Evaluation of physiological traits and identification of QTLs for drought tolerance in hexaploid wheat (*Triticum aestivum L.*)

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CHAPTER 1

GENERAL INTRODUCTION

1 Chapter 1: General introduction

Drought or water deficit stress is one of the most important limiting factors for crop production in the world, especially in Australia. Climatic changes and increasing aridity coupled with an increased demand for food by the rising population (Borlaug and Dowswell, 2003), have shifted breeders' attention to focus on producing crop plants with better adaptation and high yield under drought conditions (Bacon, 2004).

Wheat is the most widely cultivated crop in the world, which provides more than 25% of the total cereal output. It is a staple food in developing countries and provides food for almost 35% of the world's population (FAOSTAT, 2006). Wheat is mainly grown in rainfed areas of the world. In Australia, wheat is the largest grain crop and it is the second largest agricultural commodity for export. Wheat is grown in the belt of land curving across the eastern and southern regions known as wheatbelt, where winter rainfall (approximately 400 to 600 mm in average) is adequate to produce a crop. Spring wheat is grown as a winter crop sown in autumn (May to June) and harvested in early summer (November to December). Much of the wheat growing region is in the sub-humid and semi-arid zones where drought causes substantial reductions in crop production. Figure 1-1 shows the total area sown and grain crop production in Australia. The droughts in 1994, 2002 and 2006 caused a dramatic reduction in grain production, especially in wheat. Grain crop production in 2006 was severely affected by drought.

However, the occurrence of drought or water shortage is the most common constraint on all rainfed crop production in Australia. With climate change, it is also projected that rainfall across Australia continues to be dominated by high variability across regions, seasons and years. By 2030 autumn and winter rainfall is projected to decline by up to 20% and evaporation rates may increase (Gunasekera et al., 2007). In some areas around Australia, extreme changes in climate from year to year results in yield fluctuation in different years and in different regions. This causes major impacts on the nation's economy, social and environmental effects (http://www.abareconomics.com).

NOTE: This figure is included on page 2 in the print copy of the thesis held in the University of Adelaide Library.

Figure 1-1. Grain area sown and grain production in Australia from 1980 to 2007 (Colin et al., 2007)

In South Australia, the third largest wheat growing state in Australia, area of wheat production extends from the Victorian border to the Eyre Peninsula where growing wheat is also dependent on receiving adequate rainfall in the planting and growing periods from April to November. The climatic pattern in South Australia resembles that of a Mediterranean-type environment, where most precipitation falls in winter and vapour pressure deficits rise with temperature in the spring. An example of this is shown for Minnipa, Eyre Peninsula, at which field experiments for testing drought tolerance of wheat have been undertaken (Fig. 1-2). In general, rainfall in the winter months (June-August) is usually adequate for plant growth and exceeds crop demand because of mild temperatures, low evaporation, slow growth rates, and the relatively high reliability of the rainfalls. During the spring, rainfall becomes less frequent with intermittent rainy periods. Temperature and vapor pressure deficits increase and water stress of unpredictable severity, duration and timing can occur any time from September. These rainfall fluctuations during the later growing season usually cause cyclic water stress (Fig. 1-3), sometimes in conjunction with temperatures above 30°C as well as hot and dehydrating winds. Often these harsh conditions coincide with the critical developmental stages of the crop such as stem elongation, flowering and grain filling (Turner, 2004a). In addition, drought under South Australian conditions is complicated by strong genotype-by-environment interactions. Many growing areas have

hostile subsoil with salinity as well as nutrient deficiencies and toxicities being present (Rodriguez et al., 2006).

NOTE: This figure is included on page 3 in the print copy of the thesis held in the University of Adelaide Library.

Figure 1-2. The pattern of rainfall, evaporation and temperature in Minnipa, South Australia (Source: http://www.bom.gov.au)

NOTE: This figure is included on page 3 in the print copy of the thesis held in the University of Adelaide Library.

Figure 1-3. The cyclic water stress pattern during critical stages of crop production in South Australia. (Courtesy: Thorsten Schnurbusch)

Two opportunities were suggested to tackle the drought problem which faces farmers in drought-prone environments. First, improving farm management practices and second, developing drought resistant varieties via plant breeding (Richards, 1996). Both areas have had big contributions to wheat yield increases over the past century. Analyses of yield trends of wheat production in Australia suggested that at least half of the increase in yields of dryland wheat was attributed to improved agronomic management (Angus, 2001; Angus and van Herwaarden, 2001; Passioura, 2002; Turner, 2004b). However, breeding better adapted cultivars for the target environment with the appropriate agronomic packages is the most promising approach (Araus et al., 2002; Turner

2004b). Therefore, both better cultivars and better agronomy are necessary to increase crop yield under water limited conditions (Passioura, 2006).

Understanding the genetic and physiological factors which are regulating or limiting yield under water stress via tools such as quantitative trait locus (QTL) analysis coupled with improved molecular marker technology may provide an opportunity to identify chromosomal regions of traits that increase the efficiency of water use and yield under water-limited conditions. It also may lead to improved selection methods for important drought-related traits correlated with yield (Richards et al., 2002). The complex effects of drought on plants and also the complexity of yield and its components as quantitative traits have made it very difficult to improve drought tolerance of crop plants via conventional plant breeding (Reynolds et al., 2005). Through the adoption of molecular markers and QTL analysis in breeding programs, in concert with physiological studies, it has become more feasible to study and develop drought resistant crop plants.

A series of drought field experiments were carried out across the South Australian wheatbelt. Elite cultivars and breeding lines were screened and assessed based on their grain yield performance for drought tolerance by Australian Grain Technology (AGT). Grain yield data of wheat cultivars across different experiments and years showed that Excalibur and RAC875 outyielded Kukri by 10 to 40% (data kindly provided by Dr. Steve Jefferies, AGT.). The differences between lines are more obvious under drier conditions (Table 1-1). Table 1-1 shows grain yield production in 2003, 2004 and 2006 at ten different sites across the South Australian wheatbelt. Kukri showed a high yield potential similar to Excalibur and RAC875 under high rainfall conditions, while Kukri produced far less yield under severe water stress. Excalibur and RAC875, in contrast, maintained grain yield under drought stress. Figure 1-4 represents the percentage of yield production of the three wheat cultivars on the basis of site means in ten different South Australian wheat fields with an average grain yield below 1.5 t/ha.

It is hypothesised that wheat genotypes that maintain higher grain yields under Southern Australian drought conditions are those with rapid physiological responses to drought (Blum, 1999). In this study, a series of experiments were carried out aiming, firstly, to evaluate three South Australian wheat varieties for their agronomical and physiological responses to cyclic water stress under controlled conditions. These wheat cultivars, with contrasting levels of productivity under water stress, were investigated for their

underlying physiological differences to identify traits which enable these wheat cultivars to maintain higher grain yields under water stress conditions (Chapter 3). A doubled haploid mapping population was also genotyped in order to construct a genetic linkage map (Chapter 4), and this DH mapping population was phenotyped in the field in order to detect chromosomal regions or QTL associated with better performance under drought in the field (Chapter 5).

Table 1-1. Grain yield (kg/ha) of Excalibur, Kukri and RAC875 at different field sites across South Australia (2003, 2004 and 2006) (Source; AGT yield data)

	Year								
	2003			2004			2006		
Locations	Excalibur	Kukri	RAC875	Excalibur	Kukri	RAC875	Excalibur	Kukri	RAC875
Cowell	198	115	171	226	131	195	1138.6	994.5	1138.4
Kalanbi	1136	984	1145	1186	1023	1197	-	-	-
Kimba	220	161	240	232	170	253	96.0	127.9	184.9
Nujikompita	1245	1076	1225	1300	1123	1277	1570.9	1190.5	1742.6
Streaky Bay	938	813	905	947	823	921	766.1	632.9	869.4
Minnipa Early	715	450	784	773	487	851	1546.2	1336.3	1432.6
Minnipa late	431	337	553	290	226	370	552.3	507.9	635.8
Penong	-	-	-	-	-	-	424.3	326.4	463.0
Roseworthy	1147.8	953.8	1240.7	2489	2446	2899	2628.2	2287.1	2517.1
Booleroo	-	-	-	-	-	-	415.6	290.3	528.2
Average	753.9	611.2	783.0	930.5	803.6	995.2	1015.4	854.9	1056.9

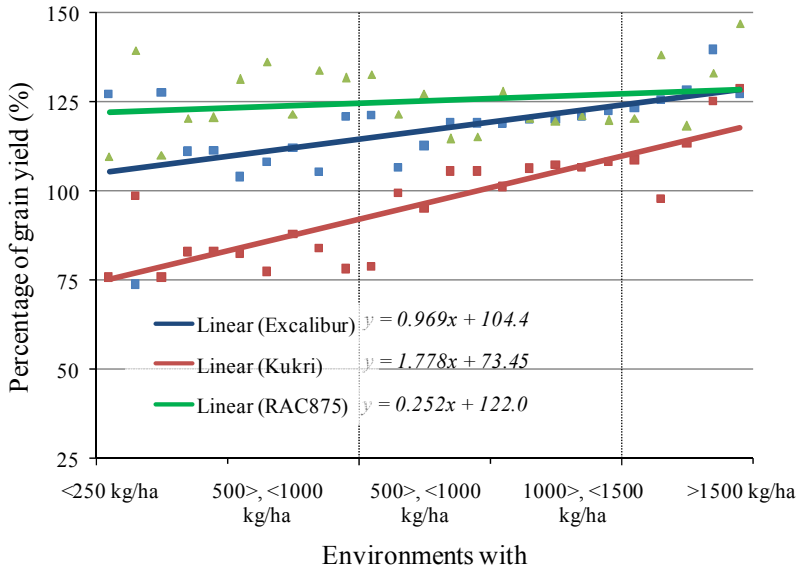

Figure 1-4. The percentage of grain yield production on average grain yield in different environments across South Australian wheatbelt.

CHAPTER 2

LITERATURE REVIEW

2 Chapter 2: Literature review

2.1 Introduction

Improving crop management and breeding techniques requires a detailed understanding of crop plants at various levels (organ, cell and gene), their environment and the interaction between these two (termed the G × E interaction). Numerous studies have been conducted to understand different aspects of plant responses by using physiological, genetic and genomics approaches in drought-stressed environments. Drought stress is a complex event, and the physiological traits and genetic mechanisms underlying drought tolerance are notoriously complex (Reynolds et al. 2005).

It is beyond the scope of this chapter to review different aspects of plant responses to drought. Rather, it intends to review the relevant literature providing background knowledge on wheat and physiology of drought tolerance as well as related terminologies. This chapter also serves to introduce the basic principles on genetic and Quantitative Trait Loci (QTL) analysis. This review first covers wheat as a staple food for much of the world's population, and it discusses the nature and genetics of wheat. The other part of the review covers drought as a main constraint limiting crop production in the world and also mechanisms of drought tolerance. It also briefly reviews screening techniques for developing varieties using traits which are supposedly good criteria for the selection of drought resistant plants. The final section, reviews the application of molecular markers in breeding programs, linkage map construction and also identification of QTLs for traits of interest.

2.2 Wheat

Bread wheat (*Triticum aestivum* L.) is a cereal and belongs to the grass family (Class *Liliopsida*, family *Poaceae*). It belongs to the tribe *Triticeae* (Briggle and Reitz, 1963). Bread wheat is the most prominent member of the tribe. It is a cultivated wheat species which is mainly grown for grain as human food source. The greatest portion of wheat production is used for bread making (Peña, 2002).

2.2.1 Importance of wheat

Wheat is the most common food for many people in the world, especially in developing countries (Curtis, 2002). It is grown on more land area than any other commercial crop and continues to be the most important food source for humans. Its production is second only to maize and greater than for any other cereal crops including rice and barley (FAOSTAT, 2006). The estimated area sown to wheat in 2006 was over 200 million hectares with average yield production of 2.8 t/ha (~600 million ton), which is about 27% of total cereal production globally (FAOSTAT, 2006). In terms of human consumption, wheat is the most important crop after rice. Wheat grain contains all essential nutrients; including carbohydrates (60-80% mainly as starch), proteins (8-17%) containing adequate amounts of all essential amino acids (except lysine, tryptophan and methionine), fats (1.5-2%), minerals (1.5-2%), vitamins (such as B complex, vitamin E) and 2.2% crude fibers (Peña, 2002).

The demand for wheat is predicted to grow faster than for any other major crop. The world population is expected to reach about 9 billion by the end of the 21st century and it has been predicted that the demand for cereals, especially wheat, will increase by approximately 50% by 2030 (Borlaug and Dowswell, 2003). The demand in developing countries is expected to raise even further (FAOSTAT, 2006). Three main ways have been suggested to maintain an adequate supply of food for future generations; firstly, expanding the land area, secondly, improving water availability and management and thirdly, improve yield stability through applying biotechnology in plant breeding. The potential for expansion in agricultural land area is limited and it is estimated that 85% of the increases in global food production must come from agricultural land that is already in use (Borlaug and Dowswell, 2003).

2.2.2 History and evolution

Cultivation of wheat began around 8000 BC along the Fertile Crescent. The domestication of wheat involved multiple polyploidization events between several species of the *Triticum* and *Aegilops* genera. Wheat is separated into three groups. Diploids (n=x=7) with 14, tetraploids (n=2x=14) with 28 and the hexaploids (n=3x=21) with 42 chromosomes. The wild and cultivated wheats can be classified based on their genomic constitution AA, AABB, or AABBDD into three species; *T. urartu*, *T.turgidum*, and *T. aestivum*. Hexaploid bread wheat (*T. aestivum*, AABBDD)

originated from three different wild wheat species. Genetic studies have revealed that, *T. urartu* was the donor of the 'AA' genome to tetraploid wheat, *T. turgidum* (AABB), and that *Ae. tauschii* was the donor of the 'DD' genome to modern bread wheat (*T. aestivum)*. The origin of the 'BB' genome remains controversial. It has been suggested that an ancestor species, *Aegilops speltoides* ssp. *speltoides,* was the donor of what became the 'BB' genome of the bread and durum wheats (Kimber and Sears, 1987; Huang et al., 2002).

2.2.3 Distribution

Wheat is cultivated over a wide range of climatic conditions all over the world, from the northern latitudes of Canada and China to the southern regions of South America and Australia. Wheat is adapted to a broad range of climatic conditions and soil fertility, and is mainly grown in rainfed areas of the world. It is best adapted to temperate regions with rainfall from 250 to 1750 mm (Curtis, 2002). In Australia, wheats are grown over a latitude range from tropical (22ºS) to temperate (30ºS to 38ºS), where rainfall distribution varies extensively over the wheat growing areas. Most areas receive less than 250 mm of rainfall during the growing season (O'Brien et al., 2001). Bread wheat is classified into two major "winter" and "spring" types, with the severity of the winter determining whether a winter or spring type is cultivated. Winter wheat is always sown in autumn. However, spring wheat is generally sown in the spring but can be sown in autumn where the winter is mild. Spring type wheat can grow quickly, moving through the vegetative stages to reproductive stages without cold treatments. In contrast, winter wheat has been selected to take advantage of a longer vegetative stage, when plant development is treated by the cooler temperatures (vernalization requirement) and shorter day length of winter. It grows slowly through the winter, is induced to flower by a period of cold and then flowers in the increasing day-length of spring. Most Australian wheats are spring types which are essentially insensitive or very slightly sensitive to vernalization. In most growing areas, wheat is sown from late autumn to mid winter (from late March to early April through to mid-July).

2.2.4 Wheat morphology and growth

An understanding of wheat growth and development is essential to achieve optimum productivity by matching management decisions (weed control) and inputs (fertilizer

application) with plant development. Also, the impact of biotic (diseases and insects) and abiotic stresses (frost, heat and drought) can be more accurately predicted with a clear picture of the relationships between growth stage and plant response to stress (McMaster et al., 2005).

Wheat is an annual grass with determinate flowering habit. Its ear or spike comprises several spikelets which alternate on opposite sides of the rachis. Within each spikelet, there are usually two to four potentially fertile florets (Kirby, 1974). Figure 2-1 shows a schematic diagram of the developmental progress of wheat. There are three major stages: the vegetative stage, the period after emergence when the crop grows vegetatively before the onset of floral initiation; the reproductive stage, when floret development occurs until the number of fertile florets is determined; and the grain-filling stage, when the grain first develops the endosperm cells and then grows to determine the final grain weight (Miralles and Slafer, 1999). The time-span of each development phase depends upon genotype, temperature, day-length and sowing date (Stapper and Fischer, 1990).

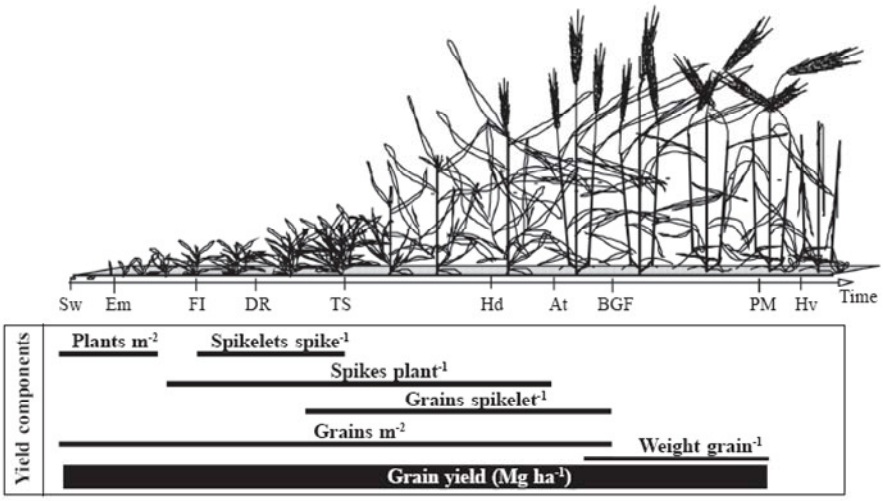

Figure 2-1. Schematic diagram of wheat growth and development stages from sowing to maturity and hravest. Sw: sowing, Em: emergence, DR: initiation of double ridge, TS: terminal spikelet initiation, Hd: heading time, At: anthesis, BGF: beginning of grain filling, PM: physiological maturity and Hv: harvest. (After Slafer 2003).

Grain yield is the final outcome of crop growth and development processes occurring throughout the growing season from sowing to harvest. Throughout the plant lifecycle many genes influence grain yield to some extent, including those genes that directly control yield potential and also those genes that are conferring adaptation through

phenological adjustment or resistance to biotic and abiotic stresses (Slafer, 2003). Grain yield (GY) in wheat is considered the product of two main yield components including the number of grain per unit land area (G/m^2) and the average individual grain weight (GW). Grain number *per se* can be broken down into its components; the number of plants per unit land area, spikes per plant, spikelets per spike and grains per spikelet (Miralles and Slafer, 1999; Slafer, 2003). Spikes per square meter is reported as the primary determinant of grain yield, followed by grains per spike, with grain weight of only minor importance (Fageria et al., 2006).

$GY = G/m^2$ ($Plant/m^2 \times Spike/Plant \times Spikelet/Spike \times Grain/Spikelet$) $\times GW$

Therefore, by studying how these yield components vary within a particular genetic background, it is possible to gain an insight into the possible function of a gene that influences yield and when, as well as how, it is likely to exert its effect. Although, these yield components are usually negatively interrelated (Slafer, 2003), by understanding the nature and genetic effects of these negative relationships, it might be possible to manipulate them in order to improve grain yield under stressed and non-stressed conditions.

2.2.4.1 Critical growth stages

Successful adaptation of a crop is largely dependent upon better adjustment of critical crop growth stages in favorable weather periods during the growing season. However, various adaptive mechanisms have also been adopted by plants that allow them to optimize growth and development while encountering environmental stresses, and eventually resulting in grain yield production (McMaster et al., 2005).

The impact of each yield component on final grain yield is determined at different stages during the growing season. Environmental conditions experienced during these developmental stages determine the magnitude of the loss in potential grain yield. Before anthesis the number of grains is determined and after anthesis the grains are filled and the individual grain weight is established. The number of grains per unit land area are formed throughout the whole pre-anthesis period, only a relatively small fraction of that period is critical, namely the period during stem elongation between terminal spikelet initiation and anthesis (Miralles and Slafer, 1999; Slafer, 2003).

Environmental stresses after terminal spikelet formation increase the rate of tiller mortality. Environmental stress prior to flag leaf appearance can result in a loss of spikelets on the developing head. Floret initiation starts in the lower central region and progresses toward the base and tip of the head. Under extreme environmental stress, all of the florets in the spikelets at the top and bottom of the head may abort prior to flowering (Edmeades et al., 1989). The final adjustments in yield production are made during the grain-filling period when grain size is determined. Generally, flowering time is the most critical period for yield determination in cereals. Drought at flowering can cause substantial yield losses through reduction in grain per spikes, and if severe can impair the development of reproductive tissues (Edmeades et al., 1989).

2.2.4.2 Flowering and maturity

Flowering time is a key adaptive trait in both crop and wild cereal species that ensures that plants set their flowers at an optimum time for pollination, seed development, and dispersal (Cockram et al., 2007). Flowering time has been particularly important for yield improvement in water-limited environments such as Australia (Siddique et al., 1989; Richards, 1991). In these environments, flowering must not only be early enough to avoid the detrimental effects of declining soil moisture and increasing temperatures, but also late enough to avoid frost.

The flowering of higher plants is a complex biological process and is regulated by both environmental and developmental factors representing a complex network of interactions between numerous genes responsible for regulating this important trait (Yong et al., 2003; Dubcovsky et al., 2007). Flowering time in wheat is determined predominantly by three sets of genes: vernalization responsive genes (*Vrn*), photoperiod responsive genes (*Ppd*) and earliness *per se* (*Eps*) genes. The *Vrn* genes regulate the requirement of a long exposure to cold temperatures to induce flowering whereas the *Ppd* genes regulate flowering time in response to day length, and *Eps* genes regulate flowering independently of environmental signals and are usually responsible for the fine-tuning of wheat flowering time (Worland, 1996; Worland et al., 1998a; Snape et al., 2001a; Bullrich et al., 2002; Shindo et al., 2002; Shindo et al., 2003). These three sets of genes influence flowering time together to determine the exact time of flowering and hence the suitability of a genotype for flowering under particular environmental conditions (Snape et al., 2001b; Dubcovsky et al., 2006).

Genetic analysis suggests that a relatively small number of conserved genes control the vernalization response (reviewed by Snape et al., 2001a; Cockram et al., 2007). The major gene for vernalization insensitive *VRN 1* and *VRN 2* which are the central genes in the vernalization pathway in wheat, barley, and other temperate cereals . *VRN1* is dominant for spring growth habit, whereas *VRN2* is dominant for winter growth habit (Yan et al., 2004). The *VRN1* is located on group 5 chromosomes in wheat (*Vrn-A1*, *Vrn-B1* and *Vrn-D1* on chromosome 5A, 5B and 5D, respectively) and most of the variation in vernalization requirement is controlled by this locus (Law and Worland, 1997; Snape et al., 2001a; Yan et al., 2004). The *VRN 1* gene has already been cloned by Yan et al. (2003). The *VRN2* gene was mapped on chromosome 5A with more than 50 cM distal to *VRN1*. These two genes have strong epistatic interactions and are likely part of the same regulatory pathway (Tranquilli and Dubcovsky, 2000). *VRN 3* (*VRN-B3* or *TaFT*) , which was previously called *VRN5* or *VRN-B4*, was also mapped on the short arm of chromosome 7B in wheat and chromosome 7HS in barley which are orthologous each other (Yan et al., 2006). *VRN3* is also an orthologue of the *FT* gene in *Arabidopsis*. The presence of *FT* gene on the short arm of the group 7 chromosomes in wheat was confirmed by Bonnin et al. (2008).

Wheat is a long-day plant and photoperiodic insensitivity is controlled by the dominant *Ppd* genes which greatly reduce sensitivity to photoperiod and confer an early flowering phenotype in short-day and long-day conditions (Welsh et al., 1973; Law et al., 1978). Photoperiod sensitivity is controlled primarily by a homoeologous series of genes *Ppd-D1* (formerly *Ppd1*), *Ppd-B1* (formerly *Ppd2*), and *Ppd-A1* (formerly *Ppd3*) located on chromosome 2D, 2B and 2A, respectively. These genes are ranked *Ppd-D1>Ppd-B1>Ppd-A1* in terms of their potency (reviewed by Worland et al., 1998; Beales et al., 2007). Comparative mapping between barley and wheat showed that the *Ppd* position on chromosome 2H in barley is homoeologous to the hexaploid wheat *Ppd* gene series on chromosome 2 (Laurie et al., 1995; Laurie, 1997; Laurie et al., 2004).

Earliness *per se* (*Eps*) is an adaptive trait that promotes flowering independently of environmental signals (Worland, 1996). The presence of earliness *per se* factors has been reported on several chromosomes of wheat (Shindo et al., 2003; Hanocq et al., 2004; Hanocq et al., 2007). By QTL analysis, Hanocq et al. (2007) showed that all chromosome groups are involved in the genetic control of earliness in bread wheat. They concluded that, not only the major genes of the *Ppd* and *Vrn* families, but also

other genome regions in groups 4 and 7 with lesser effects are influencing earliness and its components.

2.2.4.3 Plant height

Plant height is one of the most important agronomical traits in wheat cultivars. The relative yield advantage of dwarf and semi-dwarf cultivars varies with spring or winter habit, genetic background, and environmental conditions (Richards, 1992a). An important consequence of the presence of the dwarfing genes is the increase in harvest index. This is due to the production of more grain numbers, which, despite reduced grain size, results in a higher overall yield (Taiz and Zeiger, 2006). Previous genetic studies indicated that the genetic control of plant height in wheat is complex and it is controlled by a set of dwarfing genes known as reduced height (*Rht*) genes located on different chromosomes (Ellis et al., 2005). Dwarfing genes can be classified into two groups depending on the reaction to the exogenous gibberellic acid (GA) which is necessary for stem elongation (Hedden, 2003). Two genes in particular, *Rht-B1b* and *Rht-D1b* which are used in many commercial wheat varieties, are GA-insensitivity genes, while *Rht8* and *Rht9* are, GA-sensitive genes (Rebetzke and Richards, 2000). The *Rht-B1b* and *Rht-D1b* genes were mapped on homoeologous loci chromosomes 4B and 4D, respectively (Börner et al., 1997), and they were isolated from wheat (Peng et al., 1999). The *Rht-B1b* and *Rht-D1b* genes in wheat and dwarf-8 (*d8*) in maize are orthologues of the *Arabidopsis* gibberellin insensitive (*GAI*) genes which encode proteins that resemble nuclear transcription factors and contain an SH2-like domain, indicating that phosphotyrosine may participate in gibberellin signaling (Peng et al., 1999).

2.3 Drought and drought tolerance

2.3.1 Definition

There is no specific definition of drought and it can be considered from different points of view depending on diverse practitioners from meteorologists, agronomists, plant physiologists to molecular biologists and biochemists (Passioura, 2002: 2007). A meteorological drought is defined as a period of time when the amount of rainfall is less

than the long-term mean and long enough to cause moisture depletion in soil, water deficit in the plant and a decrease in the water potential of plant tissues. An agricultural drought on the other hand, is defined in terms of seasonal vegetation development. Its functional definition would be the shortage of water supply, including precipitation and soil-moisture storage capacity in quantity and distribution during the life cycle of a crop plant which restricts the expression of the full genetic potential of the plant (Swindale and Bidinger, 1981).

Nevertheless, drought is not a stable phenomenon and available soil water changes with time within a season. It often starts as mild stress and may become severe with time and/or last for a long period. In some cases, drought develops early during the vegetative stage, while in other cases it develops later, towards crop maturity (Fukai et al., 1999). The drought patterns are different between locations and years and it often does not develop at all during the growth season in areas with favorable rainfall (Fukai et al., 1999). Differences in seasonal distribution of rainfall and severity of water limitation also vary substantially from season to season for a given location. Fukai et al. (1999) pointed out that various patterns of drought have different effects on yield among genotypes; and that cultivars which have adapted to one type of drought are not necessarily tolerant to other types of drought. Therefore, identification of common drought patterns and their likelihood of occurrence are important for the development of cultivars that are suited for the region concerned. The nature of drought is complex and it can be considered as a set of climatic pressures which can be produced by several phenomena such as: heat shock, water deficiency, low air humidity, high irradiance and salinity. It also interacts with soil type. Different combinations of these phenomena cause different types of drought, each of which has led to the selection of numerous types of resistance mechanisms at different levels of organization (molecule, cell, organ and whole plant). The study of these mechanisms can provide important information for crop breeding programs (Monneveux and Belhassen, 1996).

2.3.2 The impact of drought on crop production

Drought seriously threatens the food security of people in developing countries. It is generally accepted as a major abiotic stress on crop plants and is increasingly becoming a severe problem in many regions of the world. Drought, with all its symptoms, has been with people for centuries and it is forecasted to become more serious. This is not

only because of changes in global weather patterns, but also because of the anthropogenic overuse of resources (McWilliam, 1989). Trethowan and Pfeiffer (1999) mentioned that up to approximately 70% of the area sown to wheat in developed countries and half in developing countries suffer from periodic drought, which can occur at any time during the cropping cycle in all rainfed environments. They added that terminal or post-flowering stress is typical of many Mediterranean-type environments, including North Africa, West Asia, southern Australia and southern Africa.

Due to the frequent occurrence of drought, the crop yields are limited mainly by water in most years in many environments. The challenge facing farmers in general and plant breeders in particular is to increase water productivity by producing more yield from the given limiting water supply (Passioura, 2007). Improvement of productivity of wheat under drought conditions becomes one of the most important breeding objectives. Breeding for wheat cultivars better adapted to drought is a major challenge in arid and semi-arid regions of the world due to inadequate precipitation, shortage of irrigation water and high water demand for crop evapo-transpiration in such climates.

2.3.3 Drought resistance or tolerance

In general, drought resistance 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). Turner (1979) emphasized that crop plants not only should have the ability to survive under drought but also the ability to produce a harvestable yield. Hence, drought resistance in agriculture could be defined as the ability of a crop plant to produce its economic product with minimum loss in a water-deficit environment, relative to well watered conditions. Blum (1999) mentioned that drought resistance could be considered in terms of plant water relations and plant function when the plant desiccates, as well as in terms of various traits which affect plant performance under stress, such as plant developmental plasticity and plant phenology (Blum, 1996). McWilliam (1989) pointed out that drought resistance is not a simple response of the plant to drought conditions and it is conditioned by many components. Therefore, understanding the genetic basis of drought resistance in crop plants is a pre-requisite for developing superior genotypes through conventional breeding methodology or using a biotechnological approach. Passioura (2002) argued that ‘drought resistance’ and ‘drought tolerance’ are terms that are related to survival rather than of production. They

are, thus, more relevant to natural than to agricultural environments. He pointed out that severe water deficits are rare in viable agriculture, and asking how crops respond to or survive them is unlikely to have much general impact. Passioura (2006) provided a term of water productivity or "more crop per drop". Passioura pointed out that this term can be quantified, with units of amount of crop yield per volume of water supplied or used, for example kg ha^{-1} mm^{-1}. However, we use 'drought tolerance' hereafter since it has been used frequently in the literature.

2.3.3.1 Mechanisms of drought tolerance

According to Levitt (1980), resistance to drought or water deficit stress in plants can be achieved by two main resistance mechanisms: drought escape and drought tolerance (Fig. 2-2).

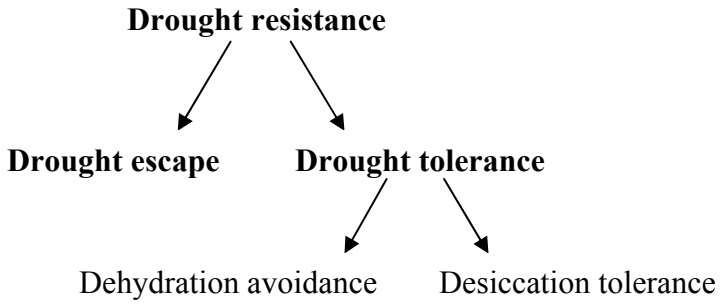

Figure 2-2. Classification of drought resistance based on Levitt's (1980) terminology (after McWilliam, 1989)

Drought escape is the ability of a plant to complete its life cycle before the onset of severe water deficit develops. This mechanism is deployed by rapid phenological development, developmental plasticity and remobilization of pre-anthesis assimilates to grain (Turner, 1979). Escape strategies rely on successful reproduction before the onset of severe stress (Chaves et al., 2003). Drought escape through early flowering is advantageous in environments with terminal drought stress and where physical or chemical barriers inhibited root growth (Turner, 1986; Blum, 1988; Bidinger and Witcombe, 1989). Breeding for shortening the crop duration has been a very successful strategy in Mediterranean conditions (Araus et al., 2002). However, early flowering

increases the risk of frost damage and in better-than-average seasons, determinate crops such as wheat and barley have little potential to take advantage of late rain. This has led to the search for cultivars that can tolerate the drought and still produce an economic yield (Turner, 2003).

Drought tolerance involves dehydration avoidance and desiccation tolerance. *Dehydration avoidance* is the ability of a plant to maintain high tissue water potential during the water stress period (Blum, 1988). Thus, it is affected either by maintaining water uptake via increased rooting and increased hydraulic conductance, or by reducing water loss via a reduction in epidermal conductance, reduction in absorbed radiation and reduction in evaporative surface.

Desiccation tolerance is the ability of a plant to endure water deficit as measured by the degree and duration of low plant water potential, and it usually involves maintenance of turgor via accumulation of solutes and increase in cell wall elasticity (Jones et al., 1981; Jones, 2004). Plants possessing these mechanisms have the ability to continue metabolizing at low leaf water potential and to maintain growth despite dehydration of the tissue or to recover efficiently after release from stress conditions (Blum, 1988).

Jones (1992) developed another classification of drought resistance for crop plants. It was slightly different from Levitt's classification, which was developed for plants in natural ecosystems. Jones used the term 'drought tolerance' instead of 'drought resistance' in his classification and suggested that drought resistance refers to the ability of plants to survive drought, but drought tolerance describes all mechanisms that tend to maintain plant survival or productivity under drought conditions.

There are genetic variations within crop species which allow plants to cope with water deficit stress under drought conditions. Blum (1999) suggested that there are three major physiological processes which allow plants to resist drought:

1) The maintenance of a high plant water status.
2) The maintenance of plant functions at low plant water status.
3) The recovery of plant water status and plant function after stress.

Drought resistance is commonly achieved by a combination of mechanisms, and different plants exhibit different strategies for survival or growth under drought conditions (Jones, 2004). Each of these mechanisms includes several traits that can be

manipulated by plant breeders, bearing in mind that not all traits are appropriate for all drought environments.

Improving drought resistance is probably one of the most difficult tasks for plant breeders. The difficulty comes from the diversity and the unpredictability of drought conditions in the field, and also from the diversity of drought resistant strategies adopted by the plants. Consequently, crop adaptation must reflect a balance between escape, avoidance and tolerance, while maintaining adequate productivity (Mitra, 2001). Richards (2004) suggested that enhanced yields in dry environments can be achieved through different mechanisms. However, as grain yield is the economic yield in most agricultural crops (like cereals), mechanisms that maintain productivity and increase reproductive efficiency under drought conditions will be important. Thus, a drought tolerant cultivar is one which gives a significantly higher yield than average under conditions where yield is limited by water availability (Quarrie and Stoja, 1999). Therefore, any other traits to be considered must be directly related to grain yield. Although much research has been done to try to increase the understanding of plant responses to drought, and probe the genetic variation in these responses, Richards (2004) reported that there are only a few examples where this research has led to improved varieties. He suggested several reasons for this:

1) Traits that contribute to plant survival, rather than maintenance of yield, in dry conditions have been considered;
2) The focus of the research has often been on isolated plants and not on the population of plants that form a crop; and
3) Physiological traits for water-limited environments are unlikely to be universal and some will be important in one region but unfavorable in another.

Therefore, considering traits which are related to productivity would probably improve drought resistant plants in target environment (Richards, 2004).

2.3.3.2 Breeding for drought tolerance

Breeding for improved drought resistance has emerged through four basic approaches. The first approach was to breed for high yield under optimum conditions. It was assumed that this would provide a yield advantage under suboptimal conditions. This approach was debated since there are G × E interactions which may limit the high

performance of genotypes under drought conditions. Blum (1988) concluded that the long range solution to yield improvement under stress cannot be sustained by improving yield potential alone. The second approach was to breed for maximum yield in the target environment (actual drought conditions) (Bidinger and Witcombe, 1989). This approach had some limitations. Drought in water-limited environments is remarkably variable from year to year and the expression of variability for yield and its components in these environments is also low (lack of yield stability). Therefore, selected cultivars should be able not only to produce outstanding yield under water-limited conditions but also have a potential to increase yield in cases when rainfall is adequate (Rosielle and Hamblin, 1981). The third approach was simultaneous selection for yield in a non-stress environment and under drought conditions, for yield stability may achieve the desired goal of creating drought-resistant genotypes with high yield (reviewed by Mitra, 2001). This approach involved the development of cultivars for water-limited environments through selection and incorporation of physiological and morphological mechanisms for drought resistance through traditional breeding programs, and to establish a drought-tolerance character which will benefit yield under water-limited conditions and then to incorporate it into the existing breeding program (Richards, 1989; Richards et al., 2001; Richards et al., 2002; Reynolds et al., 2005). The fourth and most recent approach is the multidisciplinary approach (holistic approach), achieved by integrating the physiological dissection of crop drought avoidance and tolerance traits, using various genetic and genomics tools, such as marker assisted selection, microarrays and transgenic crops, with agronomic practices that lead to better conservation and utilization of soil moisture and better matching of crop genotype with the target environment. In this approach, many factors can be considered, such as: the complexity of the genome, the nature and number of molecular markers available, the complexity of the traits to be improved, the number of plants that can be screened at each selection step, and the number of populations that can be concurrently manipulated (Serraj et al., 2005).

2.3.3.2.1 Direct selection (yield-based approach)

In conventional plant breeding, direct selection for grain yield has been used for screening drought tolerance. However, this approach is slow due to the low heritability of yield and high G × E interactions (Jackson et al., 1996; Quarrie et al., 1999; Richards,

2004). Bruce et al. (2002) pointed out that since grain yield is a complex trait for selection, increased understanding of the components that contribute to higher yields should improve the selection process and could overcome the low response to a direct selection for yield under drought conditions. Therefore, selection for other traits, which are thought to be associated with good drought tolerance and less influenced by environment than yield, might be a useful strategy for screening drought tolerant varieties (Quarrie et al., 1999). Since drought tolerance is the result of a combination of different morphological, physiological and biochemical traits, these different components could be used as selection criteria for screening plants for drought resistance. A suitable trait for screening drought tolerant plants would be:

1) Genetically associated with grain yield under drought
2) Highly heritable
3) Easy to measure and identify prior to flowering; and
4) Have a proven role in drought resistance and yield stability (Austin, 1989; Edmeades et al., 1989; Richards, 2004).

Moreover, evaluation of these traits must be rapid, easy and cheap (Araus et al., 2001, 2002). They should also allow the screening of a large population (Mitra, 2001).

2.3.3.2.2 Indirect selection (trait-based approach)

There are different approaches for identifying potential secondary traits. The most promising work in drought tolerance has occurred when physiologists and breeders have collaborated to test the validity of selecting for physiological or morphological traits in regular breeding programs (McWilliam, 1989). Araus et al. (2002) proposed that an indirect approach based on an understanding of the crop at the physiological and molecular biological levels may complement a conventional breeding program and accelerate yield improvement. In addition, they emphasized that even using molecular biological techniques for locating gene sequences, introgressing quantitative trait loci (QTL), selecting or genetically transforming economically important QTLs is strongly dependent on our understanding of yield-determining physiological processes. Therefore, by combining information on the physiological basis of yield limitation with new physiological selection tools, the probability of accelerating the rate of the genetic progress through plant breeding could be significantly increased (Richards, 1989; Reynolds et al., 1998; Richards et al., 2002; Richards, 2004; Reynolds et al., 2005).

Passioura (1996) proposed that when water is limited, then grain yield is a function of:

1) The amount of water used by the crop relative to the amount of total water supply;
2) The efficiency of the use of this water for biomass growth, i.e. the water-use efficiency (WUE); and
3) The efficiency of the conversion of the biomass into grain, i.e. the harvest index (HI).

He has formulated these factors as follow:

$$Y = T \times WUE \times HI \qquad (2\text{-}1)$$

Where Y = yield; T = fraction of available water used by the crop; WUE = water use efficiency; HI = harvest index.

Richards (1996) reported that, through using this model, it is possible to assess whether a particular trait was likely to increase any one of the components and thus increase yield under drought. Richards (2002) poited out that these three components are likely independent of each other. Thus, an improvement in any one of them could result in an increase in yield. Condon et al. (2004) suggested that crop yield could be constructed from components in the following equation:

$$Y = ET \times T / ET \times DM / T \times HI \qquad (2\text{-}2)$$

Where Y = yield; ET = transpiration efficiency; T = fraction of available water used by the crop; DM = dry matter production; HI = harvest index.

In this framework, yield is a function of:

1) The amount of water used by the crop (evapo-transpiration, ET);
2) The ratio of that water actually transpired by the crop plants (T/ET);
3) The transpiration efficiency of dry biomass production (DM/T); and
4) How effectively the achieved biomass is partitioned into the harvested product, i.e. the harvest index (HI).

Condon et al. (2004) argued that the components involved in this yield framework are not independent of each other, but each of them could, however, be considered as a target for genetic improvement.

2.3.3.3 Primary components of secondary traits

2.3.3.3.1 Evapo-transpiration (ET)

Evapo-transpiration is the term that describes the combination of evaporation (E) from soil and non-stomatal plant surfaces, and transpiration (T) from plant stomata. Improving water use is related to water uptake by the plant and reducing evaporation from the soil surface. Root traits, in this case, could maximize water extraction from soil. Richards et al. (2002) pointed out that a deep root system is associated with more water uptake from the soil and better performance under drought. However, measuring these traits is difficult. It has been suggested that the simplest way to increase rooting depth and root distribution of crops is to increase the duration of the vegetative period. This may be achieved by earlier sowing or by use of later flowering genotypes, if this is feasible. Increased early vigour may also result in both faster growth of roots which enables plants to exploit deeper soil layers, and production of more adventitious roots in the top-soil (Richards et al., 2001; 2006). Greater osmotic adjustment has also been suggested may result in more root growth and an increased ability to extract additional soil water (Morgan and Condon, 1986; Chaves et al., 2003). However, water uptake by roots may not only be limited by the genetic potential for growing deeper roots but also by soil limiting factors such as the presence of root pathogens (nematodes and root diseases), mineral deficiencies (Zn and P) or toxicities (salt, Al and B) or soil physical barriers. All of these can reduce water use, nutrient uptake and yield. Araus et al. (2002) pointed out that water use is relevant when there is still soil water available at maturity or when deep rooted genotypes can access water deep in the soil profile.

2.3.3.3.2 T/ET

The amount of water available for plant transpiration could be increased by reducing soil surface evaporation. It may be achieved by rapid canopy growth which shades the soil surface to reduce evaporation as well as to increase competition with weeds (Richards et al., 2002). Therefore, allocating soil evaporation into plant transpiration could be achieved by increasing early vigour or by adjusting phenology to match crop development and seasonal rainfall pattern (Araus, 2002; Richards et al., 2002).

In addition to better management practices to improve crop vigour (Richards and Lukacs, 2002), there are opportunities to increase vigour genetically. Richards et al. (2002) suggested that for increasing crop vigour, better seedling establishment needs to be considered, such as by finding the genetic potential for rapid early growth. Botwright et al. (2002) evaluated wheat genotypes in the field conditions for genetic improvement of early vigour and they concluded that there is potential to increase yield of wheat by selecting for greater early vigour in a wheat breeding program. To achieve better establishment and high yields, longer coleoptiles in combination with semi-dwarf stature have been suggested (Richards et al., 2002). In semi-dwarf wheats (that have gibberellic acid (GA)-insensitivity), coleoptile length and leaf area are often smaller during the early vegetative stage compared with the standard height wheats which are sensitive to GA. Short coleoptiles often result in poor emergence and therefore poor crop establishment (Rebetzke et al., 1999). Ellis et al. (2004) found a large variation in the reduction of coleoptile length between the semi-dwarf lines possessing different dwarfing genes (*Rht*). They concluded that none of GA-sensitive dwarfing genes (e.g. *Rht8*) had a major effect on coleoptile length or leaf growth, and suggested that since these *Rht* genes have effects later in development of the plant, there is an opportunity to maintaining dwarfing whilst selecting for early vigour, where fast early growth is important.

2.3.3.3.3 The ratio of dry matter to transpiration (DM/T)

Condon et al. (2004) pointed out that water use efficiency could be defined in many ways, depending on the scale of measurement and the units of exchange being considered. The agronomic WUE is the ratio of total dry matter production to evapo-transpiration, in other words, the amount of water being exchanged for the unit of production. From a physiological viewpoint, WUE could be defined as the amount of carbon assimilation through photosynthesis in exchange for water used in transpiration or net photosynthetic assimilation per unit water transpired - which is so-called ‘transpiration efficiency’ (TE). Transpiration efficiency is difficult to measure (Araus et al., 2003; Araus et al., 2004). There are many ways in which TE can be improved genetically (Richards et al., 2001; 2002). Selection for glaucousness and/or pubescence have been suggested, which increase surface reflectance to lower the surface temperature of photosynthetic tissue can increase TE in cereals (Richards et al., 1986).

However, measuring carbon isotope discrimination of plant material is the most promising method for improving TE. Two recently released commercial wheat varieties (Drysdale and Rees) have been bred for performance under water-limited environments using carbon isotope discrimination technique (Condon et al., 2006).

2.3.3.3.4 Harvest index (HI)

Harvest index is the proportion of the above-ground biomass allocated to grain. Richards (1991) pointed out that there are two separate determinants of HI for the genetic manipulation of HI in variable rainfed environments. The first determining factor is independent of drought. The second determining factor is drought-dependent and depends largely on water availability during grain filling. A high drought-independent HI has been achieved by a greater partitioning of dry matter to reproductive than to non-reproductive organs. Genes contributing to height reduction and to early flowering are simple and effective ways to increase HI. Both traits are highly heritable and both reduce the growth of vegetative organs. For genotypes with a high HI in drought-free conditions, the genetic improvement of the drought-dependent HI becomes important (Richards et al., 2001; 2002). Drought-dependent HI is often a function of post-anthesis water use. Richards and colleagues discussed some of the important traits, such as phenology (early vigour combined with early flowering), narrow xylem vessels in the seminal roots and remobilization of assimilates from stems and leaf sheaths to grain which may regulate leaf area, and thereby water use for increasing the drought-dependent HI in these conditions.

2.3.3.4 Underlying components of secondary traits – potentially useful traits for screening

2.3.3.4.1 Carbon isotope discrimination

Carbon isotope discrimination can provide an indirect measure of transpiration efficiency (TE). The theory of carbon isotope discrimination ($\Delta^{13}C$) was developed by Farquhar et al. (1982), and $\Delta^{13}C$ can provide an indirect, time-integrated estimate of internal carbon dioxide concentration (Farquhar et al., 1989). There are two stable isotopic forms of carbon (^{12}C and ^{13}C) throughout the biosphere. The most common

form is ^{12}C, which accounts for 98.9 % of atmospheric CO_2. Approximately 1.1 % of the carbon in the biosphere is in the form of the stable isotope ^{13}C. Plants discriminate against ^{13}C during the process of photosynthesis. As a result, plant dry matter contains relatively less ^{13}C than is found in the atmosphere. Variation in carbon isotope discrimination in C_3 species is greater compared with C_4 species. The association between carbon isotope discrimination and TE was well described by Condon et al. (2004; 2006). Genotypic variation in carbon isotope discrimination was found in wheat under dry environments (Condon et al., 1990; Condon and Richards, 1992; Condon et al., 1993). Farquhar and Richards (1984) suggested that $\Delta^{13}C$ could be usefully applied in breeding programs to modify TE of water-limited C3 plants. Carbon isotope discrimination has also been considered to be a good predictor of yield performance, particularly under stress conditions (Condon et al., 1987; Merah et al., 1999). Rebetzke et al.(2002) found high heritability of $\Delta^{13}C$ and its strong genetic correlation with aerial biomass and grain yield in bread wheat. They concluded that $\Delta^{13}C$ might be useful for indirect selection in a breeding program targeting increased aerial biomass and grain yield in water-limited environments, providing a rapid, cost-effective means of assessing relative differences in physiological water use efficiency (transpiration efficiency) (Condon et al., 2004).

Although measuring carbon isotope discrimination provides a powerful means of improving water-use efficiency of leaf gas exchange in C3 species, it is evident that improvements in leaf-level water-use efficiency may not always translate into higher crop water-use efficiency or yield (Condon et al., 2004). Condon et al (2004) pointed out that the variability in relationship between yield and $\Delta^{13}C$ can be related to the nature and extent of water limitation and the part of plant used for measuring $\Delta^{13}C$. They also concluded that in eastern and southern Australia with winter-dominant rainfall, breeding to improve TE does not result in higher average yield. In the northern region with summer-dominant rainfall pattern, in contrast, it appears to be more useful. The $\Delta^{13}C$ as an estimate of TE has been measured in C_3 cereals including bread wheat (Condon et al., 1987; Condon and Richards, 1992; Sayre et al., 1995), durum wheat (Merah et al., 1999; Merah et al., 2001), barley (Teulat et al., 2001b) and rice (Scartazza et al., 1998; Impa et al., 2005; Laza et al., 2006). It has allowed the compilation of relatively large databases on the genetic diversity for TE in relation to plant production in different environments (Richards, 1996). Most importantly, $\Delta^{13}C$ is easier and faster

to measure than WUE itself. Richards et al. (2002) concluded that selecting for $\Delta^{13}C$ has numerous advantages, namely that:

1) There is substantial genetic variation, the interactions between G × E are small and, therefore, heritability of $\Delta^{13}C$ is high; and
2) Its measurement is non-destructive and could be measured early in the plant's life.

2.3.3.4.2 Osmotic adjustment (OA)

Osmotic adjustment is defined as a decrease of the osmotic potential within cells, due to an active solute accumulation after water-potential reduction in response to water stress (Morgan, 1984; Blum, 1988). OA has been firmly associated with increased yield of cereal cultivars and germplasm under drought stress as seen in wheat (Morgan, 1980a; Blum and Pnuel, 1990; Blum et al., 1999), barley (Teulat et al., 1998), rice (Lilley and Ludlow, 1996; Babu et al., 1999) and sorghum (Wright et al., 1983). Morgan (1984) pointed out that OA could be considered as one of the crucial processes in plant adaptation to drought, as it can sustain tissue metabolic activity and enables re-growth upon re-watering, but OA varies greatly among genotypes. Genes or quantitative trait loci (QTLs) for OA have also been mapped in wheat (Morgan and Tan 1996), rice (Lilley et al., 1996; Robin et al., 2003) and barley (Teulat et al. 1998). Morgan and Tan (1996) mapped a osmoregulation gene (*or*) to the short arm of chromosome 7A in wheat.

Blum and Pnuel (1990) found an association between the OA capacity and yield stability under drought conditions during ear growth in wheat. They suggested that lower canopy temperatures may have partly depended on OA and concluded that if OA plays a role in sustaining leaf turgor and/or in allowing deep soil moisture extraction under drought stress (Morgan and Condon, 1986), this could be reflected in lower canopy temperatures. Teulat et al. (2001a) suggested that OA is a good physiological trait which can be considered in breeding for drought tolerance. Chaves et al. (2003) argued that OA may be critical to survival rather than to increase plant growth and crop yield under drought conditions.

Babu et al. (1999) investigated the advantages and disadvantages of four different methods for measuring OA variation in 12 rice genotypes. They concluded that

Morgan's regression method was the most comprehensive, but was time- and labour-intensive. Since, screening for osmotic adjustment requires complex measurement procedures and is time consuming as well as expensive, it is not suitable for screening a large number of genotypes (Fukai and Cooper, 1995; Zhang et al., 1999). However, the use of this trait in breeding for drought tolerance has been limited due to the nature of physiological measurements (osmometry, thermocouple psychrometry or pressure-chamber) which limit their practicality for screening large number of genotypes required in a breeding program.

2.3.3.4.3 Relative water content (RWC)

Plant water status could be measured with several major variables such as water potential (WP), osmotic adjustment (OA), turgor potential (TP) and relative water content (RWC). Although, OA is a powerful mechanism for conserving cellular hydration under water deficit stress, RWC expresses the effect of OA in this respect (Blum, 1999). Hence, RWC has been suggested as an appropriate estimate of plant water status in terms of cellular hydration under the possible effect of both leaf water potential and OA (Blum, 1999). Teulat et al. (2003) suggested that RWC is one of the traits linked to drought-tolerance which could be efficiently tracked by molecular markers. However, in the measurement of RWC adequate precautions need to be taken into account to ensure that the 'stress' treatments are truly comparable between test lines (Jones, 2007).

2.3.3.4.4 ABA concentrations

Abscisic acid (ABA) is known to affect many aspects of plant growth and development. ABA is also a stress-induced plant hormone and it has attracted much research attention as a potentially useful trait in selecting for drought tolerance in crops. ABA is generated mainly in the roots, where it stimulates growth. It passes to leaves, where it may affect on leaf rolling, stomatal closure and accelerates leaf senescence (Zhang et al., 1987). ABA is the root signal which helps the plant to reduce water loss.

Although, several studies have been conducted for screening plants for ABA concentration under drought conditions and QTLs for it have also been mapped in wheat (Quarrie et al., 1994), maize (Tuberosa et al., 1998) and rice (Quarrie et al., 1997), it is affected by genotype and the target environment (Mugo et al., 1999).

However, ABA production depends on the environment and stress occurrence, and the accumulation of it may enhance survival but reduce productivity (Leung and Giraudat, 1998; Mugo et al., 1999). Morgan (1980b) found a correlation between reduction in seed set due to water stress with an increase in the level of ABA and he assumed that water stress affects seed set through an increase in endogenous ABA. Saini and Aspinall, (1982) reported a four-fold increase in spikelet ABA concentration and a 50% reduction in grain set due to water stress. They suggested that the stages of development at or near meiosis were most susceptible to ABA, and concluded that pollen sterility induced by water deficit is mediated through endogenous ABA. Stress at the stage of meiosis reduced grain set in wheat, primarily through the induction of pollen sterility (Westgate et al., 1996; Saini and Westgate, 2000). However, using split roots to change ABA concentrations without decreasing water potential, Dembinska et al. (1992) found, in contrast to previous reports, that increased levels of ABA did not induce low grain numbers in wheat. A failure in the ability of the reproductive structures to supply necessary assimilate (sucrose) appears to be an important factor in grain abortion in maize and in the pollen sterility in wheat and rice (Saini and Westgate, 2000). Liu et al. (2005a) pointed out that ABA affects crops at early reproductive development by influencing both sink strength and assimilate partitioning from source to sink organs. ABA may affect crops at the early reproductive stage in three ways, individually or in combination. Firstly, ABA may inhibit cell division in the developing endosperm, resulting in a weak sink for assimilates. Secondly, ABA may induce earlier closure of stomata thereby decreasing photosynthesis and thus the rate of carbohydrate supply. Thirdly, ABA may disrupt carbohydrate metabolism within the ovaries by affecting carbohydrate-catalysing enzyme activity (see Liu et al., 2005a for review). However, measurement of ABA requires more time, labor, and facilities for sampling and assaying compared to traits like leaf rolling and leaf senescence.

2.3.3.4.5 Stomatal conductance

Stomatal conductance regulates CO_2 diffusion into and H_2O diffusion out of the leaf. Araus et al. (2003) pointed out that genotypes which are able to keep the stomata more open are those which are more productive. Fischer et al. (1998) found surprisingly high positive correlation between overall mean of stomatal conductance and mean yield in wheat. In wheat, stomatal conductance correlates with canopy temperature depression in

a wide range of climatic conditions (Amani et al., 1996; Lu et al., 1998). Araus et al. (2002) reported that high stomatal conductance in CIMMYT wheat cultivars was associated with cooler canopies and higher photosynthetic rates. Therefore, the hand-held infrared thermometer used for instantaneous measurement of canopy temperature could be also used for rapid, indirect determination of stomatal conductance (Fischer et al., 1998).

2.3.3.4.6 Canopy temperature depression (CTD)

A major function of transpiration in plants is leaf cooling. When plant water status is reduced and stomata close, leaf temperature rises due to the lack of transpirational cooling. Hence, canopy temperature can serve as an indirect indicator of plant transpiration and plant water status (Araus et al., 2003). Therefore, it has been suggested that selecting genotypes that maintain lower canopy temperature compared with other genotypes under the same conditions could be useful to improve drought tolerance of plants (Blum, 2004). Thus, cereal genotypes having lower canopy temperature at midday have relatively better water status and are assumed to be more drought resistant (Garrity and O'Toole, 1995; Araus et al., 2003). Canopy temperature has been shown to be negatively correlated with yield in wheat under drought stress (Blum, 1988; Olivares-Villegas et al., 2007) and hot, irrigated conditions (Reynolds et al., 2001). Olivares-Villegas et al. (2007) have recently reported lower canopy temperature as a highly heritable, drought-adaptive trait that significantly contributed to a higher crop performance under drought conditions. They assessed a recombinant inbred line population in different experiments in Mexico and under rainfed conditions in Australia. They suggested that lower canopy temperature as dehydration avoidance mechanism can be used as a selection criterion to identify high-yielding wheat genotypes or as an important predictor of yield performance under drought. However, under well-irrigated conditions, CTD showed a strong correlation with stomatal conductance and grain yield (Fischer et al. 1998).

2.3.3.4.7 Chlorophyll content

Leaf chlorophyll content can also be used as an indicator of a crop's potential photosynthetic capacity. Bolaños and Edmeades (1996) found a weak positive genetic correlation between leaf chlorophyll concentration and grain yield in maize under

different water-deficit stresses. Betran et al. (2003) pointed out that the correlation between chlorophyll content and grain yield might be related to greater radiation use efficiency in genotypes with high chlorophyll content. Zaharieva et al. (2001) reported that chlorophyll content was positively correlated with biomass and grain weight per plant in wild wheat (*Aegilops geniculata*). They also fund a positive correlation between leaf colour and chlorophyll content, and suggested that chlorophyll loss is the main factor responsible for change in leaf colour. Gutierrez-Rodriguez et al. (2004) found a strong association between chlorophyll content, photosynthetic rate and yield in wheat genotypes grown under well-irrigated and drought conditions. Therefore, the possible surrogate for photosynthetic capacity is leaf chlorophyll content, which could be measured using a hand held 'SPAD meter', such as supplied by Konica Minolta. Madhava et al. (2002) used a SPAD meter to measure the chlorophyll content in peanut. They found a significant positive relationship between transpiration efficiency and chlorophyll content. They suggested that the SPAD chlorophyll meter can be used as a rapid preliminary screening tool to select peanut genotypes with high transpiration efficiency.

Research in sorghum has showed the association between the stay-green phenotype and higher leaf chlorophyll content at all stages of development and both were associated with improved yield and transpiration efficiency under drought (Borrell et al., 2000). Stay-green is a fundamental strategy for increasing crop production, particularly under water-limited conditions. During post-anthesis drought, sorghum genotypes possessing the stay-green trait maintain more photosynthetically active leaves than genotypes not possessing the trait (Borrell et al., 2004). Richards et al. (2001) pointed out that stay-green capability might be a useful trait for environments where there is a high probability of rainfall during grain filling. Plants with stay-green characteristics and more photosynthetic tissue may further produce assimilations and extract water from soil. However, this trait may indicate the presence of drought avoidance mechanisms, but it probably does not contribute to yield if there is no water left in the soil profile by the end of the cycle to support leaf gas exchange. It may be even detrimental if it indicates lack of ability to remobilize stem reserves (Blum, 1998).

2.3.3.4.8 Chlorophyll fluorescence

Chlorophyll (Chl) fluorescence provides a fast and non-destructive tool in eco-physiological studies, and has extensively been used in assessing plant responses to environmental stress (Sayed, 2003). Baker and Rosenqvist (2004) pointed out that chl fluorescence can be a very sensitive probe of the physiological status of leaves, which can provide very rapid assessment of plant performance in a wide range of situations. Chl fluorescence techniques have successfully been used together with measurements of net CO_2 exchange and leaf water potential for rapid screening of barley genotypes for drought tolerance (Nogués et al., 1994). Araus et al. (1998) concluded that chl fluorescence measurements could be fast and useful during grain-filling in durum wheat to evaluate yield performance under Mediterranean conditions. Quenching modulated Chl fluorescence was used for screening wheat cultivars for water stress tolerance (Ali et al., 1994; Tambussi et al., 2002). In field-grown wheat cultivars growing under water stress, a high correlation existed between various Chl fluorescence indices and mean visual score for leaf vigour in the field during the anthesis-grain filling period (Balota and Lichtenthaler, 1999).

2.3.3.4.9 Stem water soluble carbohydrate (WSC)

Stem water soluble carbohydrate (WSC) is an important source of carbon for grain filling under any stresses which would inhibit current photosynthesis. Even under mild conditions, current assimilates may be limited for normal grain filling (Blum, 1998). This mechanism depends on the accumulation of reserves before flowering and the remobilization and transport of the reserves during grain filling. Reduction of assimilation during grain filling under different stresses may induce an increase in stem reserve mobilization and utilization by the grain. Blum (2002) pointed out that accumulation of WSC and remobilization of stored carbohydrates to grain are two independent processes, of which the accumulation of reserves before grain filling, despite its dependence on growing conditions before anthesis, and despite its dependence on growing conditions, is not stress responsive when stress occurs during grain filling. The capacity for high WSC accumulation in stems appears to be a genetically controlled (Blum et al., 1994; Ruuska et al., 2006; Yang et al., 2007). Yang et al. (2007) have identified several QTLs for accumulation and remobilization of WSC in wheat.

2.3.3.4.10 Leaf anatomy

Leaf waxiness, pubescence

Leaf waxiness or glaucousness, the waxy bloom on the surface of leaves and other plant parts, has been shown to be associated with grain yield in wheat in dryland field environments (Johnson et al., 1983). Johnson et al. (1983) showed that glaucous lines of wheat had increased yields under drought conditions over their non-glaucous isogenic pairs. The quantity of epicuticular waxes has showed an association with water loss through the cuticle and disease susceptibility (Clarke et al., 1994). Clarke and Richards (1988) concluded that increased epicuticular wax is associated with a large reduction in residual transpiration and glaucousness with a small reduction in residual transpiration.. In water-stressed plants, the effect of glaucousness could be greater due to its effect on reduction of leaf temperature (Richards et al., 1986), which would reduce both residual and stomatal water loss. Clarke et al. (1991) suggested that visual rating of germplasm collections under dry growing conditions for glaucousness would be an effective means of identifying genotypes worthy of further study. Clarke et al. (1993) pointed out that visual selection for glaucousness is thus likely to identify differences in approximate amounts of epicuticular wax, but the quantitative difference would have to be verified at advanced generations.

Leaf rolling and thickness

Leaf rolling is generally associated with a decline of plant water status (Courtois et al., 2000). Leaf rolling is a result of other avoidance mechanisms which result in high leaf water potential (Fukai and Cooper, 1995). It is an important trait for shedding radiant energy and is likely to result in cooler leaf temperatures, less transpiration, and lower respiratory losses. It may also be important for maximizing photosynthesis and transpiration efficiency by unrolling in the morning when the plant has a high leaf water potential and vapor pressure deficit is low, and rolling when conditions become more unfavorable (Richards et al., 2002). Champoux et al. (1995) conducted a QTL study in rice and found more than 45 QTL associated with leaf-rolling under field drought stress and root-morphology traits. Twelve of the 14 QTL associated with leaf rolling were also associated with root thickness, root/shoot ratio, or root dry weight per tiller. Courtois et al.(2000) found 11 QTLs for leaf rolling in rice in all experiments.

2.3.3.4.11 Root morphology

Roots are the main organs for plant water uptake. Despite the fact that root growth and development inevitably play a critical role in up taking water and nutrient supply to the rest of the plant, this hidden part of the plant is least studied and understood. Root development is involved in the response to many plant stresses, in particular, drought, disease and mineral deficiency, as well as toxicity. However, predicting the best rooting strategy for a particular crop will depend on the characteristics of the crop's demand for water during its life-cycle, soil depth and characteristics, as well as the seasonal rainfall. Nguyen et al. (1997) pointed out that root characteristics such as thickness, depth of rooting, root length density, root pulling force, and root penetration ability have been associated with drought avoidance in rice. Yoshida and Hasegawa (1982) used the ratio of deep-root weight to shoot weight as an index for drought resistance (avoidance) because large deep-rooted systems are able to extract more water and small shoots transpire less. Although, a deep root system contributes to maintaining good plant water status during a short stress, it can lead to faster soil water depletion which can damage plants in terminal stress situations (Ludlow and Muchow, 1990). Fukai and Cooper (1995) pointed out that a deep root system in rice with high root length density at depth was useful in extracting water thoroughly in upland conditions, but it was not appropriate for improving drought resistance in rainfed lowland rice, where the development of a hardpan may prevent deep root penetration. Price et al. (2002) argued that the contribution of deeper and thicker roots to drought resistance in rice depends on the environment in which the plants grow. Deeper roots may be less important in rainfed areas, where hardpans severely restrict root growth. Passioura (2002) pointed out that although active deep roots help reduce drainage losses, most Australian soils have hostile subsoils (saline, sodic, acidic, alkaline, high in boron, or too low in zinc and other nutrients) that are unfavorable to root growth. Passioura (2002) then pointed out that selection for deep rooting in such circumstances is well outside the scope of breeding programs for drought resistance. In general, techniques for measuring rooting depth are difficult and unsuitable for screening large numbers of breeding lines since most of the methods are destructive, elaborate and risky because of the possibility of uncontrollable losses during washing.

2.4 Quantitative Trait Loci (QTL) analysis

Plant breeders have made considerable progress in developing varieties with adaptation to water-stressed environments through pragmatic approaches, despite the lack of detailed understanding of the mechanisms underlying adaptation to water stress and the G × E interactions in the drought affected environments (Jefferies et al., 2007). The advent of molecular markers has enabled breeders to dissect quantitative traits into their single genetic components, and markers have facilitated the identification of numerous QTL controlling traits related to drought tolerance in wheat (Quarrie et al., 1994; Quarrie et al., 2006; Yue et al., 2006; Yang et al., 2007). Molecular markers have enhanced the genetic analysis of crop plants and have provided geneticists, physiologists, agronomists and breeders with valuable new tools to locate, identify and follow the numerous interacting genes that determine a complex trait in improving resistance to abiotic stresses. In QTL mapping analysis, two types of data are required: genotypic information (genotypic marker data) and phenotypic trait values. Thus, an accurate and effective QTL analysis, in addition to correct genotypic data, mostly relies on precise phenotyping methodologies.

2.4.1 Genetic linkage map

Linkage maps based on molecular markers are important tools in genetic analysis. A genetic linkage map can provide a more direct method to identify, map and measure the effects of desirable genes underlying quantitative traits via their linkage to easily detectable molecular markers (Tanksley et al., 1989). To develop a linkage map, a genetically stable population is needed. The first step in any QTL mapping is usually to construct a segregating population that originates from homozygous, inbred parental lines (Doerge, 2002). Most of the segregating populations are developed from crossing between two parental lines contrasting for the target trait(s). Among the different sorts of recombinant populations derived from variety crosses, doubled haploid (DH) and recombinant inbred lines (RILs) are more amenable to QTL mapping of traits and across different environments and years, because the same genotypes can be used in several experiments. In addition to the type of the segregating population, the size of the population has also to be carefully considered. Small populations will not allow precise gene characterization, and large populations will consume resources unnecessarily. To dissect a polygenic trait genetically, an RIL population of about 200 to 300 families is

considered suitable, but the number can be reduced if the trait is controlled by major genes (Ribaut et al., 2001).

2.4.1.1 Molecular markers

Molecular markers have been extensively used in the development of linkage maps in plants which can be used for various purposes such as identifying major genes, QTLs and map-based cloning. The molecular markers that have been used are largely referred to DNA-based markers that are derived from small regions of DNA which show sequence polymorphisms between individuals within a species. Numerous DNA-based genetic markers have been developed over the last three decades. In general, two major types of molecular marker systems are available: hybridization-based markers which rely on hybridization between a probe and homologous DNA segment within the genome, and PCR-based markers which depend upon PCR amplification reactions (reviewed by Kumar, 1999).

Restriction fragment length polymorphisms (RFLPs) marker system was one of the first techniques to have been used widely to detect variation at DNA sequence level (Botstein et al., 1980). RFLP markers have been found to be reliable and repeatable markers so that the same probe can usually be hybridized on different crop genomes. However, RFLP analysis requires large quantities of high quality DNA, and detection of RFLPs by Southern blot hybridization may be laborious and time-consuming, which makes this assay undesirable for plant breeding projects with high-throughput requirements (Kumar, 1999).

Diversity Arrays technology (DArT) have recently been used for genetic analysis in plants. This marker system is also based on the differential hybridization of cloned DNA to DNA fragments (AFLP-like genomic representations) in a sample of restriction enzyme digested DNA (Wenzl et al., 2004). DArT is based on microarray hybridizations that detect the presence versus absence of individual fragments in genomic representations (Jaccoud et al., 2001). It is essentially based on the Southern blot technique (a reverse Southern blot) where the probes are immobilized on a glass slide, and the DNA washed over the slide. Genetic marker analysis through DArT offers a low-cost high-throughput, robust system with minimal DNA sample requirement

capable of providing comprehensive genome coverage even in organisms without any DNA sequence information (Jaccoud et al., 2001).

Several types of PCR-based markers are being used in plant genome analysis, including random amplified polymorphic DNA (RAPDs), sequence-tagged sites (STSs), simple sequence repeats (SSRs) or microsatellites and amplified fragment length polymorphism (AFLP). Among the many different types of PCR-based DNA markers, SSRs are often preferred for use in plant breeding. SSRs are composed of short tandem repeats (1-6 bp) of DNA sequences, such as (AT)n, (GT)n, (ATT)n or (GACA)n, spread throughout eukaryotic genomes. These repeats are highly polymorphic, even among closely related cultivars, due to mutations causing variation in the number of repeating units (Lagercrantz et al., 1993).

The main advantages of SSRs are their reliability, co-dominance, specificity, abundance and uniform dispersal through plant genomes. Moreover, they are simple, cost-effective, robust and amenable to automation and also a small amount of DNA template is required; therefore, a large sample size can be handled efficiently. SSRs are particularly valuable in wheat mapping, as they showed a much higher level of polymorphism in hexaploid bread wheat than any other marker system (Röder et al., 1995; Chalmers et al., 2001; Gupta et al., 2002; Hayden et al., 2004).

2.4.1.2 Linkage map construction

A linkage map is a representation of the position of genetic markers within a linkage group. When a large number of markers are segregating simultaneously in a mapping population and these markers are to be placed on a linkage map, the first step is to group the markers into linkage groups. Generally, there are three steps in genetic linkage map construction: firstly, clustering markers into linkage groups; secondly, estimating pair-wise recombination frequencies in each of the linkage groups and finally optimizing the orders of markers in all linkage groups. The distances in a genetic map are determined according to the recombination fraction between two loci. The unit of measure is Morgans or centi Morgans (cM), representing the recombination frequency between the two locations. Genetic markers can be mapped relative to each other by determining recombination fractions using a mapping function.

2.4.1.2.1 Mapping function

A genetic mapping function describes the mathematical relation between recombination frequency (θ) and map distance (x). Different mapping functions can be used to relate recombination frequencies with the genetic distance between loci based on the assumption of the crossover interference. Interference is a phenomenon in which the occurrence of a crossover event in a certain region on a chromosome reduces the probability of a second crossover event in the adjacent region (Muller, 1916; Hillers, 2004).

Haldane's mapping function (Haldane, 1919) assumes that crossovers follow a Poisson distribution, with no interference between crossovers. In the absence of interference, recombination events in adjacent intervals are independent. If x is the genetic distance and θ is the recombination frequency between two loci, as a function of the genetic distance, then, the function would be as follow:

$$x = -1/2 \ln (1-2\theta) \qquad (2\text{-}3)$$

Kosambi's mapping function (Kosambi, 1944), however, allows for interference, where the occurrence of an crossover event in a given interval may reduce (positive interference) or enhance (negative interference) the occurrence of another crossover event adjacent the original locus. Positive interference results in less double recombinants (over adjacent intervals) than expected on the basis of independence of recombination events. Kosambi's mapping function is given by;

$$x = 1/4 \ln [(1+2\theta)/(1-2\theta)] \qquad (2\text{-}4)$$

Mapping methods to estimate distance between two loci rely on two point analysis. However, when large numbers of markers segregate in a mapping population, the number of possible orders increases exponentially with the number of loci to be ordered, so that for n markers the number of orders equals (n!/2) (van Os et al., 2005). To overcome this problem, multipoint linkage analysis is required (Lander et al., 1987). Therefore, finding the optimal or near-optimal ordering requires an appropriate search algorithm that avoids an exhaustive search. Finding the correct order of markers within a linkage group and calculating the map distances between them is the major job of software packages for the construction of linkage maps. Various computer packages for

linkage mapping have been developed which implement different algorithms such as the maximum likelihood method (Lander et al., 1987), the least square procedure for locus order (Stam, 1993) and the minimum number of cross-over events (van Os et al., 2005).

2.4.2 Phenotypic evaluation

In QTL mapping, regardless of the type of traits being evaluated, the quality of phenotypic data is crucial. Hence, the accurate assessment of the genotype's performance across a range of environments is important and therefore phenotypic evaluation, whether in the field, greenhouse or growth room, must be carefully planned. To evaluate a segregating population in the field in order to generate phenotypic data for QTL analysis, an appropriate experimental design should be conducted with sufficient replication to maximize the genetic gain in the segregating population (Cullis et al., 2006). In addition to applying appropriate field experimental design, a suitable statistical method for analyzing data from field experiments should also be considered (Gilmour et al., 1997). Furthermore, a proper protocol for evaluating different traits is also important to produce good phenotypic data (Ribaut et al., 2001). In general, a successful QTL mapping depends on, quality of phenotypic data, the heritability of the trait, its genetic nature (dominant, recessive or additive) and the number of genes that affect it as well as population size.

2.4.3 QTL mapping

QTL mapping has a long history, and several statistical programs have been developed to map QTLs of traits. Sax (1923) first reported the quantitative inheritance through the association of seed coat pattern and color with the seed size differences in bean. This study was one of the initial demonstrations of linkage between major genes and determinants of quantitative variation. The findings of Sax showed color to be controlled by a single gene. With the advent of molecular markers and their application in highly dense linkage maps, more sophisticated statistical approaches were developed (Lander and Botstein, 1989; Zeng, 1993; Jansen and Stam, 1994; Zeng, 1994; Stephens and Fisch, 1998). Collectively, there are several statistical methodologies for QTL analysis, including regression based methods, maximum likelihood methods, mixed model methods and Bayesian methods (reviewed by Doerge, 2002).

2.4.3.1 Single marker analysis

Single marker analysis is the earliest and simplest methods for QTL analysis. It is a regression-based method, which looks for an association between a molecular marker and trait value one by one. This approach has serious limitations which confound or may underestimate the QTL effects. These limitations can be summarized as follow: a) the method is not powerful enough - since QTLs are mapped one at a time, the effects of other mapped QTLs are ignored; b) linked QTLs can not be separated and it is difficult to distinguish a QTL with small effects from a QTL with a large effect when they are tightly linked; c) the position of the single QTL relative to the marker is not accurately defined; d) the presence of QTLs with equal sign effects can lead to the false detection of single "ghost-QTL" at an intermediate marker, while the effects of QTLs with opposite sign effects cancel so that the test for allele substitution effect at a marker locus is not even a proper test for QTL activity; and e) the error distribution is actually a mixture of normal distributions due to recombination between the marker and QTLs (Jansen, 1994).

2.4.3.2 Interval mapping (IM)

Interval mapping (IM) is an extension of single-marker analysis using maximum likelihood (Lander and Botstein, 1989). Interval mapping uses two observable flanking markers to construct an interval within which to search for QTLs. Lander and Botstein (1989) used a marker interval at a time to construct a putative QTL for testing by performing a likelihood ratio test at every position in the interval. The position with the significantly largest statistic in a chromosome region is an estimate of a QTL position. In this method, a QTL can be detected in each interval lying between any two flanking markers that individually may show no association with the trait. The interval mapping method has several advantages over the traditional approach. Firstly, the location and the effect of the QTL can be assessed more accurately; secondly, the test for identifying a putative QTL is more powerful, as the LOD (Logarithm-of-odds) threshold is the evidence for presence of QTLs at the various positions of the genome. The major shortcoming of interval mapping is that when two or more QTLs are segregating on a chromosome, the mapping of QTLs can be seriously biased and QTLs can be mapped to incorrect positions (Jansen, 1994).

2.4.3.3 Composite interval mapping (CIM)

Composite interval mapping (CIM) is a hybrid method, combining interval mapping with standard multiple regression methods (Jansen, 1993; Jansen and Stam, 1994; Zeng, 1994). CIM sought to overcome the problems described above for the interval mapping method. CIM fits parameters for a target QTL in one interval while simultaneously fitting partial regression coefficients for other markers as covariates to account for variance caused by non-target QTL (Zeng., 1993; Jansen and Stam, 1994). Theoretically, CIM gives more power and precision than simple IM because the effects of other QTLs are not present as residual variance. Additionally, CIM can remove the bias that would normally be caused by QTLs that are linked to the position being tested. However, there are some limitations to CIM: a) QTL analysis can be affected by an uneven distribution of markers in the genome, so that the test statistic in a marker-rich region may not be comparable to that in a marker-poor region; b) the difficulty of estimating the joint contribution to the genetic variance of multiple linked QTL; c) CIM is not directly extendable to analyzing epistasis; and d) the use of tightly linked markers as co-factors can reduce the statistical power to detect a QTL (Zeng et al., 1999).

2.4.3.4 Multiple interval mapping (MIM)

To address the limitations of CIM, the multiple interval mapping (MIM) method was developed (Kao and Zeng, 1997; Kao et al., 1999). For QTL mapping, the MIM method uses multiple marker intervals simultaneously to construct multiple putative QTLs. MIM is proposed as an appropriate model for the identification and estimation of genetic architecture parameters, including the number, genomic positions, effects and interactions of significant QTLs and their contribution to the genetic variance. Therefore, MIM has several advantages over IM and CIM. MIM tends to be more powerful and precise in detecting QTL, so that more minor and complex QTLs with high accuracy in position can be identified (Kao et al., 1999). In addition, MIM can search for and analyse epistatic QTLs and estimate the individual genotypic value and the heritability of quantitative traits. This estimation can also help to assess the relative contribution and importance of different genetic components, and to understand the genetic architecture of quantitative trait values and variation in a population. On the basis of the MIM result, genetic variance components contributed by individual QTLs were also estimated, and marker-assisted selection can be performed. Zeng et al. (1999)

pointed out that MIM can help to bring the three important studies (QTL mapping, genetic architecture and marker-assisted selection) together and provides a unified approach to study the genetic basis of quantitative traits.

2.4.4 Fine mapping

To refine the size of regions harboring QTLs that are detected in mapping experiments, a fine mapping experiment is necessary. Fine mapping will pave the way for marker assisted selection and introgression, and will allow positional candidate gene analyses to proceed with high levels of accuracy and precision (C.T.C. Members, 2003). Fine mapping (to less than 1-5 cM) is difficult as it requires more recombination events to separate the genes that govern the quantitative trait from closely linked markers. It might also require more sensitive phenotyping procedures, if there are several linked QTLs, because each individual QTL will probably have a smaller effect on the phenotype (Yamamoto et al., 1998). Crosses that involve many recombination events are the most successful. For instance, recombinant inbred populations are particularly useful for fine mapping because they provide high frequencies of recombination in any small chromosomal region compared to an F2 or an F1 backcross, and provide a permanent source of mapped individuals (Bennetzen, 2000).

2.4.5 Marker-assisted selection

One of the most important objectives of QTL mapping is to apply marker-assisted selection (MAS) for genetic improvement of quantitative traits. MAS could be useful for selecting traits that do not have easily scored phenotypes. And combining marker-assisted selection methods with conventional breeding programs can increase the overall selection gain and, therefore, the efficiency of breeding programs. However, one of the major difficulties in enhancing drought tolerance through MAS relates to the high QTL × environment (Q × E) interaction shown by the majority of QTLs in experiments conducted under varying water regimes and/or during different seasons (Ribaut et al., 2002).

2.5 Conclusions

Wheat is the crop that covers the largest cultivation area in the world and it is a major crop in Australia. Wheat is grown in rainfed environments of Australia where drought or water stress is the most important limiting factor for yield production. Drought itself is a complex phenomenon in terms of time, severity and duration of occurrence. Plant responses to drought are also complex and different mechanisms are adopted by plants when they encounter drought. Therefore, understanding those mechanisms which are related to plant productivity under drought conditions using physiological and molecular genetic tools may help us to dissect genetic control of traits related to plant responses under drought. In fact, a plant phenotype, in addition to the environment, is the result of a differential expression of several physiological/biochemical pathways. Hence, adopting a multidisciplinary approach by combining physiological studies, molecular genetics and QTL mapping approaches, in conjunction with functional genomics and genetic engineering, could provide useful information and tools to complement effectively conventional breeding for improving drought tolerance.

With the importance of wheat production in South Australian agriculture, this project has two main objectives. The first was to evaluate wheat varieties for their physiological responses to water stress under controlled conditions. The second objective was to identify QTLs for different agronomic and morpho-physiological traits that confer drought tolerance in wheat under field conditions.

CHAPTER 3

PHYSIOLOGICAL CHARACTERISATION OF THREE WHEAT CULTIVARS UNDER CONTROLLED DROUGHT CONDITIONS

3 Chapter 3: Physiological characterisation of three wheat cultivars under controlled drought conditions

3.1 Introduction

Drought is the most important limiting factor for crop production and it is becoming an increasingly severe problem in many regions of the world. In addition to the complexity of drought itself (Passioura, 1996; 2007), plant responses to drought are complex and different mechanisms are adopted by plants when they encounter drought (Levitt, 1980; Jones et al., 1981; Jones, 2004). These mechanisms can be (i) drought escape by rapid development which allows plants to finish their cycle before severe water stress, (ii) drought avoidance by, for instance, increasing water uptake and reducing transpiration rate by the reduction of stomatal conductance and leaf area, (iii) drought tolerance by maintaining tissue turgor during water stress via osmotic adjustment which allows plants to maintain growth under water stress, and (iv) resisting severe stress through survival mechanisms. However, this last mechanism is typically not relevant to agriculture (Tardieu, 2005). The maintenance of high plant water status and plant functions at low plant water potential, and the recovery of plant function after water stress are the major physiological processes that contribute to the maintenance of high yield under cyclic drought periods (Blum, 1996).

Understanding the physiology and genetic control of these mechanisms using physiological and molecular genetic tools will assist breeding programs seeking to improve drought resistance in crop plants. Physiological studies help to establish the precise screening techniques necessary to identify traits which are related to plant productivity. Recent studies suggest that selection of physiological traits have the potential to improve grain yield under drought in wheat (Richards, 1989; Reynolds et al., 2001; Chaves et al., 2002; Condon et al., 2002; Reynolds, 2002; Richards et al., 2002; Chaves et al., 2003; Condon et al., 2004; Richards, 2004; Olivares-Villegas et al., 2007). Therefore, understanding the physiological responses of crops under drought,

and the underlying complex genetic control of different mechanisms of drought tolerance, is crucial to enhance screening for drought tolerance.

There exist different types of drought which are related to the latitude, temperature, and seasonal precipitation of the cropping zone (Fischer and Turner, 1978). A combination of several climatic factors such as heat stress, water deficit, vapor pressure deficit, salinity and hostile subsoils can produce different types of drought. In South Australia, the target environment for this study, temporal and spatial variability in rainfall during the growing season results in cyclic water availability predominantly around anthesis and post-anthesis. In the field, there are additional factors such as high wind, low humidity, high light irradiance, soil-related constraints and biotic stresses which can confound field experiments. Several studies over the past decade have provided evidence that subsoil physical and chemical constraints across southern Australia reduce root growth and water use of crops (see Adcock et al., 2007 for review). Simulation modeling suggests that a shallow root system is advantageous in this type of environment where there are fluctuations in rainfall during the growing season (Schwinning and Ehleringer, 2001; Sadras and Angus, 2006). Shallow roots would also be advantageous in avoiding the more toxic conditions in the subsoils. Conducting drought experiments under controlled conditions (pot experiments), however, enables precise control of many of these environmental variables. Therefore, pot experiments are more reproducible, various treatments are easier to apply, and the results are easier to interpret. Unfortunately, pot experiments can have several serious disadvantages that make results difficult to extrapolate to the field (Passioura, 2006). In this study we have utilised our knowledge of the South Australian environment to replicate cyclic drought stress in the growth room. On the base of differences between three wheat cultivars (Excalibur, Kukri and RAC875) in their performance under South Australian dry environments (Chapter 1), it is assumed that a palette of traits acting in concert that may fine tune the responses to drought in Excalibur and RAC875 cultivars in such unpredictable and dynamic dry environments. Therefore, I aimed to evaluate physiological responses of these cultivars to drought, and to identify the possible palette of traits which enable these wheat cultivars to maintain high yield under cyclic drought conditions.

3.2 Materials and methods

3.2.1 Plant material/Genotypes

Three South Australian wheat cultivars, Excalibur, Kukri and RAC875, were evaluated. Excalibur (RAC177/'Monoculm'//RAC311S, released in 1991 by the University of Adelaide) is the widely adapted cultivar which has consistently yielded well over a range of environments in South Australian wheat regions but has low grain quality and is susceptible to rust. Excalibur has a good level of resistance to the root lesion nematode, *Pratylenchus neglectus,* confirmed by a gene located on chromosome 7AL (Williams et al., 2002). Excalibur is considered to be a relatively Zn-efficient genotype of bread wheat, able to exploit subsoil reserves effectively. Zubaidi et al. (1999) suggested that better root growth of Excalibur under harsh conditions might be a contributing factor to its success for this environment.

Kukri (76ECN44/76ECN36//MADDEN/6*RAC177), which was released in 1999 by the University of Adelaide, is a hard white wheat which has excellent grain quality and is rust resistant, but its yield production is low to moderate in low-rainfall environments. It is susceptible to root lesion nematodes. RAC875 (RAC655/3/Sr21/4*LANCE//4*BAYONET), a breeding line from Roseworthy Agricultural Campus, is a white wheat with high grain quality. It is a high yielding cultivar under South Australian dry environments, but it also is susceptible to rust and root lesion nematode. None of the cultivars carry any known boron tolerance genes. All three cultivars possess the *Rht2* semi-dwarfing gene and have very similar phenology (3 to 5 d difference in heading time) under field conditions (H. Kuchel, personal comunication).

3.2.2 Growth room experiments

In this study, three separate sets of growth room experiments were conducted: a preliminary experiment, Experiment I and Experiment II. Experiments were conducted in a growth room with a refrigerated cooling system at the Australian Centre for Plant Functional Genomics (ACPFG), the University of Adelaide, Australia. A mix of 400-watt high pressure sodium (HPS) and metal halide (MH) lamps provided a photon irradiance of 600 to 700 $\mu mol.m^{-2}.s^{-1}$. Temperature and humidity were controlled, and

conditions were selected to replicate the average conditions found in the northern South Australian wheatbelt during the wheat growing season. The temperature regime for these experiments was derived from Bureau of Meteorology climatic data collected at Minnipa (northern South Australia) over the period 1915 to 2004 (http://www.bom.gov.au/climate/averages/tables/ca_sa_names.shtml). Minnipa is a dry environment with the long-term average annual rainfall of ~327 mm, and the average growing season rainfall is about 242 mm (http://www.sardi.sa.gov.au). Temperature was adjusted throughout the plant growth. The mean of monthly maximum and minimum temperatures at Minnipa are given in Table 3-1, and the adjustments of temperature over time during the plant growth period under controlled conditions are also represented. Day length was 12 hours.

Table 3-1. Environmental conditions at a representative field site in the target region of interest, and the mean temperature and relative humidity in the two growth rooms during the time of the two experiments (mean ± SE).

The average temperature (ºC) in the field -Minnipa (1915-2004)			Temperature and relative humidity (RH) in growth room (cyclic drought)					
					Temp. (ºC)		RH (%)	
Month	Max./Min.	Temp. (ºC)	Growth period	Day/Night (12/12 h)	Exp. I	Exp. II	Exp. I	Exp. II
Jun-July	Max.	16.4	Weeks 1-4	Day	15.8 ± 0.1	16.5 ± 0.5	68.3 ± 1.1	50.0 ± 0.5
	Min.	6.9		Night	3.7 ± 0.2	6.0 ± 0.3	82.2 ± 1.3	83.9 ± 0.1
Aug.	Max.	17.3	Weeks 5-8	Day	17.8 ± 0.2	18.3 ± 0.2	74.0 ± 0.7	45.0 ± 0.5
	Min.	6.7		Night	5.8 ± 0.2	7.5 ± 0.3	81.1 ± 0.4	72.3 ± 0.4
Sep.-Oct.	Max.	22.3	From week 9 to maturity	Day	24.7 ± 0.2	23.8 ± 0.2	63.2 ± 0.9	55.6 ± 0.4
	Min.	9.1		Night	9.6 ± 0.1	9.5 ± 0.3	78.7 ± 0.7	81.4 ± 0.8
Average	Max.	18.7	Average	Day	18.2 ± 0.3	19.5 ± 0.3	66.1 ± 0.6	50.2 ± 0.4
	Min.	7.6		Night	7.4 ± 0.2	7.7 ± 0.3	81.6 ± 0.6	79.2 ± 0.5

Soil was collected from the field at the Roseworthy Agricultural Campus, the University of Adelaide, and air-dried. It was mixed with Waikerie sand (50:50 w/w) and basal nutrients were added to each pot at the start of the experiment (Table 3-2). The pH measured in a 1:5 dilution of soil: 0.01 M $CaCl_2$ was 6.6.

Pots were made of black plastic tubing, 15 cm diameter and 40 cm height, sealed at one end and filled with 6 to 8 kg of the soil-sand mix. To minimize evaporation from the pot surface, the soil surface was covered with a layer of white gravel.

Field capacity of the soil was determined by placing sieved (2 mm) air dried soil into a G4 sintered glass funnel holding a 100 cm water column (ψ_m = -10 KPa= -0.1 bar). The

soil was thoroughly wetted and allowed to drain for 48 h. A soil sample of approximately 30 g was oven dried at 105 ^{o}C for 24 h. The sample was placed in a desiccator and weighed to determine water content (Marshall and Holmes, 1979; Klute, 1986). Wilting point was determined using a ceramic pressure plate (Table 3-3). The pressure plate extractor was used to determine the moisture content of soils under suction of 15 bar (equivalent to 1500 KPa). The suction at 15 bar is usually applied to estimate 'wilting point' (Campbell, 1985).

Table 3-2. The recipe of nutrients that were added to pots in which the wheat plants were grown

Groups	Nutrients	(mg/kg soil)
1	$CaCO_3$	5000
2	$Ca(NO_3)_2.H_2O$	472.3
	$NH4NO_3$	350.0
3	$MgSO_4.7H_2O$	90.0
	K_2SO_4	120.0
	KH_2PO_4	75.0
	$MnSO_4.4H_2O$	3.0
	$CuSO_4.5H_2O$	5.0
4	$FeSO_4.7H_2O$	16.8
5	$CoSO_4.5H_2O$	1.0
	$H_2MoO_4.H_2O$	0.005
	$ZnSO_4.7H_2O$	4.4
	$NiSO_4.6H_2O$	0.15
	H_3BO_3 (as Boron)	0.1

Table 3-3. The percentage water content of the soil:sand mix used in the experiments to obtain particular water potentials (in bar).

Pressure plate (bar)	Water content (% oven-dry soil)	Water added to dry soil (kg)	Total weight of pot (kg)
-0.1	13.4 (Field capacity)	0.80	7.07
-1	7.6	0.46	6.73
-5	6.9	0.41	6.68
-15	4.8 (Wilting point)	0.29	6.56

The preliminary experiment was conducted with the aim of establishing growth room experiments for drought tolerance studies. Excalibur and Kukri were chosen for this experiment. Seeds were washed and soaked in water for overnight, then put onto wet filter paper in Petri dishes (20 seeds per Petri dish) to germinate. After one day, they were placed in a refrigerator (at 4 ºC) for 24 h, and then they were put into the growth room for 48 hours in the dark and then 48 h in the light. Pre-germinated seeds were planted in pots, three plants in each pot. After one week two plants were removed,

leaving one plant per pot. The experiment was carried out in a completely randomized design (CRD) with five replications. The treatments comprised five watering regimes:

1) WW; well-watered (to field capacity = 13.4%),
2) D/5; droughting to -5 bar (6.9% soil water),
3) D/rw/5; droughting to -5 bar and re-watering,
4) D/15; droughting to -15 bar (4.8% soil water),
5) D/rw/15; droughting to -15 bar then re-watering.

All plants were watered to weight daily, being maintained at field capacity for the first 40 d after planting. At the first period of drought, plants in the drought treatments were droughted by reducing water to the level of D/5 and D/15 for 10 d and then re-watered in re-watering treatments for one week. A second period of drought was imposed 10 d later. The amount of water that needed to be added to each pot for each level of droughting is given in Table 3-3. At the first period of droughting, water was gradually decreased and it took four to five days for water content to fall to the desired level of drought. In the second period, watering was stopped at the first day of the imposition of drought and it took three or four days to reach to the level of drought for D/5 and D/15, respectively. Maintaining a steady, uniform soil water deficit while the plant is using water is challenging. To keep drought constant at the level of -5 and -15 bar, a certain amount of water was added to each pot every day in the morning, as suggested by Boyer and Westgate (2004). This is done by weighing the pot and replenishing the amount of water loss up to that certain level of stress. The added water wetted a small soil volume and entered through the pot edge and soil profile, and it also penetrated slowly through a few roots, preventing the plant water potential from declining during the day. The added water was again depleted by the end of the day, and the soil water content returned to that of the previous night. In this experiment, water stress was applied based on day calendar. Since there were significant differences in flowering time between Kukri and Excalibur in the conditions of this experiment, imposing water stress based on calendar time was not appropriate.

In the light of experience from the preliminary experiment, two other experiments were conducted to investigate the responses of three parents of the mapping populations (Excalibur, Kukri and RAC875) to water deficit stress under controlled conditions, with some modifications, in particular:

- Reducing watering treatments to three and two in experiment I and II, respectively.
- Increasing planting density to three plant per pot, to be similar to the planting density used in the field
- Putting an open ended tube in the middle of the pot to help increase the distribution of water through the soil profile
- Imposing drought based on plant developmental stages, for example at the early stage of flag leaf emergence (Zadoks scale 37).
- Monitoring the soil water content, in addition to weighing and watering pots, using a soil moisture meter (TDR 100 Spectrum Technologies, Illinois, USA).
- In the re-watering treatment, stopping watering and allowing the soil to dry gradually until the symptoms of wilting in plants at midday were observed, then re-watering to full field capacity and letting plant to drying down again (Fig. 3-1).

3.2.2.1 Experiment I

Experiment I was carried out in a completely randomized design (CRD) with four replicates (pots) and three varieties (Excalibur, Kukri and RAC875). Experiment I comprised three watering regimes:

1) WW: well-watered (field capacity = 13.4%);
2) D/15: droughting to -15 bar (4.8% soil water); and
3) RW: droughting and re-watering (cyclic drought treatment).

Seeds were surface-sterilised with 0.5% sodium hypochlorite (NaOCl) for 1 minute and soaked in water overnight. Seeds were then germinated on moist filter paper in petri dishes for 24 h. Four germinated seeds were planted in each pot and thinned to three plants after establishment. In this experiment, growth room temperature was lower during the nights over the first four weeks (Table 3-1). Experiment I took 140 and 130 d from planting to maturity for WW and drought stress treatments, respectively.

3.2.2.2 Experiment II

Experiment II was conducted in a different growth room. This experiment was similar to Experiment I except the D/15 treatment was omitted. In this experiment, two watering regimes were applied - WW and RW. The same three verities, Excalibur,

Kukri and RAC875, were assessed. In contrast to Experiment I, germinated seed in this experiment was pretreated with low temperature (4^{o}C) for two weeks. As a result of this vernalisation treatment, the night temperature during the first two months of this experiment was maintained at a higher level (6 ± 0.3 and 7.5 ± 0.3 °C for the first and second months, respectively) than in Experiment I (Table 3-1). Plants in Experiment II were grown for 130 and 122 d for WW and RW treatments, respectively. Maturity in Excalibur was 10 d later than in Kukri and RAC875 in this experiment.

In both experiments, all plants were watered to weight daily with field capacity being maintained to the stage of flag leaf emergence. At the beginning of flag leaf emergence (at Zadoks scale = 37, when 1 to 5 cm of the flag leaf blade was visible, until the awns of apical florets emerged from the flag leaf sheath), water stress was imposed by gradually decreasing the amount of water to the pot for about 10 d to allow for acclimation (Fig. 3-1). For the cyclic drought treatment, plants were re-watered (RW) to the level of field capacity. Water was withheld until the symptoms of wilting appeared in the morning and also VSWC was decreased to about 7%, then plants were re-watered. Four consecutive drying cycles were imposed (60 and 55 DAP for Experiments I and II, respectively), affecting anthesis and grain filling of all cultivars, and water stress occurred in these experiments during the reproductive stages from heading to grain filling. Similar numbers of plants were maintained under non-stress treatment with regular watering (WW). Development of water stress was monitored visually (observation of leaf rolling and leaf drying) and by measurement of leaf relative water content. In addition, volumetric soil water content (VSWC) was determined with a soil moisture meter (TDR 100 Spectrum Technologies, Illinois, USA). Water uses at different stages of each experiment are given in Table 3-5. The total weight of the pot and all its contents was recorded when the soil was first brought to field capacity. When the plants were re-watered, the pots were weighed and water was added to bring the pot to the field capacity.

3.2.3 Measurements of cyclic drought experiments

Water consumption was recorded by weighing and watering the pots every second day at the early stage of development, and daily at later stages (after flag leaf emergence). Volumetric water content was measured during the entire water stress period. To control conditions in the growth room, a data logger monitored temperature and relative

humidity. A dehumidifier was used to keep the relative humidity constant (Table 3-1). Trait measurements were taken throughout the plant growth and during the cyclic water stress. How the water stress was applied and the time of trait measurements which were taken during the cyclic water stress are shown in Figure 3-1.

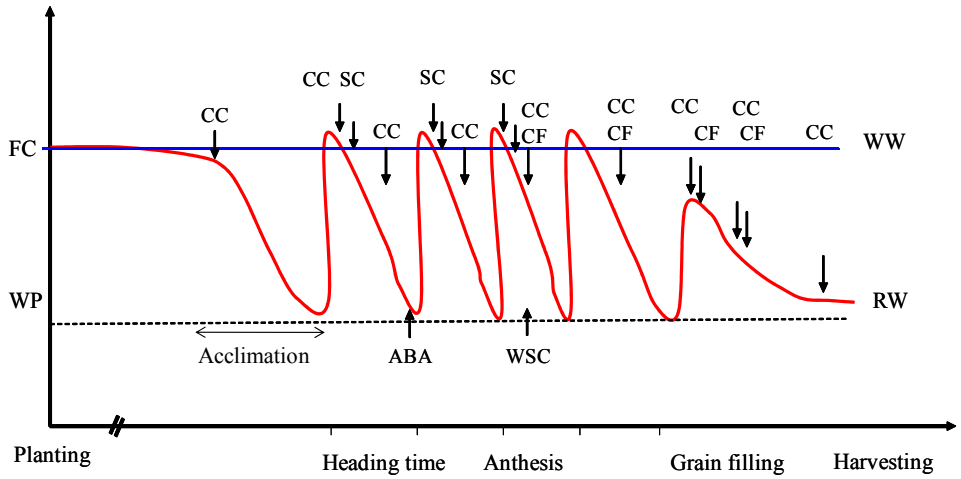

Figure 3-1. Schematic of cyclic drought application and the time of trait measurements throughout the experiment. FC =field capacity, WP= wilting point, CC=chlorophyll content, CF=chlorophyll fluorescence, SC=stomatal conductance ABA=Abscisic acid , and WSC=water soluble carbohydrates

3.2.3.1 Chlorophyll content and fluorescence

Chlorophyll content was measured (mean of four measurements) using a portable chlorophyll meter (SPAD-502, Minolta, Tokyo, Japan). Chlorophyll content was measured on the same flag leaf every 3 to 6 d to monitor the chlorophyll retention after water stress was imposed. The effect of drought on the photosynthetic apparatus of leaves was studied by measurements of chlorophyll fluorescence. Chlorophyll fluorescence parameters were determined (mean of four measurements) four times during the grain filling stages (94, 97, 103 and 109 d after planting) with a fluorimeter (PAM-2000, Walz, Germany). The maximal efficiency of PSII photochemistry (F_v/F_m) was used to study effects of water stress on photosystem II (PSII) in wheat plants (Maxwell and Johnson, 2000). To measure *Fv/Fm*, plants were kept in darkness for 20 min to allow full dark adaptation. Measurements were performed on flag leaves

between approximately 3 to 5 h after the lights were turned on (Araus et al., 1998). Moreover, photochemical quenching (*qP*) of modulated chlorophyll fluorescence was measured in light-adapted leaves, as described by Tambussi et al. (2002). *qP* gives an indication of the proportion of PSII reactions that are active (Maxwell and Johnson, 2000). Photochemical quenching was used to assess ageing of wheat flag leaf in the field (Hong et al., 1999; Lu et al., 2002), and to evaluate PSII photochemistry in wheat exposed to water stress (Lu and Zhang, 1999).

3.2.3.2 Stomatal conductance

Measurements were made on two consecutive days after re-watering between 3 to 5 h after the lights were turned on. Stomatal conductance was measured on fully expanded flag leaves from three plants in each pot with a dynamic diffusion porometer (Delta-T AP4, Delta-T Devices Ltd, UK) during the middle of the day. Two measurements from both adaxial and abaxial surfaces of the leaf were taken. The porometer was calibrated at the start of each measurement session.

3.2.3.3 ABA content

During the second period of the cyclic drought treatment, when water stress became severe (76 DAP for Kukri and RAC875 and 83 DAP for Excalibur), xylem sap and spikes were taken for ABA measurements. Xylem sap was collected by pressurizing the main stem of plant in a pressure chamber. 30 to 50 µl of sap was collected using a pipette and transferred to an Eppendorf tube. The sap was immediately frozen in liquid nitrogen and stored at -80 ^{o}C for ABA analysis (Liu et al., 2005b). Simultaneously, the spike of the main stem was sampled from control and water-stressed plants. Awns were removed from the spike and the spikelets immediately frozen in liquid nitrogen and kept at -80 °C. Frozen tissues were ground with a grinding mill covered with liquid nitrogen. Approximately 100 to 250 mg of ground tissue was used for extraction. ABA was extracted in hot water and quantified using high pressure liquid chromatography (HPLC) (Soar et al., 2004). The ABA measurements were done by Dr. Brian Loveys, CSIRO Plant Industry, Waite Campus, Adelaide.

3.2.3.4 Stem water soluble carbohydrates (WSC)

Samples for stem WSC determination were taken 5 d after anthesis (850 growing degree days, with a base temperature of 5.5 ◦C). Stems without sheaths and leaf blades were initially freeze dried then oven dried again at 70 °C for 48 h and ground in a mill to pass a 1 mm screen. WSC content was determined using the anthrone method (Yemm and Willis, 1954) with some modifications (van Herwaarden et al., 1998) as follows: carbohydrates were extracted from 50 mg of dried material with 4 mL of 80% (v/v) ethanol at 80 °C followed by two extractions with 4 mL of water at 60 °C.

3.2.3.5 Carbon isotope discrimination ($\Delta\ ^{13}C$)

Carbon isotope composition was determined on grain at harvest. Approximately 10 g of grain of each cultivar was sampled and ground to a fine powder to pass a 0.5 mm sieve and the carbon isotope composition of each cultivar was determined by mass spectrometry (Condon et al., 1987).

3.2.3.6 Leaf traits

Leaf glaucousness, leaf rolling and leaf colour were visually assessed using a 1 to 5 scale. 1 represented no visible wax on the back of the flag leaf and an unrolled leaf, while 5 indicated the highest level of wax deposition and a tightly rolled leaf (O'Toole and Cruz, 1980; Clarke and Richards, 1988; Clarke et al., 1991). In Experiment I, the flag leaf area (FLA) was measured using a portable Leaf Area Meter (CI-202, USA). Leaf turgid weight (TW) was recorded after re-hydration overnight (at 4 ºC). Finally, the leaves were oven-dried (48 h at 80°C) and dry weight (DW) was obtained. The specific leaf area (SLA, leaf area per unit leaf dry weight) and leaf dry mass content (LDMC, the ratio of leaf dry weight to its turgid weight) were calculated. Leaf thickness was then estimated from the ratio $(SLA \times LDMC)^{-1}$ as described by Vile et al. (2005).

In Experiment II, excised leaf water loss (ELWL) was measured on flag leaves 95 DAP. The excised leaves were weighed immediately after sampling to record fresh weight (FW), the incubation weight (IW) was recorded after 6 h incubation at 28 ºC at 50% relative humidity, and then dry weight (DW) was obtained after 24 h oven drying at 70 ºC as proposed by Clarke and McCaig (1982). ELWL was then calculated from the

following formula: ELWL = (FW − IW)/(FW − DW) × 100. Corresponding relative water content on a different set of leaves was also measured.

3.2.3.7 Plant dry weight determinations

The number of days from planting to heading was recorded when 50% of the plants had their ears completely emerged. At maturity, total above-ground biomass and grain yield (GY) were recorded by weighing the bulked harvested plants (three plants per pot) and seeds from each pot. Four replicate pots per cultivar and treatment were sampled for dry weight determination. Roots were sampled and carefully washed to determine dry weight after oven drying at 60°C for 48 h. Harvest index (HI) was calculated as HI = GY / total biomass (shoot mass + root mass). Grain number and grain weight per spike (determined on three main spikes), the number of spikes per plant and the number of spikelets per spike were also determined.

3.2.4 Glasshouse experiment for water status measurement

In order to determine the water status of the three different cultivars in response to water stress, a glasshouse experiment was conducted. Six plants (two of each cultivar) were grown together in black plastic pots (10-inch diameter) containing the same volume (3 kg) of standard potting mix (coco peat). Greenhouse temperatures were maintained at 24 ± 1 °C during the day and 18 ± 1 °C during the night (13/11 h day/night). Average relative humidity was 50% and 80% during the day and night, respectively. Plants were well watered until the stage of flag leaf emergence then water was withheld. Before withholding water (non-stress measurements), relative water content (RWC) and osmotic potential (OP) were determined using the flag leaf blade, and penultimate leaves were taken for leaf water potential (LWP) measurements. Two days later, water status parameters of plants were measured on a daily basis. Permanent wilting of plants occurred 12 d after the imposition of water stress. Leaves were sampled 3.5 to 4 hours after sunrise. For all three parameters, four replicates per cultivar and sampling date were taken.

3.2.4.1 Relative water content (RWC)

RWC of flag leaves was determined by the standard method (Barr and Weatherley, 1962). Leaves were cut, and collected at midday to determine fresh weight (FW). Leaf

blades were then placed with their cut end pointing down into a Falcon tube containing about 15 ml of 1 mM $CaCl_2$. The $CaCl_2$ was used to try to increase leaf cell integrity, with the aim of reducing cell lysis as a result of excessive rehydration. The turgid weight (TW) was then recorded after overnight re-hydration at 4 ºC. For dry weight (DW) determination, samples were oven dried at 70 ºC for 48 hr. RWC was calculated according to the following equation: RWC (%) = [(FW-DW)/(TW-DW)] ×100.

3.2.4.2 Osmotic potential (OP)

The OP was determined on excised flag leaves. Flag leaves were cut and placed into 10 ml screw-cap tubes (Sarstedt, Australia), immediately frozen in liquid nitrogen and stored at -80 ºC until measured. The 20 µl aliquot of cell sap, which was extracted by pressing the total leaf tissue in a 5 mL syringe containing a cellulose filter, was used for osmotic potential measurements. Osmotic potential was measured using a freezing point micro-osmometere (Fiske 210, Massachusetts, USA). Osmolality measurements in mOsm kg^{-1} water were converted to osmotic potential in MPa from the Van't Hoff relation at 20ºC (Nobel, 1999).

Osmotic adjustment (OA) was calculated using a derivation of the formula for OP (Morgan, 1992). Morgan's regression method is based upon the relationship between OP and RWC. This method compares the observed response with an expected response from an ideal osmometer. OA was estimated from the linear regressions of RWC on OP as derived from consecutive measurements during a drought stress cycle. Two regression lines were fitted for all measurements taken during the drying cycle in all plants belonging to a given replication. One regression line was derived between RWC and the measured OP, and the other between RWC and calculated OP to account for the concentration of solutes due to water loss (concentration effect). A plot of log RWC and log OP for the three varieties showed two linear phases (α and β; Fig. 3-6). Log conversion of RWC and OP was conducted to improve the linearity of the relationship. The linear form of the working formula is: $Log\pi = \log(OP_o RWC_o) - \log RWC$ where OP_o and RWC_o are the initial osmotic potential and the initial relative water content, respectively. RWC_o and OP_o data were taken after the last irrigation on well-watered plants with no history of water stress. Cultivars ranged in RWC_o from 94.5 to 97.5%. The log (OP_o RWC_o) is the intercept with the slope set to one. The slope of the observed response of log π to log RWC was evaluated. Osmotic adjustment was calculated from

the two regressions as the difference between the measured OP and predicted OP at a RWC of 70% (Morgan, 1980a, 1992; Babu et al., 1999). In this study, the slope β was used for the calculation of OA.

3.2.4.3 Leaf water potential (LWP)

LWP was estimated with a Scholander pressure chamber (Model 3000, Soil moisture Equipment Corp, Santa Barbara, CA). For the measurement of LWP, a leaf was detached from the shoot and immediately after sampling placed in the pressure chamber with the cut end protruding from the chamber and exposed to atmospheric pressure and the remainder of the leaf enclosed in the chamber. Pressure was applied to the chamber until the equilibrium pressure was indicated by the appearance of sap at the cut end of the leaf. The pressure reading at this point was recorded as the equivalent to the tension with which the plant water is held within that leaf sample. This equilibrium pressure was a measure of the leaf water potential (Boyer, 1967; Turner, 1988).

3.2.5 Data analysis

The experiments were designed in a randomised complete block. The analysis of variance (ANOVA) was performed using GenStat (Version 6.1 for Windows). Values are given as means and the standard error (s.e.) of the means which were calculated by SPSS (Version 13.0 for Windows). Graphs were constructed using SPSS and MS-Excel.

3.3 Results

Results from the preliminary experiment are given in Table 3-4. Total water consumed throughout the plant's life was significantly different between the five watering regimes. The amount of water use under stress treatments were 33, 47, 50 and 64 percent less compared to WW treatments for D/rw/5, D/5, D/rw/15 and D/15, respectively. Stress treatments were different in terms of severity and pattern in this experiment. Although, there was no difference between Excalibur and Kukri for their water use under WW treatment, these two cultivars showed different responses under stress treatments. Excalibur used significantly more water under all stress treatments compared to Kukri (Table 3-4 and Fig. 3-2). Total biomass production was also different among watering regimes. With increasing severity of water stress, biomass production decreased, on average from 114.3 to 42.7 $g.pot^{-1}$ under WW and D/15 treatments, respectively.

Correlation between total water use and total biomass was high (r = 0.97, $P < 0.001$). Water use from a pot largely depends on plant size, particularly its leaf area, with larger plants using more water daily compared with smaller ones. Water use also depends on stomatal behaviour (Fischer et al., 1998; Davies et al., 2002). Kukri produced significantly ($P < 0.05$) higher biomass under WW treatment compared to Excalibur, while Excalibur showed relatively higher biomass under all drought stress treatments. Grain yield was also higher for Kukri under WW treatment; under stress treatments, however, there were no significant differences between Kukri and Excalibur except for the D/15 treatment, where Excalibur showed significantly ($P < 0.05$) higher grain yield (Table 3-4).

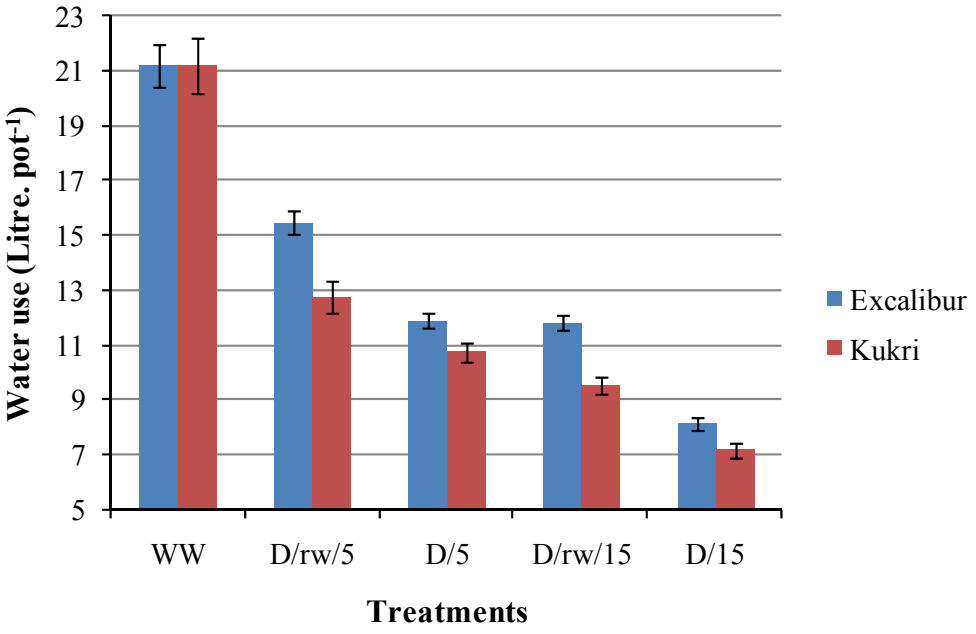

Figure 3-2. Total water use throughout the plant growth in five different watering regimes for Excalibur and Kukri.

In this experiment, two cultivars produced high numbers of tillers, on average from 21 to 10 tillers per plant under WW and D/15 (severe water stress), respectively. There were no differences between the two cultivars in terms of tiller production under different watering regimes, except for D/15, where Excalibur produced a significantly higher number of tillers than Kukri. One of the main drawbacks of this experiment was the lack of competitiveness as a result of low plant density (one plant in each pot). Low plant density in a high volume of soil (8 kg) with sufficient nutrients allowed plants to produce an excessive number of tillers.

Although heading time was not affected by different water stress treatments, the two cultivars flowered at different times. Kukri on average flowered 13 d earlier than Excalibur. These two cultivars have a tendency to flower at similar times in field conditions (H. Kuchel, personal communication; Chapter 5 of this thesis). However, they showed significantly different flowering time in this experiment. This might be related to different vernalization requirements which were not met in the growth room. The other drawback in this experiment was the way of water stress application. Water stress was imposed 40 d after planting, at the same time for both Excalibur and Kukri, despite the fact that these cultivars were flowering at different times. Consequently, inappropriate application of water stress (based on calendar time) biased cultivars assessment under stress.

Another problem was the method of stress application, where small amounts of water were being adding frequently to the soil surface. In this type of stress imposition, only the topsoil of the pot and the layer connecting to the pot edge received small amounts of water, while the rest of the soil remained dry. The frequent supply of small amounts of water to the surface of the pot resulted in a partial re-watering. In partial re-watering, a few pores can be filled and only a local volume of soil becomes wet, where the roots in the wetted part are very wet while others remain dry in the rest of the soil, which it results in partial root drying (PRD) (Boyer and Westgate, 2004). Blum (http://www.plantstress.com/methods/index.asp) pointed out that plants grown under these conditions are not actually “stressed” since they experience frequent stress-recovery cycles which are very complex physiologically and are quite different from a prolonged normal cycle of stress. He highlighted that this protocol might be correct mathematically, but it is incorrect physiologically. The problems apparent in the preliminary experiment were taken in to account for the design of Experiments I and II.

Table 3-4. Results from the preliminary experiment for Excalibur and Kukri in five different watering regimes well watered (WW), droughting to -5 bar (D/5), droughting to -5 bar and re-watering (D/rw/5), droughting to -15 bar (D/15), droughting to -15 bar then re-watering (D/rw/15). The values for total water used, heading time, total biomass (shoot and root), grain yield (GY), harvest index (HI), water use efficiency (WUE), number of tillers per plant, plant height, root dry weight and root- to-shoot ratio are given (each value represents the mean ± SE).

Treatments	WW		D/rw/5		D/5		D/rw/15		D/15	
Cultivar	Excalibur	Kukri	Excalibur	Kukri	Excalibur	Kukri	Excalibur	Kukri	Excalibur	Kukri
Water use (litre.pot^{-1})	21.2 ± 0.7	21.2 ± 1.0	15.5 ± 0.5**	12.7 ± 0.6	11.9 ± 0.3*	10.7 ± 0.4	11.8 ± 0.3**	9.6 ± 0.3	8.1 ± 0.2*	7.2 ± 0.3
Heading time (DAP)	95 ± 0.3**	80 ± 1.2	88 ± 0.8**	74 ± 0.6	86 ± 1.2*	74 ± 2.2	86 ± 0.2*	72 ± 1.3	84 ± 0.5**	72 ± 0.8
Total biomass (g)	110.8 ± 6.5	117.8 ± 5.1	78.4 ± 3.6*	65.4 ± 3.2	64.8 ± 2.6**	58.2 ± 2.4	58.6 ± 2.3*	50.6 ± 1.6	45.8 ± 1**	39.6 ± 0.8
GY (g)	27.7 ± 2.1	38.9 ± 2.5**	23.7 ± 0.9	24.3 ± 1.1	19.9 ± 2	18.7 ± 0.6	16.6 ± 0.9	16.2 ± 0.4	15.6 ± 0.5*	14.1 ± 0.4
HI	0.31 ± 0.027	0.39 ± 0.024	0.36 ± 0.016	0.42 ± 0.007	0.39 ± 0.03	0.4 ± 0.006	0.35 ± 0.015	0.38 ± 0.003*	0.41 ± 0.011	0.43 ± 0.008
WUE	0.43 ± 0.008	0.48 ± 0.018*	0.42 ± 0.011	0.46 ± 0.009*	0.43 ± 0.018	0.44 ± 0.019	0.41 ± 0.009	0.44 ± 0.013	0.47 ± 0.011	0.46 ± 0.018
Number of tillers	21.2 ± 1.5	21 ± 1.2	14.8 ± 0.8	13.4 ± 0.6	15.2 ± 0.6*	13.6 ± 0.7	12 ± 0.5	10.6 ± 0.4	13 ± 0.4*	9.8 ± 0.5
Plant height (cm)	71.4 ± 1.4	78.6 ± 1**	72.6 ± 1.4	77.4 ± 0.7*	66.8 ± 2	74.4 ± 1.6*	70.6 ± 1.7	74.6 ± 1.4*	65.2 ± 1.5	69.6 ± 2*
Root DW (g)	20.6 ± 3.2*	16.8 ± 1.2	13.2 ± 1.4*	7.4 ± 0.9	13.8 ± 0.9*	11 ± 1.6	10.6 ± 0.9*	8.4 ± 0.6	7.4 ± 0.5	6.8 ± 0.2
Root/Shoot	0.23 ± 0.029	0.17 ± 0.01	0.2 ± 0.017	0.13 ± 0.011	0.27 ± 0.023	0.23 ± 0.036	0.22 ± 0.016	0.2 ± 0.011	0.19 ± 0.012	0.21 ± 0.006

* and ** show significant differences at 0.05 and 0.001 level with ANOVA, respectively.

3.3.1 Experiment I and II

In Experiments I and II, total water consumed during plant growth was, on average, 15.7 and 9.8 litre.pot^{-1} under WW and RW treatments, respectively. Under WW conditions, RAC875 used less water (14.7 ± 0.6 litre.pot^{-1}) than Excalibur (16.2 ± 0.4 litre.pot^{-1}) and Kukri (16.1 ± 0.4 litre.pot^{-1}). The total quantity of water applied during the growth period, including before and after water stress, for the two experiments is given in Table 3-5. In Experiment I, the three cultivars used similar volumes of water before water stress. However, after the imposition of water stress, the volume of water consumed was considerably different between cultivars. During the cyclic water stress, Excalibur used significantly more water than RAC875 and Kukri (15.5% and 18.3% respectively, $P < 0.01$). There were no significant differences between Kukri and RAC875 for total water consumption. In Experiment II, Excalibur used approximately 35% more water than RAC875 and Kukri before water stress was imposed. However, this could be attributed to the later heading time of Excalibur in this experiment (see below). Analysis of VSWC during the cyclic water stress also revealed that Excalibur depleted the soil water profile faster than Kukri and RAC875 (Fig. 3-3).

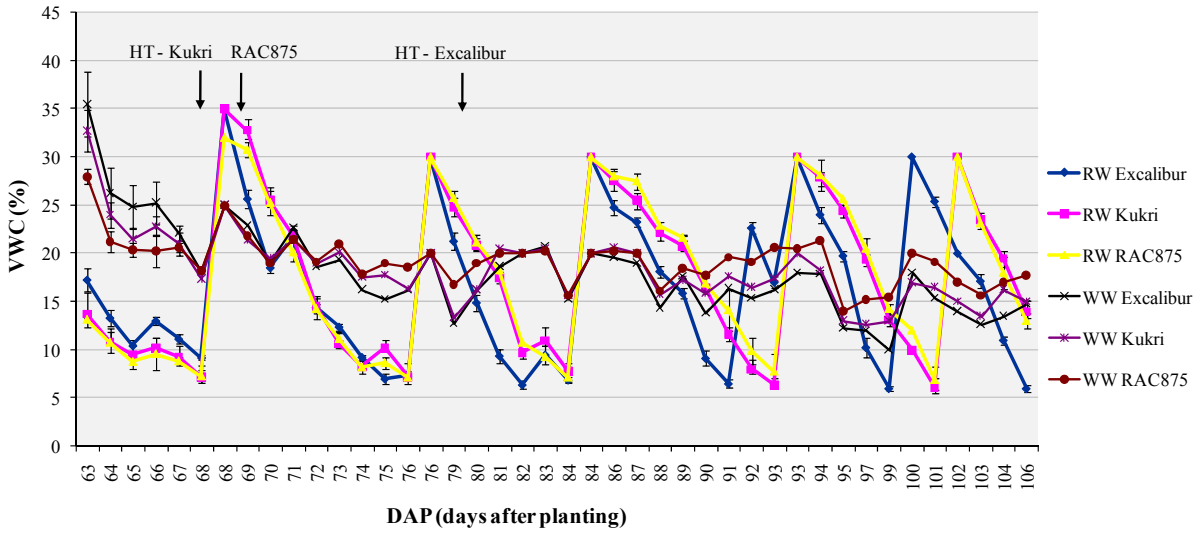

Figure 3-3. The volumetric soil water content (Vw/V) during RW and WW treatments (data from the second experiment). Water stress was started from 53 DAP for Kukri and RAC875, while it was started from 61 DAP for Excalibur. To synchronize the time of watering, all cultivars were watered at 68 DAP. VSWC measurements were started 63 DAP. HT is the heading time.

3.3.2 Agronomic traits

3.3.2.1 Heading time

In both growth room experiments, the watering regime (WW and RW) had no significant effect on heading time. Cultivars under RW treatment flowered at a similar date to those under WW treatment (Table 3-6). However, there were differences between cultivars for heading time. RAC875 and Kukri had, on average, a similar heading time under both WW and RW treatments (73 and 75 DAP for Kukri and RAC875, respectively). In Experiment I (severe water stress), Excalibur flowered 3 and 2 d later than Kukri and RAC875, respectively. In Experiment II (mild stress), however, these differences were more pronounced, with Excalibur flowering 14 and 12 d later than Kukri and RAC875, respectively.

3.3.2.2 Plant height and spike length

Under the WW treatment, Kukri was significantly ($P < 0.001$) taller than Excalibur and RAC875 (on average 75.0 ± 2.2 cm), while Excalibur was the shortest (68.9 ± 3.1 cm) cultivar in both experiments (Table 3-6). In Experiment I, where water stress was severe, there were no significant differences in plant height between cultivars under RW treatment. Kukri, which had a greater plant height under WW treatment, was similar in height compared to the other two cultivars (Table 3-6). Plant measurements revealed that the differences in plant height resulted from a reduction in peduncle length of all cultivars when exposed to drought stress. In Kukri, peduncle length was reduced on average by 37% under water stress whilst peduncle length in Excalibur and RAC875 was less affected by water limitation, with reductions of 20.2% and 24.5%, respectively. In Experiment II, where the water stress was mild, all three cultivars were approximately 10 cm taller than plants in Experiment I.

Spike length was measured in Experiment II. Under the WW treatment there was no significant difference in average spike length between the three cultivars. Conversely, under the RW treatment the spikes of Excalibur were significantly longer ($P < 0.01$) than those of Kukri and RAC875 (Table 3-6). The most significant finding was Excalibur's capacity to increase spike length under the RW treatment. In addition, the number of spikelets per spike differed significantly between cultivars under the RW treatment. Measurements of spikelet number in spikes of primary tillers revealed that

Excalibur contained approximately 30% more spikelets per spike than Kukri and RAC875 (Table 3-6).

3.3.2.3 Grain yield and its components

Under well-watered conditions, in both experiments, Kukri, Excalibur and RAC875 were found to have comparable grain yields (Table 3-6). By contrast, cyclic water stress treatment resulted in significantly different grain yields between cultivars. In Experiment I (severe water stress) the reduction in grain yield under water deficit was 83.1%, 74.7% and 67.8% for Kukri, Excalibur and RAC875, respectively. In this experiment, the drought intolerant cultivar, Kukri, yielded 44% less grain than RAC875 and 18% less grain than Excalibur. Under the milder drought stress conditions of Experiment II, the RW treatment resulted in grain losses of 49%, 57% and 36% for Kukri, Excalibur and RAC875, respectively. Notably, whilst RAC875 had the highest grain yield in both experiments (an average of 13.3 ± 2.7 $g.pot^{-1}$), under the RW treatment of Experiment II Excalibur produced less grain (11.9 ± 1.2 $g.plot^{-1}$) than Kukri. In Experiment I, performance of the cultivars was clearly comparable to their performance in the field. Excalibur and RAC875 produced more grains than Kukri under dry conditions. Additionally, the differences between three cultivars in grain yield are more evident under severe stress as it has been observed under the field conditions (Table 1-1 and Fig. 1-4, Chapter 1). In Experiment II, Excalibur produced less grain yield compared to RAC875 and Kukri. This discrepancy may be explained by variation in developmental stages between cultivars in the second experiment. Excalibur flowered much later than Kukri and RAC875 in Experiment II. Even though, water stress was imposed at the same phenological stages. In Experiment I, cultivated plants were exposed to low temperature (3-4°C at night) during the first four weeks in the growth room. In contrast, in Experiment II, to speed up plant growth, before cultivation, germinated seed were pre-treated in a cold room at 4°C for two weeks in the dark. Two weeks pre-treatment of the germinated seed at 4°C may not provide the vernalization required in Excalibur; or the shock from the transplanting of germinated seed might de-vernalise seedling.

Tiller abortion and the number of grains per spike were the components that had the greatest impact upon grain yield under RW conditions. Indeed, RAC875, the highest yielding cultivar in both experiments, produced less tillers and had a lower tiller

abortion rate than Excalibur and Kukri (Table 3-6). Under severe stress (Experiment I), the number of grains per spike recorded for Excalibur and RAC875 was significantly higher than in Kukri ($P < 0.01$). Under mild drought stress (Experiment II), however, the differences between cultivars were not significant. The reduction in grain yield under RW treatment was also closely associated with a reduction in the grain weight of main tillers. Kukri recorded lower grain weight (5.8 ± 0.2 g) in the main stems compared to RAC875 and Excalibur (7.9 ± 0.5 and 7.3 ± 0.3 g), respectively. Grain size was another yield component that was affected under cyclic drought. The drought tolerant cultivars, Excalibur and RAC875, produced larger grains on the three main tillers compared to Kukri. Although RAC875 produced the largest grain under both watering regimes and in both experiments (Table 3-6), grain produced by Excalibur was only significantly ($P < 0.01$) larger than Kukri under the RW treatment.

There were no differences in HI between cultivars under non-stress conditions, but Kukri had the smallest HI (0.14 ± 0.02) compared with Excalibur (0.16 ± 0.01) and RAC875 (0.23 ± 0.02) under cyclic drought (Experiment I). The HI in Experiment II was, in total, higher than HI in Experiment I, but Excalibur had the smallest HI (0.22 ± 0.03) of all cultivars in Experiment II. RAC875, on average, had the highest HI under cyclic drought conditions (0.31 ± 0.04). As HI was calculated from GY divided by total biomass including shoots and roots, the lower HI in Excalibur is contributed mainly due to larger root mass. Although the root mass was significantly ($P < 0.001$) lower in Experiment I than II under WW treatment, there were no significant differences among cultivars in root mass in both experiments. Under RW treatment, however, Excalibur showed larger root mass compared to Kukri and RAC875 (Table 3-6). The major determinants of yield under water stress were number of grains per spike and the percentage of aborted tillers. This finding is supported by the negative correlation between GY and the percentage of tiller abortion found in Experiment I and II ($r = -0.71$ and -0.87, $P < 0.01$) and the positive correlation between GY and number of grains per spike ($r = 0.64$ and 0.87, $P < 0.01$) under the RW treatment. There was no significant correlation between GY and grain size in both experiments.

3.3.2.4 Leaf traits

There are differences in leaf morphology between cultivars. RAC875 produces erect, rough and dark green leaves with heavy wax deposition in the abaxial surface of the leaf under both stressed and non-stressed conditions and it appears on the adaxial surface of the leaf under severe water stress. Excalibur has narrower leaves compared to RAC875 and Kukri but produces moderate amounts of waxes on the abaxial surface of the leaf under water stress. Kukri, in contrast, is a non-waxy cultivar with broad, non-erect, pale green and smooth leaves. These cultivars significantly ($P < 0.01$) differed in their level of leaf waxiness. Wax deposition typically started on the flag leaf sheath and the abaxial surface of the flag leaf blade and its expression increased with timing and water stress. Under water stress, RAC875 displayed the highest level of wax deposition (Table 3-7), with the abaxial surface completely covered with wax (score of 5.0). Kukri produced the least amount of wax in response to water deficit (score of 1.5) whilst Excalibur plants displayed an intermediate level of wax deposition. Leaf rolling was scored on the flag leaves of stressed plants during cyclic drought stress. As water stress progressed, Excalibur started leaf rolling faster than Kukri and RAC875 and had the highest value of leaf rolling during the RW treatment (score of 4). Leaf rolling was observed in Kukri (score of 3) at higher levels of water deficit than those required to elicit rolling in Excalibur, while RAC875 showed almost no leaf rolling (Table 3-7). In RAC875 the symptoms of leaf rolling were only induced when the soil water content decreased to very low levels (approximately 6% in pots, at ~ -10 bar).

Table 3-5. Water consumption during the plant growth period. Data presented as average water use per pot in litre.pot^{-1}. Total amount of water applied to each pot during the plant growth before and during the water stress imposition and the estimated water use efficiency (WUE) are given (each value represents the mean ± SE).

Treatments	WW						RW					
Experiments	I			II			I. Severe stress			II. Mild stress		
Traits	Excalibur	Kukri	RAC875	Excalibur	Kukri	RAC875	Excalibur	Kukri	RAC875	Excalibur	Kukri	RAC875
Water use before stress (litre·pot^{-1})	2.9 ± 0.2	2.8 ± 0.3	2.8 ± 0.3	4.5 ± 0.4	3.4 ± 0.1	3.8 ± 0.1	3 ± 0.2	2.8 ± 0.2	3.1 ± 0.3	5.1 ± 0.2	3.2 ± 0.1	3.3 ± 0.1
water use during stress (litre.pot^{-1})	13 ± 0.3	12.4 ± 0.1	11.5 ± 0.3	12.0 ± 0.3	13.6 ± 0.4	11.3 ± 0.5	7.1 ± 0.1	5.8 ± 0.2	6.0 ± 0.3	7.0 ± 0.2	6.4 ± 0.1	6.0 ± 0.1
Total water used (litre.pot^{-1})	15.9 ± 0.5	15.1 ± 0.4	14.3 ± 0.5	16.5 ± 0.6	17 ± 0.5	15.1 ± 0.6	10.3 ± 0.2	8.6 ± 0.2	9.1 ± 0.5	12.1 ± 0.4	9.6 ± 0.1	9.3 ± 0.1
WUE[a] (g/ litre.pot^{-1})	3.8 ± 0.1	4.2 ± 0.1	4.2 ± 0.1	5.3 ± 0.2	6.3 ± 0.1	7.2 ± 0.6	3.3 ± 0.1	3.7 ± 0.2	3.8 ± 0.2	5.5 ± 0.2	6.4 ± 0	6.4 ± 0.1

a) WUE= Total biomass/total water consumed

Table 3-6. Mean value of agronomic traits of Excalibur, Kukri and RAC875 under controlled growth room conditions. Booting, heading time and anthesis (Maturity related traits) , plant height, peduncle length and spike length (height related traits), grain yield, number of grains per spike, grain weight per spike, number of spikelets per spike, number of grains per three main spikes, thousand grain weight, number of tillers per plant, the proportion of tiller abortion (yield and its components), total biomass (shoot and root mass), root mass, root-to shoot ratio and harvest index for three cultivars grown under well watered and (WW) re-watering (RW) treatments are given (each value represents the mean ± SE).

	Treatments	WW						RW (Cyclic drought)					
	Experiments	I			II			I. Severe stress			II. Mild stress		
	Traits Cultivar	Excalibur	Kukri	RAC875	Excalibur	Kukri	RAC875	Excalibur	Kukri	RAC875	Excalibur	Kukri	RAC875
Maturity	Booting (DAP)	75.3 ± 0.3	71.3 ± 0.7	72.7 ± 0.7	78 ± 0.6	63.6 ± 1.2	68.5 ± 0.5	75.3 ± 0.7	72 ± 0.6	71.3 ± 0.3	77.5 ± 0.4	61.9 ± 0.2	65.8 ± 0.3
	Heading time (DAP)	80.3 ± 0.8	75.8 ± 1.5	79 ± 0.4	86.8 ± 0.3	68.8 ± 0.5	71.9 ± 0.4	80.6 ± 0.4	77.6 ± 0.9	78 ± 0.6	84.5 ± 0.1	69.1 ± 1.1	73.3 ± 1.1
	Anthesis (DAP)	86 ± 0.6	81.3 ± 0.9	82.7 ± 0.3	90.9 ± 3.3	77.5 ± 0.6	81 ± 0	85 ± 0	81 ± 0.6	82 ± 0.6	87.8 ± 0.1	76.4 ± 0.1	78.8 ± 1.3
Height	Plant Height (cm)	61 ± 0.8	69.8 ± 1.1	65.7 ± 1.6	76.8 ± 1.6	80.3 ± 1.7	75.1 ± 0.9	49.9 ± 1.8	51.6 ± 1.5	52.3 ± 1.2	62.5 ± 0.5	67.2 ± 1.4	63.1 ± 1.1
	Peduncle length (cm)	20.3 ± 0.7	30.8 ± 0.8	24.5 ± 1.8	26.7 ± 0.6	40.8 ± 0.7	33.6 ± 0.5	15.9 ± 0.7	19.4 ± 0.6	18.5 ± 0.3	19.5 ± 0.7	31 ± 0.7	23.2 ± 0.5
	Spike length (cm)	-	-	-	11.3 ± 0.2	11.7 ± 0.3	11.7 ± 0.1	-	-	-	13.1 ± 0.1	11.2 ± 0.2	11.5 ± 0.1
Yield & its components	GY (g.pot^{-1})	20.7 ± 0.8	21.3 ± 0.8	19.9 ± 1.5	27.7 ± 1.6	34.9 ± 1.9	31.5 ± 1	4.4 ± 0.4	3.6 ± 0.7	6.4 ± 0.8	11.9 ± 1.2	17.8 ± 0.8	20.3 ± 0.3
	Grains.spike^{-1}	-	-	-	177.0 ± 7.8	180.5 ± 6.9	138.8 ± 1.9	-	-	-	160 ± 7.2	161 ± 5	165.8 ± 7.6
	Grain weight.spike^{-1} (g)	-	-	-	5.6 ± 0.2	5.5 ± 0.3	6.2 ± 0.1	-	-	-	7.3 ± 0.3	5.8 ± 0.2	7.9 ± 0.5
	Spikelet No.	-	-	-	21.8 ± 1.9	20.3 ± 1.6	19.4 ± 1.5	-	-	-	26.7 ± 0.2	18.5 ± 0.2	19 ± 0.2
	Seeds/head	71.1 ± 7.2	93.6 ± 1.2	82.9 ± 4.7	59 ± 2.6	60.2 ± 2.3	46.3 ± 0.6	66.7 ± 2.8	44.5 ± 11.6	64.3 ± 4.7	53.3 ± 2.4	53.7 ± 1.7	55.3 ± 2.5
	TGW (g)	40 ± 0.9	36.9 ± 0.7	48.6 ± 1.1	31.7 ± 2	30.2 ± 0.7	44.7 ± 0.7	37 ± 0.8	34.8 ± 2.4	45.3 ± 1.3	45.8 ± 2.7	35.8 ± 1.1	47.9 ± 1.3
	Tillers.plant^{-1}	8.5 ± 0.5	6.9 ± 0.1	5.6 ± 0.3	8.5 ± 0.4	8.8 ± 0.4	7 ± 0.2	8 ± 0.4	7 ± 0.3	4.8 ± 0.3	4.9 ± 0.2	5.3 ± 0.1	3.6 ± 0.1
	Aborted tillers.plant^{-1}	1.3 ± 0.5	0.8 ± 0.2	0.6 ± 0	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	6.2 ± 0.3	4.5 ± 0.2	2.6 ± 0.4	2.1 ± 0.4	0.9 ± 0.4	0.2 ± 0.1
	Abortion (%)	14.5 ± 5.2	10.8 ± 2.2	11.4 ± 1.3	2.1 ± 1.2	1 ± 1	2.5 ± 2.5	77.8 ± 0.7	64.4 ± 2	53.4 ± 6.4	75.6 ± 15.7	20.8 ± 7.7	4.9 ± 3.4
Biomass	Biomass (g.pot^{-1})[a]	59.6 ± 1.7	64.2 ± 1.6	59.9 ± 2.6	90.5 ± 3.1	111.2 ± 3.8	112.3 ± 4.8	33.7 ± 1.7	32.1 ± 0.5	34.6 ± 0.8	66.9 ± 2.5	61.9 ± 1.4	58.1 ± 1.4
	Root mass (g)	5.6 ± 0.5	7.9 ± 0.3	6.7 ± 0.3	10.8 ± 0.3	10.7 ± 1.1	9.4 ± 0.5	6.5 ± 0.2	6.1 ± 0.4	6.4 ± 0.2	12.6 ± 0.9	7.6 ± 0.3	7.1 ± 0.4
	Root/shoot ratio (%)	10.8 ± 0.8	13.97 ± 0.3	12.67 ± 0.4	11.95 ± 0.53	9.61 ± 0.8	8.4 ± 0.2	23.98 ± 1.1	23.5 ± 2.1	22.9 ± 0.5	17.8 ± 0.97	12.3 ± 0.6	12.2 ± 0.91
	HI_B	0.38 ± 0.02	0.38 ± 0.01	0.37 ± 0.01	0.35 ± 0.01	0.35 ± 0.01	0.31 ± 0.01	0.16 ± 0.01	0.14 ± 0.02	0.23 ± 0.02	0.22 ± 0.03	0.33 ± 0.01	0.4 ± 0.01
	HI_TB	0.35 ± 0.02	0.33 ± 0.01	0.33 ± 0.01	0.31 ± 0.01	0.31 ± 0.01	0.28 ± 0.01	0.13 ± 0.01	0.11 ± 0.02	0.18 ± 0.02	0.18 ± 0.02	0.29 ± 0.01	0.35 ± 0.01

a) Biomass=Shoot mass + Root mass, HI_B= GY/above ground biomass and HI_TB=GY/Total biomass

The average number of green leaves that were maintained during the grain filling period at 86, 90, 95 and 105 DAP were different between treatments and cultivars. The number of green leaves was significantly ($P < 0.001$) reduced under RW treatment than under WW treatment. Nevertheless, under both WW and RW treatments, Excalibur and RAC875 had significantly ($P < 0.001$) more green leaves compared to Kukri (Table 3-8). The relative number of green leaves maintained (%) under RW compared to WW treatment is shown in Figure 3-4. On days 86 and 90, the percentages of retained green leaves in Excalibur plants were higher (90.3 %) compared to RAC875 and Kukri (about 87.5%). Five and ten days later, the percentage of retained green leaves were similar in Excalibur and RAC875 (88.5 % and 85.2 % for day 95 and 105, respectively), which were higher than those in Kukri (84.3 and 81.3 %, Fig. 3-4). These data suggest that Excalibur and RAC875, in addition to having higher chlorophyll content, they can maintain more green leaves compared to Kukri with increasing water stress.

Water stress affected individual flag leaf area (FLA). Plants under RW treatment showed significantly ($P < 0.001$) smaller FLA compared to plants under WW treatment (Table 3-8). Significant differences between cultivars under RW and WW treatments were observed. Under WW treatment, Kukri showed significantly ($P < 0.001$) higher FLA (39.2 ± 1.3 cm^2) compared to Excalibur and RAC875 (32.6 ± 1.3 and 34.6 ± 1.3 cm^2, respectively). However, under RW treatment, RAC875 had the smallest FLA (21.6 ± 1.1 cm^2), which was highly significant ($P < 0.001$). The specific leaf area (SLA, $cm^2.g^{-1}$) and the leaf dry matter content (LDMC, $mg.g^{-1}$) were used to estimate leaf thickness (LT). Under WW, although, the LT for RAC875 was larger than Excalibur and Kukri, it was not statistically significant. Under WW treatment, although the LT for RAC875 was bigger than Excalibur and Kukri, the difference was not statistically significant. However, under RW treatment, RAC875 showed significantly ($P < 0.001$) higher leaf thickness compared to Excalibur and Kukri (Table 3-8). Water stress had no effect on leaf thickness in Excalibur, whilst it led to a 13.4 % and 30.5 % increase in leaf thickness in Kukri and RAC875, respectively. Water loss from excised flag leaf was significantly ($P < 0.001$) higher in Kukri compared to Excalibur and RAC875 under both WW and RW treatments. Although RWC was higher (95.1 ± 0.2 %) in non-stressed plants than stressed ones (90.8 ± 0.5 %), there were no significant differences between cultivars at these levels of RWC.

Under water stress, collectively, RAC875 showed higher chlorophyll content, greater leaf waxiness, smaller flag leaf area and thicker leaves compared to Kukri and Excalibur. High chlorophyll content in RAC875 might be a reflection of leaf thickness in this cultivar. Thicker leaves would have a higher density of chloroplasts per unit area, and therefore chlorophyll content per unit leaf area (Araus et al., 1986). Excalibur, on the other hand, showed more leaf rolling, was moderate in leaf waxiness, retained more green leaves same as RAC875, larger leaf area and smaller leaf thickness. Both drought tolerant cultivars had lower excised-leaf water loss, reflecting lower residual transpiration.

Table 3-7. Leaf traits from Experiment I and II, where plants were subjected to well watered (WW) and cyclic drought (RW) treatments. Values for chlorophyll content (ChlC), leaf waxiness, leaf rolling and the average number of green leaves on 95 DAP of Excalibur, Kukri and RAC875 representing averages of combined Experiment I and Experiment II. Values for flag leaf area (FLA), specific leaf area (SLA), leaf dry matter content (LDMC) and leaf thickness (LT) were measured in Experiment I, while excised leaf water loss (ELWL) was measured in Experiment II (each value represents the mean ± SE).

	WW			RW		
Traits	Excalibur	Kukri	RAC875	Excalibur	Kukri	RAC875
ChlC (SPAD unit)	49.1 ± 1.7	51.1 ± 0.4	57 ± 0.7**	54.8 ± 1.7	54 ± 0.9	61.7 ± 1.4**
Waxiness (1-5)	1.5 ± 0.1	1.0	4.0 ± 0.1**	3.0 ± 0.1*	1.5 ± 0.2	5.0 ± 0.2**
Leaf rolling (1-5)	1.0	1.0	1.0	4.0 ± 0.1**	3.0 ± 0.2*	1.4 ± 0.1
Retained green leaves	3.6 ± 0.2*	2.8 ± 0.09	3.7 ± 0.1*	3.3 ± 0.1*	2.2 ± 0.05	3.2 ± 0.1*
FLA (cm^2)	32.6 ± 1.3	39.2 ± 1.3**	34.6 ± 1.3	29.8 ± 0.9**	26.8 ± 2.5**	21.6 ± 1.1
SLA ($cm^2.g^{-1}$)	243.6 + 12.4	250.1 + 11.8	234.1 + 11.8	258.9 + 9.4	231.4 + 7.7	209.9 + 8
LDMC ($mg.g^{-1}$)	268.4 + 11.0	273.5 + 10.8	260.5 + 10.1	261.7 + 7.0**	255.1 + 7.1*	221.1 + 4.8
LT	15.6 + 1.5	14.9 + 1.4	16.7 + 1.6	15.1 + 1	16.9 + 1.0	21.8 + 1.3**
ELWL (%)	43.9 ± 1.4	49.0 ± 0.6**	41.7 ± 1.1	35.3 ± 3.4	57.7 ± 3.0**	47.6 ± 2.6

* and ** show significant differences at 0.05 and 0.001 level with ANOVA, respectively.

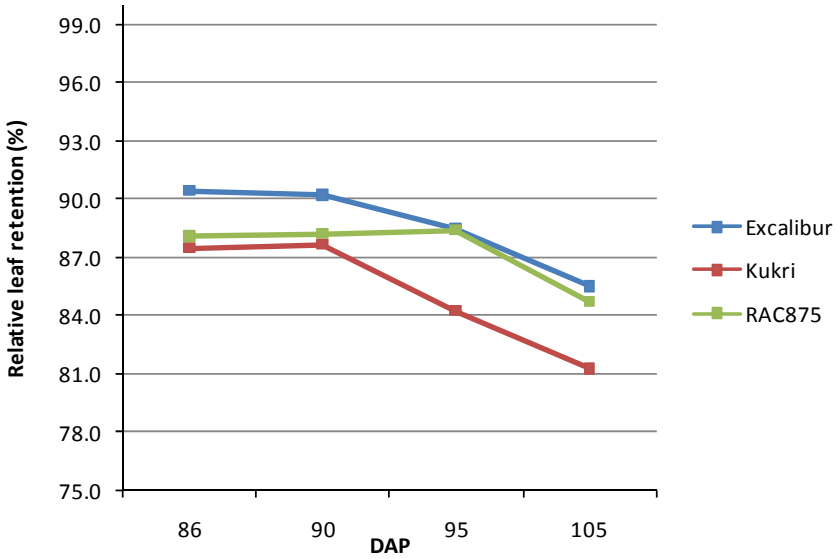

Figure 3-4. Proportion of retained green leaves under water stress (RW) compared to control (WW) in Excalibur, Kukri and RAC875. The number of retained green leaves was counted 86, 90, 95 and 105 days after planting (DAP) in Experiment I.

3.3.3 Physiological traits

3.3.3.1 Water status measurements from glasshouse experiment

The average osmotic potential (OP) of leaves of all cultivars at the initiation of withholding water was -1.55 MPa and decreased to -3.03 MPa by the end of the experiment. After six day of drying, the differences between cultivars became apparent, with OP in Kukri and RAC875 becoming progressively less negative than Excalibur in successive drying conditions (Fig. 3-5). After withholding water from the pot, it took 14 d to reach to the level of RWC = 60%. It seems that the soil water depleted gradually since the coco-peat retains water for longer compared with the sandy-clay soil in the field.

To determine the OA for the three cultivars, two regression lines were calculated based on logarithmic conversion of RWC and OP values (see Materials and Methods). Regressions of RWC on OP for the three cultivars are presented in Figure 3-6. Figure 3-6 shows the relationship between RWC and OP which represents a biphasic (phase α and β in Fig. 3-6) response (Wright et al., 1983). The magnitude of the first phase differed substantially between cultivars. There was a clear response in Excalibur and RAC875. Both showed small changes in RWC as OP declined. The initial response did

not follow the behavior of an ideal osmometer (dashed line), which can be mainly attributed to OA (Fig. 3-6). In the second phase, however, OP declined linearly with RWC. Therefore, decline in OP in the second phase (β) can be attributed to an increase in solute concentration due to water loss (Morgan, 1980a; Wright et al., 1983). The OA value for Excalibur (0.846 MPa) was greater than for RAC875 (0.364 MPa) and Kukri (0.097 MPa), indicating a greater degree of solute accumulation (Table 3-9). The calculation of OA based on Morgan's method showed that Excalibur had the highest and Kukri the lowest OA; RAC875 was intermediate (Table 3-9).

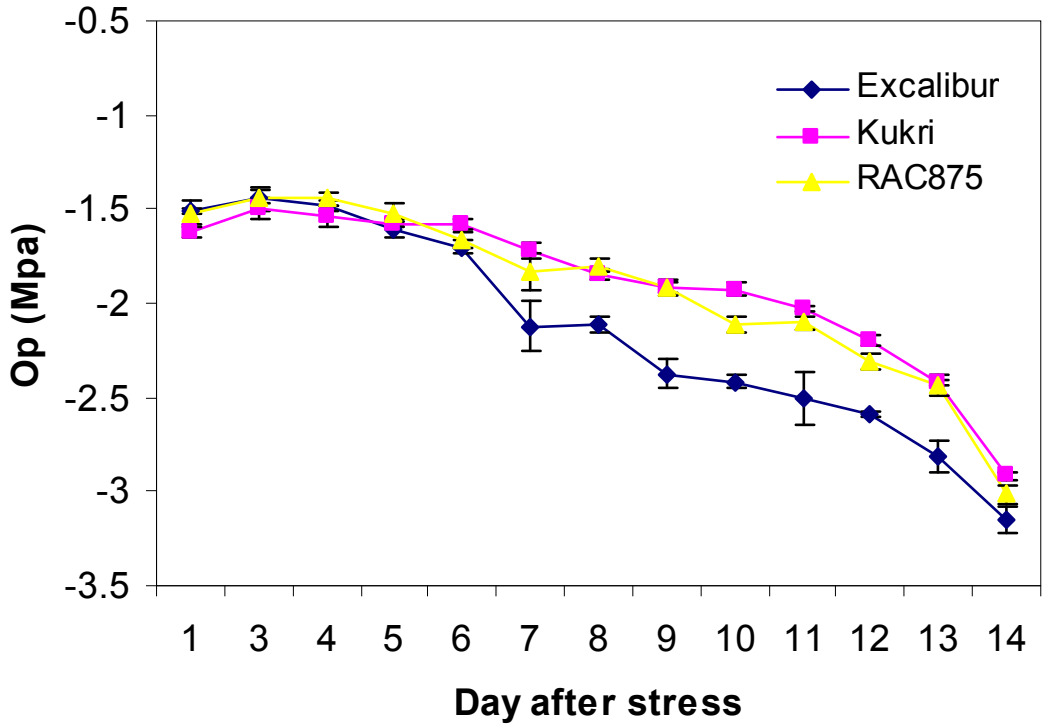

Figure 3-5. Decrease in osmotic potential with successive water stress in Excalibur, Kukri and RAC875 in glasshouse Experiment.

Table 3-8. The calculated OA and the predicted OP at 70% relative water content for the concentration effect and expressed sap osmotic potential for Excalibur, Kukri and RAC875.

Cultivars	OA (MPa)	OP due to concentration effect (MPa)	OP (MPa)	Regression model for phase β (RWC and OP)	r^2
Excalibur	0.846	-0.31 (-2.04) [a]	-0.46 (-2.89)	*y = 1.08x - 2.46*	0.84
Kukri	0.097	-0.35 (-2.25)	-0.37 (-2.35)	*y = 0.79x - 1.82*	0.97
RAC875	0.364	-0.31(-2.05)	-0.38 (-2.42)	*y = 1.05x - 2.32*	0.90

a) Data in parenthesis are anti logarithmic values of OP

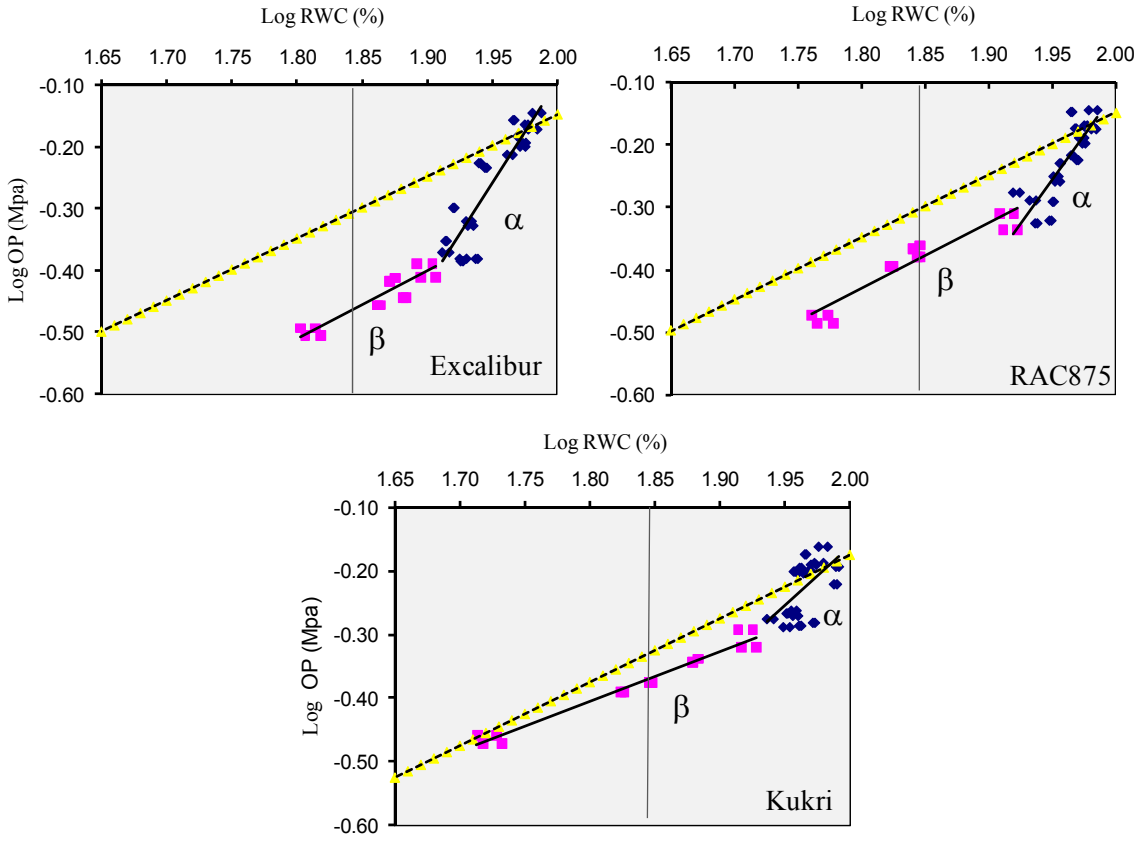

Figure 3-6. The linear regressions of osmotic potential (OP) and relative water content (RWC) in log scale. Relationship between RWC and OP for flag leaves of Excalibur (a), Kukri (b) and RAC875 (c). The dashed line is the response of an ideal osmometer and solid line is the actual fit for RWC vs. OP. the vertical gray line is the logarithm of RWC=70%. There are two linear phases which in the first phase (α) there was little changes in RWC as OP declined, while at the second phase (β), RWC declined linearly with OP. Three cultivars are grouped according to their response to high (a), low (b) and medium (c) osmotic adjustment (OA).

3.3.3.2 Stomatal conductance

Stomatal conductance was measured once in Experiment I and three times in Experiment II 24 h and 48 h after re-watering to investigate plant recovery. Average stomatal conductance and leaf temperature measurements from Experiments I and II are given in Figure 3-7. These measurements revealed that Excalibur has an intrinsically higher stomatal conductance than Kukri and RAC875. Excalibur showed significantly ($P < 0.01$) higher stomatal conductance than Kukri and RAC875 at both sampling time points and under both watering regimes. 24 h after re-watering, the stomatal conductance of plants in the RW treatment was lower than those in the WW treatment. However, by 48 h after re-watering stomatal conductance of all cultivars had returned to levels exhibited by plants in the WW treatments. Excalibur had the highest stomatal conductance under RW treatment ($P < 0.001$). Kukri had the lowest stomatal conductance on the second day after re-watering. Excalibur recovered much more rapidly after re-watering than the other two cultivars. 24 h after re-watering the stomatal conductance of RW Excalibur was about 57% of the level of the WW Excalibur, whereas the stomatal conductance of RW Kukri and RAC875 stomatal conductance was only about 33% and 28% of the WW plants, respectively. The recovery rates after 48 h of re-watering were 95%, 88% and 118% for Excalibur, Kukri and RAC875, respectively (Fig. 3-7a).

Plants subjected to cyclic drought showed lower stomatal conductance during stress and recovery of stomatal function occurred two d after re-watering. Our data show that stressed plant had more stomata closure in the first day after re-watering and they were recovered at the second day. The stomatal conductance results from all series of measurements showed that Excalibur had the highest stomatal conductance at the first and the second day after re-watering. RAC875 had similar response as Kukri at the first day, but it showed slightly higher stomatal conductance at the second day after re-watering.

The leaf temperature of re-watered Excalibur plants was significantly ($P < 0.01$) lower than Kukri and RAC875 at both time points reflecting the higher transpiration rate of Excalibur following re-watering (Fig. 3-7b). The relationship between stomatal conductance and leaf temperature was highly significant ($P < 0.01$) under cyclic drought

treatment (r = -0.72 and r = -0.73, 24 and 48 h after re-watering, respectively), but there were no significant correlations between stomatal conductance and leaf temperature under WW treatment.

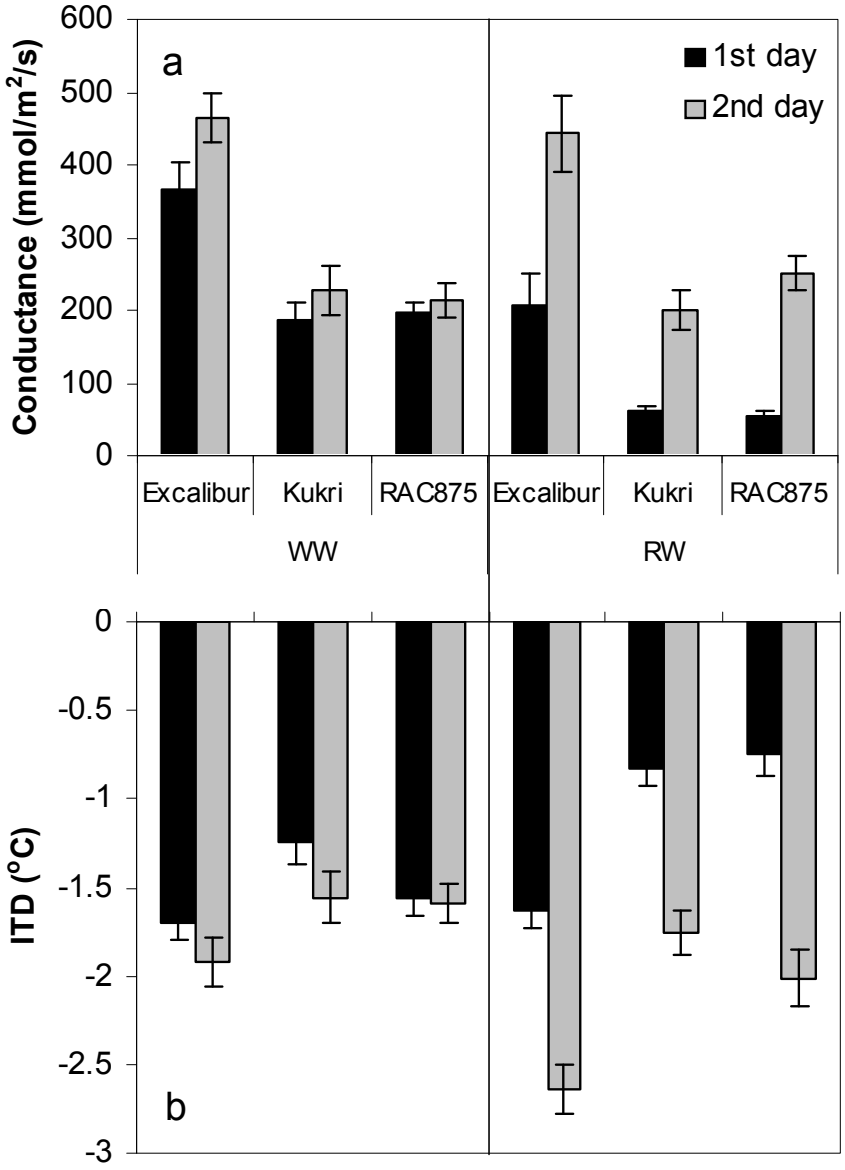

Figure 3-7. The average stomatal conductance in experiments I & II for the first and second day after re-watering. in experiment I, measurements were done in one period, but in experiment II three periods of measurements were performed (a). Leaf initial temperature differences (ITD) after re-watering (b). ITD was calculated by subtracting the temperature of the ambient air of the leaf temperature. Error bars are SE of means.

3.3.3.3 Chlorophyll content and fluorescence

Chlorophyll content and fluorescence of plants under WW and RW treatments were investigated in Experiment II. RAC875 had the highest chlorophyll content (55 to 65 SPAD units) under both stressed and non stressed conditions. In all cultivars the imposition of drought stress resulted in an increase in chlorophyll content until plants reached anthesis (Figure 3-8a and b). Following anthesis (80 DAP), the chlorophyll content of droughted Kukri and Excalibur plants decreased, whilst the chlorophyll content of RAC875 leaves continued to increase for a further 19 d. Drought had the most pronounced effect on chlorophyll content in Kukri plants, with leaves of drought-treated plants containing approximately 29% less chlorophyll than well-watered plants at 108 DAP. Conversely, at 108 DAP the chlorophyll content of drought stressed RAC875 and Excalibur plants was similar to well-watered plants at the same stage of development. A significant positive correlation was found between chlorophyll content and grain size ($r = 0.52$ and $r = 0.87$, $P < 0.001$).

The *Fv/Fm* ratio, which is an indication of the maximum yield of photosystem II photochemistry, ranged between 0.82 and 0.78. There were no significant differences over time among cultivars under WW treatment (Fig 3-8c). Under RW treatment, Fv/Fm did not change (0.81) at the first measurement date (87 DAP) and cultivars displayed similar photosynthetic responses. Although, Fv/Fm did not change in Excalibur and RAC875 at the time of measurements 97, 104 and 109 d after planting, it decreased dramatically in Kukri plants (from 0.82 to 0.65) under the RW treatment (Fig. 3-8d). This decrease was nearly 6% on day 97, 20% on day 104 and remained steady until day 109. Despite considerable differences in the dynamics of leaf chlorophyll content under the WW conditions, the fluorescence measurements revealed no significant differences between cultivars over a period of 27 days post anthesis (Fig. 3-8c). Chlorophyll fluorescence from light-adapted measurements, photochemical quenching (*qP*), showed a similar pattern of Fv/Fm, with slight differences in the WW treatment. Under the WW treatment, RAC875 with high chlorophyll content showed a high level of *qP* at 87 d compared to Kukri and Excalibur (Fig. 3-8e). At 109 d, RAC875 and Excalibur were similar, while *qP* in Kukri decreased by about 5%. Despite a decrease in the chlorophyll content on day 109 in Kukri, *Fv/Fm* and *qP* showed little changes during senescence of flag leaves. These data suggest that the large decrease in

chlorophyll content of Kukri leaves post anthesis is not pre-programmed leaf senescence. It might rather be related to leaf aging (Lu and Zhang, 1998; Lu et al., 2002).

However, under the RW treatment, chlorophyll fluorescence measurements were found to reflect the chlorophyll content observations. Chlorophyll fluorescence of RAC875 and Excalibur were unaltered by the drought treatment consistent with our observation that both cultivars maintained chlorophyll content under these conditions. In contrast, chlorophyll florescence of Kukri was significantly reduced under the RW treatment indicating that leaf senescence was accelerated under drought. This may be associated with a programmed (drought-induced) leaf senescence phenomenon (Munne-Bosch and Alegre, 2004). Although programmed leaf senescence and leaf abscission contribute to plant survival under drought conditions in nature (Munne-Bosch and Alegre, 2000, 2004), this strategy can lead to yield loss in economically important annual crops (Borrell et al., 2000; Jiang et al., 2004; Rivero et al., 2007). On the basis of the assumption that leaf senescence is a type of programmed cell death (PCD) that is inappropriately activated during drought stress, Rivero et al. (2007) generated transgenic tobacco plants expressing an isopentenyltransferase gene driven by a stress- and maturation-induced promoter. They found that the suppression of drought-induced leaf senescence resulted in excellent drought tolerance. The transgenic plants maintained relatively high water content and retained photosynthetic activity during the drought compared to control plants. Additionally, the transgenic plants grown under restrictive water supply showed a minimal yield loss when watered with only 30% of the amount of water used under control conditions.

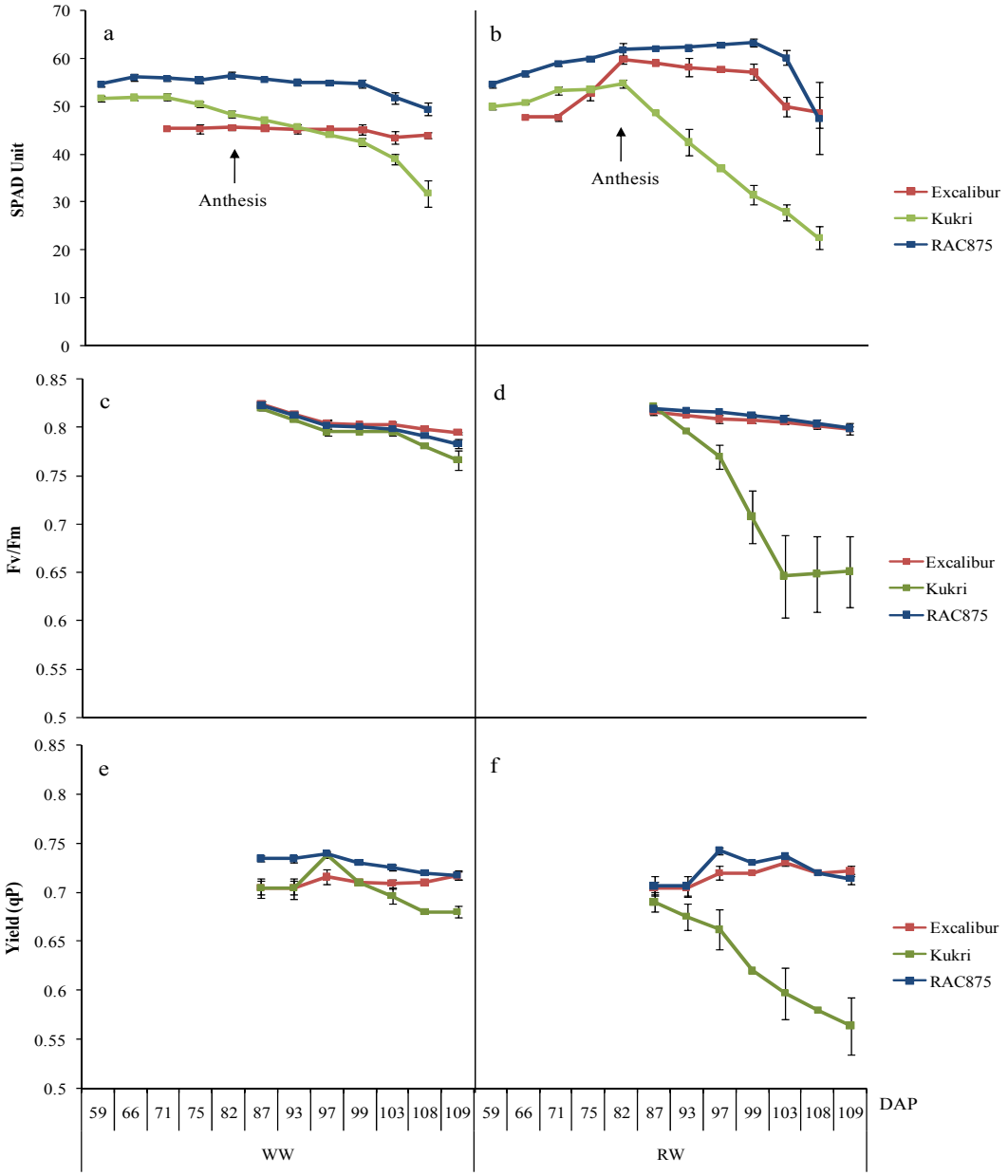

Figure 3-8. Chlorophyll content and fluorescence (Fv/Fm ratio) in Excalibur, Kukri and RAC875 for experiment II. The chlorophyll content under WW and RW treatments (a and b), large error bar on day 108 for RAC875 under RW treatment is because of senescence starting in flag leaves, Fv/Fm under WW and RW treatments (c and d) and photochemical quenching (qP) under WW and RW treatments (e and f). The measurements were done on the same flag leaves during the plant growth and during the grain filling period. Each value represents the mean of four measurements. The error bars are SE of means.

3.3.3.4 Water soluble carbohydrates (WSC)

Fig. 3-9a shows the WSC of plants 5 d after anthesis. Stem WSC of all cultivars increased under RW relative to the WW treatment ($P < 0.01$). Under the WW treatment, RAC875 had the highest WSC content (193.1 ± 22.8 mg.g-1 DW), whereas the WSC content of Kukri and Excalibur was similar (111.7 ± 20.8 and 119.6 ± 40.8 mg.g-1 DW, respectively). However, under the RW treatment the WSC content of RAC875 and Kukri increased to a similar level (246.4 ± 21.0 and 241.1 ± 21.8 mg.g-1 DW, respectively), whilst Excalibur recorded a lower WSC content (159.7 ± 18.7 mg.g-1 DW). WSC in Excalibur did not change in stressed plants compared to non-stressed ones. Comparing the three cultivars, RAC875 and Kukri had higher WSC values compared to Excalibur under stressed conditions.

3.3.3.5 ABA assay

ABA levels were markedly higher in droughted plants compared with WW plants ($P < 0.001$, Fig. 3-9b). The ABA concentration in xylem sap and floral tissues of all cultivars increased under the RW treatment for the three cultivars in both xylem sap and floral tissues. However, the level of ABA was 3-fold higher in floral tissues compared to xylem sap. ABA content increased 3.8 and 11.4 times in spikes and xylem sap due to water stress respectively. Although, there were no differences between cultivars under WW treatment for ABA content, the cultivars showed significant differences ($P < 0.05$) under RW treatment. Excalibur showed lower ABA content in spikelets and xylem sap compared to Kukri and RAC875. One data point in Excalibur was removed from the analysis as it was an outlier observation (181.5 $\mu g.g^{-1}$, versus the mean of 34.5 ± 8.0181.5 $\mu g.g^{-1}$). Water stress caused a 3.3-fold increase in the ABA content of spikes, but no correlation was found between ABA content and grain set and increased levels of ABA did not induce low grain number in this germplasm (Dembinska et al., 1992). This result shows that Excalibur accumulates less ABA under RW treatment indicating that ABA may be a primary controller of stomatal behavior in these plants.

3.3.3.6 Carbon isotope discrimination ($\Delta^{13}C$)

Carbon isotope discrimination ($\Delta^{13}C$) value from grain was highly affected by water stress. In Experiment II, there were no genotypic differences among cultivars for $\Delta^{13}C$

under WW and RW treatments. Plants under WW treatment had significantly (P < 0.001) higher $\Delta^{13}C$ value compared to plants under the RW treatment. The three cultivars exhibited similar $\Delta^{13}C$ values under WW treatment. However, under RW treatment, Excalibur showed a slightly greater $\Delta^{13}C$ value but the differences were not statistically significant (Fig. 3-9c). In general, there were no clear genotypic differences between cultivars under both WW and RW treatment in Experiment II. Highly significant correlations (r = 0.87, P < 0.01) were found between $\Delta^{13}C$ and total water consumed, and $\Delta^{13}C$ and stomatal conductance at the first day after re-watering when plants had experienced water stress under RW treatment (r = 0.8, P < 0.01). Cultivars which showed higher stomatal conductance used more water and had higher $\Delta^{13}C$.

Water use efficiency (WUE), as the ratio between the volume of water consumed and the total biomass (shoot and root) produced, is shown in Table 3-5. ANOVA showed that in Experiment I, WUE was significantly (P < 0.0001) lower compared to Experiment II. In Experiment I, there was a significant difference (P < 0.001) between treatments, while the difference was not significant in Experiment II (Fig. 3-9d). In both experiments, Excalibur showed significantly (P < 0.01) lower WUE compared to Kukri and RAC875. Correlations between WUE and $\Delta^{13}C$ were not found under both WW and RW treatments in Experiment II.

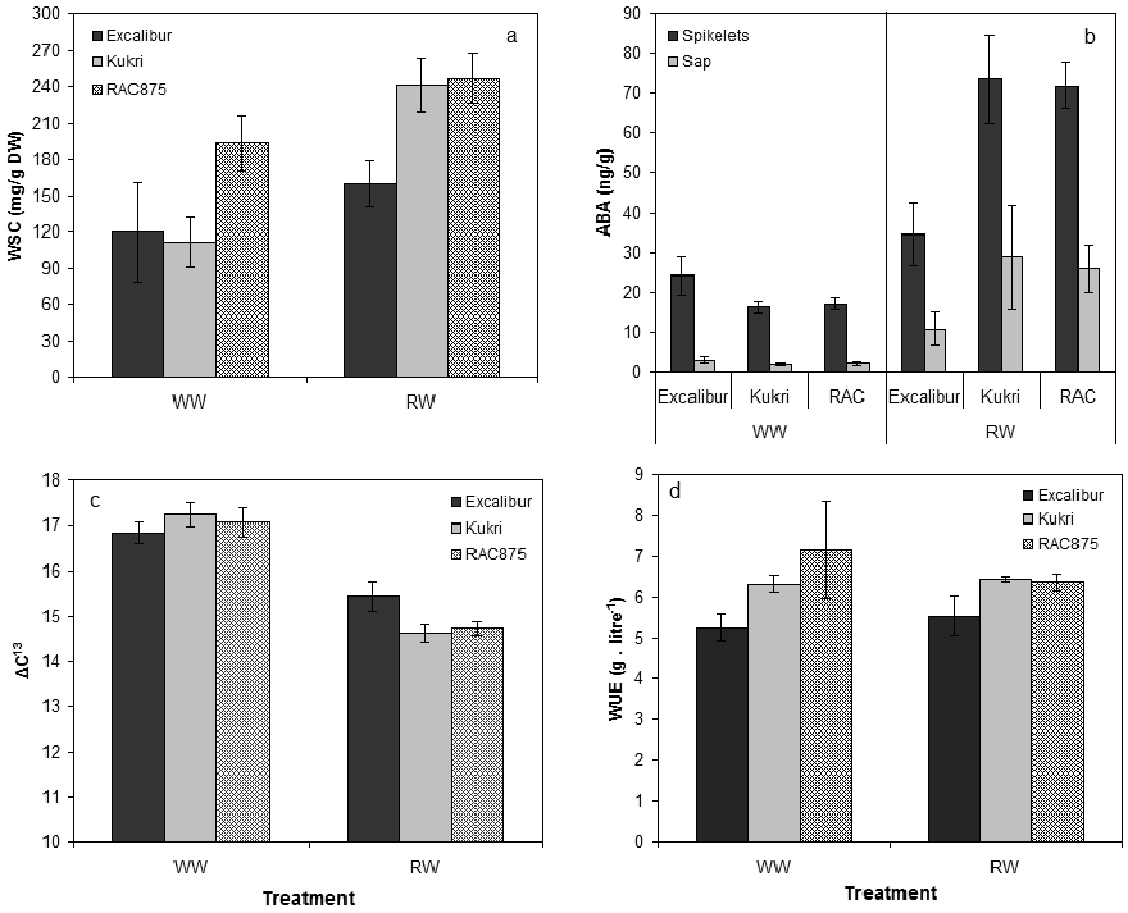

Figure 3-9. Drought related traits for Excalibur, Kukri and RAC875 under WW and RW treatments in experiment II. (a) Values for water soluble stem carbohydrates (WSC), stem samples were taken 5 days after anthesis. (b) ABA concentration in floral tissue and xylem sap. (c) Values for carbon isotope discrimination on grains and (d) Values for agronomic WUE for Excalibur, Kukri and RAC875 under WW and RW treatments Error bars are SE of means.

3.4 Discussion

3.4.1 Grains per spike and tiller number are the major yield components for higher grain yield under cyclic drought

The three cultivars performed similarly in the field and under controlled condition experiments. Under cyclic water stress, both RAC875 and Excalibur, as the more drought tolerant cultivars, outyielded Kukri. The mechanisms underlying drought stress responses of RAC875 and Excalibur seemed different, as evidenced from their agronomic performance in growth room experiments. The main yield components associated with yield reduction across the experiments were grain number per spike and number of fertile tillers. Reduction in grain number and number of fertile tiller was mainly associated with floret sterility and tiller abortion under water stress. Blum and Pnuel (1990) reported that a reduction in spike number was a consequence of tiller abortion under drought stress in wheat. Water stress occurring during later stages of development caused greater reduction in grains per spike, and in total number of tillers per plant. They concluded that maintaining the potential grain number per spike under stress appeared to be more important than the tillering ability (Blum et al., 1990). In our study, RAC875 had the ability to produce less tillers and maintained higher numbers of grains per tiller. Excalibur showed higher tiller numbers and had the ability to recover under cyclic water stress more rapidly and produced more grains on recovered tillers. Kukri did not possess any of these mechanisms. It had high numbers of tillers and did not recover after water stress to produce grains resulting in high numbers of aborted tillers under RW treatment.

3.4.2 Desiccation tolerance via OA

The results from the glasshouse experiment showed that there are different responses among the genotypes for OA capacity. However, at this stage it cannot be fully ruled out that the maturity differences between Excalibur and the other two cultivars also influenced differences for OA. The three cultivars could be ranked from high to low OA. Excalibur showed high, RAC875 moderate and Kukri low OA. These differences in OA capacity may contribute to plant productivity due to fast recovery from water stress after re-watering. Meiotic stage in pollen mother cell and anthesis are more sensitive to water stress, and water deficit during these stages significantly reduces grain

set as a result of male sterility (Saini and Aspinall, 1982). The capacity to adjust osmotically may enhance spikelet fertility due to pollen development. Under severe drought stress, plants with the ability to adjust osmotically can maintain turgor when leaf water potential is reduced (Morgan, 1980a; Blum et al., 1999; Blum, 2006). Morgan and Condon (1986) found a positive relationship between seed set and turgor maintenance, and concluded that genotypes with low turgor maintenance produced lower seed set. OA appears to be associated with extended root growth (Sharp et al., 2004), sustained leaf gas exchange, sustained cellular membrane function, protein function and chloroplast volume and function (reviewed by Blum, 1988; Zhang et al., 1999). OA, in addition to sustaining cellular function under stress, might allow the plants to recover faster from water stress. It also allows plant to maintain turgor and cell function for a longer time under drought conditions.

3.4.3 The role of OA in stomatal conductance and recovery

Stomatal conductance regulates CO_2 diffusion into and H_2O diffusion out of the leaf. Stomatal behavior is closely related to changes in soil water availability, hormonal factors and xylem sap pH (Dodd, 2003; Davies et al., 2005). Plants initially respond to soil water deficits by closing stomata, which is initiated by hormonal accumulation (ABA) and signaling (Goodger et al., 2005; Liu et al., 2005b). Plants subjected to cyclic drought showed lower stomatal conductance during stress, and recovery of stomatal function occurred two days after re-watering. Our data show that stressed plants had more stomatal closure in the first day after re-watering, but they were recovered by the second day. The stomatal conductance results from all series of measurements showed that Excalibur had the highest stomatal conductance at the first and second days after re-watering. RAC875 had a similar response to Kukri on the first day, but then showed slightly higher stomatal conductance by the second day after re-watering.

The genotypic variation in stomatal response in these cultivars may be partly explained by their variation in OA capability. Excalibur, with a high level of OA, showed higher stomatal conductance after recovery, while Kukri had very low OA and kept stomata more closed. Osmotic adjustment in leaves has been demonstrated to maintain stomatal opening (see Turner, 1986 for review). Wright (1983) observed that sorghum genotypes with higher OA capability had the ability to maintain stomatal conductance compared to those with low OA. OA resulted in a lower osmotic potential for a given level of leaf

RWC at a given level of soil water content. Hence it sustained leaf turgor pressure during soil drying (Turner, 1986). Therefore, genotypes with high OA and more leaf turgor maintenance may become less stressed at a given level of water stress. Consequently they produce or accumulate less ABA, resulting in higher stomata activity relative to those genotypes with lower leaf turgor (Ali et al., 1999). In this study ABA was measured in the stressed and non-stressed plants. In Kukri and RAC875, samples for ABA measurements were taken on 75 DAP at a VSWC of 10.0 ± 0.4 % and 8.8 ± 0.5 %, respectively. Excalibur samples were taken on day 83 when VSWC for this line was 9.5 ± 1.0 %. This indicated that pots were very similar in soil water content, but cultivars showed significantly ($P < 0.05$) different ABA contents in both xylem sap and spikelets. Although the cultivars were at the same stress level in terms of water availability, their ABA response appeared different. It might be concluded that higher OA capability in Excalibur allowed these plants to become less stressed which resulted in low ABA contents, higher stomatal conductance and rapid recovery after stress. Turgor maintenance might also delay irreversible cell membrane damage and sustain cell functions which are critical for rapid recovery after relief from severe drought stress (Elmi and West, 1995).

In C3 plants, discriminating against ^{13}C is dependent on the ratio of intercellular to atmospheric carbon dioxide partial pressure (Pi/Pa), a higher $\Delta^{13}C$ resulting from higher Pi/Pa due to higher stomatal conductance (Araus et al., 2002). Environmental conditions such as high light intensity, water deficit stress, salinity and air pollution can affect $\Delta^{13}C$ (Farquhar and Richards, 1984; Condon et al., 1990). This effect is primarily attributed to the decreased stomatal conductance (reviewed by Farquhar et al., 1989). Condon et al. (1992) reported a decrease in $\Delta^{13}C$ in wheat which was attributed to stomatal closure in response to increasing vapor pressure deficit (VPD). Condon et al. (1992) pointed out that variation in $\Delta^{13}C$ among genotypes may be attributed to genotypic variation in the greater extent of soil water extraction near anthesis and variation in the response of stomata to soil water depletion and/or to increasing VPD. Condon et al. (1992) acknowledged that the measurement of $\Delta^{13}C$, in spite its application, has several shortcomings. It does not provide information on the magnitude of either the assimilation rates or transpiration of the stomata, nor whether variation in $\Delta^{13}C$ is being driven by variation in stomatal conductance or photosynthetic capacity. For example, a variety with high stomatal conductance and high photosynthetic capacity would give

similar $\Delta^{13}C$ values as a variety with low conductance and low capacity. However, a segregation population may have all combinations of high, low and intermediate conductance and capacity lines, and therefore a range of possible values of $\Delta^{13}C$. Further investigatins would be required to evaluate for $\Delta^{13}C$ in the mapping populations.

3.4.4 Stay-green trait and its effect on grain filling

The result from chlorophyll content measurements during plant growth indicates that cultivars with high chlorophyll content seem to stay green for longer. RAC875 was a dark green plant with higher chlorophyll content at all times and it stayed green after water stress occurred. Excalibur was ranked moderate for chlorophyll content. However, Kukri was a pale green plant with lower chlorophyll content under both non-stressed and stressed treatments, and leaf chlorophyll content decreased dramatically after anthesis. Chlorophyll retention or stay-green in sorghum was associated with higher leaf chlorophyll content at all stages of development and both were associated with improved yield and transpiration efficiency under post-anthesis drought (Borrell et al., 2000). Betran et al. (2003) pointed out that the correlation between chlorophyll content and grain yield might be related to greater radiation use efficiency in cultivars with higher chlorophyll content. Borrell et al. (2000) found that stay-green sorghum hybrids maintained more photosynthetically active leaves than senescent hybrids under post-anthesis drought conditions.

In our experiments, pale leaves with less chlorophyll content senesced early while dark green leaves with high chlorophyll content consistently stayed green. Kukri showed leaf deterioration and drying soon after anthesis and during grain filling. High chlorophyll content and stay-green in RAC875 likely contributed to its high yielding capacity. Gutierrez-Rodriguez et al. (2004) found a strong association between SPAD reading, photosynthesis rate and yield in wheat cultivars grown under well-irrigated and drought conditions. Zaharieva et al. (2001) reported that chlorophyll content was positively correlated with biomass and grain weight per plant in wild wheat (*Aegilops geniculata*). They also found a positive correlation between leaf color and chlorophyll content, and suggested that chlorophyll loss is the main factor responsible for change in leaf color. Nevertheless, leaf color has been reported to be an important trait in heat stress avoidance. Reduced leaf chlorophyll content can decrease radiation absorbed by the leaf

surface, thereby reducing the risk of desiccation (Blum, 1988; Reynolds et al., 2005). However, the high SPAD values reflect not only an increase in chlorophyll content but also an increase in leaf thickness. Work in rice (Peng et al., 1993) and maize (Chapman and Barreto, 1997) have demonstrated that thicker leaves (high SLW) can have greater SPAD values for the same chlorophyll content of thinner leaves (low SLW). Quarei et al. (2005) have also reported that higher flag leaf chlorophyll content in wheat was associated with leaf thickness.

Our results from chlorophyll fluorescence measurements as a reflection of photosynthetic capacity shows that the flag leaf of the stay-green cultivar remained functional during the grain filling period. The decrease in *Fv/Fm* of Kukri is consistent with chlorophyll content degradation. Differences in photosynthetic capacities during the grain filling period could be due to different amounts of chloroplasts per unit leaf area. Therefore, total chlorophyll content is an indication of the potential photosynthetic capacity and these two traits are correlated. These results suggest that chlorophyll content per unit leaf area could be a good indicator of the activity of photosynthetic tissue. A positive correlation was found between chlorophyll content and total grain weight (TGW) only under water stress treatment. RAC875, with a higher chlorophyll content, had higher grain size, while Kukri with lower chlorophyll content had small and more shrunken grains. The retention of photosynthetic capacity under water stress conditions of stay-green cultivars ensures continued availability of new assimilates and is associated with increased nitrogen uptake during grain filling in sorghum (Borrell and Hammer, 2000), and can potentially improve grain size. Spano et al. (2003) reported a 10-12% increase in grain size of the stay-green mutants of durum wheat. They concluded that the extended period of flag leaf photosynthetic capacity during the phase of grain filling is associated with the production of larger grains. Richards et al. (2001) pointed out that stay-green capability might be a useful trait in environments with a high probability of rainfall during grain filling. Plants with stay-green characteristics and more photosynthetic tissue may further produce assimilates and extract further water from the soil.

3.4.5 WSC as a source for grain filling

Water soluble carbohydrates (WSC) stored from pre-anthesis plant assimilation for remobilization to the grain during grain filling have increasingly been recognised as an

important contributor to grain yield when photosynthesis is inhibited by drought, heat or disease stress during this stage (Nicolas and Turner, 1993; Blum, 1998; van Herwaarden et al., 1998). The stem reserve accumulation, as a backup source during grain filling, is a genetically controlled constitutive trait (Blum, 2002) which is not stress-responsive, while the signal for reserve (e.g. fructans) conversion into soluble fractions that can be translocated from stem to the grain can be stress-responsive (Dubois et al., 1990; Virgona and Barlow, 1991).

The capacity of a genotype to accumulate WSC as a source on one hand, and the demand of the sink for remobilization of the WSC on the other hand are important. Dubois et al. (1990) suggested that enzymatic activity involved with remobilisation may be a function of source-sink interactions. While the size of the storage is important, the strength of the sink organs and thier capacity to utilise the imported carbon is also important for allowing grain filling from stem reserves (Cook and Evans, 1978). Source activity could be partitioned into two components, namely, current photosynthetic assimilates and stem reserve mobilisation. The contribution of current photosynthesis to grain filling in different plant parts (e.g. leaves, ear and awns) is related to their potential photosynthetic activity (Bewley and Black, 1994), which could be affected by environmental stresses such as heat and drought (Blum, 1986). During the grain filling period, therefore, stem reserves can serve as a buffer between the supply of current photosynthetic assimilates produced by the source and the demand by the sink (Borras et al., 2004). The rate of grain growth, endosperm cell number and duration of grain growth are three broad determinants of grain size (Gleadow et al., 1982). The development of the grain can be divided into two stages, grain enlargement and grain filling (Bewley and Black, 1994). Grain enlargement is the result of cell division and number which is set 5 to 15 d after anthesis. The sink size of the grain largely determines the subsequent rate of dry matter accumulation (Nicolas et al., 1984). Therefore, cultivars which had potentially larger grain size and more grain per spike possess a larger sink size.

Blum et al. (1994) suggested that during grain filling when grain growth depends on remobilised stem reserves, a longer grain-filling duration is also more important than a high rate of filling. Although, longer grain filling duration may increase the risk of severe drought and heat stress during the later stages of the grain filling period, remobilization of large amounts of WSC to grain complements the longer grain-filling

duration despite the harsh environment. Shorter grain filling durations may allow some avoidance of terminal stress while longer duration may allow greater utilisation of stem reserves for grain filling under stress (Blum, 1998). Ehdaie et al.(2008) found no correlation between the amount of stem reserves and the amount of current assimilates contributed to grain yield. They concluded that selecting genotypes that simultaneously remobilize relatively greater stem reserves and current assimilates to grain yield under drought could reduce the adverse effect of drought.

In this study, the average WSC in the stem of stressed plants was 1.5-fold higher than in the stem of non-stressed plants. These results are in agreement with Goggin and Setter (2004), who found significantly higher average concentrations of total carbohydrates (1.8-fold) and fructans (2.5-fold) in the stems of rainfed compared to irrigated plants. They speculated that the fructan accumulation in rainfed stems increased in response to water deficit (Goggin and Setter, 2004). Therefore, the synthesis of fructan is an important process in accumulation of WSC in wheat stem (Dubois et al., 1990; Goggin and Setter, 2004). Our results show that RAC875 had a constantly greater capacity for WSC storage under both stress and non-stress treatments. A greater capacity for maximising carbon assimilation during the pre-anthesis period in RAC875 resulted in high WSC concentration. Irrespective of the environment during grain filling, RAC875 had more available WSC than Excalibur and Kukri. While under stress treatment, both RAC875 and Kukri showed higher WSC content compared to Excalibur. In this study, it was impossible to evaluate the proportion of available WSC that contributed to grain filling. It is speculated that cultivars with high WSC at anthesis remobilize high proportion of WSC to the grain during grain-filing even under non stress conditions.

3.4.6 Leaf morphology and its effect on drought tolerance

The wide variation in leaf morphology (e.g. leaf waxiness and leaf rolling) and differences in leaf thickness within cultivars were observed. Leaf glaucousness or waxiness is one characteristic that has been shown to be associated with drought tolerance in wheat (Richards et al., 1986; Blum, 1988; Clarke and Richards, 1988), rice (Haque et al., 1992) and barley (Febrero et al., 1998). An important function of leaf waxiness is to increase the efficiency of stomatal control by reducing water loss after stomatal closure. Clarke and Richards (1988) indicated that leaf waxiness is associated with a large reduction in residual transpiration in durum wheat. Richards et al. (1986)

suggested that in water stressed plants, the effect of waxiness could be greater because of its effect on reduction of leaf temperature which reduces both residual and stomatal water loss. A transgenic study has also shown that over-expression of the *WXP1* gene in alfalfa led to a significant increase in cuticular wax production and the accumulation of more cuticular waxes reduced water loss and increased plant drought tolerance (Zhang et al., 2005). It can be concluded that leaf waxiness in RAC875 is a trait which may reduce water loss through the epicuticular wax layer. This trait might be more advantageous when water stress is intensified with high temperature and light intensities. Leaf waxiness, and pubescence have been reported to contribute to stress avoidance, by reducing radiation absorbed by the plant (Blum, 1988). The leaf waxiness and leaf angle (erectness) in combination may improve radiation use efficiency (RUE). Reynolds et al. (2000) pointed out that photosynthetic rate of the whole canopy can be enhanced by manipulation of leaf angle. Waxiness increases radiation reflectance, reduces leaf and spike temperature and therefore increases leaf and florets survival (Johnson et al., 1983; Richards et al., 1986) and erectophile leaf canopy can use radiation efficiently, while intercepting less radiation (Reynolds et al., 2000). Richards et al. (1986) argued that increasing leaf reflectance should not affect the yield potential of the crop, despite the reduction in photosynthetic radiation penetrating the leaves. They concluded that a 0.5 °C reduction in leaf temperature for six hours per day would be sufficient to extend grain filling by more than three days, depending on the water availability. Wheat genotypes with erect leaves outyielded prostrate genotypes under water stress (Innes and Blackwell, 1983). Leaf rolling can reduce effective leaf area and hence reduce radiation intercepted and consequently reduce transpiration under water stress (Loss and Siddique, 1994).

Leaf growth, size, shape and thickness are greatly affected by the environment, especially by irradiance and water deficit (Araus et al., 1986; Tardieu, 2006). Reduction in leaf growth, which results in a smaller transpiring leaf area, is an adaptive response to water deficit (Tardieu, 2005). Reduction in leaf growth is an avoidance mechanism that prevents cell water stress in both the short and long term (Tardieu, 2006). Tardieu (2006) pointed out that short term reduction in leaf area has a similar role to stomatal closure, which allows plant to avoid damaging leaf water potential in leaves by reducing the water flow through the leaf surface. In the longer term, a reduced leaf area can save soil water for later stages of plant development via reduction in transpiration.

3.4.7 Cyclic drought in pot experiments

The South Australian environment as target region for the current study is characterized by fluctuation in rainfall, shallow high calcareous soils with increasing pH, salinity and sodicity at depth (Rengasamy, 2002). These physical and chemical constraints directly inhibit a deep root system (Rengasamy et al., 2003). In shallow soil, moisture fluctuations are also most rapid and extreme, and every rain event generates a pulse of moisture that, depending on the event, size and evaporative demand of the atmosphere, can last from a few hours to many weeks (Schwinning and Ehleringer, 2001). Our data from the growth room experiments showed that, with target environment considerations, drought experiments under controlled conditions can relate to field performance, as plants were grown in 35 cm long pots and cyclic drought during reproductive stages of development. In addition to reducing unpredictable variables under controlled conditions, it is possible to determine the extent of the drought stress. It also makes it possible to measure extensive traits throughout the experiment. Although, cyclic drought experiment in a growth room worked for a few number of cultivars, there are limitations. In particular, controlling watering regimes is very labor intensive, which makes it difficult to assess large number of genotypes or mapping populations. Also, the described conditions might not work for all genotypes (e.g. genotypes which rely on a deep root system to exploit water from depth, unless deep soils are mimicked (1-2 m long PVC cylinders).

3.5 Conclusions

Cyclic drought is a frequent event in South Australian environments, occurring during pre-anthesis, post-anthesis and grain-filling period. We showed that cyclic water stress under growth room conditions mimicking the drought of the target-environment, working consistently to distinguish drought tolerant and intolerant cultivars. Therefore, data from pot experiments based on this system can be extrapolated with some confidence to the field.

Although Excalibur and RAC875 are evidently more drought tolerant cultivars compared to Kukri, it would not mean that Kukri (a drought susceptible cultivar) does not possess any “drought tolerance traits”. It must be borne in mind that the cultivars which were evaluated in this study (Excalibur, Kukri and RAC875) have been bred

CHAPTER 4

GENETIC LINKAGE MAP CONSTRUCTION FOR THE RAC875/KUKRI DH POPULATION

4 Chapter 4: Genetic linkage map construction for the RAC875/Kukri DH population

4.1 Introduction

A genetic linkage map based on molecular markers is an important tool in genetic analysis and it is a necessary prerequisite to study the inheritance of both qualitative and quantitative traits. A linkage map with sufficient genome coverage and high confidence in locus order is very useful for the assessment of gene effects on phenotypic traits, marker assisted breeding and map-based gene cloning.

Bread wheat (*Triticum aestivum* L.) is an allohexaploid (2n = 6x = 42) with the three distinct but related genomes AA, BB, and DD. Each genome comprised seven chromosomes. It has an extremely large genome of ~16 × 10^9 bp per chromosome (Bennett and Smith, 1976). The size and structure of the wheat genome makes it one of the most complex crop species for genetic analysis. The wheat genome is highly complex and it possesses a high percentage (> 90%) of repetitive sequences (Langridge et al., 2001).

One of the main objectives of this study was to provide a genetic linkage map of the RAC875/Kukri DH mapping population of wheat. The approach for constructing the genetic map was based on using diversity array technology (DArT; http://www.diversityarrays.com) markers to provide a skeleton map to which locus-specific SSR markers were added. This map can be used for the identification of associations between markers, major genes, and QTLs.

4.2 Materials and methods

4.2.1 Plant material

A DH population comprising 368 individuals from a cross between Kukri (76ECN44/76ECN36//MADDEN/6*RAC177) and RAC875 (RAC655/3/Sr21/4*LANCE//4*BAYONET) was used to construct a genetic linkage map. Kukri was released in 1999 by the University of Adelaide. It is a hard white wheat which has excellent grain quality and is rust resistant, but its yield production is low to moderate in low-rainfall environments. It is susceptible to root lesion nematodes. RAC875 is a breeding line from Roseworthy Agricultural Campus. It is a white wheat with high grain quality and a high yielding cultivar under South Australian dry environments. However, it also is susceptible to rust and root lesion nematode. The two cultivars do not carry any known boron tolerance genes. The estimated coefficient parentage for the two parents was 0.08.

4.2.2 DNA extraction

DNA extraction was performed using a DNA midi-prep method (Rogowsky et al., 1991) with minor modifications. About 2g of young leaf material form adult plants were collected into a falcon tube. Collected leaf tissues were stored at -80 °C and then freeze-dried overnight. For grinding, 2 large and 4 medium stainless steel ball bearings were put into the tube and using a flask shaker to grind the tissue to a fine powder for 1 min. Ball bearings were then removed from the tube. For DNA extraction, 4.5 mL of DNA extraction buffer and 4.5 mL of phenol-chloroform-isoamyl alcohol (25:24:1) were added. The extraction buffer was 0.1 *M* Tris-HCl (pH 8.5), 10 m*M* EDTA, 0.1 *M* NaCl, 1% sarkosyl and 2% PVPP (polyvinyl-polypyrrolidone, insoluble). Tubes were vortexed and well mixed for 20 min. The content of the falcon tubes were transferred to yellow capped tubes (10 mL, Sarstedt, Australia) and centrifuged at 4000 rpm for 10 min. The supernatant was then transferred into a silica matrix tube (BD Vacutainer®). The aqueous phase was re-extracted with 4 mL phenol-chloroform-isoamyl alcohol for 5 min on the orbital mixer and centrifuged for 5 min. The supernatant was transferred to a clean yellow capped tube and DNA was precipitated by adding of 400 µL 3 M Na-acetate (pH 4.8) and 4 mL of isopropanol. DNA was collected and transferred into 2 mL Eppendorf tubes, washed with 70% ethanol, dried, and re-suspended in 350 µL of R40

(40 µg/ml heat-treated RNase A in TE buffer [10 mM TRIS-HC1 (pH 8.0), 1 mM EDTA]).

High-throughput DNA extraction from freeze-dried wheat leaf was used to extract DNA of recombinant inbred lines (RILs). Fresh leaf tissues (4 weeks old) of the individuals from each of the RILs were collected into a well of a micro-titre plate and they were stored at -70°C and freeze-dried for overnight. Freeze-dried leaf tissue was ground to a fine powder with the Qiagen grinder (Retsch mill, MM 300) for 5 minutes. The powdered tissue was mixed with 600 µl extraction buffer (0.1 M Tris-HCI pH 7.5, 0.05 M EDTA pH 8.0 and 1.25% SDS) and incubated at 65°C for 1 h, followed by the addition of 300 µl of 6 M ammonium acetate. The mixture was then centrifuged at 3000 rpm for 30 minutes, 600 µl of the supernatant was recovered into a new deep-well micro-titre plate containing 360 µl iso-propanol in each well, mixed thoroughly and centrifuged for 30 minutes at 3000 rpm. Precipitated DNA was rinsed in 500 µl of 70% ethanol and re-suspended in 400 µl of milli-Q water for overnight, then centrifuged to spin down the undissolved debris. Recovered DNA was then diluted 1 in 2 milli-Q water to use in PCR reaction.

4.2.3 Molecular marker analysis

4.2.3.1 SSR markers

Multiplex-ready marker technology (MRT, http://www.genica.net.au) was used for polymorphism screening and also genotyping the mapping population. Multiplex-ready PCR assays and post-PCR pooling of multiplexed assays were performed as described by Hayden et al. (2007). A detailed protocol is provided at http://www.genica.net.au. Briefly, the multiplex PCR amplification takes place in two stages (Fig. 1). In the first stage, SSR loci are amplified with locus-specific primers tagged at their 5' ends with generic sequences. In the second stage, dye labeled primers complementary to the tag sequences amplify the first stage products. By dye-labeling one of the tag primers, the PCR products can be fluorescently labeled during amplification and separated in a detection channel of choice on a DNA fragment analyzer. One of the SSR primers labeled with VIC, FAM, NED and PET for separation and visualization using an ABI 3730 capillary sequencer (Applied Biosystems, Foster City, California, USA).

The multiplex PCR amplification is achieved in a single-step, closed-tube assay. The following PCR profile was used to amplify all multiplex-ready markers, irrespective of whether markers are deployed in the marker screening or genetic mapping. The PCR amplification started with an initial denaturation step of 10 min at 95°C to heat activate the DNA polymerase. PCR was performed in two phases for a total of 65 cycles. The first PCR phase was 25 cycles; 60s at 92°C, 90 s at 50°C and 60 s at 72°C for five cycles. The next 20 cycles were with 30s at 92°C, 90 s at 63°C, and 60 s at 72°C, followed by the second phase with 40 cycles; 15 s at 92°C, 60 s at 54°C, and 60 s at 72°C, and a final extension step of 10 min at 72°C.

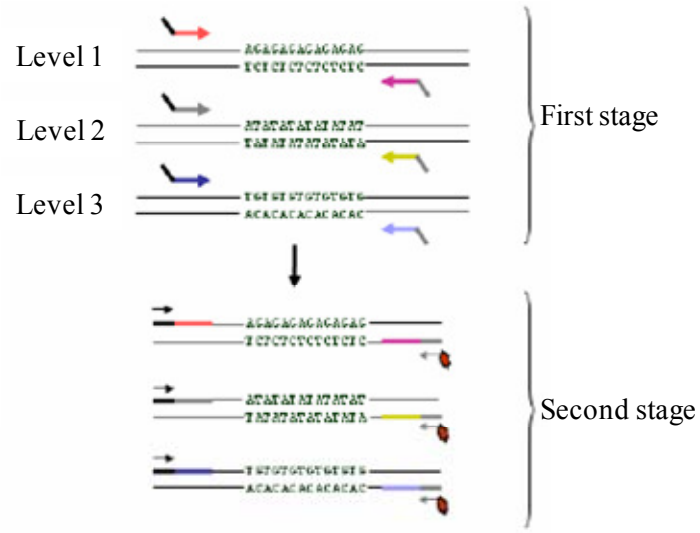

Figure 4-1. Diagram representing multiplex-ready PCR (after Hayden et al., 2007)

4.2.3.1.1 Marker screening

To find polymorphic markers in the population, an experiment was designed using Automated Designer for Marker Screening which was developed by Dr. Matthew Hayden, The University of Adelaide. A set of 850 SSR markers were selected using the Multiplex-Ready Marker database and Multiplex-Ready CMAP Interface. The marker panels comprised of SSRs with non-overlapping allele sizes were created for the selected markers using the BINNER software (http://www.genica.net.au). The DNA samples were the parental lines and DNA pool of bulk 6 DH lines. A pipetting robot Biomek 3000 (Beckman Coulter, Fullerton, CA, USA) was used for dispensing DNA template to 384-well plate to automate PCR setup.

PCR was performed in a 6 µl reaction mixture containing 3 µL master mix and 3 µL of diluted primers (0.4 µM locus-specific primer in sterile water). The master mix containing 1.2 µl 5x Mpx-Rdy Buffer, 0.03 µl Immolase (5U/µl), 0.045 µl 10µM dye-labeled tagF primer, 0.045 µl 10µM tagR primer , 1.0 µl genomic DNA (50 ng/ µl) and 0.68 µl sterile water. The multiplex-ready PCR buffer contains 0.2 mM dNTPs, Immolase buffer (Bioline, Luckenwalde, Germany), 1x buffer contains 16 mM (NH4)2SO2, 0.01% Tween-20, 100 mM Tris-HCl, pH 8.3, 1.5 mM magnesium chloride (MgCl2) and 100 ng/ µl bovine serum albumin Fraction V (Sigma-Aldrich, Tanfkirchen, Germany). The latter reagent is used to help stabilize Immolase DNA polymerase during PCR.

4.2.3.1.2 Genetic mapping

For genetic mapping, markers with known allele size for parental lines were uploaded to BINNER software (http://www.genica.con.au) and used to develop marker panels for the population. The use of the BINNER software allows constructing multiplex-ready marker panels in which markers within the same dye detection channel do not have overlapping PCR fragment sizes. PCR was performed for each marker in a 7 µl reaction mixture containing 4 µL master mix and 3 µL of genomic DNA (25 ng/µL). The master mix containing 1.2 µl 5× Mpx-Rdy Buffer, 0.03 µl Immolase or Tif DNA polymerase (Bioline), 0.045 µl 10µM dye-labeled tagF primer, 0.045 µl 10µM tagR primer and an appropriate concentration of locus-specific Primer.

To prepare PCR products for DNA fragment analyzer, post-PCR protocol was followed (Hayden et al., 2007). The post-PCR procedure covered four steps, firstly, pool PCR products labeled with the same fluorescent dye, secondly, multi-pooling to combine pooled PCR products labeled with different dyes, thirdly desalt PCR products and fourthly, prepare samples for DNA fragment analysis. To pool markers with the same dye, PCR products of each marker were diluted with an appropriate volume of sterile water. Twelve µl of PCR product of each marker was pooled in a 384-well plate. Then a standardized procedure of multi-pooling was used to prepare SSR diluted PCR products labeled with different fluorescent dyes and were pooled at a ratio of 2:2:1:2 for VIC:FAM:NED:PET, and then desalted by ultra-filtration using an AcroPrep 384 filter plate with 10 kDa Omega membrane according to the manufacturer's instructions (PALL Life Sciences, Surry Hills, NSW, Australia). Three µl of desalted PCR product

resuspended in water was added to 8 µl of deionized formamide containing 0.8 µl of GeneScan500 LIZ size standard (Applied Biosystems). The mixture was heated uncovered at 90°C for 5 min to evaporate the water. This multi-pooling procedure resulted in 0.03 µl of each PCR being electrophoresed by ABI 3730. Semi-automated SSR allele sizing was performed using GeneMapper v4 software (Applied Biosystems, Foster City, California, USA).

4.2.3.2 DArT markers

DArT is a high-throughput genotyping system based on a microarray platform which was generated by Triticarte Pty. Ltd. (http://www.diversityarrays.com). DArT as a practical and cost-effective whole-genome fingerprinting tool is described by Jaccoud et al. (2001), Kilian et al. (2005) and Akbari et al. (2006). DArT was successfully applied to rice (Jaccoud et al., 2001), barley (Wenzl et al., 2004), cassava (Xia et al., 2005) and wheat (Akbari et al., 2006; Semagn et al., 2006). The DArT protocol for wheat is well described in Akbari et al. (2006). In brief, a genomic representation of DNA/lines was produced after *Pst*I–*Taq*I digestion, spotted on microarray slides and the individual genotypes screened for polymorphism based on fluorescence signals. The DArT analysis was performed on Triticarte's wheat array version 2.3. This array contains 5,000 wheat clones from the libraries described in Akbari et al (2006). Two hundred and ninety-six loci were scored as polymorphic or present (1) / absent (0), respectively. DArT markers consisted of the prefix "wPt", followed by numbers corresponding to a particular clone in the genomic representation, where w stands for wheat, P for primary restriction enzyme used (*Pst*I) and t for secondary restriction enzyme (*Taq*I).

4.2.3.3 Constructing a genetic linkage map

The scores of all DArT markers (1 and 0) were converted into genotype codes ('A' and 'B') according to the scores of the parents. DArT data were then merged with the segregation data for SSR markers. Map Manager versions QTXb20 (Manly et al., 2001) were used in linkage analysis of the markers. The Kosambi mapping function was used to calculate distances derived from recombination values (Kosambi, 1944). Linkage groups were established by considering all estimates of recombination frequencies. An LOD-score above 3 was used as critical value. If two markers are significantly linked (by LOD value) they belong to the same linkage group. For each segregating marker, a

χ^2 analysis ($P < 0.01$) with 1 d.f. was performed to test for deviation from the 1:1 expected segregation ratio. Markers showing the highest segregation distortion were inspected by looking at the graphical genotype and were, if necessary, removed from linkage groups.

The CMap comparisons were used to identify errors in the genetic map. CMap is graphical viewing tool, which provides an efficient interface for comparing maps among different populations, homoeologous chromosomes within polyploid species, or homologous regions from other species (Carollo et al., 2005). A linkage group was then assigned to a chromosome, when it contained SSR loci that had been assigned to a particular chromosome in previously published genetic maps (http://www.genica.net.au/cmap/crcmpb-live) and also based on map positions of DArT loci on wheat genetic maps (http://www.triticarte.com.au/content/wheat_diversity_analysis.html). Unlinked linkage blocks with anchor markers on the same chromosome were then forced into one linkage group and oriented relative to each other according to the consensus map for SSR markers. The final map was drawn using the MapChart program, v. 2.1 (Voorrips, 2002).

The order of markers in the linkage groups was calculated with RECORD (van Os et al., 2005). After ordering of the markers, data were displayed in a map order as a colour-coded graphical genotype in Microsoft Excel using the conditional cell formatting formula. Singletons (a single locus in one progeny line that shows double recombination with both its directly neighbouring loci) and other potential errors in the marker segregation data were identified by visual inspection of graphical genotypes. SSR markers were reevaluated by visual inspection of the GeneMapper file and corrected if necessary. The identified singletons were replaced by missing values as suggested by Van Os et al. (2005). The corrected data were ordered for a second time with RECORD. Van Os et al. (2005) suggested that in large mapping populations, missing observations do not severely harm the marker order. However, markers with high missing observations should be treated with care (Hackett and Broadfoot, 2003). The number of double crossover events was then calculated for each linkage group. An excess of 5 double crossover events was identified, the segregation data were re-inspected and, if necessary, corrected.

4.3 Results

4.3.1 Molecular markers

4.3.1.1 DArT assay

The analysis has been performed in the population of 368 doubled haploids and 2 parental lines. Two hundred and ninety six DArT markers were scored on 370 lines. 27% of the population (82 individuals) showed higher level of missing values, and out of which 39 samples did not pass all of the quality requirements. The first 43 samples had missing values because these sampled were analysed with different version of array containing 3000 clones, while the rest were analysed with array containing 5000 clones. The overall call rate was 90.1% which was in the normal range compared to the average hexaploid wheat analysis (Akbari et al., 2006). The 39 samples had a relatively high number of missing values and an accordingly lower sample call rate.

The parents of a cross did not always score differently, even when a marker was segregating properly in the progeny. In this case, there were 17 markers out of 296 that did not differentiate the parental lines. There may be various reasons for this, heterogeneity of parental stocks, when the parents of the cross were not the plants from which the DNA is extracted, can cause some scoring difference. It is expected that some of the Mendelian-type DArT markers may be due to stable methylation polymorphisms (Wenzl et al., 2004). Reversion in methylation polymorphism pattern of the DNA in different individuals in the population might be the case. Isidore et al. (2003) suggested that changes in parental *Pst*I-site methylation patterns in the population contributed to the error frequencies observed in the data. Wenzl et al. (2004) reported that unstable methylation caused non-Mendelian behavior of some DArT markers. And scoring error in one parent, especially if DNA quality of parents was not as good as DNA of the progeny (for example DNA was extracted from parents earlier).

4.3.1.2 Microsatellite assay

Out of 850 SSR markers screened on the parental lines, 231 (27%) markers were detected being polymorphic. Of 231 markers, 220 were mapped on 368 DH lines. Few SSR markers (about 5%) that showed polymorphic patterns in parental lines, they were found monomorphic in the progenies. This could be due to heterogeneity of parental

lines or contamination in DNA stock. Some SSR markers showed multi locus pattern varied from 1 to 3 loci. Those multi locus markers were named with suffix (a, b, c…) to create a divided form of marker.

4.3.2 Map construction

A total of 519 markers were obtained, including 296 DArTs and 223 SSRs. These markers were initially mapped in 36 linkage groups, each with 2-20 markers. Markers that were unlinked and highly distorted were excluded from the data set, leaving 495 markers (296 DArTs and 199 SSRs) that were used to assemble the genetic linkage map (Table 4-1 and Fig. 4-3). Final mapping was performed by combining two or more linkage groups that belong to the same chromosome (Fig. 4-3).

4.3.2.1 Distribution of markers

The RAC875/Kukri map was extensively mapped, with at least one linkage group assigned to each of the 21 linkage groups. The map accounted for a total length of 3,156.7 cM, with an average density of one marker per 6.4 cM (Table 4-1). The B genome was the densest, containing 213 anchor markers (74SSRs and 139 DArTs) that accounted for 1,201 cM of genetic distance (5.6 cM per marker). The A genome linkage groups spanned 1,198.3 cM with 182 anchor markers (75 SSRs and 107 DArTs) with average 10.0 cM per marker. The D genome was less densely covered, with 100 anchor markers accounting for 757.4 cM (7.6 cM per marker). Chromosome 3B harbored the highest percentage (11.5 %) of all markers and was the largest linkage group with 239.7 cM, while chromosome 5D, 4D, and 6D had the lowest percentage of markers 1.0%, 1.2% and 1.6%, respectively. The shortest chromosome was 4D with 8.1 cM in length (Fig. 4-3 and Table 4-1). However, several areas showed very limited genome coverage. The short arms of chromosome 2D and 7A, chromosome 4D, 5D and 6D are poorly represented. The B genome chromosomes had the highest frequency of polymorphism among the three genomes and possessed 43% of the markers (Table 4-1). The distribution of markers among the genomes was not uniform. The number of markers mapped on the A, B and D genomes were 182 (37%), 213 (43%) and 100 (20%), respectively.

There was variation in the number of markers, map length and marker density on the basis of the homoeologous group. Marker number and density were the highest in

homoeologous groups 1 and 3 (106 loci and 100, respectively), while homoeologous groups 1 and 7 showed the highest marker density (5.7 and 5.5 cM/marker, respectively). Homoeologous groups 4 and 5 had the lowest number of markers (47 and 36 loci, respectively), the shortest map length (114.9 and 103 cM, respectively) and homoeologous group 5 had the lowest marker density (8 cM/marker). Although both DArTs and SSRs were distributed randomly along the maps, DArT markers contributed most markers per linkage group. However, homoeologous group 5 showed fewer DArT markers compared to SSRs (Fig. 4-2). DArT markers tended to cluster in almost all linkage groups (e.g 1AL, 1D, 2B, 2D, 3BL, 6B, 7A, 7B and 7DS). These clusters consisted of many markers, tightly linked with each other. Most of DArT markers clustered on telomeric region of the chromosomes (Fig. 4-3). One possible explanation for this could be that DArT markers have been developed from gene-rich telomeric region (Wenzl et al., 2004; Akbari et al., 2006).

Table 4-1. The distribution of mapped molecular markers, their chromosomal locations, and their genomic coverage across the 21 linkage groups.

Chromosome	SSRs	DArTs	Marker		Size (cM)	cM/Marker
			No.	%		
1A	14	30	44	8.9	202.5	4.6
1B	17	19	36	7.3	205.4	5.7
1D	11	15	26	5.3	177.4	6.8
2A	9	13	22	4.4	165.2	7.5
2B	5	24	29	5.9	139.4	4.8
2D	4	12	16	3.2	165.9	10.4
3A	12	10	22	4.4	175.8	8.0
3B	19	38	57	11.5	273.2	4.8
3D	13	8	21	4.2	189.4	9.0
4A	9	13	22	4.4	187	8.5
4B	8	11	19	3.8	149.6	7.9
4D	3	3	6	1.2	8.1	1.4
5A	9	3	12	2.4	142.3	11.9
5B	10	9	19	3.8	144.6	7.6
5D	2	3	5	1.0	22.3	4.5
6A	6	16	22	4.4	172.2	7.8
6B	6	26	32	6.5	160	5.0
6D	7	1	8	1.6	81.7	10.2
7A	16	22	38	7.7	153.3	4.0
7B	9	12	21	4.2	128.8	6.1
7D	10	8	18	3.6	112.6	6.3
A	75	107	182	36.8	1198.3	6.6
B	74	139	213	43.0	1201	5.6
D	50	50	100	20.2	757.4	7.6
	199	296	495	100	3156.7	6.4

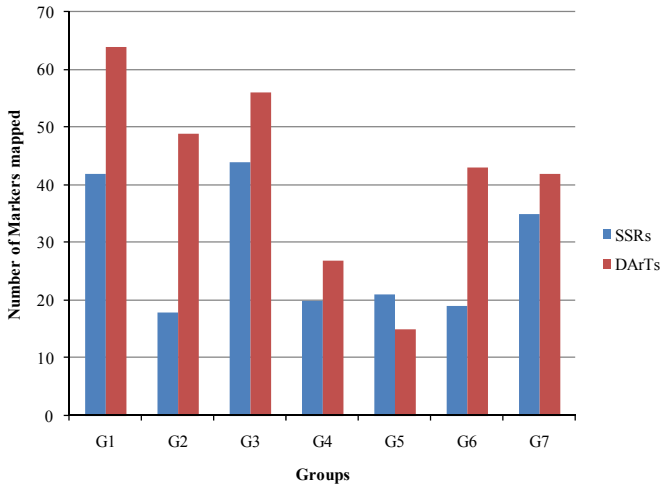

Figure 4-2. Distribution of SSR and DArT markers across the seven groups in wheat.

4.3.2.2 Segregation distortion

The chi-square (χ^2, $P < 0.05$) analysis for assessing segregation patterns of markers in the RAC875/Kukri linkage map revealed significant segregation distortion from the expected 1:1 ratio. Out of the markers used for mapping, 112 (22.2%) showed segregation distortion. Among those, 37 (7.3%) and 17 (3.4%) were highly affected at the $P < 0.01$ and $P < 0.001$ significant levels, respectively (Table 4-3). Markers with the greatest degree of deviation within each region of strong distortion at the $P < 0.01$ and $P < 0.001$ significant levels, as well as their Chi-square values, are given in Table 4-3. Among 54 (10.7%) of highly distorted segregations (Table 4-3), different proportions of DArT and SSR markers were observed. DArT markers showed higher level of segregation distortion compared with SSR markers (80% (43) and 20% (11), respectively). Markers showing the highest segregation distortion ($P < 0.001$) were removed from linkage groups.

From total SSR and DArT markers over 368 individual loci, DArT markers showed high level of missing values compared to SSR markers. On average, 10.5% were missing values of which 8.6% and 1.9% were DArT and SSR markers, respectively (Table 4-2). In total, the 368 lines inherited 51% of their alleles from the male parent "Kukri" and 49% from the female parent "RAC875". For the DArT markers, 50.8% (159 individuals) and 49.2% (154 individuals) inherited from "Kukri" and "RAC875", respectively. These values in SSR markers were 51.4% and 48.6% which inherited from kukri and RAC875, respectively (Table 4-2). Markers exhibiting segregation distortion in favor of 'Kukri' alleles were more frequent (67%) than those in favor of 'RAC875' alleles (33%). This result shows that the population was skewed in favor of the Kukri allele as the male parent in this population (Table 4-3).

Table 4-2. Average number of missing values and inherited alleles from parental lines 'Kukri' and 'RAC875'.

Marker	Kukri (A)	RAC875 (B)	A+B	Missing	paired t-test	P-value
SSRs	180	171	351	17	5.20	0.0001
DArTs	159	154	313	55	2.74	0.007
Average	168	161	329	39	5.21	0.0001

The marker loci with distorted segregation ratios were distributed on all of the chromosomes with the exception of 4D. However, they were not randomly distributed.

A chromosomal region with skewed segregation of four or more closely linked markers was considered as significant segregation distortion. Accordingly, chromosomes 1B, 1D, 3A, 4A, 4B, 5A and 6A, 6B, 6D, 7A and 7D showed distorted segregations in clusters. The distorted segregation loci in chromosome 1B and 5A were skewed in favor of 'RAC875', while in the nine other chromosomes they were skewed for the 'Kukri' allele. About 25% of the DArT markers were clustered on chromosomes 1B, in favor of the 'RAC875' allele (Table 4-3).

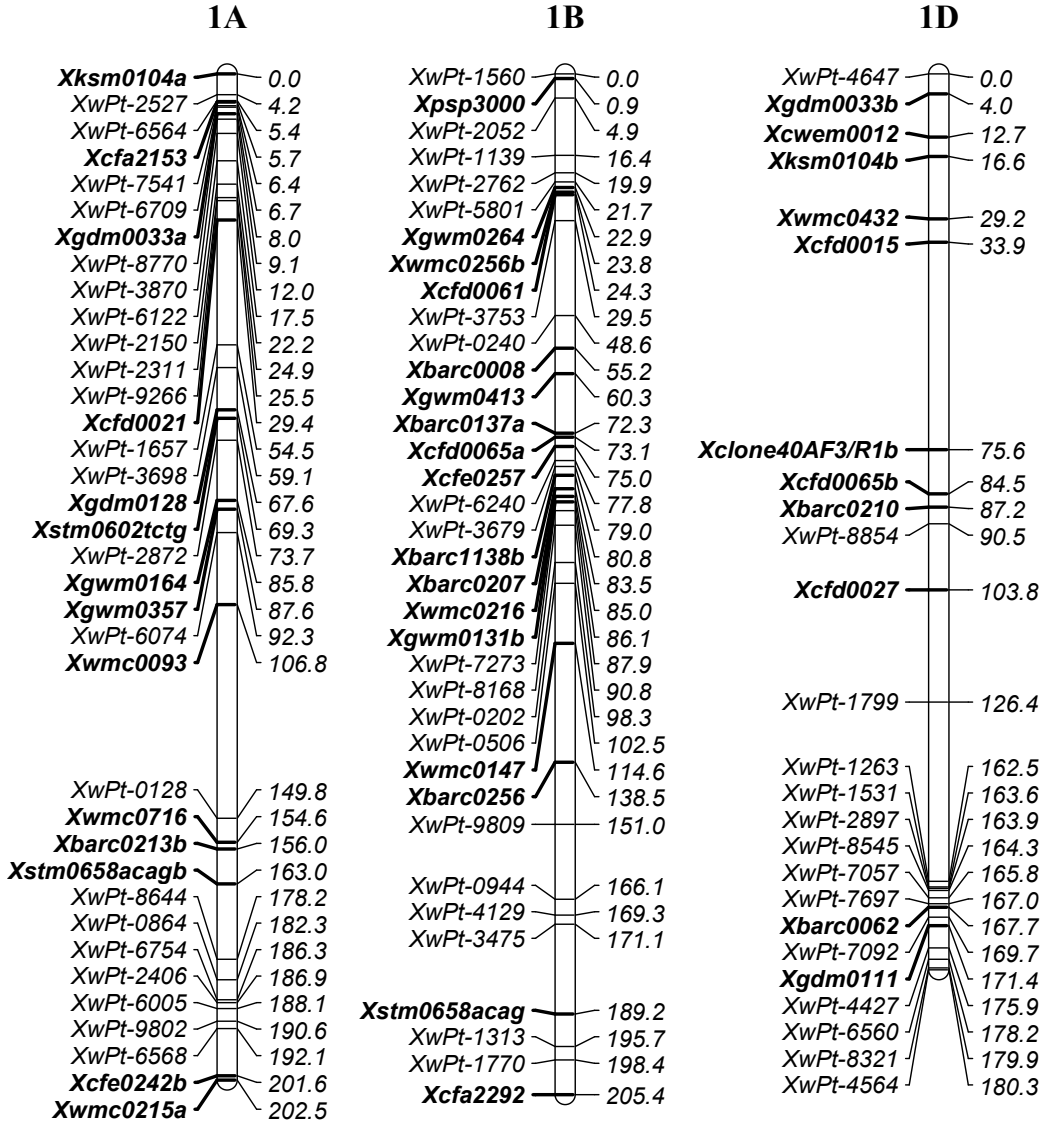

Figure 4-3. Genetic linkage groups constructed in the 368 doubled-haploid lines population derived from a cross between'RAC875' and 'Kukri'. Markers are placed in their most likely positions compared to CMap. The SSR markers are presented in bold across linkage groups. SSR primers with different mapped loci have a suffix of either "a", "b" or "c". Cumulative map distances are shown in centiMorgan (cM) on the right side of the linkage groups and was calculated using the Kosambi mapping function. The broken line indicates the lack of linkage on chromosomes.

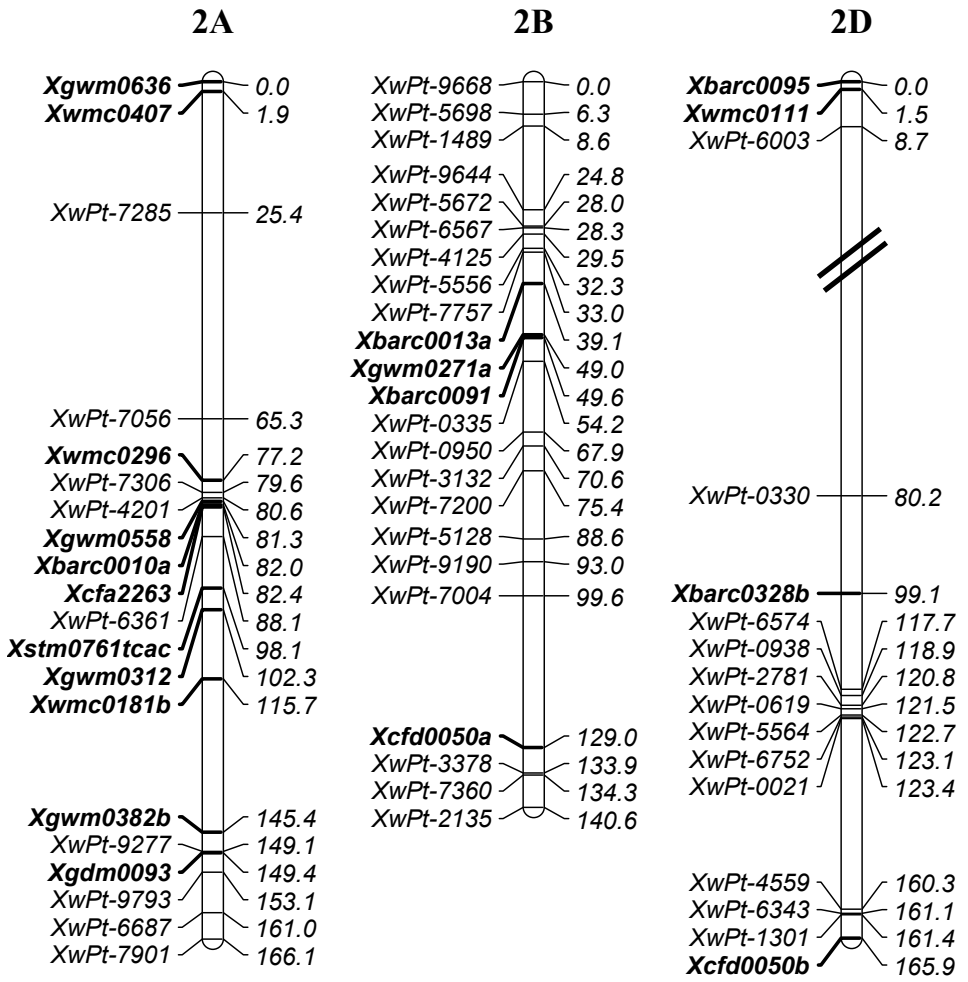

Figure 4-3. Continued

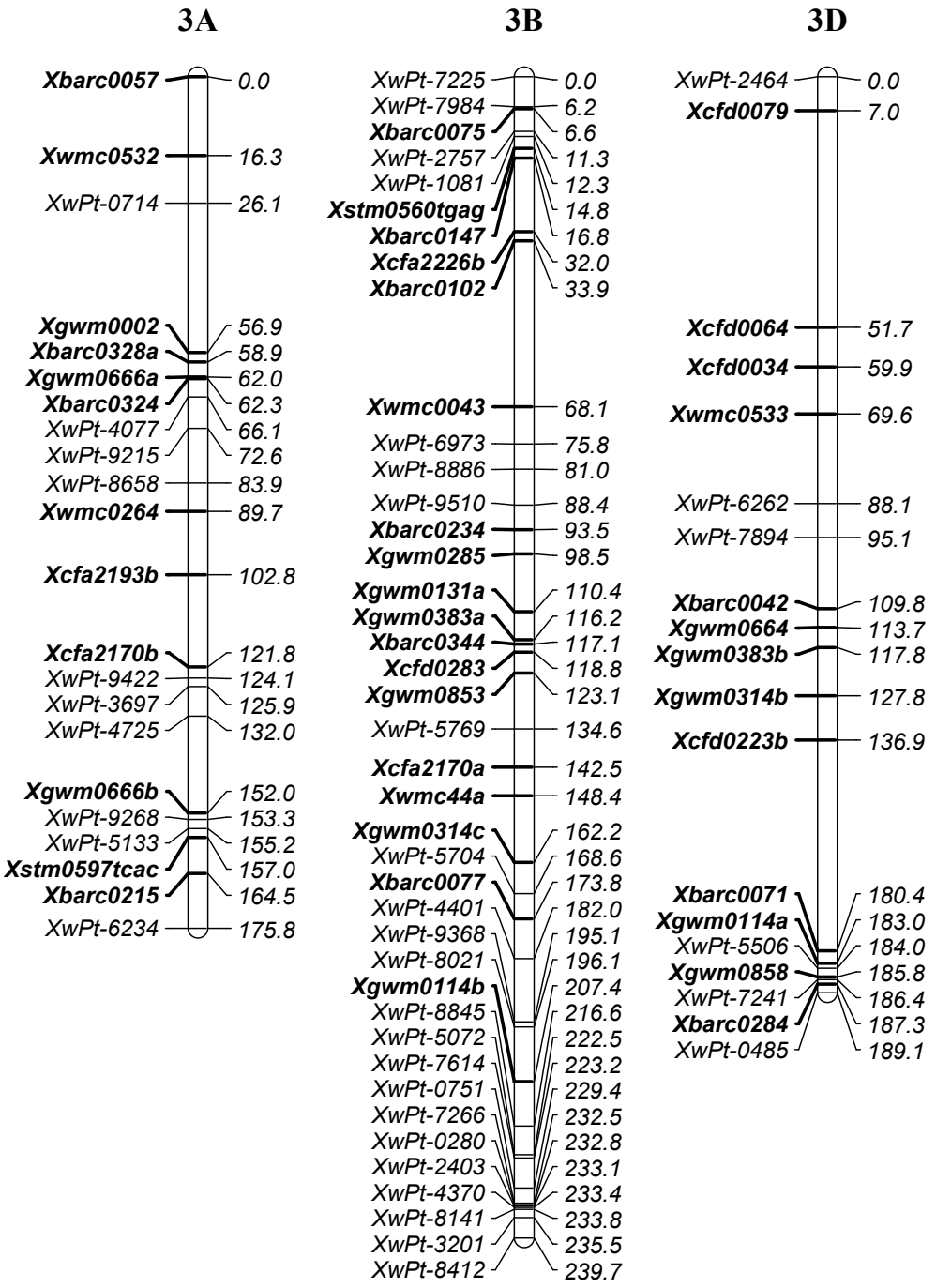

Figure 4-3. Continued

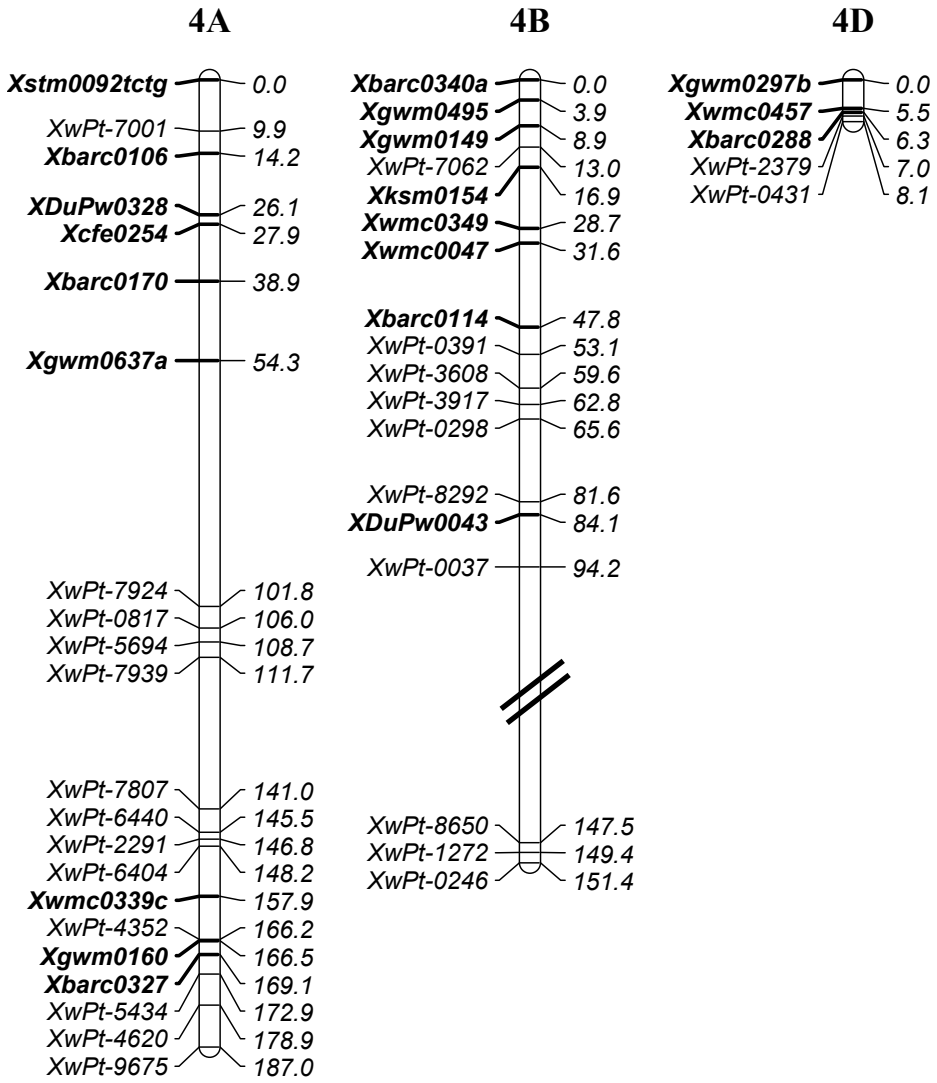

Figure 4-3. Continued

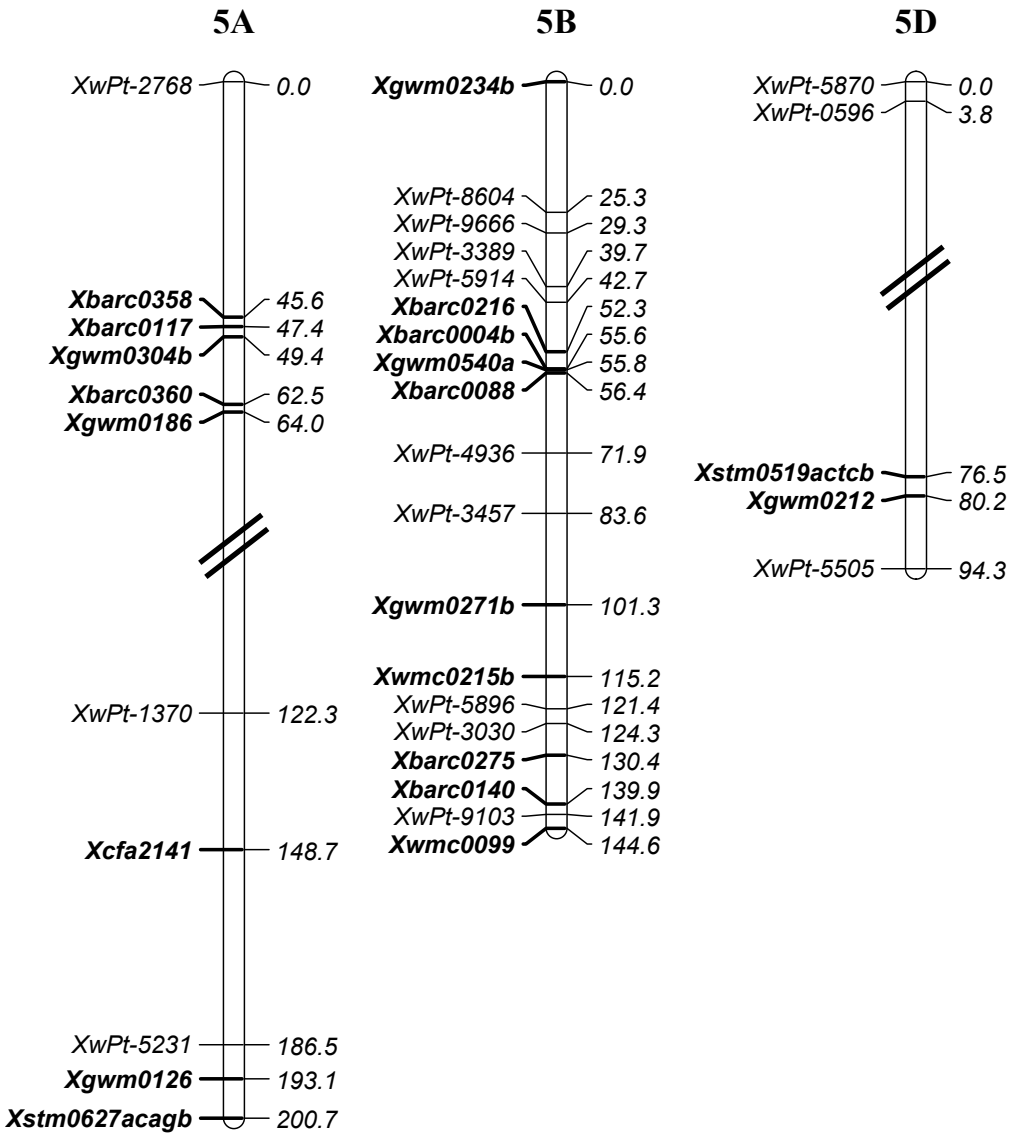

Figure 4-3. Continued

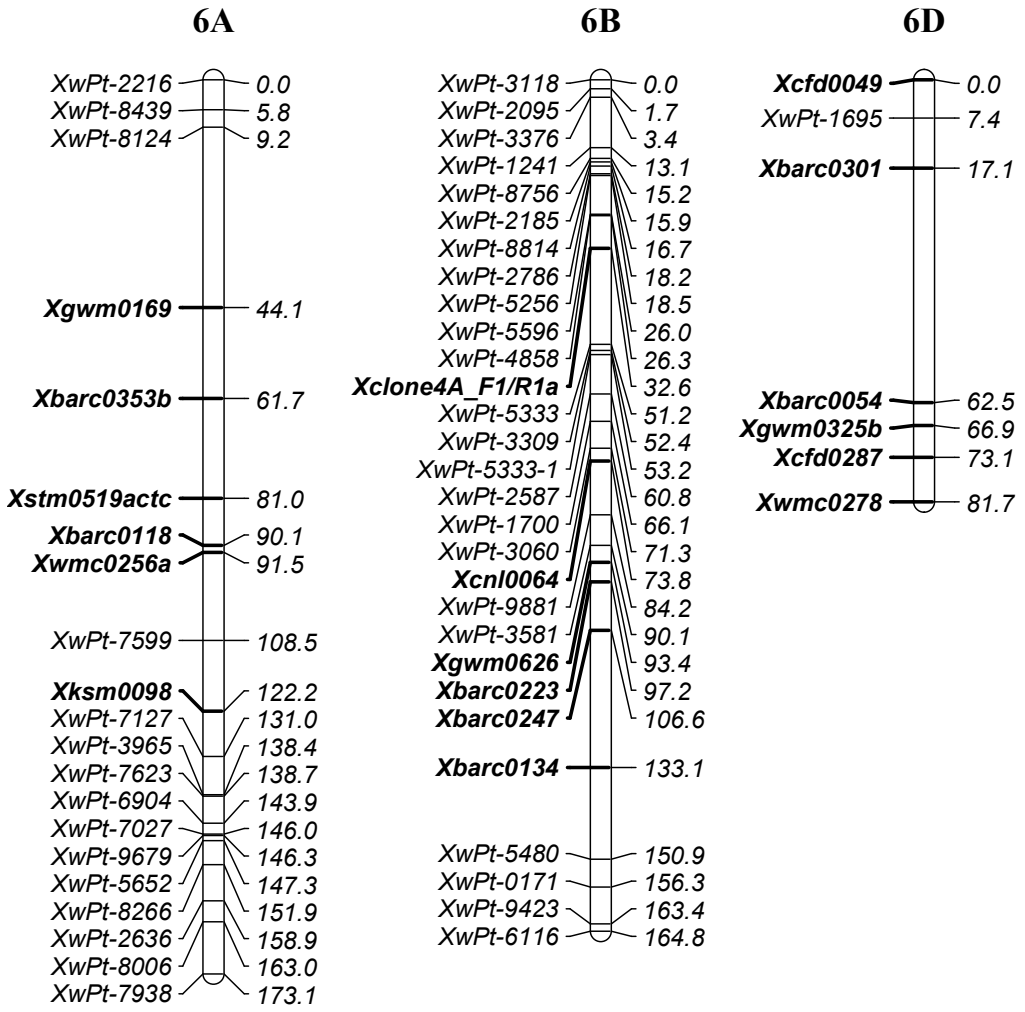

Figure 4-3. Continued

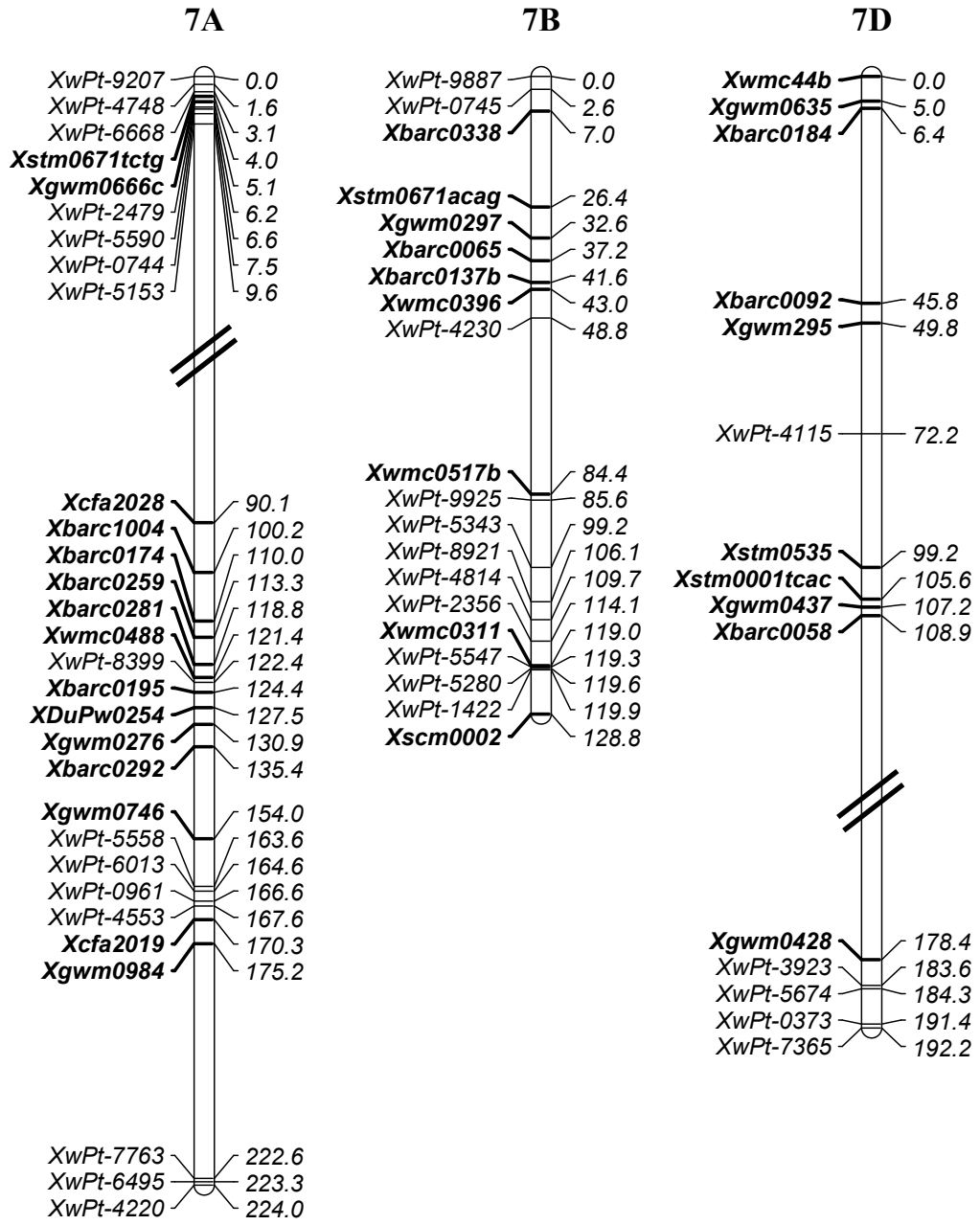

Figure 4-3. Continued

Table 4-3. Distorted segregation of marker loci across the genetic map of Kukri/RAC875.

Chromosome	Type	Marker name	A	B	Missing	χ^2
1A	DArT	*XwPt-6122*	183	130	55	9.0**
	DArT	*XwPt-6074*	119	168	81	8.4**
1B	DArT	*XwPt-2052*	196	131	41	12.9***
	DArT	*XwPt-1139*	186	130	52	9.9**
	DArT	*XwPt-3753*	131	183	54	8.6**
	DArT	*XwPt-0240*	114	163	91	8.7**
	DArT	*XwPt-6240*	125	170	73	6.9**
	DArT	*XwPt-0506*	178	132	58	6.8**
	DArT	*XwPt-4129*	130	178	60	7.5**
	DArT	*XwPt-3475*	131	177	60	6.9**
	DArT	*XwPt-1313*	137	196	35	10.5**
	DArT	*XwPt-1770*	139	186	43	6.8**
1D	SSR	*Xwmc0215c*	222	137	9	20.1***
	DArT	*XwPt-4427*	177	118	73	11.8***
	DArT	*XwPt-6560*	161	113	94	8.4**
2A	SSR	*Xbarc0010b*	72	216	80	72.0***
	DArT	*XwPt-6361*	188	134	46	9.1**
2B	DArT	*XwPt-2135*	117	175	76	11.5***
2D	SSR	*Xgwm0382a*	260	105	3	65.8***
3A	DArT	*XwPt-2866*	192	96	80	32.0***
	DArT	*XwPt-9215*	211	126	31	21.4***
	DArT	*XwPt-8658*	171	111	86	12.8***
	SSR	*Xwmc0264*	208	153	7	8.4**
	DArT	*XwPt-5133*	167	122	79	7.0**
3B	SSR	*Xwmc0307*	200	149	19	7.5**
	DArT	*XwPt-8412*	210	113	45	29.1***
3D	DArT	*XwPt-7894*	124	195	49	15.8***
4A	SSR	*Xgwm0637a*	142	193	33	7.8**
	DArT	*XwPt-7924*	208	114	46	27.4***
	DArT	*XwPt-7939*	126	183	59	10.5**
	DArT	*XwPt-7807*	134	183	51	7.6**
	DArT	*XwPt-6440*	182	135	51	7.0**
4B	DArT	*XwPt-7062*	201	117	50	22.2***
	DArT	*XwPt-0391*	202	137	29	12.5***
	DArT	*XwPt-3608*	190	140	38	7.6**
5A	SSR	*Xbarc0360*	147	205	16	9.6**
	SSR	*Xbarc0141*	122	168	78	7.3**
	SSR	*Xgwm0186*	148	210	10	10.7**
5B	DArT	*XwPt-3389*	167	122	79	7.0**
	DArT	*XwPt-4936*	142	192	34	7.5**
	DArT	*XwPt-9103*	174	125	69	8.0**
6A	SSR	*Xwmc0256a*	207	156	5	7.2**
	DArT	*XwPt-7599*	187	133	48	9.1**
	DArT	*XwPt-8266*	115	167	86	9.6**
6B	DArT	*XwPt-2185*	174	122	72	9.1**
	DArT	*XwPt-8814*	190	125	53	13.4***
	DArT	*XwPt-6116*	167	121	80	7.3**
7A	DArT	*XwPt-5153*	182	134	52	7.3**
	SSR	*Xbarc0174*	178	128	62	8.2**
	DArT	*XwPt-8492*	178	128	62	8.2**
	DArT	*XwPt-4796*	175	102	91	19.2***
7B	DArT	*XwPt-8921*	127	185	56	10.8**
	DArT	*XwPt-4814*	130	187	51	10.2**
7D	DArT	*XwPt-4115*	174	112	82	13.4***

** and *** showing significance of χ^2 at 0.01 and 0.001 level, respectively.

4.4 Discussion

The map generated in this study was produced with the aim of identifying QTLs of economically important traits such as yield and its components segregating in the population. The population size in this study was relatively large, with 368 DH lines. This population size can offer the potential for fine-mapping major genes and QTLs. In general, larger populations have a beneficial effect on the mapping result. In larger populations, more recombination events between a pair of markers can occur, which can lead to the higher resolution between the markers. Therefore, the positioning of the markers will be more accurate, and the relative impact of missing observations and scoring errors decreases (van Os et al., 2005). It might also be possible to detect epistasis. The power to efficiently detect epistasis varies with the size of the population and the precision of statistical methods with which the phenotypic data are analysed (Carlborg and Haley, 2004; Carlborg et al., 2006; Yang et al., 2007). In addition to large population size, uniform genome coverage and marker density are required for an accurate identification of QTLs (Chalmers et al., 2001).

Several linkage maps have already been published in wheat, most of them are summarized at: (http://wheat.pw.usda.gov/ggpages/map_summary.html). The ITMI population (W7984 × Opata 85) is the most comprehensive, fully mapped population which comprises approximately 1065 markers with total genome coverage of 5256 cM (reviewed by Langridge et al., 2001). The SSR consensus map comprising 1,235 microsatellite markers covered 2,569 cM without any gaps across the genome (Somers et al., 2004). Overall, our map, in comparison, had a reasonably good coverage 3,156.7 cM, representing 60% of the ITMI population with 5256 cM (Langridge et al., 2001) and 86% of the Courtot × Chinese Spring map with 3,876 cM (Sourdille et al., 2004). It is also comparable to recently published maps in wheat (Chalmers et al., 2001; Quarrie et al., 2005; Akbari et al., 2006; Semagn et al., 2006). However, there are some gaps in the map and there are also some chromosomes which lack markers. This can be explained by a lack of polymorphism in the corresponding regions of the map (Messmer et al., 1999; Paillard et al., 2003). The chromosomes 4D, 5D, and 6D are only partly covered. In published maps by Chalmers et al. (2001), Paillard et al. (2003) and Semagn et al.(2006), chromosome 4D is either missing or only partially covered. The relative order of markers within linkage groups is largely in agreement with other published

mapping information. The polymorphism levels between the three genomes A, B and D is entirely consistent with mapping studies in other wheat populations (Röder et al., 1998; Messmer et al., 1999; Chalmers et al., 2001; Kammholz et al., 2001; Paillard et al., 2003; Liu et al., 2005c; Quarrie et al., 2005; Semagn et al., 2006), in which the B genome had the highest and the D genome the lowest polymorphism and coverage. To obtain an improved coverage of the D genome and gaps in the linkage groups, additional markers would be need to be added to the map.

The ideal set of molecular marker data for linkage mapping has no missing values, no genotyping errors and the markers segregate in the expected ratio for that type of population. However, in practice, mapping data is complicated by all of these factors (Hackett and Broadfoot, 2003). Several regions of the map showed significant segregation distortion. DArT markers had higher percentage of distortion and that could mostly be related to missing values. However, several reasons have been suggested for explaining segregation distortion within wheat × maize-derived doubled haploid populations. These include heterogeneity within the parents, selection associated with the doubled haploid production process, outcrossing and admixture of seed during increase for experiments, and errors in scoring of markers and map construction (Kammholz et al., 2001). If distortion is the result of failure or errors scoring in the process of data production, that may create a serious problem in map construction. In a smaller population size, the effects of error on mapping could be even more severe (Hackett and Broadfoot, 2003). Therefore, these types of markers should be excluded from the data set (Jansen et al., 2001).

In this study, we found distorted segregations in clusters of four or more markers in chromosomes 1B, 1D, 3A, 4A, 4B, 5A and 6A, 6B, 6D, 7A and 7D. Faris et al. (1998) analyzed the degree and direction of segregation distortion in an *Aegilops tauschii* F2 population using molecular markers and found regions with significantly skewed ratios on chromosomes 1D, 3D, 4D, 5D, and 7D. The most severely distorted regions were found on chromosome 5D. It has been shown that the homoeologous group 5 chromosomes of wheat and its relatives possess factors (segregation distortion regions, SDRs) associated with segregation distortion (Faris et al., 1998; Faris et al., 2000; Peng et al., 2000; Kumar et al., 2007). Campbell et al. (1999) found segregation distortion on chromosomes 2B and 6B. Kammholz et al. (2002) also found distorted markers that were located on chromosomes 1D, 2B, 4D and 7A. Paillard et al. (2003) reported

clusters of distorted regions on chromosomes 1A, 1D, 2B, 3A, 5A and 5B, of which chromosome 2B was severely affected by segregation distortion.

There are several mechanisms that can cause segregation distortion. Taylor and Ingvarsson (2003) suggested two generalizing statement explaining segregation distortion factors which act in the male gametes and have the effect of reducing male fertility. First, mechanisms that they do not distort meiosis *per se,* but rather alter the products of meiosis by causing chromosome breakage and/or aborting gametes that do not carry the driving allele. Second, genetic conflict could arise due to meiotic drive elements having deleterious effects, which would then cause inadvertent selection for alleles at other loci that suppress their effects.

Jansen et al. (2001) and Hackett and Broadfoot (2003) pointed out that markers with similar distorted segregation ratios will tend to cluster together on the map and distorted segregation ratios, theoretically, should not give difficulties in mapping unless there are two linked loci, both of which affect viability. However, in QTL analysis, distorted segregation is unlikely to be problematic since QTLs will generally have the same segregation ratios as closely linked markers (Jansen et al., 2001; Hackett and Broadfoot, 2003).

The constructed map will be the base for QTL identification. In the present study, QTLs associated with agronomic traits and some physiological traits under water stress and non-stress environments are localized and, in the future, this population will be used for identification of QTLs linked to other traits such as biotic and abiotic stress resistance.

under South Australian dry conditions. Kukri is also an adapted cultivar which may show some drought tolerance traits. However, what make the other two cultivars more productive are their differences in a palette of traits. These differences could be leaf structure and morphology (e.g. leaf waxiness, leaf rolling, thick and erect leaves), capacity of tilling, chlorophyll retention and stay-green, OA capability, water soluble carbohydrates accumulation and root traits.

Based on the results of this study, it can be concluded that different mechanisms are involved to confer drought tolerance in RAC875 and Excalibur. RAC875 outyielded Kukri on average by 24.3 %; it possesses mechanisms which help to maintain a high tissue water status by reduced water loss through leaf surface by reducing leaf area, reducing residual transpiration and, therefore, avoiding deleterious leaf water potential in leaves. RAC875 is a more conservative cultivar which shows lowest tiller number per se, moderate OA, low stomatal conductance and therefore slower recovery, thick green leaves, stronger leaf waxiness, stay-green phenotype and high WSC. However, RAC875 is potentially sink-limited due to its lower tiller number.

Excalibur, on the other hand, seems to be a more responsive cultivar under drought conditions. It showed a strong interaction with environmental conditions. Excalibur produced more tillers (high pre-anthesis biomass) at the first place and aborted tillers under stress and concentrated on the main stems (higher number of spikelet per spike). It produced greater total biomass and had a higher root-shoot ratio under water stress. It showed leaf rolling and moderate leaf waxiness under stress. Excalibur showed the highest OA capability, highest stomatal conductance and rapid recovery after re-watering. Excalibur possesses mechanisms which allow the plant to endure low tissue osmotic potential and to recover rapidly after re-watering. These three cultivars with different responses to drought stress were selected to develop mapping populations for genetic studies to identify genomic regions controlling drought tolerance in wheat.