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QTL identified for stay-green in a multi-reference nested association mapping population of wheat exhibit context dependent expression and parent-specific alleles

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ABSTRACT

A stay-green phenotype is useful for adaptation of wheat to end-of-season drought conditions. We identified quantitative trait loci (QTL) for stay-green traits, as well as for height, days to anthesis and yield, in a multi-reference nested association mapping (MR-NAM) population of wheat (*Triticum aestivum* L.) in two environments differing in degree of drought stress experienced post-anthesis. The MR-NAM population consisted of three inter-related nested association mapping populations developed by nesting 11 diverse adaptation donors within three common reference parents, adapted to the northern, southern and western cropping regions of Australia, respectively. The construction of the MR-NAM population enables the assessment of the effect on a trait of multiple alleles at any particular locus, in different genetic backgrounds, and facilitates concurrent QTL mapping and germplasm development. This approach enabled identification of parent-specific alleles and context dependent expression. Using a new statistical method specifically developed to identify QTL in MR-NAM populations, we identified 65 QTL for stay-green traits. Co-location was observed between (i) trait by loci associations for some of the different stay-green traits, (ii) for QTL between the two environments, and (iii) between QTL for stay-green traits, plant height and grain yield. Some QTL co-located with those identified in other studies however, others are likely novel. Genetic markers associated with QTL for stay-green can be applied in breeding to enrich populations for stay-green traits in early generations of selection, prior to field testing in yield plots, in particular for the development of wheat cultivars targeted to end-of-season drought-stressed environments. This information is important for breeders, because it facilitates identification of the sources of the most promising alleles at particular loci for specific genetic backgrounds and growing environments.

Abbreviations: °Cd, degrees Celsius days; Alternate allele, an allele that is alternate to that of the wheat reference used by DArT (DArT, 2018); BC_1, first backcross; BLUEs, best linear unbiased estimators; BLUPs, best linear unbiased predictors; CAIGE, CIMMYT-Australia-ICARDA Germplasm Evaluation; CIMMYT, International Centre for Maize and Wheat Improvement; DAF, Queensland Government, Department of Agriculture and Fisheries; DArT, Diversity Array Technology Pty Ltd; DH, doubled haploid; Donors, population parents contributing one or more target trait; EndS, end-senescence; ET, environment type; F_1, F_2, F_4, $F_{3:4}$, $F_{3:5}$, $F_{3:6}$, indicate successive breeding generations; Founders, all population parents including donors and reference parents; G × E, genotype by environment interaction; ICARDA, International Centre for Agricultural Research in Dry Areas; LD, linkage disequilibrium; LG, linkage group; Matching allele, the allele matching the wheat reference used by DArT (DArT, 2018); MidS, mid-senescence; MR-NAM, multi-reference nested association mapping; Ma-NAM, Mace derived nested association mapping; NAM, nested association mapping; NDVI, normalised difference vegetation index; Nmax, maximum greenness; OnS, onset-senescence; QTL, quantitative trait loci; Reference parents, adapted population reference parents; RILs, recurrent inbred lines; Sc-NAM, Scout derived nested association mapping; SGint, stay-green integral; SR, maximum senescence rate; Su-NAM, Suntop derived nested association mapping.

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1. Introduction

Common bread wheat (*Triticum aestivum* L.) is one of the world's major grain crops. It is a staple food for around 2.5 billion people, more than 30 % of the world's population (FAO (Food and Agriculture Organization), 2017). Increasing grain yield is important to ensure global food security in the face of a rapidly growing population and a changing climate (Reynolds et al., 2009). Wheat is important, not only as a major food, feed and industrial crop, but also for the livelihood of millions of farmers.

Limited water availability during growth is the primary constraint to wheat grain yield under rain-fed conditions. Drought stress affects 42 % of the 218.5 million hectares of wheat farmland worldwide (Crespo-Herrera et al., 2017; FAO (Food and Agriculture Organization), 2017; Kosina et al., 2007). This limitation is likely to be experienced more often and with greater severity under predicted climate-change scenarios in major production areas (Chenu et al., 2013; Watson et al., 2017).

Grain producing plants that stay green for longer during their development can continue to photosynthesise and produce sugars (Thomas and Howarth, 2000; Thomas and Smart, 1993). This can lead to an increase in yield, particularly under water-limited conditions during grain filling, compared with plants that senesce sooner (e.g. Christopher et al., 2016; Ullah and Chenu, 2019). This trait is regulated by complex genetic controls in wheat (Kumar et al., 2010) and many other crops, such as sorghum (*Sorghum bicolor* (L.) Moench; Harris et al., 2007), millet (*Pennisetum glaucum* LR Br.;Tharanya et al., 2018), barley (*Hordeum vulgare* L.; Gous et al., 2016) and rice (*Oryza sativa* L.; Ba Hoang and Kobata, 2009).

Lopes et al. (2012) showed that stay-green was a primary physiological trait contributing to the continuing genetic gain for yield in wheats from the International Centre for Maize and Wheat Improvement (CIMMYT). Similarly, Kitonyo et al. (2017) showed that selection for yield of Australian wheat varieties was associated with changed patterns of canopy senescence. Moreover, further improvements in yield are likely by further introgression of stay-green traits into new cultivars, unlike some other primary physiological traits, such as thousand kernel weight and height (Lopes et al., 2012).

A better understanding of the genetic basis for stay-green will help determine which traits can be selected to manipulate the timing and rate of senescence, in specific genetic backgrounds for particular types of environments. This will allow better matching of adaptation to key target environments with regard to the pattern of water availability during the critical grain-filling period, to maintain grain quality as well as to improve grain yield and yield stability. Identification of quantitative trait loci (QTL) for stay-green is an important step in understanding the underlying genetics and physiology of this complex phenotype.

Previous studies have identified genetic regions associated with various measures of stay-green, under a range of conditions with regard to moisture stress, in a range of wheat germplasm, and in different kinds of populations (Table S1). These include measures based on green leaf area duration, green leaf area, various greenness measures and chlorophyll content. Studies have been conducted in irrigated and rain-fed field trials (though most often without specific information about the extent and/or timing of moisture stress), as well as under glasshouse and laboratory conditions. Germplasm investigated include, spring and winter wheats; elite genotypes, synthetic-hexaploid wheats and trait donors; including both durum and bread wheats. Generally, populations studied were derived from bi-parental crosses, such as recombinant inbred line (RIL) or doubled haploid (DH) populations (e.g. Christopher et al., 2018; Graziani et al., 2014; Mason et al., 2018; Peleg et al., 2009; Pinto et al., 2010; Vijayalakshmi et al., 2010; Wang et al., 2015; Yang et al., 2007). Others were collections of breeding lines and/or commercial cultivars, with unknown population structure, when genome wide association mapping was often applied (e.g. Daba et al., 2020; Sinha et al., 2018). Meta-analyses have also been reported (e.g. Acuña-Galindo et al., 2015; Zhang et al., 2010).

To understand the genetics of stay-green traits and to identify QTL, we used a multi-reference nested association mapping (MR-NAM) population, constructed to investigate drought and heat stress. The utilisation of nested association mapping (NAM) populations aims to overcome the limitations of both RIL populations and association mapping approaches (McMullen et al., 2009). Recombinant inbred line populations are generally bi-parental and sample a limited number of recombination events, thus limiting the resolution of QTL detection. Association mapping capitalizes on historical recombination events and low linkage disequilibrium within populations of diverse individuals, which increases allele diversity and mapping resolution (Gage et al., 2020). However, association mapping can be confounded by population structure, which is generally unknown, so different approaches need to be followed to estimate and account for it. Nested association mapping populations, on the other hand, have known population structure, relatively high allelic diversity and many recombination events. Genetic analysis of NAM populations needs to account for population structure.

Nested association mapping populations are commonly generated by crossing a diverse array of trait donors to a single, usually well-adapted and well characterised, reference parent. This facilitates the detection of QTL of small effect, with relatively high certainty, as well as the detection of parent-specific alleles, which may be present in only one or two founders. Similarly, multi-parent advanced generation inter-cross (MAGIC) populations are made by inter-crossing a set of parental genotypes (Gage et al., 2020). They have potential for more diverse recombinant haplotypes, but generally involve fewer founders, compared with NAM populations. The MR-NAM population strikes a balance between these two approaches, combining multiple donors, with several reference parents (Richard, 2018). Multi-reference nested association mapping RILs can be inter-crossed to achieve the diverse haplotypes of MAGIC genotypes. Further, it has the extra advantage of allowing the addition of new reference parents to the population, as well as new donors, to address new breeding goals (Richard, 2018).

In a previous study, we identified QTL for stay-green traits in a bi-parental population (Christopher et al., 2018). Here we extended this by examining more genetic diversity, seeking to identify multiple alleles at stay-green trait QTL using a wheat MR-NAM population. The MR-NAM population, constructed to investigate drought and heat stress, was utilized with three main objectives, to:

1 identify QTL associated with stay-green traits,
2 determine how different alleles at these QTL influence plant phenotype when in different genetic backgrounds and in two different environments, and
3 develop germplasm suitable for wheat breeders to introduce new genetic variability to enhance stay-green into their populations with the aim to improve tolerance to drought stress during grain development.

The overall aim was to provide a package of new potential parent germplasm, and matched selection tools, to enable breeders to incorporate stay-green traits into wheat cultivars targeting end-of-season drought-stressed environments. Additionally, this study will enhance understanding of the genetic control of these important traits in adapted germplasm and in relevant growing environments.

2. Materials and methods

2.1. Experimental environments

Experiments were conducted at two locations geographically near each other, but in two different seasons, under natural rain-fed conditions at the Queensland Government Department of Agriculture and Fisheries, Hermitage Research Facility, Warwick, Australia (WAR; 28.21 °S 152.10 °E, 480 m above sea level). These experiments were

designated WAR15 and WAR16. They were conducted in the wheat growing seasons in the winter of 2015 and 2016, respectively. Soils were deep, heavy black cracking clay, with high moisture holding capacity (vertisols), which are able to store moisture during fallow for subsequent crop growth. In Queensland, in-season rainfall is typically highly variable, with winter crops relying heavily on soil moisture stored from summer rainfall. Limited in-crop rainfall, especially around anthesis and during grain filling, can significantly reduce crop yields (Chenu et al., 2011, 2013).

Wheat genotypes were sown at 25 cm row spacing with a target population density of 100 plants m^{-2} and harvested from 2m × 4m plots. Ample nutrients were available, with 120 kg ha^{-1} urea applied prior to sowing and 40 kg ha^{-1} of Incitec Pivot Starter Z® (containing 10.5 % N, 19.5 % P, 2.2 % S, and 2.2 % Zn) at sowing. Weeds and diseases were controlled as necessary. Immediately after planting, 20 mm of irrigation was applied to ensure good crop establishment. Details of further attributes of the two experimental environments are provided in Table 1, Fig. 1 and Fig. S1.

Both experimental environments were classified into one of four previously-identified main environment types (ETs) for Australian wheat cropping, based on the severity and timing of moisture stress, as defined by Chenu et al. (2013). The four ETs are ranked from ET1 to ET4 in ascending order of moisture stress, with ET1 experiencing little moisture stress, ET2 experiencing stress during the grain filling period, ET3 experiencing stress during the period just prior to anthesis to mid-grain filling, and ET4 experiencing stress throughout most of the growing season (Chenu et al., 2013).

2.2. Crop measurements and traits

Plants established well in each experiment. For each plot, Zadoks stages were recorded weekly to determine days to anthesis (GS65; Zadok et al., 1974). Normalised difference vegetative index (NDVI) was measured weekly for each plot from prior to anthesis until after maturity using a hand-held 'Greenseeker' model 505 (NTech Industries, Ukiah, California) which was conveyed at 0.8 m s^{-1}, 0.5 m above the canopy. Mature grain was harvested using a small plot harvester to estimate yield. The grain was held in cold storage to reach a uniform moisture content of approximately 15% prior to weighing.

Up to nine traits (Table 2; Fig. 2) were either measured or estimated for each plot in each environment as described by Christopher et al. (2014, 2016). Sowing date, seedling establishment, anthesis date, crop height and grain yield, at approximately 15 % moisture, were measured directly. Stay-green traits were estimated from a logistic function fitted to NDVI data centred at anthesis for each plot (for more details see Christopher et al., 2014).

For each plot, NDVI data were collected approximately weekly from near ear emergence until maturity. A logistic function was fitted,

$$NVDI = N_{final} + \left(\frac{N_{green\_max}}{1 + \left(\frac{t}{MidS^{SR}} \right)} \right) \quad (1)$$

where N_{final} is the NDVI of the fully senesced plants; N_{green_max} is the difference in NDVI between the maximum and final values (i.e. Nmax - $N_{final} = N_{green\_max}$); MidS is the thermal time from anthesis to loss of 50 % of N_{green_max}; SR is an indicator of the senescence rate; and t is thermal time from anthesis of the plot (Table 2). In general terms, this is an overall measure of greenness from around anthesis until maturity.

Onset-senescence (OnS) is the thermal time from anthesis to loss of 90 % of N_{green_max}. The stay-green integral (SGint) was calculated as the integral from anthesis to 1500 day degrees (°Cd) after anthesis. Thus, SGint corresponds to the area under the stay-green curve from anthesis until the completion of senescence and is related to the overall green-leaf area retention of the crop after anthesis. In general, greater values for each trait contribute to greater greenness, later senescence and a greater integral of leaf greenness, which are all favourable for producing a stay-green phenotype (Christopher et al., 2016). Fig. 2 presents a typical dynamic of NDVI and the different stay-green traits.

2.3. Plant material

The MR-NAM population used in this study was described by Richard (2018) (Fig. 3). It included eleven donors crossed with up to three reference parents, Suntop, Scout and Mace, which are key cultivars for the Australian northern, southern and western wheat production regions respectively (GRDC (Grains Research and Development Corporation), 2020; Table 3). Of these, Suntop is considered stay-green, with Scout and Mace senescent types.

Eleven trait and adaptation donors were selected for traits including stay-green, favourable root architecture, tolerance to drought and heat, as well as disease resistance, acid soil tolerance, pre-harvest sprouting resistance and yield. The group including all 14 parents, both the 11 donors and 3 reference parents, is referred to here as the "founders" (Richard, 2018).

The donors, Dharwah dry, Drysdale, SeriM82, SB062, ZWB10.37 and ZWW10.50 were selected for favourable traits related to drought and/or heat adaptation. Dharwah dry and SeriM82 both have a stay-green phenotype (Christopher et al., 2008; Manschadi et al., 2010; Olivares-Villegas et al., 2007). SeriM82 has a dense root system at depth (Christopher et al., 2008; Manschadi et al., 2006; Manske and Vlek, 2002). The rest of the donors are considered moderate to intermediate with regard to expression of stay-green. Drysdale has superior transpiration efficiency (Condon et al., 2004; Tausz-Posch et al., 2012; Fletcher and Chenu, 2015; Fletcher et al., 2018) and SB062 is tolerant to heat stress, has low canopy temperature, and high levels of water soluble stem carbohydrates (Dreccer et al., 2009; Olivares-Villegas et al., 2007; Ullah and Chenu, 2019). Donors ZWB10.37 and ZWW10.50 produced high yield in the CIMMYT-Australia-ICARDA (International Centre for Agricultural Research in Dry Areas) Germplasm Evaluation (CAIGE) trials conducted in Australia. The donors EGA Gregory and EGA Wylie express multiple disease resistances, both being moderately tolerant to the root lesion nematode, *Pratylenchus thornei*, while EGA Wylie is also moderately resistant to *Fusarium* crown rot and black point (GRDC and DAFF (Grains Research and Development Corporation and the Queensland Department of Agriculture, Fisheries and Forestry), 2014; Zheng et al., 2014). The donor Westonia was selected for tolerance to acid soil and to manganese and aluminium toxicities (Khabaz-Saberi et al., 2010; Tang et al., 2003). The donor UQ114 is resistant to pre-harvest sprouting, with high levels of grain dormancy (Hickey et al., 2009).

Bulked F_2 generation families were subjected to a moderate selection pressure (around one plant in four selected) for plant-height and

Table 1
Attributes of two field experiments, experiment identifier (Expt), average number of replicate plots per genotype (Ave reps), soil moisture at planting, in-crop rainfall, sowing and harvest dates and mean in-crop daily maximum (max.) and minimum (min.) temperatures (temp.).

Expt	Ave reps	Soil moisture at planting (mm)[a]	In-crop rainfall (mm)	Sowing date	Harvest date	Mean max. temp. (°C)	Mean min. temp. (°C)
WAR15	1.40	668	103	11 June	27 Nov	21.6	7.3
WAR16	1.46	377	303	22 July	15 Dec	23.2	8.8

[a] Soil moisture at planting is moisture content in the soil profile to a depth of 1200 mm at planting.

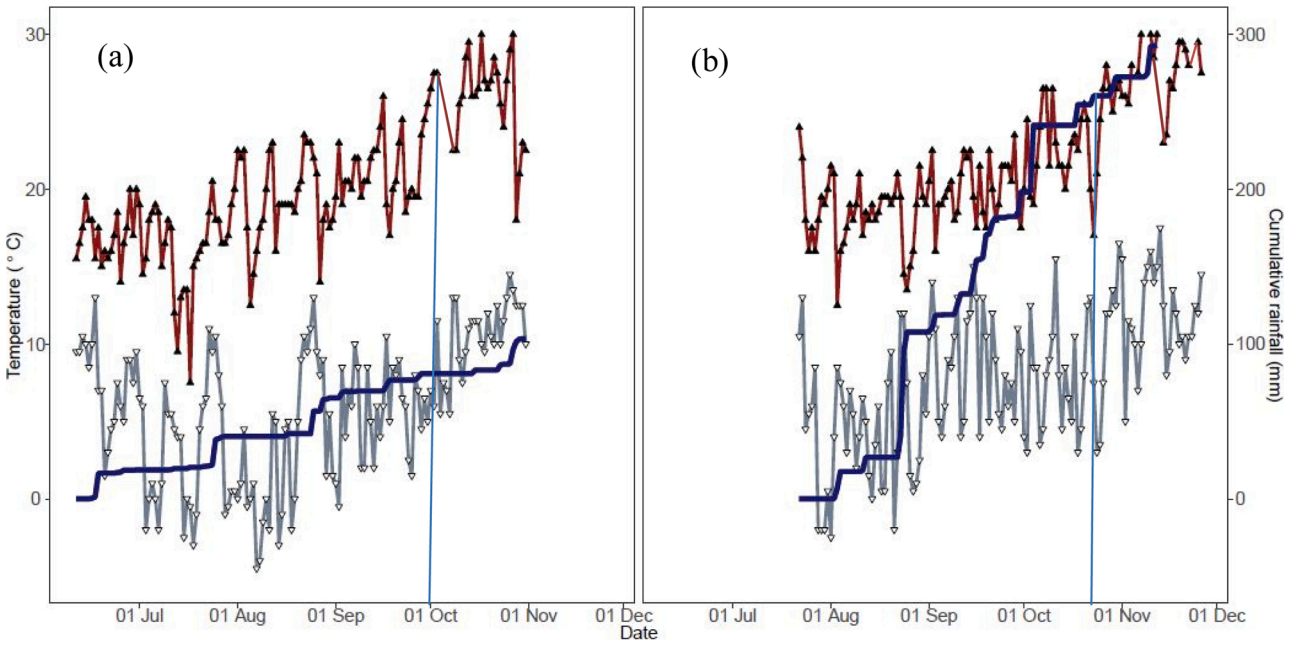

Fig. 1. Daily maximum temperature (red; °C), minimum temperature (grey, °C) and cumulative rainfall (navy, mm), from planting to harvest date for (a) WAR15, and (b) WAR16. Mean anthesis date is indicated by the vertical blue line for each experiment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 2
Traits measured ([m]) in field experiments or calculated ([c]) from in-field measurements.

Abbreviation	Trait	Description
Nmax[c]	Maximum greenness	Maximum NDVI value near anthesis for each genotype
OnS[a, c]	Onset-senescence	Thermal time from anthesis to 90 % of maximum leaf greenness; degrees day (°Cd)
MidS[c]	Mid-senescence	Thermal time from anthesis to 50 % of maximum leaf greenness; degrees day (°Cd)
EndS[c]	End-senescence	Thermal time from anthesis to 10 % of maximum leaf greenness; degrees day (°Cd)
SR[c]	Maximum senescence rate	Indicator of the maximum rate of NDVI decrease
SGint[c]	Stay-green integral	Integral of leaf greenness from anthesis to 1500 °Cd after anthesis
Yld[m]	Yield	Grain yield at approximately 15 % moisture (t ha^{-1})
DTA[m]	Days to anthesis	Days to Zadok 65 from planting (days)
Ht[m]	Plant height	Measured to top glume (cm) after anthesis

[a] Note that OnS, MidS, and EndS, named in Christopher et al. (2016), were previously named TFN90, TFN50, and TFN10, respectively, in Christopher et al. (2014).

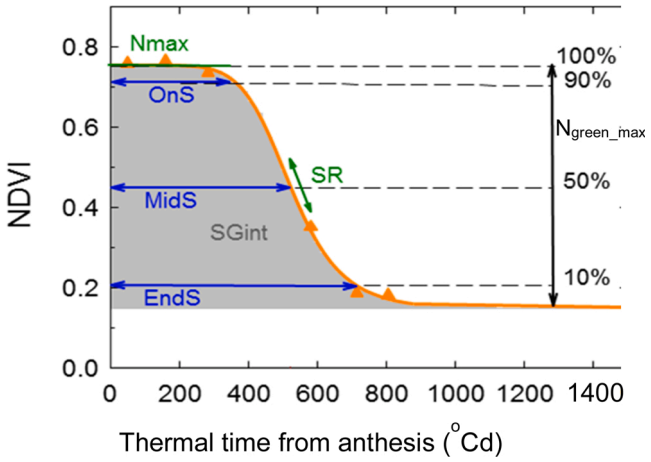

Fig. 2. A generalised model for describing canopy greenness in wheat over time, showing a fitted logistic curve and estimated stay-green traits (modified from Christopher et al., 2016). Descriptions of the stay-green traits are given in Table 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

maturity to resemble the respective reference parent. Populations were then subjected to two generations of self-fertilization with single seed descent in each generation. Eight hundred and forty five RILs were genotyped, using a single plant sample, at the $F_{3:4}$ generation. Bulked $F_{3:5}$ populations were phenotyped at Warwick in 2015 (WAR15) with a subset of these phenotyped as $F_{3:6}$ at Warwick in 2016 (WAR16) (Richard, 2018; Table 3).

For each experiment, WAR15 and WAR16, along with the MR-NAM population and their parents, additional wheat genotypes, such as breeding lines and cultivars associated with other studies, were grown. All of the genotypes grown at the site in that year, referred to as the "trial", were used to characterise the site, for example in the measures reported in Tables 4 and 5. For the QTL analysis, only the MR-NAM population and the founders were included, as described in section 2.5 Experimental design and statistical analysis.

2.4. Genotyping

Multi-reference nested association mapping RILs were genotyped in the F_4 generation, along with founders using the Diversity Arrays Technology Pty Ltd (DArT) wheat genome-by-sequencing platform. Genomic DNA was extracted using the recommended CTAB-based extraction protocol (DArT (Diversity Array Technology), 2018). Genotype-by-sequencing of the MR-NAM RILs generated 18,827 single nucleotide polymorphisms (SNPs). Markers were positioned on the wheat DArT consensus map, version 4 (DArT (Diversity Array Technology), 2018).

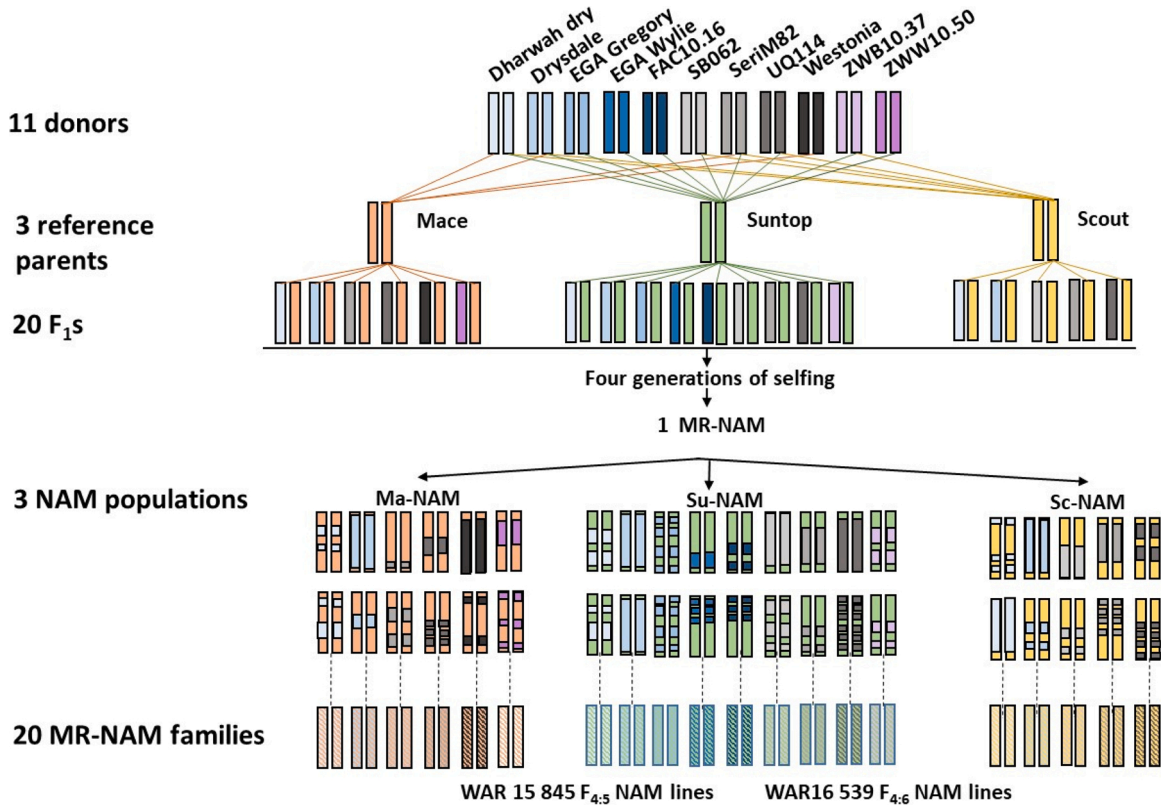

Fig. 3. The multi-reference nested association mapping population comprised 11 donors and three reference parents. Genome reshuffling occurred throughout crossing and development of recombinant inbred lines via four generations of self-fertilization through single seed descent. In WAR15 the MR-NAM population had 845 $F_{4:5}$ lines across six Mace-derived families (Ma-NAM), five Scout-derived families (Sc-NAM), and nine Suntop-derived families (Su-NAM). A subset of 539 $F_{4:6}$ lines were grown in WAR16, which did not include Sc-NAM. As Scout-derived families were of lesser interest for the targeted Australian northern cropping region, few lines from this family were included in WAR16. In WAR16, there were five Ma-NAM and nine Su-NAM grown. (Modified from Richard, 2018).

Table 3
The incomplete factorial crossing design generated 845 MR-NAM recombinant inbred lines from 20 families grown in experiments WAR15 and WAR16. The number of individuals in each of the 20 families ranged from 30 to 51. All Mace and Suntop families were represented in both environments, with the same number or fewer genotypes per family represented in WAR16. Scout families were not represented in WAR16.

Donors	WAR15				WAR16		
Reference parent	Mace	Scout	Suntop	Total	Mace	Suntop	Total
Dharwah dry	43	50	42	135	37	32	69
Drysdale	45	42	50	137	45	46	91
EGA Gregory			37	37		37	37
EGA Wylie			37	37		30	30
FAC10.16			39	39		39	39
SB062	44	40	51	135	40	49	89
SeriM82	42	50	46	138	38	44	82
UQ114		42	40	82		39	39
Westonia	33			33	31		31
ZWB10.37			34	34		32	32
ZWW10.50	38			38			
Total	245	224	376	845	191	348	539

2.5. Experimental design and statistical analysis

Genotypes in the experiments were randomised to plots in two replicate blocks as a partially replicated (*p-rep*) design (Cullis et al., 2006). Experimental MR-NAM lines were replicated 1.40 and 1.46 times in WAR15 and WAR16, respectively. Plots were arranged in the field as a rectangular array of 36 columns by 38 rows in WAR15 and as 20 columns by 63 rows in WAR16, where entries were latinized across rows and columns. All designs were generated using the optimal design methods described in Butler et al. (2008) using the R statistical package od, available from https://mmade.org/optimaldesign/ (Butler, 2018) .

A linear mixed model was fitted to the response data for the nine traits listed in Table 2. The model included fixed effects for environment means, and random effects for genotype by environment effects, replicate blocks and any extraneous spatial field trend aligned with rows and columns in the field. The residual covariance model included a heterogeneous residual variance for each environment and a separable spatial covariance model across rows and columns following the methodology of Gilmour et al. (1997). An unstructured variance matrix was fitted to the genotype by environment effects (Kelly et al., 2007), and the genetic correlation was derived from this variance matrix. Variance parameters were estimated using residual maximum likelihood estimation (Patterson and Thompson, 1971) and analyses were conducted in the ASReml-R package (Butler et al., 2009; R Core Development Team, 2020). Best linear unbiased predictors (BLUPs) of RIL performance for each trait were estimated, including the founder lines. Heritability was calculated as one minus the prediction error variance divided by twice the genotypic variance at each experiment, as described by Cullis et al. (2006).

2.6. QTL analysis

Of the 18,827 SNPs reported, 3401 (~18 %) were used in the QTL analysis. Single nucleotide polymorphisms were removed when they were not positioned on the DArT consensus linkage map (v4; DArT (Diversity Array Technology), 2018). Single nucleotide polymorphisms where a founder was determined to be heterozygous were considered missing data. Markers with a minor allele frequency of less than 0.01 across families were excluded. Single nucleotide polymorphisms with a

Table 4
Founders of the multi-reference nested association mapping (MR-NAM) population were tested along with the MR-NAM populations, standards and other experimental genotypes in two field environments, WAR15 and WAR16. Best linear unbiased predictors, and standard errors (s.e.) are presented for days to anthesis, plant height and yield for MR-NAM founders, along with the trial mean and heritability for each trait for all genotypes tested in each trial. Neither ZWB10.37 nor ZWW10.55 were grown in WAR15.

Genotype	Days to anthesis (days)				Plant height (cm)				Grain yield (t ha^{-1})			
	WAR15	s.e.	WAR16	s.e.	WAR15	s.e.	WAR16	s.e.	WAR15	s.e.	WAR16	s.e.
Dharwah dry	115.0	0.8	87.3	1.1	105	3	110	2	6.0	0.3	4.5	0.2
Drysdale	112.3	0.8	82.6	1.1	82	3	83	2	6.7	0.3	4.8	0.2
EGA Gregory	114.5	0.8	87.2	1.1	88	3	88	2	6.3	0.3	4.9	0.2
EGA Wylie	113.4	0.9	85.9	1.6	82	4	81	2	6.8	0.3	4.6	0.2
FAC10.16	113.4	0.8	84.0	1.1	94	3	91	2	7.1	0.3	5.0	0.2
Mace[R]	111.6	0.8	83.8	1.1	80	3	81	2	6.7	0.3	4.9	0.2
SB062	111.4	0.8	83.2	1.1	88	3	88	2	7.0	0.3	4.6	0.2
Scout[R]	113.0	0.8	82.8	1.1	84	3	85	2	6.7	0.3	4.9	0.2
SeriM82	113.2	0.8	86.0	1.1	78	3	80	2	6.3	0.3	4.2	0.2
Suntop[R]	112.4	0.8	83.2	1.1	87	3	87	2	6.9	0.3	5.1	0.2
UQ114	112.4	0.8	83.1	1.1	86	3	85	2	7.0	0.3	5.1	0.2
Westonia	113.4	0.8	85.2	1.1	81	3	86	2	6.7	0.3	5.3	0.2
ZWB10.37			84.4	1.2			93	2			5.2	0.2
ZWW10.50			86.2	1.2			89	2			5.0	0.2
Trial mean	112.7		83.9		88		89		6.6		4.8	
Heritability in trial	0.65		0.67		0.82		0.85		0.65		0.63	
Genetic correlation between WAR15 & WAR16 trials	0.72				0.89				0.59			

[R] Reference parent.

Table 5
Mean and heritability of stay-green traits was calculated for each trial, as well as genotypic correlation between trials. For comparison between experimental environments, these included all genotypes tested in each environment, including multi-reference nested association mapping recombinant inbred lines, standards and other experimental genotypes.

Trait[a]	Nmax		OnS (°Cd)		MidS (°Cd)		EndS (°Cd)		SR		SGint	
	WAR15	WAR16	WAR15	WAR16	WAR15	WAR16	WAR15	WAR16	WAR15	WAR16	WAR15	WAR16
Trial mean	0.798	0.749	227	225	438	378	743	577	6.77	8.93	513	437
Genotypic variance	0.00018	0.00028	2642	1338	1536	539	2760	11,378	1.22	2.68	651	352
Error variance	0.00025	0.00027	3418	960	2450	829	2242	1746	0.39	1.26	11,170	702
Population range[b]	0.760–0.828	0.701–0.781	40–352	113–304	302–544	317–429	606–911	514–664	3.61–10.07	5.74–13.76	446–577	400–483
Heritability	0.25	0.19	0.28	0.21	0.26	0.15	0.25	0.15	0.28	0.24	0.24	0.13
Genotypic correlation	0.55		0.35		0.27		0.64		0.53		0.31	

[a] Maximum leaf greenness (Nmax); onset-senescence (OnS); mid- senescence (MidS); end-senescence (EndS); maximum senescence rate (SR); stay-green integral (SGint).

[b] Population range for MR-NAM only.

missing rate across all families greater than 10 % were excluded, as were SNPs with a missing rate of over 50 % within a family. Some SNPs were excluded based on inconsistencies between family and donor genotypes. Single nucleotide polymorphisms for some genotypes were imputed based on family information (identity by descent) or, if this was uncertain, by following a random forest process imputed in NAM package (Xavier et al., 2015) in R (R Core Team, 2019).

Quantitative trait loci analysis was then conducted on 20 families, including up to 845 MR-NAM RILs, depending on the experiment and trait. A single-stage analysis of raw phenotypic data, molecular markers and population design was used for the genetic analyses for each experiment, using a QTL analysis method that was developed to take the structure of the MR-NAM population into account. This QTL method used markers from the whole genome to analyse phenotypic and genotypic data in a single process called whole genome nested association mapping (WG-NAM; Paccapelo et al., 2018). The method was based on an analytical approach applied to MAGIC populations (Verbyla et al., 2014), and adapted for the structure in the MR-NAM population. It is based on a model for genetic effects, $\boldsymbol{g}$,

$$\boldsymbol{g} = \boldsymbol{P}_A\boldsymbol{a} + \boldsymbol{u}_p$$

where $\boldsymbol{P}_A$ is the (n_g x n_f n_m) matrix of allele probabilities for n_g RILs (with marker data), n_f founders and n_m markers, $\boldsymbol{a}$ is the (n_f n_m x *1*) vector of effects for each founder by marker allele and $\boldsymbol{u}_p$ is the (n_g x *1*) vector of polygenic effects. Additionally,

$$\boldsymbol{a} \sim N\left(0, \sigma_a^2\boldsymbol{I}\right) \text{ and } \boldsymbol{u}_p \sim N\left(0, \sigma_p^2\boldsymbol{I}\right)$$

A linear mixed model was fitted to the response variable for each of the stay-green traits in Table 2. The linear mixed model included an overall mean, as well as a fixed effect with a level for each genotype in the experiment without marker data. The model also included the above term for genetic effects **g** as a random effect, together with random effects for design terms and extraneous spatial effects, with a residual spatial covariance structure described in the statistical methods. All designs were generated using the optimal design methods described in (Butler et al., 2008) using the R statistical package od, available from https://mmade.org/optimaldesign/ (Butler, 2018).

The use of whole genome analysis in the initial step avoids

drawbacks arising from multiple sampling, occurring in many other QTL analysis methods (Paccapelo et al., 2018). These drawbacks led to a requirement for extreme probability thresholds for testing QTL significance. By analysing the phenomic and genomic data in a one-step process, precision of QTL detection is also improved by taking into account error in both data sets. Alternative, two-step QTL analyses take predicted means from phenotypic analysis and use these in the genomic analysis, usually without adequately accounting for the error around each phenotypic mean. The WG-NAM method also enables calculation of the QTL effects in each NAM family. This information is important for breeders, because it facilitates identification of the sources of the most promising alleles at particular loci for specific genetic backgrounds.

A significance level of 0.05 was used to identify QTL. A significance level of 0.10 probability of association was used to identify parents that were the primary contributors of either favourable or unfavourable alleles.

Single nucleotide polymorphism markers associated with the identified QTL were mapped to the International Wheat Genome Sequencing Consortium (IWGSC) wheat sequence v1.0 (URGI, (Unité de Recherche Génomique Info, French National Institute for Agriculture, Food and Environment; INRAE), 2020) by using the BLAST algorithm on the Graingenes (2020) platform.

Linkage disequilibrium was calculated between pairs of SNPs associated with QTL using the R-package snpStats (Clayton, 2019). Single nucleotide polymorphisms were considered to be associated with the same QTL when linkage disequilibrium was greater than 0.50. R-squared was the statistic of choice to measure linkage disequilibrium.

3. Results

3.1. The two experiments experienced end-of-season water stress with important differences

In both WAR15 and WAR16 plants experienced moderate to severe water-stress of short duration, mainly after anthesis (Fig. 1; Fig. S1). Thus they were both classified into the same broad environment type, ET2, as defined by Chenu et al. (2013).

However, closer examination of the conditions in the two experiments revealed important differences, with WAR16 plants experiencing a more severe stress (Fig. S1). We examined days to anthesis, plant height and grain yield expressed by the founders at each site (Table 4). Environment mean was calculated using all genotypes grown in each trial, which included founders, MR-NAM lines, and standard and other experimental genotypes grown at that site in that year. WAR15 was sown 41 days earlier in the year (Table 1, Fig. 1) and the mean duration to flowering was 29 days longer than for WAR16 (Table 4). Thus, it is likely that the greater soil moisture at planting (669 mm compared with 377 mm), as well as the longer crop duration and cooler conditions, particularly late in the season, were major contributors to the 1.7 t ha^{-1} higher mean yield in WAR15, despite the greater in-crop rainfall experienced by WAR16 (Fig. 1; Tables 1, 4). Heritability for these traits (for all genotypes grown at each trial), was similar between experiments and was greater for plant height than for days to anthesis or grain yield (Table 4). Genetic correlations between the WAR15 and WAR16 trials, based on all genotypes, were moderate to high, ranging from 0.59 for grain yield to 0.89 for plant height. Thus, genotype performance was more consistent for plant height between the two environments, compared with days to anthesis and yield (Table 4).

Mean and heritability of stay-green traits for each trial, as well as genotypic correlation between trials are shown in Table 5. The mean maximum greenness was similar in the two trials, as was time to onset-senescence. However, as senescence progressed through the mid-point to completion of leaf senescence the difference between the two experiments increased, as a result of increased post-anthesis water stress experienced in WAR16 (Fig. S1). Leaf greenness was retained for longer in WAR15 than in WAR16 (end-senescence, Table 5). This was also reflected in the greater senescence rate for WAR16 and greater stay-green integral for WAR15.

Heritability for all traits was lower in WAR16 than WAR15. Genetic correlation expressed among the two trials varied considerably between traits, ranging from a low of 0.27 for mid-senescence to a high of 0.64 for end-senescence.

3.2. Quantitative trait loci identified for stay-green traits were widely distributed across the genome

Twenty-seven QTL were identified in WAR15 and 41 in WAR16. They were distributed across 17 linkage groups. Some QTL were associated with more than one stay-green trait. Thus, there were a total of 82 stay-green trait by loci associations (Table S2). They were (chromosomes in parenthesis and number of QTL where more than one was identified): maximum greenness (4A × 2, 4B, 6A, 6B × 2, 7A × 2, 7B), onset-senescence (1A × 3, 1D, 2B × 2, 3A, 3B, 4A, 4B, 4D × 2, 5A × 2, 5B × 4, 6B, 7B), mid-senescence (2A, 3A, 3B, 4A × 2, 4B, 5A × 2, 6A, 7B × 2, 7D × 2), end-senescence (2B, 3A, 3B × 2, 4A × 2, 4B, 5A × 2, 5B, 6B × 2, 7A, 7B, 7D), maximum senescence rate (1A, 2B, 3A, 4A, 4D, 5B × 3, 6B, 7D) and stay-green integral (1A, 3A, 3B, 3D, 4A × 2, 4B × 2, 5A, 5B, 6A, 7A, 7B × 2, 7D) (Fig. 4, Table 6).

In several instances, despite close linkage being indicated by the marker location on the consensus map, linkage disequilibrium was low (<0.5). For example, CI3027174, CI1102883 and CI1092615 on linkage group 6B are indicated to lie within a 0.51 cM span on the consensus linkage map, but no linkage disequilibrium was indicated between these markers when tested pairwise. This is reflected in the large separation of these markers on the sequence map (over 1.4 Mbp; Table S2), and is likely the result of the different germplasm contributing to the linkage map compared with the MR-NAM population.

Stay-green QTL of interest were defined as either (i) those explaining greater than five percent of variation for the trait or (ii) those that coincided with QTL for yield or height (Table 6). As the allele probabilities were calculated from information for the smaller numbers of genotypes in the families of a particular donor parent (Totals column, Table 3), the allele probability values for each donor are generally larger than those calculated using all genotypes in the whole MR-NAM population.

At least some of the MR-NAM founders carry inter-specific translocations, particularly for rust resistances. For example, Drysdale, EGA Gregory, EGA Wylie, Mace and Suntop carry Sr2 from *T. turgidum* on 3BS. EGA Wylie carries Sr36 from *T. timopheevi* on 2BS. Mace, Scout and Suntop carry Sr38 (with Lr37 and Yr17) from *V. ventricosum*on 2AS (Borlaug Rust Initiative, 2021; Park et al., 2020; Wellings et al., 2011). It is not always reported or known if the breeding line founders carry these or other translocations. Drysdale does contribute a stay-green allele on chromosome 3B (QSG.qwr-3B.4), which may be associated with the translocation. Neither of the translocations associated with Sr36 or Sr38 appear to be associated with stay-green alleles in that no QTL of interest appear on those chromosomes contributed by the relevant parents.

There was generally a clear distinction between the likelihood of association between the allele of the contributing donors of the favourable or unfavourable alleles and the other donors. For example, for QSG.qwr-1A.4 associated with stay-green integral, the values for families with significant allele probability ($P < 0.1$) were for the Drysdale families ($P = 0.090$, effect +4.385) and the Mace families ($P = 0.002$, effect -8.807), while for other donors the probabilities ranged from 0.191 to 0.497 (not shown in Table 6). Exceptions included QSG.qwr-4A.1 and QSG.qwr-4B.3, for which no individual donor had an allele probability of < 0.10, despite significant associations at the whole population level (Table 6; Table S2). Some of the QTL had more than one parent showing a significant allele probability for either favourable or unfavourable alleles. For example, QSG.qwr-3A.2 had favourable alleles contributed by both Mace and SeriM82, that is, they both had allele probabilities of less than 0.10.

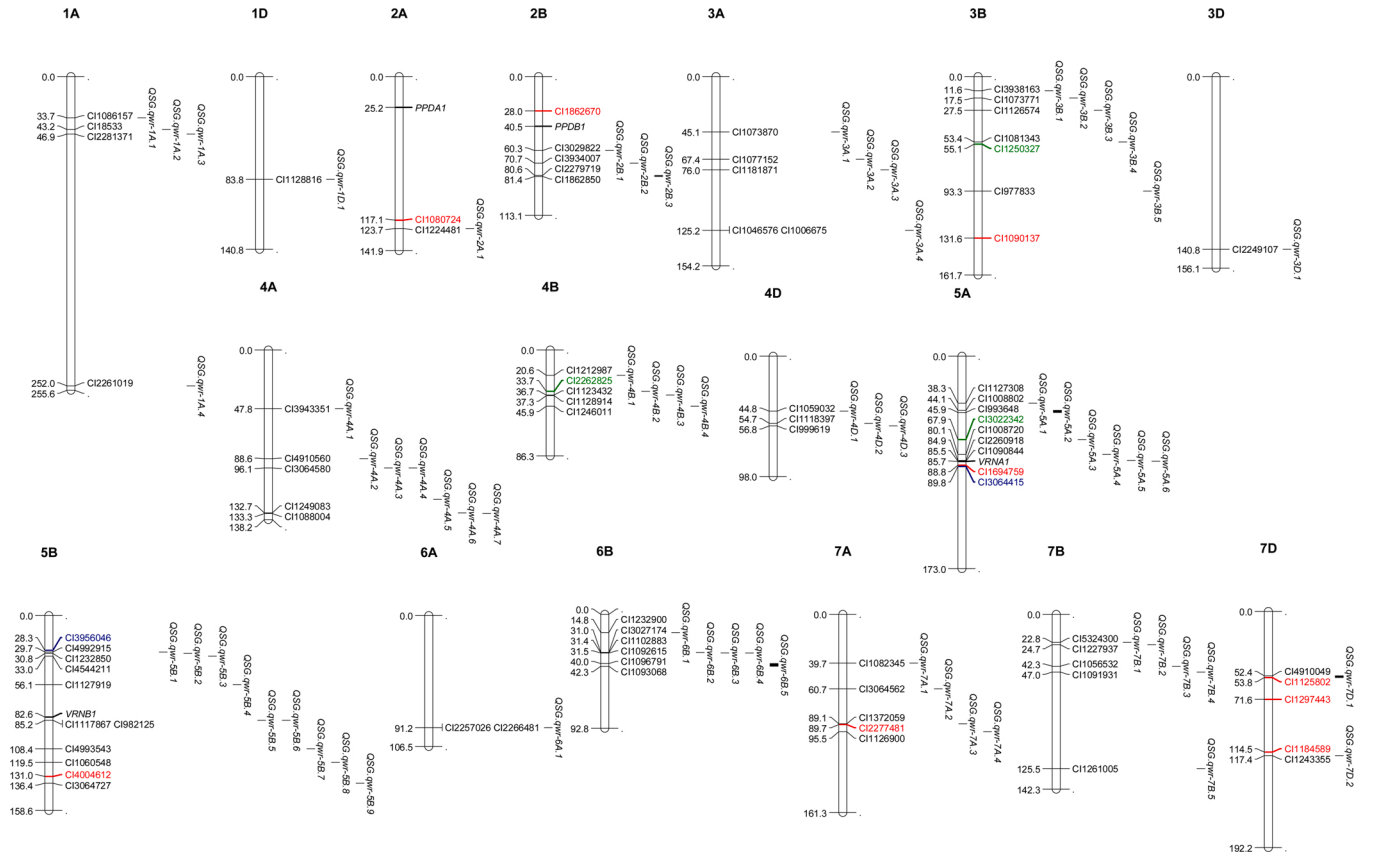

Fig. 4. Stay-green quantitative trait loci are distributed across 17 linkage groups. Some co-located with quantitative trait loci for plant height (Ht; green), and grain yield (Yld; red). Clone locations (DArT consensus linkage map v.4) harbouring SNP associated with days to anthesis (DTA; navy) identified in this study (refer to Table S2), as well as vernalisation (VRN) and photoperiod response (PPD) consensus map locations are included. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

SNP markers associated with stay-green QTL were mostly positioned in the expected order on the IWGSC v1.0 sequence (Table S2), but there were some discrepancies between the linkage and sequence maps. For example, QSG.qwr-4A.7, identified in both WAR15 and WAR16 for three different stay-green traits, located to chromosome 7A on the IWGSC but the 4A linkage group on the DArT reference linkage map (DArT (Diversity Array Technology), 2018). Further, there are several examples of markers for QTL mapping to linkage groups homoeologous to the identified chromosome location, or disparate orders within chromosomes and linkage groups. These discrepancies may be partly the result of different wheat germplasm used to develop the consensus map and the sequence, some are likely to be assembly or mapping errors, which will be reduced as maps and sequences are refined over time.

3.3. *Some stay-green QTL were associated with multiple traits and/or experiments*

Of the 82 associations identified for stay-green traits in this study, 12 were associated with more than one stay-green trait (Table 6; Table S2). Two of these QTL were identified in both experiments, QSG.qwr-4A.7 and QSG.qwr-7D.1. Co-location of stay-green traits was not unexpected due to the clear inter-relatedness of the SG traits calculated for the fitted NDVI logistic curve (Table 2; Fig. 2; Christopher et al., 2016).

3.4. *Some stay-green QTL were associated with QTL for yield or height, but not days to anthesis*

In total, fourteen QTL were detected for yield and two for days to anthesis (Fig. 4; Table S2). Thirty five QTL were identified for plant height,with only those associated with stay-green QTL shown inFig. 4 and Table S2. Thus, despite moderate selection, during population development, for days to anthesis and plant height similar to the reference parent, QTL for these traits were still evident.

Three stay-green QTL were co-located with yield QTL. QSG.qwr-4A.4 in WAR15 and QSG.qwr-4A.5 in WAR16 each associated with maximum greenness co-located with QYld.qwr-4A.2 and QYld.qwr-4A.3 respectively (Table 6; Fig. 4). QSG.qwr-7D.1 associated with multiple stay-green traits in both WAR15 and WAR16 coincided with QYld.qwr-7D.1 in WAR16 (Table 6; Fig. 4). Correlations (R^2) between maximum greenness and yield of genotypes were of 0.31 and 0.25 in WAR15 and WAR16, respectively.

Two stay-green QTL were located near QTL for height in WAR15. QSG.qwr-3B.4 and QSG.qwr-4B.2, each associated with end-senescence, were linked with QHt.qwr-3B.1 and QHt.qwr-4B.1 respectively. QHt.qwr-4B.1 and/or QHt.qwr-4B.2 are likely to coincide with the major dwarfing gene *Rht-B1*. QSG.qwwr-5A.3 identified in WAR16 coincided with QHt.qwr-5A.1 in WAR15 (Table 6; Fig. 4).

Three *VRN* genes are major regulators of the response of wheat to temperature and day-length, which influences phenology, particularly spring growth habit. *VRN* genes have been shown to have pleiotropic effects on root architecture in wheat and barley (Voss-Fels et al., 2018), which is intrinsically associated with drought tolerance. QDTA.qwr-5A.1 mapped close to *VRNA1* on the DArT reference linkage map, but QDTA.qwr-5B.1 was distant from *VRNB1*. No days to anthesis or stay-green QTL were located on linkage group 5D, which carries the third major vernalisation gene, *VRND1*. No stay-green QTL exhibited linkage disequilibrium with identified days to anthesis QTL, but several mapped close by (Fig. 4; Table S2). The other major gene series determining plant response to day length are the photoperiod sensitivity genes (*Ppd*). No stay-green QTL showed association with either *PPDA1*

Table 6

Quantitative trait loci (QTL) of interest identified for stay-green traits and QTL with which they co-located. Stay-green QTL of interest were defined as either (i) those explaining greater than five percent of variation for the trait or (ii) those that coincided with QTL for yield or height. Quantitative trait loci were identified in the multi-reference nested association mapping population in at least one of two experiments, WAR15 and WAR16. Linkage group (LG) position and experiment in which they were identified are indicated. Boxes indicate linkage disequilibrium > 0.5. Range of significant effects from minimum (Min) to maximum (Max) as well as contributing founders and probabilities (Allele prob) for favourable and unfavourable alleles were derived from the whole genome nested association mapping analysis of the QTL effects of the identified maximum probability single nucleotide polymorphism (SNP). Parental allele designations (Allele) are "0" =matching allele homozygote, "1"= alternate allele homozygote. A complete list of QTL identified for stay-green and agronomic traits is presented in Table S2.

QTL[a]	LG	Position (cM)	Trait[b]	Experiment	SNP				Range of significant effects[d]		Favourable allele[e]			Unfavourable allele[e]		
					Clone ID	Allele	SNP pos	% var[c]	Min	Max	Contributor[f]	Allele[g]	Allele prob[h]	Contributor[f]	Allele[g]	Allele prob[h]
QSG.qwr-1A.1	1A	33.69	OnS	WAR16	1,086,157[ps]	C/T	50	13.8	−20.318					Dharwah dry	1	0.013
	1A	33.69	SR	WAR16				4.4	−0.468					Dharwah dry	1	0.026
QSG.qwr-1A.4	1A	252.03	SGint	WAR16	2,261,019	T/C	29	8.6	−8.807	4.385	Drysdale	1	0.090	Mace	0	0.002
QSG.qwr-2B.2	2B	70.74	OnS	WAR16	3,934,007	G/A	7	18.3	−14.459	11.218	Suntop	0	0.040	Mace	0	0.021
QSG.qwr-3A.2	3A	67.38	SGint	WAR16	1,077,152	G/C	37	7.8	−6.262	4.299	Mace	1	0.062	SB062	0	0.049
										7.039	SeriM82	0	0.038			
QSG.qwr-3B.2	3B	17.45	SGint	WAR16	1,073,771	T/G	51	9.7	−5.187	8.49	Dharwah dry	0	0.017	Drysdale	0	0.096
									−6.283					Mace	1	0.016
QSG.qwr-3B.3	3B	27.51	MidS	WAR16	1,126,574	A/G	37	7.2	−7.140	8.085	Dharwah dry	1	0.037	Mace	0	0.026
QSG.qwr-3B.4	3B	53.40	EndS	WAR15	1,081,343 [ps]	A/C	21	2.1	−11.800	12.026	Drysdale	0	0.034	Mace	1	0.073
QHt.qwr-3B.1	3B	55.13	Ht	WAR15	1,250,327 [ps]	A/T	15	3.1	−3.215	2.164	Drysdale	1	0.050	Scout	0	0.024
QSG.qwr-4A.1	4A	47.80	MidS	WAR16	3,943,351 [ps]	C/G	12	7.6	−5.368	7.148	[f](Suntop	0	0.106)	(EGA Wylie	1	0.174)
	4A	47.80	SGint	WAR16				5.3	−4.571	5.773	(Suntop	0	0.116)	(EGA Wylie	1	0.172)
QSG.qwr-4A.4	4A	96.08	Nmax	WAR15	3,064,580	T/A	8	2.7	−0.002	0.003	Drysdale	1	0.023	Suntop	0	0.092
QYld.qwr-4A.2	4A	96.08	Yld	WAR15				3.2	−12.803	13.462	Drysdale	1	0.021	Suntop	0	0.041
QSG.qwr-4A.5	4A	121.70	Nmax	WAR16	1,695,994 [ps]	C/T	41	26.6		0.015	Mace	0	0.006			
QYld.qwr-4A.3	4A	121.70	Yld	WAR16				1.4		7.689				Drysdale	1	0.049
QSG.qwr-4A.7	4A	133.29	EndS	WAR15	1,088,004 [ps]	T/A	22	14.2	−16.385	32.612	Mace	0	0.002	Suntop	0	0.078
	4A	133.29	OnS	WAR16				4.0	−8.997	10.526	Dharwah dry	1	0.052	(Mace	0	0.101)
	4A	133.29	SR	WAR16				5.7	−0.440					Mace	0	0.046
QSG.qwr-4B.2	4B	33.74	EndS	WAR15	2,262,825	A/G	43	3.5	−13.014	16.282	Mace	1	0.003	Dharwah dry	0	0.047
QHt.qwr-4B.1	4B	33.74	Ht	WAR15				1.0		1.6	Mace	1	0.025			
QSG.qwr-4B.3	4B	36.73	MidS	WAR16	1,123,432 [ps]	C/G	9	5.3		7.638	(EGA Gregory	1	0.103)			
	4B	36.73	SGint	WAR16				3.1		5.73	(EGA Gregory	1	0.117)			
	4B	37.29	OnS	WAR16	1,128,914 [ps]	G/C	37	1.4	−6.035	5.468	(EGA Gregory	1	0.178)	(Suntop	0	0.155)
QSG.qwr-4D.3	4D	56.76	SR	WAR16	999,619	A/C	17	6.1		0.373	Suntop	0	0.008			
QHt.qwr-5A.1	5A	67.87	Ht	WAR15	3,022,342	G/A	18	0.2	−0.818					Mace	1	0.057
QSG.qwr-5A.3	5A	67.87	MidS	WAR16				3.6	−5.042					Mace	1	0.023
QSG.qwr-5A.4	5A	80.08	EndS	WAR15	1,008,720 [ps]	G/T	57	5.1	−18.531	23.535	Mace	1	0.037	Westonia	0	0.078
QSG.qwr-6A.1	6A	91.17	MidS	WAR16	2,257,026 [ps]	C/T	66	5.9	−5.384					SB062	1	0.087
	6A	91.17	Nmax	WAR16	2,266,481 [ps]	C/T	54	1.9		0.006	SB062	1	0.055			
	6A	91.17	SGint	WAR16				3.9	−6.430					SB062	1	0.04
QSG.qwr-6B.4	6B	31.46	Nmax	WAR15	1,092,615 [ps]	C/T	26	7.2	−0.003	0.004	Suntop	0	0.032	Drysdale	1	0.086
														Mace	0	0.093
QSG.qwr-7B.2	7B	24.71	MidS	WAR16	1,227,937 [ps]	A/G	58	9.0		9.510	Mace	0	0.076			
	7B	24.71	OnS	WAR16				4.5	−10.373	10.877	Mace	0	0.099	(Westonia	1	0.111)
QSG.qwr-7B.3	7B	42.30	EndS	WAR15	1,056,532 [ps]	A/G	60	11.2		31.182	Scout	0	0.003			
QSG.qwr-7B.4	7B	47.00	SGint	WAR16	1,091,931 [ps]	T/C	13	11.5	−6.891	8.459	Mace	0	0.048	Dharwah dry	1	0.068
	7B	47.00	MidS	WAR16				2.6	−5.373					Dharwah dry	1	0.079
QSG.qwr-7D.1	7D	52.39	EndS	WAR15	4,910,049	T/C	10	6.1	−20.882	22.489	ZWW10.50	0	0.016	SB062	0	0.004
	7D	52.39	MidS	WAR15				8.0	−13.205	11.449	UQ114	1	0.058	SB062	0	0.002
									−8.177	9.767	Suntop	1	0.020	SeriM82	0	0.054
	7D	53.84	SGint	WAR15	1,125,802	A/G	21	7.9	−9.314	5.133	Mace	0	0.067	SB062	1	0.001
									−5.474	5.887	Suntop	0	0.033	SeriM82	1	0.053
									−5.565					ZWB10.37	1	0.085
	7D	53.84	EndS	WAR16				9.3	−9.973	11.352	Suntop	0	0.033	SB062	1	0.062
									−14.494					ZWB10.37	1	0.028

(*continued on next page*)

Table 6 (*continued*)

QTL[a]	LG	Position (cM)	Trait[b]	Experiment	SNP			% var[c]	Range of significant effects[d]		Favourable allele[e]			Unfavourable allele[e]		
					Clone ID	Allele	SNP pos		Min	Max	Contributor[f]	Allele[g]	Allele prob[h]	Contributor[f]	Allele[g]	Allele prob[h]
	7D	53.84	SGint	WAR16				6.7	−5.141	6.278	Suntop	0	0.018	SB062	1	0.056
			SR	WAR16				4.3	−0.382	0.364	ZWB10.37	1	0.050	Suntop	0	0.014
QYld.qwr-7D.1	7D	53.84	Yld	WAR16				1.7	−5.444	5.929	Suntop	0	0.056	SB062	1	0.088
										0.443	Mace	0	0.088			

[a] QTL were named according to McIntosh et al. (2003) (Table 6). SG represents stay-green; qwr represents Queensland wheat research; followed by chromosome number and an Arabic numeral when multiple QTL were detected on one linkage group.

[b] Maximum leaf greenness (Nmax), onset-senescence (OnS), mid-point of senescence (MidS), near completion of leaf senescence (EndS), maximum senescence rate (SR), stay-green integral (SGint).

[c] The percentage variance explained overall refers to the proportion of variance for the entire MR-NAM population explained.

[d] The range of effects is the range represented by the alleles with probabilities of < 0.10 within donor families. For the MR-NAM population as a whole, all QTL had probability < 0.05.

[e] Greater values for each trait generally contribute to greater greenness, later senescence and a greater integral of leaf greenness, which are all favourable for producing a stay-green phenotype. Donors for favourable or unfavourable alleles listed with allele probability of < 0.10.

[f] Contributors listed for favourable or unfavourable alleles were identified as those with allele probabilities of < 0.10. When no donors were identified with allele probabilities of < 0.10, the donor with the lowest allele probability was cited in brackets.

[g] Alleles were identified as either "matching" ("0"), when sequence matched the wheat reference used by DArT, or "alternate" ("1"), which carries the alternate base pair at that locus (Li et al., 2015). Allele probability refers to the allele within the NAM donor families contributing favourable or unfavourable alleles.

[h] Allele probability refers to the allele within the NAM donor families contributing positive or negative alleles. For example, for QSG.qwr-1A.4, it refer to the values for the Drysdale and Mace families respectively. [ps] parent-specific allele.

or *PPDB1* (Fig. 4).

Earliness *Per Se* (EPS) are another class of genes that affect aspects of development rate, which are independent of responses to vernalisation and photoperiod (Komugi, 2012). Loci associated with EPS have been identified on linkage groups 1B, 1DL, 3AS, 3AL and 5BL (Blake et al., 2019). Of these regions, only 3A has been identified to carry a stay-green QTL of interest in this study (QSG.qwr-3A.2). This locus was previously mapped at the end of linkage group 3AS (0.0 cM) by Shah et al. (1999) while QSG.qwr-3A.2 is located at 67.4 cM.

3.5. Sources of promising alleles for stay-green traits could be identified

Information on the sources of the most promising alleles at particular loci for specific genetic backgrounds is important for breeders. The MR-NAM population in combination with the WG-NAM QTL analysis could be used to do this. "Parent-specific" alleles were defined as those for which fewer than 200 of either the "1" or "0" alleles were represented in the 845 RILs, where for homozygous genotypes both alleles were counted, and for the heterozygous genotypes one of each allele was counted. All parent-specific alleles identified were contributed by either one or two of the 12 founders (data not presented). Overall, 30 of the SNPs associated with stay-green QTL expressed alleles that were parent-specific in this MR-NAM population (Table S2). Thirteen of those SNPs were associated with 11 stay-green QTL of interest (Table 6).

For example, the identification of QSG.qwr-3B.4 was based on 138 RILs from three bi-parental crosses, the parent-specific allele being contributed by Drysdale in WAR15 (Table 3). However, some parent-specific QTL were identified in a relatively small number of RILs, particularly if the parent-specific allele contributor was a "donor", for example, QSG.qwr-4B.3 identified in WAR16. EGA Gregory was the only parent contributing the alternate "1″ allele, and is represented in only one bi-parental cross, with Suntop, in a family of 37 RILs (Table 3). These QTL should be considered with greater caution by breeders but can be a useful indicator for further research where of sufficient interest.

Thus, the detection of uncommon alleles is one of the strengths of using a multi-parent population for identification of QTL. In most cases, the contributor of the parent-specific allele was identified as a donor for the QTL (i.e. having allele probability of < 0.10), for either the favourable or unfavourable allele. However, there were exceptions, for example, QSG.qwr.4A.5, which is represented by CI1695994, and associated with maximum greenness. For this marker, Drysdale was the donor of the donor-specific "1″ allele, but only Mace, with the "0" allele, showed a significant positive effect of 0.015. Suntop, also expressied the "0" allele, but in Suntop families this allele had a negative effect (-0.003), with a probability of 0.106. Drysdale had the next greatest (non-significant) allele probability of 0.179, and a negative effect (-0.005) (data not shown). Another example was QSG.qwr-7B.3, associated with EndS. Dharwah dry was the only donor of the rare "1" allele for CI1056532 (data not shown), but only when crossed with Scout, expressing the "0" allele, was the allele probability significant, with a relatively large effect (11.2 %). This situation, where significant allele probabilities depend on the parents, is indicative of how these alleles can perform differently in different genetic contexts (Table 6).

3.6. QTL were identified which explained greater than 5% of variation for stay-green traits

Eighteen stay-green QTL explained more than 5% of the variation for at least one stay-green trait, six of which explained more than 10 % of the variation. The variation explained for stay-green traits by the identified QTL ranged between 0.8 % for QSG.qwr-4D.1, associated with onset-senescence, and 26.6 % for QSG.qwr-4A.5, associated with maximum senescence rate (Table 6; Table S2). The average overall variation explained across traits was 4.3 %, with the average in WAR15 being 3.4 % and for WAR16 being 5.2 % (Table 6; Table S2).

The ranges in QTL effects for stay-green traits were, maximum leaf greenness −0.010 to +0.015; onset- senescence −20.3 to +11.2 °C d; mid-senescence −13.2 to +11.4 °C d; end-senescence −20.9 to +32.6 °C d; maximum senescence rate −0.47 to +0.47; and stay-green integral −9.3 to +8.5 (Table S2). The range of significant effects of alleles is presented in the units used to measure that trait. Comparisons can be made within a stay-green trait. If comparing between traits these ranges should be viewed in the context of the overall variation for that trait expressed in each experiment (Table 5).

3.7. Both favourable and unfavourable alleles were contributed by both donors and reference parents

The greatest values for individual stay-green traits were not consistently expressed by particular founders (Table S1). Furthermore, the donor with the greatest value for each trait was not consistent between environments. The exception was for maximum leaf greenness, where Westonia expressing the greatest maximum leaf greenness and Mace the least in both experiments.

Parents identified as the primary contributor for either favourable or unfavourable alleles were those with probability of association of < 0.10 in the families to which they contributed (Table 6). Most founders contributed both favourable and unfavourable alleles for stay-green traits, even for the same trait. For example, Drysdale is the donor of the favourable allele for QSG.qwr-1A.4 and the unfavourable allele for QSG.qwr-3B.2, both associated with stay-green integral.

In cases of an allele being contributed by only one or two founders, the relationship between the allele and the effect appears straightforward in most cases. For example, for QSG.qwr-3B.4 associated with end-senescence, SNP CI1081343 was represented by the alternate allele "1" in all donors except Drysdale, which had the "0" allele. The favourable effect of the alternate allele "1" on end-senescence was significant in families with Drysdale as a parent. Hence, in general, the "1" allele should be selected for when trying to increase the duration from flowering to end-senescence, at least where Drysdale is a parent.

In other situations, it is clear that alleles are behaving differently in different genetic backgrounds. For example, for QSG.qwr-4A.7, the SNP CI1088004 was represented by the matching "0" allele in all donors except Dharwah dry "1". However, a favourable effect was contributed to end-senescence by Mace, and an unfavourable effect by Suntop in WAR15, both of which express the matching allele "0". This QTL is also associated with onset-senescence and maximum senescence rate in WAR16. In this experiment, Dharwah dry had a significant favourable effect on onset-senescence and Mace the unfavourable effect for both onset-senescence and maximum senescence rate. This suggests that the effect of this QTL is influenced strongly by both the genetic context and physical environment in which it is expressed. In this case, the choice of which allele to select to improve end-senescence will depend on the specific parentage of the genotype and the target environment.

4. Discussion

4.1. Environment influences stay-green trait expression

The expression of stay-green, and individual stay-green traits is strongly influenced by drought environment type (Christopher et al., 2016). In both WAR15 and WAR16, plants experienced moderate to severe water-stress of short duration, mainly after anthesis (Fig. S1), leading to their classification as the same environment type (ET2; Chenu et al., 2013). Christopher et al. (2016) found that in ET2 environments, maximum greenness was the stay-green trait which tended to be positively associated with yield. The current study was consistent with this finding, in that of the three stay-green QTL co-located with a yield QTL, two (QSG.qwr-4A.4 and QSG.qwr-4A.5, identified in WAR15 and WAR16 respectively) were associated with maximum leaf greenness. Maximum greenness and yield of genotypes were moderately, positively correlated at the two sites.

Quantitative trait loci were identified for each stay-green trait in both experiments, but only two stay-green QTL were detected in both experiments (Table 6; Table S2). While the two sampled environments had similar timing of crop stress relative to anthesis, more severe drought stress was experienced by plants in WAR16 (Fig. S1). Furthermore, differences in other environmental factors, including stored soil moisture at planting, planting time, in-crop rainfall and temperature, may have also influenced expression of stay-green (Tables 5, 6), as well as agronomic traits (Table 4). Thus, as has been found previously (Christopher et al., 2018), genotype by environment interactions can be large for stay-green.

As the season progressed, differences between expression of stay-green traits at each experimental site increased, with expression of stay-green greater in WAR15 for all traits except senescence rate (Table 5). This is likely due to WAR15 experiencing considerably less in crop rainfall, so onset of water deficit may have been sooner, and a longer season, so the rate of senescence could be slower, compared with WAR16 (Table 1; Fig. 1; Fig. S1). This further emphasises the strong association between stay-green expression and the environment. Heritabilities for all stay-green traits were greater for the WAR15 trial than the WAR16 trial, suggesting more within-site variability in WAR16, but were of moderate magnitude for all traits (Table 5). This indicates that selection for these traits can be successful but the effect will likely vary with environment. Thus, characterisation of selection environments is valuable to understand which stay-green traits contribute most to the stay-green phenotype in different environment types, and which, if any, may be detrimental in particular target environments (Chenu, 2015; Chenu et al., 2011, 2013).

The ability of a genotype to modify phenotypic expression in response to different environmental conditions can also be referred to as phenotypic plasticity (Ungerer et al., 2003), and arises from interactions between QTL and environments. Thus, although relatively large genotype by environment interactions make selection for stay-green difficult for breeders, such genotype by environment interactions may work in favour of adaptability of genotypes to unpredictable conditions regarding water availability.

4.2. Some QTL co-located with those identified in other studies, while others are likely novel

We utilised an MR-NAM population, constructed specifically to develop breeding lines for tolerance to high temperatures and water deficit, and to identify QTL for adaptation traits. This permitted the investigation of a wide range of potential QTL conferring drought tolerance, in different combinations, in multiple elite adapted genetic backgrounds. As mentioned above, the MR-NAM design simultaneously exploits the advantages of both linkage analysis and association mapping to enable precise identification of QTL (Gage et al., 2020). It also assesses their expression in commercially relevant genetic backgrounds and growing conditions (Yu et al., 2008; Richard, 2018 for description of the MR-NAM used in this study).

Quantitative trait loci associated with stay-green traits were identified on seventeen chromosomes. Fifty-eight of the 65 QTL identified were from the A and B genomes, with only seven identified from the D genome (with QSG.qwr-4D.3 and QSG.qwr-7D.1 the most significant) (Fig. 4; Table S2), the D genome being the least diverse of the bread wheat genomes (Mirzaghaderi and Mason, 2019). This suggests that the genetic determination of stay-green may be different between the ancestral progenitors represented by the three genomes. This could be utilized, by exploiting wheat progenitors for novel alleles or loci influencing stay-green. This finding is contrary to the report of Acuña-Galindo et al. (2015), who found in a meta-analysis of 25 studies investigating hexaploid wheat, that QTL associated with adaptation to drought and heat stress were evenly distributed among the three wheat genomes. Further, it contrasts with reports of a higher density of stress-related expressed sequence tags mapping to the D genome (Ramalingam et al., 2006). It has been suggested that the D genome progenitor of wheat, *Aegilops tauschii*, is a rich source of stress-tolerance alleles in synthetic-hexaploid wheats (Trethowan and Mujeeb-Kazi, 2008). These authors described several studies indicating derived synthetic-hexaploids, which were derived from a diverse set of *A. tauschii* D genome donors, were often superior in yield to their adaptive recurrent parents and best local check cultivars under drought stress conditions (e.g. Azizinya et al., 2005; Dreccer et al., 2007; Ogbonnaya et al., 2007; Trethowan et al., 2000, 2003).

It is difficult to compare QTL locations identified in different studies. Firstly, because stay-green is a complex phenotype to measure, and a wide range of proxy traits have been used to measure it, such as flag leaf characteristics, chlorophyll content and NDVI. Measurements can also be made at various stages of plant development. Secondly, because of the rapid development in marker technologies and genotyping-by-sequencing technologies, different marker systems and maps have been used to locate QTL, including microsatellite-based maps, maps based on SNP chip technology, both array and sequence based diversity array technology (namely DArT P/L), and now whole genome sequencing (IWGSC v1.0). Some consensus maps have been developed as well as maps made using combinations of technologies, but alignment is still often difficult. Thirdly, environments in which populations were tested varied considerably in the degree of water stress experienced, and when that stress was experienced by the plants, in relation to time of anthesis. Finally, all of these factors interact with the specific genetic material tested. Comparisons between winter- and spring-wheats in relation to expression of stay-green is especially difficult, due to the different phenologies of these types and their different response to environmental cues. Further, because of its hexaploid nature, wheat has a tendency to retain both natural and induced genetic insertions, deletions and inversions, which means that the order of genes may vary considerably, even within the relatively narrow base of cultivated wheat (e.g. Liu 2019).

A selection of studies reporting locations of QTL relevant to stay-green in wheat has been summarised in Table S1. All linkage groups have been identified as harbouring stay-green related traits in at least one of these studies. Those QTL that explain a greater percentage of variation for a trait or are associated with multiple traits, including grain yield, and are more straightforward regarding identifying the origin of the favourable allele and consistency of how alleles behave in different backgrounds, are likely to be those of most immediate interest to breeders (Table 6).

QSG.qwr-1A.1, for example, explained 13.8 % of variation for onset-senescence and 4.4 % of variation for senescence rate in WAR16. When locations were compared by projecting maps, using total length of the 1A linkage group, several stay-green related QTL from other studies were identified in a similar location. For example, QTL have been identified for green leaf number (Shi et al., 2019) and for NDVI (Li et al., 2015; Shi et al., 2017; Gizaw et al., 2018) under well-watered conditions in a similar genetic location. The closest stay-green related QTL was one identified for transpiration efficiency, QTe.qwr-1A.1, 2.4 cM away in a subset of the Su-NAM population used in the current study (Fletcher, 2020).

QSG.qwr-2B.2 explained 18.3 % variation in onset-senescence in WAR16. Studies have identified stay-green relevant QTL on linkage group 2B for NDVI (Mason et al., 2018; Pinto et al., 2010), for NDVI and canopy temperature (Liu et al., 2019), for NDVI and SPAD (Graziani et al., 2014), for chlorophyll content (Shi et al., 2017), for leaf senescence (Li et al., 2015) and for stay-green *per se* (Gizaw et al., 2018). Acuña-Galindo et al. (2015) identified a MetaQTL, MQTL18, on 2B, which included a SG QTL, which was around 6 cM from QSG.qwr-2B.2.

QSG.qwr-4A.4 was associated with maximum greenness and yield. (Pinto et al., 2010) identified a QTL at this location associated with NDVI, under heat and drought conditions, in a population of spring wheats derived from SeriM82/Babax. This QTL may also correspond with the MQTL32 (Acuña-Galindo et al., 2015), which encompasses QTL

for stay-green and other drought related traits, for example QMRS.vij-4A (Vijayalakshmi et al., 2010).

QSG.qwr-4A.5 explained 26.6 % of variation of maximum leaf greenness and was associated with a yield QTL in WAR 16. Pinto et al. (2010) reported a QTL related to NDVI during grain-filling close to this QTL and Wang et al. (2015) reported QMrs-4A.1 for maximum rate of senescence under well-watered conditions. QSG.qwr-4A.7 explained 14.2 % of variation for end of senescence in WAR15, and was associated with onset-senescence and rate of senescence in WAR16. Both QSG.qwr-4A.5 and QSG.qwr-4A.7 co-locate with QSG.qgw-4A.2 identified by Christopher et al. (2018) for maximum greenness, onset-senescence, mid-point of senescence, stay-green integral and maximum senescence rate as well as yield and root number (qRN.qgw-4A.2), in a Ser-iM82/Hartog RIL population, in both water-stressed and unstressed environments. This QTL also may be associated with a transpiration efficiency QTL, QTe.qwr-4A.2 identified in the Su-NAM included in this study (Fletcher, 2020).

QSG.qwr-4B.3 was associated with mid-senescence, stay-green integral and onset-senescence. This QTL co-locates with QSG.qgw-4B, reported by Christopher et al. (2018), which also encompassed the *Rht-B1* dwarfing gene locus. It also co-located with 4B-b QTL associated with canopy temperature identified by Pinto et al. (2010) and MQTL34 (Acuña-Galindo et al., 2015) associated with QTL for stay-green and other traits expressed under drought and heat conditions (Vijayalakshmi et al., 2010; Zhang et al., 2010).

QSG.qwr-7B.3 explained 11.2 % of variation in end-senescence in WAR15 and QSG.qwr-7B.4 explained 11.5 % of variation for stay-green integral and 2.6 % variation for mid-senescence in WAR16. Both QSG.qwr-7B.3 and QSG.qwr-7B.4 may correspond with QSG.qgw-7B identified by Christopher et al. (2018), which encompassed QTL for maximum greenness, onset-senescence, mid-senescence, stay-green integral and senescence rate. They may also correspond with QTL identified by (Gizaw et al., 2018) for NDVI in a winter wheat association-mapping panel. Meta-QTL62 and 63 (Acuña-Galindo et al., 2015) are around 6 cM from QSG.qwr-7B.4 on the DArT reference linkage map and encompassed QTL for chlorophyll, time to maximum rate of senescence and 25 % leaf greenness (Vijayalakshmi et al., 2010). Pinto et al. (2010) also identified QTL for NDVI at grain filling on 7B.

QSG.qwr-7D. 1 was associated with multiple stay-green traits in both WAR15 and WAR16, as well as yield QTL in WAR16. This is near MQTL64 encompassing QTL for photosynthesis and water-soluble carbohydrates, but not stay-green *per se* (Acuña-Galindo et al., 2015). It is also near Q25 %G^h.ksu-7D and QTmrs^h.ksu-7D for time from 100 % to 25 % leaf green area and time to maximum rate of senescence (Vijayalakshmi et al., 2010). Other nearby QTL were QTs-7D for time of onset leaf senescence (Wang et al., 2015) and possibly Qv/Fo.cgb-7D for potential quantum yield of photosystem two photochemistry (Yang et al., 2007) and QTe.qwr-7D.1 for transpiration efficiency (Fletcher, 2020).

4.3. Some stay-green QTL were associated with multiple traits

The fact that some QTL were associated with multiple stay-green traits is not surprising, since either these traits are correlated (e.g. the three measures of timing of senescence) or input into the calculation of others (e.g. SGint is effectively a combination of the other stay-green traits). On the other hand, the traits do each contribute to the stay-green genotype in different ways, so genetic coincidence, and general consistency of parents contributing the favourable or unfavourable allele, does add some weight to the utility of the QTL for selection. QSG.qwr-7D.2 is of particular interest, as it was identified in both experiments, for multiple traits and explaining between 4.3 and 9.3 % of variance for these traits, and is one of the few QTL associated with stay-green from the D genome.

4.4. Stay-green QTL coincide with QTL for yield, plant height and days to anthesis

Three stay-green QTL coincided with yield QTL, two associated with maximum greenness, and a third associated with multiple stay-green traits (Fig. 4; Table 6; Table S2). This contrasts with the findings of Acuña-Galindo et al. (2015) who found in a meta-analysis of 30 studies, that half of QTL identified for stay-green were associated with yield QTL. It was shown by Christopher et al. (2016) that in environments classified as ET2 it is maximum greenness, of the stay-green traits, which is most clearly positively associated with yield. The current study was consistent with this finding.

Major genes associated with height and phenology, particularly *Rht* and *Ppd* genes, are frequently associated with stress-tolerance genes, either through direct association of the traits (for example, interaction between height and partitioning of carbohydrates) or through pleiotropic effects, as discussed by Acuña-Galindo et al. (2015). We found some associations.

QSG.qwr-4B.2, which is associated with end-senescence, co-locates with QHt.qwr-4B.1. This chromosome harbours one of the major dwarfing genes, *Rht-B1*, which does not appear on the DArT consensus linkage map. By combining information from Christopher et al. (2018) and the DArT consensus linkage map, *Rht-B1* is likely to be located at around 20 cM, which coincides with QSG.qwr-4B.1. QSG.qwr-3B.4 associated with end-senescence is in linkage disequilibrium with a QTL for plant height QHt.qwr-3B.1 in WAR15. QSG.qwr-5A.3 associated with mid-senescence in WAR16 coincides with QHt.qwr-5A.1 detected in WAR15.

4.5. Some "parent-specific" alleles associated with stay-green traits were identified

Favourable stay-green alleles were identified for some traits from multiple NAM families contributed by multiple donors (Table 5). In contrast, the favourable alleles for other traits came from only single families and single donor genotypes. Furthermore, it was possible to identify specific contributors for some QTL (by identifying those with allele probabilities < 0.10) while for other QTL there were no clear contributors. This demonstrates the power of the whole NAM to identify QTL that might not have been identified in individual families. This result confirms the utility of the MR-NAM approach to identify multiple sources of favourable alleles, as well as to identify relatively uncommon alleles coming from a single source. Those QTL identified in small families however may require validation before breeders will be confident in applying them. This information is important to direct stacking of multiple favourable alleles for target traits using marker-assisted selection strategies.

4.6. The percent of variation explained by the QTL was small to moderate

The percentage variation explained by each QTL for each stay-green trait was generally small to moderate, mostly in the order of 3–4%, so alleles for each trait, and different traits, should ideally be stacked to achieve the stay-green phenotype. This contrasts with other cereal plants, like sorghum and barley, which tend to have fewer loci of relatively large effect associated with stay-green (Gous et al., 2016; Harris et al., 2007). Six QTL were identified which explained more than 10 % and up to 27 % variation for stay-green traits, which alone could have significant impacts on the expression of the stay-green phenotype.

In wheat, the stay-green phenotype will need to be combined with drought avoidance strategies, such as root architecture or transpiration efficiency, to achieve a phenotype with tolerance to water deficit post-anthesis. To some extent these traits may be drivers of some of the

stay-green traits assessed in this study, as stay-green is an emergent consequence of the water supply-demand balance in crops. In both sorghum and barley, root architecture is closely associated with the stay-green phenotype (Mace et al., 2012; Borrell et al., 2014; Gous et al., 2016). In wheat, QTL for transpiration efficiency co-located with stay-green QTL in this population (Fletcher, 2020).

4.7. Senescent founders may harbour favourable stay-green alleles

Ranking amongst founders showed little consistency between traits (Table S1). The greatest values for individual stay-green traits were often not associated with the lines considered the most "stay-green". This suggests that it is a combination of traits, expressed at a moderate level, that contribute to the stay-green phenotype. This reinforces the belief that stay-green is a complex phenotype, and that multiple combinations of these stay-green traits can result in an overall stay-green phenotype in the plant.

Rankings amongst founders for expression of stay-green traits also differed between experiments (Table S3). Maximum leaf greenness was the only stay-green trait for which the founders expressing most and least greenness was consistent between the two experiments. This is consistent with this trait being the most dominant in driving stay-green in these types of environment, and with the two studied environment varying in drought intensity later during the grain-filling period. This further demonstrates the strong influence of genotype by environment interactions, and that stay-green traits may be expressed in different combinations to achieve a stay-green phenotype under different environmental conditions.

The impact of particular QTL on trait expression was context dependent. In some cases, the same allele could be associated with either a favourable or an unfavourable stay-green phenotypic effect, depending on genetic background. For example, for QSG.qwr-2B.2 founders contributing positive and negative effects on the stay-green trait, Suntop and Mace respectively, are both homozygous for the "0" matching allele. In other cases, like QSG.qwr-3A.2, both alleles were associated with favourable phenotypes. Both Mace and SeriM82 contributed alleles with favourable effects on the stay-green integral, but carried the "1" and "0" alleles respectively (Table 6). Thus, the expression of the stay-green phenotype is dependent on genetic background, that is, most likely due to epistatic and/or pleiotropic effects.

Many founders, whether stay-green or senescent types, contributed both favourable and unfavourable alleles for stay-green traits, even for the same trait (Table 6). This suggests there is considerable scope, through transgressive segregation, to develop germplasm with a more stay-green habit in elite backgrounds by combining alleles identified within this MR-NAM population. Further, it will be beneficial to intercross selected, promising MR-NAM RILs to combine positive alleles at different loci from multiple donors.

5. Conclusions

Stay-green is an important trait that can be utilised by wheat breeders to improve drought tolerance. The inheritance of this trait is complex in wheat compared with other crop plants, in that it is highly polygenic and subject to considerable genotype by environment interactions and epistatic effects. We have shown, that despite these challenges, breeding for stay-green, in combination with associated drought tolerance mechanisms, like root architecture or transpiration efficiency, can be effective. By using the MR-NAM population and some novel approaches to identifying QTL, a package of genetic material, matched selection tools and information about the impact of different alleles in different backgrounds for enhanced drought tolerance, was delivered to Australian plant breeders. These will contribute to improving resilience of wheat cultivars targeting environments likely to experience end-of-season water stress in the face of a drier future climate in Australia and globally.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.fcr.2021.108181.

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