops have been deciphered for sorghum, pearl millet and foxtail millet (Table 13.3). In sorghum, more than 85% of genome is sequenced covering 699 Mb of 800 Mb genome; 29,488 genes, 5,599 SSR markers sites and 38,85,829 SNP sites were identified. In pearl millet, 90% of the genome is mapped, using whole genome data and GBS (genotyping-by-sequencing) of 994 lines constituting 260 B lines, 320 R lines and 345 Pearl Millet inbred Germplasm Association Panel (PMiGAP); 88,256 SSR motifs are tagged (Varshney et al. 2017). Using these

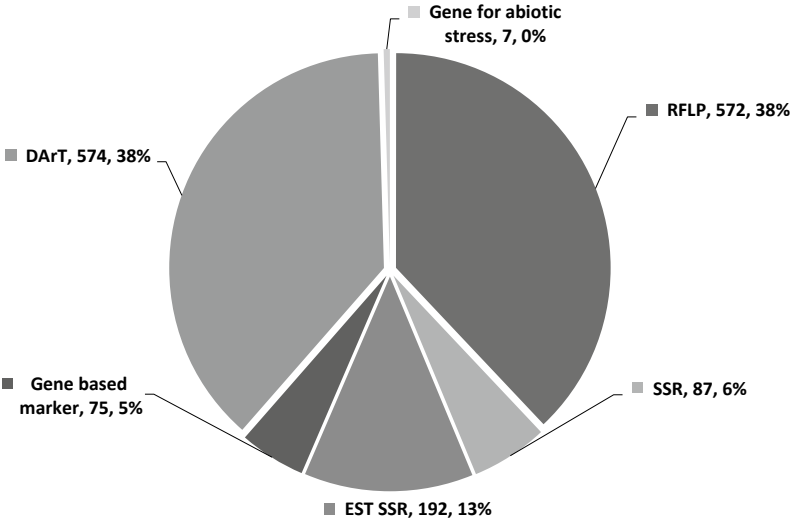

Fig. 13.2 Information on number of molecular markers identified in pearl millet (Lata 2015; Bandyopadhyay et al. 2017)

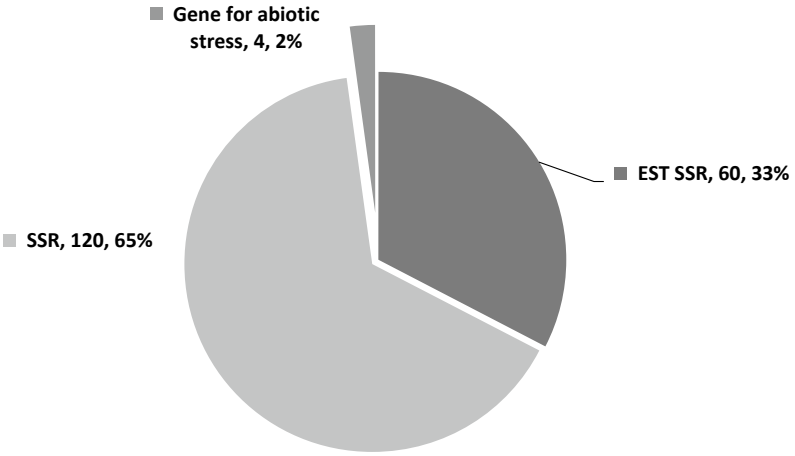

Fig. 13.3 The number of molecular markers identified in finger millet (Lata 2015; Bandyopadhyay et al. 2017)

SSR motifs, 74,891 SSR site-specific primers are designed, which will be helpful in MAS for strengthening the breeding programs in pearl millet. In foxtail millet, more than 85% of the genome has been mapped accounting for 423 Mb; repeat elements comprised of 29% of the genome and 38,801 genes have been mapped in the genome (Zhang et al. 2012a, b). The genomic homology among millet crops has produced interesting results. A close genomic synteny is observed among pearl

Table 13.3 Genomic information extracted from whole genome data in millet crops

Crop	Genome size	Genome sequences	Total genes	SSR identified	SNP identified	References
Pearl Millet	1.96 Gb	1.79 Gb	38,579	88,256	29,542,173	Varshney et al. (2017)
Foxtail Millet	490 Mb	423 Mb	38,801	–	–	Zhang et al. (2012a, b)
Sorghum	800 Mb	699 Mb	29,448	5599	38,85,829	Devos (2010), Yonemaru et al. (2009), Bekele et al. (2013)

Table 13.4 List of genomic databases developed in millets

Pearl Millet	Pearl Millet Drought Transcriptome database (PMDTDb)	http://webtom.cabgrid.res.in/pmdtdb/ (Unpublished)
Foxtail millet	Foxtail millet Marker Database (FmMDb):	http://www.nipgr.res.in/foxtail.html
	Foxtail millet Transcription Factor Database (FmTFDb)	http://59.163.192.91/FmTFDb/
	Foxtail millet miRNA Database (FmMiRNADb)	http://59.163.192.91/FmMiRNADb/
	Foxtail millet Transposable Elements-based Marker Database (FmTEMDb)	http://59.163.192.91/FmTEMDB/
Sorghum	Sorghum Functional Genomics Database	http://structuralbiology.cau.edu.cn/sorghum/index.html
	Sorghum transcriptome database	http://sorghum.riken.jp/morokoshi/Home.html
	SorGSD—Sorghum genome SNP database	http://sorgsd.big.ac.cn/
	Sorghum Transcription Factor Database	http://www.planttfdb_v1.cbi.pku.edu.cn:9010/web/index.php?sp=sb

millet, sorghum and foxtail millet genomes- with 14,398 common genes. Pairwise, pearl millet and sorghum have 15,078 common genes, pearl millet and foxtail millet have 15,887 common genes and sorghum and foxtail millet share 16,688 genes in common (Varshney et al. 2017). The genomic information generated in these crops is also available in public domain (Table 13.4).

13.3 Status of Genomic Understanding of Millets for Abiotic Stress Tolerance

13.3.1 Sorghum

In semi arid regions of world sorghum is mainly cultivated as a staple food and fodder crop. Molecular studies have provided valuable information on gene(s)/QTL's existing in sorghum conferring tolerance against drought and other abiotic stress.

13.3.1.1 QTL Mapping

In sorghum, stay green trait is the most important trait strongly associated with terminal drought tolerance. Crasta et al. (1999) developed recombinant inbred line (RIL) mapping population between B35 and Tx430. Based on simple interval mapping using RFLP markers, 3 major stay green QTLs (SGA, SGD and SGG) were tagged contributing 42% of phenotypic variation. Further composite interval mapping-based and QTL × environment interaction-based studies validated the QTL's association with stay green trait. A mapping population developed between B35 x Tx7000 was tested over environment by Subudhi et al. (2000) for four stay green QTLs (Stg1, Stg2, Stg3 and Stg4) identified earlier by Xu et al. (2000), which suggested importance of *Stg2* for control of stay green trait. Near isogenic lines (NILs) carrying stay green QTL in rabi sorghum were studied by Chaudhari and Fakrudin (2017). They identified 16 genes linked with three QTLs $qSTG_1$, $qSTG_2$ and $qSTG_3$, which were reported to have significant role in drought tolerant pathways.

13.3.1.2 Transcriptome Analysis

Transcriptome analysis for heat and drought in sorghum revealed role of unique transcription factors MYB78 and ATAF1, heat shock proteins and polyamine biosynthetic pathways in abiotic stress tolerance (Johnson et al. 2014). Aquaporin gene (*AQP*) families are responsible for water transportation in plants. Genome wide survey deciphered 41 non-redundant *AQP* genes belonging to 4 families- plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin 26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs) in sorghum, and *SbAQP* gene has been identified as a valuable resource for stress adaptation (Reddy et al. 2015).

13.3.2 Pearl Millet

Pearl millet is highly tolerant to drought (2.5 tolerance degree in a scale of 3; Creswell and Martin 1993) but terminal drought during reproductive stage significantly reduces

yield and in-turn productivity. The crop has the inherent ability to recover from intermittent drought due to its fast growth rate and asynchronous tillering habit (Bidinger et al. 1987). Breeding for drought resistance or tolerance is one of the major breeding objectives for pearl millet breeders. The complex nature of drought has provided limited scope in characterizing the genetic stock for drought resistance, which has limited use of trait-based plant breeding strategy to develop drought resistant genotypes. Molecular breeding using genomic tools have certainly made us understand the genetics behind drought tolerance/resistance.

13.3.2.1 QTL Mapping

Drought resistant/tolerant QTL have been mapped in the crop responsible for yield under drought conditions. In 2004 and 2005, a major QTL, DT-QTL, was located on linkage group (LG) 2 in two independent mapping populations i.e., H77/833-2 × PRLT 2/89-33 and ICMB 841 × 863 B (Bidinger et al. 2005; Serraj et al. 2005). This QTL was validated by developing NILs of H-77/833-2 using MABC (markers assisted back crossing) approach. DT-QTL is reported to confer high leaf abscisic acid (ABA) content and limit the transpiration rates at high vapor pressure deficits, hence conferring drought tolerance (Kholová et al. 2010). Yadav et al. (2016) reported the advances made in utilization of genomic tools in tagging the DT-QTL, its validation and fine mapping. Another drought tolerance QTL have been reported using mapping population ICMB 841 × 863B (Yadav et al. 2004) on LG 5 and LG 6. Bidinger et al. (2007) reported QTLs on LG 3 and LG 4 for late drought stress conditions. Among all QTLs, DT-QTL on LG 2 has the highest LOD score (6.3–6.9) and represents 32% of phenotypic variation (Yadav et al. 2004)). Further, absence of QTL × environment interaction (Yadav et al, 2004) and its consistency in two genetic backgrounds has made it a major target for marker-assisted breeding for enhancing yielding ability of pearl millet under terminal or post flowering drought stress.

13.3.2.2 Transcriptome and Germplasm Association Panel Studies

Sehgal et al. (2012) reported 75 new EST gene-based markers for identifying candidate genes underlying the DT-QTL in pearl millet. Eighteen new gene-based markers were mapped having association with DT-QTL on LG-2, which earlier had only five EST-SSR marker loci (Rajaram et al. 2010). The study by Sehgal et al. (2015) involving 37 SSR and CISP markers on 250 lines of PMiGAP revealed that in DT-QTL (LG 2) UBC (ubiquitin conjugating enzyme), LHCP (light-harvesting chlorophyll a/b-binding proteins) and PhyC (phytochrome C)were the key regulatory and downstream genes, which were associated with the stay green trait. Most importantly, significant association of a SNP in putative acetyl CoA carboxylase gene was found with grain yield, grain harvest index and panicle yield under all water stress treatments.

13.3.3 Foxtail Millet

Foxtail millet has inherent ability to withstand against drought. The phenotypic makeup of the plant makes it efficient to use water better than other cereals and millet crops. The plants of foxtail millet have relatively small leaf area, cells walls are thick and the root system is dense which makes it a model crop for studying drought tolerance mechanism.

13.3.3.1 QTL Mapping

Dehydration responsive element binding (DREB) proteins play a critical role against abiotic stress-mediated gene expression. In foxtail millet, DREB homologs have been tagged and a SNP is reported in *SiDREB2* gene and an allele-specific marker (ASM) has been developed, which can be used for identifying putative drought tolerant genotypes (Lata et al. 2011). The *SiDREB2-ASM* was validated on 122 foxtail millet accessions (Lata and Prasad 2014), which were characterized as highly tolerant, tolerant, sensitive and highly sensitive based on PEG induced dehydration stress. The *SiDREB2-ASM* is found to be controlling 20% of phenotypic variation and hence important for MAS application in foxtail millet for screening drought tolerant genotypes. Wild progenitor i.e., Green foxtail (*Setaria viridis*) (from Uzbekistan) and *S. italica* cv. Yugu 1 (from China) were crossed to develop a mapping population to identify QTL for early seedling osmotic adjustment. Out of the18 QTL characterized for the target trait, 8 QTLs were contributed by wild progenitor *S. viridis* (Qie et al. 2014) showing the importance of wild species in identifying valuable genes in millets. Wang et al. (2014) characterized a novel late embryogenesis abundant (LEA) gene *SiLEA14* in foxtail millet. Transgenic foxtail millet and Arabidopsis plants with *SiLEA14* gene showed high degree of tolerance to salt and osmotic stress. Overexpression of abscisic acid stress ripening gene *ASR1* in tobacco enhanced drought and oxidative tolerance in transgenic plants showing the potential of transgenic approaches for developing abiotic stress tolerance in foxtail millet (Feng et al. 2016).

13.3.3.2 MicroRNA and Genome Studies

MicroRNAs role in drought stress is also tagged in foxtail millet. Yadav et al. (2016) based on genome wide survey identified 14 known and 29 novel miRNA families responsive to dehydration stress. Genome wide analysis assisted in identifying 124 C_2H_2 type zinc finger transcription factor (TFs) in the foxtail genome. Of these, expression profile of candidate SiC_2H_2 genes were studied in response to drought, salinity and cold stress, which showed differential pattern of the genes at a given point of time of stress (Muthamilarasan et al. 2014)

13.3.4 Finger Millet

Finger millet is considered abiotic stress tolerant, however, the information pertaining to application of genomics in finger millet is very limited (Gupta et al. 2017).

13.3.4.1 Transcriptome and Genome Analysis

In few of the recent studies, expression analysis done under drought conditions revealed presence of genes induced under stress viz., EcDehydrin7, Ec-apx1, Metallothionein, Farnesylated protein ATFP6, Farnesyl pyrophosphate, Protein phosphatase 2A, RISBZ4, NAC 67, EcNAC1. RNA sequencing, assembling and qRT-PCR in finger millet cultivar ML-365 identified 2866 drought responsive genes, of which the major genes were MYB, WRKY, ZFHD, MYC, ABF, NAC, GRF, AREB, and NF-Y transcription factors (Hittalmani et al. 2017).

13.3.5 Proso Millet

Genomic information pertaining to proso millet is very meager; a few studies are reported on ESTs and gene expression. Saha et al. (2016) studied 211 EST which were derived from stress induced leaf tissues. Thirty two *PmWRKY* genes are reported to be involved in abiotic stress response (Yue et al. 2016). The first linkage map in proso millet was reported by Rajput et al. (2016). They made an F_2 mapping population of Huntsman × Minsum cross and genotyped it with GBS markers and phenotyped for agro-morphological traits including water use efficiency. Eighteen QTLs were mapped on 14 linkage groups.

13.4 Salinity Stress

13.4.1 Pearl Millet

The response of the major DT-QTL identified on LG2 in pearl millet was evaluated in a range of salinity and alkalinity stress conditions, which showed that this QTL also governs tolerance effect against saline and alkaline conditions (Sharma et al. 2011). The study also determined that DT-QTL limits the accumulation of Na^+ ions in the leaves. Genome scan studies have shown that wild pearl millet populations possess valuable genes for abiotic stress tolerance (Salazar et al. 2016).

13.4.2 Finger Millet

Trancriptome analysis of leaf from salt tolerant genotype Trichy 1 showed upregulation of various functional group of genes belonging to families of transporters, transcription factors, cell signaling, osmotic homeostasis and biosysntheis of compatible solutes, whereas down regulation of flavonoids biosysthesis was also observed in salt tolerant genotype (Rahman et al. 2014).

13.4.3 Sorghum

In Sorghum, meager information is available on genomic application for salt tolerance. Buchanan et al. (2005) studied transcriptome following exposure of seedlings to high salinity (150 mM NaCl) in addition to osmotic stress (20% polyethylene glycol) or abscisic acid (125 mM ABA). Authors demonstrated that there exists a complex gene regulatory network that differentially modulates gene expression in a tissue- and kinetic-specific manner in response to ABA, high salinity and water deficit.

13.5 Conclusion

Next generation sequencing technologies have provided an insight into the nucleotide arrangements of a crop as well as have allowed generation of large number of markers required for genomics-assisted breeding in many crops. In millets too, whole genome sequence information of sorghum, pearl millet and foxtail millets have been made available recently, which has been utilized efficiently to identify candidate genes for abiotic stress tolerance and for advancing breeding strategies such as genomic selection. QTLs conferring stress tolerance have been identified in few of the major millet crops but fine mapping and development of gene specific markers for high throughput selection needs emphasis. Though efforts are going on for identification of genomic regions conferring stress tolerance against salinity and heat stress but the available information in millet crops is meager. Hence, it is necessary to give importance to these traits also besides drought to design climate resilient genotypes.

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