Genetic and genomic tools to improve drought tolerance in wheat

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Abstract

Tolerance to drought is a quantitative trait, with a complex phenotype, often confounded by plant phenology. Breeding for drought tolerance is further complicated since several types of abiotic stress, such as high temperatures, high irradiance and nutrient toxicities or deficiencies can challenge crop plants simultaneously. Although marker-assisted selection is now widely deployed in wheat, it has not contributed significantly to cultivar improvement for adaptation to low yielding environments and breeding has relied largely on direct phenotypic selection for improved performance in these difficult environments. The limited success of the physiological and molecular breeding approaches now suggests that a careful rethink is needed of our strategies to better understand and breed for drought tolerance. A research program for increasing drought tolerance of wheat should tackle the problem in a multi-disciplinary approach, considering interaction between multiple stresses and plant phenology, and integrating the physiological dissection of drought tolerance traits and the genetic and genomics tools, such as quantitative trait loci (QTL), microarrays and transgenic crops. In this paper, recent advances in the genetics and genomics of drought tolerance in wheat and barley are reviewed and used as a base for revisiting approaches to analyze drought tolerance in wheat. We

then describe a strategy where we target a specific environment and select appropriate germplasm adapted to the chosen environment based on extensive definition of the morpho-physiological and molecular mechanisms of tolerance of the parents. This information was used to create structured populations and develop models for QTL analysis and positional cloning.

The complexity of drought tolerance

Drought tolerance is defined as the ability of a plant to live, grow and reproduce satisfactorily with limited water supply or under periodic conditions of water deficit (Turner, 1979). Crop plants should not only have the ability to survive under drought but also the ability to produce a harvestable yield. Research into the molecular aspects of drought tolerance has tended to focus on plant survival at the expense of yield. However, severe water deficits are rare in viable agriculture, and asking how crops respond to or survive extreme drought is unlikely to have much of a practical impact (Passioura, 2002). The aim is not to 'convert wheat to a cactus' but to allow wheat to continue to grow and yield grain under water limited conditions.

Drought tolerance is a quantitative trait, with complex phenotype and genetic control (McWilliam, 1989). Understanding the genetic basis of drought tolerance in crop plants is a pre-requisite for developing superior genotypes through conventional breeding. Given the complexity of the genetic control of drought tolerance (multigenic, low-heritability and high G x E interactions), marker assisted selection has not contributed significantly to cultivar improvement for dry environments and breeding has relied on direct phenotypic selection. There are additional problems in investigating the genomics of drought tolerance in species such as wheat: most pathways and candidates can be more effectively studied in model species with

smaller and sequenced genomes such as *Arabidopsis* and even amongst the cereals there is more extensive data available for rice and maize when compared to wheat. However, recent technological advances and the imperative to ensure sustainable food production has driven research programs to genetically improve this crop despite the size and complexity of the genome (bread wheat is hexaploid with 16 Gb; Feuillet *et al.*, 2008).

Breeding for drought tolerance is further complicated by the fact that several types of abiotic stress can challenge crop plants simultaneously. High temperatures, high irradiance, scarcity of water and nutrient deficiencies are commonly encountered under normal growing conditions but may not be amenable to management through traditional farm practices. Certain soil properties such as composition and structure can also affect the balance of these different stresses (examples reviewed by Whitmore and Whalley, 2009). Higher plants have evolved multiple, interconnected strategies that enable them to survive unpredictable environmental fluctuations. However, these strategies are not always well developed in the cereal cultivars grown by farmers. At the molecular scale, pathways and gene networks between abiotic stresses overlap; for example, about 40% of drought or high salinity inducible genes are also induced by cold stress in rice (Shinozaki and Yamaguchi-Shinozaki, 2007). Some biochemical mechanisms may have opposing effects under different stresses; therefore tackling tolerance to one stress may lead to sensitivity to another. For example, some plants avoid heat stress by increasing stomatal conductance and consequently evaporative cooling. However, closing the stomata helps to decrease the loss of water and maintain turgor under conditions of low soil water potential. The two mechanisms will conflict when high temperature and drought occur simultaneously, which is frequently the case in a Mediterranean climate. Moreover,

74 the osmo-protectant amino acid proline has a toxic effect under heat stress and its
75 accumulation may not be an appropriate tolerance mechanism in field conditions
76 when heat and drought stresses are combined (Rizhsky *et al.*, 2004; Salekdeh *et al.*,
77 2009). Although the reductionist approach of studying isolated stress has
78 considerably increased our knowledge of tolerance mechanisms, interaction between
79 multiple stresses and stress combinations should be studied so as to make progress
80 relevant to field conditions.

81 In addition to these confounding environmental factors, a drought research program
82 should also consider plant phenology. By completing its life cycle before the onset
83 of severe water deficit, plants are often able to escape drought (Chaves et al., 2003).
84 This mechanism of avoidance is deployed by rapid phenological development,
85 developmental plasticity and remobilization of pre-anthesis assimilates to grain
86 (Turner, 1979). A short life cycle is particularly advantageous in environments with
87 terminal drought stress or where physical or chemical barriers inhibit root growth
88 (Bidinger and Witcombe, 1989; Blum, 1988; Turner, 1986). The plant's response to
89 drought can be confounded by the environmental covariates as a result of differing
90 phenology. Plant maturity strongly influences grain yield under dry conditions (Jiang
91 and Zeng, 1995; Ouk et al., 2006). A further confounding factor is plant
92 morphology, particularly plant height and tillering. Small plants with few tillers can
93 show higher Water Use Efficiency (WUE, ratio of the volume of water consumed to
94 the total biomass produced, or ratio of biomass to total evapotranspiration) than tall
95 multi-tillered plants. Since the genotypic variation of WUE is mainly driven by
96 variations in water use rather than by variations in plant assimilation, the selection
97 for high WUE may result in smaller plants, instead of high yield under drought
98 (Blum, 2005). Some QTLs for carbon isotope discrimination (a measurement of

WUE) in wheat were actually associated with variation in heading date and plant height (Rebetzke *et al.*, 2008). Breeding for a shortened crop lifecycle has been a very successful strategy in Mediterranean conditions (Araus *et al.*, 2002). However, in well-developed agricultural regions, crop flowering time has already been optimised by breeders so that the plant's phenology matches its environment (Passioura, 2007). Therefore research should now focus on optimizing vegetative development to manage biomass and ensure effective assimilates remobilization to grain when water supply becomes limiting.

QTLs of drought tolerance in wheat and barley

Most QTLs for drought tolerance in wheat and its close relative barley have been identified through yield and yield component measurements under water limited conditions (Maccaferri *et al.*, 2008; Mathews *et al.*, 2008; McIntyre *et al.*, 2009; Quarrie *et al.*, 2006; von Korff *et al.*, 2008). Although yield is the most relevant trait to breeders, it is very difficult to accurately describe with respect to water use and to identify candidate regions for positional cloning. Few studies have identified QTLs associated with specific components of drought response (Table 3). Although the development of gene-based molecular markers and genome sequencing should accelerate positional cloning (reviewed in (Collins *et al.*, 2008), the genomic regions associated to individual QTL are still very large and are usually unsuitable for screening in a breeding program.

Despite their importance in drought tolerance, reproductive organs and the root have attracted little attention in genetic studies. The effect of drought on reproductive processes has been extensively described in cereals (see review by Barnabas *et al.*, 2008). Passioura (2007) suggested that floral infertility resulting from water deficit

could be a promising target for improvement but no QTL studies for this trait have been published in wheat or barley. Improving the competence of the root systems to extract water from the soil also seems an obvious target for genetic analysis. A simulation analysis of root trait modification suggested that an extra 10 mm of water extracted during the grain filling would increase yield by 500 kg.ha^{-1}, representing a 25% increase of the average wheat yield in Australia (~2000 kg.ha^{-1}) (Manschadi *et al.*, 2006). The identification of markers or genes associated with root growth and architecture would be particularly useful for breeding programs to improve root traits by molecular marker-assisted selection. Few papers have described work on the identification of QTLs for root traits in wheat. Ma *et al.*, (2005) found a QTL for root-growth rate under Al treatment. QTLs of root traits (primary/lateral root length and number, root dry matter) under control conditions and during nitrogen deficiency were identified in wheat (Laperche *et al.*, 2006). Relative root growth was also used by Jefferies *et al.*, (1999) to map QTL for tolerance to toxic levels of soil boron. However, QTLs corresponding to root architecture in dry environments are yet to be discovered in wheat and barley.

Over the past few years there have been several mapping studies that have targeted drought tolerance and other abiotic stress tolerance loci associated with performance in low yielding environments (Table 4). However, despite this substantial research effort the only markers that have found their way into practical plant breeding programs are those for boron and aluminium tolerance (Gupta *et al.*, 2010).

'Omics of drought tolerance

The tools of genomics offer the means to produce comprehensive datasets on changes in gene expression, protein profiles and metabolites that result from

147 exposure to drought. Comparison of gene expression in Arabidopsis and rice showed
148 that the two species share many common stress-inducible genes (Shinozaki and
149 Yamaguchi-Shinozaki, 2007). Abiotic stress tolerance involves similar transcription
150 factors in both dicotyledonous and monocotyledonous plants and some molecular
151 mechanisms of drought tolerance have been extensively described (reviewed by
152 Yamaguchi-Shinozaki and Shinozaki, 2006). It includes signal transduction cascade
153 and activation/regulation of transcription, functional protection of proteins by late-
154 embryogenesis abundant proteins (*eg* dehydrins) and chaperon proteins (*eg* heat
155 shock proteins), accumulation of osmolytes (proline, glycine betaine, trehalose,
156 mannitol, myo-inositol), induction of chemical antioxidants (ascorbic acid and
157 glutathione) and enzymes reducing the toxicity of reactive oxygen species
158 (superoxide dismutase, glutathione S-transferase). Homologous genes of these
159 different classes were also identified in transcriptomic experiments comparing wheat
160 lines grown under well watered and water stressed conditions (Aprile *et al.*, 2009;
161 Ergen and Budak, 2009; Ergen *et al.*, 2009; Mohammadi *et al.*, 2007, 2008a; Xue *et*
162 *al.*, 2006).

163 Despite the existence of common regulatory mechanisms across species, the
164 conservation of the molecular response to dehydration across experiments (Aprile *et*
165 *al.*, 2009; Mohammadi *et al.*, 2007) is low due to variation in stress dynamics, stage
166 of development and tissue analyzed. Interestingly, microarray assays revealed
167 unexpected results such as a decrease in the expression of glutathione-related genes
168 following withholding of water in a tolerant synthetic wheat line (Mohammadi *et al.*,
169 2007), or the accumulation of proline in drought sensitive emmer wheat line (Ergen
170 and Budak, 2009), suggesting that some pathways/mechanisms are dependent upon
171 genotype and the duration, intensity and type of stress applied.

Comparison of transcript profiles between tolerant and susceptible lines under water stress has revealed differences in regulatory pathways. A drought tolerant emmer wheat genotype showed induction in *bZIP* and *HD-ZIP* gene expression (transcription factors known to be related to the ABA regulatory pathway) in response to shock-like drought stress, whereas the sensitive genotype induced some genes encoding transcription factors that bind to ethylene responsive elements. The two genotypes also showed differences in expression of the *phospholipase C* gene, involved in 1,4,5-triphosphate (IP3) signaling, and MAPK cascade elements (Ergen *et al.*, 2009).

A direct link between a gene expression profiles and specific physiological mechanisms has not been demonstrated, probably because the drought stress scenario and the germplasm used for microarrays and physiological experiments are seldom the same. Mohammadi *et al.* (2007) identified drought responsive genes in the root of a synthetic hexaploid wheat line considered as a drought avoider. This line retained a higher net photosynthetic rate and relative water content compared to a sensitive line, Opata. However, these researchers could not establish a causal relationship between the transcripts and the maintenance of relative water content. In another experiment, the synthetic line showed higher cell membrane stability and a lower rate of water loss from excised leaves, longer roots, larger root:shoot ratios and a larger number of seminal roots than Opata (Mohammadi *et al.*, 2008a).

A broad strategy for studying the genetics of drought tolerance in low yielding environments

Three major approaches for improving drought tolerance in wheat have been employed for many years:

1. The empirical selection for yield under water limited condition. This has been widely used and the good performance of modern cultivars is testimony to the success of this approach. However, there are clear signs that the rates of gain are declining and are insufficient to meet demand (Tester and Langridge, 2010).
2. Define physiological ideotypes for improved yield under water limited conditions, identify sources of variation for these traits and introduce these traits into elite varieties (Reynolds *et al.*, 2009; Richards *et al.*, 2010). Although this approach has been followed for several decades it has met with only limited success. The use of carbon isotope discrimination to screen for WUE is probably the only trait to lead to new cultivars.
3. Marker assisted selection based around screening for desirable alleles at QTL for drought tolerance. Despite many publications of QTL associated with drought tolerance (Table 4) a recent survey of molecular markers being deployed in wheat breeding programs failed to identify a single case where a drought or drought related marker was being implemented (Gupta *et al.*, 2010). The survey found that almost 50 loci were currently being tracked with molecular markers but the only loci associated with performance in low yielding environments were for tolerance to high soil boron (*Bo1*), aluminium toxicity (*Almt-1*), nematode resistance (cereal cyst and root lesion nematodes) and plant height. This strongly implies that the previous drought QTL studies have failed to identify loci of value to wheat breeding programs.

The limited success of the physiological and molecular breeding approaches until now suggests a careful rethink is needed of our strategies to better understand and breed for drought tolerance. Some of the new plant genomic techniques and

platforms may allow us to overcome the previous limitations but some may simply lead us further down the tried and failed paths.

A research program for increasing drought tolerance of wheat should tackle the problem in a multi-disciplinary approach, integrating the physiological dissection of drought tolerance traits and the genetic and genomics tools, such as quantitative trait loci (QTL), microarrays and transgenic crops. To do so, teams should include molecular biologists, physiologists and breeders. This is the approach taken in major companies which have successfully demonstrated application of patented technologies for drought tolerance in the field (Passioura, 2007). In this paper, we describe a strategy where we target a specific environment, select appropriate germplasm adapted to the given environment to create structured populations, extensively describe the morpho-physiological and molecular mechanisms of tolerance of the parents and combine this knowledge in models for QTL analysis and positional cloning (Figure 1). These various components of the drought genetics strategy are discussed below.

Target a realistic and specific environment

Firstly, if the strategy is to have practical relevance it should look at drought tolerance under field conditions. Molecular biologists have often reported the effect of genes on drought tolerance in unrealistic environments (dessication of detached leaves or of seedlings transferred from hydroponics to air, osmotic shock by applying polyethylene glycol) and rarely proved their phenotype in the field and their expected value in breeding (Blum, 2005; Passioura, 2007).

Secondly, it is clear that there are different types of drought and that plants have developed different morpho-physiological mechanisms to address them. For

example, low water use by moderated growth is an advantage in dry land conditions of stored soil moisture (eastern Australia type) but might be detrimental in other environments where drought may develop at any time during the season (Western Australia type) (Blum, 2005). Plants have developed different root architecture to optimize the timing of water extraction from soil depending of the drought conditions. A compact and deep root system with a uniform root branching pattern reduces water use early in the season but increases access to water during grain filling and increases yield in conditions of stored soil moisture (Manschadi *et al.*, 2006). In the Mediterranean environment, with seasonal rainfall and terminal drought, a large and shallow root system allows water extraction early in the season in the top soil layers where the water is available (Manschadi *et al.*, 2006).

Due to strong G x E interactions, a QTL can have positive, null or negative additive effects depending on the drought conditions (Collins et al., 2008). Many QTLs identified for response to drought are not "stable" in different environments. Yang *et al.*, (2007) found that 7 of 10 significantly additive QTLs for stem water-soluble carbohydrates content (compound which are stored for further grain filling in dry conditions) in wheat interacted with the environment. Thus, a research program on drought tolerance should first define a target drought scenario. Salekdeh *et al.* (2009) proposed the creation of a Minimum Information About Drought Experiment (MIADE) as a standard for the drought community, similar to the MIAME standard for transcriptomics data. The environment of South Australia, which is the focus of the strategy presented in this paper, is a Mediterranean-type of drought (Izanloo *et al.*, 2008). Wheat production relies on rainfall during the growing season when precipitation decreases with the rise of temperature during spring. The availability of water is cyclic with a succession of precipitation and drought periods from anthesis

to grain-filling stages. The drought is combined with a cyclic heat stress between rainfall events. Additional stress factors such as high wind, high irradiance, low air humidity, hostile subsoil with salinity, boron toxicity and nutrient deficiency are common in Southern Australia.

Population structure for genetic study of drought tolerance

The size and structure of the population used for genetic analysis must be carefully assessed. Previous attempts by our group to use association mapping or multiparental populations to study the genetics of drought were not regarded as successful. These studies simply identified loci controlling phenology, largely maturity, height and tillering, as major components of the drought response. The loci identified were all previously known and had little practical relevance. As described below, our approach was to use several large populations based exclusively on parents adapted to our targeted environment but differing in drought responses.

Germplasm should be selected based on the likelihood that the lines will produce valuable new genetic combination of direct and immediate relevance to breeding programs developing cultivars for the target environment. Selection for drought tolerance should not have a significant negative effect on other selection targets in a breeding program, such as maturity, height, disease resistance and grain quality. The use of elite varieties in the targeted environment has some benefits: the lines can be used directly in breeding program. Moreover, alleles discovered in non-elite germplasm might not lead to improvement because it was already selected during the development of elite wheat cultivars (Collins et al., 2008).

Based around comprehensive field data generated during a severe drought in the 2001/2002 season, a genetics-based drought strategy was devised with Australian

wheat breeders to select parental lines for the development of segregating populations. Elite cultivars and breeding lines were screened and assessed based on their grain yield under severe water limitation in South Australian environment. A trait summary for the parental lines described below is presented in Table 1. The two lines Excalibur and RAC875 represent major sources of drought tolerance in the Southern Australian environment. RAC875 was also believed to show superior tolerance to heat stress during grain filling relative to other material in the breeding programs. In the severe drought over the 2006 season where average yields at our field sites were only 0.8 t/ha, Excalibur and RAC875 were consistently higher yielding than other varieties (116% and 122% of the site means, respectively). Excalibur and RAC875 show similar behaviour under drought and out-yielded the variety Kukri, which was chosen as drought sensitive parent, by 10 to 40% under severe water stress (data provided by Dr. Steve Jefferies). Fig. 2 represents the percentage of yield production of four of the wheat cultivars on the basis of site means with an average grain yield below 3.0 $t.ha^{-1}$ (the data is based on the 2009 National Varietal trials and did not include RAC875). The parental line Drysdale was selected based on the carbon isotope discrimination screen, while Gladius is a new variety released in 2007 and is based on Excalibur and RAC875 (http://pbr.ipaustralia.plantbreeders.gov.au/). Gladius has been the highest yielding variety in the severe droughts of 2001/2002 and 2006/2007. Thus, this population will allow us to investigate the significance of the C-isotope discrimination trait relative to other factors and also allow confirmation of loci identified from the other two populations. Importantly, Gladius also shows the heat tolerance seen in RAC875. This is likely to be significant both in determining general tolerance to

drought stress but may also be of importance in lifting yield and grain quality in irrigated environments.

For two populations, Excalibur x Kukri and RAC875 x Kukri, doubled haploid (DH) populations (approximately 300 for each cross) and genetic maps have been generated, and seeds have been multiplied in Australia, in India (DRW, Karnal), in Mexico (CIMMYT) and Syria (ICARDA). Field data was collected from trials sown in Australia and Mexico in 2006, 2007 and 2008. Both crosses have also been used to generate large single seed descent populations (SSD or recombinant inbred lines, RILs) of 3,000 lines, which have been taken to F5. For the population of Gladius x Drysdale, 5,000 RILs have been produced, and a subset of 250 lines used for preparation of a preliminary map. In total, over 10,000 lines have been developed for the drought work. This represents a unique resource for studying the genetic control of drought tolerance in wheat. The nature of the populations means that they are of immediate relevance to wheat breeding programs targeted to low yielding Mediterranean type environments and they are being used directly for selecting new breeding lines.

The basic strategy for deploying these populations is to use the small doubled haploid populations and 250 randomly selected SSD lines for the Gladius x Drysdale for preliminary mapping of a wide range of drought related traits under controlled glasshouse and field conditions. In addition, confounding factors such as boron and salinity tolerance and nematode resistance and tolerance (both cereal cyst and root lesion nematodes) have been assessed. Since the major loci controlling boron tolerance and nematode resistance are known (Gupta *et al.*, 2010), these can be readily accounted for but salt and nematode tolerance must be measured. The field

data have been generated at multiple sites in Australia and at droughted and irrigated trials at the CIMMYT station at Obregon in Mexico.

The SSD populations are sufficiently large (3,000 for each cross) to permit immediate transfer to positional cloning projects and detailed genetic dissection of traits. They also provide an opportunity to explore epistatic interactions. As loci influencing particular components of drought tolerance are identified these will be used to divide the population for a second round of analysis. With populations of 3,000 lines we can explore the behaviour of five or six loci alone or in combination. This will allow complex genetic interactions to be explored. In particular, this approach may permit analysis of genome interactions. Subsets of lines can also be selected to fix genes of major effect such as the flowering time genes *Vrn* or *Ppd* to overcome the confounding effect of maturity on other traits (Distelfeld *et al.*, 2009).

Extensive description of the drought tolerant and sensitive parental lines

During the period of population development, detailed analyses of the parents can help define the physiological, biochemical and molecular components of drought response and the difference in behavior of parents. This information provides an opportunity for determining the relative significance and reliability of different phenotyping options in the segregating populations and also provides valuable resources to support gene discovery work.

The growth of the three key parents, Kukri, Excalibur and RAC875 has been analysed in detail in controlled conditions and under a cyclic drought regime that reproduces the Southern Australian environment or Mediterranean type drought stress (Izanloo *et al.*, 2008). Interestingly, Excalibur and RAC875 showed different strategies of tolerance to the same drought scenario. In the drought treatment,

RAC875 produced fewer tillers, maintained a higher number of grains per tiller and showed moderate osmotic adjustment (Table 2). The mechanism of tolerance of RAC875 seemed more conservative: under both well-watered and dry conditions, plants stored more water soluble carbohydrates in the stem, and leaves were more waxed and thicker, showing constitutive aspects of tolerance to drought. The genotype Excalibur showed higher osmotic adjustment, low ABA content, high stomatal conductance and rapid recovery after stress. The plant morphology and yield component reflected this responsive mechanism: leaves rolled in dry conditions reducing radiation interception; a high number of tillers were produced and then aborted in water-limiting conditions; the plants were able to compensate for the tiller abortion by producing more grains per tiller after recovery. Excalibur showed a more responsive mechanism with a strong interaction with environmental conditions.

Another component of the parental analysis is the generation of molecular and biochemical data on the drought responses. 'Omics profiles of the wheat parents Excalibur, RAC875 and Kukri have been developed during a cyclic drought under the same conditions as used for the morpho-physiological study by Izanloo *et al.* (2008). We have used the *Triticum aestivum* 17K oligo microarray developed by the Genome Canada/Genome Prairie/Genome Quebec program Functional Genomics of Abiotic Stress (FGAS), the USDA-ARS-Genomics and Gene Discovery Research Unit and the Australian Centre Plant Functional Genomics. The probe sequences of the microchip were from cold-stressed cv Norstar crown and leaf, cold and salt stressed root, unstressed controls, NSF-mapped ESTs, wheat zygotic and early embryo and ESTs of genes involved in cell-wall metabolism. Two treatments (cyclic drought and well-watered) of the three wheat lines, 5 sampling time-points (during development of drought, before and after rewatering) with 5 biological replicates per

391 sampling time-point and 3 tissues per replicate (leaf, stem and spike) generated more
392 than 450 samples. Preliminary results of leaf samples showed a total of 6,537
393 differentially expressed genes. A database, DroughtComparator, is being developed
394 to support analysis and interpretation of the data. This database will be publicly
395 released on completion of the experiment. A cDNA series, for use in mRNA
396 quantification by Q-PCR, is now being used to validate the gene expression profiles.

397 Metabolite profiles of leaves and grains have also been generated during the same
398 experiment. The results showed significantly different responses to drought stress for
399 RAC875, Kukri and Excalibur. Interestingly, the control and water-stress data
400 formed distinct clusters for Excalibur, the drought responsive genotype. Other cell
401 components such as fructans, betaines, lipids, waxes are being measured. Detailed
402 comparison with the transcript data should help us to understand the biochemical
403 bases and regulation of the different tolerance mechanisms of Excalibur and
404 RAC875.

405 **Use of mathematical models for QTL analysis**

406 A growing concept in biology is the use of mathematical models to understand
407 complex trait such as yield during drought. The procedure consists of dissecting the
408 phenotype and the response to environment into elementary and simpler responses
409 (Reymond *et al.*, 2003; Tardieu, 2003). Such modeling has been successfully used to
410 study leaf growth in maize (Chenu *et al.*, 2009). Manschadi *et al.*, (2006) proposed
411 integration of a physiological understanding with plant breeding to develop
412 mechanistic crop models and design ideotypes to targeted environments.

413 The problem of non-stable QTL because of the differences of environmental
414 conditions between experiments (Reymond *et al.*, 2003) may be overcome by

measuring accurately the environmental variables and using ecophysiological models. Combining QTL and ecophysiological models can help predict a phenotype from combinations of alleles by analyzing QTL for each parameter of the model. For example, the response of leaf growth to temperature and water deficit in maize has been broken down into traits such as intrinsic elongation rate (Reymond *et al.*, 2003; Tardieu, 2003). The QTL for each trait did not coincide with the same regions of the genome suggesting that the traits were regulated by different genes. In this approach the QTL correspond to well-defined functions and hypotheses about the function of the genes underlying the QTL are likely to be more accurate and the number of candidate genes narrower than in convention QTL mapping.

The gene networks are regulated and coordinated so that a plant react in a predictable way to a given environmental condition (Tardieu, 2003). The 'omics datasets serve to help develop models of networks and pathways that are triggered in particular genotypes in response to drought. The network information can feed directly into building models of the relationship of specific pathways and processes to the physiological responses to drought. The comparison of gene regulatory networks and QTL for each parameter of the model could also help identify candidate genes for QTL cloning. By studying all possible aspects from molecular aspects to plant physiology in the same germplasm (RAC875, Excalibur, Kukri) and in a specific drought scenario (terminal drought of South Australia), we should be able to dissect the phenotype from gene to a plant mechanism and build the appropriate models. The approach is empowered by the use of the same germplasm for creating the genetic populations (DH and RIL) and allowing the analysis of different allelic combinations.

The QTL under fine-mapping at the ACPFG

Genetic maps using DArT and SSR markers have been constructed for Excalibur x Kukri, RAC875 x Kukri and Gladius x Drysdale populations. For each population, between 600 and 1,000 markers have been included in the maps. The two Kukri populations were grown at over ten sites in Southern Australian and at the CIMMYT field site in Obregon, for the past three years. There are now data available for over 20 sites per year. In addition to standard assessment of lines during the growing season and recording of detailed climatic data at each site, the soil at the field sites was sampled extensively and assessed for nutrient levels and the presence of soil pathogens and pests. The sites range from severe drought stress with average yields at below 0.5 $t.ha^{-1}$ to sites where average yields were around 7 $t.ha^{-1}$. The Excalibur x Kukri population has also been screened for tolerance to resistance to nematodes (both cereal cyst and root lesion), boron toxicity, sodium exclusion and high pH using established lab based hydroponic assays.

The first targets for positional cloning were common loci that appeared in both Kukri populations. For the target loci around 6,000 RILs are available to support fine mapping. Four loci have been identified for positional cloning: a locus on chromosome 1B associated with yield under drought, a locus on 3B associated with yield under heat stress and also canopy temperature suppression, a 6A locus that shows a correlation between flag leaf width and grain size and a 7A locus that shows increased spike length, higher grain number and increased yield, particularly under severe drought stress (Izanloo et al., unpublished results). Loci of potential value to the breeding programs will be confirmed using the larger RILs populations and through analysis of selected lines from the Gladius x Drysdale population. These will be made available to breeding programs as rapidly as possible.

Moving forward to positional cloning of drought tolerance QTLs

Fine mapping of a drought response may define a QTL to a region containing a large number of genes. Genes that show a drought responsive expression pattern or may encode an enzyme or other protein involved in a metabolic pathway that responds to drought stress, would become strong candidates for further analysis. In maize, 22 differentially expressed genes were identified in a microarray experiment on four susceptible and tolerant recombinant inbred lines, co-located on the genetic map with QTLs for drought tolerance (Marino *et al.*, 2009). A physical map of genes involved in drought tolerance has been attempted in wheat by Ramalingam *et al.* (2006) who assigned 259 EST (811 loci) to chromosome deletion bins of wheat. However, the analysis of transcript profiles in wheat is impaired by the absence of a genome sequence and knowledge of homeologous sequences. The comparison of QTL and microarray data is also difficult due to the low number of sequence-based markers in the wheat genetic map. To overcome the problem, a SNP database is under

construction based on deep sequencing (over 1 million reads) of normalized full-length cDNA of the Australian parental lines described above. Preliminary results indicated that this work will generate around 30,000 SNPs per cross, which should greatly facilitate the fine-mapping of the QTL and the use of grass synteny to select candidate genes for cloning. It will also facilitate the genetic mapping of drought responsive genes in Excalibur and RAC875 identified by the microarray and reverse genetics programs.

Major changes in gene expression, protein or metabolite profiles can be directly mapped onto the mapping populations. Coincidence of loci controlling gene expression (eQTL), protein (pQTL) or metabolite (mQTL) with physiological or yield related loci, indicates possible biochemical processes underlying the physiological response. The co-localization of eQTL and physiological QTL for diverse traits showed that it may facilitate the identification of candidate genes and accelerate positional cloning (Kliebenstein, 2009). The gene expression profile of lines in a mapping population using Affymetrix microarrays successfully identified eQTL associated with seed development in wheat (Jordan *et al.*, 2007) and resistance to leaf rust in barley (Chen *et al.*, 2010b). This method also allows the identification of *trans* eQTL and provides valuable information on the regulatory network involved in different tolerance mechanisms. However, the cost of microarray analysis for a segregating population is prohibitive. To reduce the cost of such experiment, Xue *et al.* (2006) compared the transcript profiles of selected progeny lines showing difference in transpiration efficiency (carbon isotope discrimination). They identified 93 differentially expressed genes between high and low transpiration efficiency lines of a wheat progeny Quarrion/Genaro. In a similar approach, recombinant lines of the Excalibur/Kukri and RAC875/Kukri population will be selected based on the

503 outcomes of field screening and will also feed into the metabolomics and
504 transcription profiling projects.

505 **Conclusions**

506 Our knowledge of the mechanism of drought tolerance has been enhanced by
507 research programs targeting specific physiological, genetics or molecular aspect of
508 the drought response. However, in wheat these approaches have not lead to an
509 increase in tolerance over that already achieved by breeders using empirical
510 selection. Although the idea of linking physiology, 'omics and quantitative genetics
511 have been already proposed, only few research programs have taken this integrative
512 approach. The great strength of genetic and genomics analysis in wheat has been the
513 ability to generate large populations and well developed field phenotyping
514 capabilities. However, genetic studies have often been too ambitious and not
515 permitted effective dissection of the drought response or focused on specific drought
516 scenarios or regimes. Analysis of response to drought has been further complicated
517 by the absence of a genome sequence and the generally poor genomics resources has
518 been limiting. New developments in sequencing, marker development and genome
519 analysis have created the opportunity to revisit the way in which we structure
520 populations for analysis and tackle specific components of drought tolerance.
521 Phenotyping has now become the major cost and rate-limiting step in the genetic
522 analysis of drought tolerance and many other traits, and the development of rapid
523 and cheap procedures to characterize components of the drought response will be
524 critical in improving genetic resolution.

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753 **Table 1**. Characteristics of wheat parents used for the drought mapping populations.
754 The traits listed are expected to affect field performance of these lines under water
755 limiting conditions.

Traits	RAC875	Kukri	Excalibur	Drysdale	Gladius
Drought tolerance	high	low	high	high	high
Heat tolerance	high	low	intermediate	-	high
Boron uptake	Low	Intermediate	Very high	Intermediate-High	Intermediate-High
Sodium uptake	Low-Intermediate	Intermediate	Low	High	Low-Intermediate
Zn efficiency	Low	Low	Intermediate-High	Low	Unknown
Nematodes					
Cereal cyst	Unknown	Susceptible	Susceptible	Susceptible	Unknown
Root lesion	Susceptible	Susceptible	Moderately resistant	Susceptible	Unknown
High pH	Intermediate	Intermediate	Intolerant	Unknown	Unknown

756

757 **Table 2.** Characteristics of the "drought tolerant" wheat genotypes RAC875 and

758 Excalibur under a cyclic terminal drought regime.

RAC875	Excalibur
Lowest tiller number *per se*	High tiller number, more tiller abortion under stress
Thick green leaves	Higher total biomass
Stronger leaf waxiness	Higher root-shoot ratio under water stress
Stay-green phenotype	Highest osmotic adjustment
Moderate osmotic adjustment	Low ABA content under stress
Low stomatal conductance	Highest stomatal conductance
Slower recovery in stomatal aperture	Rapid recovery after re-watering
High water soluble carbohydrate	

759

760

761 **Table 3.** QTLs of physiological responses to drought stress identified in wheat and
762 barley.

Trait	Species	Drought condition	Chromosome location	Reference
Water soluble carbohydrate	wheat	Rainfed field	1A, 1D, 2D, 4A, 6B, 7B, 7D	Yang *et al.*, 2007
Carbon isotope ratio, osmotic potential, chlorophyll content, flag leaf rolling index	durum wheat	Rainfed field	2B, 4A, 5A, 7B	Peleg *et al.*, 2009
Grain carbon isotope discrimination	barley	Mediterranean rain fed field	2H, 3H, 6H, 7H	Teulat *et al.*, 2002
Relative water content	barley	Mediterranean rain fed field	6HL	Teulat *et al.*, 2003
Leaf osmotic potential, osmotic potential at full turgor, osmotic adjustment, carbon isotope discrimination	barley	Water-deficit in growth chamber	6HL	Teulat *et al.*, 2001; Diab *et al.*, 2004
Water soluble carbohydrate	barley	Water-deficit in growth chamber	4H	Diab *et al.*, 2004
Chlorophyll and chlorophyll	barley	Post-flowering	2H, 4H, 6H, 7H	Guo *et al.*, 2008

fluorescence parameters				
Relative water content	barley	Water-withholding	1H, 2H, 6H	Chen *et al.*, 2010a

763

764 **Table 4.** QTLs identified for tolerance to several abiotic stresses in wheat and barley
765 (update of the review by Langridge *et al.* (2006) which covered studies earlier that
766 2006).

Stress	Chromosome location	Reference
wheat		
Drought	20 QTLs	Kirigwi *et al.*, 2007; Mathews *et al.*, 2008; Salem *et al.*, 2007
Cold	5A, 1D	Baga *et al.*, 2007
Copper toxicity	1AL, 2DS, 3DS, 4AL, 5AL, 5DL, 5BL and 7DS	Balint *et al.*, 2007; Balint *et al.*, 2009
Aluminium toxicity	4DL, 3BL, 2A, 5AS and 2DL	Cai *et al.*, 2008; Ma *et al.*, 2006
Salinity	47 QTLs	Ma *et al.*, 2007
Heat	1B, 5B and 7B	Mohammadi *et al.*, 2008b
Nitrogen deficiency	2D, 4B and 5A	Laperche *et al.*, 2008
barley		
Drought	38 QTLs	von Korff *et al.*, 2008
Salinity	30 QTLs	Witzel *et al.*, 2010; Xue *et al.*, 2009
Water-logging	20 QTLs	Li *et al.*, 2008
Aluminium toxicity	2H, 3H and 4H	Navakode *et al.*, 2009

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Figure legends

Fig. 1. Schematic representation of the pathway from parental lines selection to gene discovery. The diagram shows the role of physiological analysis, population development and phenotyping and the various 'omics technologies in supporting a gene discovery path. Given the complexity and variability of the droughted environment, the first task is defining the type of drought regime or regimes that are being investigated. The second issue is selecting germplasm that will be suitable for the target environments and is likely to reveal major loci associated with tolerance. These lines are used to develop the segregating populations that form the base for the genetic analysis. Mathematical models of physiological traits and omics of parental and selected recombinant lines provide functional data to select candidate genes for the QTL. A further component in the process of defining target regions and candidates for drought tolerance in wheat is the use of the sequenced cereal genomes. The strong conservation of gene order between the grasses means that the rice and *Brachypodium* genomes provide a valuable resource in developing markers for fine mapping in a target region and for identifying candidate genes for the QTL.

Fig. 2. Relative yield of mapping parents in low yielding environments in 2009. The data are taken from the National Variety Trials (http://www.nvtonline.com.au/home.htm). The yields of the four varieties are expressed as a percentage of the site mean. RAC875 was not included in these trials.