Genes, haplotypes and physiological traits associated with a chromosome 3B locus for wheat improvement in hot climates

By

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Abstract

Crop productivity in many wheat cultivation areas is severely affected by dry and hot conditions, increasing the yield gap between potential and actual yield. To close this gap, the identification of genes contributing to yield in stressed conditions is one key to the breeding of tolerant cultivars. However, due to low stability across environments, no genes have yet been identified for yield variation. In this project, we focussed on the positional cloning of a quantitative trait locus (QTL), *qYDH.3BL,* located on the wheat chromosome arm 3BL. The QTL has been identified by the multi-environment analysis of the double-haploid (DH) population of the cross from the drought tolerant line RAC875 and the susceptible variety Kukri. The QTL was constitutively expressed in the Mexican environment with the positive allele from RAC875 associated with an increase in yield, thousand grain weight and early vigour under dry and hot conditions. Greater allele effect dependent on temperature has been observed at *qYDH.3BL*, suggesting that the QTL is heat related.

To fine map *qYDH.3BL*, we developed a deep-soil platform using 1 m deep wheelie bins placed in the polytunnel to increase temperature. We confirmed the expression of *qYDH.3BL* using a set of RAC875 x Kukri RILs. Single marker analysis showed the positive allele from RAC875 associated with spike length and biomass, early vigour and stem biomass. The development of a high-density genetic map of *qYDH.3BL* in RAC875, combined with the deep-platform experiment, narrowed the QTL interval to a 690 kbp sequence. The anchoring of the interval in the physical assembly of the wheat cv. Chinese Spring reference genome (IWGSC Ref 1.0) identified 12 candidate genes. The study of the allelic diversity at *qYDH.3BL* in a wheat diversity panel of 808 accessions identified four haplotypes. Haplotype I, the RAC875 allele was over-represented in the CIMMYT germplasm suggesting that the allele may have been selected for yield in a Mexican environment-type.

To study the physiological mechanisms under *qYDH.3BL* control, we developed heterozygous inbred families (HIF). The lines were phenotyped in the deep soil platform. The lines with the RAC875 allele among the HIFs increased biomass, single grain weight and number of spikelets per spike. Water use was also measured in the deep soil platform using sap flow sensors. RAC875 had an increased water use compared to Kukri. A similar pattern was observed in the HIFs, the lines with the RAC875 allele had a higher water use compared to those with the Kukri allele. As *qYDH.3BL* was constitutively expressed in the Mexican environments characterised

by a deep soil and RAC875 was shown to have a lower root conductivity than Kukri due to its root anatomy, we also phenotyped the roots of the HIFs. We did not identify root traits that could contribute to the heat tolerance mechanism associated with *qYDH.3BL*.

Finally, we studied the expression of the 12 candidate genes within the 690 kbp interval in the HIFs. Expression analysis identified a strong candidate gene, *Seven in absentia* (*TaSINA*). The gene was up-regulated in Kukri compared to RAC875 and in the HIFs containing the Kukri allele compared to those with the RAC875 allele. *TaSINA* is annotated as an E3 ubiquitin ligase protein, a family involved in ubiquitin pathway. To study the role of *TaSINA* in drought and heat tolerance, we screened the Gladius TILLING population and identified two missense variants. Study of the phylogenetic relationship of *TaSINA* with published *SINA* genes in plants and animals revealed that *TaSINA* is specific to the Triticeae. This is the first report of a wheat SINA gene and a gene associated to yield variation under heat in wheat. Discovery of new alleles of *TaSINA* could lead to the breeding of new varieties able to maintain yield under heat stress conditions.

Thesis declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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List of abbreviations

A	Leaf photosynthesis
Ci	Internal CO_2 concentration
CID	Carbon isotope discrimination
CIMMYT	International maize and wheat improvement center
CS	Chinese Spring
CSS	Chromosome survey sequence
DAS	Days after sowing
DAWN	Diversity among wheat genomes
DMSO	Dimethyl sulfoxide
DREB	Dehydration responsive element binding
E	Transpiration
EMS	Ethylmethane sulfonate
eQTL	Expression quantitative trait locus
GBS	Genotyping-by-sequencing
GEBV	Genomic estimated breeding value
gs	Stomatal conductance
GS	Genomic selection
HC	High confidence
HIF	Heterozygous inbred family
InDel	Insertion/Deletion
IWGSC	International Wheat Genome Sequencing Consortium
KASP	Kompetitive allele specific PCR
LC	Low confidence
MAGIC	Multi-parent advanced generation intercrossed
MAS	Marker-assisted selection
ME	Mega-environment
mQTL	Metabolomic quantitative trait locus
NAC	NAM, ATAF/ ATAF2, and CUC2
NAM	Nested-association mapping
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pQTL	Proteomic quantitative trait locus
QTL	Quantitative trait locus
RIL	Recombinant inbred line
RING	Really interesting new gene
RDD	Root DNA density
SINA	Seven in absentia

SNP	Single nucleotide polymorphism
TILLING	Targeting induced local lesions in genomes
VPD	Vapour pressure deficit
WGS	Whole genome shotgun sequencing
WT	Wild-type

General introduction

The challenge of the 21st century is to maintain sustainable agricultural production while producing more food to feed the increasing world population. Wheat is the most important cereal in terms of consumption and as a protein source for the human diet. Its production alone must increase by 60 % by 2050 to maintain food security based on production data of 2005-2007 (Alexandratos & Bruinsma, 2012). At the same time, efforts in reducing the negative impact of agriculture on the environment are required as agriculture already accounts for 24 % of total greenhouse gas emissions and uses 70% of freshwater resources (FAO, 2011). Several solutions for sustainable agriculture able to produce more food have been proposed and detailed in Foley et al., (2011). These include limiting agricultural land extension and re-allocating cultivated lands to food production, increasing agricultural resource efficiency (e.g. water, fertilizer), improving food distribution by shifting diets and reducing food waste but also closing yield gaps. As each solution presents benefits and disadvantages, developing sustainable agriculture for the 21st century can only be achieved through integrative solutions.

Closing yield gaps in regions of low average yield is likely to have a great impact on total cereal production as they are often regions with less favourable climates (Tester & Langridge, 2010). Licker et al., (2010) developed a method to estimate yield gaps by first estimating the climatic yield potential in regions of similar climate. Yields gaps were then determined by comparing yield potential with actual yields. They estimated that production of the three most important cereal crops (maize, rice and wheat) could increase by 50, 40 and 60 %, respectively, if 95 % of the cultivated lands reached their climatic yield potential. In Australia, where wheat is the number one cereal, relative wheat yield ranged between 49% and 57 % between 2000 and 2014 with actual yields ranging between 1.6 and 1.8 t/ha while predicted yield ranged between 3.0 and 3.3 t/ha (http://www.yieldgapaustralia.com.au/). Closing yield gaps to reach optimal yield will required better crop management through new farming-systems along with the breeding of cultivars. To breed high-yielding cultivars adapted to different climates, it is essential to first identify the genetic factors contributing to yield as the molecular basis of grain yield remains to be characterized in wheat.

A number of regions of the wheat genome have been identified through QTL analyses for their role in controlling yield variation. However, genes underlying these regions remain to be identified and their function characterised. The focus of this study was to identify the genetic variation underlying *qYDH.3BL*, a yield QTL identified on the long arm of chromosome 3B in wheat grown in hot and dry climates (Bennett et al., 2012; Bonneau et al., 2013). Additionally, this study aimed to discover the physiological traits contributing to the QTL expression. Chapter I reviews the advance and strategies of positional cloning in wheat combined with an overview of studies conducted on drought and heat tolerance. Chapter II focusses on the fine mapping of *qYDH.3BL* in RAC875 to narrow down the locus and identify underlying genes. The chapter also focusses on the study of the allelic diversity at *qYDH.3BL* in a worldwide collection of wheat. Chapter III describes the physiological study of HIFs to decipher the mechanisms of drought and heat tolerance associated with *qYDH.3BL*. Chapter IV focusses on the expression analysis of candidate genes using HIFs to identify the potential causal gene(s) associated with *qYDH.3BL*. Finally, Chapter V concludes the thesis by presenting the main outcomes of this research and includes some perspectives for future research leading towards the cloning of the first gene controlling yield in a hot and dry climates.

Chapter I

Chapter I: Literature review

1. Introduction

Bread wheat is the most produced cereal in Australia and the third most produced cereal in the world after maize and rice. In 2016-2017, approximately 760 million tons of wheat were produced on a total surface area of 220 million hectares (FAOSTAT). Cultivated on every continent except Antarctica, wheat is the most widely grown cereal. It is grown in a wide range of climates and environments which results in a large variation of yield from low yielding environments producing less than 1.3 t/ha to high yielding environments producing more than 7.6 t/ha (Figure 1). Wheat plays a major role in food security, as it is a staple food for millions people providing approximately 20 % of the food supply (Shiferaw et al., 2013). It is also the primary source of protein for the human diet, providing 21 % of protein, while rice provides 13 % and maize 4%.

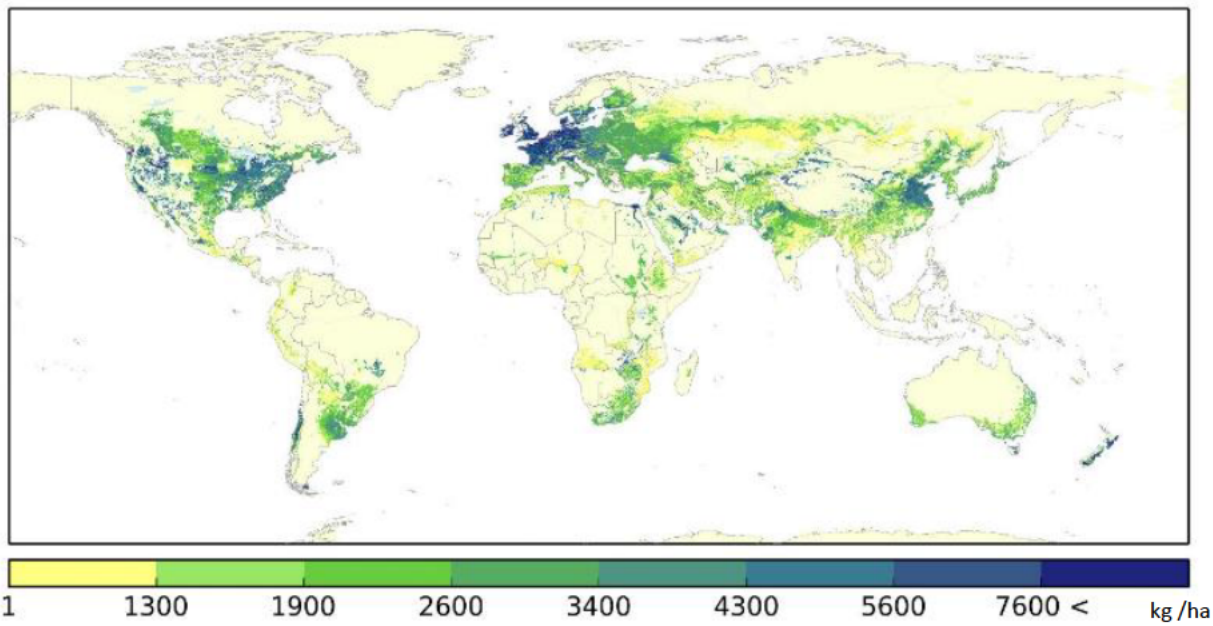

Figure 1. Wheat yield in different world regions. Yield varies from less than 1 t/ha to more than 7.6 t/ha (You et al., 2014; mapspam).

The United Nations have estimated that the world population will reach 9.6 billion people by 2050. This drastic increase of population will raise the demand on food and cereal prices with

consequent distress to millions of people and worsening an already existing food crisis (Alexandratos & Bruinsma, 2012). Today more than 1 billion people are lacking access to food or are malnourished, but this situation could worsen over time as developing countries rely on wheat imports (Foley et al., 2011). The yield improvement of wheat, currently at 1 % per year, is insufficient to meet the predicted food demand (Ray et al., 2013). To maintain food security, wheat yields must increase at a rate of 1.6 % per year to meet the predicted requirements of 2050 (Lucas, 2013).

The beginning of agriculture 10,000 years ago in the Fertile Crescent was an major event in human history (Lev-Yadun et al., 2000). It marked the beginning of plant and animal domestication that gave rise to the domesticated species that we have today (Purugganan & Fuller, 2009). Some the characteristics of domesticated cereals originate from the early selection for traits including grain size, non-shattering wheat type, reduced tillering, and increased selfing rates. At the beginning of agriculture, einkorn wheat and emmer wheat were the cultivated species (Lev-Yadun et al., 2000). Modern bread wheat (*Triticum aestivum L.*) (hexaploid genome AABBDD) which is cultivated today, originated from two separate inter-specific hybridizations of three genomes at different periods of time (Petersen et al., 2006). The first hybridization happened between *Triticum urartu* (A genome) and a relative of *Aegilops speltoides* (S genome ancestor of the B genome) which generated the emmer wheat (*Triticum turgidum* subsp. dicoccoides, AABB). Later on, emmer wheat hybridized with *Aegilops tauschii* (DD genome).

The major shift in yield improvement of modern wheat happened during the 1960's and is referred as the "green revolution". Between 1966 and 1999, total wheat production increased by 91 %, the production was about 200 million tons in 1966 and reached 576 million tons by 1999 (Kush, 2001). During the green revolution, cultivars were selected for high yield potential with responsiveness to fertilisers, wide adaptability across environments, shorter life cycle, resistance to biotic and abiotic stresses and improved grain quality. The discovery of dwarfing and early flowering alleles through the introduction of dwarf genotypes such as Norin 10 originating from Japan, contributed to the success of the green revolution and enabled the breeding of high-yielding wheat varieties (Borlaug, 1968). Semi-dwarf varieties showed improved performance in the field due to improved resistance to lodging and increased harvest index through reduced vegetative biomass. The substantial increase of cereal production was also due to new farm management practices, particularly the use of fertilisers, and the expansion

of cultivated areas (Kush, 2001). Effects of the green revolution lasted until the 1990's; then yield improvement started to slow and reached a plateau in major wheat producer regions of Northern Europe, India and Australia (Grassini et al., 2013).

Increasing crop productivity to feed the future world population will require us to close the gap between optimal wheat yield and actual, realised yield. Wheat production is affected by many factors, of which drought episodes and hot temperatures have a major impact, contributing significantly to the yield gap. In the USA, between 1980 and 2012, combined drought and heat stress led to $200 billion agriculture loss (Suzuki et al, 2014). In Australia, the wheat harvest value during the 2013-2014 season was about $8 billion. During the 2014-2015 season, the wheat harvest value dropped by 10.9% from $8 billion to $7.1 billion, due to poor water accumulation during the winter season and early heat waves (http://www.abs.gov.au). The increased occurrence and severity of drought and heat episodes during the growing season in major wheat producing regions in the USA, India, Australia and the Mediterranean are a serious concern for wheat production (Asseng et al., 2011; Lobell et al., 2012). Another alarming aspect is climate change. A study of yield potential in water-limited conditions at 50 sites in Australia observed a 27 % reduction of yield potential between 1990 and 2015 due to reduced rainfall and rising temperatures, compared to what could be achieved through ideal farming practice and the use of adapted modern cultivars (Hochman et al., 2017). Future seasonal temperatures have been predicted to exceed the extreme temperatures recorded between 1990 and 2006 (Battisti & Naylor, 2009). Improving crop productivity in stress-prone environments, which account for the largest part of the total cultivated area, is thought to be the best strategy to reach the 1.6 % yield improvement rate required to feed the future world population (Tester & Langridge, 2010).

Conventional breeding had an important contribution to the yield increase achieved during the green revolution. However, the shift from landraces to modern cultivars at that period created a genetic bottleneck by reducing the gene pool available to breeders. Moreover, conventional breeding is a long and tedious process, which requires many generations of crosses to obtain pure lines and observation of performance of the cultivars in the field. Improving plant tolerance in dry and hot climates requires cultivars able to maintain their yield under stressful conditions. The discovery and use of new alleles conferring drought and / or heat stress tolerance is a key to improve grain yield in hot and dry climates. However genetic determinants of yield are still unknown.

2. Plant physiology of wheat under drought, heat and drought and heat combined

a. Plant responses to individual drought or heat stress

Plant responses to individual drought or heat stress have been extensively studied in wheat. Many reviews focussing on plant responses under drought and the strategies to improve drought tolerance have been published (Araus, 2002; Richards, 2006; Reynolds & Tuberosa, 2008; Bacelar et al., 2012). Under drought stress, plants have developed different mechanisms to cope with the stress and complete their life cycle. The escape mechanism enables plants to achieve their reproduction before the onset of severe stress by shortening their life cycle through early maturity (Ludlow & Muchow, 1990). Dehydration avoidance describes a mechanism where plants maintain water uptake through an improved root system combined with mechanisms to prevent water loss. The last mechanism that has been observed in plants is the ability to tolerate the stress, often associated with osmotic adjustment through changes of turgour pressure with osmoprotectants.

Models have been established to decipher phenotypic traits important for maintaining yield under drought stress. Passioura, (1977) described water-limited yield as a result of the interaction between water uptake x water use efficiency x harvest index (Figure 2). Water uptake depends on stored water in the soil and the amount of rainfall combined with the capacity of plants to avoid evaporation from the soil through rapid cover of the soil surface or improved water extraction by the roots. Harvest index is the ratio of grain yield to the total biomass produced and depends on the water used after anthesis rather than total water used (Passioura, 1977). The efficiency of water use is defined as the ratio of biomass to water transpired. Successful breeding of drought tolerant cultivars has been achieved in wheat by targeting traits such as transpiration efficiency and the related trait of water use efficiency (Richards, 2006). Carbon isotope discrimination (CID) is the ratio of ^{13}C / ^{12}C present in the air and in the plant and it is negatively correlated with transpiration efficiency (Farquhar et al., 1989). CID for grains, flag leaf and awns have been positively correlated with grain yield and harvest index in some water-limited environments. High CID in mature grain is associated with an increase in grain yield under post-anthesis water stress (Merah et al., 2001; Monneveux et al., 2005). The Australian wheat cultivar, Drysdale, developed by backcrossing of the wheat cultivar Quarrion (low CID) into the wheat cultivar Hartog (intermediate CID), improved yield by 23 % on

average in New South Wales over 12 trials (Richards, 2006). However, Blum (2005) commented on the fact that selecting cultivars with high WUE using CID was not suitable to all environments as it resulted in small or earlier maturing plants with reduced water use. In dry environments with stored soil moisture, high WUE cultivars will have an advantage.

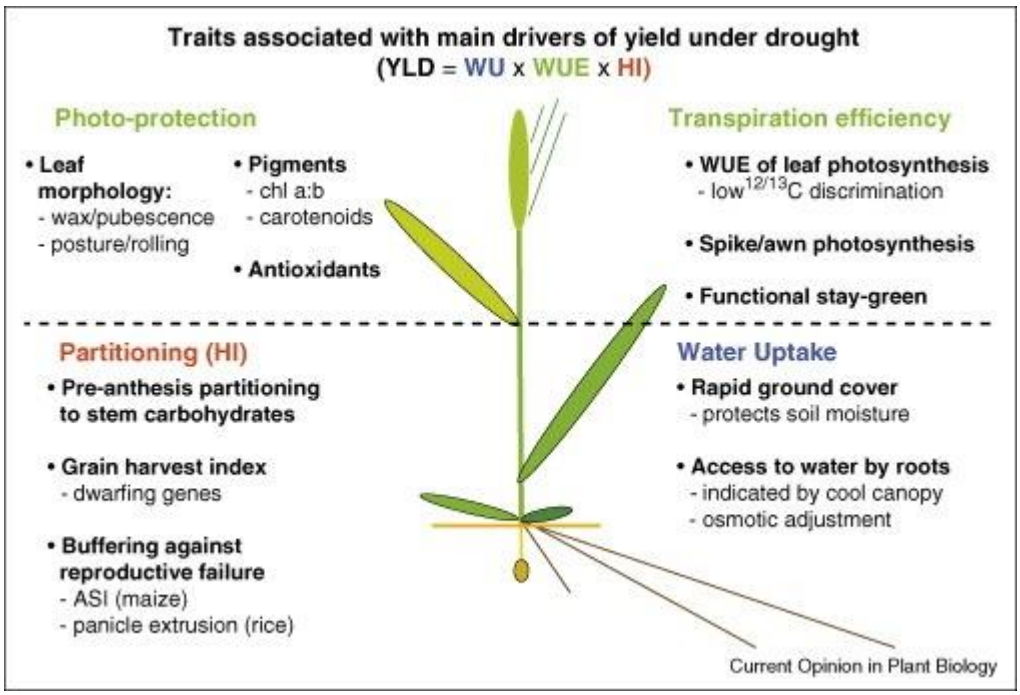

Figure 2. Traits associated with main drivers of yield under drought. Sourced from Reynolds and Tuberosa (2008).

Heat stress has also been extensively studied in wheat and reviewed (Wahid et al., 2007; Cossani & Reynolds, 2012). Wheat is a temperate cereal with optimal growth when temperatures range between 12 °C and 27 °C. Heat stress occurs when temperatures are above the optimal temperature threshold, impairing plant development and reproduction. Heat stress can occur in the field as chronic high temperatures during grain filling with temperature ranging between 18 - 25 °C and maximum day temperature up to 32 °C in temperate environments (Wardlaw & Wrigley, 1994). In warmer environments, chronic high temperatures are above 32 °C during grain filling; in Australia, temperatures at grain filling are often above 30 °C and can go up to 40 °C. Short periods of high temperatures (< 32 °C) or heat waves also impact grain development, starch and protein accumulation and can induce early senescence (Wardlaw & Wrigley, 1994). The effects of heat stress on final grain yield depend on the plant developmental stage when temperatures rise. When heat stress occurs before or during anthesis, grain yield

was reduced due to a decrease in fertilization and grain set (Ferris et al., 1998). Heat stress at the early stage of grain filling reduced grain weight and dry matter content (Stone and Nicolas 1996). As for yield under drought, models have been developed to decipher the traits contributing to yield under heat stress (Cossani & Reynolds, 2012). Yield under heat is determined by three components when water and nutrients are not limiting factors: light interception (LI), radiation use efficiency (RUE) and harvest index (Figure 3). LI depends on canopy development and architecture but also a stay-green phenotype while RUE depends on the difference between carbon assimilation and use for the growth (Cossani & Reynolds, 2012).

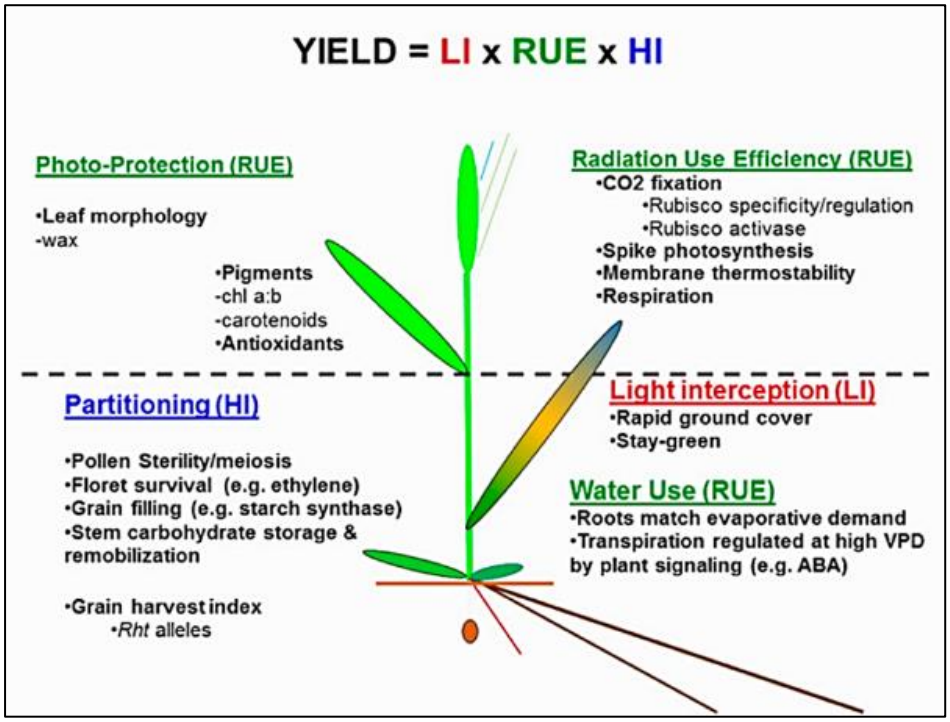

Figure 3. Main drivers of yield under heat stress sourced from Cossani & Reynolds (2012).

b. Plant responses to a combination of drought and heat stresses

In the field, drought and heat often occur at the same time. Although plant's responses to combined drought and heat stress have not been extensively investigated, first studies showed that responses to the combined stresses cannot be determined by studying the individual stress (Mittler, 2006). Few studies have investigated the effects of combined drought and heat stress and, in parallel, have looked at the effects of the individual stress in wheat (Machado and Paulsen 2001; Mahrookashani et al., 2017; Nicolas et al., 1984; Prasad et al., 2011; Shah and

Paulsen, 2003; Wang et al., 2010; Wardlaw, 2002). The same conclusion was drawn in these studies, that damage was more dramatic under combined stresses than induced by individual stress. Mahrookashani et al. (2017) observed that single grain weight was reduced by up to 27 % under drought stress while it was up to 83 % under drought and heat stress combined. One of the reasons is that drought and heat stresses may have opposite effects on a plant's water management. A study of two wheat lines showed that plants under drought reduced their transpiration rate, stomatal conductance and intercellular CO_2 concentration while heat stress had positive effects on these traits (Wang et al., 2010). Plants under drought close their stomata and reduce their transpiration to avoid water loss decreasing the fixation of CO_2 while under heat stress plants cool down by opening their stomata and transpiring. Recently, Tricker et al. (2018) reviewed the physiological traits that could be relevant to breed for improved tolerance to the combined stresses in wheat. They concluded that improving water management during plant growth through fine-tuning gas exchange could maintain CO_2 accumulation while protecting against photosynthetic damage. It remains that the physiological basis of combined drought and heat stresses is not fully understood in wheat and this is the same at the genetic level.

3. Use of omics to identify stress-regulated genes

Omics tool are useful to study stress-regulated changes of the transcriptome, proteome and metabolome. Studying the transcriptome profile of cultivars under stressed conditions enables the identification of stress-regulated transcripts and pathways contributing to the tolerance mechanisms of plants under drought and/ or heat stress. Many studies have been conducted in wheat and barley to identify transcripts affected by drought stress (Aprile et al., 2009; Khoshro et al., 2013; Mohammadi et al., 2008; Ozturk and Talamé 2002; Talamè et al., 2007), heat stress (Qin et al., 2011) and both stress combined (Liu et al., 2015).

Omics data can also be used to identify regions of the genome associated with drought and/or heat stress tolerance by mapping variations in transcripts (eQTL), metabolites (mQTL) and proteins (pQTL) (Langridge & Fleury, 2011). A study of 205 metabolites in flag leaf tissues along with 29 agronomic traits in the Excalibur / Kukri (RILs) population enabled the identification of mQTL for 95 metabolites and QTLs for 22 agronomic traits (Hill et al., 2013). Out of 95 metabolites, mQTLs for 38 metabolites were co-located with QTLs for agronomic traits. Integrated approaches combining QTL mapping and mapping of differentially expressed

genes (eQTL) could lead to the identification of causal gene(s) responsible for the phenotype at a QTL. In durum wheat, combined QTL mapping with the mapping of differentially expressed genes and known genes identified six candidate genes and 19 differentially expressed sequences associated with QTL for drought tolerance traits (Diab et al., 2008). Combined QTL mapping and transcript profiling enabled the identification of QTLs for drought response co-locating with 88 candidates genes impacted by drought stress in maize (Marino et al., 2009). In rice, a microarray study narrowed down the list of candidate genes to 8 genes differentially expressed between tolerant and susceptible cultivars and associated with a QTL for grain number on chromosome 4 (Deshmukh et al., 2010). The mapping of expression data can also lead to the identification of both cis-and trans-acting eQTLs (Jordan et al., 2007). Expression analysis of developing seeds allowed the identification of 542 eQTLs at a single location, in a wheat DH population cv. RL4452 × 'AC Domain'. The mapping of the eQTLs combined with the physical mapping of wheat expressed sequence tags identified both cis-acting eQTLs and trans-acting eQTLs.

Omics have also been shown to potentially help hybrid breeding by predicting hybrid performance and heterosis. In maize, transcriptome profiling of 21 parental lines used for the breeding of 98 hybrids were used to predict transciptome-based distances; comparison with genetic distances showed a high correlation (Frisch et al., 2010). In rice, a metabolomic study has also been used to predict the performance of 18 inbred lines for three agronomic traits: plant height, heading date and grain yield (Dan et al., 2016). Using information on 525 metabolites in the parental lines combined with a partial least square regression method, they observed high predictive abilities for the three traits and a high efficiency as not affected by population structures.

a. Genes conferring drought or heat tolerance in wheat

So far, only a few genes have been functionally characterized in wheat for their role in drought or heat tolerance. Gene engineering techniques have been used in each case to generate transgenic lines either overexpressing gene targets under the control of a constitutive or stress-inducible promoter, or by silencing gene targets. Phenotyping of the stable transformants under drought or heat stress was used to validate gene targets as actually contributing to drought or heat tolerance. None of these genes was identified through genetic mapping. However, it is encouraging to see that these genes have been shown to be associated with drought and / or heat tolerance in wheat. Examples are described below.

b. Genes associated with drought stress tolerance in wheat

Many of the genes associated with drought tolerance in wheat are transcription factors regulating the expression of downstream genes through interactions with cis elements present in the gene promoter regions (Nakashima et al., 2014). A few transcription factors of the *DREB* (*dehydration responsive element binding*) gene family have been characterized in wheat for their role in improving drought tolerance. Transgenic plants overexpressing *TaDREB* showed a stay-green phenotype of leaves several days after stopping watering and remained green days after rewatering in comparison to non-transgenic plants (Wang et al., 2006). Transgenic plants had also more than a two-fold increase in leaf content of the osmolyte proline. *TaDREB2* and *TaDREB3* overexpressing plants had an improved survival rate under severe drought compared to the wild-type (WT) but were delayed in their development and had a reduced grain yield compared to the WT (Morran et al., 2011). Similarly, wheat transgenic plants overexpressing the *A. thaliana DREB1A* gene under the control of a stress-inducible promoter showed improved survival rate and increased resistance to water stress in glasshouse conditions (Pellegrineschi et al., 2004) but no yield advantage in field conditions compared to null plants (Saint Pierre et al., 2012).

Transcription factors from other families have been characterized in wheat. Transgenic plants overexpressing *TaNAC69*, a transcription factor of the *NAC* (*NAM*, *ATAF/ATAF2*, and *CUC2*) family, displayed increased shoot biomass under mild salt stress and water-limited conditions compared to WT (Xue et al., 2011). Silencing of *TaBTF3* (*basic transcription factor 3*) in transgenic wheat reduced survival rates, proline content and water content but also increased salt accumulation and water loss under both cold and drought stresses (Kang et al., 2013). Finally, transgenic wheat plants overexpressing *TaERF3* (ethylene-response factor) accumulated more proline and chlorophyll while H_2O_2 content and stomatal conductance were reduced under drought stress compared to WT plants (Rong et al., 2014).

c. Genes conferring heat stress tolerance in wheat

Genes conferring heat stress tolerance have been characterized only recently in wheat. Transcription factors of the heat shock protein (HSP) family have been identified for their ability to protect plants against stress by maintaining protein functional conformations (Wang et al., 2004). Wheat plants overexpressing *TaHSfA6f,* an A6 subclass of a heat shock transcription factor, exhibited improved thermotolerance under heat stress when plants were

pre-treated with polyethylene glycol (PEG) before exposure to extreme heat temperature (> 45 °C) compared to the WT (Xue et al., 2015). *TaHsfC2a,* a heat shock transcription factor, acted as a transcriptional activator of heat shock protein genes in grains, improving thermotolerance. Genes involved in different tolerance mechanisms have been also characterised in wheat (Zang et al., 2017). Plants overexpressing *TaFER-5B,* a ferritin gene mapped on chromosome 5, showed improved thermotolerance associated with reactive oxygen species (ROS) scavenging. Under stressed conditions, the concentration of ROS can dramatically increase and induce irreparable cell damage. Recently, the gibberellic acid-stimulated transcript gene, *TaGASR1*, has been characterised in wheat as a positive regulator of heat stress tolerance in overexpressing lines under the control of a maize ubiquitin promoter (Zhang et al., 2017). The transgenic wheat lines had an improved grain weight on the main spike and an improved oxidative stress tolerance compared to control plants under heat stress.

In almost all the case, the genes characterised above have been identified through transcriptome analysis. Reverse genetics were then used to validate their role in conferring stress tolerance. Many of the genes associated with stress tolerance are transcription factors that are involved in signalling cascades. Identified genes at the beginning of the cascade of signals can lead to the identification of downstream genes responsible for stress tolerance. However, inconsistent results have been observed between glasshouse conditions and field environments (e.g. *DREB1A*) due to the nature of the stress that plants are exposed to under controlled conditions (e.g. used of PEG to improve thermotolerance). Yield is a complex trait influenced by the interaction between the Genotype x Environment (G x E) that results, sometimes, in a strong allelic effect in contrasting environments (Parent et al., 2017). A cultivar that performed well in one environment will not necessarily perform the same way in a different environment. To characterise the molecular basis of yield in dry and hot climates, forward genetics is more appropriate as the trait is controlled by multiple genes. Forward genetics start with an observed phenotype and aims to identify the gene(s) responsible for the variation.

4. Genetic mapping of regions of the wheat genome controlling yield and yield components

a. Importance of defining a target environment

To help breeding high-yielding cultivars adapted to a wide range of environments, CIMMYT classified the wheat cultivated area into 12 mega-environments (ME): 6 ME for spring wheat, 3 ME for facultative wheat and 3 ME for winter wheat (Rajaram et al., 1994) (Figure 4). The ME were defined by the type of biotic and abiotic stresses occurring, cropping system requirements, consumer preferences and volume of production. This classification helps breeders to select germplasm and target physiological and morphological traits that could confer adaptation to their target environment.

Crop modelling is a powerful tool to predict yield in target environments by combining large amounts of data collected over multiple growing seasons or field trials and mathematical equations. Chenu et al. (2017) have reviewed the use of crop modelling for wheat and its benefit for breeding in the context of climate change. By combining information on genotypes, meteorological and soil data but also farm management data, crop modelling can anticipate the effects of future seasonal changes for specific breeding programs.

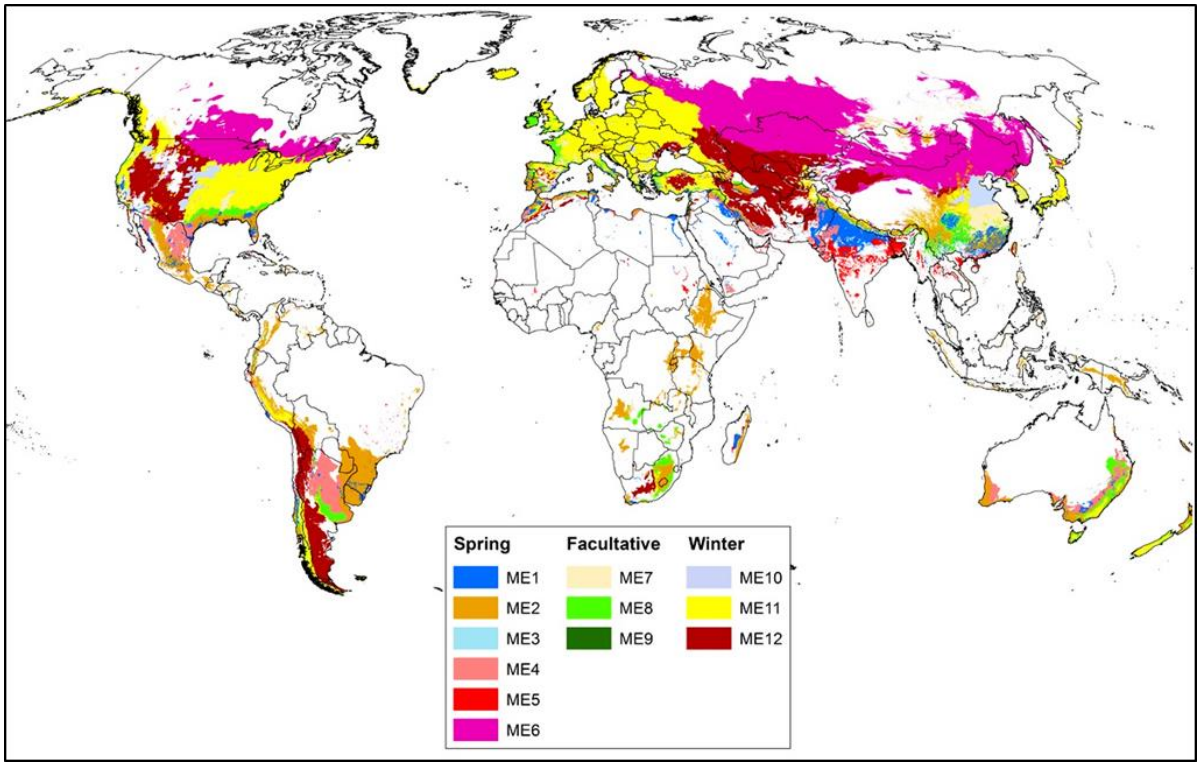

Figure 4. Geographic location of the 12 mega-environments described by Rajaram et al., (1994). Image from sourced from Reynolds et al., (2012).

b. Genetic mapping of yield and yield component traits in wheat

Yield is a complex quantitative trait, difficult to breed for as it is highly variable across environments. The trait is driven by a strong G x E interaction resulting in low yield stability. Yield and yield components are controlled by multiple genes. Consequently, the identification of the genetic determinants of yield in dry and hot environments for the breeding of high-yielding cultivars is difficult (Fleury et al., 2010). Quantitative trait locus (QTL) analysis has been used extensively in plant breeding to identify regions of the genome controlling important agronomic traits. QTL analysis has been described as the mapping of the genetic factors contributing to the variation of a quantitative trait using genetic markers in chromosomes (Geldermann, 1975). It enables us to associate genetic regions to phenotypic variation. The development of high throughput molecular markers technologies in wheat contributed to the study of genetic diversity through genome-wide genotyping. Technologies such as Kompetitive Allele Specific PCR (KASP) (He et al., 2014), genotyping arrays for high-throughput single nucleotide polymorphism (SNPs) assay using different technologies including Affymetrix Axiom (Winfield et al., 2016; Allen et al., 2017) or Illumina Infinium beadChip (Cavanagh et al., 2013; Wang et al., 2014) but also genotyping by sequencing methods (reviewed in Scheben et al., 2017) enabled the development of high resolution genetic maps for QTL analyses.

Wheat QTLs have been identified through the combination of high density genetic maps and the phenotyping of segregating populations. Genes underlying QTL can be identified by studying large populations representing a great number of recombination events. *Gpc-B1*, a gene controlling protein, zinc and iron content, was fine mapped to a 7.4 kb physical interval after the genotyping of 9,000 gametes (Uauy et al., 2006).

QTL analysis has been extensively used in wheat to identify genetic factors associated with drought and / or heat tolerance in wheat. A recent review, highlighted the QTLs identified so far in wheat under drought or heat stress but also under drought and heat combined (Tricker et al., 2018). The QTLs were associated with numerous phenotypic traits but only a few were co-located with QTLs for yield and yield-component traits (Bennett et al., 2012; Czyczylo-Mysza et al., 2011; Dashti and Yazdi-Samadi 2007; Golabadi et al., 2011; Kadam et al., 2012; Kirigwi et al., 2007; Maccaferri et al., 2008; Merchuk-Ovnat et al., 2017; Ogbonnaya et al., 2017; Paliwal et al., 2012; Pinto et al., 2010; Quarrie et al., 2005; Shirdelmoghanloo et al., 2016; Tahmasebi et al., 2016; Xu et al., 2017; Yang et al., 2007; Maphosa et al., 2014; McIntyre et al., 2010).

Molecular markers associated with agronomic traits can be used for marker-assisted selection (MAS) to track desirable alleles in germplasm (Langridge & Fleury, 2011). MAS has been used in different breeding programs to target traits such as disease / insect / virus resistance but also quality traits (reviewed by Gupta et al., 2010). MAS has been also used for the pyramiding of QTLs or genes. Tyagi et al. (2014) reported the pyramiding of eight QTLs/genes for grain quality traits and rust disease resistance in the wheat line PBW383 using MAS. Although MAS is successful for loci of major effect, MAS for yield and yield components is still limited by the fact that the traits are controlled by multiple genes of both major and minor effects, limiting the deployment of QTL in breeding programs. Moreover, as the trait is influenced by G x E, MAS for yield and yield components should be addressed to a specific target environment.

5. Positional cloning of genes in wheat: a long but possible process

a. Wheat genes identified by positional cloning of QTLs

Positional cloning consists of the identification and analysis of genes underlying a genomic region controlling the variation of traits. The positional cloning of QTLs in wheat is a long and laborious process due to the complexity and size of the bread wheat genome (17 Gb; IWGSC, 2014), the high number of repetitive regions and its hexaploid nature (2n = 6x = 42). The lack, until recently, of a reference sequence for the wheat genome was one of the limitations to the positional cloning of genes. Nevertheless, major genes have been cloned in wheat through positional cloning including genes controlling photoperiod (*Ppd-B1* and *Ppd-D1*, Beales et al., 2007), vernalization (*Vrn1* and *Vrn2*, Yan et al., 2003; Yan et al., 2004), plant height (*Rht1* and *Rht2*, Peng et al., 1999), boron tolerance (Pallotta et al., 2014), male fertility (*Ms1*, Tucker et al., 2017) and many genes determining resistance to pathogens (Krattinger et al., 2009). The cloning of these genes has been possible through the combination of genetic and physical mapping, and using the genome synteny between wheat and other grass species (Moore et al., 1995). The positional cloning of *Ph1*, a gene controlling chromosome pairing, was achieved through the development of molecular markers using the gene synteny between wheat and the rice and brachypodium genomes (Griffiths et al., 2006). Positional cloning of *Ms1* was assisted through map-based cloning using a population of 7000 gametes segregating for a male sterile mutant *ms1d*, that narrowed the locus to 0.5 cM (Tucker et al., 2017). Markers designed in the interval enabled the identification of bacterial artificial chromosome (BAC) sequence where eight genes were present of which one was proved causal.

The cloning of genes for complex traits such as yield variation in drought and/or heat stress tolerance has not yet been achieved in wheat as most of the QTLs identified control only a small proportion of the variance for the trait, combined with the instability of QTLs across environments especially under strong G x E. However, genes associated with yield related traits have been identified in wheat (reviewed by Nadolska-Orczyk et al., 2017), of which *TaGW2*, an orthologue of the rice gene *GW2* that encodes for an E3-ubiquitin ligase and negatively regulates grain size. Silencing of *TaGW2* in wheat transgenic lines decreased grain size and knock-down of the gene reduced endosperm cell number suggesting an opposite effect of *TaGW2* compared to its orthologue in rice (Bednarek et al., 2012). In another experiment, silencing of the three homologues of *TaGW2* in wheat located on chromosomes 2A, 2B and 2D resulted in transgenic lines with bigger grain (Hong et al., 2014).

b. Resources for gene functional validation

The functional characterisation of the causal gene(s) or causal sequence variant(s) conferring drought and/or heat stress tolerance in wheat is essential to validate their function and understand the mechanisms associated with the phenotype. Gene engineering has commonly been used in plants to investigate the function of genes of interest through the generation of transgenic lines, by either overexpressing or silencing the gene of interest. Complementation of knock-out genes to restore the wild-type phenotype is also an essential step in validating gene function (Varshney et al., 2011)

Reverse genetic tools have also been used to study genes of interest through the characterisation of gene variants. Techniques such as TILLING (Targeted Induced Lesions IN Genomes), which combines the generation of a mutant population by either chemical or physical treatments and the high-throughput detection of mutations in target genes (McCallum et al., 2000), are commonly used in plants. A recent example used a TILLING mutant to confirm the gene identified for male sterility (*Ms1)* in wheat. The mutant, *ms1h,* carried a mutation in the lipid binding domain contributing to the functional characterisation of *Ms1* (Tucker et al., 2017).

Targeted induced mutation through gene editing is also increasingly used to functionally characterise genes in crops (Belhaj et al., 2015). One of the most promising techniques is the CRISPR (Clustered regularly interspaced short palindromic repeats) /Cas (CRISPR-associated) 9 system that was first identified in bacteria and archea as a RNA-mediated defence mechanism against viruses and plasmids (Jinek et al., 2012). The technique enables the editing of specific sequences through the generation of single guide RNA (sgRNA) of 20 nucleotides binding to

gene(s) target via a PAM sequence (5'-NGG). The PAM motif is recognized by the Cas 9 protein that induces DNA double-strand breaks that are repaired either by nonhomologous end-joining or homology-directed repair (Shan et al., 2014). CRISPR-Cas9 has been successfully used in maize to generate variants of the *ARGOS8* gene which regulates ethylene responses (Shi et al., 2017). The new *ARGOS8* allele increased grain yield under drought stress at flowering while no yield penalty was observed under well-watered conditions compared to WT.

c. A reference genome sequence for wheat

Combining different approaches such as genomics, transcriptomics, metabolomics and proteomics, is expected to accelerate the identification of candidate genes underlying major QTL (Langridge & Fleury, 2011). Next-generation sequencing (NGS) contributed to the acceleration of the sequencing of complex genomes (Shendure & Ji, 2008). The first whole-genome shotgun sequencing (WGS) of the hexaploid wheat cv. Chinese Spring by used the Roche 454 pyrosequencing technique (Brenchley et al., 2012). Using the synteny with related genomes such as rice, sorghum and Brachypodium, between 94 000 and 96 000 genes were identified and located on the three sub-genomes. To investigate the genetic diversity of the Australian germplasm and de-novo SNPs discovery, an initiative combining researchers and Bioplatforms Australia led to the shot-gun sequencing of 16 wheat lines including RAC875, Kukri, Drysdale and Gladius (Edwards et al., 2012). These lines were sequenced using Illumina technology with a 6 - 30 X raw coverage. Data were curated to remove low quality reads and made publicly available (http://wheatgenome.info). The selection of these cultivars for WGS was particularly relevant as QTLs for yield and yield components have been identified in the doubled-haploid lines (DH) population of the cross RAC875 x Kukri (Bennett et al., 2012) and RIL population of the cross Drysdale x Gladius (Maphosa et al., 2014).

In a recent review, it has been estimated that the number of published papers on gene cloning in rice increased from 40 – 80 per year between 1995 and 2001 to more than 100 articles per year after the release of the rice genome (Jackson, 2016). A high quality reference sequence is essential for the identification of genes underlying agronomic traits and to study the genome-wide genetic diversity. Sequencing the wheat genome has been one of the biggest challenges of the crop science community in the past decade. The flow sorting of wheat chromosomes has greatly contributed to the sequencing of the bread wheat genome by separating the genome into single chromosomes and chromosomes arms reducing genome complexity (Kubaláková et al., 2002). The development of a draft sequence for the 3B chromosome was a first step towards

the sequencing of the wheat genome (Paux et al., 2008). The construction of the 3B physical map was based on a chromosome-specific bacterial artificial chromosomes (BAC) library obtained from the flow-sorting of chromosome 3B followed by the digestion and cloning of the chromosome fragments (Šafář et al., 2004). The 3B physical map covered 1 Gbp (82 % of 3B chromosome) assembled into 1,036 contigs anchored with 1,443 markers (Paux et al., 2008). The sequencing of the 3B chromosome was part of the work of International Wheat Genome Sequencing Consortium (IWGSC) to raise awareness of the needs and challenges in sequencing the wheat genome and to pool resources from various countries. The IWGSC initiative led first to the release of the chromosome survey sequence (CSS) cv. Chinese Spring representing 61 % of the genome (IWGSC, 2014). The CSS was obtained by shot-gun sequencing of chromosome sorted DNA using Illumina technology and de-novo assembled. This assembly was followed by the IWGSC Ref v 0.4 released in June 2016 (https://wheat-urgi.versailles.inra.fr/). This assembly was obtained by de-novo assembly of Illumina shot-gun sequencing data using NRGene DeNovoMAGIC software (www.nrgene.com) that produced scaffolds for a total size of 14.5 Gbp. Scaffolds have been assigned to chromosomes using POPSEQ that allows to genetically order WGS data independently of a physical map (Ariyadasa et al., 2014) and a Hi-C map which exploits the folding properties of DNA to identify interaction between genome regions (Lieberman-Aiden & Berkum, 2009). The latest wheat reference sequence, IWGSC Ref 1.0, combines all data available for the wheat genome including the IWGSC Ref 0.4, physical maps for each chromosome, BAC sequences for eight chromosomes, BioNano optical maps for chromosome group 7 and genotyping by sequencing (GBS) to map the Chinese Spring x Renan RILs population (www.wheatgenome.org). Another reference sequence of the wheat genome cv. Chinese Spring and covering almost the entire genome, ~ 15 Gbp was obtained by de-novo assembly of 100 bp short Illumina reads library combined with a library of long Pacific Biosciences reads (Zimin et al., 2017). The availability of a reference sequence is a step forward toward the cloning of genes in wheat.

The increased amount of genomic data along with the development of bioinformatic tools should accelerate the identification of sequence variants and help to the positional cloning of genes. A few bioinformatics tools are now available in wheat to integrate different datasets for the identification of de-novo SNPs from large NGS datasets. The Diversity Among Wheat geNones database (DAWN) combined the IWGSC Ref 1.0 cv. Chinese Spring with the gene annotation, WGS data of 16 wheat lines but also RNA-seq data and markers datasets. The

genome browser, GBrowse, is another application that allow the visualization of genomic data and annotations (Stein et al., 2002). This application enables the visualization of large regions of a genome and enables the integration of additional tracks for data mining and identification of sequence variants. Ensembl is another project that has been created to group information on sequenced genomes in one location (Birney et al., 2004). An Ensembl for wheat is available (http://plants.ensembl.org/Triticum_aestivum/Info/Index). It allows the integration of any data set that could be mapped into reference genomes. Ultimately, the international Wheat Initiative aims to create a wheat information system to support the wheat research community and integrate in a unique database all the genetic resources and bioinformatic tool available for wheat (www.wheatinitiative.org).

6. The wheat *qYDH.3BL* locus for yield in hot and dry environments

a. Identification of a constitutive QTL on chromosome 3BL

This study focusses on a previously reported QTL, *qYDH.3BL*, associated with yield and canopy temperature in a hot and dry climate and located on the long arm of chromosome 3B (Bennett et al., 2012). The QTL has been identified in the DH population of the cross between the drought tolerant line RAC875 and the drought susceptible variety Kukri. The QTL was identified in a multi-environment analysis of 16 field trials conducted in South Australia and Mexico (Cuidad de Obregon) over three seasons. In northern Mexico, plants were phenotyped under three different conditions: drought (conventional sowing time and flooding irrigation), heat (late sowing and flooding irrigation) and fully-irrigated (conventional sowing time and drip irrigation). Bonneau et al., (2013) narrowed down the QTL to a 5 cM interval by combining a high-density genetic map of the RAC875 x Kukri DH population and an extended multi-environment analysis combining 21 field trials. *QYDH.3BL* was associated with an increase in yield, thousand grain weight (TGW) and early vigour. They also found that the QTL was under strong G x E control. The allele from RAC875 was positively linked with these traits in almost all the Mexican field trials, while the allele from the other parent "Kukri" was positively associated to three irrigated trials in South Australia. Overall the QTL had no effect in rain-fed environments of South Australia (Bonneau et al., 2013). A study of the environmental constraints governing the G x E interaction at *qYDH.3BL* identified an allele effect dependent on high temperatures, suggesting that *qYDH.3BL* is a heat-related QTL (Parent et al., 2017). The QTL has also been identified in the recombinant inbred lines (RILs) population of a cross

from the two wheat varieties, Gladius and Drysdale. The positive allele from Drysdale was associated with an increase in TGW in five field trials conducted at Leeton in New South Wales, Australia and Obregon in northern Mexico (Maphosa et al., 2014). A QTL associated with canopy temperature and co-locating with the genetic interval of *qYDH.3BL* has also been found in a RILs population of the cross between the two wheat varieties Seri and Babax (Pinto et al., 2010).

In South Australia, crops are grown in a Mediterranean-type climate characterized by a cyclic drought, with alternate periods of rainfall and hot and dry weather, then followed by a terminal drought (Izanloo et al., 2008). Recorded temperatures at flowering time ranged between 19.3 to 22.6 °C in the field trials (Bonneau et al., 2013). In northern Mexico, in the Sonora region, the weather is characterized by a very hot climate with almost no rainfall during the growing season (Olivares-Villegas et al., 2007). Irrigation by flooding is practiced in this region, also characterized by high irradiation (20.8 $MJm^{-2}day^{-1}$) (Lopes & Reynolds, 2010). Temperatures at flowering time ranged from 27.1 and 30 °C (Bonneau et al., 2013). The two environments also contrasted in their soil type, in South Australia, the soil is characterized as shallow (Bennett et al., 2012) while the soil in Mexico is composed of sandy clay and has a deep profile with water available at depth (Verhulst et al., 2009). Leeton is located in New South Wales (NSW), Australia. Temperatures during summer can reach up to 42 °C and the average rainfall is around 433 mm per year (Bonneau, 2012). The soil is characterised as grey vertosol allowing genotypes to grow roots at depth with observed penetration rate up to 155.6 ± 3.0 cm (Wasson et al., 2014).

Northern Mexico and Leeton (NSW) are both characterised by a deep soil profile that favours genotypes with deep root systems able to reach stored soil moisture at depth. In these two environments, the RAC875 allele was positively associated to yield and yield components at *qYDH.3BL*. Based on these observations, we hypothesized that the deep soil profile contributed to the tolerance mechanism associated with the RAC875 allele at *qYDH.3BL*.

7. RAC875, a water conservative wheat line under water-limited environments

RAC875 (RAC-655//SR21/4*Lance/3/4*Bayonet) and Kukri (Madden/6*RAC-177//Grajo/76-ECN-44) were chosen as parental lines for the development of a mapping population to identify regions of the wheat genome associated with yield under drought stress (Fleury et al., 2010).

The response of RAC875 in water-limited environments was associated with a reduction of its water loss through reduction of its total leaf area surface and residual transpiration in the early stages of development (Izanloo et al., 2008). A shotgun proteomic study of the same cyclic drought experiment described in Izanloo et al. (2008), showed that RAC875 exhibited a large number of changes in protein level occurring quickly after the beginning of the stress, particularly proteins involved in oxidative stress metabolism and scavenging of reactive oxygen species (Ford et al., 2011). At the metabolic level, RAC875 exhibited an increase level of amino acids of which proline was the most significant (Bowne et al., 2012).
Study of the root anatomy and root hydraulic conductivity of RAC875 and Kukri, showed a lower root conductivity in RAC875 due to smaller stele and metaxylem diameters in the seminal roots (Schoppach et al., 2014). Smaller stele and metaxylem diameters were also observed in nodal roots of RAC875, independently of the environment where plants were grown (Steinemann et al., 2015). While the diameter and area of metaxylem vessels were reduced in RAC875, the number of vessels was more important compared to Kukri.

Combining the data of the wheat reference sequence cv. Chinese Spring, IWGSC Ref 1.0 with the WGS data of the parental lines enabled the de-novo identification of SNPs for the fine mapping of *qYDH.3BL* and the determination of its genomic structure in RAC875. The study of the *qYDH.3BL* in a deep-soil profile allowed us to test our hypothesis and validate what characteristic(s) of northern Mexico and Leeton environments trigger the QTL's expression. Root anatomy and morphology traits but also water management were investigated to see if the QTL plays a role in these traits. Finally, by integrating the genetic and physiological study at *qYDH.3BL*, we identified possible causal gene(s) contributing the yield variation at *qYDH.3BL* in hot climates.

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Chapter II

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Name of Principal Author (Candidate)	Pauline Thomelin
Contribution to the Paper	Conceived the experiment, performed experiment and analysed data and wrote the manuscript.
Overall percentage (%)	65 %
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 12.06.18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
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Contribution to the Paper	Provided help and critical comments for the bioinformatics analysis
Signature	Date 30/5/2018

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Contribution to the Paper	Supervised development of the work, contributed to the analysis and interpretation of the data. Reviewed and edited the chapter.
Signature	Date 29/05/2018

Chapter II: Fine mapping of *qYDH.3BL*, a QTL associated with heat tolerance in bread wheat (*Triticum aestivum L.*)

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1. Abstract

Crop productivity in hot climates is severely, negatively impacted by heat stress. To breed new cultivars able to tolerate hot conditions, it is essential to identify and understand the contribution of genes controlling yield variation. We focussed on the identification of the genetic factors associated with *qYDH.3BL,* a QTL located on the bread wheat chromosome arm 3BL. The QTL was identified in the doubled-haploid population from the cross between the drought tolerant line RAC875 and the susceptible variety Kukri. The QTL was constitutively expressed in the deep soil of Mexico, with the RAC875 allele positively associated with an increase in grain yield, thousand grain weight and early vigour under dry and hot conditions. We confirmed these effects using a deep-soil platform. We fine mapped the QTL interval using RAC875 x Kukri RILs to a 690 kbp sequence with 12 genes in the wheat cv. Chinese Spring reference genome, IWGSC Ref 1.0. The study of a wheat diversity panel of 808 worldwide accessions identified four haplotypes. We identified two major haplotypes, haplotype I shared by RAC875, and haplotype IV shared by Kukri and two minor haplotypes. Haplotype I was over represented in CIMMYT germplasm suggesting that this variant had been selected for in a global wheat breeding program due to its influence on yield under heat stress.

2. Introduction

Bread wheat is the world's third largest crop providing 20 % of food and has an important role in global food security (Shiferaw et al., 2013). It is also the most widely grown crop worldwide in a wide range of climates, including in many regions where the climate is challenging. Major yield increases have been achieved through the use of dwarfing and maturity alleles. These alleles drove major advances in wheat breeding that led to the high yielding varieties of the Green revolution of the 1960s (Dalrymple, 1978). Since then, yield improvement has slowed to about 1% increase per year and the introduction of novel, high-yielding varieties is urgently required to ensure food security (Atlin et al., 2017).

Wheat productivity in the field is affected by many factors of which drought and heat are the most devastating abiotic stresses. Due to the increasing occurrence of drought and heat stress particularly in the Mediterranean region, the USA, India and Australia, which are amongst the largest producers of bread wheat in the world, improving yield in these stressful conditions is a priority. Targeting crop productivity in regions affected by drought and heat is believed to be one of the best strategies to reach the 1.6 % yield improvement per year required to face the needs of an increasing world population (Asseng et al., 2011; Lobell et al., 2012).

The identification of genes associated with yield variation in stress-prone environments is crucial to the breeding of new high yielding varieties. Many genetic studies have focussed on the identification of QTL associated with yield variation in dry and hot conditions in wheat using bi-parental populations (Bennett et al., 2012; Diab et al., 2008; Kirigwi et al., 2007; Maccaferri et al., 2008; Maphosa et al., 2014; McIntyre et al., 2010; Pinto et al., 2010; Shirdelmoghanloo et al., 2016). Although many QTL for yield have been identified, few have been deployed in breeding programs due to the complexity of the trait, its high variability across environments and its multigenic nature. Positional cloning of QTL is difficult due to the complexity of the bread wheat genome and the absence, until recently, of a reference genome. The joint efforts of scientists through the International Wheat Genome Sequencing Consortium (IWGSC) have led to the release of a reference genome, IWGSC Ref 1.0 cv. Chinese Spring (http://www.wheatgenome.org/). The increasing amount of genetic data available will greatly aid the cloning of QTL associated with yield variation.

In this study, we focussed on a QTL located on chromosome 3BL, *qYDH.3BL*, identified in a doubled-haploid population from the cross between RAC875 and Kukri. They had been selected for their contrasting physiological responses in Mediterranean-like climatic conditions. RAC875 has been characterised as a water conservative cultivar while Kukri depletes water

more quickly under stress (Izanloo et al., 2008). A multi-environment analysis of 21 field trials described in Bonneau et al. (2013) showed a strong G x E interaction at *qYDH.3BL*. The QTL was constitutively expressed in the deep soil of northern Mexico with the RAC875 allele positively contributing to yield, thousand grain weight and early vigour. However, in rare conditions, the Kukri allele increased yield at *qYDH.3BL* when irrigation was supplied in South Australian fields (Bennett et al., 2012; Bonneau et al., 2013). Parent et al. (2017) observed an allele effect at *qYDH.3BL* dependent on temperature, indicating that *qYDH.3BL* is a heat related QTL. This QTL was also identified in the Drysdale x Gladius RILs population with the positive allele from Drysdale contributing to grain width, thickness, thousand grain weight and yield in deep soils of New South Wales (Maphosa et al., 2014).

Here, we report (i) the fine mapping of *qYDH.3BL*, a QTL previously described on chromosome 3B that increases yield and yield components in hot and dry climates (Bonneau et al. 2013); (ii) the identification of a list of 12 candidate genes in a narrowed sequence interval of 690 kbp; (iii) the worldwide allelic distribution of *qYDH.3BL* across continents and through breeding history.

3. Results

The QTL, *qYDH.3BL*, originally found in the spring wheat RAC875 x Kukri DH population (Bonneau et al., 2013) is also present in Drysdale x Gladius (Maphosa et al., 2014). Multi-environment analysis in Australia and Mexico showed that the allele from RAC875 and Drysdale is the same and increased yield in comparison with the Kukri and Gladius allele (Figure 1a and b). To fine map *qYDH.3BL* in RAC875, we anchored the interval defined by the multi-environment analysis onto the IWGSC v 1.0 reference sequence of Chinese Spring. The whole genome shotgun sequencing data of the four parent lines (Edwards et al., 2012) were aligned against the reference sequence to identify SNPs. Comparison between the haplotypes RAC875-Drysdale vs Kukri-Gladius enabled us to find new sequence variants. Seventy-four SNP-based markers (AWG- and ADW-) and InDel markers were added to the RAC875 x Kukri RIL map previously described in Bonneau et al. (2013) and to the Drysdale x Gladius RIL map described in Bonneau (2012) to identify new recombination points.

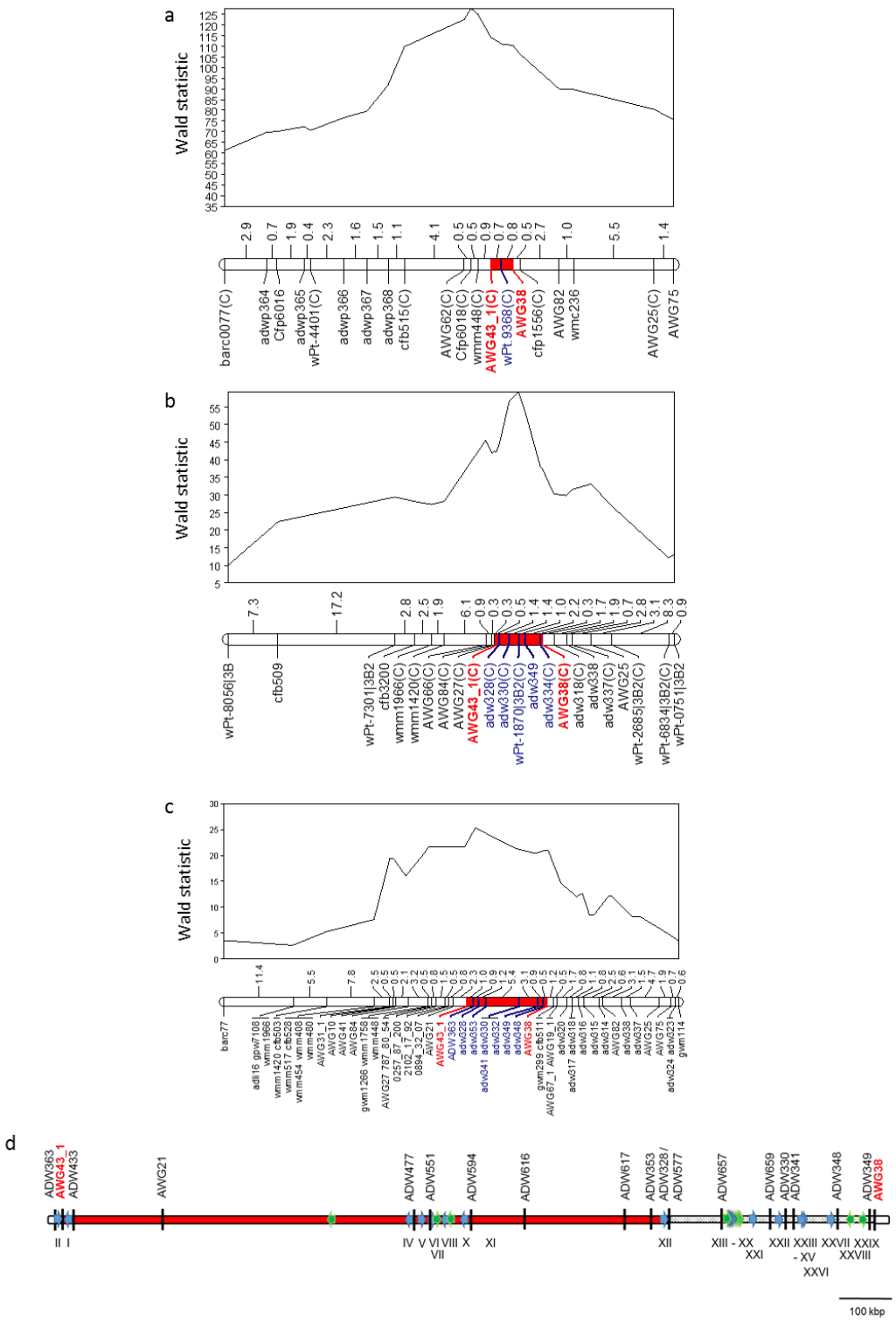

Figure 1. Genetic mapping of *qYDH.3BL* in RAC875 x Kukri and Drysdale x Gladius populations and its genomic structure in RAC875. a, QTL analysis of the RAC875 x Kukri DH population in 27 field trials located in northern Mexico and Australia (Bennett et al., 2012; Bonneau et al., 2013). b, QTL analysis of the Gladius x Drysdale RILs in 5 field trials in 2009 and 2010 located in NSW and northern Mexico . c, QTL analysis of RAC875 x Kukri recombinant inbred lines (RILs) in 4 field trials conducted in 2011 and 2012 in Cuidad de Obregon, Mexico. b,c, QTL analysis delimited a new interval located between the markers AWG43_1 and AWG38. d, Physical map of *qYDH.3BL* in RAC875. The blue arrows represent the high confidence genes and the green arrows the low confidence genes.

The multi-environment analysis of the RAC875 x Kukri RILs using the high-density genetic map refined the QTL interval delimited by the flanking markers AWG43_1 and AWG38. The QTL was significant ($P < 0.001$) for yield in 2012 in Mexican environments after late sowing heat treatment. The RAC875 allele was positively associated with a 10 % increase of yield compared to the Kukri allele (Figure 1c).

We developed a deep soil-mimic platform to test the hypothesis that a deep soil profile, characteristic of the Mexican environments, triggered *qYDH.3BL* expression. RAC875 x Kukri RILs previously grown in Mexican field trials (Bonneau et al., 2013) were phenotyped in 2014, in the deep soil platform with two treatments, drought alone and combined drought and heat. The analysis of variance of the drought and heat combined treatment showed a significant positive effect ($P < 0.05$ and $P < 0.01$) of the RAC875 allele on most traits. The RAC875 allele at *qYDH.3BL* increased spike length, stem biomass, seminal roots number, spikelets per spike and many other traits by more than 20 % compared to Kukri allele (Figure 2). No effects were observed on stomatal density, nor on carbon and nitrogen isotope discrimination in mature grains.

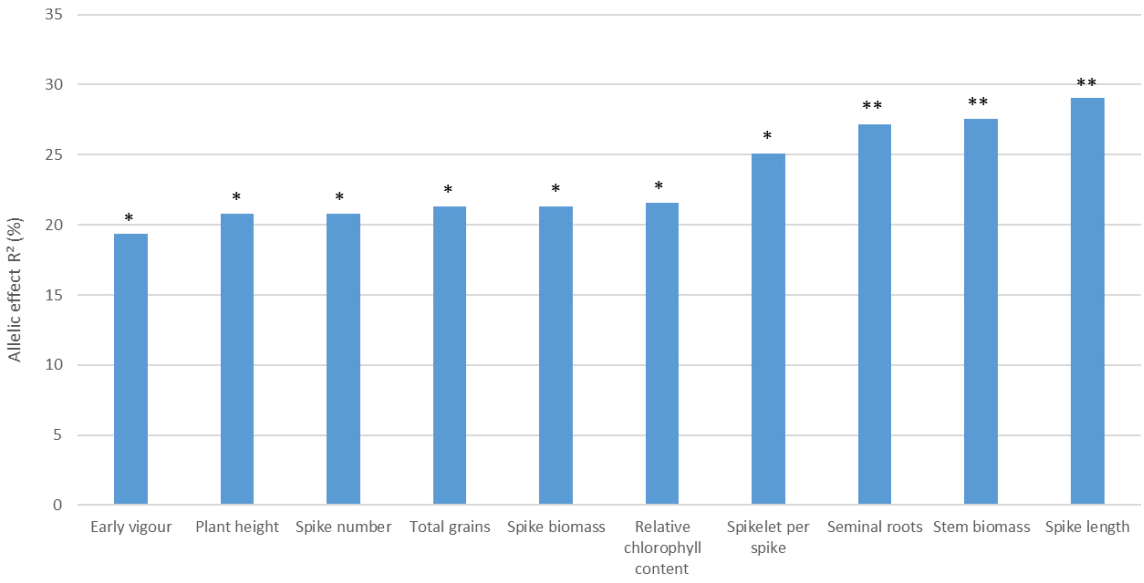

Figure 2. Positive effect associated with the RAC875 allele at *qYDH.3BL* in 20 RAC875 x Kukri RILs grown in the deep soil platform under drought and heat in 2014.
* : p value < 0.05; ** : p value < 0.01

The single marker analysis of the 2014 drought treatment showed the RAC875 allele was positively associated with a small increase in plant biomass and tiller number (Supplementary Figure 1). The Kukri allele was associated with an increase in root growth angle with 14.3% of

the variance explained by ADW341. The results from the 2014 field trials showed that *qYDH.3BL* expression in the deep soil platform was similar to the Mexican trial results. As expected, the effects were larger under dry and hot conditions than under drought alone.

In 2015, 30 RILs with recombination points in the QTL interval were phenotyped in the deep soil platform to fine map *qYDH.3BL*. In 2015, temperatures were higher than in 2014 with a maximum temperature at flowering time of 33.4˚C for 2014, while it ranged between 34.8˚C and 38.8˚C in 2015 (Supplementary Figure 2). Spike length and biomass, stem biomass and early vigour were significantly higher ($P < 0.05$ and $P < 0.01$) in plants containing the RAC875 allele at the ADW594 – ADW577 interval (Figure 3 a-d).

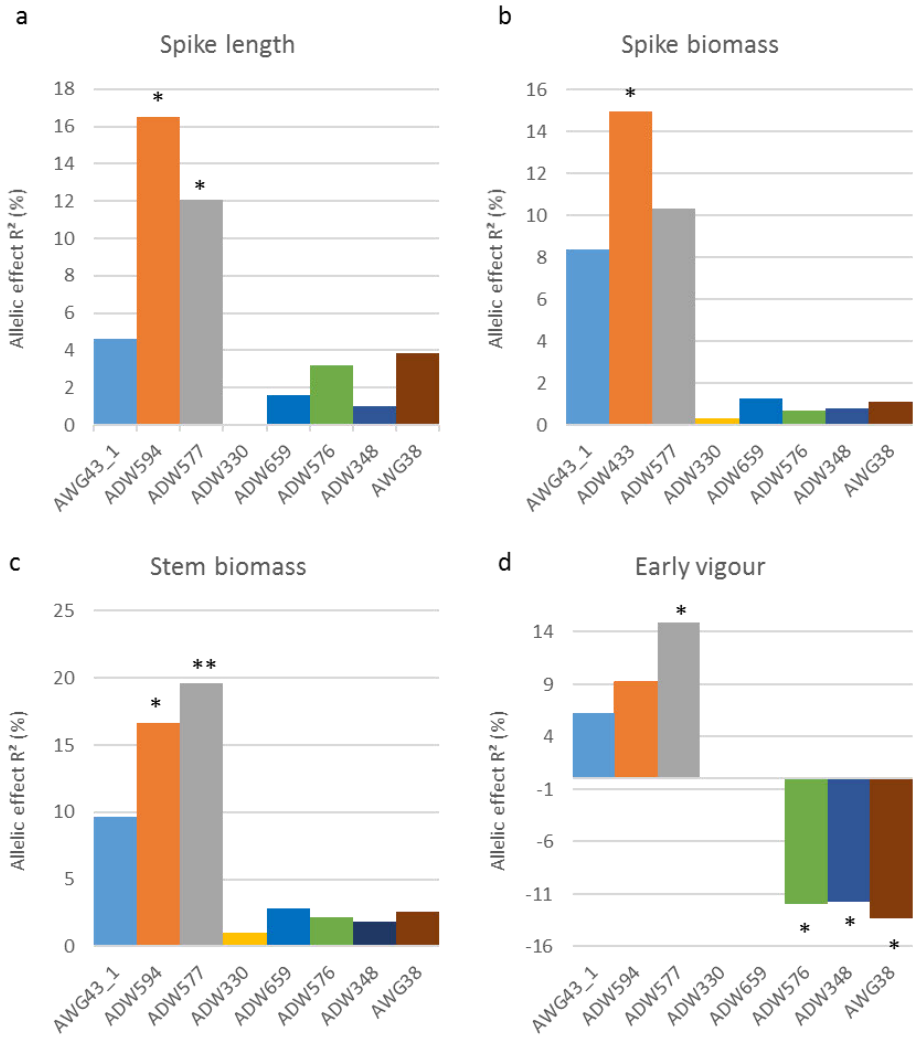

Figure 3. Single marker analysis of the RAC875 x Kukri 2015 experiment showed that the QTL was associated with spike length (a) and biomass (b), stem biomass (c) and early vigour (d). Positive effects at the QTL were associated with the RAC875 allele, negative effects with the Kukri allele.
* : p value < 0.05; ** : p value < 0.01

In 2015, 40 Drysdale x Gladius RILs were also phenotyped under dry and hot conditions using the deep soil platform. The 20 lines defined two recombination points in the QTL interval. The single marker analysis showed the positive allele from Drysdale associated with a significant increase ($P < 0.05$) in flag leaf length and single grain weight (Figure 4a and b).

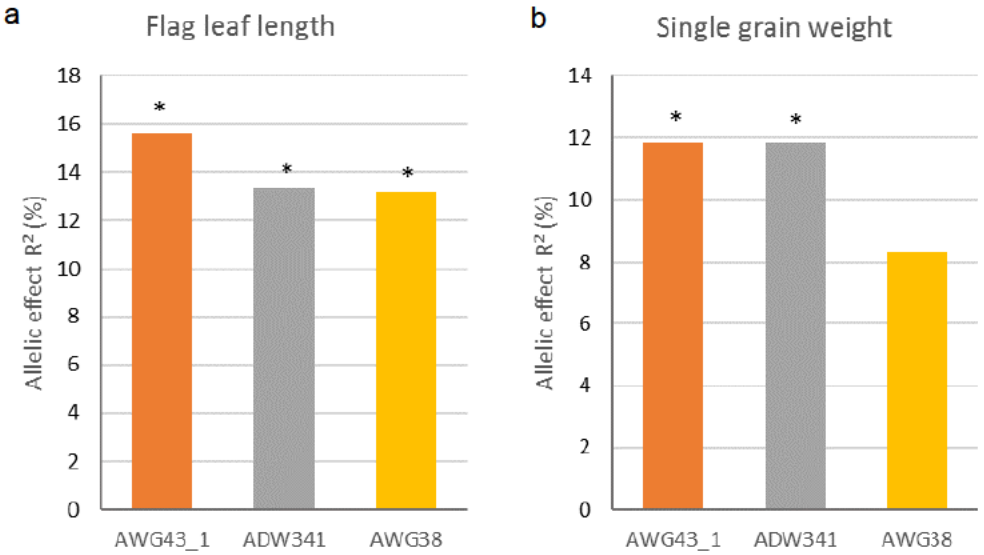

Figure 4. Single marker analysis of the Drysdale x Gladius 2015 experiment showed that the QTL was associated with flag leaf length (a) and single grain weight (b). Positive effects at the QTL were associated with the Drysdale allele.
* : p value < 0.05

The interval delimited by AWG43_1 and AWG38 defined a ~1.5 Mbp sequence on the bread wheat reference sequence of Chinese Spring (Figure 1d). However, the order of the SNP-based markers located in the AWG43_1 and AWG38 interval on the reference genome Chinese Spring is different from the one in the RAC875 x Kukri RILs genetic map. The QTL interval delimited by AWG43_1 and AWG38 was estimated to be smaller in RAC875. DAWN showed a low coverage between the markers ADW477 and ADW594 (Supplementary Figure 4), so we did a local assembly of RAC875 at this interval. The local assembly showed that the low coverage did not mean that the region was missing in RAC875. Moreover, we did not identify additional genes in RAC875.

The eight recombination points defined by the 30 RAC875 x Kukri RILs in the QTL interval helped to further fine map *qYDH.3BL*. We narrowed down the interval to ~ 690 Kbp sequence delimited by the SNP-based markers ADW594 and ADW577 and containing 12 genes, eight high confidence (HC) and four low confidence (LC) (Table I). The putative functions of the eight HC genes were retrieved by homology with the Brachypodium and rice genomes.

Table I. Genes annotated in the Ref v 1.0 and present in the interval between ADW594 - ADW577. The putative function of each gene was retrieved by homology with rice and brachypodium. HC = High confidence gene; LC = Low confidence gene

	Gene ID	Position RefSeq v1.0	Confidence	Strand	Putative function based on rice orthologues	E value	Putative function based on brachypodium orthologues	E value	Expression data*
I	TraesCS3B01G570900	802845950 - 802848503	high	+	LOC_Os10g33910.1_ Mitochondrial import inner membrane translocase subunit Tim16	4.1 e-42	Bradi2g61480_mitochondrial import inner membrane translocase subunit TIM16	1.7 e-55	expressed
II	TraesCS3B01G571000	802848874 - 802852363	high	-	LOC_OS02g17280_Gamma-secretase subunit APH-1B	4.3 e-131	Bradi3g10110_endopeptidase activity	8.5 e- 135	expressed
III	TraesCS3B01G851200LC	803363524 - 803364407	low	-	-	-	-	-	-
IV	TraesCS3B01G572500	803511461 - 803513157	high	-	LOC_Os11g14410_ Polygalacturonase	8.5 e-142	Bradi2g57427_Polygalacturonase / Pectinase	8.2 e- 87	-
V	TraesCS3B01G572600	803523701 - 803525213	high	+	LOC_Os01g72720_expressed protein	8.9 e-11	Bradi2g61275_PF03478 Protein of unknown function	4.8 e-56	expressed
VI	TraesCS3B01G572700	803540158 - 803540583	high	+	-	-	Bradi1g36100_Glycosyl hydrolase, subfamily GH 28	1.6 e-22	-
VII	TraesCS3B01G851300LC	803580461 - 803581032	low	+	LOC_Os01g72620_expressed protein	1.6 e-22	-	-	-
VIII	TraesCS3B01G572800	803583095 - 803583731	high	-	LOC_Os01g59540_GRF zinc finger family protein	1.1 e-4	Bradi5g21617	1.5 e-24	-
IX	TraesCS3B01G851400LC	803586310 - 803587263	low	-	LOC_Os07g42000_expressed protein	1.8 e-14	-	-	-
X	TraesCS3B01G572900	803628595 - 803630088	high	-	LOC_Os01g03170_seven in absentia protein family protein	3.3 e-34	Bradi1g27970_ubiquitin-protein ligase activity	6 e-57	expressed
XI	TraesCS3B01G851500LC	803715648 - 803716915	low	-	-	-	-	-	-
XII	TraesCS3B01G573000	804008796 - 804009926	high	+	LOC_Os01g03170_seven in absentia protein family protein	2.7 e-31	Bradi1g27970_ubiquitin-protein ligase activity	1.7 e-48	-

* Expression data extracted from www.wheat-expression.com and in-house

To identify SNPs between the parental lines RAC875 and Kukri in the coding sequence of the genes, we used whole genome shotgun sequencing of the parental lines, with putative SNP calls aligned and visualized in comparison with IWGSC Ref 1.0 of cv. Chinese Spring and each other in the software DAWN. We did not find any missense SNP in the coding sequence of the HC genes that could explain the phenotype associated with the QTL. Using the whole genome shotgun sequencing data, we found that gene XII had a low coverage in the promoter and coding sequence suggesting a potential deletion in the gene sequence of RAC875 and Drysdale. We then searched in a transcriptomic database (www.wheat-expression.com) to find evidence of expression of the candidate genes (Table I). Expression data were only found for four of the HC genes. These genes are a mitochondrial inner import subunit PAM16 (gene I), a gamma-secretase Aph-1 protein (gene II), a seven in absentia (gene X) and a gene of unknown function (gene V).

Allelic distribution at qYDH.3BL in a worldwide diversity panel

Genotyping of a worldwide wheat collection with SNP in the interval ADW594 - ADW577 identified four haplotypes (Figure 5a). Two haplotypes were over represented in the accessions, 32.86 % of the accessions carried the RAC875 allele (haplotype I) and 50.31 % the Kukri allele (haplotype IV) (Figure 5a). The other two haplotypes were carried by a smaller number of accessions, 6.5 % for haplotype II and 10.3 % for haplotype III (Figure 5a). We looked at the origins and years of release of the accessions in each haplotype to identify a potential pattern of selection. Haplotype I was over-represented in accessions originating from the CIMMYT program compared to the three other haplotypes and equally present in the European and Australian germplasm (Figure 5b). We observed that haplotype II was mostly present in the European germplasm while haplotype III was mostly present in the Australian germplasm. Haplotype IV was more abundant in landraces originating from the Middle-East and historical wheat lines such as Du Toits, Federation, Ward's Prolific and Hudson Early Purple Straw, released before 1900. Haplotype I appeared later in the panel's chronology with most of the lines released after 1950.

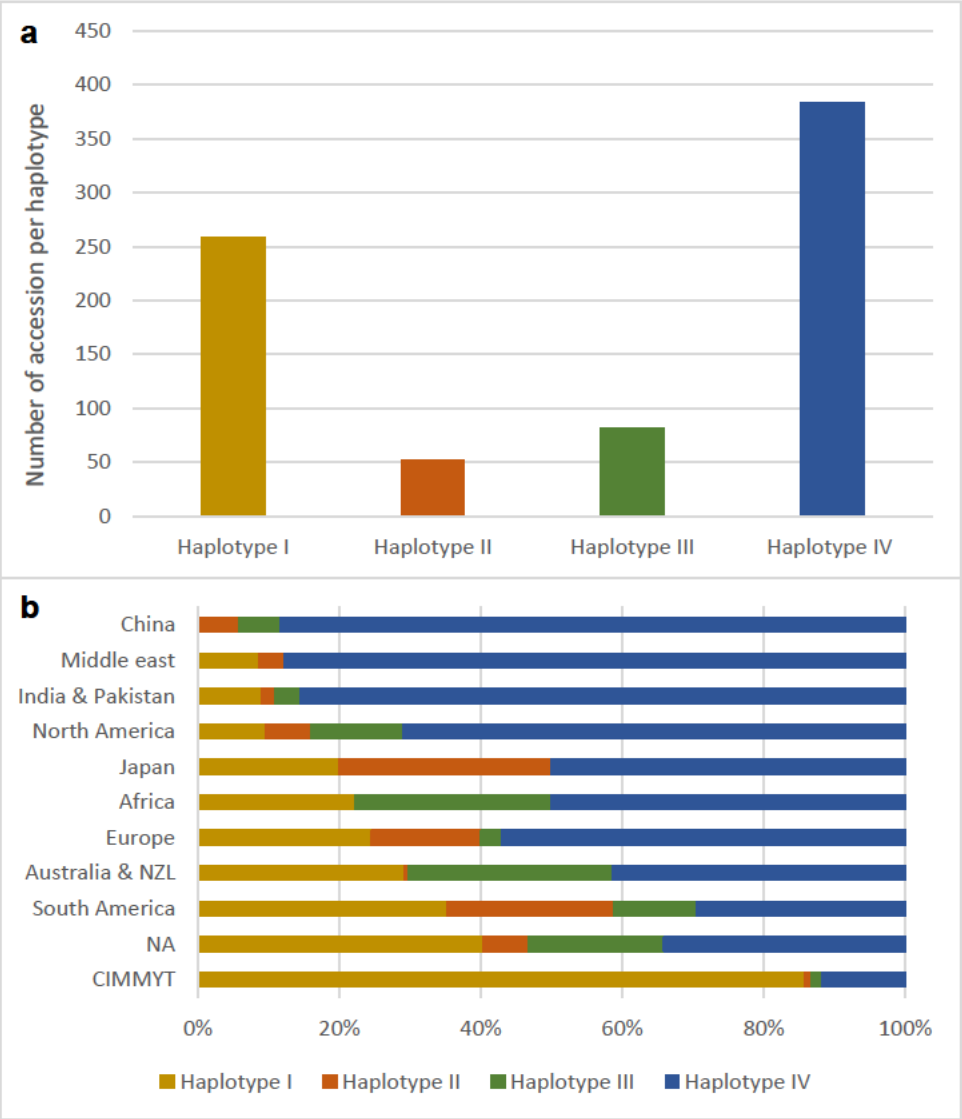

Figure 5. Haplotype analysis of a diversity panel of 808 accessions at the interval between ADW594 – ADW577. a) Four haplotypes were identified, with haplotype I being RAC875 (light brown) allele and haplotype IV being Kukri allele (blue). b) Geographical distribution of the accessions within each haplotype.

4. Discussion

Yield variation in stress-prone environments is a complex trait. It has been intensively studied at the genetic and physiological levels but genic determinants remain unknown in wheat. *QYDH.3BL* was identified in the spring wheat RAC875 x Kukri and the Drysdale x Gladius populations (Maphosa et al., 2014). The multi-environment analysis of 31 field trials located in Mexico and Australia (Figure 1) showed that the RAC875 allele was positively associated with the QTL in almost all Mexican environments. The Mexican field trials were conducted in the

northern part of Mexico in the Sonora region. The climate in the Sonora region is very hot with temperatures at flowering time around 25 - 28 ˚C for conventional sowing date, and 30 - 35 ˚C for late sowing trials, and dry as there is no rainfall during the growing season. The soil profile at the CIMMYT station, where the trials were conducted, is deep with an effective soil depth of 110 – 120 cm (Verhulst et al., 2009). The QTL was the most significant when flood irrigation was used in comparison to drip irrigation in the Mexican field trial. Bonneau et al. (2013) suggested that flood irrigation had an advantage over drip irrigation under drought in northern Mexico due to the soil profile. The deep soil would favour water storage at depth, which could be available for plants at critical growth stages (Pinto & Reynolds, 2015). Bonneau et al. (2013) also found that the Kukri allele was positive in two irrigated trials located in South Australia, where soil is shallow (50-60 cm depth). The quantitative analysis of soil water potential and air temperature as co-variates identified a switch of allele effect at *qYDH.3BL* in response to temperature. Parent et al. (2017) showed that the RAC875 allele increased individual seed weight, biomass and harvest index when temperatures were above 25 ˚C. The 2014 experiment in the deep-soil platform showed that the RAC875 allele positively affected a larger number of traits under drought and heat combined than when drought alone was applied, confirming that RAC875 allele effects are heat-related as found by Parent et al. (2017).

The RAC875 allele contributed to an increase in early vigour, plant height, total grain number, seminal root number and spike length under heat stress (Figure 2). The RAC875 allele also increase stem and spike biomass, spike number and spikelet per spike under combined drought and heat stress. *QYDH.3BL* has already been associated with early vigour in six of the environments where the trait has been analysed (Bonneau et al., 2013). The association of *qYDH.3BL* with early vigour suggests that the mechanisms underlying the QTL are effective at the early stage of plant development, before any treatment has been applied. Early vigour is an important trait associated with water uptake, minimising water loss due to soil evapotranspiration to the profit of transpiration (Ludlow & Muchow, 1990). The RAC875 allele also contributed to an increase of the above ground biomass suggesting that *qYDH.3BL* is potentially associated with whole plant development. Following the conclusions of Parent et al. (2017), we hypothesize that the mechanisms associated with the RAC875 allele at *qYDH.3BL* were potentially detrimental or had no effect under optimal conditions as observed in South Australian field trials in 2009 (Bonneau et al., 2013) but were beneficial to the plant when temperatures increased.

Using eight SNP markers in the ADW594 - ADW577 interval, we found four haplotypes at the QTL in a worldwide collection of 808 wheat accessions. The Kukri allele (or haplotype IV) was the most common in the panel, with half of the accessions showing this haplotype. The haplotype was also the most widely distributed globally and shared by historical wheat lines suggesting that the Kukri allele is an historical allele in wheat breeding programs with no yield penalty. The RAC875 haplotype (haplotype I) was over-represented in the CIMMYT germplasm and equally represented in the European and Australian germplasm. The over-representation of the haplotype in CIMMYT material suggests positive selection in the CIMMYT breeding program. This haplotype first appeared in the panel at the beginning of the 20th century in Australia, but remained rare until the 1960s when it became more common in CIMMYT germplasm and worldwide. The 1960s correspond to the Green revolution which led to a major shift in wheat breeding with high yielding cultivars, showing improved disease resistance and less lodging (Borlaug, 1968). This period also corresponded with the dominance of shuttle breeding at CIMMYT, where lines were selected for high yield in the irrigated environment of Obregon followed by selection under high disease pressure at high altitudes near Mexico City Toluca. Haplotype I may have contributed to the breeding of high yielding wheat varieties resilient to heat stress with no yield penalty in dry conditions. The haplotype I would have then be distributed to other continents with the Green revolution germplasm (Borlaug, 1968).

The fine mapping of the QTL defined a list of 12 candidate genes of which eight were HC in the physical interval. We found evidence of expression for four genes, a mitochondrial import inner membrane translocase subunit TIM16, a gamma secretase subunit APH-1B, a seven in absentia gene, and a gene of unknown function.

Mitochondrial import inner membrane translocase subunit Tim16

Tim 16, also called Pam 16, was first identified in *Saccharomyces cerevisiae* as part of the pre-sequence translocase-associated protein import motor (PAM). The PAM complex is composed of five subunits, the matrix of heat shock protein (mtHsp70) and four co-chaperones, Mge1, Tim44, Pam18 and Pam 16, which all contribute to the transport of pre-protein from the cytosol to the mitochondrial matrix (Frazier et al., 2004). The Pam 16 subunit is a J-like protein, which interacts with the J domain of the co-chaperone Pam 18 and forms a heterodimer. Pam 16 has been proposed to regulate the activity of Pam 18 which activates the ATPase activity of mtHsp70 (Mokranjac et al., 2006). Pam16 does not seem to interact directly with mtHSP70 but

participate in its regulation via its interaction with Pam18. Pam16 was shown to be essential for yeast cell viability (Frazier et al., 2004). Mutations preventing the formation of the heterodimer between Pam 16-Pam18 were also lethal for yeast cells (Mokranjac et al., 2006). *AtPAM16*, an orthologue of the yeast *Pam16* gene, was identified in Arabidopsis (Huang et al., 2013). The double mutant plants, *AtPAM16* and *AtPAM16l* (paralogue of *AtPAM16*) did not survive while *AtPAM16* mutant plants were smaller, were more pathogen resistant but accumulated more reactive oxygen species (ROS) (Huang et al., 2013). *AtPAM16* was proposed as a regulator of ROS accumulation. *AtPam16L1* and *AtPam16L2,* the two orthologues in Arabidopsis, have been shown to be preferentially expressed at senescence and up-regulated under abiotic stress (Chen et al., 2013). Based on the information on the role of PAM 16 in different species, putative *TaPAM16* at *qYDH.3BL* could potentially act as a regulator of the redox state of plants under dry and hot conditions by controlling the production or scavenging of ROS. However, we did not identify any missense SNPs in the coding sequence of the gene between the parental lines although the gene is expressed which suggests that the gene remains a candidate.

Gamma-secretase subunit APH-1B

The gamma secretase subunit APH-1B (Anterior Pharynx-defective 1) is part of the gamma secretase complex, an intramembrane-cleaving protease (Kimberly et al., 2003). The complex is composed of three other subunits: presenilin (PS), nicastrin (NCT) and PEN-2 (presenilin enhancer 2). *APH-1* was first identified in *Caenorhabditis elegans* embryos where it codes for a multipass membrane protein essential for the Notch signalling pathway that controls cell interactions for cell fate speciation (Goutte et al., 2002). Gamma-secretase subunits have also been identified in plants (Smolarkiewicz et al., 2014). The four subunits have been shown to be highly conserved between plant and animal species. Gamma-secretases were localised in the endomembrane of protoplasts in Arabidopsis. The role of the gamma-secretase subunit *APH-1B* is plants is still unclear, therefore it is difficult to hypothesise a potential role of the gene at *qYDH.3BL*.

Seven in absentia gene family (SINA)

SINA genes encode E3 ubiquitin ligase proteins. First reported in Drosophila, *SINA* were associated with the development of photoreceptor cell, R7, localized in the eye (Carthew et al., 1990). These proteins are involved in post translational regulation pathways through the degradation of target proteins. E3 ubiquitin ligase proteins are involved in an ubiquitination

complex formed by the E1 ubiquitin-activating enzyme and the E2 ubiquitin-conjugating enzyme which transfer the ubiquitin to the E3 enzyme that recognizes the target protein (Mazzucotelli et al., 2006). A genome-wide analysis of the gene family identified several copies of *SINA* in Arabidopsis, *Populus trichocarpa, Oryza sativa, Zea mays* and *Physcomitrella patens* (Wang et al., 2008). Each gene contained two domains, a C3HC4 RING domain and a SINA domain that were highly conserved among species (Wang et al., 2008). Subsequently, a few *SINA* genes have been reported for their role in developmental processes and abiotic stress tolerance (Recchia et al., 2013; Welsch et al., 2007) but no *SINA* genes have been reported in wheat so far. The ectopic expression of a dominant negative form of the Arabidopsis *SINAT5* gene negatively regulates plant growth and nodulation in transgenic *Medicago truncatula* (Den Herder et al., 2008). The *SINAT5* gene directly interacts with a *NAC1* transcription factor to regulate auxin signals. Arabidopsis plants overexpressing *SINAT5* had fewer lateral roots compared to the wild type (Xie et al., 2002). In rice, the overexpression of *OsDIS1* encoding a SINA E3 ligase reduced drought tolerance (Ning et al., 2011). Suppression of gene expression via RNAi promoted drought tolerance indicating that *OsDIS1* acts as a negative regulator of drought tolerance. Opposite effects were observed in Arabidopsis, where overexpression of *SINA2* increased drought tolerance by reducing water loss (Bao et al., 2013). This gene is an interesting candidate for *qYDH.3BL*.

In this study, the fine mapping of *qYDH.3BL* defined a list of genes in a 690 kbp physical DNA sequence, likely to contribute to heat tolerance. Further work will be required to verify that no gene has been missed in the sequence interval. Variations in the presence absence of exist among cultivars of the same species (Springer et al., 2009). However, the reads coverage in DAWN was good at the QTL interval. Moreover, the local assembly in RAC875 at the low read coverage interval did not identify additional genes. Follow-up experiments will aim to determine the potential association of the individual candidate genes with the phenotype at *qYDH.3BL*. Expression analysis will be conducted to identify any regulation pattern that could link a candidate gene to the phenotype even if their potential function remains unknown. Ultimately, functional validation of the gene(s) is required to confirm the role of a potential causal gene(s) with the phenotype through gene engineering or reverse genetic techniques.

5. Material & Methods

Plant materials

A recombinant inbred lines (RILs) population of 2000 individuals of the cross between RAC875 (RAC-655//SR21/4*Lance/3/4*Bayonet) and Kukri (Madden/6*RAC-177//Grajo/76-ECN-44) were used for the fine mapping of *qYDH.3BL*. A set of 70 RAC875 x Kukri RILs were selected for their contrasted yield in the 2011 (Bonneau et al., 2013) and 2012 field trials conducted in Mexico and based on their recombination point in the QTL interval (Supplementary Table I). In 2014, 44 RILs were tested under dry conditions and a subset of 20 lines were phenotyped in combined dry and hot conditions.

A second set of 46 RILs from the cross between Drysdale (Hartog*3/Quarrion) and Gladius (RAC875/Krichauff//Excalibur/Kukri/3/RAC875/Krichauff/4/RAC875//Excalibur/Kukri) (DG) was also studied. The lines were selected based on their extreme yield values during the 2009 and 2010 Mexican and New South Wales field trials (Bonneau, 2012). The four parental lines RAC875, Kukri, Drysdale and Gladius were included in each trial.

Genotyping and genetic map construction

Gene-based markers were designed using the whole genome sequencing (WGS) data of the parental lines (RAC875, Kukri, Drysdale and Gladius) generated by BioPlatform Australia using whole genome next-generation sequencing (Edwards et al., 2012). Sequence reads (100 bp) were aligned against the reference sequence IWGSC Ref 1.0 of Chinese Spring (https://www.wheatgenome.org/) and visualized using DAWN. DAWN is a visualizing tool that integrate multiple datasets including the wheat reference sequence IWGSC Ref 1.0, RNA sequencing data, WGS data of 16 Australian cultivars and several marker database. SNPs within *qYDH.3BL* interval were identified with a minimum of five reads per line at a SNP position. Regions with a high density of reads are more likely to be repetitive; therefore, SNPs with a coverage higher than 50 reads were discarded.

Kompetitive Allele Specific PCR (KASP) markers were designed with the Kraken software (LGC genomics, Middlesex, UK) on sequences of at least 100 bp spanning a SNP. Two allele-specific forward primers were designed for each SNP in combination with a common reverse. Each forward primer had a tail attached at the 5'end specific to a fluorophore. The hybridization of one specific forward primer to the target sequence allowed the pairing of the fluorophore present in the KASP mix to the primer tail releasing the quencher allowing emission of

fluorescence (Semagn et al., 2014). KASP assays were performed using the thermocycler Hydrocycler^{-16} thermal cycler (LGC genomics, Middlesex, UK) following these steps: 94 °C for 15 min; followed by 10 touchdown cycles at 94 °C for 20 sec, an annealing step starting at 61 °C and reducing by 0.6 °C every cycle; followed by 26 cycles at 94 °C for 20 sec, 55 °C for 1 min. After the initial run, KASP assays were read using the microplate reader PHERAstar^{Plus} (BMG LABTECH, Ortenberg, Germany). If KASP assays were not completed after the initial run, three rounds of three cycles at 94 °C for 20 sec, 55 °C for 1 min were added. KASP primers were prefixed AWG- and ADW- (Supplementary Table II).

Chromosome specific primers were designed using primer 3 (Untergasser et al., 2012) available in Geneious (Biomatters, Auckland, New Zealand) to specifically target InDel on chromosome 3B (Supplementary Table III). InDel were amplified by multiplexing PCR using the primers pair: of a primer pair, 5'- GGACAGCTTAGGCGAGGAAT-3' and 5'- GCTGGGGCTTCCTTAATCTC- 3', targeting the ubiquitous gene *TaWin*, a gene of the 14-3-3 like protein family (Tenea et al., 2011). Nulli-tetrasomic lines specific to the group 3 chromosome (Sears, 1966) were used to verify that the primers targeting the InDel were chromosome 3B specific. PCR reaction were performed in a 10 µl volume reaction with 0.5 µl of 10 µM *TaWIN* primers, 0.3 µl of 10 µM InDel gene primers, 2 µl 2 mM dNTPs, 0.4 µl of 50 mM $MgCl_2$, 1 µl of 10X high fidelity buffer, 0.1 µl of 5 U / µl Platinum® *Taq* DNA polymerase high fidelity, DNA template and H_2O. PCR amplification were performed using the Tetrad® 2 thermocycler (Bio-Rad, Hercules, California, USA) following these steps: initial denaturation at 94 °C for 1 min follow by 35 cycles at 94 °C for 20 sec, annealing step depending on the melting temperature of each primer pair for 30 sec, 72 °C for 1 min and final elongation at 72 °C for 5 min.

The genotyping data of the KASP and InDel markers were added to RAC875 x Kukri and Drysdale x Gladius RILs genetic maps generated by Bonneau et al., 2013. Genetic maps were constructed using ASMap package (Taylor & Butler, 2017) available in the software R (https://cran.r-project.org/). Genetic distance between each marker was calculated using the Kosambi mapping function (Kosambi, 1943) which is based on a new algorithm available in the web tool MSTmap (Wu et al., 2008). This algorithm can determine the markers order efficiently by establishing a minimum spanning tree.

Deep soil phenotyping platform

Wheelie bins (100 x 57.5 x 51 cm) were filled with a mixed soil (1/3 Coco peat, 1/3 sand and 1/3 clay). Each bin contained 25 plants: 24 RILs and parental lines plus one filler plant placed

in the centre of the bins. Nine bins were used in 2014 and 12 bins in2015. In 2014, six bins were allocated to the drought treatment and three bins to combined drought and heat. In 2015, six bins were allocated to the RAC875 x Kukri RILs and six bins to the Drysdale x Gladius RILs. Each line used in 2014 and 2015 had three biological replicates allocated in different bins. Plants were ordered in a 5 x 5 plant grid with 10 cm space between each plant. The experimental design was a resolved latinized incomplete block using CycDesigN (Whitaker et al., 2002), taking into account the position of the lines according to a west/east axis and split the bins between an inner and outer layer.

Trials were conducted at the Waite Campus, Urrbrae, Australia, in a polytunnel to ensure optimal conditions at the right developmental stage (Supplementary Figure 5). Two trials were conducted in 2014 and 2015. In 2014, plants were phenotyped under drought, and combined drought and heat. Plants were sown on the 11th of August 2014 for the drought treatment (Supplementary Figure 2). For the combined drought and heat treatment, plants were sown later, 8th of September, to maximize the chance of heat stress during the growth. Plants were well-watered during the first month for both treatments and then watering was stopped for the rest of the treatments to let the bins dry and impose drought stress at early grain filling. In 2015, both trials were conducted under dry and hot conditions. Plants were sown on the 10th of July for the RAC875 x Kukri plants. The Drysdale x Gladius plants were sown on the 18th of August. Plants were watered until late booting stage to let the bin dry and impose a drought stress at early grain filling (Supplementary Figure 2). Meteorological data, air temperature and relative humidity were recorded with a data logger (Kongin KG100, China). Gypsum blocks (MEA, Magill, SA, Australia) were placed in the bins at two depths (10 and 40 cm) to record soil moisture tension, kPa (Supplementary Figure 3).

Phenotypic evaluation in the wheelie bin system

The developmental stage of each plant was scored using the Zadoks'scale from booting stage to grain filling two times a week (Zadoks et al., 1974). Early vigour was scored using two methods. In 2014, during the first month after planting, photographs were taken for each bin every week and early vigour were scored using a 1 to 5 scale, with 1 minimal growth and 5 maximal growth. In 2015, early vigour was monitored by measuring the leaf area when a plant reached the four-leaves stage. The lengths and widths of each leaf on the main tiller were measured to determine the individual leaf area. The total leaf area was calculated as the sum of the leaf area (leaf width x leaf length x 0.8) of the second and third leaves using the method described by Richards (2002).

Chlorophyll content of the flag leaf was evaluated with a SPAD-502L meter (Ozaka, Japan) at three stages: booting, anthesis and grain filling. Three measurements per sample at each stage were taken to estimate the average value.

Flag leaf area was measured from booting to flowering time using the LI-3000C portable leaf area meter (LI-COR Inc., USA) in 2014. In 2015, flag leaf length and width were measured at flowering stage with a ruler. Stomatal density was measured by taking an imprint of the flag leaf adaxial face at anthesis using translucent nail polish to fix an impression to a glass slide. The slides were then analysed by microscopy using the Leica AS-LMD Laser Microdissection Microscope (Wetzlar, Germany). Three pictures per sample were taken and the guard cells number was counted for each picture as described by Shahinnia et al. (2016).

Tiller number was recorded twice, at four-leaf stage and before harvesting. Tiller abortion was calculated at the end of the trial by subtracting the spike number from the tiller number. After harvest, spikes were manually harvested and counted to evaluate the number of spikes per plant. Spikes and stem biomass were weighed separately and harvest index was determined by dividing grain biomass by total above-ground biomass. Spike lengths were measured using a ruler before counting the number of spikelets per spike for each spike. Seeds number was measured using a seed counter (Pfueffer GmBH, Germany) and then weighed to determine single grain weight. In 2014, carbon and nitrogen isotope discrimination of mature grains were measured on five grains per plant. Grains were dried for one week at 60˚C and finely ground to 1 to 4 mg of powder stored in tin aluminium capsules (Sercon, gateway Crewe, UK). The samples were sent to the Stable Isotope Facility, University of California, Davis, for analysis using continuous flow Isotope Ratio Mass Spectrometer (IRMS).

Root traits including root growth angle, number of seminal roots and number of adventitious crown and brace roots were also evaluated in 2014 by digging out the roots at 10 cm depth after harvesting. The number of both seminal and nodal roots were measured. Photographs of the root system were taken for each plant and analysed using Image J software (Schneider et al, 2012). The root growth angle of the angle formed by seminal roots and soil surface was measured.

QTL analysis

The best predicted unbiased estimates (BLUEs) data were generated for each trait under model (1) using asreml (Butler et al., 2007) and asremlPlus (Brien, 2015), packages for the R statistical environment (R Core Team, 2014) . The normal distribution of the BLUEs was then evaluated

using the Shapiro-Wilk test (Shapiro & Wilk, 1965). Single marker analysis at each marker position was performed to test each marker's association with the studied traits. Single marker analysis was performed with a one-way ANOVA in R. For the traits which did not follow a normal distribution, a Kruskal-Wallis test was used (Kruskal & Wallis, 1952)

(1) E[Y} = (Rep*Zone)/Side + Line,

Var[Y] = Rep:Zone:Side:Bin/Position

The multi-environments analysis of the RAC875 x Kukri RILs combined four field trials conducted in Mexico, Cuidad Obregón, in 2011 (Bonneau et al., 2013) and 2012 (Supplementary Table I) and was performed as described in Bonneau et al. (2013).

Anchoring of qYDH.3BL onto the wheat reference sequence

Flanking markers at the QTL were used to find sequence similarity with the reference sequence IWGSC Ref 1.0 of Chinese Spring using in-house BLAST portal (e-value -10; # hit return =100). The 1.5 Mbp sequence delimited by AWG43_1 and AWG38 was extracted using Fetch-seq, an in-house sequence retriever that allow the extraction of sub-sequences from the IWGSC Ref 1.0 using coordinates. Gene annotations were retrieved using the coordinated interval in the browser available on the URGI website (https://wheat-urgi.versailles.inra.fr/*). The* IWGSC Ref 1.0 was annotated using both TriAnnot (Leroy et al., 2012) *and PGSB pipelines (*mips.helmholtz-muenchen.de/plant/index.jsp). Genomic sequences of the annotated gene were used to find similarities with *Brachypodium distachyon* v 3.1 and the *Oryza sativa* v 7 reference genomes to identify putative gene function by BLAST (default settings) in phytozome (https://phytozome.jgi.doe.gov/).

Local assembly of RAC875 sequence

Initial alignment of the WGS reads data of RAC875 indicated that approximately 200 kpb region in IWGSC Ref v 1.0 located between the markers ADW477 and ADW594 was either absent or highly divergent in RAC875 (Supplementary Figure 4). Further alignments of RAC875 paired-end (PE) and mate-pair (PE) data to IWGSC Ref 1.0 failed to produce any evidence for the region being deleted. Reads and their mates which remained unaligned and those which aligned within the region of interests were assembled using in-house software into unitigs i.e. contigs unambiguously supported by k-mers. Unitigs were scaffolded with the

complete MP and PE datasets using space. The resulting scaffolds were aligned to IWGSC Ref 1.0 using BLASTn to exclude those derived from other regions of the genome and to identify a subset likely to be derived from the region of interest. The total length of 80 scaffolds putatively identified as coming from the region of interest adds up to close to 200 kbp and the longest scaffold covers 36 kbp. The scaffolds contain almost 60% ambiguous bases.

In silico gene expression analysis

Genomic sequences of the annotated genes were BLAST against transcriptomic databases available online to obtain existing information on their expression profiles. Transcriptomic data were collected from WheatExp (http://wheat.pw.usda.gov/WheatExp/) that combined six independent transcriptomic experiments. Two of them were of particular interest, the first one is a transcriptomic analysis conducted in five tissues at different Zadoks' stages (Zadoks et al., 1974) in the cultivar Chinese Spring (Choulet et al., 2014). The second experiment is a drought and heat experiment conducted on wheat seedlings cv. TAM 107 (Liu et al., 2015). Seedlings were exposed to three different stresses: drought, heat and drought and heat combined. Genomic sequence of the annotated genes in IWGSC Ref 1.0 were BLAST against the TGAC gene annotations (*https://plants.ensembl.org/index.html).* Not all the annotated genes from IWGSC Ref 1.0 were annotated in the TGAC assembly, genes VIII and IX were not found in the TGAC annotations. The TGAC gene ID were then used to retrieve data from the wheat-expression database (www.wheat-expression.com/) which is an open-access platform that combines RNA-seq data (Borrill et al., 2016).

Haplotype analysis

A diversity panel combining 808 landraces and modern varieties of hexaploid wheat was used to identify haplotypes at the QTL. 544 accessions were spring wheat type originated from world-wide locations including more than one quarter from Australia. We also used the INRA core collection which combines 372 worldwide accessions of winter wheat coming mostly from Europe (Balfourier et al., 2007).

Each accession was genotyped using eight markers located at the QTL peak. Markers were ordered following their genetic positions, and lines sharing the same allele were grouped together. Pedigree, year of release and geographical origin of the lines were collected through the wheat pedigree portal, www.wheatpedigree.net, and personal communications (Peter Langridge, Margaret Pallotta).

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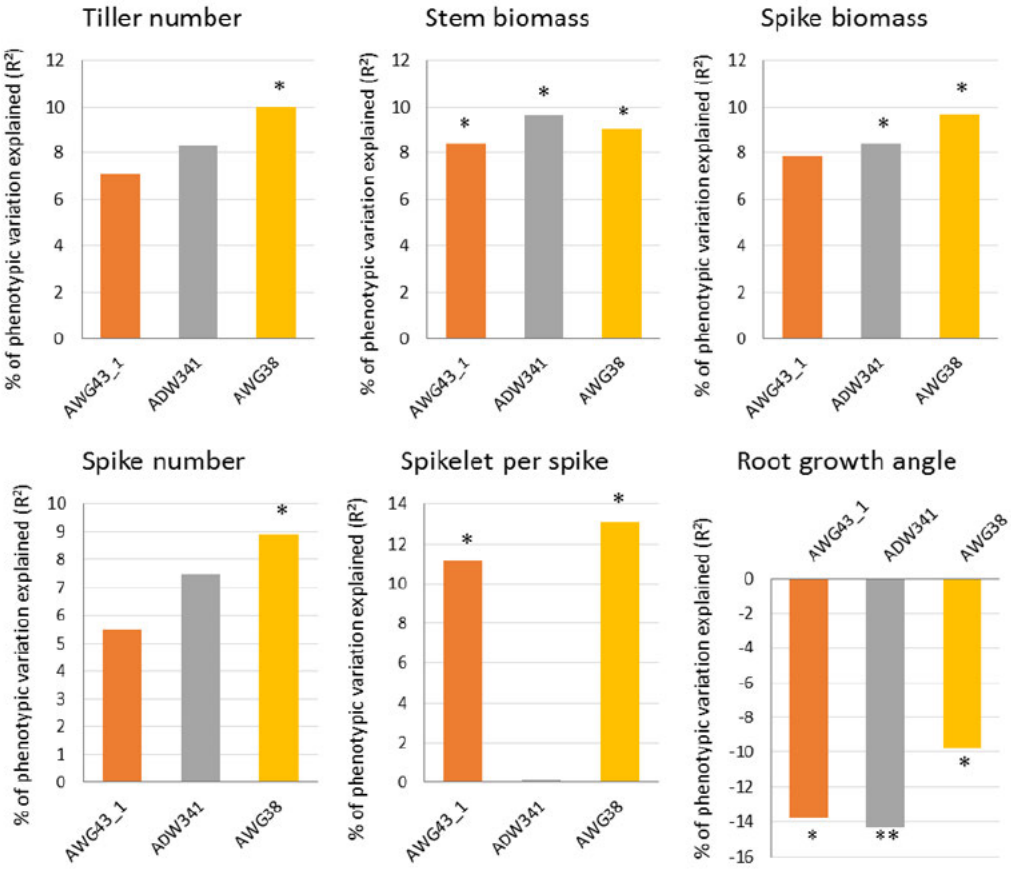
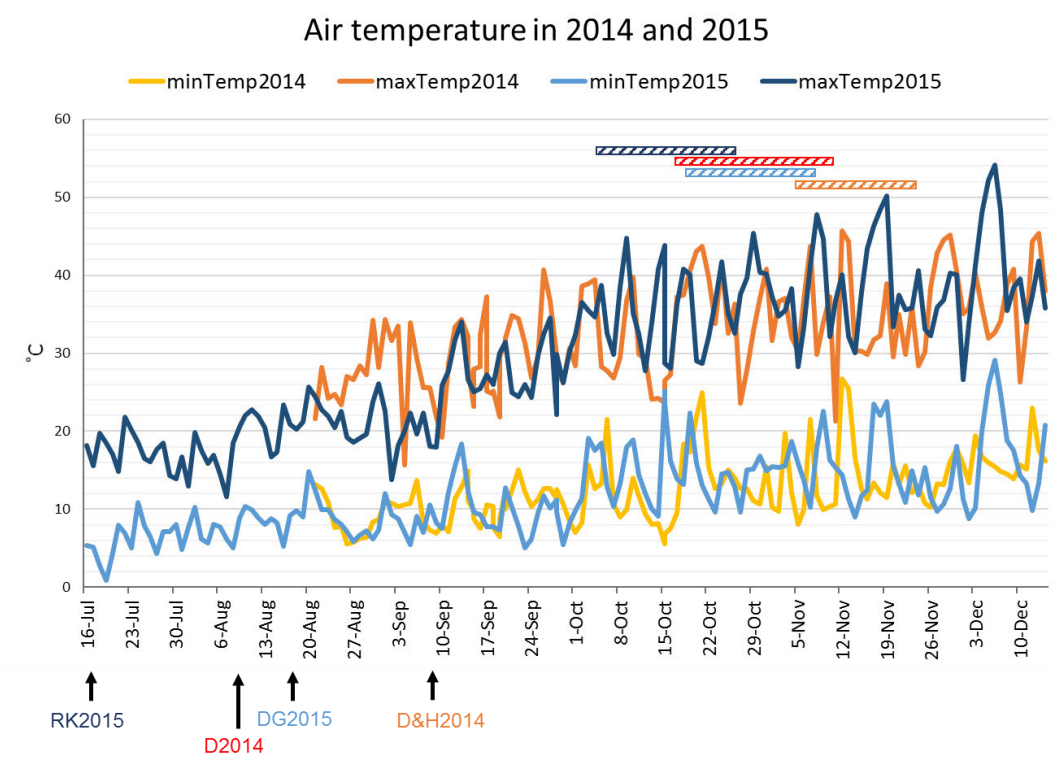
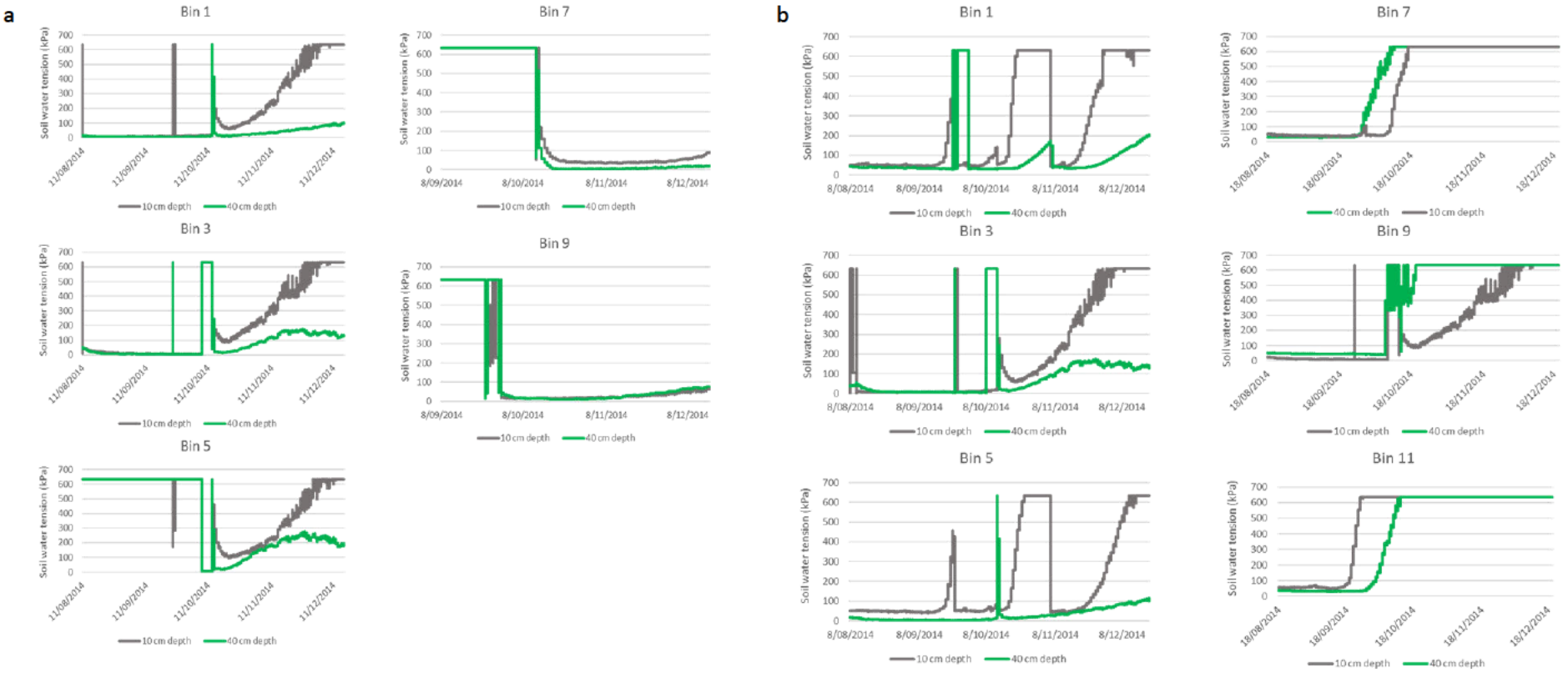
8. Supplementary data



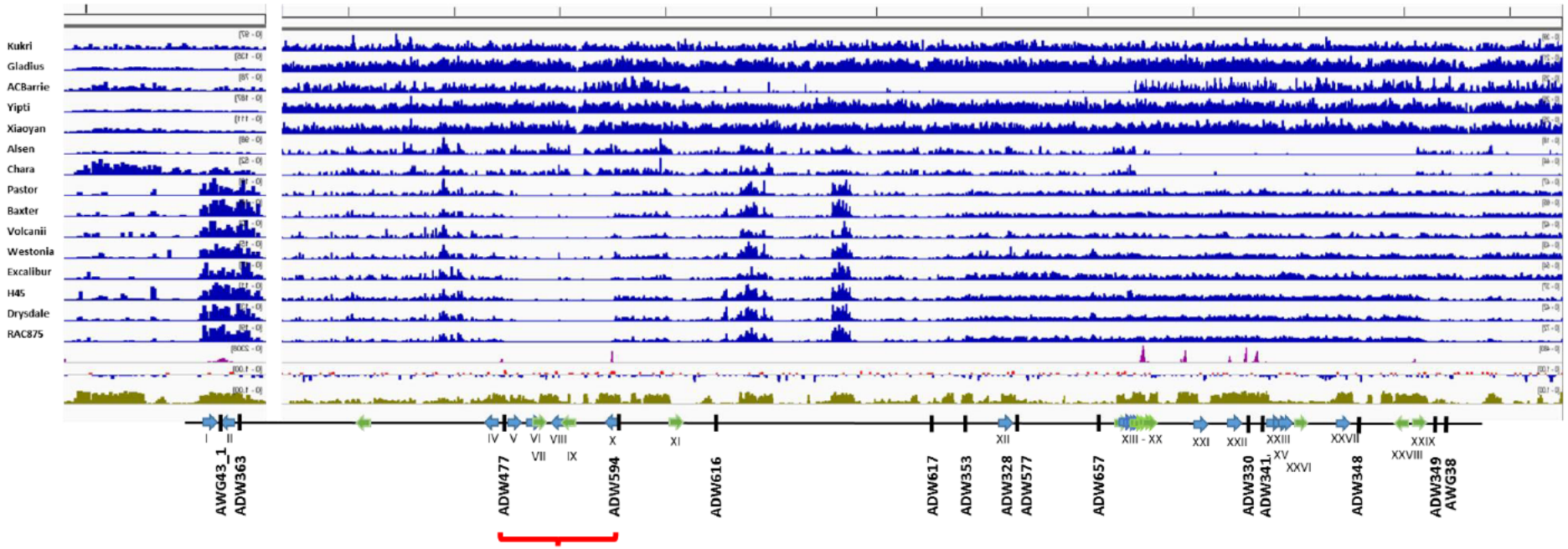
Supplementary Figure 1. Single marker analysis of 44 RAC875 x Kukri RILs phenotyped under drought conditions in the deep soil platform in 2014. Positive effect indicates that the RAC875 allele is associated with the trait while a negative effect indicates that the Kukri allele is associated with the trait.
* : p value < 0.05; ** : p value < 0.01;



Supplementary Figure 2. Meteorological data of 2014 and 2015. The graph combined minimum and maximum temperatures in Celsius degrees for the two seasons. Black arrows indicate the sowing date for each experiment: RK = RAC875 x Kukri; D = drought; DG = Drysdale x Gladius; D&H = drought and heat. Flowering periods have been highlighted for each experiment: dark blue for RK2015; red for D2014; light blue for DG2015; orange for D&H2014.



Supplementary Figure 3. Soil water tension data of 2014 and 2015 wheelie bin experiments. Soil water tension was recorded in five bins in 2014 (a) and six bins in 2015 (b). In 2014, bins 1, 3 and 5 were allocated to the drought and heat treatment while bins 7 and 9 were allocated to the drought and heat treatment. In 2015 bins 1, 3 and 5 were allocated to the RAC875 x Kukri RILs and bins 7, 9 and 11 allocated to the Drysdale x Gladius RILs.



Supplementary Figure 4. Screenshot of the DAWN software at *qYDH.3BL* interval. The blue peaks indicate the WGS read coverage for each cultivar at a specific position of a scaffolds from IWGSC Ref 0.4. The interval highlighted in red indicates the interval that has been assembled in RAC875.

Supplementary Table I. Descriptions of the four environments where the RAC875 x Kukri RILs field trials were conducted, showing locations, water supply from rainfall and/or irrigation and RAC875 x Kukri DH and RI lines (from Bonneau, 2012).

Environment [a]	Location	Latitude	Longitude	Altitude (M)	Lines tested [b]	Sowing density seed m^{-2}	Rainfall and/or irrigation mm	Mean temperature around flowering time [c]	
								Sept	**Oct**
MexObr11_DI_CS	Cuidad de Obregon	27˚28' N	109˚56' W	38	34 DH/77 RI	200	150	24.5	29.3
MexObr12_DI_CS	Cuidad de Obregon	27˚28' N	109˚56' W	38	27 DH/ 40 RI	200	100	24.7	26.6
								April	**May**
MexObr11_FI_LS	Cuidad de Obregon	27˚28' N	109˚56' W	38	34 DH/ 109 RI	200	1050	30.7	34.8
MexObr12_FI_LS	Cuidad de Obregon	27˚28' N	109˚56' W	38	27 DH/ 40 RI	200	900	30.5	37.8

a Mex Mexico, DI drip irrigation, FI for flooding irrigation, LS for late sowing, CS for conventional sowing date.
b DH doubled haploid lines, RI recombinant inbred lines.
c The temperature data for Mexico were collected at www.agroson.com.mx

Supplementary Table II. KASP markers for fine mapping *qDHY.3BL*. Each marker had two forward primer targeting a different allele and a common reverse primer.

Marker name	Allele 1 primer sequences	Allele 2 primer sequences	Common reverse
AWG61	GAAGGTGACCAAGTTCATGCTACCCCGAGACAGGCGAGG	GAAGGTCGGAGTCAACGGATTGACCCCGAGACAGGCGAGA	ACGGCAGGAGGGCCAACTGTAA
AWG13	GAAGGTGACCAAGTTCATGCTACGCTATCAACTTCCCGATCCAA	GAAGGTCGGAGTCAACGGATTCGCTATCAACTTCCCGATCCAG	CGATGCTCTGTGCCTGCAGGAA
AWG66	GAAGGTGACCAAGTTCATGCTGGCAGGACCCAGAGACACTG	GAAGGTCGGAGTCAACGGATTGGCAGGACCCAGAGACACTA	GGACATGCACAGTGACCACGTTAAA
AWG10	GAAGGTGACCAAGTTCATGCTGTGGAGCAGCCTACGGCCAA	GAAGGTCGGAGTCAACGGATTGGAGCAGCCTACGGCCAC	TTTTGCTCCTTCAACGCGACAGCTA
AWG31_1	GAAGGTGACCAAGTTCATGCTGGCTTGGCTCTTTCCAGATTGATT	GAAGGTCGGAGTCAACGGATTGGCTTGGCTCTTTCCAGATTGATA	GTAGCTGTGGGAGTGAGTGATGAA
AWG41	GAAGGTGACCAAGTTCATGCTCGCCGGCGAGAGGT	GAAGGTCGGAGTCAACGGATTGCTCGCCGGCGAGAGGC	CGTGTGCTGGAAGAAGGCGTCAT
AWG84	GAAGGTGACCAAGTTCATGCTGTTTGCCACTAGTTCTGACTGGAT	GAAGGTCGGAGTCAACGGATTTGCCACTAGTTCTGACTGGAC	GTAACCAGCCACATCAGCATGACAA
AWG27	GAAGGTGACCAAGTTCATGCTTCGTGTTTGCTGCGGTCTGCT	GAAGGTCGGAGTCAACGGATTCGTGTTTGCTGCGGTCTGCG	AAAACAAAAAGAAGAGGAAGCCCAAGCTA
AWG57	GAAGGTGACCAAGTTCATGCTGTTTGGACGAAAGCCCTATTTTCTG	GAAGGTCGGAGTCAACGGATTAGTTTGGACGAAAGCCCTATTTTCTA	ACGAGAGCCGCTCACCCACAAT
AWG47_2	GAAGGTGACCAAGTTCATGCTAATAATGGTGTCCATGCACCGTTTC	GAAGGTCGGAGTCAACGGATTCAATAATGGTGTCCATGCACCGTTTT	CTCCTGACAACCATGCCTTCAACAA
AWG62	GAAGGTGACCAAGTTCATGCTCCTCGGCGGCGGCTGTAG	GAAGGTCGGAGTCAACGGATTCCTCGGCGGCGGCTGTAT	CAAACGTTGTCGCCCGGCCAT
AWG21	GAAGGTGACCAAGTTCATGCTCCAAACAGCCACTAAGTGAGGAG	GAAGGTCGGAGTCAACGGATTCCAAACAGCCACTAAGTGAGGAA	AGCGATCCCCCCTTTGACCGAA
AWG43_1	GAAGGTGACCAAGTTCATGCTGATGTTGACACTTTGTGTTGTTGCC	GAAGGTCGGAGTCAACGGATTGATGTTGACACTTTGTGTTGTTGCG	AGCTGCTACAACAATCAAACATTGCAGAT
AWG69_1	GAAGGTGACCAAGTTCATGCTCGCTAGGTTTCTGCGACCGC	GAAGGTCGGAGTCAACGGATTCCGCTAGGTTTCTGCGACCGT	CGCAGCAGCCCAGGCGCAA
AWG75	GAAGGTGACCAAGTTCATGCTGGTTCAAGATCAGGTTCATGATCAG	GAAGGTCGGAGTCAACGGATTGGTTCAAGATCAGGTTCATGATCAC	AATTGTACTTGCATGGTAAGCTCTGGAAT
AWG19_1	GAAGGTGACCAAGTTCATGCTCCTGGTGTCCATCCATCGTCTT	GAAGGTCGGAGTCAACGGATTCTGGTGTCCATCCATCGTCTC	GATTTATGAGCGCGCTGAATGGGTT
AWG67_1	GAAGGTGACCAAGTTCATGCTGGGGAGTGGCCTCTGCCTT	GAAGGTCGGAGTCAACGGATTGGGAGTGGCCTCTGCCTC	CACCACCACGAGTGCTGCGAT
AWG38	GAAGGTGACCAAGTTCATGCTACTGTGAATTGGCCATTCTTCTCTG	GAAGGTCGGAGTCAACGGATTAAACTGTGAATTGGCCATTCTTCTCTA	TTTTGGGGATGATTTCATTGCCTGGAAA
AWG82	GAAGGTGACCAAGTTCATGCTCGGCAGCAGATTGAGCGTCG	GAAGGTCGGAGTCAACGGATTCCGGCAGCAGATTGAGCGTCA	GACTCTCCGTCGCCTCTGGAT
AWG25	GAAGGTGACCAAGTTCATGCTGTTGTTGAGGATGCGGGTT	GAAGGTCGGAGTCAACGGATTCTGTTGTTGAGGATGCGGGTG	TGTTGCTCCTTGAATTCACTTATGCAGAT
ADW313	GAAGGTGACCAAGTTCATGCTCCATGTTAAGATATAGTCATCTAACTGC	GAAGGTCGGAGTCAACGGATTCCATGTTAAGATATAGTCATCTAACTGT	TCTTCCTATCAAATCGGTCTTTTGGGATA

ADW314	GAAGGTGACCAAGTTCATGCTTTTTCTTTTGATGGCTACTATGGTGC	GAAGGTCGGAGTCAACGGATTTTTCTTTTGATGGCTACTATGGTGG	TGACACAGACGCCCGTGACCTA
ADW316	GAAGGTGACCAAGTTCATGCTTGGTCTGTGCCTCTGTGATCA	GAAGGTCGGAGTCAACGGATTGGTCTGTGCCTCTGTGATCG	GGCGACGCTTGGATCTCTGGTT
ADW317	GAAGGTGACCAAGTTCATGCTCGATGCTCACGCCAGACAACTAA	GAAGGTCGGAGTCAACGGATTGATGCTCACGCCAGACAACTAC	GTCGAGGATCGCTCTGCCTTGAT
ADW318	GAAGGTGACCAAGTTCATGCTCCCCTCGTGCTACATCTCGA	GAAGGTCGGAGTCAACGGATTCCCCTCGTGCTACATCTCGC	TCGGCATGACAGGCAGCGACAA
ADW319	GAAGGTGACCAAGTTCATGCTAATCTATGATCACCGGTGCCCA	GAAGGTCGGAGTCAACGGATTAATCTATGATCACCGGTGCCCG	GCAGAGTTGGAGAAGTCGAAGCTTT
ADW320	GAAGGTGACCAAGTTCATGCTAATAATTAAATGGAAGAGGCCTATGAGATA	GAAGGTCGGAGTCAACGGATTAATTAAATGGAAGAGGCCTATGAGATG	GATGGCATCCACATTTAGCTTTTACCAT
ADW322	GAAGGTGACCAAGTTCATGCTGAAGCAACATCATGAATTCATGCGG	GAAGGTCGGAGTCAACGGATTAAGAAGCAACATCATGAATTCATGCGT	GGCAGTCTGATAGCCCAAAGAACTT
ADW323	GAAGGTGACCAAGTTCATGCTAATCCCAGTGGCATCATTTACAGC	GAAGGTCGGAGTCAACGGATTGAATCCCAGTGGCATCATTTACAGT	GCTTGCTGTACAAAAGCATGGTACCAT
ADW324	GAAGGTGACCAAGTTCATGCTCCGCCAAACGACCCAG	GAAGGTCGGAGTCAACGGATTTGCTCCGCCAAACGACCCAT	TATGGCCTTGTCTACCCTGGAGTT
ADW328	GAAGGTGACCAAGTTCATGCTCAGCCATGCTGCTTGTAGCGG	GAAGGTCGGAGTCAACGGATTCAGCCATGCTGCTTGTAGCGA	CTCAGTCGAGTGACGTTAGCATCTT
ADW334	GAAGGTGACCAAGTTCATGCTGTTGTACCCGGTCTAGGATCGT	GAAGGTCGGAGTCAACGGATTGTACCCGGTCTAGGATCGC	CCTACGGTGCGTTCAAGTTGATGTA
ADW330	GAAGGTGACCAAGTTCATGCTCGGTTAGGTATTGTGAGGCAACG	GAAGGTCGGAGTCAACGGATTACGGTTAGGTATTGTGAGGCAACA	CGCCCACTGCTGCCGCCAT
ADW332	GAAGGTGACCAAGTTCATGCTGGTTTGGGCCTGTCCAACCG	GAAGGTCGGAGTCAACGGATTCGGTTTGGGCCTGTCCAACCA	GTCCTTATATAGGAGGTCAGAAACCAA
ADW315	GAAGGTGACCAAGTTCATGCTCAATATCTTGAATTCCAAGGCCCCA	GAAGGTCGGAGTCAACGGATTAATATCTTGAATTCCAAGGCCCCC	GGTGGAGTGTATTATGCCGACCTAA
ADW337	GAAGGTGACCAAGTTCATGCTCGCATATCCTCCAATTTCGCTTTCT	GAAGGTCGGAGTCAACGGATTGCATATCCTCCAATTTCGCTTTCG	CACCGAGAATCTCATTGATGAGGAAATAT
ADW338	GAAGGTGACCAAGTTCATGCTGTCTCCATGCATGAGAAATCCAG	GAAGGTCGGAGTCAACGGATTACGTCTCCATGCATGAGAAATCCAA	CGGGGCGAAAGCCTTTTTCGATAAT
ADW341	GAAGGTGACCAAGTTCATGCTCAAGATGTCGACACCCACCCTT	GAAGGTCGGAGTCAACGGATTCAAGATGTCGACACCCACCCTA	TCATCGGCCGGTGGCACACATA
ADW342	GAAGGTGACCAAGTTCATGCTCATCATCGAAACAGGGGCTAGGA	GAAGGTCGGAGTCAACGGATTATCATCGAAACAGGGGCTAGGC	GGCGTCTCCCTGAAGATGGAATAAA
ADW343	GAAGGTGACCAAGTTCATGCTGGGGACCCCATAAAGTTCATCTG	GAAGGTCGGAGTCAACGGATTGGGGACCCCATAAAGTTCATCTC	GTTCTTGCGCAGGGTGTTGACGAT
ADW345	GAAGGTGACCAAGTTCATGCTGGCCGGCGCTTGGCTCG	GAAGGTCGGAGTCAACGGATTATGGCCGGCGCTTGGCTCA	CTCGTCTTCCGAGTCCATTAAACGAA
ADW347	GAAGGTGACCAAGTTCATGCTGTGTATGCGATCGGTCCGCTG	GAAGGTCGGAGTCAACGGATTGTGTATGCGATCGGTCCGCTT	CTGCTCTGGGCACCTAAAGATATCTT
ADW348	GAAGGTGACCAAGTTCATGCTGTCGGCCAAGCTAGATCGCTC	GAAGGTCGGAGTCAACGGATTGTCGGCCAAGCTAGATCGCTT	CGTAATGTCGACGTTTGCAGCGAT
ADW349	GAAGGTGACCAAGTTCATGCTAGCGAAGTGGGCCGTCCAG	GAAGGTCGGAGTCAACGGATTGAGCGAAGTGGGCCGTCCAA	AACCGGAAACAGAAAGCCTTGCGTA
ADW351	GAAGGTGACCAAGTTCATGCTCATTTAATTTTCTGCATGTATCAAATTTGTAGA	GAAGGTCGGAGTCAACGGATTCATTTAATTTTCTGCATGTATCAAATTTGTAGT	GAGCCTAAATTAGTATGAGTTCTACCACAT

ADW353	GAAGGTGACCAAGTTCATGCTCCACATGCCACTTCCTCTTTTTTTTTA	GAAGGTCGGAGTCAACGGATTCCACATGCCACTTCCTCTTTTTTTTTT	GACATGATCTACTTTGCATACACAATGTAT
ADW363	GAAGGTGACCAAGTTCATGCTCGAAGTCACCATAGTCCACAGAC	GAAGGTCGGAGTCAACGGATTACGAAGTCACCATAGTCCACAGAT	TGTGAGGAGACATTGCCGTCGATTA
ADW364	GAAGGTGACCAAGTTCATGCTCCTCATCCCACATCTGCTGAG	GAAGGTCGGAGTCAACGGATTGCCTCATCCCACATCTGCTGAT	GAAATGGGTGGATAGGGAACTCGAT
ADW371	GAAGGTGACCAAGTTCATGCTAACTTATTGGGAAGGAGCGCCATT	GAAGGTCGGAGTCAACGGATTCTTATTGGGAAGGAGCGCCATC	GGTCGTGTGTTGGAGTTGTCCTA
ADW373	GAAGGTGACCAAGTTCATGCTGAGGTGGGTTGCTTCCAAACG	GAAGGTCGGAGTCAACGGATTGGAGGTGGGTTGCTTCCAAACA	CATCAATTGGTGGCTGCTCCAAGTA
ADW374	GAAGGTGACCAAGTTCATGCTCGTGCGTCCGTTTCACGTAAGA	GAAGGTCGGAGTCAACGGATTGTGCGTCCGTTTCACGTAAGG	GGGTGCAGCCCAGTAAACAAAAGAA
ADW431	GAAGGTGACCAAGTTCATGCTCCTCTATGAACGCATGCACACAC	GAAGGTCGGAGTCAACGGATTACCTCTATGAACGCATGCACACAT	GAGAAGCTCGCAGAGACGGCAT
ADW432	GAAGGTGACCAAGTTCATGCTGAGCGTTTGTGCGTGAACTTTTTTTTT	GAAGGTCGGAGTCAACGGATTAGCGTTTGTGCGTGAACTTTTTTTTC	GAGAGCTCATCCAACTGGTTTTCCAA
ADW433	GAAGGTGACCAAGTTCATGCTATGACGTGGAACCCAATATTCACATTT	GAAGGTCGGAGTCAACGGATTGACGTGGAACCCAATATTCACATTG	GACATGTGGGCCCTACTCGACAT
ADW469	GAAGGTGACCAAGTTCATGCTACTCGACATGACGGTCCACTT	GAAGGTCGGAGTCAACGGATTACTCGACATGACGGTCCACTC	CATTGTAAATGATGACGTGGAACCCAATA
ADW477	GAAGGTGACCAAGTTCATGCTCACAGTATAGCAGTTCATTCGGACAT	GAAGGTCGGAGTCAACGGATTACAGTATAGCAGTTCATTCGGACAG	CGCCAATATTTGGCAATTAGATGAACCAT
ADW576	GAAGGTGACCAAGTTCATGCTACACAGATCCTCCATAAAACAGAATTTTTA	GAAGGTCGGAGTCAACGGATTACACAGATCCTCCATAAAACAGAATTTTTT	TATTATTATTAGGGCACATTTTCCGAATTT
ADW577	GAAGGTGACCAAGTTCATGCTGTAGTCTCACTGGAGCTCTCCA	GAAGGTCGGAGTCAACGGATTAGTCTCACTGGAGCTCTCCG	CAAGCTTGTTGGACATCATAGGAAAGTT
ADW594	GAAGGTGACCAAGTTCATGCTGATGATGTCCTTAGACGTGGGC	GAAGGTCGGAGTCAACGGATTGATGATGTCCTTAGACGTGGGT	TATTATGGTAGACCCTTCTTTCATTAAGAT
ADW595	GAAGGTGACCAAGTTCATGCTTTGGGATAATCTACAGTATGTTTAACTCA	GAAGGTCGGAGTCAACGGATTTGGGATAATCTACAGTATGTTTAACTCT	CTTTATTCGGCATGGATGCTATTCCATA
ADW616	GAAGGTGACCAAGTTCATGCTGCCACGGGGTGTTCGTGGAAA	GAAGGTCGGAGTCAACGGATTCCACGGGGTGTTCGTGGAAC	CTCTCATGCCCTAAGCCAACCTAA
ADW617	GAAGGTGACCAAGTTCATGCTTCAAGCACTCTAATTATCATCCTTC	GAAGGTCGGAGTCAACGGATTCTTCAAGCACTCTAATTATCATCCTTG	GCCCAGATGGTTTTCCCGCATATTT
ADW657	GAAGGTGACCAAGTTCATGCTAAAAAATGCAGACCACAATTGTCTGTC	GAAGGTCGGAGTCAACGGATTAAAAAATGCAGACCACAATTGTCTGTG	GTAGCGGTACACCCAGTGGCAA
ADW659	GAAGGTGACCAAGTTCATGCTATACCAGTGTCATCCATGCACTAG	GAAGGTCGGAGTCAACGGATTCATACCAGTGTCATCCATGCACTAA	ACTGCATTTATCTGGCCGGACCAAA

Supplementary Table III. Chromosome specific primer sequences used for multiplex PCRs. Gene IDs are from the IWGSC chromosome arm survey sequence cv. Chinese Spring.

Gene ID	Marker name	Primer sets
TRAES3BF210200010CFD	Indel-1	Forward: 5' - TCTGAACCTCTGCCATTGCT – 3' Reverse: 5' - AGAAGTCACGGGCTTTGAGT – 3'
TRAES3BF025700020CFD	Indel-2	Forward: 5' - CGGTGAAACGTCAATTGGGC – 3' Reverse: 5' - TGTGTTGACGAAGCAGCTTT – 3'
TRAES3BF078700070CFD	Indel-3	Forward: 5' – ACAGGAACGTCACTTCCTCTTC – 3' Reverse: 5' – ATACACGGGAGAAGGATGAGGA – 3'
TRAES3BF089500010CFD	Indel-4	Forward: 5' – GAACCTCTGCCATTGCTTGC – 3' Reverse: 5' – TTGCTAGGCCGTTCTGGATG – 3'
TRAES3BF089400020CFD	Indel-5	Forward: 5' – ACGTCTACCCCTACTTCGCT – 3' Reverse: 5' – TCTCGACCTCATCCCCTGGT – 3'
TRAES3BF089400030CFD	Indel-6	Forward: 5' - GACATAGCTGCCAGTGGGTT – 3' Reverse: 5' - CCCGGCACAGCACTTAAGTA – 3'
TRAES3BF057400070CFD	Indel-7	Forward: 5' – ACTTTTTCTTAGCCGATGCA – 3' Reverse: 5' - TTGAAGTAGGCATTCTCAAC – 3'
TRAES3BF057400130CFD	Indel-8	Forward: 5'- GTCAAATGCATGAGCTTTAC – 3' Reverse: 5' – GTGTGGATGCACGTGTATTT – 3'

Supplementary Figure 5. Set up of the deep soil platform. On the left, 1 m deep wheelie bins were used to mimic a deep soil profile as in natural conditions in Northern Mexico. On the right, wheelie bins were located under a polytunnel to avoid any rainfall and increase temperature. Plants were irrigated until booting to let the bins dry up. Soil moisture was recorded with gypsum blocks located at 10 and 40 cm depth. Temperature and humidity were recorded using dataloggers.

Chapter III

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Pauline Thomelin
Contribution to the Paper	Conducted the study from the phenotyping to the analysis and interpretation of the data. Wrote the manuscript
Overall percentage (%)	80 %
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 12.06.18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Paul Eckermann
Contribution to the Paper	Developed the experimental design of the deep-soil experiment in 2017 and provided support for the data analysis.
Signature	Date 28/5/18

Name of Co-Author	Affiliate Professor Peter Langridge
Contribution to the Paper	Contributed to the analysis and interpretation of the data. Reviewed and edited the chapter.
Signature	Date 16 June 2018

Name of Co-Author	Dr. Penny Tricker		
Contribution to the Paper	Supervised development of the work, contributed to the analysis and interpretation of the data. Reviewed and edited the chapter.		
Signature		Date	30/5/18

Name of Co-Author	Assoc. Prof Delphine Fleury		
Contribution to the Paper	Supervised development of the work, contributed to the analysis and interpretation of the data. Reviewed and edited the chapter.		
Signature		Date	29/05/2018

Chapter III: Study of the physiological basis of *qYDH.3BL* using heterozygous inbred families

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1. Abstract

We investigated the mechanisms triggering *qYDH.3BL* expression, a QTL associated with high biomass and yield in hot climates. Using a deep-soil platform, we studied the physiological responses of four heterozygous inbred families (HIF) segregating for the QTL and grown under combined drought and heat stresses. We observed a strong allele effect at the QTL dependent on temperature when comparing the 2016 and 2017 experiments. Under cooler temperatures observed in 2016, HIFs containing the allele from the drought sensitive parent Kukri showed higher tiller numbers and flag leaf width compared to the HIFs containing the allele from the drought tolerant parent RAC875. In 2017, which was a warmer season than 2016, we observed that the lines containing the RAC875 allele within tree HIFs were associated with an increase in biomass. We also studied the root morphology and anatomy of the HIFs and parental lines. RAC875 had a higher number of central metaxylem vessels than Kukri but with a smaller diameter. We also observed that Kukri had more protoxylem vessels than RAC875 and was significant in one HIF with the lines containing the Kukri allele. We also used sap flow sensors to measure water use in the stem during days and nights and total transpiration per day. RAC875 used more water than Kukri and was responsive to temperatures variations. We observed a similar pattern in the HIFs regarding transpiration per day. The lines containing the RAC875 allele within the HIFs varied more between days and nights but also responded to temperatures.

2. Introduction

Drought episodes and heat waves are among the most devastating environmental events affecting crop productivity and threatening food security. Episodes of extreme weather conditions have been occurring more often and severely due to climate change and its direct effects on precipitations (Trenberth, 2011). Understanding the mechanisms of tolerance developed by plants under combined drought and heat stress should contribute to breed more effectively high-yielding cultivars adapted to stress-prone environments. Ultimately, the breeding of cultivars with improved tolerance should have a great impact on crop productivity and yield improvement as stressed-prone environments account for a large portion of cultivated areas (Tester & Langridge, 2010).

Grain yield is highly variable across environments, limiting yield stability. The definition of the target environment is first required for breeding tolerant cultivars. The QTL *qYDH.3BL* contributed to an increase of yield, TGW and early vigour in the Australian spring wheat population RAC875/Kukri (Bonneau et al., 2013). In the Sonora region in northern Mexico (27° latitude) where *qYDH.3BL* was constitutively expressed, the weather is semi-arid with almost no rainfall during the growing season (Olivares-Villegas et al., 2007; Pinto et al., 2010). This cropping environment is characterised by high radiation. Irrigation by flooding is used and the soil is composed of sandy clay with water holding capacity at depth that favour cultivars with deep root systems.

Wheat with a deep root system has been associated with improved tolerance to drought stress when water was available at depth (Lopes & Reynolds, 2010). Manschadi et al. (2006) found that root architecture and vertical distribution in the soil layers are the main traits contributing to improved tolerance in water-limited environments. In rice, *DEEPER ROOTING 1 (DRO1)*, a gene involved in root architecture has been associated with dehydration avoidance and yield increase under drought (Uga et al., 2013). The gene controls the root growth angle allowing deep root growth to reach water at depth. Study of root morphology and anatomy of the parental lines, RAC875 and Kukri, showed that RAC875 had a lower root hydraulic conductivity than Kukri (Schoppach et al., 2014). Differences were observed in root anatomy: RAC875 had smaller stele and reduced metaxylem diameter in seminal roots (Schoppach et al., 2014) and nodal roots but had more vessels independently of the environment compared to Kukri (Steinemann et al., 2015). RAC875 has been characterised as a water-use conservative cultivar under cyclic drought that limits water loss by reducing its leaf area and residual transpiration

(Izanloo et al., 2008). High hydraulic resistance in seminal roots has been shown to reduce water use at the early stages of development, which will be used at later stages when stored water is available (Passioura, 1972). Cultivars with reduced xylem vessel diameter, from 65 µm to 55 µm, showed a yield advantage, up to 11 %, compared to non-selected cultivars in dry environments with no yield penalty in well-watered environments (Richards & Passioura, 1989). Early seedling vigour is also a drought tolerance trait as it limits water losses from the soil surface by reducing evapotranspiration (Richards & Lukacs, 2002). Improved early vigour contributes to the growth of more vigorous roots able to exploit water available at depth (Richards et al., 2002).

In this study, we focussed on the phenotyping of four HIFs derived from RAC875 x Kukri RILs using the deep-soil platform described in chapter II. We phenotyped the HIFs roots to look for associations between *qYDH.3BL* alleles and anatomical and morphological root traits that were previously associated with the water conservative behaviour of RAC875. We also studied the transpiration and water use of the HIFs using sap flow sensors.

3. Material & Methods

Plant materials

Four HIFs were developed from F2:6 recombinant inbred lines (RILs) of the cross between the parental lines RAC875 (RAC-655//SR21/4*LANCE/3/4*BAYONET) and Kukri (MADDEN/6*RAC-177//GRAJO/76-ECN-44). RILs with residual heterozygosity at the locus were selfed to obtain HIFs where lines have a homogenous background and only segregate at *qYDH.3BL*.

Experimental design in deep soil platform

In both 2016 and 2017, 12 wheelie bins were used to phenotype the HIFs under drought and heat. For the 2016 experiment, two bins were allocated to each HIF and each bin contained 24 lines. Each bin was divided in four blocks, each containing 6 plants (Supplementary Figure 1a). Two blocks were allocated to the lines containing the RAC875 allele and two blocks to the lines containing the Kukri allele (Supplementary Figure 1b). Black double walled plastic boards of 2.5 mm (Bunnings warehouse, Melbourne, Australia) were used to physically separate the

blocks within a bin to measure root DNA density (RDD). The HIFs were sown on the 15th of August and watered until mid-October. Watering was stopped when plants reached mid-booting stage.

In 2017, the HIFs were grown in a single plant plot design. The design was developed using the function "prDiGGer" in the DiGGer package in R for partially replicated designs (Supplementary Figure 1c). Twenty-five lines were grown per bin, in a 5 rows x 5 columns grid. RAC875 and Kukri had 15 replicates each while each HIF had 54 replicates, 27 replicates for the lines with the RAC875 allele and 27 for for those with the Kukri allele. Replicates were distributed within the bins in a way that the same genotype was not present twice in the same row or column. The plants were sown the 31st of July. Watering was maintained until the beginning of October and then stopped when plants reached mid-booting stage.

Soil moisture, air temperature and relative humidity were recorded in both experiments (see Chapter II) (Supplementary Figure 2). The setup of the deep-soil platform was identical as described in Chapter II, except for the plot design.

Phenotypic evaluation in the deep-soil platform

Plant developmental stage was scored in both 2016 and 2017 as described in Chapter II, as well as early vigour which was measured as early leaf area. In 2016, root density was estimated using the root DNA density (RDD) method described by Huang et al. (2013). Four soil cores were collected per depth, at two depths, 10 and 60 cm and for each genotype. Samples were placed in plastic bags and kept on ice. DNA extraction and QPCR analysis were performed by the PIRSA-SARDI department, Waite campus. RDD was evaluated by QPCR using amplification refractory mutation system primers for wheat *internal transcribed spacers 2* (*TaITS2)* and the TaqMan probe after scaling the RDD of each genotype to the reference cultivar Krichauff as described in Huang et al., 2013.

In 2016, reactive oxygen species (ROS) production was evaluated during early grain filling in leaf samples for each HIF family. Hydrogen peroxide (H_2O_2) production was measured by staining leaves in a 1 % (w/v) 3, 3'-diaminobenzidine (DAB) solution adjusted to pH 3.0. Leaves were vacuum infiltrated for 20 min and incubated overnight at room temperature, with agitation and protected from light. The following day leaves were placed in a de-stain solution of ethanol, acetic acid and glycerol and boiled for two-hours to bleached away the chlorophyll. If samples were not totally free of chlorophyll, the de-stain solution was replaced by a fresh

batch and samples were boiled for an additional hour. Samples were then kept in a 60 % glycerol solution in the dark and scanned with the EPSON EXPRESSION 10000 XL scanner. H_2O_2 was also quantitatively measured using the Amplex®UltraRed Reagent (Invitrogen). Briefly, leaf samples were collected at early grain filling stage and ground to a fine powder. Samples were prepared by adding 500 µl of 50 mM potassium phosphate pH 7 to 100 mg of ground tissues and centrifuged for 20 min at maximum speed. Supernatants were then collected and mixed to a working solution containing the Amplex®UltraRed reagent and the horseradish peroxidase. The fluorescence emission was then captured to determine the amount of H_2O_2 using the microplate reader POLARstar OPTIMA (BMG LABTECH, Ortenberg, Germany).

Superoxide (O_2^-) production was determined by staining leaves in 0.1 % of nitroblue tetrazolium (NBT) pH 7.8. As for H_2O_2 production, leaves were first vacuum infiltrated with the staining solution before incubation overnight at room temperature, with agitation, protected from the light. The day after, the staining solution was replaced by the de-staining solution before boiling the samples for two hours. Samples were then kept in a 60 % glycerol solution in the dark for scanning.

Gas exchanges were measured at two development stages, late booting and early grain filling. At early grain filling, plants were experiencing severe stress and no measurements could be recorded. Stomatal conductance (g_s) internal CO_2 concentration (Ci), saturated leaf photosynthesis (A), vapour pressure deficit (VPD) and transpiration (E) measurements were collected on flag leaves between 11:00 am to 2:00 pm using the CIRAS-3 portable photosynthesis system (PP systems, Amesbury, Massachusetts, USA) under controlled light: 1250 umol m^{-2} s^{-1}.

Before harvesting the plants, plant height and tiller number were recorded. Tiller abortion was later calculated by subtracting the number of spikes from the number of tillers at maturity. After harvest, spike and stem biomass were weighed separately. The harvest index was determined by dividing the grain biomass by the total plant biomass. Yield as weight of grain per plant and yield components such as number of spikelets per spike, single grain weight (grain weight divided by grain number per plot) and number of spikes per plant were recorded.

Measurement of plant water use using sap flow sensors

In 2017, SF-4/5 Micro Stem Sap Flow sensors (Edaphic Scientific, Port Macquarie, NSW, Australia) were used in the wheelie bins on the HIFs to measure sap flow through the main stem and calculate plant water use (Supplementary Figure 3). Each sensor contains a heater

located between two temperature probes. Data were automatically collected every 15 min after the initially warm up of the stem by the heater for five min. The output voltage was proportional to the difference of temperature between the two probes. Sensors were installed on plants from mid-October when watering was stopped until harvest and placed between the first and second node. Data were recorded from the beginning of grain filling. Sensors were isolated with aluminium foil to avoid temperature fluctuations. Sensors were calibrated by measuring gs ($mmol/m^{-2}/s^{-1}$) of plants every two hours from pre-dawn until after sunset with an AP4 leaf porometer (Delta-T devices, Cambridge, UK). The stomatal conductance data were compared to the sap flow data and a linear regression were applied to the data. A calibration equation (1) for each genotype was obtained and used to normalise the raw sap flow data. The data were then averaged for every hour to calculate the mean average of the sap flow data for each allele within a HIF. The data were then plotted with the daily mean temperature. We also calculated the total transpiration rate per day and compared it within each HIF between the lines containing the RAC875 allele and those with the Kukri allele.

$$(1)\quad y = \alpha + \beta_{gs}x$$

Root morphological and anatomical measurements

In 2017, HIFs were grown in black plastic pots (9 cm diameter x 18 cm high) filled with a 50:50 soil mix of cocopeat and sand, in greenhouse conditions with a photoperiod of 12 hours. Six replicates per line were grown until sampling. Plants were harvested at two time points, 21 and 42 days after sowing (DAS). At 21 DAS, total leaf area of all the leaves from the main tiller was measured. Shoots were collected at both 21 and 42 DAS and dried at 50 ˚C for a week and weighed. Roots were gently rinsed to remove any excess of soil and then placed in 80 % ethanol before storing them at 4 ˚C until measurements. Roots were separated from the root-shoot junction and aligned on a tray filled with water to scan them. Roots were scanned using the EPSON EXPRESSION 10000 XL scanner (Epson, Suwa, Japan). Each scan was then analysed using the RootGraph software (Cai et al., 2015; http://www.plantimage-analysis.org/software/rootgraph) to measure morphological traits including total root volume, total root length, total root area and root tip number (Figure 1).

After scanning, samples of seminal roots were collected at two positions: 5 cm away from the tip (TIP) and 2 cm away from the root-shoot junction (RSJ). Once collected, samples were stored overnight in an aqueous fixative solution: 0.1 M phosphate buffer pH 7.4, 4%

formaldehyde and 0.25% glutaraldehyde. Root samples were then embedded in 5 % agarose gel for sectioning using a Leica VT1200 S fully automated vibrating blade microtome (Leica, Wetzlar, Germany). Cross sections as fine as 40 µm were stained in 0.5 % toluidine blue and mounted with 90 % glycerol. Slides were analysed with the Nikon Ni-E optical microscope (Nikon, Tokyo, Japan) and pictures were taken with a DS-Ri1 colour cooled digital camera (Nikon, Tokyo, Japan). Three technical replicates for both RSJ and TIP at both 21 and 42 DAS were analysed per genotype. Root anatomy features including root diameter, stele diameter, central metaxylem vessels (CMX) diameter and number, protoxylem diameter and number and thickness cortexwere measured using the Image J software (Schneider et al., 2012). A t-test in the R statistical environment (R Core Team, 2014) was used to determine any significant differences between the lines containing the RAC875 and the lines containing the Kukri allele for each trait measured.

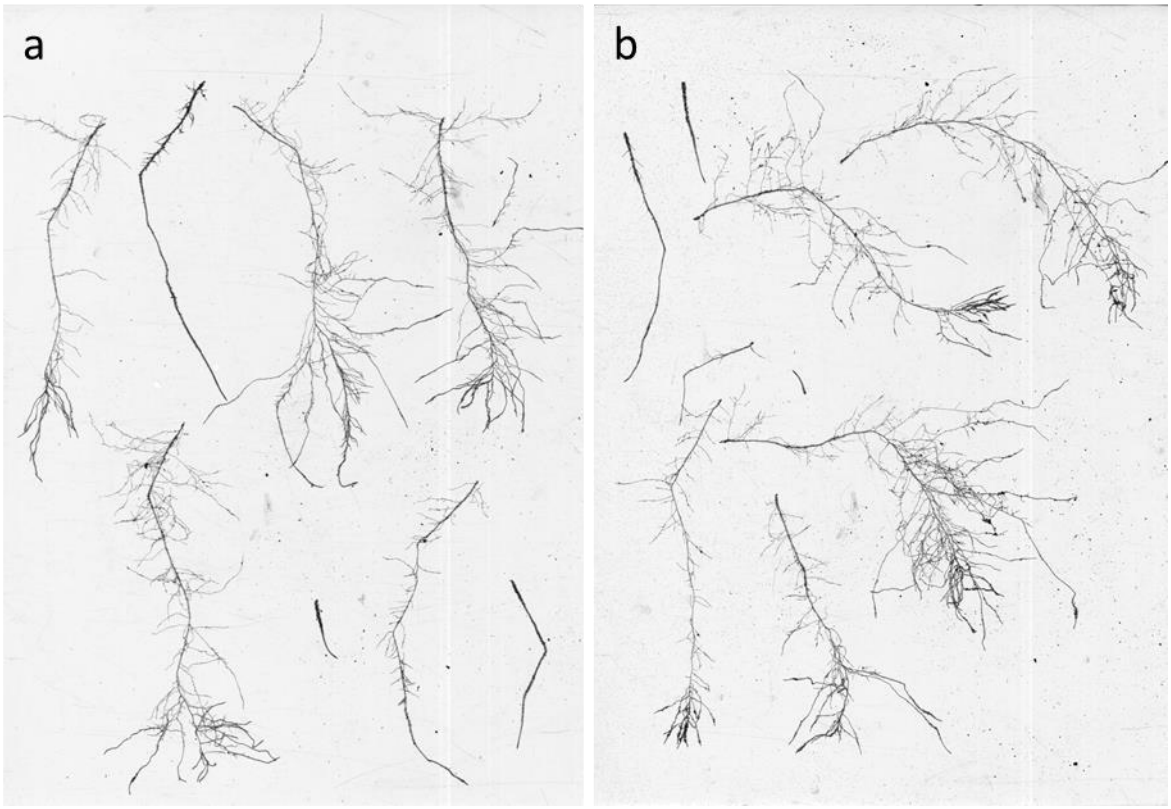

Figure 1. Root morphology of RAC875 (a) and Kukri (b) grown in pots in glasshouse at 21 DAS. Roots were separated at the root-shoot junction and spread on a tray containing water to capture most of the roots.

4. Results

To understand the physiological mechanisms associated with *qYDH.3BL*, we developed four HIFs. We used F_5 RAC875 x Kukri single plant RILs that showed residual heterozygosity in the interval delimited by the two flanking markers AWG43_1 and AWG38. The selected plants were selfed (Figure 2a) to generate HIF that segregated for RAC875 and Kukri alleles at the QTL (Figure 2b). The progeny were genotyped to verify the haplotype they contain (Figure 2c). The HIFs were phenotyped using the deep-soil platform described in Chapter II. The seeds for each HIF family were grown homogenously across the treatments, the planted seeds were selected based on their visual aspect (shape and size). In 2016, no significant differences were detected between AA and BB alleles for each HIF, except for flag leaf width and tiller number. The lines containing the Kukri allele in the HIF4 contributed to an increase of tiller number (Tables I). We also analysed H_2O_2 and O^- content at early grain filling, gas exchanges and RDD for both the parental lines and the HIFs; no significant changes were observed for RDD and gas exchange measurements (Supplementary Table I). In 2016, the environmental conditions were cooler compared to the previous seasons of 2014 and 2015 (Supplementary Figure 4). In 2014, maximum temperature at flowering time reached 33.4°C, and ranged between 34.8 °C and 38.8 °C in 2015, while in 2016, maximum temperature at flowering time ranged between 22.8 °C and 33.3 °C. The analysis of variance between parental lines in the experiment conducted in 2016 showed that Kukri was positively associated with a number of traits including early vigour, flag leaf width and length, plant height, tiller number, spike number, stem and spike biomass, spike number, grain number and number spikelets per spike (Tables I).

A second experiment was conducted in 2017 using the same HIFs under combined drought and heat stress. The experimental design was changed from a six-plants per plot to a single plant design as in 2014 and 2015 to avoid any bin effects. HIF1 plants started to flower 5 days after the end of the flowering period for the other HIFs analysed and the parental lines. As these plants encountered more severe stress, higher temperature at flowering time (+ 3°C compare to the other HIFs) and more severe drought (Supplementary Figure 2), we removed this family from the analysis to avoid any confounding effects due to differences in phenology. The analysis of the overall experiment using data from HIF2, 3 and 4, showed strong differences among bins, particularly among bins with different soil moisture. We therefore looked at the phenotypic variation per HIF in each individual bin (Supplementary Figure 2b). We observed important variations from bin to bin; therefore, we removed bins that did not show significant differences between A and B alleles within a HIF as those would dilute the variance. The

analysis of variance of HIF 2 showed that the lines containing the RAC875 allele had an increased spike and stem biomass and average number of spikelets per spike (Table III). The analysis of variance of HIF3 showed that the lines containing the RAC875 allele had a higher single grain weight than Kukri lines. Finally, HIF 4 lines with RAC875 allele had an increased spike and stem biomass, grain number, number of spikelets per spike, and spike number.

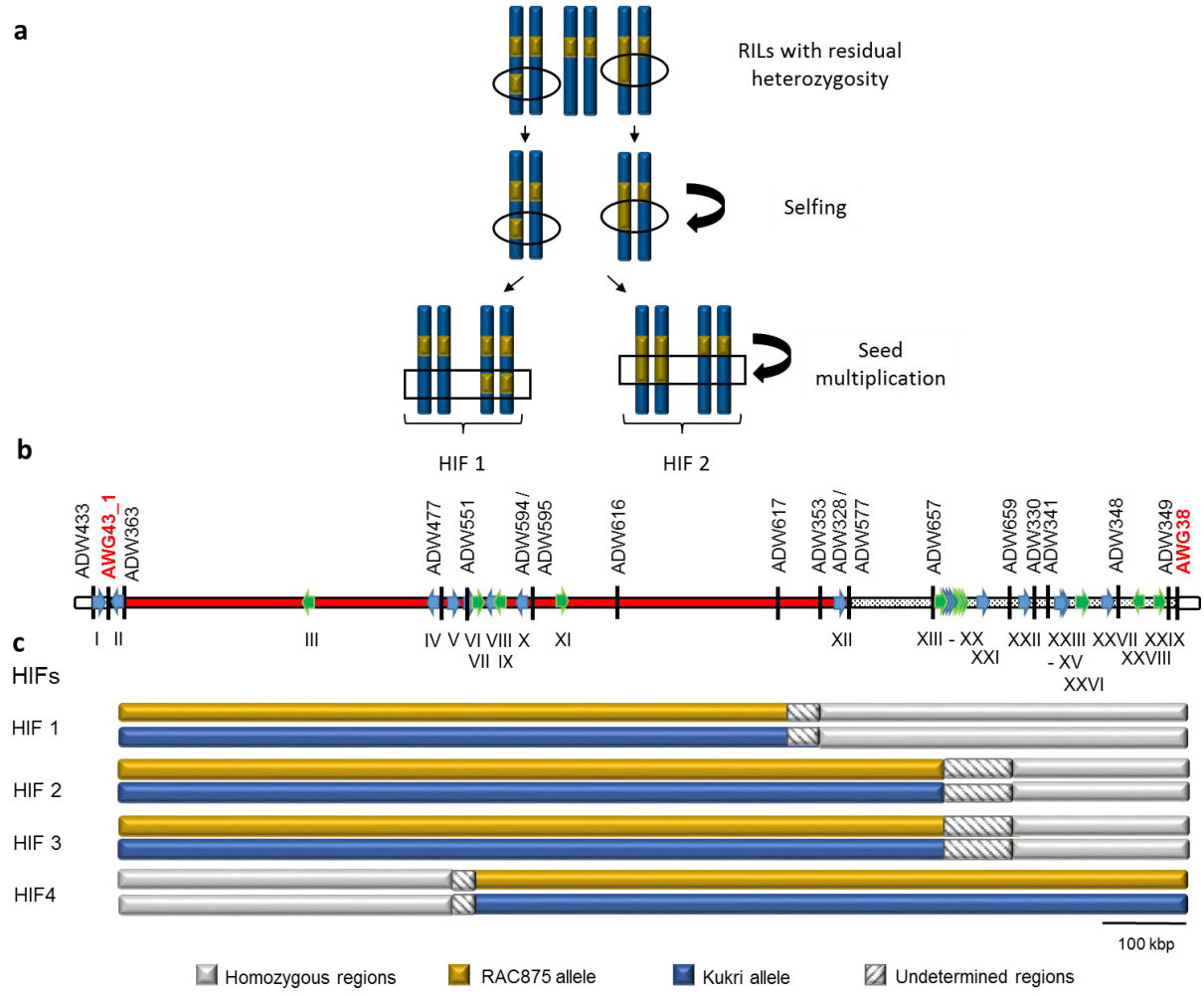

Figure 2. HIFs developed from F2:6 RAC875 x Kukri RILs. a & b, the HIFs with residual heterozygosity were selected using the two flanking markers at the QTL, AWG43_1 and AWG38. Single plants were then selfed. The descent was genotyped to distinguish lines containing RAC875 and Kukri alleles at the QTL, and seed multiplied. b, Haplotype of the four HIFs developed.

Shoot biomass and root morphology and anatomy

In 2017, shoot biomass and root morphology and anatomy traits were phenotyped in the HIFs under controlled conditions with plants grown in pots. The traits were measured at 21 and 42 DAS without stress, as the QTL is associated with an increase of early vigour before the onset of stress. The roots had reached the bottom of the pots when samples were harvested 42 DAS.

Early vigour was measured at 21 DAS but was not significantly different between the AA and BB alleles among the HIFs and parental lines (Table III). Dry shoot biomass and dry root biomass were measured at 21 and 42 DAS. We did not observe any differences in shoot or root biomass at either 21 or 42 DAS for any of the HIFs or parental lines (Table III).

Table I. Analysis of variance among HIFs containing the RAC875 vs Kukri allele in the 2016 experiment in wheelie bins. Data were recorded per plant for early vigour, flag leaf length and width, plant height, tiller numbers, spike number and tiller abortion under combined drought and heat. Data were recorded per plot for stem and spike biomass, grain number, single grain weight and number of spikelets per spike. AA indicates that the HIF lines containing the RAC875 allele; BB, HIF lines containing the Kukri allele.

Traits	Early vigour (cm^2)	Flag leaf length (cm)	Flag leaf width (cm)	Plant height (cm)	Tiller numbers	Spike number	Tiller abortion	Spike length (cm)	Stem biomass (g)	Spike biomass (g)	Grain number	Single grain weight (g)	Spikelet per spike	H_2O_2 content (µM / 100 mg FW)
RAC875	16.06	23.52	2.26	49.52	3.74	2.22	1.52	8.98	18.02	10.36	306.75	0.02	17.09	0.98
Kukri	23.06	27.91	2.11	58.47	4.88	3.63	1.25	9.66	24.57	18.37	522.75	0.02	20.30	0.60
T-test	***	***	**	****	***	****	ns	*	**	***	***	ns	***	****
HIF1-AA	17.97	29.91	2.36	57.09	4.17	2.67	1.5	10.04	28.91	11.63	304.25	0.02	17.93	0.04
HIF1-BB	15.76	29.95	2.3	59.92	3.74	2.29	1.71	10.51	26.44	12.82	341.25	0.02	17.7	0.05
T-test	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
HIF2-AA	22.41	24.61	2.33	56	3.58	2.71	0.86	8.88	18.44	15.86	436	0.02	18.01	0.02
HIF2-BB	23.94	25.1	2.3	55.2	1.43	3.04	1.08	8.99	21.76	16.27	484.25	0.02	18.09	0.03
T-test	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
HIF3-AA	20.23	26.74	2.21	62.94	4.08	2.71	1.38	9.49	23.7	14.63	434.25	0.02	17.9	0.08
HIF3-BB	19.36	24.89	2.1	61.5	4.08	2.63	1.46	9.04	21.88	14.36	425.5	0.02	17.05	0.09
T-test	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
HIF4-AA	13.11	24.08	1.93	55.84	3.52	3.33	0.46	7.87	17.19	19.75	508.75	0.02	15.96	0.56
HIF4-BB	15.6	25.6	2.03	55.6	4.38	3.63	0.75	7.95	20.29	21.75	599.25	0.02	16.55	0.49
T-test	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns

AA: RAC875 allele; BB: Kukri allele; ns = non significant; FW = fresh weight; significance of the allele is indicated by the p value (* < 0.05; ** < 0.01; *** < 0.001; **** < 0.0001)

Table II. Analysis of variance in the HIFs containing the RAC875 vs Kukri allele in the 2017 experiment in wheelie bins under combined drought and heat (each value represent the mean ± sd)

Traits	Early vigour (cm^2)	Spike biomass (g)	Stem biomass (g)	Grain number	Number of spikelets per spike	Single grain weight (g)	Average spike length	Spike number	Tiller number	Plant height (cm)
HIF2-AA	25.52 ± 3.59	1.95 ± 0.74	2.74 ± 0.72	49.63 ± 16.30	18.04 ± 0.48	0.02	9.23 ± 0.30	1.78 ± 0.83	3.67 ± 0.87	52.22 ± 3.85
HIF2-BB	23.75 ± 4.05	1.21 ± 0.34	1.81 ± 0.43	37.75 ± 7.70	17.48 ± 0.47	0.02	8.87 ± 0.51	1.38 ± 0.52	3 ± 0.53	48.39 ± 4.91
df	16	16	16	15	16	16	16	16	16	16
F-test	ns	*	**	ns	*	ns	ns	ns	ns	ns
HIF3-AA	19.40 ± 2.81	1.77 ± 0.46	3.01 ± 0.78	40.63 ± 10.2	16.25 ± 0.94	0.03 ± 0.01	8.89 ± 2.98	1.58 ± 0.51	3.62 ± 0.51	54.68 ± 5.11
HIF3-BB	17.66 ± 2.63	1.46 ± 0.97	2.58 ± 1.26	31.08 ± 17.6	16.07 ± 1.17	0.02 ± 0.01	8.64 ± 2.94	1.64 ± 0.84	3.5 ± 1.02	50.9 ± 6.01
df	26	25	25	23	25	24	25	25	26	25
F-test	ns	ns	ns	ns	ns	**	ns	ns	ns	ns
HIF4-AA	24.81 ± 2.71	2.38 ± 0.91	3.05 ± 0.9	67.36 ± 13.74	16.3 ± 0.39	0.02	8.03 ± 0.41	2.75 ± 0.62	3.58 ± 0.79	54.05 ± 4.56
HIF4-BB	22.49 ± 2.76	1.69 ± 0.54	2.26 ± 0.55	51.45 ± 14.44	15.74 ± 0.81	0.02	7.88 ± 0.44	1.91 ± 0.7	3.27 ± 0.79	51.73 ± 5.62
df	22	22	22	21	22	22	21	22	22	22
F-test	ns	*	*	*	*	ns	ns	**	ns	ns

AA: RAC875 allele; BB: Kukri allele; ns = non significant; Significance of the allele is indicated by the p value (* < 0.05; ** < 0.01)

We did not observe any significant differences between the parental lines or among the HIFs for the root morphology traits including total root volume, total root length, total root surface and number of root tips.

We studied the root anatomy of the parental lines along with the four HIFs using microscopic analysis of cross sections taken at two locations on the seminal roots: 2 cm away from the RSJ and 5 cm away from the tips. At 21 DAS, we observed that Kukri had significantly ($P < 0.05$) more protoxylem than RAC875 at both the TIP and RSJ (Table IV). We observed the same effect in the HIF 3 when the line containing the Kukri allele was compared to the line with the RAC875 allele. We also observed that RAC875 had more central metaxylem vessels than Kukri but the diameter of the metaxylem vessels were smaller in RAC875 compared to Kukri at RSJ (Table V; Figure 3). We did not observe any significant differences at the metaxylem level in the HIFs except for HIF 3 where the lines containing the Kukri allele had smaller central metaxylem vessels than those with the RAC875 allele. We did not observe any significant differences between RAC875 and Kukri among the HIFs at 42 DAS at the RSJ. No significant differences between alleles was observed for HIFs 2 and 4 for any of the traits studied.

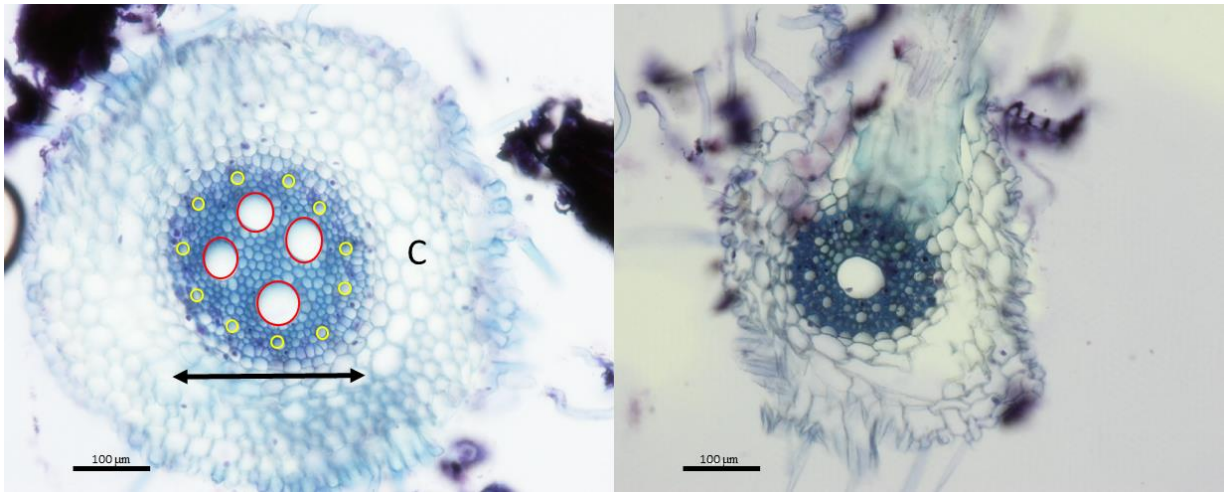

Figure 3. Root anatomy of RAC875 and Kukri at 21 DAS at the RSJ. Left, RAC875; right, Kukri; cross sections of roots were obtained using a vibrotome and stained with toluidine blue before observing them under microscope. Red circles indicate the CMX; the yellow circles indicate the protoxylem vessels; the black arrow highlights the stele and the c indicates the cortex-epidermis.

Table III. Analysis of variance root and shoot traits measured in the HIFs and the parental lines grown in pots in glasshouse in 2017. Traits were measured per plant at 21 and 42 DAS.

	21 DAS							42 DAS					
	Early vigour	Dry root biomass (g)	Dry shoot biomass (g)	Total root length (cm)	Total root area (cm^2)	Total root volume (cm^3)	Number of toot tips	Root biomass (g)	Shoot biomass (g)	Total root length (cm)	Total root area (cm^2)	Total root volume (cm^3)	Number of root tips
RAC875	25.31	0.06	1.61	1069.67	153.09	2.58	804.83	0.66	2.87	2187.51	1293.27	21.38	7847.83
Kukri	25.58	0.07	1.88	1024.61	149.63	2.48	932.33	0.66	3.48	2237.9	1314.73	19.47	8517.17
T-test	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
HIF1-AA	26.59	0.06	1.87	998.63	142.05	2.13	921.5	0.74	3.86	2501.01	1477.98	24.19	8938.17
HIF1-BB	28.44	0.05	1.81	1008.31	145.63	2.22	831.67	0.62	3.38	2301.35	1351.45	21.5	8210.5
T-test	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
HIF2-BB	19.17	0.05	1.15	851.68	141.43	2.33	582	0.69	3.43	2074.47	1235.68	18.66	7561.67
HIF2-BB	22.36	0.06	1.62	832.61	143.05	2.42	625.17	0.61	3.71	2161.51	1274.81	19.65	7562.17
T-test	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
HIF3-AA	23.23	0.06	1.91	1122.94	174.6	2.82	849.83	0.52	3.08	1916.83	1145.45	16.48	8427
HIF3-BB	20.05	0.05	1.48	982.58	156.15	2.45	706	0.60	3.66	2262.58	1354.65	20.82	8024.8
T-test	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
HIF4-AA	24.09	0.06	1.73	1021.11	162.81	2.65	771.17	0.40	2.97	1446.82	867.02	12.8	6588.33
HIF4-BB	22.06	0.05	1.83	898.2	143.41	2.37	742	0.57	30.3	1675.76	1002.51	14.72	7386.5
T-test	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

AA: RAC875 allele; BB: Kukri allele; ns = non-significant

Table IV. Analysis of the root anatomy traits measured at 21 DAS in the HIFs and the parental lines grown in pots in a glasshouse. Cross sections were done on six plants with three technical replicates per plant.

	RSJ							TIP						
	Root diameter (µm)	Stele diameter (µm)	CMX diameter (µm)	Protoxylem diameter (µm)	Thickness cortex (µm)	CMX number	Protoxylem number	Root diameter (µm)	Stele diameter (µm)	CMX diameter (µm)	Protoxylem diameter (µm)	Thickness cortex (µm)	CMX number	Protoxylem number
RAC875	519.22	221	48.31	12.96	298.22	1.69	9.25	480.75	193.06	56.68	12.63	287.69	1.14	11.47
Kukri	515.58	224.64	56.19	13.24	290.94	1.14	13.17	427.14	177.89	55.90	13.81	249.25	1.08	14.33
T-test	ns	ns	*	ns	ns	**	*	ns	ns	ns	ns	ns	ns	*
HIF1-AA	517.06	243.14	50.88	15.46	273.92	1.97	10.56	436.25	178.67	54.13	15.03	257.58	1.08	10.50
HIF1-BB	517.67	221.22	53.11	15.26	296.44	1.44	8.72	474.94	180.97	57.24	16.29	293.97	1.17	11.11
T-test	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns
HIF2-AA	507.39	225.28	51.94	15.03	282.11	1.56	10.89	511.36	180.02	56.96	13.78	331.33	1.08	10.33
HIF2-BB	535.52	230.61	46.78	13.17	304.92	2.00	10.14	518.83	191.81	55.69	12.22	327.03	1.61	10.14
T-test	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
HIF3-AA	506.4	219.7	49.25	15.71	286.7	1.63	7.53	447.83	180.25	66.25	15.78	267.58	1.33	7.94
HIF3-BB	441.42	194.66	47.63	14.63	246.75	1.42	10.00	413.86	179	52.5	13.93	234.86	1.22	10.86
T-test	ns	ns	ns	ns	ns	ns	*	ns	ns	**	ns	ns	ns	*
HIF4-AA	479.64	208.56	46.90	12.8	271.1	1.56	10.33	438.36	177.53	63.33	14.42	260.83	1.06	10.31
HIF4-BB	519.36	223.64	47.64	14.14	295.72	1.69	10.08	440.47	185.33	61.25	14.01	255.14	1.25	10.08
T-test	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

AA: RAC875 allele; BB: Kukri allele; ns = non-significant; CMX = central metaxylem vessels; significance of the allele is indicated by the p value (* < 0.05; ** < 0.01).

Table V. Analysis of the root anatomy traits measured at 42 DAS in the HIFs and the parental lines grown in pots in a glasshouse. Cross sections were done on six plants with three technical replicates per plant.

	RSJ							TIP						
	Root diameter (µm)	Stele diameter (µm)	CMX diameter (µm)	Protoxylem diameter (µm)	Thickness cortex (µm)	CMX number	Protoxylem number	Root diameter (µm)	Stele diameter (µm)	CMX diameter (µm)	Protoxylem diameter (µm)	Thickness cortex (µm)	CMX number	Protoxylem number
RAC875	588	302.1	49.76	13.46	285.9	3.40	10.10	481.67	190.61	61.36	14.39	291.36	1.14	12.67
Kukri	523.67	158	48.75	11.83	365.67	2.67	11.33	455.1	184.3	56.8	12.34	270.8	1.27	13.63
T-test	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns
HIF1-AA	573.92	280.25	51.86	17.15	293.67	3.42	10.17	503.37	211.3	61.06	16.41	292.07	1.40	8.40
HIF1-BB	556.2	251.4	53.3	15.93	304.8	2.90	11.30	549.75	231.5	22.53	16.25	318.25	2.08	11.17
T-test	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
HIF2-AA	536.75	257.67	57.98	14.48	279.01	2.50	10.50	555.75	233.56	63.44	14.81	322.19	1.97	11.89
HIF2-BB	575.58	270.67	54.44	13.91	304.92	2.83	12.67	487.14	203.73	71.89	14.60	283.42	1.22	13.58
T-test	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
HIF3-AA	427.31	219.47	49.54	13.57	207.83	2.11	11.94	440.22	204.28	58.85	13.46	235.94	1.72	11.72
HIF3-BB	510.6	255.7	45.38	14.44	254.9	3.10	10.40	472.4	201	64.47	15.66	271.4	1.47	11.20
T-test	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns
HIF4-AA	528.25	234.83	50.17	13.69	293.42	2.75	10.17	602	205.67	61.8	15.96	396.33	1.58	11.17
HIF4-BB	605.83	265.25	51.3	13.14	340.58	2.92	10.17	536.7	209.3	60.48	14.85	327.4	1.70	10.90
T-test	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

AA: RAC875 allele; BB: Kukri allele; ns = non-significant; CMX = central metaxylem vessels; significance of the allele is indicated by the p value (* < 0.05).

Sap flow data

We plotted the normalised sap flow data of the parental lines along with the mean daily temperatures and observed that variations in sap flow mass for RAC875 overlapped temperature variations. This result suggests that RAC875 water use responds to daily temperatures (Figure 4). By contrast, Kukri sap flow mass data did not vary with the temperature; instead, it was constant during the whole experiment. When we looked at the total transpiration per day, Kukri transpired approximately the same amount of water every day while we observed much more variations in water use for RAC875. We then looked at the sap flow data and water use in the HIFs by plotting the normalised sap flow data in each HIF and compared the lines containing the RAC875 allele and those with the Kukri allele (Figure 5 & 6). We observed the same water management behaviour for the HIF3 than the parental lines even though HIF3 seems to transpire a lot more water (Figure 6). Sap flow mass varied greatly between day and night for the lines containing the RAC875 allele while it was constant for the lines containing the Kukri allele. We observed the same trend in transpiration rate per day: the lines containing the RAC875 allele used much more water than the lines containing the Kukri allele, which transpired approximately the same amount of water every day. Variations of sap flow mass were even higher in HIF2-AA (Figure 5a). However, when we looked at the total transpiration per day, there was not much difference between HIF-2AA and HIF-2BB, even if the lines containing the RAC875 allele seems to be more responsive to temperature variations (Figure 5b). Finally, the HIF4 was quite different compared to the parental lines or the other HIFs. There was not much variation in sap flow mass between day and night whether the lines carried the RAC875 or Kukri. Moreover, the total transpiration per day was quite similar and very low for either HIF4-AA or HIF4-BB, it seems strange that a plant would not have transpired (data not shown).

5. Discussion

We studied the physiological basis of HIFs to understand the mechanisms of tolerance associated with RAC875 allele at *qYDH.3BL* in a hot climate. We used the same deep-soil platform as described in Chapter II as it has proven to be an efficient platform to study the mechanisms triggering *qYDH.3BL* expression.

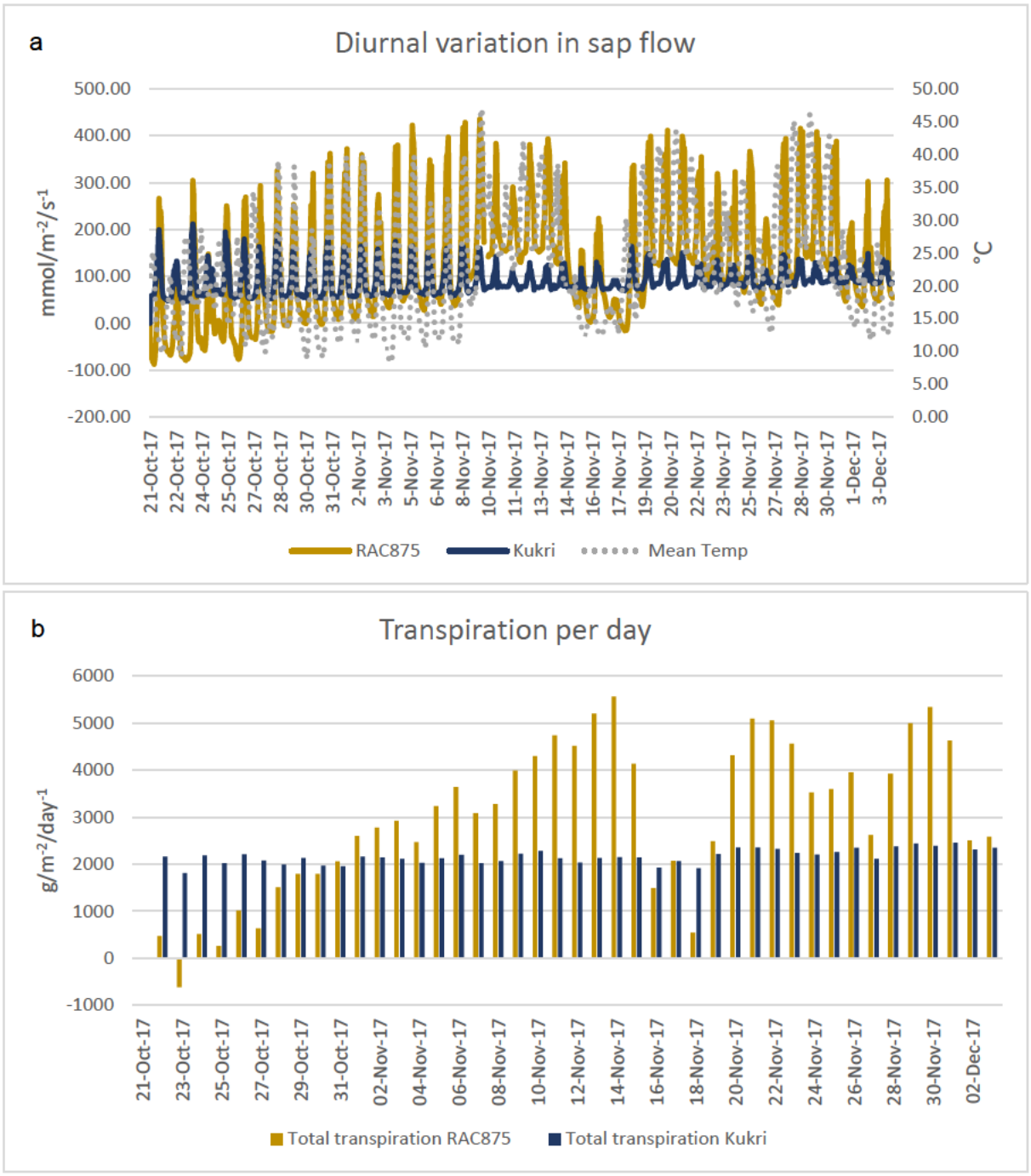

Figure 4. Diurnal variation in sap flow and transpiration per day in parental lines. Sap flow data were normalized and averaged hourly to calculate water use. Data were collected every 15 minutes each day and night from the 21st of October to the 3rd of December. One sensor per parental line was used in the experiment. a, diurnal variation in sap flow mass average hourly; b, measure of total transpiration rate per day for both RAC875 and Kukri.

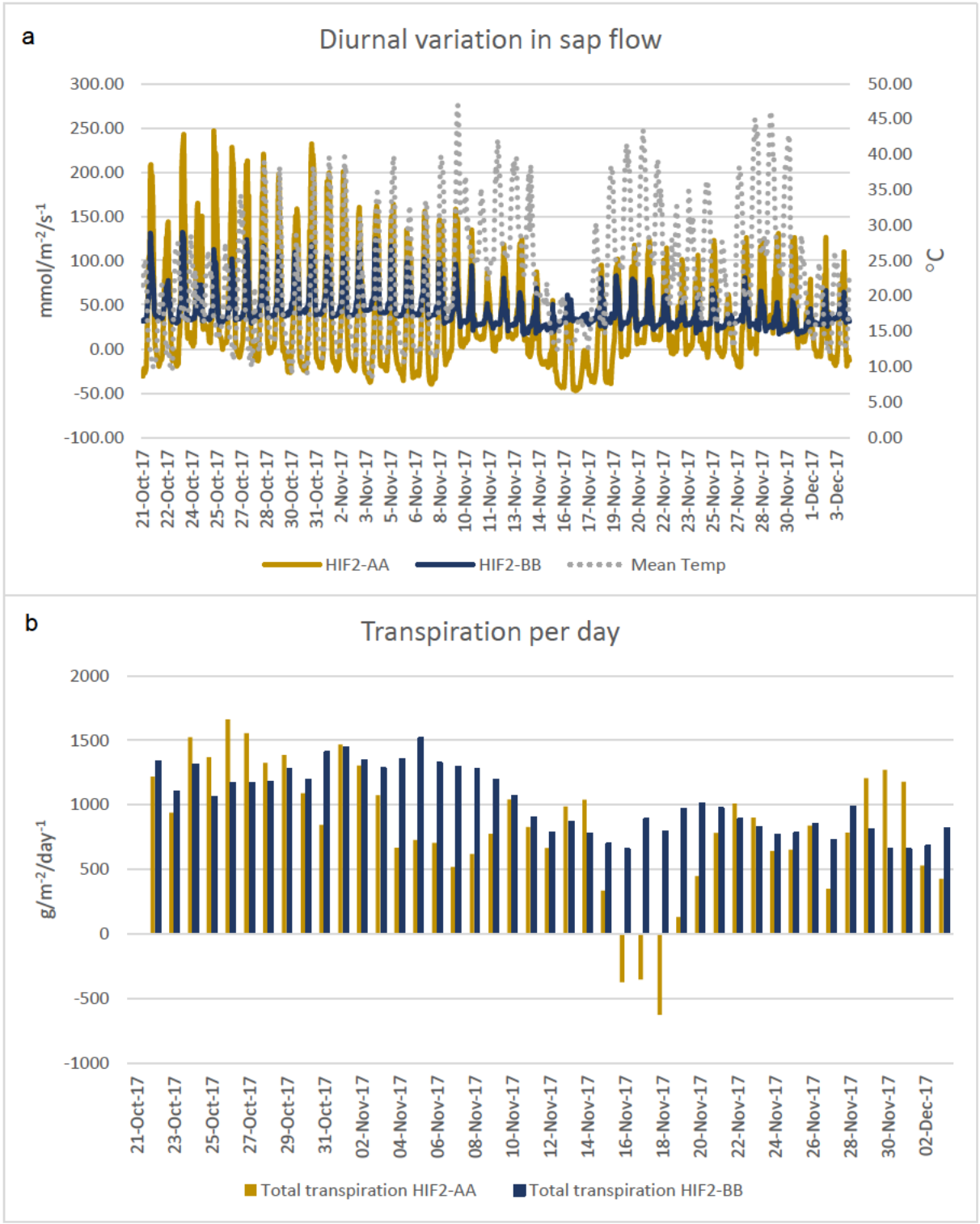

Figure 5. Diurnal variation in sap flow and transpiration per day in HIF2. Sap flow data after normalization and averaged hourly. Data were collected every 15 minutes each day and night from the 21st of October to the 3rd of December. Six sensors were used for each HIF: three sensors for the lines containing the RAC875 and three for the ones containing the Kukri allele. a, diurnal variation in sap flow mass average hourly; b, measure of total transpiration rate per day for HIF2-AA and HIF2-BB.

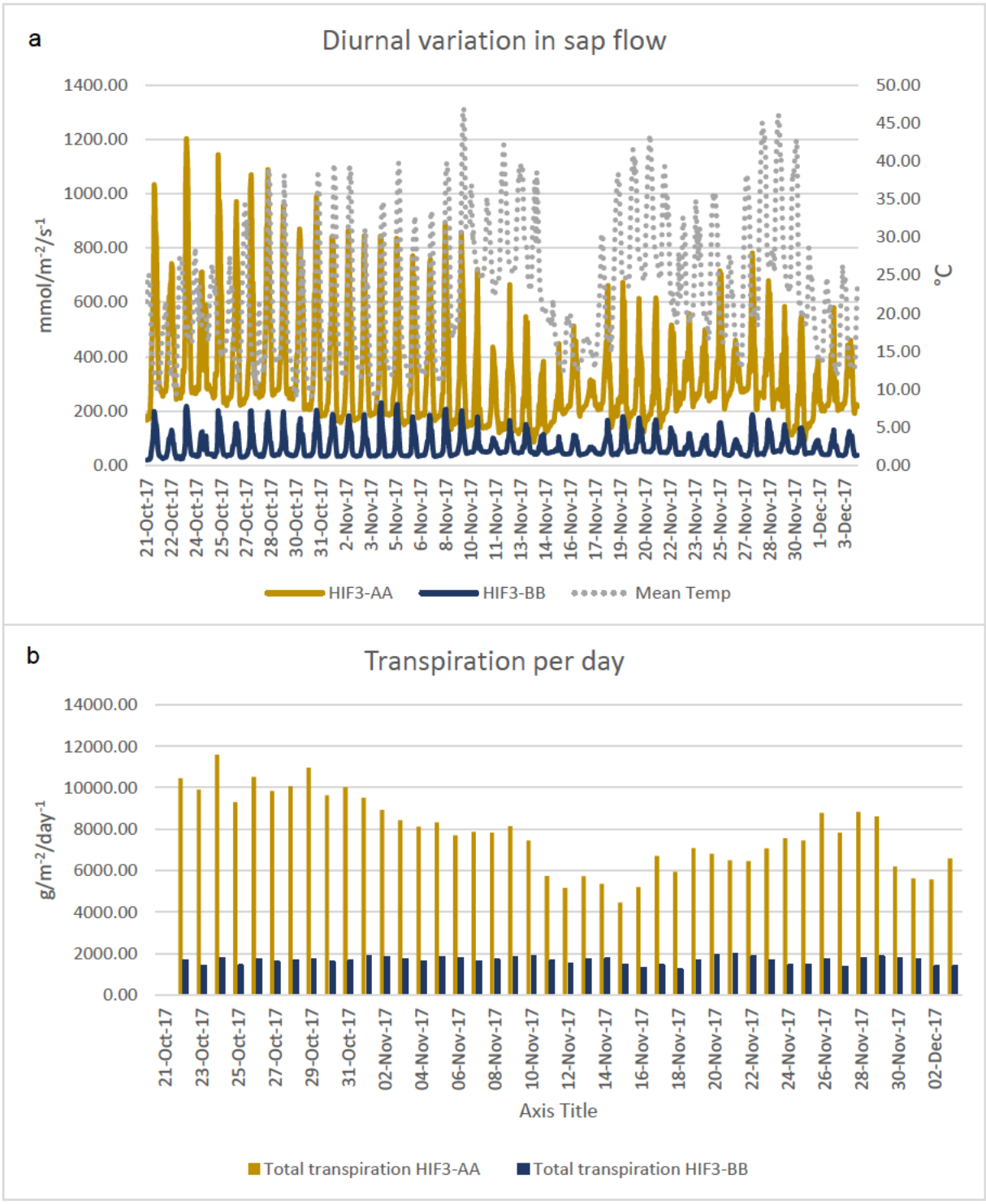

Figure 6. Diurnal variation in sap flow and transpiration per day in HIF3. Sap flow data after normalization and averaged hourly. Data were collected every 15 minutes each day and night from the 21st of October to the 3rd of December. Six sensors were used for each HIF: three sensors for the lines containing the RAC875 and three for the ones containing the Kukri allele. a, diurnal variation in sap flow mass average hourly; b, measure of total transpiration rate per day for HIF3-AA and HIF3-BB.

In 2016, the environmental conditions were cooler than in 2014 and 2015 (Supplementary Figure 4). The average minimum temperatures at flowering time was 11 °C (3 and 4 °C less than in 2014 and 2015 respectively) and average maximum temprature was 28.5 °C (5 to 6 °C less than in 2014 and 2015 respectively). The 2016 experiment confirmed the effects of the Kukri allele previously observed by Bonneau et al. (2013) and Parent et al. (2017). Bonneau et al. (2013) observed that the Kukri allele was positive in irrigated trials of South Australia. Parent et al. (2017) looked at the environmental factors contributing to the allele effects and concluded that the positive allele at the QTL was temperature-dependent; the Kukri allele is positive when temperatures at flowering time average between 18 and 25°C while the RAC875 is positive when temperature are above 25°C. In 2017, average minimum temperatures at flowering time was about 15 °C and average maximum temperature 31 °C and the positive allele was RAC875 among the HIF confirming that the switch of allele effect associated with *qYDH.3BL* is heat dependent. We also observed a strong *qYDH.3BL* x G x E interaction in 2017, confirming that the QTL effect is highly variable across environments or bins here. Lines containing the RAC875 within HIF 2, 3 and 4 were associated with an increase in stem and spike biomass but also single grain weight. We could not conclude on ROS and gas axchange in HIF as 2016 conditions were too cold.

We hypothesized that the water conservative behaviour of RAC875 under cyclic drought could be associated with its lower root hydraulic conductivity (Schoppach et al., 2014). Steinemann et al., (2015) also observed that RAC875 had smaller metaxylem vessels diameter than Kukri but more central metaxylem vessels independently of the water regimes. We analysed root morphology and anatomy of the HIFs at early stage, before the onset of stress to see if *qYDH.3BL* affects root development along the crop cycle. As previously observed, RAC875 had smaller metaxylems vessels diameter as Kukri at 21 DAS at the RSJ. However, we did not observe any differences for the stele diameter (Tables IV and V). We also observed that RAC875 had significantly ($P < 0.01$) more metaxylem vessels than Kukri at the RSJ as observed by Steinemann et al. (2015) (Table IV). Even though we have been able to confirm the results of Steinemann et al. (2015) and Schoppach et al. (2016), we did not observe any significant difference in the HIFs.

We also used sap flow sensors to measure relative variations in xylem sap flow at the stem level. Sap flow sensors have been developed to evaluate the transpiration rate of whole plants by measuring the ascending sap in the stem (Smith & Allen, 1996). The measurements are non-destructive and continuous. We observed that the water consumption of RAC875 responded to

daily temperature variations while water use in Kukri did not seem to be responsive. Moreover, the daily transpiration rate of RAC875 was more important than Kukri most of the time, except for a few exceptions. These results contrast with the observations of Izanloo et al. (2008) on the water-management behaviour of RAC875 and Kukri under cyclic-drought. They concluded that RAC875 was reducing its water consumption at early stages to use it later when stress become more severe. The experiment of Izanloo et al. (2008) was conducted in pots under cyclic-drought, while we used a deep-soil platform with water available at depth under combined drought and heat stress. It seems that under heat stress RAC875 is using water available at depth during his whole development. The analysis of the sap flow variations and transpiration rate per day showed similar pattern in the HIFs. We observed strong variations in diurnal sap flow mass in the lines containing the RAC875 allele compared to those with the Kukri allele. This was particularly striking in HIF3: AA lines respond to temperature while BB lines water use was constant as Kukri parent. Overall, HIF3-AA containing RAC875 allele transpired three to five times more than HIF3-BB. The physiological analysis of lines that segregate only at *qYDH.3BL* contributed to a more detailed study of the physiological basis of the QTL. We have confirmed that the QTL is heat dependent with the positive allele from RAC875 when temperatures are high around anthesis. The RAC875 allele increase plant biomass from early stage of development and seems to be involved in the dynamic water-use in response to heat.

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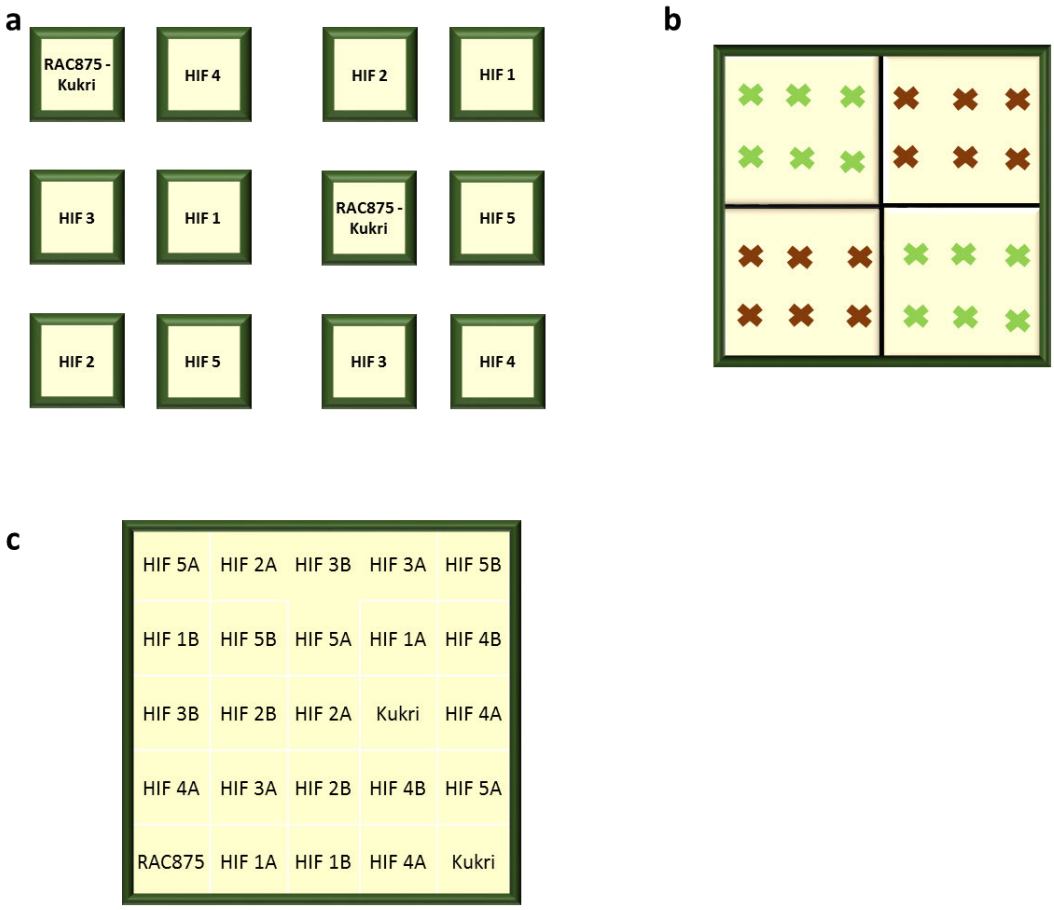
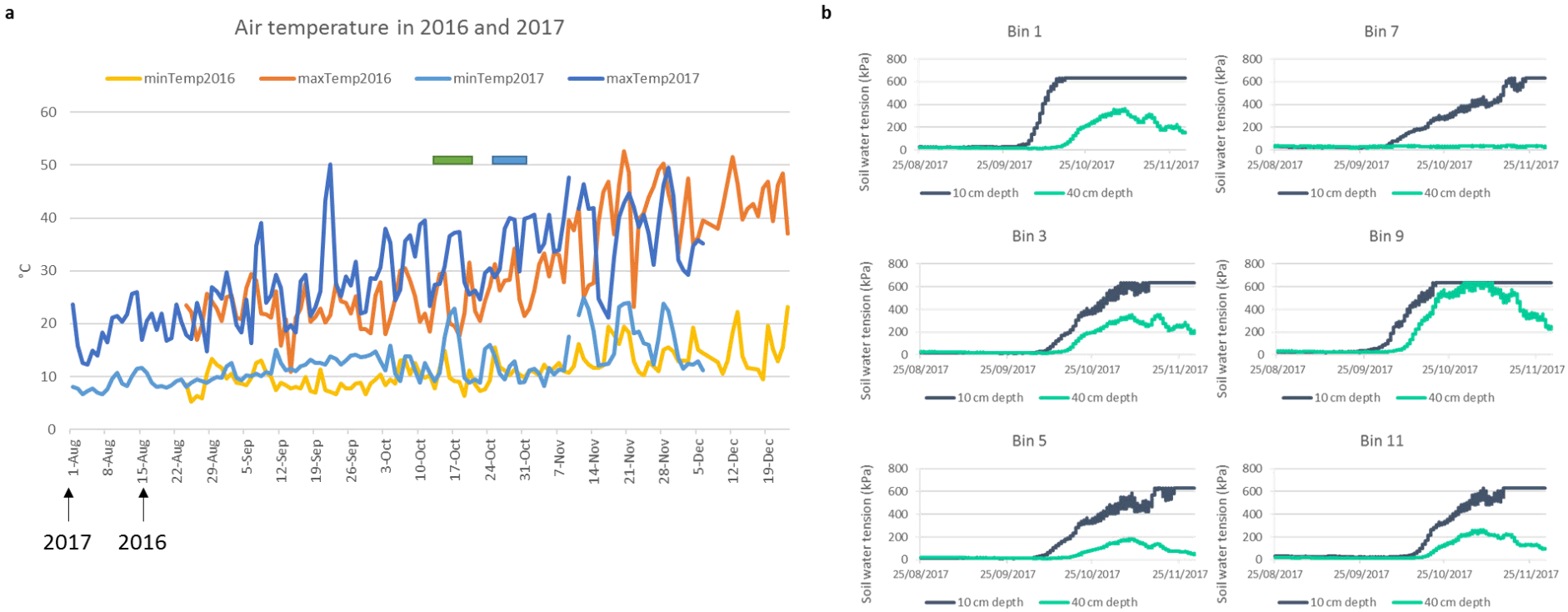
7. Supplementary data

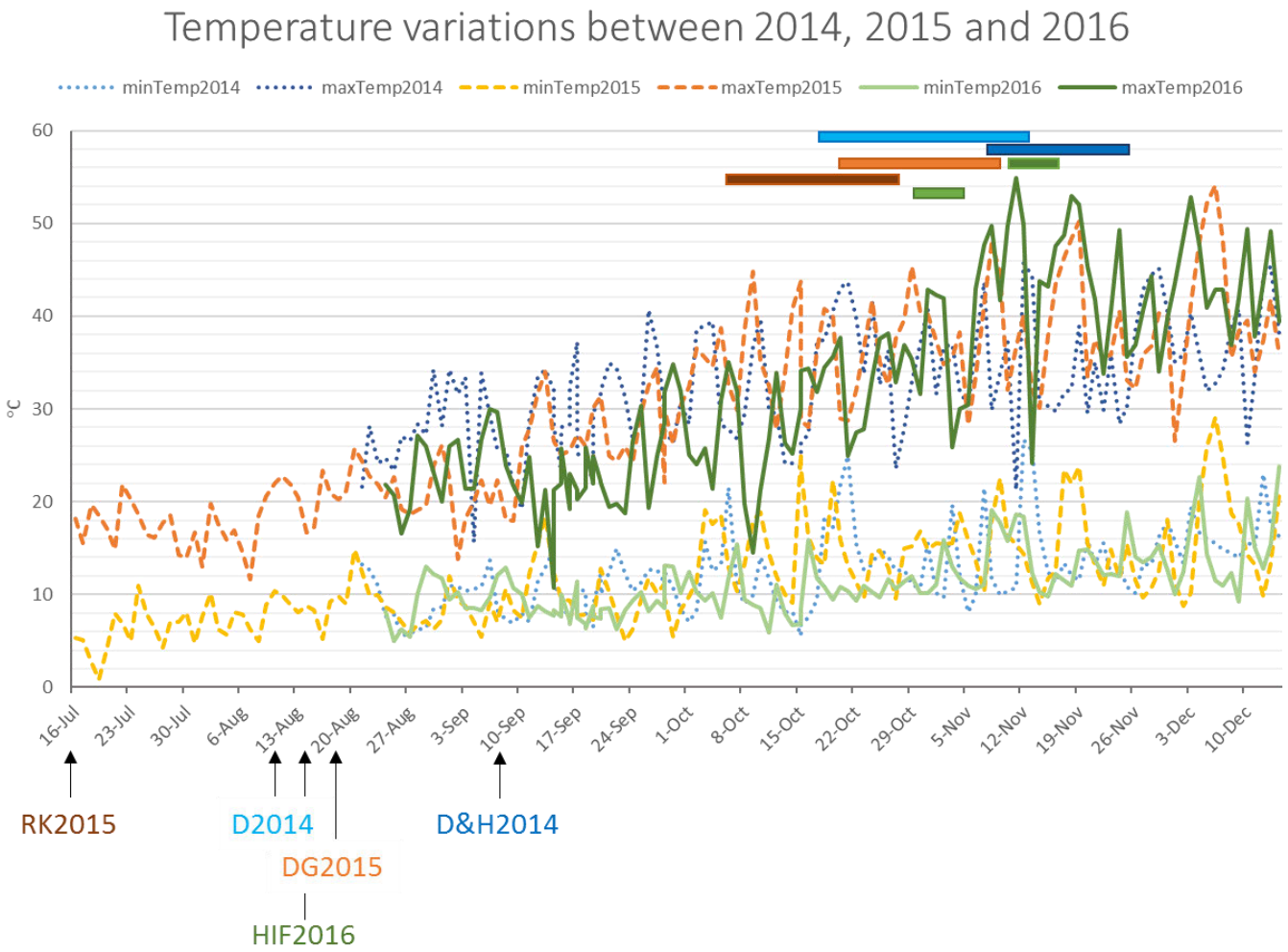


Supplementary Figure 1. a, In the 2016 wheelie bin experiment, two bins were allocated for each HIF; b, Each bin were physically divided by a plastic layer placed horizontally to define 4 blocks, each containing 6 plants. Two blocks were allocated for the lines containing the RAC875 allele (green crosses) and the two others to the lines containing the Kukri allele (brown crosses). The blocks were distributed according to a west-east axis; c, randomized design developed for the 2017 wheelie bin experiment.



Supplementary Figure 2. a, Meteorological data of 2016 and 2017 wheelie bin experiments. The graph combined minimum and maximum temperatures; the green rectangle indicates the flowering date range of the parental lines but also of the HIF2, HIF3 and HIF4 in 2017; the blue rectangle indicates the flowering range of HIF1 in 2017. Black arrows indicate the sowing date of each experiment; b, soil moisture data recorded in 2017 for six bins of the experiment. Data were collected at 10 and 40 cm depth using soil moisture sensors.

Supplementary Figure 3. Sap flow sensors, located on the main tiller, were used to measure heat pulse velocity every 15 minutes at the stem level during both days and nights.



Supplementary Figure 4. Meteorological data during the 2014, 2015 and 2016 experiments. Temperatures in 2016 were cooler than 2014 and 2015. At the beginning of November 2016, we created a chamber around the wheelie bins using plastic shelter to increase temperatures under the polytunnel. Black arrows indicate the sowing date for each experiment: RK = RAC8785 x Kukri; D = drought; DG = Drysdale x Gladius; D&H = drought and heat. Flowering periods have been highlighted for each experiment: light blue for D2014; dark blue for D&H2014; orange for DG2015; brown for RK2015; light green for HIF 2, 3 and 4 and the parental lines in 2016 and dark green for HIF 1 in 2016.

Supplemental Table I. Analysis of variance among HIFs containing the RAC875 vs Kukri allele in the 2016 experiment in wheelie bins. Data were recorded per plant for relative chlorophyll content at booting stage and early grain filling. Data were recorded per block for gas exchange measurements and RDD; AA indicates that the HIF lines containing the RAC875 allele; BB, HIF lines containing the Kukri allele.

	Booting stage						Early grain filling	RDD (ng DNA / ng samples)	
Traits	Relative chlorophyll content	Ci ($\mu mol.mol^{-1}$)	gs ($mmol\ m^{-2}\ s^{-1}$)	VPD	A (µmol.m-2.s-1)	E (mmol m-2 s-1)	Relative chlorophyll content	10 cm	60 cm
RAC875	54.07	193.3	68.67	2.17	7.53	1.38	54.95	93.93	22.76
Kukri	47.68	192.6	66.55	2.15	7.36	1.34	52	79.78	39.31
T-test	***	ns	ns	ns	ns	ns	ns	ns	ns
HIF1-AA	48.42	222.13	88.75	2.11	7.91	1.74	57.07	126.05	99.15
HIF1-BB	48.56	222.63	94.5	2.1	7.825	1.77	55.025	179.19	111.05
T-test	ns	ns	ns	ns	ns	ns	ns	ns	ns
HIF2-AA	50.5	204.56	66.33	2.22	6.88	1.38	51.68	82.54	27.96
HIF2-BB	46.74	189.88	55.5	2.29	5.96	1.19	51.35	79.4	22.38
T-test	ns	ns	ns	ns	ns	ns	ns	ns	ns
HIF3-AA	50.26	201.3	64.7	2.2	6.81	1.33	53.98	99.28	49.02
HIF3-BB	50.08	199.88	72.13	2.17	7.41	1.47	53.97	104.22	29.57
T-test	ns	ns	ns	ns	ns	ns	ns	ns	ns
HIF4-AA	53.29	233	95.88	2.12	8.16	1.88	53	82.32	24.64
HIF4-BB	53.49	223.38	93.63	2.14	8.3	1.84	55.44	85.61	26.52
T-test	ns	ns	ns	ns	ns	ns	ns	ns	ns

AA: RAC875 allele; BB: Kukri allele; ns = non-significant

Chapter IV

Statement of Authorship

Title of Paper	Study of a wheat, *Seven in Absentia (TaSINA)* gene underlying *qYDH.3BL*
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Principal Author

Name of Principal Author (Candidate)	Pauline Thomelin
Contribution to the Paper	Conducted the experiment, performed molecular and bioinformatics analysis. Wrote the manuscript
Overall percentage (%)	75
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 12.06.18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature	Date 30-05-2018

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Contribution to the Paper	Contributed to the analysis and interpretation of the data. Reviewed and edited the chapter.
Signature	16 June 2018

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Contribution to the Paper	Supervised development of the work, contributed to the analysis and interpretation of the data. Reviewed and edited the chapter.		
Signature		Date	30/5/18

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Contribution to the Paper	Supervised development of the work, contributed to the analysis and interpretation of the data. Reviewed and edited the chapter.		
Signature		Date	29/05/2018

Chapter IV: Study of a wheat, *Seven in Absentia (TaSINA)* gene underlying *qYDH.3BL*

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1. Abstract

Water limitation and extreme temperature are the most common environmental limitations for crop productivity. The identification of genes associated with drought and heat tolerance in plants assists the breeding of elite cultivars able to maintain yield in stress-prone environments. The fine mapping of *qYDH.3BL*, a QTL associated with an increase of biomass and yield in a hot climate delimited a region containing 12 candidate genes. The expression analysis of the candidate genes in four heterozygous inbred families (HIFs) revealed a strong candidate, *Seven in Absentia* (*TaSINA).* This gene was significantly more strongly expressed in Kukri compared to RAC875 and in the HIFs containing the Kukri allele than the RAC875 allele. SINA have been characterized as E3 ubiquitin ligase proteins involved in the ubiquitin pathway for the degradation of target proteins. To validate the role of *TaSINA* in drought and heat tolerance, we identified missense variants predicted to affect the protein function. We also studied the phylogenetic relationship of *TaSINA* with the previously published plant and animal *SINA* genes. We observed that *TaSINA* had only low sequence homology with the SINA gene family and had a modified RING finger domain missing a conserved cysteine. Taken together these results are a step toward the functional characterisation of a candidate gene for yield variation in a hot climate in wheat.

2. Introduction

Crop productivity is highly variable and dependent on the environmental conditions during growth. Bread wheat is the most widely grown cereal, in a wide range of climates that are not always suitable for optimal growth. Among the limitations to optimal crop productivity, drought and extreme heat have been responsible for dramatic losses. Between 1964 and 2007, 9 to 10 % of national crop productions around the world have been lost due to a combination of drought and extreme hot temperatures (Lesk et al., 2016). Climate change is predicted to lead to an increased frequency of extreme weather episodes in the future. Breeding of cultivars adapted to a changing environment is essential to increase cereals production and maintain food security (Battisti & Naylor, 2009).

To maintain wheat productivity in hot climates, the identification and characterisation of new alleles controlling yield variation would be highly beneficial to the breeding of new, more tolerant cultivars. So far, only a few genes have been characterised and validated for their role in heat tolerance in wheat, and reviewed in Chapter I. Most of these genes have been characterised under controlled conditions, except for the heat-responsive gene *TaGASR1,* a gibberellic acid-stimulated transcript (Zhang et al., 2017). Transgenic wheat lines overexpressing *TaGASR1* were tested in the field for their response to heat stress. The characterisation under field conditions of genes potentially associated with stress tolerance is an important step to validate their role in improving stress tolerance but also characterise their breeding value (Fleury et al., 2010). Even though a few wheat genes have been associated with improved heat tolerance, little is known about the mechanism controlling yield variation in hot climates.

Genetic studies have in the past successfully identified genomic regions of the wheat genome associated with yield and yield components variation under heat stress (reviewed by Tricker et al., 2018) but none of them has led to the identification of the underlying genes. A multi-environment analysis of a double haploid population of the cross between the wheat lines, RAC875 and Kukri, identified a quantitative trait locus (QTL), *qYDH.3BL,* located on the chromosome arm 3BL. This QTL has been reported to increase yield, thousand grain weight and early vigour in hot climates (Bonneau et al., 2013). Fine mapping using SNP markers combined with the physiological study of a set of recombinant inbred lines narrowed the QTL interval to a list of 12 annotated genes in the reference sequence of Chinese Spring (IWGSC Ref 1.0) (see Chapter II).

In this study, we identified a strong candidate gene, *Seven in Absentia* (*TaSINA*) by expression analysis of the high confidence genes at the narrowed QTL interval. To confirm the role of *TaSINA* in conferring heat tolerance, we genotyped the hexaploid wheat TILLING population cv. Gladius to identify variants of the gene. We also studied the phylogenetic relationships of *TaSINA* with the previously published SINA genes in plants.

3. Material & Methods

Plant material

Four HIFs were developed from F2:6 recombinant inbred lines (RILs) from the cross between RAC875 (RAC-655//SR21/4*Lance/3/4*Bayonet) and Kukri (Madden/6*RAC-177//Grajo/76-ECN-44) and these were used to study the expression of the candidate genes underlying *qYDH.3BL* expression. The HIFs is one of the method used to develop near isogenic lines from RILs with residual heterozygosity.

Gene expression analysis

Seedlings were collected for gene expression analysis. Seedlings of the HIF families were grown in an air-conditioned glasshouse in small pot trays filled with a potting mix (coco-peat). Total RNA for both root and shoot tissues were extracted from 2 week-old seedlings using the Spectrum™ Plant Total RNA Kit (Sigma-Alridch, Carlsbad, California, USA). cDNAs were synthetised using the SuperScript IV First-Strand Synthesis System (Invitrogen, Sigma-Alridch, Carlsbad, California, USA) and used for QPCR. QPCR primers targeting 70-200 bp amplicon sequences were designed to target chromosome specific gene copies using Primer 3 (Untergasser et al., 2012) available in Geneious (Biomatters, Auckland, New Zealand). Specificity of the primers was tested with nulli-tetrasomic lines (Supplementary Table I). QPCR analyses were performed using the Kapa SYBR Fast Universal 2X qPCR Master Mix (Geneworks, Thebarton, South Australia, Australia) on the QuantStudio 6 Flex (Applied Biosystems, Foster City, CA, USA). Three technical replicates per sample and per gene and four wheat reference genes were included in the analysis for normalization of gene expression: wheat *actin* (*TaActin*), wheat *cyclophilin* (*TaCyclophilin 2*2*), wheat *glyceraldehyde-3-phosphate dehydrogenase* (*TaGAPdH 2*2*) and wheat *elongation factor* (*TaEFA2*2*) (Supplementary Table II).

The "wheat-drought cDNA series" of RAC875 and Kukri described by Bowne et al., 2012 was also used for QPCR of the gene *TaSINA*. The cDNA series were from plants grown in pots filled with a potting mix (coco-peat) in an air-conditioned glasshouse. The drought experiment started when the first flag leaf emerged. Five flag leaf samples were collected per treatment. Samples were collected at 5, 9 and 14 days after withholding watering. Plants were re-watered to field capacity, leaf samples were collected when plant reached a wilting point 23 days after beginning of the stress. The last sampling was 25 days after the beginning of the stress, after the second re-watering.

Gene sequencing and promoter analysis

TaSINA chromosome specific primers were designed with Primer 3 available in Geneious (Biomatters, Auckland, New Zealand). Primers were conserved among Kukri and Chinese Spring (Supplementary Table III). The software LinRegPCR (Ruijter et al., 2009) was used to test the QPCR efficiency and determine the quantity of starting material using the raw data of fluorescence of each sample assay where *TaSINA* was amplified.

Promoter sequences of the RAC875 and Kukri allele at *TaSINA* were annotated using the Plant Cis-acting Regulatory DNA Elements (PLACE) database which includes sequence motifs reported as cis-acting regulatory DNA elements (Higo et al., 1999). Protein domains, residues and motifs of *TaSINA* were identified using Prosite (Sigrist et al., 2002), Pfam (http://beta.supfam.org/) and NLS mapper (Kosugi et al., 2009).

Identification of Gladius TILLING mutants

The TILLING (Targeting Induced Lethal Lesions IN Genome) population of the hexaploid wheat cv. Gladius was screened to identify mutations in the coding sequence of *TaSINA* and its homeologues located on chromosomes 3A and 3D. The mutant population is composed of ~ 1,600 individuals obtained after mutagenesis with ethylmethane sulfonate (EMS). DNA of each individual of the M3 generation was extracted and later pooled in a two-way strategy in a plate to enable the detection of mutants as described by Sharp et al., (2014).

The online program Codons Optimized to Discover Deleterious Lesions (CODDLE) (http://www.proweb.org/coddle) was used to determine regions of *TaSINA* protein sequence most likely to carry mutations with deleterious effects based on the properties of EMS to preferentially induce transition mutations (G:C becomes A:T). Chromosome specific primers were manually designed using Primer 3 (Untergasser et al., 2012) available in Geneious (Biomatters, Auckland, New Zealand) to amplify the coding sequence of *TaSINA* and its

homeologues on chromosome 3A and 3D. Primers were designed to amplify only one homeologue and identify genome specific mutations (Supplementary Table IV, Supplementary Figure 1).

TaSINA and its homeologues were first amplified by polymerase chain reaction (PCR) using chromosome specific primers for mutation screening. PCR amplification were performed using the T100^{TM} Thermal Cycler (Bio-Rad, Hercules, California, USA) following these steps: 95 ºC for 3 min; followed by 7 touchdown cycles at 95 ºC for 30 sec, an annealing step starting at 70 ºC and reducing by 1 ºC every cycle for 30 sec and 72 ºC for 1 min and 30 sec; followed by 28 cycles at 95 ºC for 30 sec, 64 ºC for 30 sec, 72 ºC for 1 min and 30 sec, and a final elongation step at 72 ºC for 5 min. PCR amplification conditions were adapted to amplify the 3A and 3D homeologues with respectively 6 and 8 touchdown cycles followed respectively by 29 cycles at 65 ºC annealing temperature and 26 cycles at 62 ºC. PCR amplification for mutation detection were performed in a 10 µl volume reaction with 0.4 µl 10 µM primers, 1 µl 2 mM dNTPs, 0.5 µl dimethyl sulfoxide (DMSO), 0.8 µl 50 mM $MgCl_2$, 2 µl 5X colorless GoTaq® reaction buffer (Promega, Madison, Wisconsin, USA), 0.05 µl GoTaq® DNA polymerase (Promega), pooled DNA and H_2O.

The amplification was followed by a denaturation / re-annealing step: 95 º for 5 min, 90 °C for 1 min, then 99 cycles of 89 °C for 30 sec with each cycle reducing by 0.5 °C. After PCR, products were digested with the CEL 1 enzyme, which specifically recognizes mismatches induced by amino acid change and cleaves single-strand DNA containing a single base pair change. Finally, samples were electrophoresed on a thin 2 % agarose gel of ~ 5 mm to separate digested fragments. Mutants were detected when two pools had the same digestion profiles and shared a common fragment. The presence of mutation was then confirmed by mixing mutant DNA with wild-type DNA in a 1:1 ratio to create DNA heteroduplexes detectable by the CEL1 endonuclease. PCR products of the mutated gene were purified using the NucleoSpin® gel and PCR clean-up kit (Scientifix, Cheltenham, Victoria, Australia) and Sanger sequenced at the Australian Genome Research Facility Ltd (http://www.agrf.org.au/). Sequencing results were visualised and analysed in Geneious 11.0.2 (Biomatters, Auckland, New Zealand). For non-silent mutations, effects of each amino acid change were evaluated using the online tool SIFT (Sorting Tolerant from Intolerant) (Kumar et al., 2009).

M3 seeds of each Gladius TILLING mutant predicted to carry a deleterious mutation were individually grown in pots (8.9 x 18 cm). Leaf samples were collected to extract DNA as described in Shavrukov et al., (2010). KASP markers targeting single nucleotide mutations were designed to genotype mutants and determine their zygosity using the Kraken software

(LGC genomics, Middlesex, UK). The software allowed the design of two forward primers for each SNP allele and a common reverse primer.

In silico screening of Kronos and Cadenza TILLING mutants

A BLAST search was performed using the coding sequence of *TaSINA* and its homeologues on chromosome 3A and 3D against the Wheat TILLING database (http://www.wheat-tilling.com/) to identify mutant lines. Blast settings: e value = 10; # hit = 250. The database combined the re-sequencing data of 1,535 mutants of the tetraploid wheat cv. Kronos (APB MSFRS POP Sel (D03-12)) originated from USA and 1,200 mutants of the hexaploid wheat cv. Cadenza (AXONA/TONIC) originated from Great Britain (www.wheatpedigree.net; Krasileva et al., 2017).

Sequence retrieval of the SINA gene family

Protein sequences of the already published *SINA* genes in *Arabidopsis thaliana*, *Oryza sativa*, *Zea Mays*, *Medicago truncatula*, *Populus trichocarpa*, *Solanum Lycopersicum*, *Drosophila melanogaster* and *Homo sapiens* were retrieved using their gene identifiers in both phytozome (https://phytozome.jgi.doe.gov/) and NCBI (National Centre for Biotechnology Information) (https://www.ncbi.nlm.nih.gov/) (Supplementary Table V). SINA genes in wheat were identified in phytozome (https://phytozome.jgi.doe.gov/). The protein sequence of *TaSINA* translated from DNA sequence was used as a query to identify its closest orthologues in *Brachypodium distachion*, *Oryza sativa*, *Hordeum vulgare*, *Aegilops tauschii* and *Tritricum urartu* (https://phytozome.jgi.doe.gov/). Protein sequences were aligned using MUSCLE (Edgar, 2004) available in Geneious 11.0.2 (Biomatters, Auckland, New Zealand).

Phylogenetic analysis of TaSINA

Phylogenetic analysis of *TaSINA* and its homeologues along with the SINA genes previously published was performed using a neighbour joining approach (Saitou et al., 1987) in MEGA 7.0.26. (Kumar et al., 2016). The phylogenetic tree was based on both the RING-finger and the ZINC-finger domains as described in Wang et al. (2008). To evaluate the confidence of the inferred tree, the bootstrap method was used with 100 bootstraps (Felsenstein, 1985). The generated tree in Newick format was then uploaded in iTOL (Interactive Tree Of Life) (Letunic et al., 2016).

4. Results

We studied the expression profile of the eight high confidence (HC) genes present in the narrowed interval delimited by ADW594 and ADW577 in four HIFs (Figure 1). The HIFs were developed from RAC875 x Kukri RILs single plants with residual heterozygosity at *qYDH.3BL*. The plants were selfed to create heterozygous inbred families (HIFs) where lines segregate at *qYDH.3BL* (Figure 1c). We did not analyse the expression profile of the four LC genes present in the interval (genes III, VII, IX and XI) as no evidence of gene expression could be found in the wheat-expression database (http://www.wheat-expression.com) or in our in-house database (Suchecki et al., 2017). As the QTL was previously associated with early vigour, when no treatment had yet been applied, we studied gene expression at an early stage, in two-week old seedlings grown in glasshouse conditions. Out of the HC genes analysed, gene I had relatively high expression in both shoot and root tissues. However, the expression profile of the gene in RAC875 and the HIFs containing the RAC875 allele was not significantly different from Kukri and the lines containing the Kukri allele (Figure 2a).

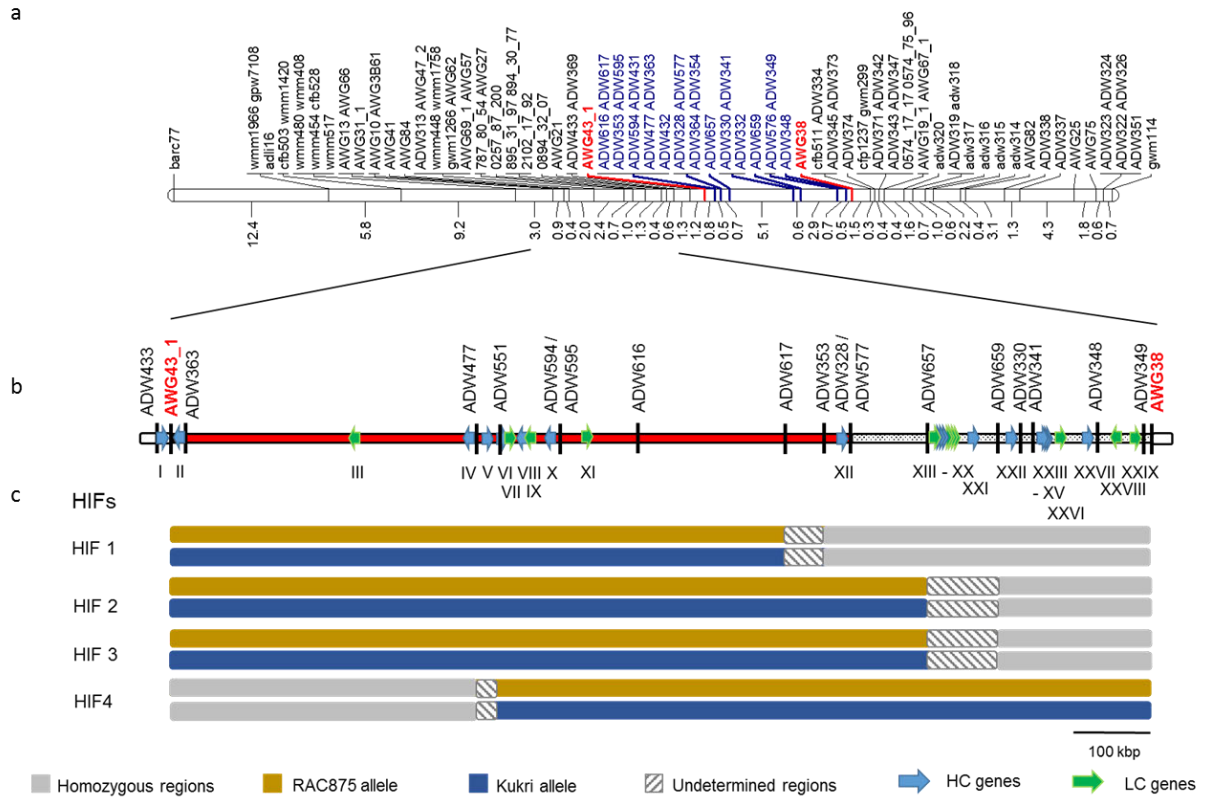


Figure 1. Annotated genes in the interval delimited by ADW594 - ADW577 and haplotypes of the heterozygous inbred families (HIFs). a, RAC875 x Kukri high density genetic map. The fine mapping of *qYDH.3BL*, narrowed the interval delimited by ADW594 - 577. b, Twelve genes have been annotated in the interval delimited by ADW594 – ADW577 highlighted in red. c, Haplotypes of RAC875 x Kukri recombinant inbred lines with residual heterozygosity at *qYDH.3BL* used to develop the HIFs.

1

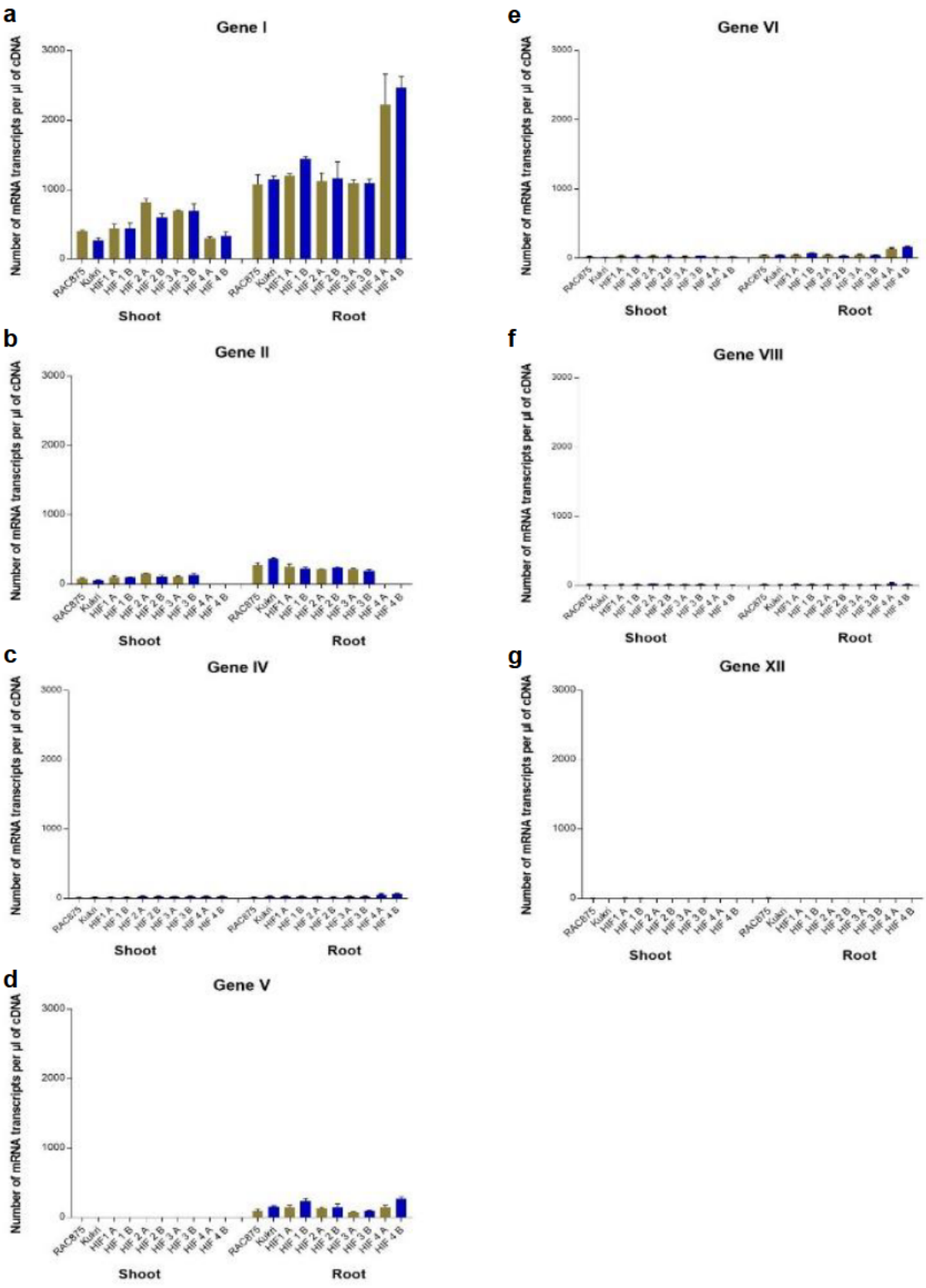


Figure 2. Expression analysis of the candidate genes in the genetic background of the HIFs. **a-g** Expression of the genes present in the interval delimited by ADW594 and ADW577 interval. No significant difference in the expression profile of these genes could be observed between parents and among the HIFs for each gene in either tissue. "A" corresponds to the RAC875 allele and the "B" corresponds to the Kukri allele

Nor was any allele-specific pattern of expression observed for genes II, IV, V, VI, VIII and XII (Figure 2a-g). Gene X was the only gene with differential expression between the parental lines and among the HIFs (Figure 3a). The gene was consistently significantly ($P < 0.01$ to $P < 0.05$) over-expressed in Kukri compared to RAC875, in both shoot and root tissues and in the lines containing the Kukri allele compared to the lines containing the RAC875 allele. By contrast, the homeologous genes on chromosomes 3A and 3D had the same expression profile in the two parental lines and the HIFs independently of the allele that they carried and the tissue analyzed (Figure 3c & 3d). We also analyzed the expression profile of gene X in a wheat-drought cDNA series available in-house for RAC875 and Kukri. Gene X had the same expression profile in both drought and controlled conditions (Figure 3b). Gene X has been annotated in the reference sequence of the wheat genome, IWGSC Ref 1.0, as a *seven in absentia (SINA)* gene that encodes an E3 ubiquitin ligase protein. Gene X will be referred to hereafter as *TaSINA*.

The sequencing of *TaSINA* from parental lines showed polymorphisms in the promoter sequence. Annotation of the *cis*-acting elements present in the promoter sequence of *TaSINA* in the parents revealed four elements specifically present in the promoter sequence of only one of the parents and absent in the other (Figure 3e). CANBNNAPA (CNAACAC) and HDZIP2ATATHB2 (TAATMATTA) motifs were identified in the promoter sequence of *TaSINA* in Kukri. These *cis* elements were absent in the RAC875 promoter. Two different cis-acting elements were found only in the promoter sequence of *TaSINA* in RAC875. The insertion of a CCAC domain, 713 bp before the starting codon, created a SBOXATRBCS (CACCTCCA) motif. This element has been identified in several promoters of *RBCS* photosynthetic genes in Arabidopsis (Acevedo-Hernández et al., 2005). The second cis-acting element present in the RAC875 promoter of *TaSINA* is a -10PEHVPSBD (TATTCT) motif. This cis-acting element present in the promoter sequence of the barley chloroplast *psbD* gene coding for a photosystem II reaction chlorophyll-binding protein, has been characterized as a light responsive regulator (Thum et al., 2001).

One of the SNP identified was located at the 3'end of the forward qPCR primer for *TaSINA*. To know if the SNP polymorphism could have had an impact on the final expression data of *TaSINA*, we compared the PCR efficiency using raw fluorescence data of each sample. The analysis of variance did not show any significant difference in qPCR efficiency or starting concentration per sample (N_O) between the AA and BB lines (Table I) so this SNP did not affect the results.

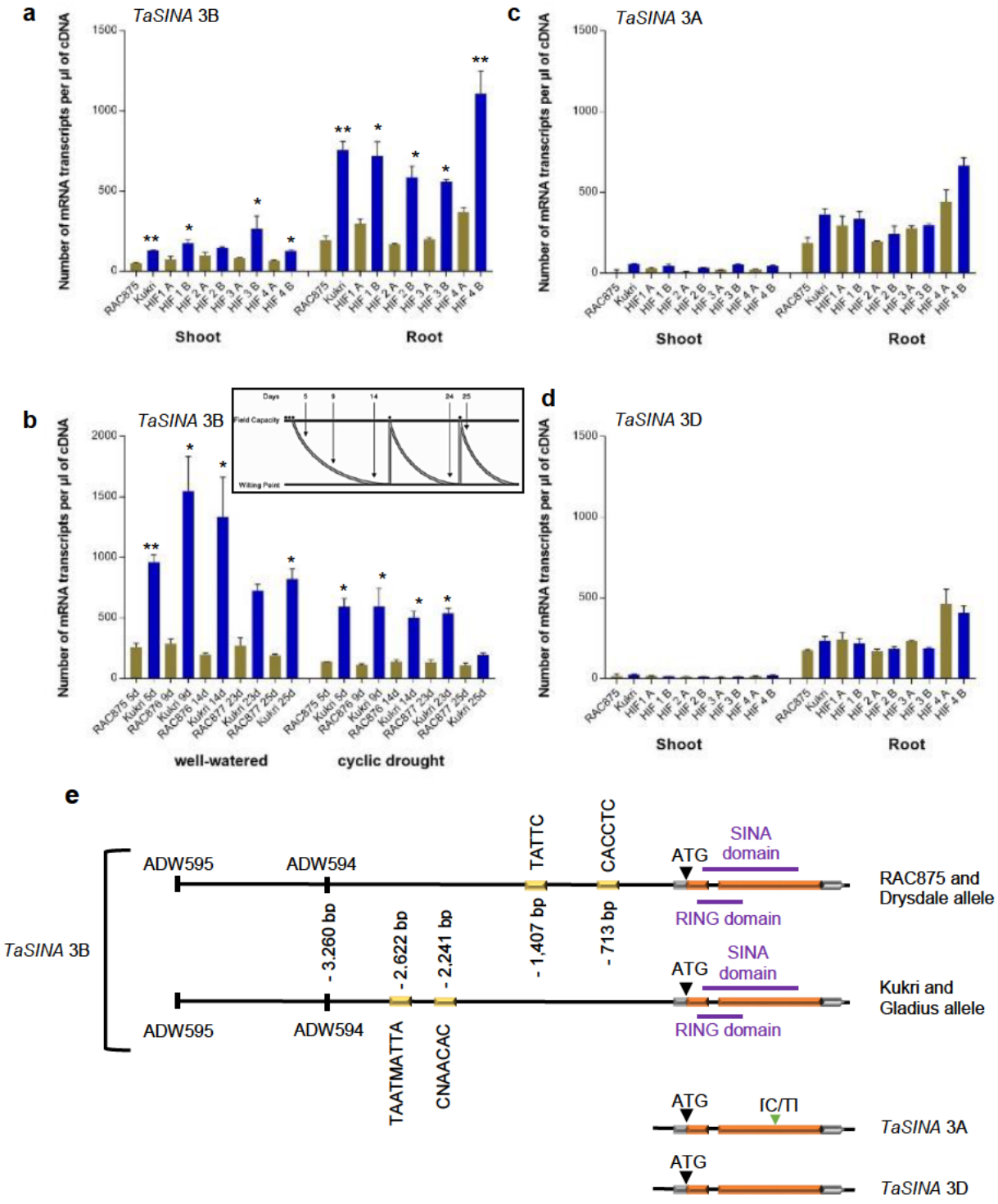


Figure 3. Expression analysis of *TaSINA* and its homeologues in the genetic background of the heterozygous inbred families (HIFs).
a, c, d, Expression analysis in shoot and root tissues of (**a**) *TaSINA 3B*, (**c**) *TaSINA 3A* and (**d**) *TaSINA 3D*, "A' corresponds to the RAC875 allele and the "B" corresponds to the Kukri allele. **b,** Expression of *TaSINA 3B* in RAC875 and Kukri under well-watered and cyclic drought conditions described in Lobell et al., 2011. *: p value < 0.05; **: p value < 0.01; ***: p value < 0.001. **e**, Exon-intron structure of *TaSINA 3B* and its homeologues. Yellow boxes indicate the position of cis-acting elements present in the promoter region of *TaSINA 3B* for the RAC875 and Kukri allele; green triangle, SNP position inducing the alanine to valine amino acid change; orange boxes represent the exons and the grey boxes the UTRs.

Table I Analysis of variance of the PCR efficiency and starting concentration per samples in the lines containing whether the RAC875 or Kukri allele at *TaSINA***.**

	PCR efficiency	N_O
RAC875 allele at *TaSINA*	1.9 ± 0.01	2.3E-08
Kukri allele at *TaSINA*	1.9 ± 0.02	8.2E-09
df	179	179
F-test	ns	ns

The sequencing of *TaSINA* gene in RAC875 revealed two amino acid substitutions between the RAC875 protein sequence and Kukri (Figure 5). The conserved domains of the protein sequence were retrieved and annotated (Figure 5).

The sequencing of the coding sequence of the *TaSINA* homeologues on chromosome 3A and 3D showed that they are monomorphic between RAC875 and Kukri, which correlates which the fact that no QTL were identified on either chromosome 3A or 3D. The alignment of the sequences of *TaSINA* and its homeologues on chromosome 3A and 3D revealed a SNP [C/T] in the second exon. This SNP would change an amino acid at the position 227 from an alanine to a valine, and was predicted to affect the 3A protein in both RAC875 and Kukri. (plant.ensembl.org/Triticum_aestivum; Figure 3e).

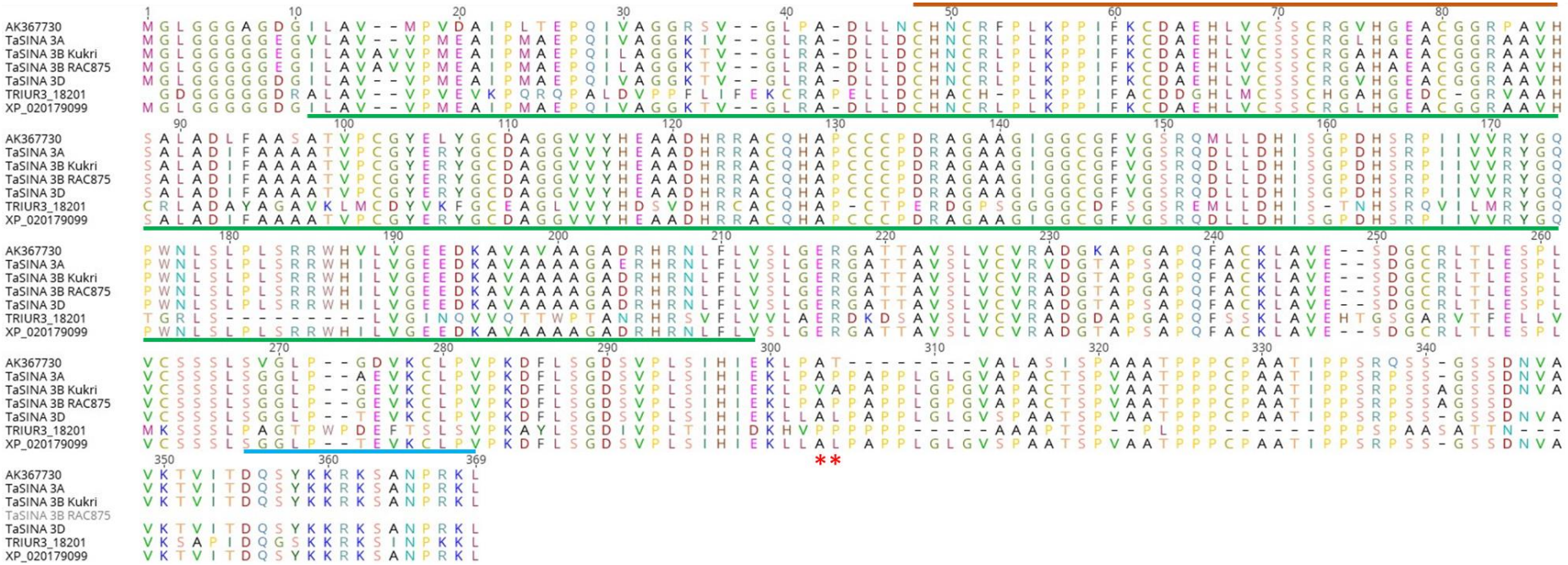


Figure 5. Amino acid alignment of *TaSINA* gene in RAC875 and Kukri and its homeologues with SINA genes from barley (AK), *Triticum urartu* (TRIUR) and *Aegilops tauschii* (XP). The conserved domains were identified and highlighted: the orange line indicates the position of the conserved amino acid in RING finger domain; the green line indicates the position of the TRAF-like domain of the Seven in absentia homolog family; the blue line indicates the position of a nuclear localization signal; the red star indicates the amino acid substitutions between *TaSINA* in Kukri and *TaSINA* in RAC875.

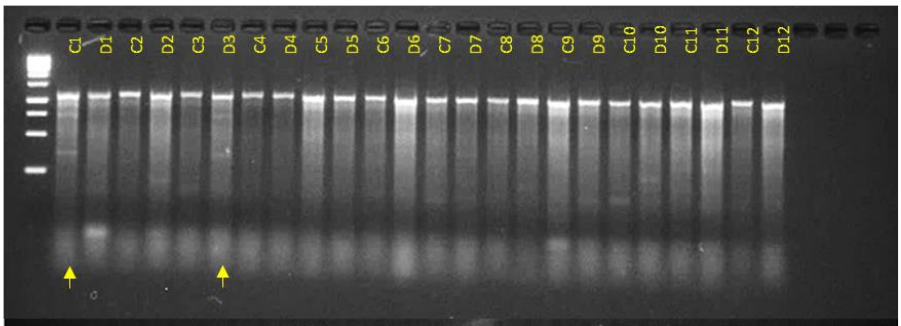
We then screened the Gladius TILLING collection to find EMS mutants of *TaSINA* and its homeologues. The web-based tool CODDLE was used to identify regions of *TaSINA* gene most likely to contain mutations that will affect protein function. Based on the properties of EMS to preferentially induce transition mutations combined with the online tool SIFT that predicts the effect of amino acid substitution, CODDLE identified a region of 140 bp located in the second exon where mutations were more likely to have a deleterious effect (Supplementary Figure 1). The detection method used to identify mutants of *TaSINA* gene and its homeologues was based on a two-way pooling strategy (Sharp & Dong, 2014) (Figure 4a). A mutant was found when the same profile of digestion was observed in two distinct pools and shared a common mutant (Figure 4b). To eliminate potential false positives, individual mutant DNA was mixed with Gladius wild-type DNA to create DNA heteroduplexes that could be detected by the CEL 1 enzyme. After digestion, samples that had the same digestion profile as during the first round of identification were retained (Figure 4c). The genotyping of the 1,600 individuals enabled us to identify 62 potential mutants in the coding sequence of the three copies of *TaSINA*. These mutants were Sanger sequenced to confirmed mutations. In total 34 mutants were validated, eight mutants for the A copy, 10 for the B copy and 16 for the D copy. Most of the nucleotide changes resulted from transitions, G to A (32.3 %) and C to T (58.8 %). Out of 34 mutants, 11 carried a silent mutation and 23 a non-synonymous mutation; no splice-junction mutations were identified. Using the online tool SIFT, two of these mutations were predicted to affect the protein function of *TaSINA 3B* and its homeologue on chromosome 3A (Table II). These two mutant lines were seed multiplied for future studies.

The *in silico* screening of the durum wheat cv. Kronos and the bread wheat cv. Cadenza TILLING database allowed us to identify more mutants of *TaSINA.* Mutations were identified and annotated based on the IWGSC Chinese Spring CSS while we used the gene model from the IWGSC Ref 1.0, TraesCS3B01G572900, as gene model for *TaSINA*. The genotyping of Kronos and Cadenza using three KASP markers (ADW477, ADW594 and ADW595; Figure 1c) indicated that they carried the RAC875 allele at *TaSINA*. The coding sequence of *TaSINA* was used as a query and identified a total of 101 Cadenza mutants and 39 Kronos mutants. Variants were manually located into the coding sequence of *TaSINA* to determine the nature of the amino acid substitutions. Out of 101 Cadenza mutant lines, 57 variants showed non-synonymous substitutions with 11 predicted to affect the protein sequence with high confidence and 30 with low confidence. The mutants will be ordered for further characterisation.

a

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1 B1 C1 D1	A2 B2 C2 D2	A3 B3 C3 D3	A4 B4 C4 D4	A5 B5 C5 D5	A6 B6 C6 D6	A7 B7 C7 D7	A8 B8 C8 D8	A9 B9 C9 D9	A10 B10 C10 D10	A11 B11 C11 D11	A12 B12 C12 D12
B	A1 A2 A3 A4	B1 B2 B3 B4	C1 C2 C3 C4	D1 D2 D3 D4	A5 A6 A7 A8	B5 B6 B7 B8	C5 C6 C7 C8	D5 D6 D7 D8	A9 A10 A11 A12	B9 B10 B11 B12	C9 C10 C11 C12	D9 D10 D11 D12
C	E1 F1 G1 H1	E2 F2 G2 H2	E3 F3 G3 H3	E4 F4 G4 H4	E5 F5 G5 H5	E6 F6 G6 H6	E7 F7 G7 H7	E8 F8 G8 H8	E9 F9 G9 H9	E10 F10 G10 H10	E11 F11 G11 H11	E12 F12 G12 H12
D	E1 E2 E3 E4	F1 F2 F3 F4	G1 G2 G3 G4	H1 H2 H3 H4	E5 E6 E7 E8	F5 F6 F7 F8	G5 G6 G7 G8	H5 H6 H7 H8	E9 E10 E11 E12	F9 F10 F11 F12	G9 G10 G11 G12	H9 H10 H11 H12
E												
F												
G												
H												

b



c

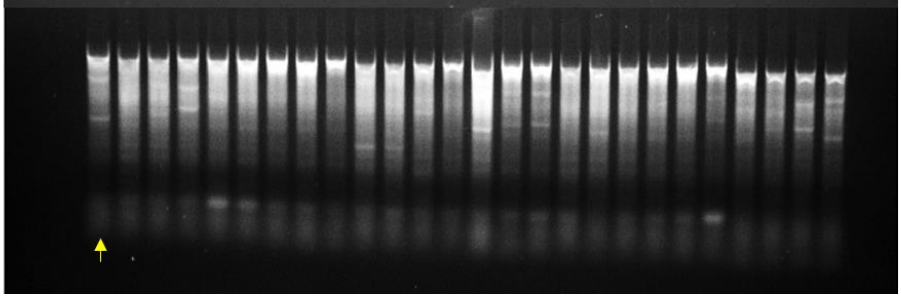


Figure 4. DNA pooling strategy for mutation screening. a, DNA samples were pooled following a 2-way strategy. Each mutant line was present in two different pools, each containing 4 mutants (Sharp & Dong, 2014). b, Example of mutation detection in the hexaploid TILLING population cv. Gladius. The same digestion pattern in the C1 and D3 wells indicated the potential presence of a common mutant within the pools. c, DNA of the previously identified mutant present in the C1 and D3 wells, was mixed with Gladius wild-type DNA. After the CEL 1 digestion step, the same profile of digestion was observed confirming the presence of a mutation.

Table II. Summary of the detected variants in the Gladius TILLING population for *TaSINA* and its homeologues on chromosome 3A and 3D. Number between brackets indicate the SIFT score of the missense mutations predicted to affect protein function

	Number of variants detected	Silent mutations	Non-synonymous mutations	SIFT predictions [a]	
				Tolerated by protein	Affect protein function
TaSINA 3A	8	2 (25 %)	6	5	1 (0.03)
TaSINA 3B	10	3 (30 %)	7	6	1 (0.02)
TaSINA 3D	16	6 (37.5 %)	10	10	0

a Numbers in parentheses indicate the probability that a missense mutation affects the protein function, value < 0.05 substitution predicted to be deleterious

We studied the homology of *TaSINA* with *SINA* genes known in plant and animal species. Eighteen SINA genes were retrieved in *Arabidopsis thaliana,* seven in *Oryza sativa,* six in *Zea mays,* six in *Medicago truncatula,* 10 in *Populus trichocarpa,* six in *Solanum lycopersicum,* one in *Drosophila Melanogaster* and two in *Homo sapiens*. In wheat, 36 SINA genes were retrieved from phytozome using the *Triticum aestivum v2.2*. The amino acid alignment showed that *TaSINA* had low sequence homology with the SINA gene family (Supplementary Figure 1). We identified one orthologue to *TaSINA* in brachypodium, bradi1g27970.1, and in rice, LOC_Os01g03170.1, with pairwise identity of 36 % and 27.1 % respectively. The closest orthologues to *TaSINA* were the barley AK367730 and *Aegilops tauschii* XP_020179099 which suggests that *TaSINA* is specific to the Triticeae.

We also observed that *TaSINA* has a modified C3HC4 RING finger domain: the sixth cysteine residue was replaced by an aspartic acid modifying the C3HC4 RING-finger domain to a C3DHC3 type (Supplementary Figure 1). This modified RING finger is shared among the homeologues and the close orthologues in barley, *Aegilops tauschii* and *Triticum urartu* (Supplementary Figure 1).

The phylogenetic analysis of the relationship among the SINA gene family was based on the conserved RING-finger and ZINC-finger domains. The tree included the SINA genes known in plant and animal species and the closest orthologues and paralogues of *TaSINA*. We identified four clades in Figure 6. The first clade contains SINA genes from *Arabidopsis*

thaliana, *Oryza sativa*, *Zea mays*, *Medicago truncatula*, *Populus trichocarpa* and *Solanum lycopersicum.* The second clade contains the first *SINA* gene identified in *Drosophila melanogaster* and *Homo sapiens.* The third clade is composed of *TaSINA* 3B with its homeologues with the closest orthologues in barley and *Aegilops tauschii*. Finally, the 4th clade contains the 11 SINA-like genes that have been identified in Arabidopsis (Figure 6). *TaSINA* gene seemed to be closer to the Arabidopsis SINA-like than the SINA genes known in other plant species (Figure 6).

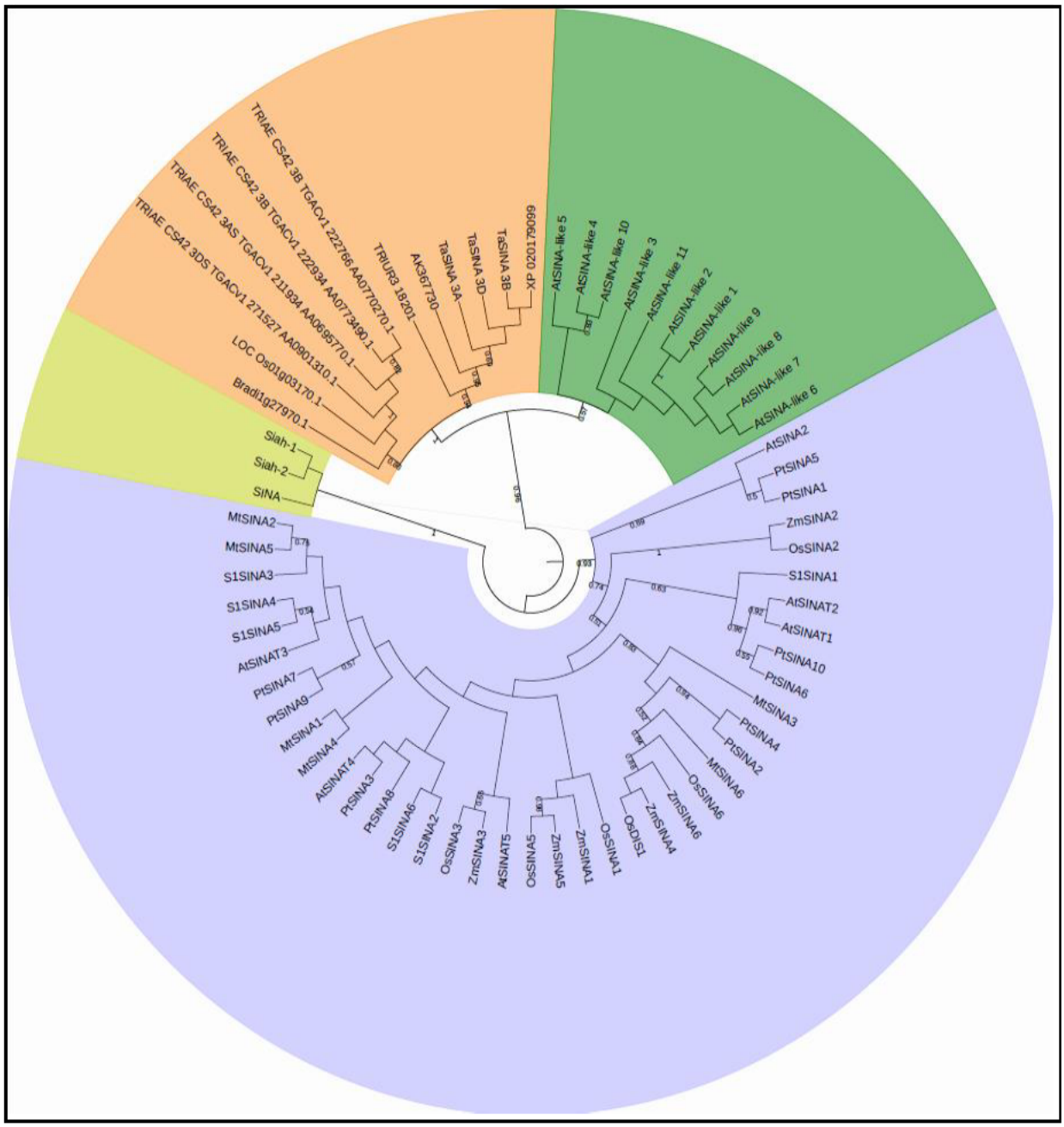


Figure 6. Phylogenetic relationship of *TaSINA* with the previously published *SINA* genes from Arabidopsis (*AtSINA*), Drosophila (*SINA*), human (*Siah*), barley, (*AK*), maize (*ZmSINA*), poplar (*PtSINA*), tomato (*SiSINA*), rice (*OsSINA, LOC*), medicago (*MtSINA*) and closest orthologues in *Triticum urartu* (TRIUR), brachypodium (Bradi), *Triticum aestivum* (TRIAE) and *Aegilops tauschii* (XP). The neighbour joining tree identified four clades: plant *SINA* genes (clade I, purple), animal *SINA* genes (clade II, dark yellow), *TaSINA* (clade III, orange) and Arabidopsis *SINA*-like genes (clade IV, green).

5. Discussion

The QTL, *qYDH.3BL*, associated with an increase of biomass and yield in a hot climate, has been narrowed down to a sequence interval containing eight HC genes in the wheat cv. Chinese Spring reference genome (Chapter II). The expression analysis of these genes revealed a strong candidate gene, *Seven in Absentia TaSINA*, predicted to encode an E3 ubiquitin ligase protein. E3 ubiquitin-ligase proteins are involved in post-translational regulation via degradation of target proteins (Mazzucotelli et al., 2006). E3 ubiquitin ligase proteins form a complex with the E1 ubiquitin-activating enzyme and the E2 ubiquitin-conjugating enzyme and recognize target proteins for degradation.

TaSINA was significantly over-expressed in Kukri and in the HIFs containing the Kukri allele compared to RAC875 allele. The subsequent sequencing of *TaSINA* in RAC875 revealed a SNP between the alleles located in the qPCR primer. The analysis of the raw fluorescence data did not show any difference in PCR efficiency whether the lines carried the RAC875 or the Kukri allele. Nonetheless, results will be re-confirmed.

SINA genes were first identified in Drosophila for their role in the speciation of R7 precursor into R7 photoreceptor cell localized in the eye (Carthew et al., 1990). *SINA* genes have been also reported in plant species with a role in plant development. In Arabidopsis, *SINAT5* negatively regulates lateral roots production through ubiquitin-mediated proteolysis of its target protein, *NAC1* (NAM, ATAF/ ATAF2, and CUC2) (Xie et al., 2002). In *Medicago truncatula*, the ectopic expression of a negative dominant form of the Arabidopsis *SINAT5* reduced root nodulation, but both root and shoot growth were more vigorous in young (in vitro) plants after 20 days and older plants, after eight weeks' growth (Den Herder et al., 2008). *SINAT5* has been also reported as a potential regulator of flowering time in Arabidopsis through direct interaction with *Flowering Locus C* as a target protein for ubiquitination (Park et al., 2007) but also *Long Elongated Hypocotyl*, a circadian oscillator gene (Park et al., 2010). Another Arabidopsis *SINA*, *SINAT2*, is involved in carotenoid biosynthesis via the post-translational regulation of AtRAP2.2 transcription factor (Welsch et al., 2007). *SINAT2* with *SINAT1* act also as negative regulators of autophagy by ubiquitin-mediated proteolysis of AUTOPHAGY PROTEIN 6 (ATG 6) (Qi et al., 2017).

The *SINA* gene family has a role in biotic and abiotic stress tolerance. OsDIS1, a SINA protein, is a negative regulator of drought tolerance in rice (Ning et al., 2011). *SINA2*, a tumor necrosis factor receptor-associated factor (TRAF)-like, increased drought tolerance in Arabidopsis while its mutation reduced drought tolerance (Bao et al., 2013). *SINA* genes have been also associated

with disease resistance in tomato where different copies of *SINA* have opposite effects on defence signalling for hypersensitive response cell death (Wang et al., 2018). In this study, we describe a potential role of *SINA* gene with heat tolerance in wheat. As observed under drought stress in rice (Ning et al., 2011), *SINA* genes in wheat are likely to act as negative regulators of heat tolerance via degradation of target proteins as we observed that *TaSINA* was down-regulated in RAC875 compared to Kukri.

Sequencing of the promoter sequence of RAC875 *TaSINA* revealed a *cis*-acting element, SBOXATRCS (CACCTCCA) also called S box, absent in the promoter of Kukri *TaSINA* (Figure 7). This motif is a putative ABSCISIC ACID INSENSITIVE-4 (ABI4) -binding site which negatively regulates the *Conserved Modular Arrangement 5* genes, *CMA5*, in Arabidopsis in response to sugar and ABA signals (Acevedo-Hernández et al., 2005). A truncated version of *CMA5* lacking the S box element showed that ABI4 acts as a repressor of *CMA5* activity, mediating ABA and sugar signaling. In Arabidopsis, the ABI4 transcription factor regulates nuclear genes expression by retrograde signal from plastids (Koussevitzky et al., 2007). ABA is a phytohormone that has been shown to play a central role in different physiological processes of plant such as seed dormancy, senescence, root architecture and abiotic stress (Sah et al., 2016). ABA has been also shown to mediate gene expression in response to environmental stimuli such as heat (Xue et al., 2015), is a positive regulator of ABA signalling and regulates ABA-dependent responses in heat stress (Huang et al., 2016). Sugar signalling also plays an important role in mediating plant responses to environmental stimuli and mediates gene expression of photosynthesis genes, carbon and nitrogen metabolism and secondary metabolisms (Rolland et al., 2002). We hypothesize that the presence of the S box element, ABI4 binding site, in the promoter sequence of *TaSINA* contributes to the down-regulation of its expression in RAC875 and might affect ABA and sugar signalling. In Kukri, the absence of the motif would prevent the regulation of *TaSINA* and lead to gene expression and consequently, the degradation of its target proteins (Figure 7).

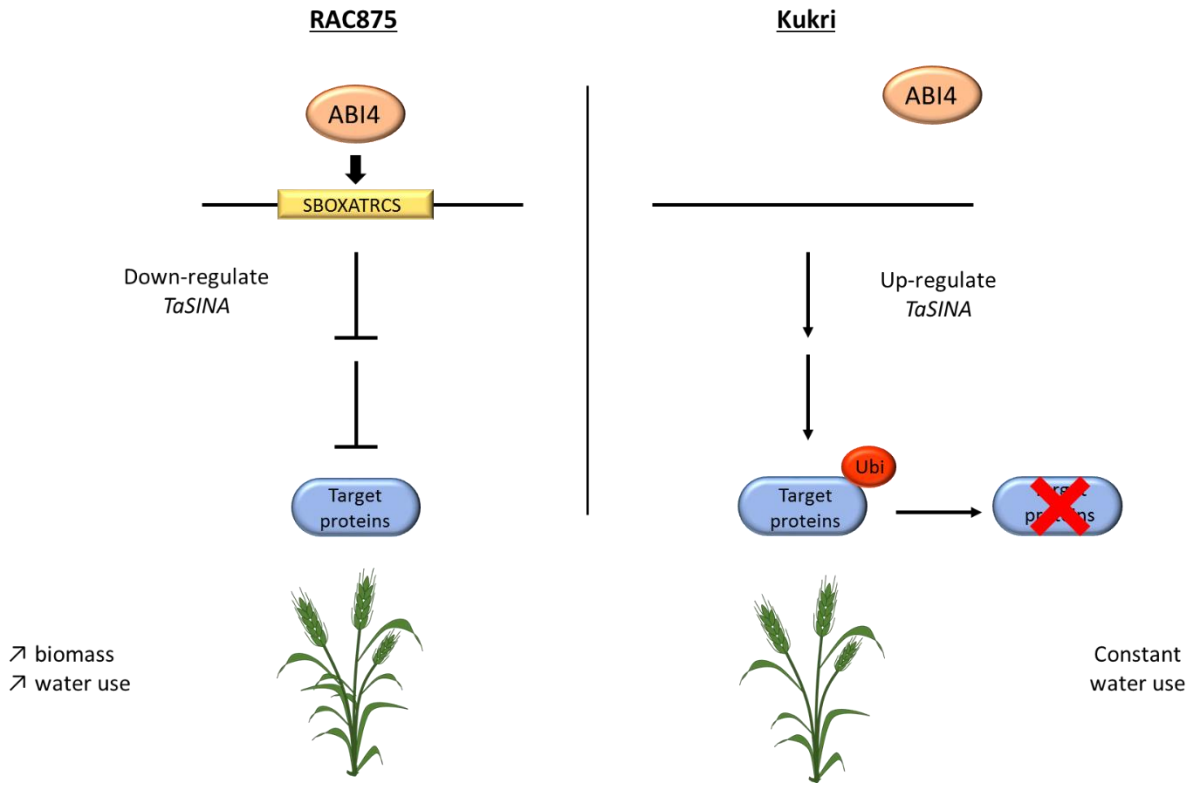


Figure 7. Model of the potential function of TaSINA in RAC875 vs. Kukri.

Genome-wide analysis previously identify several copies of SINA gene family in *Arabidopsis thaliana*, *Populus trichocarpa*, *Oryza sativa*, *Zea mays* and *Physcomitrella patens.* The *SINA* gene family has been also reported in *Medicago truncatula* (D'haeseleer et al., 2011) and more recently in tomato (*Solanum lycopersicum*) (Wang et al., 2018). The gene structure among species indicated a highly conserved gene family in plant species (Wang et al., 2008). SINA gene family is characterised by a conserved N-terminal C3HC4 RING (Really Interesting New Gene) finger domain followed by a C-terminal SINA domain that regulates interactions with target proteins (Hu et al., 1999). We found that *TaSINA* and its closest orthologues in Triticeae have a modified RING-finger domain, C3DHC3 type. The RING type genes in Arabidopsis include two canonical RING domains (C3H2C3 or C3HC4) and a few types of modified RING domains that differ by the positions and number of the cysteine or histidine residues (Stone et al., 2005). The fourth or eighth zinc ligand of the conserved RING domain can be replaced by an aspartic acid without affecting the function (Stone et al., 2005). Both *Triticum urartu* and *Aegilops tauschii*, the ancestors of bread wheat share the same modified RING domain than

TaSINA indicating that the speciation of the *SINA* genes in Triticeae happened a long time ago. This speciation of *SINA* genes could indicate a potential new role of SINA genes in Triticeae.

To confirm the role of *TaSINA* in the increase of biomass and yield in a hot climate we identified new mutations in wheat TILLING collections. TILLING is a high-throughput technique to identify mutations in genes of interest. This method combines inducing hundreds or thousands of mutations chemically or physically and high-throughput screening for mutations in genes of interests (McCallum et al., 2000). TILLING mutants have been already used successfully in wheat to characterise the *MS1* gene, a male fertility gene (Tucker et al., 2017). We used Gladius TILLING population was used here as Gladius shared the same allele at *qYDH.3BL* and the same *TaSINA* sequence as Kukri. A knock-out mutant should mimic RAC875 – Drysdale response. We did not identify any mutation inducing a premature stop codon for either *TaSINA* or its homeologues. However, one missense mutation of *TaSINA 3A* (P96L) and one for the *TaSINA 3B* (R226W) were predicted to affect the protein function based on sequence homology. Both missense mutations were located in the predicted SINA domain, which could potentially affect the binding to its target proteins. A loss of function mutation of *TaSINA* in the Gladius background where the gene is normally expressed will help to functionally characterise *TaSINA* and prove the role of RAC875 *TaSINA* in conferring heat tolerance.

Additional mutations of *TaSINA* were identified in Kronos and Cadenza TILLING collections. Kronos is a tetraploid wheat originating from California and Cadenza is a hexaploid wheat originating from England. The mutants would enable us to test if *qYDH.3BL* can improve heat tolerance in durum wheat and in American and European wheats. Additionally, the database used the old gene models from the IWGSC Chinese Spring chromosome arm survey sequence (IWGSC, 2014). The screening of *TaSINA* mutants will be updated when the database incorporates the new gene models from the latest reference genome, IWGSC Ref 1.0.

In this study, expression analysis identified a strong candidate gene *TaSINA* with a unique feature that seems to be specific to the triticeae as showed by the phylogenetic study. Future experiments will determine if *TaSINA* acts as an E3 ubiquitin ligase protein with an ubiquitination assay. If the ubiquitin ligase function of *TaSINA* is confirmed, the next step will be to find the protein targeted by *TaSINA* to understand its mechanism of action. Taken all together, this study is the first step towards the functional characterisation of *TaSINA* gene in wheat.

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7. Supplementary data

Supplemental Table I. Chromosome specific QPCR primers designed to the study the expression profiles of the high confidence gene.

Primer name	Forward primer 5' → 3'	Reverse primer 5' → 3'
TraesCS3B01G570900	ATCCATACTTCCTCGATGATC	CCAGGAGTAGTACAAGGTCAC
TraesCS3B01G571000	ATTCTTTGGCCCGGTGGA	CGAGTTGGGAAGAGATCGTAA
TraesCS3B01G572500	CTTGACATTAGCCAATCCAAA	GGATGCCGTCGGTGTTGC
TraesCS3B01G572600	CAATGAATATACCATGATGTTGG	GGTAATAGACGCAGTTGGCAT
TraesCS3B01G572700	GTACACTGACATCAAGGGCTT	TGACCTCTCCGGACGCGA
TraesCS3B01G572800	GGATAAGGAGAAGGATGTGCA	CCTCCTTCTCACGACGCTCA
TraesCS3B01G572900	ATCGAGAAGCTCCCGGTT	TTGCCTTGATCTGTCCCT
TraesCS3B01G573000	TCCTTCTGACACTACTCCTCCTG	TTGCCTTGATCTGTCCCT

Supplemental Table II. Primer pairs of the four reference genes used to normalize gene expression.

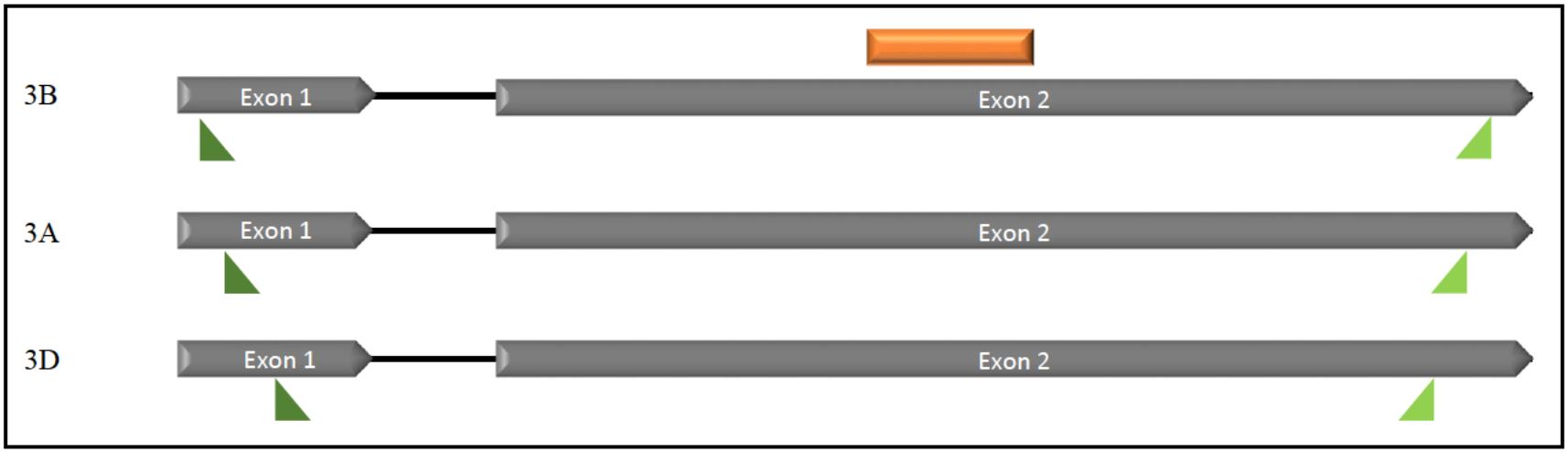
Primer name	Forward primer 5' → 3'	Reverse primer 5' → 3'
TaActin	GACAATGGAACCGGAATGGTC	GTGTGATGCCAGATTTTCTCCAT
*TaCyclophilin 2*2*	CAAGCCGCTGCACTACAAGG	AGGGGACGGTGCAGATGAA
*TaGAPdH 2*2*	TTCAACATCATTCCAAGGAGCA	CGTAACCCAAAATGCCCTTG
*TaEFA2*2*	CAGATTGGCAACGGCTACG	CGGACAGCAAAACGACCAAG

Supplemental Table III. Primer pairs used to Sanger sequence *TaSINA.* The promoter sequence, approximately 5,000 bp before the start of the CDS, and the CDS were Sanger sequenced.

Primer name	Interval	Gene location	Forward primer 5' → 3'	Reverse primer 5' → 3'
ADW611	- 4,742 to - 2,780 bp	promoter	GTTTATATGGTCTAGATGGTTGAAC	ATGATAATATCCATCGAGACAGG
ADW612	- 3,088 to - 1,911 bp	promoter	AAACTCGAATTCCTCCTAT	GTGATGAGCTATCAATGG
ADW613	-2,394 to -293 bp	promoter	CTAAAAGTGAGTCTCTCTGG	TACCATCCACCTAACCTA
ADW664	- 1,052 bp to +273 bp	promoter and CDS	TCAAACCAAAGACGGAACCGAG	CAAGATTCATGCACACCAGATAC
ADW615	+ 42 to + 1,167 bp	CDS	GCCGTGGTGCCCATGGAG	CGTTTCTTGTAGCTCTGATCGGTG

Supplemental Table IV. Chromosome specific primer pair designed to amplify only one homeologue at the time. Primer pairs were tested with nulli-tetrasomic lines to validate their specificity.

Primer pairs	Chromosome	Primer sequences
Adw615	3B	GCCGTGGTGCCCATGGAG
		CGTTTCTTGTAGCTCTGATCGGTG
Adw621	3A	GAAGCCATCCCGATGGCC
		GCCGGGCAAGGAGGAGGA
Adw622	3D	GGACCTGCTCGACTGCCAT
		GAGGAGCAGGGAGAGCCA



Supplemental Figure 1. Schematic representation of gene VII and its homeologues on chromosome 3A and 3D. The dark green triangles are the forward primers designed to identify genome specific mutation; light green triangles are the reverse primers. The orange box highlight the region of the coding sequence of gene VII most likely to contain mutations due to the nature of EMS mutation induced.

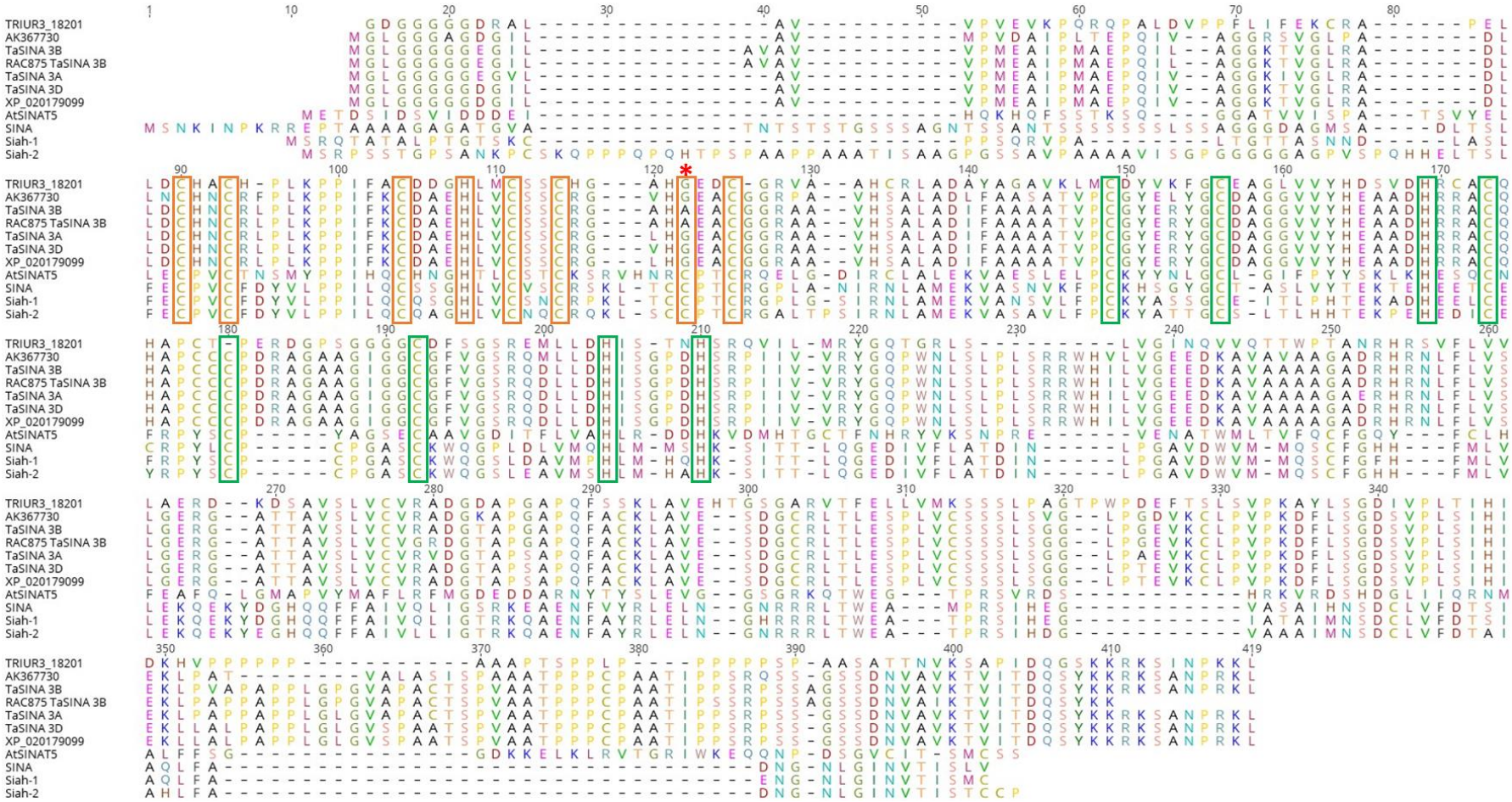
Supplemental Table V. *SINA* gene and genebank accession in Arabidopsis, Drosophila, Human, barley, maize, poplar, tomato, medicago, rice, *Triticum urartu*, *Aegilops tauschii*, brachypodium and *Triticum aestivum*.

Species	Gene name	Accession	Length
Arabidopsis	AtSINAT1	NP_181729.1[a]	305
	AtSINAT2	NP_191363.1[a]	308
	AtSINAT3	NP_567118.1[a]	326
	AtSINAT4	NP194517.1[a]	327
	AtSINAT5	AAM11573[a]	309
	AtSINA2	NP_001319542	243
	AtSINA-like 1	NP_176834	366
	AtSINA-like 2	NP_176835	313
	AtSINA-like 3	NP_176836	303
	AtSINA-like 4	NP_849853	330
	AtSINA-like 5	Q7XA77	328
	AtSINA-like 6	NP_198603	281
	AtSINA-like 7	NP_198605	287
	AtSINA-like 8	Q9FKD6	263
	AtSINA-like 9	NP_198607	277
	AtSINA-like 10	NP_198609	350
	AtSINA-like 11	NP_001331378	314
Drosophila	SINA	XP_001973003.1	314
Human	Siah-1	NP_003022	282
	Siah-2	NP_005058	324
Barley	AK367730	BAJ98933	351
Maize	ZmSINA1	EF434384[a]	300
	ZmSINA2	EF434387[a]	313
	ZmSINA3	EF434383[a]	341
	ZmSINA4	EF434385[a]	302
	ZmSINA5	EF434382[a]	349
	ZmSINA6	EF434386[a]	302
Poplar	PtSINA1	XP_002299139[a]	286
	PtSINA2	XP_002299212[a]	296
	PtSINA3	XP_002302632[a]	330

	PtSINA4	XP_002303919[a]	305
	PtSINA5	XP_002303919[a]	311
	PtSINA6	XP_002308357[a]	307
	PtSINA7	XP_002318423[a]	332
	PtSINA8	XP_002320867[a]	330
	PtSINA9	XP_002321406[a]	332
	PtSINA10	XP_002323331.2[a]	304
Tomato	SiSINA1	AK324518[b]	308
	SiSINA2	BT013026[b]	333
	SiSINA3	AK322153[b]	331
	SiSINA4	AK320390[b]	318
	SiSINA5	AK321160[b]	313
	SiSINA6	XM_004248034[b]	316
Medicago	MtSINA1	EU189945[c]	333
	MtSINA2	EU189946[c]	323
	MtSINA3	EU189947[c]	304
	MtSINA4	EU189948[c]	324
	MtSINA5	EU189949[c]	309
	MtSINA6	EU189950[c]	304
Rice	OsSINA1	XP_015640386[a]	347
	OsSINA2	XP_015623254[a]	308
	OsSINA3	XP_015626757[a]	349
	OsDIS1	AK058336[a]	301
	OsSINA5	XP_015640384[a]	361
	OsSINA6	XP_015644782[a]	302
	LOC_Os01g031701.1	BAD52674	652
Triticum urartu	TRIUR3_18201	-	337
Aegilops tauschii	XP_020179099	XP_020179099	359
Brachypodium	Bradi1g27970.1	XP_003560221	315
Triticum aestivum	TRIAE_CS42_3AS_TGACv1_211934_AA0695770.1	-	353
	TRIAE_CS42_3B_TGACv1_222766_AA0770270.1	-	357
	TRIAE_CS42_3B_TGACv1_222934_AA0773490	-	357

Triticum aestivum	TRIAE_CS42_3DS_TGACv1_271527_AA0901310.1	-	353
	TAsINA 3A	-	360
	TaSINA 3B	-	362
	TaSINA 3D	-	360

a. Wang et al., 2008
b. Wang et al., 2018
c. Den Herder et al., 2018



Supplemental Figure 1. Amino acid alignment of *TaSINA* gene and its homeologues with SINA genes from Arabidopsis (AtSINA), Drosophila, human (Siah), barley (AK), *Triticum urartu* (TRIUR) and *Aegilops tauschii* (XP). The conserved domains were identified and highlighted: the orange rectangles indicate the position of the conserved amino acid in RING finger domain; the green rectangles indicate the position of the zing-finger domain; the red star indicates the residue modified in the RING domain of *TaSINA.*

Chapter V

Chapter V: Perspectives and conclusion

Closing yield gaps through the breeding of high-yielding cultivars for hot climates is one strategy advanced to improve wheat production. Our project was designed with this mission in mind. Specifically we aimed to identify the genetic factors associated with a yield QTL located on the chromosome arm 3BL of wheat. The QTL was associated with the maintenance of yield under heat stress. This project led to the identification of a candidate gene, *TaSINA*, predicted to encode for an E3 ubiquitin-protein ligase. We proposed a model to explain the mechanism of stress tolerance associated with *qYDH.3BL* in Chapter IV and initiated the functional characterisation of this candidate gene through the identification of loss-of-function mutants by TILLING population screening. The identification of *TaSINA* is a step forward toward the cloning of the first gene controlling yield under hot environments and the breeding of heat tolerant cultivars. This work was assisted by the release of a wheat reference genome sequence and assembly. In this chapter, I discuss the opportunities that a reference sequence offer for wheat and comment on the benefits of positional cloning.

1. A wheat reference sequence: impact on wheat genomics

The release of a near-complete wheat reference sequence of cv. Chinese Spring (CS), RefSeq v 1.0, along with its annotation, has largely contributed to the identification of *TaSINA.* A reference sequence for wheat was hotly anticipated by the wheat community to unlock the genetics of this complex genome. The sequencing of the wheat genome through the IWGSC has been achieved after more than 10 years of research and the joint efforts of international research teams. Today two high-quality reference sequences are available for the wheat genome cv. CS (IWGSC, 2014; Zimin et al., 2017). The development of next-generation sequencing (NGS) technology has dramatically decreased sequencing cost and contributed to the re-sequencing of genomes as observed for human. A first draft sequence of the human genome was published in 2001, using a whole-genome shotgun sequencing approach based on the Sanger sequencing method (Venter et al., 2001). At that time, the estimated cost for sequencing a human genome was about $100 million (Wetterstrand, 2018). Sequencing costs have dramatically decreased with the introduction of NGS; the cost of re-sequencing a human

genome was about $1,000 in July 2017 and achieved in eight days. In wheat, the 454 pyrosequencer that increased by 100 fold the number of bases sequenced compared to Sanger sequencing, allowing the sequencing of 25 million bases in a four-hour run (Margulies et al., 2005), contributed to the whole genome sequencing (WGS) of CS at a coverage of 5X (Brenchley et al., 2012). The first reference draft was generated by Illumina shot gun sequencing of isolated chromosome arms and unrevealed most genic content of the wheat genome (IWGSC, 2014). The release of the IWGSC Ref 1.0 has been possible with the development of a new assembler, DeNovoMAGIC™ (www.nrgene.com) that allows *de novo* assembly of Illumina short reads. The development of "third generation" technologies including PacBio, enables the sequencing in real time of long molecules of DNA and can generate long reads up to several kbp that greatly assists correct assembly (Eid et al., 2009). This technology was used in combination to short Illumina reads to generate a near-complete wheat reference sequence of CS (Zimin et al., 2017). The improvement of sequencing technologies over time should contribute to improve sequencing accuracy and continue to reduce the costs. Moreover, along with the development of powerful assemblers, it should decrease the time required for the re-sequencing and assembly of wheat genomes and accelerate the re-sequencing of a large number of varieties to identify the diversity of the wheat genetic pool. It remains that the wheat genome accounts for a large number of repetitive regions and a high level of copy variation along the genome.

The availability of a high-quality reference genome will accelerate the discovery of genes through positional cloning as observed in other species and contribute to the characterisation of important biosynthesis pathways and genome-wide analysis of gene families. This project is a good example of the benefits of having a reference genome sequence. The 3B QTL was initially identified 10 years ago and after three PhD thesis, we are now able to pinpoint it at the gene level and have identified a strong candidate gene. However, this gene is still to be functionally characterized and lot of works needs to be done to validate its role in improving heat tolerance. The deployment of genes in breeding programs is a long process, which can take up to 10 years from the initial cross to the release of varieties. This means that from the identification of a QTL to the release of a variety, the whole process can take up to 20 years. The availability of a reference sequence should greatly reduce the pre-breeding time from the identification of QTL to the cloning of the underlying genes.

One of the strategy that should contribute to the identification of gene variants underlying important traits is the development of pan-genomes. A pan-genome aims to identify the structural and genetic variations in a species, or group of closely related species that cannot be captured with only one genome sequence (www.wheatinitiative.org). In 2016, the 10+ Wheat Genomes project was created as an associated programme of the Wheat Initiative. The aims of the 10+ Wheat Genomes is to sequence, assemble and annotate at least 10 wheat genomes (www.10wheatgenomes.com). Another wheat pan-genome project has been developed with the aim to re-sequence the eight founders of a multi-parent advanced generation intercrossed (MAGIC) population in winter wheat (Mackay et al., 2014) along with two international CIMMYT lines (http://gtr.ukri.org). Their objectives are to create high quality sequences and annotations for each cultivar, but also to survey sequence 94 accessions of a diversity panel and catalogue sequence variants for genetic analysis.

A pan-genome has been already published in wheat (Montenegro et al., 2017). It was based on an improved reference of the IWGSC CS chromosome survey sequence, with a larger assembly size and reduction of duplicated regions. The improved reference was then used to study the diversity in 18 cultivars using the WGS data of 16 Australian cultivars (Edwards et al., 2012) and the WGS data publicly available for the synthetic wheat W7984 and OpataM85. The study of this pan-genome predicted 128 656 genes: 64.3 % were the core genome, present in all the cultivars, and the remaining 35.7 % varied in their presence from one genome to another. Moreover, 12,150 genes were absent in Chinese Spring and present in all the other genotypes which proved that with only one reference genome we cannot identify all the genetic pool of wheat. The development of pan-genome through the re-sequencing of varieties will largely contribute to gene discovery that is essential for the breeding of high-yielding cultivars and increase the wheat improvement rate.

2. Towards the breeding of high yielding cultivars under heat stress

This study aimed to identify genes underlying a QTL associated with high biomass and yield under heat stress in a bi-parental population. Bi-parental populations have been commonly used in genetic studies to identify genomic regions associated with important agronomic traits (Tricker et al., 2018). They are generated from the cross of two genotypes contrasting in their phenotype for a trait of interest. The use of bi-parental populations for genetic mapping has contributed to the identification of a large number of QTL; it was estimated that approximately 1,200 QTL and approximately 10,000 marker-trait associations have been identified in 12 crop

plants including maize, rice, barley and wheat (Bernardo, 2008). However, bi-parental populations are limited in allelic diversity; they contain only two allelic versions of a gene in the population, reducing the phenotypic diversity that can be detected. They are also limited in the recombination frequency. Multi-parent populations have been developed to overcome these limitations. MAGIC populations have been developed for spring wheat from the cross of four Australian elite cultivars (Huang et al., 2012) but also for winter wheat through the cross of eight winter wheat cultivars (Mackay et al., 2014). MAGIC populations are created from multiple generation of intercrossing among the founders of the population. Another type of multi-parent population is a nested-association mapping (NAMs) population. NAM populations are developed from a panel of founders crossed with recurrent parents to develop related subpopulations (Yu et al., 2008). A wheat NAM population is being developed by the University of Adelaide by combining 75 wheat accessions selected from a worldwide diversity panel and crossed with two Australian cultivars selected as recurrent parents based on their improved tolerance to drought and heat and their nitrogen use efficiency (www.wheathub.com.au). Between 100 – 150 subpopulations, each containing ~100 individuals are being generated, representing in total a population of more than 10,000 individuals. These multi-parent populations present some advantages over bi-parental populations including an increased recombination frequency that benefits the accuracy of QTL position and an increased number of allelic variants at a same locus, increasing the phenotyping diversity that can be evaluated (Gardner et al., 2016). These multi-parent populations enable to detect the position and effect of QTL more accurately and contribute to the deployment of exotic alleles in breeding programs. In maize, the development of NAM populations has successfully contributed to the identification of key genes, including genes controlling leaf size and angle, determinant for the global plant architecture (Tian et al., 2011). The NAM population was developed from the cross of 25 diverse lines with one reference line with generated approximately 5,000 RILs (McMullen et al., 2009). The high-throughput phenotyping of the of 50,000 plots across 9 environments enabled the detection of 30-36 QTLs for each leaf related traits studied which controlled 74.8 - 80.35 % of phenotypic variation (Tian et al., 2011).

Even though many QTL associated with yield under drought and heat have been identified in wheat, none has been deployed in breeding program through MAS. MAS for quantitative traits is limited by the fact that QTL are in general of small effects and are affected by G x E. Therefore, breeders are limited by the number of loci they can select for, as the more loci you are selecting for, the more lines are needed to find the right combination of alleles. Since then,

new breeding tools have been implemented in wheat breeding including genomic selection (GS). GS uses genome-wide markers to predict the genomic estimated breeding value (GEBV) of individuals (Meuwissen et al., 2001). GS was initially used in animal breeding where it has proven its efficacy in predicting breeding value of animals (Bassi et al., 2015). GS is based on the high-throughput genotyping and phenotyping of a training population to predict the GEBV of a related breeding population (Meuwissen et al., 2001). Close markers are combined into haplotypes and the phenotypic variation explained by each haplotype is estimated. GS is a promising tool for selecting cultivars with high GEBV and ultimately breed high-yielding cultivars. It remains that this technique has some limitations including the size of the training population that affect recombination frequency and linkage disequilibrium (Bassi et al., 2015). GS requires a high-density of genetic markers to increase accuracy and increase the linkage disequilibrium between QTL and markers. If QTL and markers are not closely linked, small-effect QTL may be missed. Additionally, one can only recombine what is present in a breeding program; when all the main haplotype blocks would have been recombined by GS, novel alleles are still needed to breed future varieties. Linkage drag is also a limitation to GS. Moreover, when studying complex trait such as grain yield, GS can also be limited by a low genetic correlation of the trait across different environments, especially under rainfed/heat-affected conditions (Bassi et al., 2015). In maize, the study of eight tropical populations across four drought stress environments showed an average gain of 0.086 $Mg.ha^{-1}$ per cycle of GS (Beyene et al., 2015).That is why the mapping of QTL followed by positional cloning to find genes and their function is still important. New sequences variants can be found in mutant collections or created by genome editing technology when the gene is known.

The deployment of high-throughput phenotyping technologies is also increasingly relevant to breeding. As abiotic stress tolerance results from complex interaction of multiple genes and are highly dependent on the environment, phenotyping is quite complex and require new, non-destructive tools to help the identification of genetic factors associated with abiotic stress tolerance. Phenotyping platforms combining fully automated facilities in glasshouses (e.g. DroughtSpotter®, Phenospex, Heerlen, The Netherlands) and high-throughput imaging have now been developed (rewieved by Araus & Cairns, 2014). These technologies enable to capture novel, dynamic traits such as plant growth and transpiration rate, which gives a novel understanding of plant functions. Those methods have successfully been used to identify regions of the wheat genome controlling plant growth and transpiration and associated with yield under drought stress (Parent et al., 2015). High-throughput phenotyping technologies have

also been developed for field phenotyping using remote sensing technologies such as multispectral cameras or near-infrared-image capture attached to unmanned aerial vehicle (rewieved by Araus & Cairns, 2014). Field phenotyping is important to evaluate the real crop performance in field conditions in comparison to controlled environments that enable to isolate single stress effects. The development of high-throughput, cost-effective platforms enables to phenotype large populations such as NAM and also breeding programmes.

The identification of a gene, *TaSINA*, for a QTL controlling yield under hot climate is an important contribution to the wheat genetics. It is a step towards the cloning of heat tolerance genes and elucidate the molecular basis of grain yield. The release of a near-complete reference sequence of the wheat genome combined with the advance in sequencing and assembly technologies should increase the number of re-sequenced accessions and accelerate the characterisation of the wheat gene pool. Translating this information into a breeding program would contribute to breed more efficiently and accurately varieties adapted to target-environments.

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