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ORIGINAL PAPER

Identification of novel quantitative trait loci for days to ear emergence and flag leaf glaucousness in a bread wheat (*Triticum aestivum* L.) population adapted to southern Australian conditions

Dion Bennett · Ali Izanloo · James Edwards · Haydn Kuchel · Ken Chalmers · Mark Tester · Matthew Reynolds · Thorsten Schnurbusch · Peter Langridge

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Abstract In southern Australia, where the climate is predominantly Mediterranean, achieving the correct flowering time in bread wheat minimizes the impact of in-season cyclical and terminal drought. Flag leaf glaucousness has been hypothesized as an important component of drought tolerance but its value and genetic basis in locally adapted germplasm is unknown. From a cross between Kukri and RAC875, a doubled-haploid (DH) population was developed. A genetic linkage map consisting of 456 DArT and SSR markers was used to detect QTL affecting time to ear emergence and Zadoks growth score in seven field experiments. While ear emergence time was similar between the parents, there was significant transgressive segregation in the population. This was the result of segregation for the previously characterized *Ppd-D1a* and *Ppd-B1* photoperiod responsive alleles. QTL of smaller effect were also detected on chromosomes 1A, 4A, 4B, 5A, 5B, 7A and 7B. A novel QTL for flag leaf glaucousness of large, repeatable effect was detected in six field experiments, on chromosome 3A (*QW.aww-3A*) and accounted for up to 52 percent of genetic variance for this trait. *QW.aww-3A* was validated under glasshouse conditions in a recombinant inbred line population from the same cross. The genetic basis of time to ear emergence in this population will aid breeders' understanding of phenological adaptation to the local environment. Novel loci identified for flag leaf glaucousness and the wide phenotypic variation within the DH population offers considerable scope to investigate the impact and value of this trait for bread wheat production in southern Australia.

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D. Bennett and A. Izanloo contributed equally to this work.

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D. Bennett · A. Izanloo · J. Edwards · K. Chalmers · M. Tester · T. Schnurbusch · P. Langridge
Australian Centre for Plant Functional Genomics, Waite Campus, University of Adelaide, PMB1, Glen Osmond, SA 5064, Australia

D. Bennett (✉) · J. Edwards · H. Kuchel
Australian Grain Technologies, Perkins building, Roseworthy Campus, Roseworthy, SA, Australia
e-mail: dion.bennett@ausgraintech.com

Present Address:
A. Izanloo
Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Birjand, Birjand, Iran

M. Reynolds
International Maize and Wheat Improvement Center (CIMMYT), Int. AP 6-641, 06600 México, D.F., Mexico

Present Address:
T. Schnurbusch
Leibniz-Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstr. 3, 06466 Gatersleben, Germany

Introduction

In Australia, bread wheat (*Triticum aestivum* L.) has the highest production of all cereal grains and contributes significantly to Australia's world trade. However, the wheat growing areas are largely rain-fed and water availability is a major limitation on production. The southern Australian or Mediterranean-type climate is characterized by cyclical (intermittent periods of water stress occurring pre- and/or post-anthesis) and terminal drought, in combination with

increasing temperatures, which all limit production in these areas.

In this environment, flowering time must be early enough to minimize the detrimental effects of declining soil moisture and increasing temperatures late in the season, but must be also late enough to avoid reproductive frost damage. The correct flowering time has been particularly important for yield improvement in water-limited environments (Loss and Siddique 1994; Richards 1991). Flowering time is a highly heritable trait in wheat and is determined predominantly by three well-characterized groups of loci: vernalization requirement (*Vrn*), photoperiodic response (*Ppd*) and earliness per se (*Eps*) (Bullrich et al. 2002; Shindo et al. 2002, 2003; Snape et al. 2001a; Worland 1996; Worland et al. 1998).

The *VRN* genes regulate the requirement of a long exposure to cold temperatures to induce flowering, with *VRN 1* and *VRN 2* being the central genes in the vernalization pathway in wheat and barley (Fu et al. 2005; Stelmakh 1993; Trevaskis et al. 2006). *VRN1* is dominant for spring growth habit, whereas *VRN2* is dominant for winter growth habit (Yan et al. 2003, 2004b). The *VRN1* genes are located on the group 5 chromosomes in wheat (*Vrn-A1*, *Vrn-B1* and *Vrn-D1* on chromosome 5A, 5B and 5D, respectively) and most of the variation in vernalization requirement is controlled by recessive alleles at these loci (Law and Worland 1997; Snape et al. 2001b; Yan et al. 2004a). The *VRN2* gene is more than 50 centiMorgans (cM) distal to *Vrn-B1* on chromosome 5B and these two genes show strong epistatic interactions. They are part of the same regulatory pathway (Trevaskis et al. 2007; Yan et al. 2006) although *VRN1* can still be functional when a non functional allele of *VRN2* is present (Stelmakh 1993; Tranquilli and Dubcovsky 2000). *VRN3* (*VRN-B3* or *TaFT*), has been mapped on the short arm of chromosome 7B in wheat (Kuchel et al. 2006) and appears to have an epistatic interaction with other *VRN* genes, while also acting independently when only the vernalization sensitive allele is present (Yan et al. 2006).

Wheat is a long-day plant and this is controlled by the dominant *PPD* genes which greatly reduce sensitivity to photoperiod and confer an early flowering phenotype under short-day and long-day conditions (Worland et al. 1994). Photoperiod sensitivity is controlled primarily by a homoeologous series of genes which are *Ppd-D1, Ppd-B1*, and *Ppd-A1* located on chromosome 2D, 2B and 2A, respectively, and ranked *Ppd-D1* > *Ppd-B1* > *Ppd-A1* in terms of their potency (Beales et al. 2007; Worland et al. 1998). In addition, QTL of minor effect that respond to daylength have been identified on chromosomes 1A, 7A and 7B (Kuchel et al. 2006).

Earliness per se is an adaptive trait that promotes flowering independent of vernalization and photoperiod response (Worland 1996). The presence of earliness per se loci has been reported on several chromosomes of wheat (Hanocq et al. 2007; 2004; Shindo et al. 2003). By Quantitative Trait Locus (QTL) analysis, many studies have revealed that all chromosome groups are involved in the genetic control of earliness per se in bread wheat and not just the major genes of the *PPD* and *VRN* pathways (Chen et al. 2010; Griffiths et al. 2009; Hanocq et al. 2007; 2004; Kulwal et al. 2003; Law et al. 1998; Snape et al. 2001a; Toth et al. 2003).

Leaf waxiness or glaucousness has been reported to protect plants against high radiation, reducing canopy temperatures, increasing water use efficiency, and improving yield in barley and wheat in certain environments (Gonzalez and Ayerbe 2010; King and von Wettstein-Knowles 2000; Richards 1984). However, there is evidence that in some environments, leaf waxiness can have a negative effect on yield (Simmonds et al. 2008) and biomass production (Merah et al. 2000). Merah et al. (2000) also established that genotypes with greater glaucousness had reduced transpiration efficiency, so the precize characteristic conferred by the glaucousness trait remains uncertain.

Inheritance studies for glaucousness have demonstrated that the expression of the waxiness gene (*W1*) is dominant over non-waxy, but is inhibited by the epistatic influence of the dominant inhibitor of the waxiness gene (*Iw1*). These genes were located on the short arm of chromosome 2B (Driscoll 1966; Tsunewaki and Ebana 1999) and additional waxiness and inhibitor genes (*W2* and *Iw2*) are located on the homoeologous chromosome arm 2DS (Liu et al. 2007; Tsunewaki and Ebana 1999; Watanabe et al. 2005). Mason et al. (2010) identified a single QTL for flag leaf glaucousness on chromosome 5A, with the additive allele coming from a heat tolerant parent. Additional genetic studies by Borner et al. (2002) and Kulwal et al. (2003) have identified numerous QTL of minor effect in the ITMI Recombinant Inbred Line (RIL) population, on chromosomes 1A, 1D, 2DL, 4A, 4B, 6A, 7A and 7D, indicating complex genetic control. A number of these loci mapped independent of flowering loci.

To date, numerous studies have identified the impact of vernalization and photoperiod response on phenological adaptation in southern Australia but, with the exception of Eagles et al. (2009) and Kuchel et al. (2006), relatively few studies have been published investigating the underlying genetic basis. Furthermore, the genetic analysis of variation for flag leaf glaucousness has revealed numerous loci influencing this trait, indicating complex genetic control, and the extent of deployment of these loci within locally adapted germplasm is unknown. The development of appropriate populations would aid plant breeders by improving their understanding of the genetic basis of these important traits with consequent benefits for bread wheat production.

The main objectives of this study were to (I) provide a framework genetic linkage map for a doubled haploid (DH) population developed from a cross between RAC875 (female) and Kukri (male)and (II) identify QTL for days to ear emergence and flag leaf glaucousness under southern Australian conditions.

Materials and methods

Plant materials, DNA extraction and marker screening

A DH population comprising 368 individuals from a cross between Kukri (male) and RAC875 (female) were used to construct a genetic linkage map. Table 1 summarizes some of the key physiological differences between the two parents (from field data and Izanloo et al. (2008), who physiologically described and characterized the parents in more detail). The strategy for the development of the populations is described in more detail by Fleury et al. (2010).

DNA extraction was performed using a DNA midi-prep method outlined in Rogowsky et al. (1991) with modifications as described by Pallotta et al. (2000). Multiplex-ready marker technology (MRT) was used for polymorphism screening and also genotyping the mapping population. Multiplex-ready PCR assays and post-PCR pooling of multiplexed assays were performed as described by Hayden et al. (2008).

To find polymorphic markers in the population, an experiment was designed using Automated Designer for Marker Screening which was developed by Dr. M. Hayden, University of Adelaide. A set of 850 SSR markers was selected using the Multiplex-Ready Marker database and Multiplex-Ready CMAP Interface. Marker panels comprising SSRs with non-overlapping allele sizes were created for the selected markers using the BINNER software (Hayden et al. 2008). Initial screening for polymorphisms within the population was conducted on the parental lines and a DNA bulk of six randomly sampled DH lines. The PPD-D1 marker was screened following the protocol of Beales et al. (2007). To prepare PCR products for DNA fragment analyser, the post-PCR protocol of Hayden et al. (2008) was followed. Diversity Array Technology® (DArT) marker assays were performed by Triticarte Pty Ltd (Australia). The DArT protocol for wheat is described by Akbari et al. (2006) and analysis was performed on Triticarte's wheat array version 2.3. DArT markers consist of the prefix "wPt", followed by numbers corresponding to a particular clone in the genomic representation, where w stands for wheat, P for primary restriction enzyme used (*Pst*I) and t for secondary restriction enzyme (*Taq*I).

Constructing a genetic linkage map of the DH population

Map Manager version QTXb20 (Manly et al. 2001) was used in linkage analysis of the markers. The Kosambi mapping function was used to calculate distances derived from recombination values (Kosambi 1944). Linkage groups were established by considering all estimates of recombination frequencies. A LOD-score above 3 was used as critical value. Where two markers are significantly linked (by LOD value) they were considered to belong to the same linkage group. For each segregating marker, a chi-squared (χ^2) analysis ($P < 0.01$) with 1 d.f. was performed to test for deviation from the 1:1 expected segregation ratio, with markers showing significant segregation distortion removed from linkage groups where necessary. For linkage analysis in RILs, the Haldane mapping function (Haldane 1919) was used to estimate distances between markers.

CMAP was used to compare published maps and identify errors (marker order and chromosome orientation). Linkage groups were assigned to a chromosome when it contained SSR and DArT loci previously on published genetic maps assigned to a particular chromosome (http://www.genica.net.au/cmap/crcmpb-live and http://www.triticarte.com.au/content/wheat_diver-sity_analysis.html). Linkage blocks from the same chromosome that were statistically unlinked were forced into one linkage group and oriented relative to each other according to the consensus map for SSR markers

Table 1 Summary of some key developmental and physiological differences between Kukri and RAC875 (from Izanloo et al. 2008), the parents of the DH population. Additional agronomic measurements and observations from a field experiment (RAC07)

	Growth room measurements, under cyclical drought treatment							Field observations		
	Ear emergence days after planting	Leaf rolling (1–5)	Osmotic adjustment flag leave (Mpa)	Chlorophyll content (SPAD)	Grain size (TGW, g)	Tiller number (plant^{-1})	Tiller abortion (%)	Auricle colour	Stripe rust reaction	WSC (g/m^2)
Kukri	73	3.0*	0.097*	54.0*	35.3*	6.2*	42.6*	Purple	Resistant	107.0*
RAC875	75	1.5	0.364	61.7	46.6	4.2	29.2	White	Susceptible	135.0

Traits marked with a (*) indicates the difference was significant at $P < 0.05$

WSC water soluble carbohydrates, *TGW* thousand grain weight

(Somers et al. 2004). The order of markers in the linkage groups was also checked with RECORD (van Os et al. 2005). Visual inspection of graphical genotypes identified singletons and any other errors in marker segregation data. Any identified singletons were replaced by missing values as suggested by Van Os et al. (2005). The corrected data were again ordered for a second time with RECORD and double-crossover events calculated for each linkage group. Where an excess of five double crossover events was identified, the segregation data were re-inspected and, if necessary, corrected. The final map was drawn using the MapChart program, v. 2.1 (Voorrips 2002).

Genotyping the RIL population

RILs (F_2 derived F_4:F_5; 380 lines in total) were randomly sampled from 2,976 RILs that were generated by single-seed-descent from a cross between Kukri and RAC875. These were used for genotyping as well as phenotyping for leaf waxiness. To identify additional markers on chromosome 3A in the region of interest, which had not previously been assessed for polymorphism in the population, DNA samples from the two parents (Kukri and RAC875) and two bulks of DNA from DH lines contrasting in leaf waxiness were used.

Field experiments

For all experiments, the design was a two-replicate randomized complete block. Parental lines and up to 10 check varieties with a broad range of drought tolerance (data unpublished) were included and in the CIMMYT experiments, parents and two local varieties were used as checks. The RACMET trial used data from 2006, 2007 and 2008 experiments (Table 2). Sites in 2006 were selected to represent three subtly different environments in South Australia (location and key climatic variables are summarized in Table 3). Roseworthy Agricultural College (RAC) is a relatively high rainfall and reliable site, while Booleroo (BOL06) was selected as a site with a relatively cool winter and hot spring, with low rainfall. Minnipa (MIN06) and Booleroo are representative of a large proportion of the South Australian grain belt, frequently experiencing strong cyclical and terminal droughts. In 2006, all sites experienced severe drought, with in-season rainfalls between 29 and 71 percent of the long term average (Table 2).

In the CIMMYT fully irrigated (CIMI07) experiment, four irrigation applications were applied: at germination, 42, 78 and 130 days after germination. In the CIMMYT managed drought (CIMD07) experiment, the crop was drip-irrigated and received a total of 153 mm of water over three applications: at germination, 28 and 40 days after germination. The CIMI07 trial was conducted to establish the yield potential of the population with no water limitation and the CIMD07 trial was used to obtain further data of performance under water limitation, with these field conditions previously characterized as a successful selection tool for improving drought tolerance (Kirigwi et al. 2004).

Trait evaluation

Ear emergence time and anthesis date were recorded as the number of calendar days after sowing when 50% of the

Table 2 Location of experimental sites, elevation, long term average growing season rainfall, actual growing season rainfall and average vegetative and reproductive minimum and maximum temperatures

Site	Abbreviation	Year	Longitude	Latitude	Site		Trial		
					Elevation (m above sea level)	Long term May–October rainfall (mm)	May–October rainfall (mm)	Vegetative[a] min/max (°C)	Reproductive[a] min/max (°C)
Booleroo	BOL06	2006	32.88 S	138.35 E	405	232.9	86	0.7/15.0	6.9/25.1
Minnipa	MIN06	2006	32.84 S	135.15 E	168	238.1	68	5.6/15.7	9.5/24.0
Roseworthy	RACMET[b]	2006	34.53 S	138.69 E	68	335.8	130.8	4.1/15.6	5.4/22.4
		2007					228.8	5.1/15.1	6.7/21.6
		2008					239.8	7.1/16.7	5.8/19.9
CIMMYT-Irrigated	CIMI07	2006/7	27.25 N	109.54 W	38	500[c]	[c]	6.5/23.7	9.7/29.2
CIMMYT-Drought	CIMD07	2006/7	27.25 N	109.54 W	38	150[c]	[c]	7.2/23.7	8.2/28.4

Source: Australian Bureau of Meteorology

[a] Ear emergence estimated as being at 1200 degree days after sowing on average

[b] Three seasons treated as a multi environment trial for phenotypic data

[c] Trials fully irrigated

Table 3 Summary of the marker coverage over the three bread wheat genomes in terms of average markers per chromosome, average genetic distance of each chromosome (in cM) and average interval between markers (cM)

Genome	Average markers	Average cM	Average interval
A	25.00	187.99	8.74
B	26.71	164.20	6.43
D	13.43	129.59	10.18
All	21.71	160.59	8.45
Total	456	3372.4	

spikes or anthers had emerged from the boot or spike, respectively. Maturity differences were also scored in the Australian experiments (excluding RAC08) by recording plant growth stages following the Zadoks scale (Zadoks et al. 1974), at a single time point when the trial was visually assessed to be at an average growth stage of anthesis.

Leaf waxiness was assessed visually using a 1–6 scale in the 2006 Australian experiments and expanded to a 1–9 score in 2007 and 2008 for the CIMMYT and Australian experiments. A score of 1 occurred when no wax was observed on the abaxial surface of the flag leaf and 6 (or 9 for 2007 and 2008) occurred when wax was visible on 100% of the abaxial and adaxial surface of the flag leaf (Fig. 1). In the CIMI07 trial, leaf waxiness was scored on flag leaves at the trial average growth stages of booting, ear emergence and anthesis, with the largest of the three scores assigned to a line used for mapping.

The evaluation of RILs for leaf waxiness was conducted from the stage of stem elongation in the glasshouse. Excess tillers were removed, maintaining the main stem for each line. Leaf waxiness was scored on the flag leaf sheath and blade twice throughout the experiment, at ear emergence and anthesis for each individual line, on the 1–9 scale.

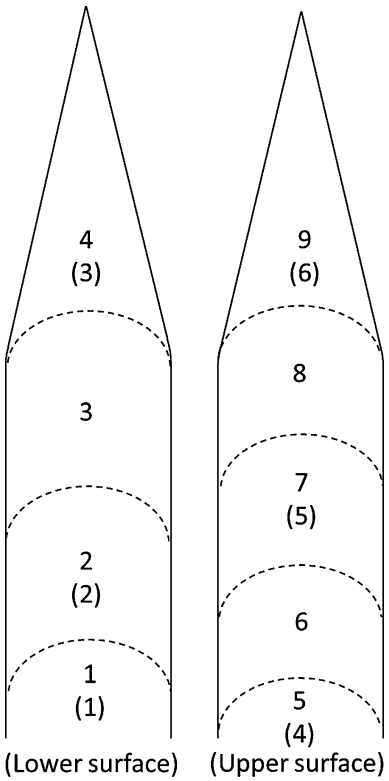


Fig. 1 Approximate flag leaf area with visual glaucousness and the appropriate corresponding score. 2006 score included in parentheses for comparison

Statistical analysis

Spatial methods developed by Gilmour et al. (1997) were followed to minimize or remove spatial effects of field variation. For each trait in each environment, mixed linear model analysis using the method of residual maximum likelihood (REML) was performed in GenStat release 8.2 (Lawes Agricultural Trust, 2005). Genotype was firstly fitted as a random effect, to assess the proportion of variance accounted for by genotype and to subsequently calculate broad sense heritability. Then with Genotype as a fixed effect, the data was reanalysed to produce the best linear unbiased estimates (BLUEs), which were used for QTL mapping. With a high genetic correlation and detecting the same QTL (data not shown), the Roseworthy experiments were treated as a multi environment trial (MET, RACMET) and analysed following the methods of Mathews et al. (2008).

Ear emergence time was also fitted as a covariate in the glaucousness analysis for each experiment. When the effect of ear emergence time was not significant, it was excluded from the model. Fitted data based on the best possible model for each experiment were then used for QTL analysis.

QTL mapping

The QTL analysis was performed by the mixed-model based composite interval mapping (CIM) using QTLNetwork v2.0 (Yang et al. 2007). To estimate the empirical significance thresholds for detecting putative QTLs of each trait, 1,000 permutations with the experimental type I error $P = 0.05$ significance level were defined (Churchill and Doerge 1994). The significant threshold was also estimated at $P = 0.1$ level to detect potential QTL. The QTL that were at or above the significance threshold with $P = 0.05$ value for one or more environment are reported as '*putative* QTL' and those that were at or above the significance threshold with $P = 0.1$ for two or more environments are referred as '*suggestive* QTL'. Trait abbreviations and QTL designations were defined adopting the nomenclature

suggested by the wheat catalogue of gene symbols (McIntosh et al. 2003), with '*aww*' signifying 'Australia Wheat Waite'. Since there are no previous reports of mapping Zadoks score, we suggest the designation '*QZad.*-' for this trait. Where multiple QTL were detected for the same trait on the same chromosome, the designation 'chromosome'-1, 'chromosome'-2 etc was used.

Results

Genotyping and map construction

A total of 456 markers, consisting of 246 DArTs and 210 SSRs, were used to assemble the final genetic linkage map (Fig. 2). Both the *Ppd-B1* and *Ppd-D1* markers were segregating within the population but *Ppd-B1* mapped to the same locus as *barc0013a*. None of the *VRN1* diagnostic markers (Yan et al. 2004b) were polymorphic in this population (data not shown). The genetic length of the map was 3372 cM with an average marker interval of 8.5 cM. The B genome had the greatest coverage, with 187 polymorphic markers accounting for 1,149.4 cM of the total map (6.4 cM per marker), while the D genome had the poorest coverage (Table 2). In particular, chromosomes 4D, 5D, and 6D were covered by 1.1, 1.3 and 1.5% of markers, respectively (Supplementary Table 1). Chromosome 7A contained the highest percentage (9.2 %) of markers and was the largest linkage group at 229.6 cM (Supplementary Table 1). Chromosomes 5A, 5D, 7A and 7D were characterized by large genetic gaps between two or more linkage groups and were forced together.

Phenotypic variation for ear emergence time, Zadoks growth stage and flag leaf glaucousness

The ear emergence dates for the two parents were not significantly different, except for CIMI07, for which RAC875 was earlier. In the DH population there was a large range in ear emergence time. Across the experiments, the average range in days to ear emergence from the earliest to the latest genotype was 44 days. Ear emergence was always reached by the parents before the average of the population and the population displayed significant transgressive segregation for this trait. Ear emergence date followed a bimodal inheritance pattern (Fig. 3), indicating the presence of two major genes and followed a 3:1 early:late segregation pattern (282 DH lines classified as early with < 117 days to ear emergence, 86 classified as late with ear emergence time > 121 days), as determined by a chi-square test (chi-square $= 0.522$ with 1 d.f., $P = 0.47$). The broad sense heritability of both days to ear emergence and Zadoks score was very high, ranging from 0.95 for BOL06 Zadoks score to 0.99 for BOL06 days to ear emergence, with the exception of CIMMYT managed drought environment (CIMD07), which had a heritability of 0.86 (Table 4). Given their inverse relationship, days to ear emergence and Zadoks score were highly negatively correlated in each experiment (Table 5).

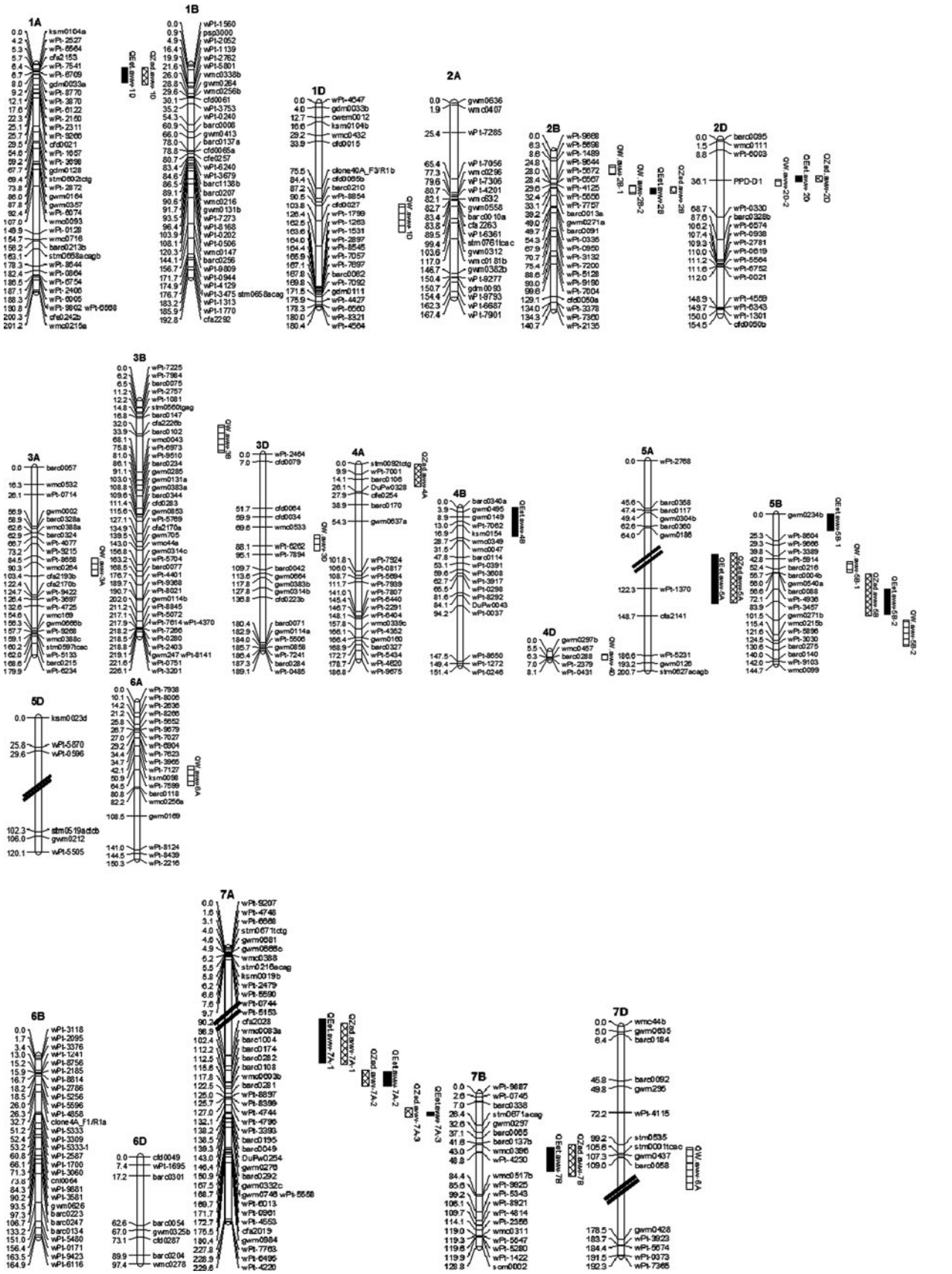
Fig. 2 Genetic linkage groups constructed in the 368 line doubled haploid population derived from a cross between 'RAC875' and 'Kukri'. Estimated QTL positions for each trait are illustrated by a *solid bar* (days to ear emergence), *cross hatched bar* (Zadoks score) or *horizontal lined bar* (flag leaf glaucousness), with the length of the bar indicating the location of the peak at $P < 0.05$. Chromosomes 5A, 5D, 7A and 7D were characterized by large genetic gaps between two or more linkage groups and were forced together ▶

Flag leaf glaucousness scores also varied widely in the DH population and were significantly different ($P \leq 0.001$) between the parents. RAC875 displayed a significantly ($P \leq 0.01$) higher level of glaucousness than Kukri in all experiments (Table 4). Broad sense heritability for this trait was again high, ranging from 0.72 to 0.88. The frequency distribution of flag leaf glaucousness indicated that this trait was quantitatively inherited (Fig. 4). In the Minnipa (MIN06) experiment, glaucousness was significantly correlated with all ear emergence time and Zadoks score measurements, which was also the case with the CIMD07 experiment.

QTLs for days to ear emergence and Zadoks score

A total of 44 suggestive and putative QTL were detected for phenological development, with 27 for days to ear emergence (in five experiments) and 17 for Zadoks score (in three experiments, Supplementary Table 2). A QTL on chromosome 2D was designated *QEet.aww-2D* and accounted for the greatest percentage of genetic variance (up to 50.3 percent). This locus also had the largest LOD score and additive effect at all sites, with a maximum of 105.1 and 8.1 days, respectively. This QTL also had the largest effect on Zadoks score at all three sites and was coincident with the diagnostic *PPD-D1a* marker (Fig. 2). The RAC875 allele at this locus was associated with a delay in ear emergence, and therefore, a lower Zadoks score.

Another QTL detected in all experiments, on chromosome 2B and designated *QEet.aww-2B*, also had a large effect, with the Kukri allele increasing days to ear emergence and reducing Zadoks score. This locus accounted for up to 24.9% of variance with an additive effect of between 3.8 and 5.2 days. It is very likely that these two loci are mainly responsible for the bimodal inheritance pattern within the population (Fig. 3). In addition, the day length sensitive alleles at these loci had a significant interaction (data not shown), which accounted for up to 12% of genetic


1A
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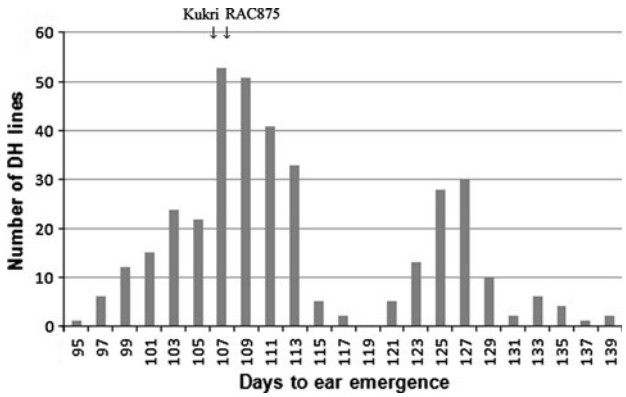

Fig. 3 Phenotypic frequency distribution of days to ear emergence within the doubled haploid population developed from a cross between Kukri and RAC875, grown at Roseworthy, South Australia, in 2007

variance for ear emergence time and Zadoks score. Three more QTL, designated *QEet.aww-7A-1, QEet.aww-7A-2* and *QEet.aww-7A-3*, were also detected in most experiments although these had a much smaller effect; but still significant effects on both days to ear emergence and Zadoks score.

Further QTL were detected on chromosomes 1A, 4A, 4B, 5A, 5B and 7B in one or more environments but not all, with each having small but significant effects (ranging from one to two days). QTLs *QEet.aww-1A*, *QEet.aww-4A* and *QEet.aww-4B* were detected only in the RACMET trial, with *QZad.aww-4A* detected for Zadoks score only and *QEet.aww-4B* for days to ear emergence only. A QTL on 7B (*QEet.aww-7B*) was detected only in the BOL06 trial, for both days to ear emergence and Zadoks score. All Zadoks score QTL, with the exception of *QZad.aww-4A*, were co-located with ear emergence time QTL.

All minor QTL for days to ear emergence, with the exception of *QEet.aww-5A* and *QEet.aww-5B*, had a negative additive effect, indicating that Kukri was the parent with the later allele at these loci. The *Ppd-D1* allele carried by RAC875 had a larger effect on days to emergence than the *Ppd-B1* photoperiod sensitive allele carried by Kukri and as such, RAC875 required fewer of these loci of smaller effect, to achieve a similar ear emergence date to Kukri.

The importance of consistent methodology and accurate phenotyping cannot be over stated. At one severely water stressed site (CIMD07), anthesis date was used as a measurement of time to ear emergence. Due to the severity of the water stress experienced by the later maturing lines, many flowered either in the boot or failed to reach ear emergence, making scoring this trait difficult and introducing greater error. This was reflected through the lower heritability at this site and subsequent detection of only two QTL.

QTLs for flag leaf glaucousness in the RAC875/Kukri DH population

A major novel glaucousness QTL close to the marker locus *wmc264* on chromosome 3A was detected in all environments and designated *QW.aww-3A*. The LOD score ranged

Table 4 Days to ear emergence, Zadoks and glaucousness scores for parents, the mean and range within the doubled haploid population and broad sense heritability at five experiments in South Australia and northern Mexico

Trait	Trial	Kukri	RAC875	Min–Max	Mean	H^2
Days to ear emergence	RACMET	109.3	110.9	99.4–137.9	114.2	0.98
	BOL06	110.2	109.0	94.1–140.0	111.1	0.99
	MIN06	93.4	93.8	85.4–126.7	99.7	0.97
	CIMI07	76.0	72.1*	59.8–105.4	79.2	0.97
	CIMD07[a]	79.5	77.5	53.2–101.6	81.3	0.88
Zadoks score	RACMET[b]	67.0	68.6	45.2–75.4	64.5	0.96
	BOL06	71.0	74.3	40.1–84.5	70.2	0.95
	MIN06	71.4	73.8	37.2–81.0	66.3	0.98
Glaucousness	RACMET[b]	4.1	7.0**	3.6–7.8	5.7	0.86
	BOL06	1.5	5.3**	1.1–5.8	3.2	0.88
	MIN06	2.5	3.6**	1.1–4.7	2.7	0.72
	CIMI07	4.1	7.0**	2.9–7.2	5.0	0.83
	CIMD07	4.1	5.9**	3.05–6.6	4.5	0.84

[a] Days to anthesis

[b] Roseworthy 2006 and 2007 data only

* Significant ($P \leq 0.01$) difference between Kukri and RAC875

** Significant ($P \leq 0.001$) difference between Kukri and RAC875

Table 5 Phenotypic correlations between all experiments (BOL06, MIN06, RACMET, CIMI07, CIMD07) and traits (glaucousness, days to ear emergence, Zadoks score) in the RAC875/Kukri doubled haploid population containing 368 lines

		Glaucousness					Days to ear emergence					Zadoks score		
		MIN06	BOL06	RACMET	CIMD07	CIMI07	BOL06	MIN06	RACMET	CIMD07	CIMI07	BOL06	MIN06	RACMET
Glaucousness	MIN06	1												
	BOL06	0.65*	1											
	RACMET	0.41*	0.53*	1										
	CIMD07	0.13	0.41*	0.64*	1									
	CIMI07	0.52*	0.54*	0.71**	0.67**	1								
Days to ear emergence	BOL06	0.64*	0.28	0.23	−0.17	0.26	1							
	MIN06	0.67**	0.29	0.19	−0.21	0.25	0.94**	1						
	RACMET	0.69**	0.30	0.20	−0.19	0.27	0.94**	0.95**	1					
	CIMD07	0.65*	0.34*	0.23	−0.16	0.27	0.77**	0.79**	0.81**	1				
	CIMI07	0.66*	0.28	0.19	−0.21	0.26	0.90**	0.92**	0.92**	0.81**	1			
Zadoks score	BOL06	−0.60*	−0.19	−0.19	0.21	−0.23	−0.96**	−0.92**	−0.92**	−0.72**	−0.89**	1		
	MIN06	−0.60*	−0.155	−0.10	0.27	−0.21	−0.89**	−0.94**	−0.93**	−0.76**	−0.91**	0.90**	1	
	RACMET	−0.68**	−0.26	−0.18	0.21	−0.25	−0.94**	−0.96**	−0.98**	−0.81**	−0.92**	0.92**	0.96**	1

* Significant at $P < 0.05$, ** significant at $P < 0.01$

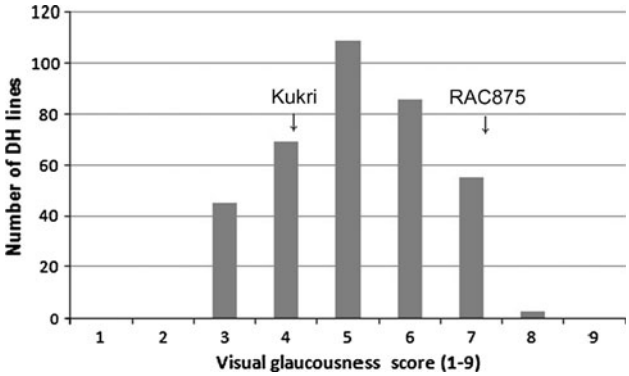

Fig. 4 Phenotypic frequency distribution of flag leaf glaucousness score within the doubled haploid population developed from a cross between Kukri and RAC875, grown at Roseworthy, South Australia, in 2007

from 3.1 to 71 (Supplementary Table 2) and the large additive effect at this locus was positive, indicating RAC875 was the donor of the allele increasing flag leaf glaucousness score.

A second glaucousness QTL, designated *QW.aww-2D* was also detected in all environments and was located at a similar locus to *QEet.aww-2D*. The additive effect ranged from 0.5 to 1.1, with Kukri being the favourable parent. *QW.aww-6A* was detected in all of the Australian experiments with a positive additive effect ranging from 0.13 to 0.31. Four other QTL were detected in two experiments on chromosomes 1D, 2B-1, 4D and 5B (designated *QW.aww-1D*, *QW.aww-2B-1*, *QW.aww-4D* and *QW.aww-5B*, respectively) and were of minor, but significant effect (Supplementary Table 2). Further QTL identified in only one trial and of minor effect were identified on chromosomes 2B, 3B, 3D, and 7D, with *QW.aww-2B-2* coincident with *QEet.aww-2B*. No other QTL identified for flag leaf glaucousness were coincident with QTL identified for days to ear emergence or Zadoks score. The RAC875 allele contributed increased glaucousness at seven of the 11 QTL detected.

Validating QW.aww-3A using RILs

A bulk segregant analysis revealed no segregating markers additional to those on the DH map in the *QW.aww-3A* region of the RILs. The region between *barc324* and *cfa2123b* spanned around 35.9 cM, compared with 40.8 cM in DHs (Figs. 2, 5). The marker locus *wmc264* showed the closest association with *QW.aww-3A*, similar to the mapping in the DH lines; it explained 27% of the phenotypic variation. Composite interval mapping in the RILs using the greatest flag leaf score achieved by each line detected a significant QTL, *QW.aww-3A*, with a LOD value of 23.8 at *wmc264* in the *barc324-cfa2123b* interval

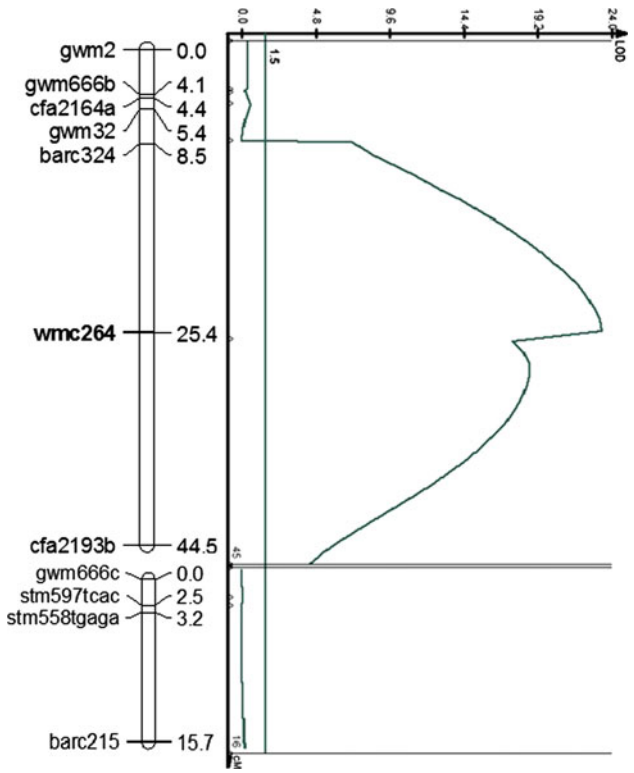

Fig. 5 The estimated position of SSR markers on chromosome 3A, with the centromere located between the two linkage groups, in 380 RILs from a cross between Kukri and RAC875. The detected QTL, centred over the marker locus *wmc264*, is for flag leaf glaucousness, scored visually in a glasshouse experiment

(Fig. 4), which explained 34.7% of the phenotypic variance.

Discussion

The genetic linkage map for QTL analysis

A DH population, from a cross between bread wheat lines 'RAC875' and 'Kukri' has been developed to investigate phenotypic and genotypic relationships in important agronomic traits under southern Australian wheat production conditions. RAC875 is a breeder's line, previously identified as having relatively stable grain yields in water limited environments and a conservative response to cyclical drought stress (Izanloo et al. 2008). In the same study, Kukri showed a relatively intolerant response.

The genetic linkage map generated in this study was produced with the aim of identifying QTL for physiologically and developmentally important traits such as phenological adaptation and flag leaf glaucousness. The population size in this study comprized of 368 DH lines was relatively large, with this offering the potential for fine-mapping QTL and genes of interest. In general, large populations have a beneficial effect on the mapping result as more recombination events can be assessed between pairs of markers. This increases genetic resolution and increases the power to detect QTL of minor effect (Vales et al. 2005). In addition, the positioning of the markers will also be more accurate and the relative impact of missing observations and scoring errors decreases as there are more replicates of individual alleles (van Os et al. 2005). Map size was comparable to recently published genetic linkage maps of hexaploid wheat (Mason et al. 2010; Somers et al. 2004).

Genetic variation for phenological development in the population

Time to ear emergence is one of the most important adaptive traits for production in the southern Australian environment. Nine QTL for time to ear emergence were found on chromosomes 1A, 2B, 2D, 4B, 5A, 5B, 7A and 7B. Ear emergence time in the field is influenced by interactions between genetic factors responsible for photoperiod sensitivity (*Ppd*), vernalization requirement (*Vrn*) and earliness per se (*Eps*) (Dubcovsky et al. 2006; Shindo et al. 2003). In this study, the most significant QTL were located on the short arm of chromosomes 2B and 2D. These two QTL are coincident with the previously characterized homoeoallelic loci *Ppd-B1* and *Ppd-D1* (Beales et al. 2007; Worland 1996; Worland et al. 1998). Two QTL for earliness per se on 2B, distal to *Ppd-B1*, have also been reported (Kuchel et al. 2006; Shindo et al. 2003) but do not appear to be segregating in this population.

The photoperiod responsive allele *Ppd-D1a* plays a major role in regulating flowering time in wheat (Worland 1996; Worland et al. 1998). The dominant *Ppd* genes reduce sensitivity to photoperiod and accelerates flowering under short and long-day conditions (Cockram et al. 2007). In this population, it is very likely that the presence of both photoperiod sensitivity alleles at '*Ppd-B1*' and '*Ppd-D1*' from 'Kukri' and 'RAC875', respectively, were associated with the extreme delay in ear emergence time in approximately one quarter of the population. A similar result was found by Quarrie et al. (2005) in the CS/SQ1 population, in which different alleles of both *Ppd-B1* and *Ppd-D1* from both parents (CS and SQ1) were associated with large variation for time to ear emergence.

A significant QTL for ear emergence time was detected on the long arm of chromosome 5B (*QEet.aww-5B*), and one suggestive ear emergence time QTL was also identified in a poorly covered region on chromosome 5A (*QEet.aww-5A*) in the *gwm0186-wPt-1370* interval. The gene *Vrn-B1* has previously been mapped on the long arm of chromosome 5B, closely linked to the marker locus *gwm408* (Leonova et al. 2003). QTL for earliness per se have also

been detected on chromosome 5B in fully vernalized plants, independent of the *Vrn-B1* locus and proximal to the centromeric region (Hanocq et al. 2004; Toth et al. 2003; Worland 1996; Worland et al. 1998). A review of common markers between these two studies and our present results, as well as a comparison of the reported effect on time to ear emergence, suggest that *QEet.aww-5B* identified in these studies is most likely to be the same QTL and not *Vrn-B1*.

In addition to the *VRN-A1* and *VRN2* genes being located on chromosome arm 5AL in previous studies (Yan et al. 2003), Kato et al. (1999) reported a locus for earliness per se on chromosome arm 5AL of hexaploid wheat. In the present study, *QEet.aww-5A* was located in a region of poor marker density on the short arm, near the centromere. Additional marker coverage, particularly using markers from published maps would help narrow down the region associated with this QTL. However, given the relatively minor effect on time to ear emergence of the two alleles at this locus, this may be of negligible value for plant breeding.

QTL for ear emergence time were also identified on the homoeologous group 7 chromosomes. *QEet.aww-7A-1, QEet.aww-7A-2* and *QEet.aww-7A-3* were detected in most environments while *QEet.aww-7B* was detected in only one experiment but in a position syntenic to *QEet.aww-7A-1*. These QTL are possibly associated with *TaFT (VRN3,* previously called *VRN5 or VRN4*) on the short arm of the group 7 chromosomes (Bonnin et al. 2008; Yan et al. 2006). *TaFT* is located 1 cM distal to *abc158-7B* on 7BS (Yan et al. 2006). In both wheat and barley, *FT* is associated with a flowering promoter gene orthologous to the Arabidopsis *FT* gene (Yan et al. 2006). The *TaFT-A*, *TaFT-B* and *TaFT-D* were physically assigned to the short arm of chromosomes 7A, 7B and 7D, respectively, where QTL for time to ear emergence have also been detected in wheat.

TaFT-A was assigned to the 7AS8-0.45-0.59 bin, at an estimated position near the marker *barc154,* 0.4 cM from *cfa2028.* The marker locus *cfa2028* was assigned to the C-7AS8-0.45 bin, close to the centromere and in the RAC875/Kukri population showed a significant association with time to ear emergence across all environments. Studies by Quarrie et al. (2005) and Kuchel et al. (2006) both identified a QTL for flowering time on the short arm of chromosome 7A, possibly in the same bin as Ta*FT-A*. Hanocq et al. (2007) reported two QTL on the short arm of chromosome 7A, one for days to ear emergence and one for earliness per se. The loci from these three studies were all located in a 30 cM region around *barc154*. For chromosome 7B, a number of meta-QTL for ear emergence date and earliness per se have been identified (Griffiths et al. 2009; Hanocq et al. 2007) and one QTL for ear emergence date (Kuchel et al. 2006) has been located in a similar region to *QEet.aww-7B* from the present study.

Kuchel et al. (2006) reported a photoperiod-responsive locus on the long arm of chromosome 1A in wheat, which did not respond to vernalization and was in a position homoeologous to the photoperiod sensitive locus *Ppd-H2* on chromosome 1H (Laurie et al. 1995; Law et al. 1998). Law et al. (1998) concluded that genes for time to ear emergence were probably present on the short arm of homoeologous group 1 chromosomes in wheat, but they were unable to genetically map these loci. In the present study, *QEet.aww-1A* was detected in just one South Australian experiment, indicating G × E interaction. To the best of our knowledge, this is the first example of the genetic mapping of this locus, which is likely to be a locus similar to that postulated by Law et al. (1998).

To date, no loci responding to day length or vernalization have been identified on the group 4 chromosomes. From meta-QTL analyses conducted by Hanocq et al. (2007) and Griffiths et al. (2009), one earliness per se locus was detected on chromosome 4B, in a similar region to *QEet.aww-4B*. Hanocq et al. (2007) also identified a small meta-QTL on 4AL. However, there are no other reports of loci on chromosome 4A which affect time to ear emergence. The *QZad.aww-4A* locus, located on chromosome arm 4AS, was identified in the RACMET trial by Zadoks score and significantly influenced the development of plants in this environment. We suggest that this is a new *Eet* locus, which given its position on the chromosome, is potentially homoeologous to the *QEet.aww-4B* locus.

Strategies to deal with variation in time to ear emergence in the RAC875/Kukri population

When these populations were developed, markers for most vernalization and both major photoperiod genes were not available. Whilst care was taken to select parents with similar phenology, variation observed for days to ear emergence in this population was large. In a population designed to investigate drought tolerance and QTL analysis of associated traits, this is undesirable, as early lines are able to escape severe water deficit, while late lines experience a more intense stress at a different growth stage (Pinto et al. 2010; Reynolds et al. 2009). The effect of this on QTL mapping was highlighted in the correlation between MIN06 flag leaf glaucousness and phenology and subsequent detection of the PPD-D1 QTL for leaf glaucousness. However, there are a number of techniques that can be employed to minimize the confounding effects of flowering time on drought tolerance.

The first approach involves adjusting data for phenology prior to QTL analysis, using either days to ear emergence, Zadoks score or the genotype for the marker nearest to a given phenology QTL (where closely linked markers are available) as a covariate. The Zadoks score can be used as a

surrogate for time to ear emergence, where it may not be practical to measure days to ear emergence (for example, due to lack of resources at a site or distances required to visit an experiment regularly). Zadoks score was found to show high heritability and detected most or all of the same QTL detected by days to ear emergence. However, fitting QTL allele scores for each genotype would be most accurate for this approach, particularly when diagnostic marker scores are available, as is the case for *Ppd-D1a*.

Unfortunately, adjustment of data for phenology does not address the problem since late lines experience a dramatically different environment compared to early lines and phenotypic comparison can be difficult. Therefore, a second approach would be to reduce the population size based on phenology. Given the large size of the DH population, lines with phenology extremes could be omitted from future experiments. Within the population, a distinct sub-population of approximately 110 late lines exists, which could be omitted to minimize the strength of phenology effects on future results, as well as reduce time and resources required for phenotyping experiments. This has proven to be a successful strategy for QTL detection (Reynolds et al. 2009). Given the size of the population, it could be further divided up into even smaller phenological sub-groups and QTL analysis conducted on lines experiencing similar water deficits at similar growth stages. However, QTL mapping with smaller numbers of lines can fail to detect QTL of smaller, yet still significant effects, even with a greater number of replicates or environments (Schon et al. 2004). Importantly, selecting out lines with similar phenology also means that regions of the genome associated with flowering time are being fixed. Consequently, QTL in the vicinity of flowering time loci will probably escape detection. It is therefore likely that a combination of these strategies will be most effective to reduce the influence of phenology on genetic mapping results within this population in the future.

The identification of novel loci influencing flag leaf glaucousness and validation of a novel QTL of large effect on chromosome 3A

The large variation for flag leaf glaucousness observed in this study, as well as the large population size offers considerable scope to investigate the precize impact of this trait within bread wheat and its value in the southern Australian environment. Johnson et al. (1983) found significant grain yield increases in glaucous Near Isogenic Lines (NILs) of wheat over their non-glaucous pair. These glaucous lines had greater leaf surface reflectance; and studying a closely related set of germplasm, Richards (1984) and Richards et al. (1986) identified improved water use efficiency, reduced leaf temperatures as well as increased biomass production and improved flag leaf green area retention under heat and drought stressed conditions, in glaucous genotypes. However, Merah et al. (2000) identified a negative effect of glaucousness on biomass production in durum wheat in one season, where drought was not experienced during vegetative growth. These authors also identified significantly reduced transpiration efficiency, this also being observed by Febrero et al. (1998) in barley.

Richards et al. (2010) suggested that visual selection for glaucousness is straightforward under favourable conditions but less so when phenotyping large populations in dry environments. This study identified and validated under glasshouse, high- and low-yielding field conditions, the presence of a major, novel leaf waxiness QTL (*QW.aww-3A*) in the RAC875/Kukri DH and RIL population, which indicates a robust, repeatable effect from this locus. Richards (1984) concluded from a diallel cross that glaucousness in durum wheat was under the control of both major and minor genes. Subsequent QTL studies have reported up to eight loci of minor effect and two or major effect, none of which were previously detected at the *QW.aww-3A* locus (Borner et al. 2002; Kulwal et al. 2003; Mason et al. 2010). In this study we also detected the loci *QW.aww-3B* and *QW.aww-3D*. These have also not been previously reported and appear to be at similar locations on the short arm of their respective chromosomes, suggesting these three QTL are potential homoeologous loci of *QW.aww-3A*. However, *QW.aww-3A* is the major locus controlling glaucousness in this population.

QW.aww-2B-1 was located distal to *QW.aww-2B-2* and is in a similar position to the previously reported *W1* gene (Driscoll 1966; Tsunewaki and Ebana 1999). QTL on chromosomes 1D, 6A and 7D detected in this study, have also been reported in previous studies (Borner et al. 2002; Kulwal et al. 2003). Despite being of relatively small influence, two other loci (*QW.aww-4D* and *QW.aww-5B*), which have not previously been reported, had a significant effect on flag leaf glaucousness. The genetic basis of leaf waxiness in the South Australian cultivars used in the present study is genetically distinct from the materials used in previous studies.

Conclusions

We have developed a mapping population and presented the framework for a linkage map to investigate the genetic basis of traits exerting a significant effect on grain yield in the southern Australian environment. The large variation for flag leaf glaucousness within this population offers the opportunity to investigate the effect and value of this trait in water-limited environments of southern Australia.

Previous studies suggest that the QTL controlling glaucousness will exert significant influence over traits such as anthesis biomass, harvest index, grain size, spikelet fertility and ultimately, grain yield. Although this is yet to be confirmed, further work to fine map and identify genetic markers linked with the *QW.aww-3A* locus can be undertaken using the RIL population but may be of negligible value as molecular markers in breeding given the ease and accuracy of visually scoring this trait. However, this study has identified several new loci controlling glaucousness and confirmed the complexity of this trait. If leaf waxiness proves to be a valuable component of stress tolerance, as proposed by others, this new understanding will be important in optimizing glaucousness and determining the best loci and alleles to deploy.

While the wide variation for time to ear emergence could potentially cause considerable confounding effects on other traits measured within this population, there are various strategies available to deal with this. The identification of loci influencing phenological development in this population, including one not previously reported, will aid in interpretation of yield performance and improve knowledge of crop production in the Mediterranean-type environment.

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