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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

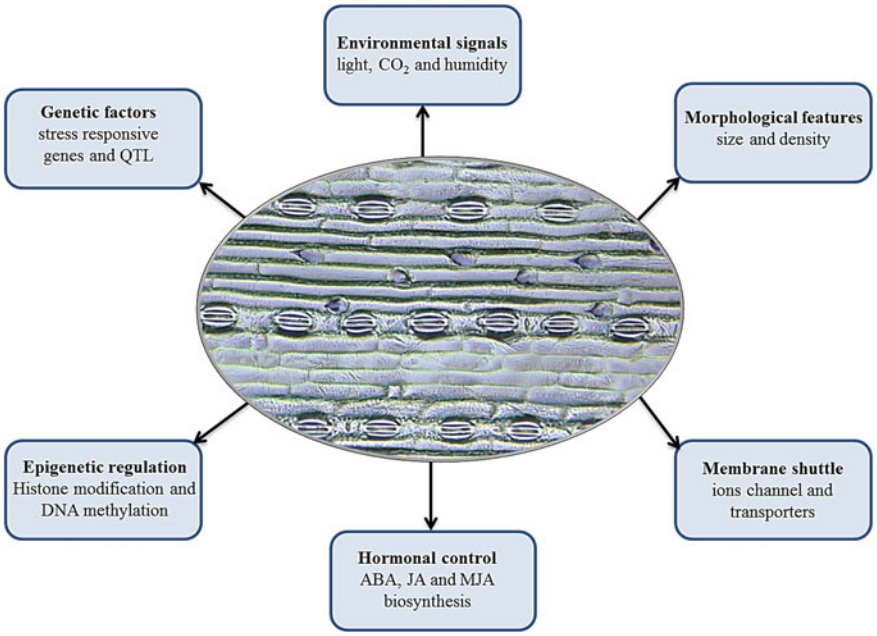

Fig. 1.1 Schematic model for processes regulating stomata during development and under stress. Leaf imprint was taken from the adaxial leaf surface of the RAC875 bread wheat cultivar (Shahinnia et al. 2016)

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