The genetic improvement of wheat and barley for reproductive frost tolerance

By

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Chapter 4: Fine mapping the reproductive frost tolerance locus on chromosome 5HL in barley

Introduction

The initial mapping study identified two genetic loci associated with Reproductive Frost Tolerance (RFT) traits in three different doubled haploid populations (Chapter 2). The tolerance allele at the 5H RFT locus was closely linked in coupling with winter alleles of the major developmental gene *vrn*-H1 (Chen *et al.*, 2009b), and this might constrain its use in most Australian barley production environments where a winter growth habit is undesirable. In the major barley productions zones of Australia, a vernalisation requirement is inappropriate because the cold requirement for floral initiation is rarely met during the mild Australian winter period, leading to flowering late in the season when severe heat and drought stresses are encountered.

The 5H QTL identified in the three populations spanned approximately 10-15cM. Further refinement of this region using the DH populations would be difficult because these include few recombinants for the critical region (Chapter 2). The Galleon×Haruna Nijo DH population had 13 recombinants in this region and the Amagi Nijo×WI2585 had 15, and many of these recombinants were missing FIS data. The presence of the second major RFT locus segregating in these populations also contributes to error as average effects of the second locus need to be removed from the phenotypic data when completing single point marker regression analysis. The average molecular marker coverage of the most recent Galleon×Haruna Nijo and Amagi Nijo×WI2585 maps is low, with 1 marker per 3.2 and

10.7 cM respectively. This can also limit the ability to more accurately delimit the locus and to dissect closely linked loci.

One strategy to better define a target locus is to develop a mapping population that focuses on specific genomic regions of interest. A focussed mapping strategy has been effectively utilised when attempting to fine map a trait with prior knowledge of the target genetic location (Francia *et al.*, 2007). Populations can be selected that are homozygous for other potentially confounding loci. These potentially confounding loci may include other loci directly or indirectly involved in the expression of the trait of interest.

Access to reliable and repeatable phenotyping is an important part of any fine mapping study. The development of field based methods for RFT screening has enabled the identification of genetic variation for this trait, as well as its genetic analysis (Chapter 2). Although this has been the most reliable method of screening for RFT, there are limitations to the number of lines that can be tested and the throughput is also restricted to one cycle per year. Screening populations that are segregating for plant height or maturity are difficult to phenotype using the field screening system because these traits can confer frost avoidance. The unreliable nature of the severity and timing of natural frost events can mean discriminating data capture may not occur every season.

Controlled environment rooms have been used extensively to simulate frost events and to phenotype germplasm at the vegetative stages of development (Saulescu and Braun, 2001). Such facilities have been based on convective cooling which, when used in the context of RFT at the heading stages of development, have not been able to produce reliable results (Fuller *et al.*, 2007). The recent construction of a growth chamber at the Australian Genome Research Facility in Adelaide that is equipped with radiative cooling and detailed temperature control provides an opportunity to reliably screen more diverse germplasm that

are required for detailed genetic analysis. The application of a reliable and accurate temperature profile allows material to be treated under reproducible conditions in the absence of many of the potentially confounding effects inherent in field based screening.

The research described in this chapter was conducted to refine the genetic interval of the 5HL RFT locus. Germplasm and markers developed during this study may be useful to barley breeding programs for the more efficient selection of RFT. The genetic analysis may also provide an understanding of the relationship between the RFT phenotype and the *vrn*-H1 gene present within the target interval, and provide a platform for positional cloning of the gene(s) controlling RFT at this locus.

Materials and Methods

Marker development using rice synteny

The genomic sequence from the syntenous genetic region in rice was used to increase the marker coverage across the 5H RFT QTL interval. Sequences of known genes, including the cloned vernalisation responsive gene *vrn*-H1 and of RFLP probes mapped in the region (Yan *et al.*, 2003) were aligned to the complete rice genome sequence using BLASTn at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The physical location of these genes in rice and their mapped locations in barley could be determined, enabling the coliniarity between rice and barley for this region to be defined. Good coliniarity between rice and barley in this genetic region was expected based on a previous study (Sorrells *et al.*, 2003) which mapped wheat derived ESTs on the rice physical map.

Genomic sequences of other rice genes within the colinear 5H frost tolerance genomic region were also used for marker development. These sequences were BLASTed against the Triticeae EST database to identify ESTs from barley or wheat orthologues and to predict

intron position and size. Primers were designed within exon regions, matching sequence of the barley ESTs to amplify the putative introns identified in rice, targeting predicted barley genomic amplification product sizes in the range of 1000 -1500 base pairs. Primers were used to amplify a fragment from each of the parents of the mapping populations by PCR.

Reagents for 10µl PCR reaction:

DNA (75µg/µl approx.)	2µl
10x Immolase^{TM} buffer (incl. 15mM $MgCl_2$)	1µl
dNTPs (5µM)	1µl
$MgCl_2$ (50mM)	0.1µl
Primer-F (5µM)	0.5µl
Primer-R (5µM)	0.5µl
Immolase^{TM} DNA Polimerase (5µ/µl)	0.1µl
H_2O	4.8µl

PCR protocol:

1) 95°C for 7 minutes

2) 94°C for 10 seconds

3) 60°C for 30 seconds

4) 68°C for X minutes

5) Step 2-4 34 more times

6) 68°C for 10 minutes

7) End

X = Refer to table 1 for specific extension times for each marker used

The amplified fragments were then purified with a Qiagen MinElute® PCR purification kit using the protocol outlined by the manufacturer. Purified fragments were prepared using

the protocol outlined in the document titled 'GUIDE TO AGRF SERVICE SEQUENCING' (http://www.agrf.org.au) and then submitted to the Australian Genome Research Facility for automated capillary separation.

Sequences from both parents were aligned using the Vector NTI Advance 9.1.0 program ContigExpress to identify polymorphisms. In all cases a single nucleotide polymorphism was identified which could be recognized by digestion with a restriction enzyme. SNPs within restriction enzyme sites were used to produce different fragment sizes so either parental genotype could be identified by gel separation. Amplified parental fragments were digested with the identified restriction enzyme to confirm the polymorphism. Digestion conditions for each restriction enzyme (Table 1) were completed as recommended by the manufacturer (http://www.neb.com). A summary of the markers is provided in Table 1.

Table 1. Details of PCR markers used to fine map the RFT genomic region on chromosome arm 5HL. Markers developed in this study are indicated by 'JR'. Several PCR markers were previously developed by Anita Brule-Babel (Unpublished) and Fu *et al.* (2005). The BACs from the syntenous genomic region in rice used to predict intron location, sizes and the presence of the gene in this interval in barley are also noted. Specific PCR extension times, restriction enzymes and the base pair position of the restriction site where the SNP polymorphism resided are listed for each marker.

Marker	Source	Frag bp	R.Enz	Poly pos bp	Rice Chr.	Rice BAC	Forward Primer 5'-3'	Reverse Primer 5'-3'	Extn time
CK881	JR	1460	*HincII*	495	3	OJ1365_D05	CACCGTATTCATATTCATCCTCCAC	CAGATGGTATCCATACTGTAGTCCG	2:20
TC09	JR	1530	*RsaI*	775	3	OJ1124_H03	GGTGGAGGCAGAGAAGCTTAATGAG	GCAAGTCTCTTGCGTTATGTCATGG	1:40
TLK	JR	914	*AseI*	229	3	OSJNBa0069E14	CTTCTTGGAAAGGGAGGGTTTAGTG	CCATACAGATTCTCACACCTACTCC	1:40
ADA	ABB	1613	*MspI*	61	3	OSJNBa0047E24	CTCCTTTCTCCTCTTTTGCTCTATG	GGAGAAGGATCTAACAAATGAAGAC	2:20
Vrn1_Del	Fu *et al.* (2005)	474	-	-	3	OJ1112_G08	GCTCCAGCTGATGAAACTCC	CTTCATGGTTTTGCAAGCTCC	0:40
Vrn1_Int	Fu *et al.* (2005)	403	-	-	3	OJ1112_G08	TTCATCATGGATCGCCAGTA	AAAGCTCCTGCCAACTACGA	0:40
644	ABB	1214	*Msp I*	306	3	OSJNBb0081K01	ACACCATTTACATGCTTTGCACCTC	CAAAGAACTTAATGGATGTGTCCCC	1:40
GN3	ABB	1519	*Hae III*	298	3	OSJNBb0081K01	TCTACTGACTGAGCAAGGCGAGGTG	CTAGATTACTATGTTAGCAACAGTG	2:20
GN1	ABB	1157	*Hpy188 I*	232	3	OSJNBb0081K01	GATACTCCTAGTCAGTTGAAAATAG	GAGGGACATCAATGAACTTCAAGAC	1:40
PAGP	JR	1107	*AciI*	241	3	OSJNBa0040E01	GCCAAGCAAGAAAACCAAGTGGGTG	GACTGAAATGCACACAGTGATTGTG	1:40
CKAS	JR	1507	*AseI*	200	3	OSJNBa0002J24	GTGTGTAATACCTTTAGATATCGGG	GATCTAATGCCTGGAATGGTATAAC	2:20
AJ927	JR	1266	*HaeIII*	714	3	OSJNBb0106M04	CTTCACCTTGCGCCGATGGCCGAAG	CATGTACTCTAGATAACCATGCTTG	1:40
BJ928	JR	1255	*DdeI*	327	3	OSJNBa0070N04	CATCACGATGACACAGATCAACGGC	GCTGTTGACATGGCAATTTGACTGG	1:40

Mapping the new markers in Amaji Nijo×WI2585 and Galleon×Haruna Nijo DH populations

The markers were mapped in barley by using them to genotype individuals from the Galleon×Haruna Nijo and Amagi Nijo×WI2585 DH mapping populations. Marker order was determined by examining the graphical genotype and arranging markers in the order that gave fewest double recombinants. Genetic distances were determined by converting recombination fractions to cM using the Kosambi mapping function (Kosambi, 1944).

Amagi Nijo×WI2585 fine mapping population development

A cross between the frost tolerant cultivar Amagi Nijo and the frost intolerant breeder's line WI2585 was screened at the F_3 generation to identify lines for fine mapping. Individuals were screened for markers linked to loci that may confound the phenotyping of the population except for *vrn*-H1 as it was within the 5HL RFT QTL. The F_3 plants named P-122-2 and P-144-4 were selected that were homozygous for the developmental gene *eps*2 (Coventry *et al.*, 2003, Pallotta *et al.*, 2003) and the frost tolerance locus on chromosome 2H whilst being heterozygous across the 5H frost tolerance QTL region. F_4 progeny of these F_3 plants (the P-122-2 and P-144-4 families) were screened with markers flanking the 5H frost tolerance region. Plants displaying recombination between the flanking markers were selected for progeny testing in the F_5 generation. The recombinant F_4 individuals were also genotyped using markers in the 5H RFT QTL region to identify the position of the recombination event. The progeny of each recombinant F_4 plant were screened with the flanking 5H markers to identify individuals homozygous for recombinant or non recombinant chromosomes within the critical 5H RFT region.

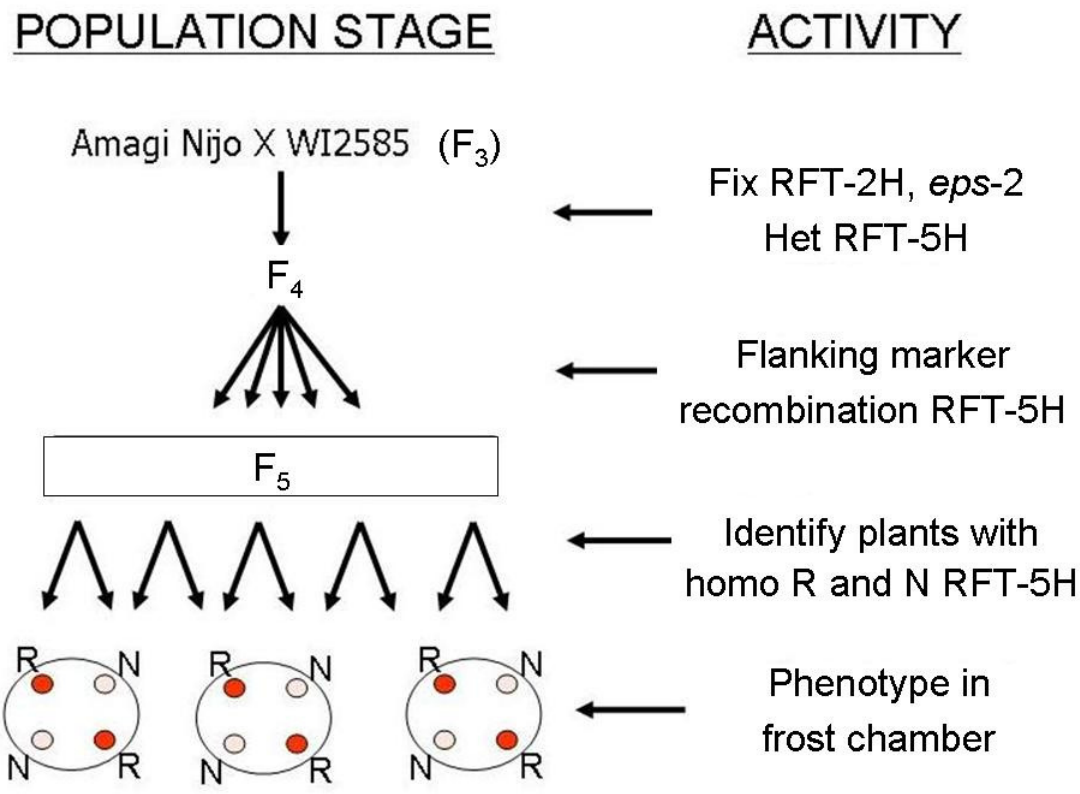

Figure 1. A schematic illustration of germplasm development for detailed genetic analysis of the 5H RFT locus using a cross between Amagi Nijo and WI2585. R= individual with recombination within critical 5H RFT region. N= individual with no recombination within the critical 5H RFT region. 'homo' refers to a homozygous chromosome segment in the critical 5HL RFT region. FIS data was generated when the plants were at the F_5 stage. Pictures at the bottom show the arrangement of plants in pots for frosting.

A third population was developed to separate *vrn*-H1 from the RFT locus on chromosome 5HL. Plant P-122-2-68 was homozygous for the spring *Vrn*-H1 allele, contributed by the frost susceptible parent WI2585, but showed recombination between *Vrn-H1* and the marker PAPG, so that it was heterozygous distal to *vrn*-H1, at least for the 5cM region from PAPG to AJ927. P-122-2-68 was homozygous for the WI2585 allele at *eps*2 and the 2HL RFT locus. Identified homozygous recombinants and homozygous non recombinants individuals

from this population were phenotyped for days to head emergence and RFT as described below.

Effect of vrn-H1 on flowering time

Mapping of flowering time in the Amagi Nijo×WI2585 and Galleon×Haruna Nijo DH populations revealed major QTLs for maturity segregating within the critical 5H RFT regions (Chapter 2). To further define the location of the earliness locus, detailed heading notes were recorded for all plants used in the genetic analysis. The date of awn emergence of the primary tiller of each plant in Julian days was recorded to facilitate fine mapping.

Phenotyping $F_{4:5}$ populations for frost tolerance

The Australian Genome Research Facility frost chamber was used for phenotyping F_5 homozygous recombinant and homozygous non recombinant plants. Plants were sown in 10cm × 10cm × 20cm deep pots during June 2006 and leaf tissue was sampled at the 1 leaf stage for genotyping. Individuals homozygous for the parental recombinant or non recombinant chromosomes were identified using flanking markers CK881 and AJ927 and transplanted into 8 inch pots containing UC soil in a pair wise manner as illustrated in Figure 1. Sibs from each heterozygous recombinant individual were grown in the same pot to reduce the physical distance between plants that were being compared for RFT to minimize effects of any spatial variation in conditions within the frost chamber. Before and after frost treatments the plants were placed outside during the 'normal' crop production period to ensure that day length and temperatures were as close as possible to those normally experienced in the field. Head emergence occurred in early October. When the majority of plants reached head emergence, individual tillers were tagged according to their specific developmental stage in order to account for any flowering time differences between

the plants. Tillers were categorized into four developmental stages according to the scale of (Zadoks *et al.*, 1974):

Stage 1: Booting/pre anthesis (Z45)

Stage 2: Awns emerging/pre anthesis/anthers green (Z49)

Stage 3: Head emerging/pre anthesis/anthers yellow (Z55)

Stage 4: Head fully emerged/post anthesis (Z59 to Z69)

Pots were placed in the frost chamber in a completely randomised design as illustrated in Figure 2. The coordinate (range and row) was recorded for each pot to account for any spatial variation in the chamber when conducting the data analysis. Pots with plants that were not to be used in the analysis were placed either side of the plants to be tested, with the aim of reducing variation in heat load and subsequent temperature variation within the chamber. Plants were exposed to a 24 hour treatment that simulated a single radiative frost event (Figure 3). The temperature profile programmed in the frost simulation chamber was established from field based temperature data and from validation work previously conducted (data not shown). After the frost treatment, pots were returned to the pre-treatment conditions until the plants reached maturity. Each individual spike was scored for sterility level, defined as the number of sterile florets expressed as a percentage of the total number of florets (Chapter 3).

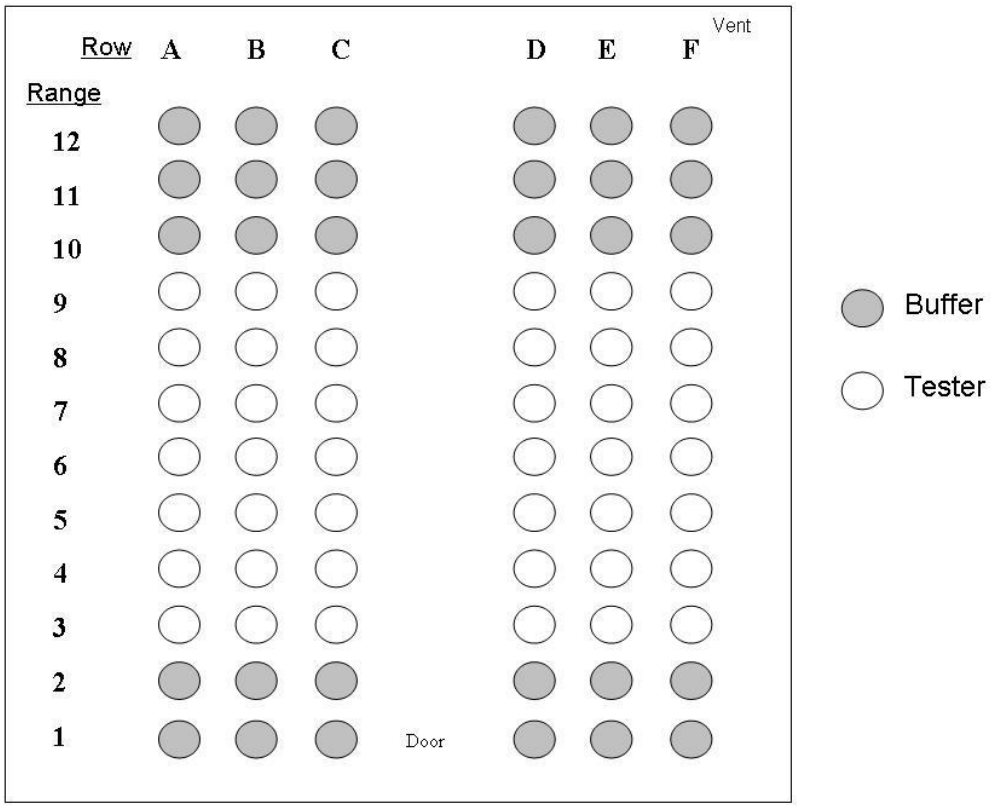

Figure 2. The layout of tester and buffer pots within the AGRF frost chamber.

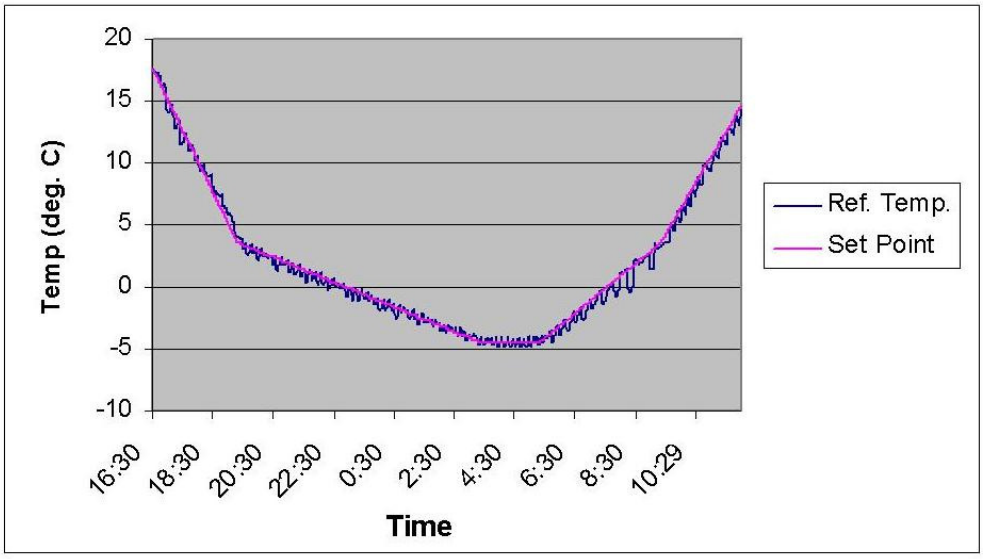

Figure 3. Temperature profile of simulated frost events. The 'Set Point' temperature is the temperature profile programmed into the AGRF frost simulation chamber. The 'Ref. Temp' refers to the actual temperature recorded in the centre of the frost simulation chamber at average spike hight.

Data collected were spatially analysed via REML (Genstat© Version 8) to take into account any spatial temperature variation that may have occurred during the frost treatment. Maturity scores were included in the analysis to identify any maturity effects. Means and standard errors were obtained to test for statistically significant differences. Each marker developed within the critical 5H region was fitted to the analysis model to determine associations of the phenotypes (heading date in Julian days and FIS) with each marker allele.

Results

Marker development using rice colinearity

Marker development within the 5H RFT region was initially undertaken using information from sequenced genes located around the cloned *vrn*-H1 gene (Yan *et al.*, 2003). Genes 644, GN1, GN2 and CYB, identified by Yan *et al.* (2003) as being close to *vrn*-A1 in *T. monococcum* were targeted for CAPS marker development. All CAPS markers that were developed from genes in the *vrn*-H1 region (Table 1) were perfectly collinear between barley and rice, as determined from mapping in the Galleon×Haruna Nijo and Amagi Nijo×WI2585 DH populations (data not shown, and Fig. 4, respectively). These were also collinear between *Triticum monococcum* and rice (Yan *et al.*, 2003). The most proximal marker chosen was CK881 and the most distal marker chosen was AJ927 (BJ928 was considered to be distal to the 5H RFT QTL). These 2 markers, that appeared to flank the RFT QTL peak, spanned a corresponding region in rice of approximately 146kb covering 12 rice BACs on the physical map (Saisho *et al.*, 2002).

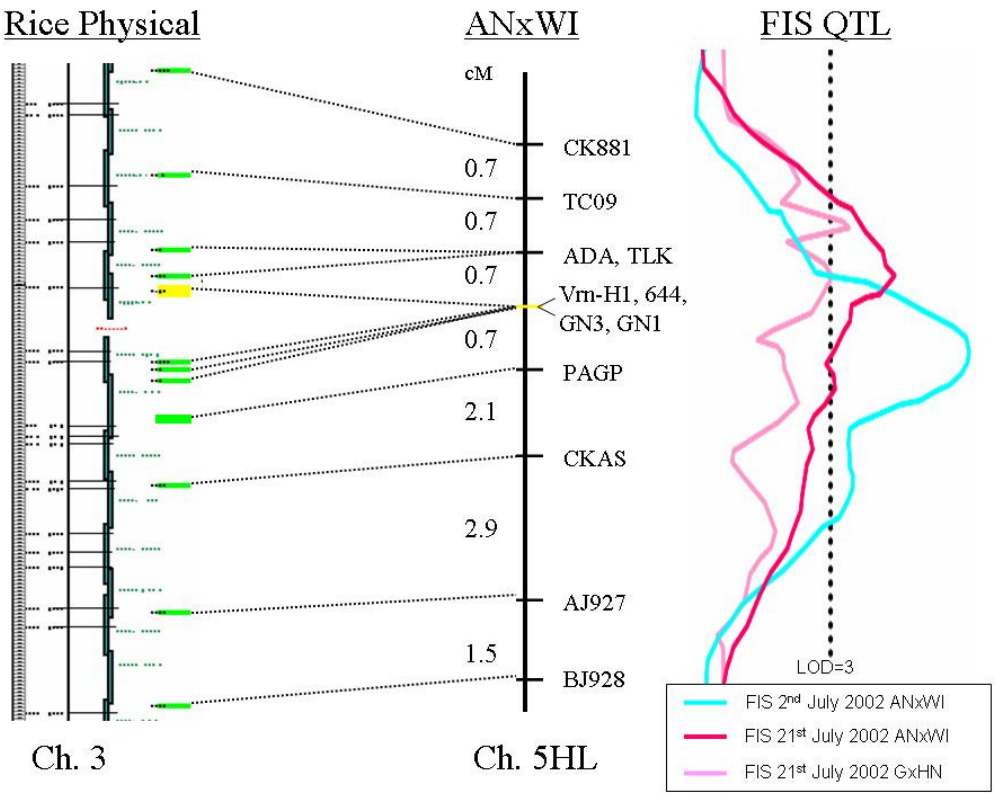

Figure 4. The location of genes in rice used for CAPS marker development and their positions in barley. The QTL identified in Chapter 2 are aligned with the 5HL linkage map of the Amagi Nijo×WI2585 population. The location of the FIS QTL from the Galleon×Haruna Nijo population is estimated from common molecular marker positions.

Initial Amagi Nijo×WI2585 fine mapping population development

Marker screening at the F_3 stage identified two plants that were suitable for subsequent fine mapping the 5H RFT locus. These two F_3 plants were homozygous across the 2H RFT QTL region and *eps*2, a developmental gene segregating in this population (Pallotta *et al.*, 2003), (Chapter 2), whilst being heterozygous across the 5H RFT region (Table 2). These two individuals, called P-122-2 and P-114-4, carried alternate fixed alleles at the 2H RFT region and *eps*2. 96 F_4 progeny from each F_3 plant were screened as seedlings with the AJ927 and CK881 markers flanking the 5H RFT locus to identify individuals resulting from recombination in the 5H QTL interval. 9 P-122-2 F_4 recombinant plants and the 10 P-144-4

F_4 recombinant plants were identified, giving a genetic distance between these markers of 8.1cM and 12.2cM, respectively. The genetic distance between these 2 markers in the Amagi Nijo×WI2585 DH population was calculated to be 7.8cM. The F_4 recombinant plants were also screened with the markers between CK881 and AJ927 to better define the location of each recombination event. The F_4 recombinant plants were progressed to F_5. The 9 P-122-2 F_4 recombinant plants and the 10 P-144-4 F_4 recombinant plants were allowed to self and the resulting F_5 plants screened with markers CK881 and AJ927 to select individuals homozygous for the recombinant or non recombinant chromosomes for phenotyping. The *vrn*-H1 marker was also scored on the F_5 plants. All other marker genotypes for the F_5 plants were inferred from the genotypes of the F_4 plants.

The frost tolerance phenotype and maturity were recorded on the selected F_5 homozygous plants. None of the F_5 progeny families from the selfed F_4 recombinants significantly deviated from the expected ratio of 1:2:1 of homozygous recombinants:heterozygotes:homozygous non recombinants respectively ($P<0.05$).

Table 2. Molecular marker genotypes at loci linked to maturity and RFT loci in the 2 F_3 parent plants (P-114-4 and P-122-2) of populations used for initial fine mapping of the 5H RFT locus. Markers for *eps2* and the 2H RFT locus were supplied by Anita Brule-Babel (Unpublished). 'HET'= Heterozygous, 'AN'=Amagi Nijo, 'WI'=WI2585.

Chromosome	Region	Marker	P-114-4	P-122-2
5H	FT QTL	CK881	HET	HET
5H	FT QTL	*Vrn-H1*	HET	HET
5H	FT QTL	644	HET	HET
5H	FT QTL	AJ927	HET	HET
2H	*eps2*	355	AN	WI
2H	FT QTL	694	AN	WI
2H	FT QTL	680	AN	WI
2H	FT QTL	AO	AN	WI
2H	FT QTL	CAR	AN	-

Effect of vrn-H1 chromosome region on flowering time

The difference in time to head emergence between the earliest and latest lines in the P-122-2 and P-114-4 F_5 families was 16 and 20 days, respectively. Effects of flowering time were analysed separately within each population. There was a significant $F_{4:5}$ family effect on flowering time ($P<0.001$) within both the P-114-4 and P-122-2 populations. A single marker regression was employed on the P-122-2 and P-114-4 populations to better delimit the locus controlling maturity on chromosome 5HL. All 8 markers within the 5H RFT region had a significant association with days to awn emergence in the P-114-4 population ($P<0.001$), while all but the CK881 marker showed such an association in the P-122-2 population ($P<0.05$). Of all the loci assayed, *vrn*-H1 had the strongest association with days to awn emergence (Figure 5). The winter *vrn*-H1 allele was associated with the earlier phenotype, with an average effect of 11.2 days in the P-114-4 population and 5.5 days in the P-122-2 population (Figure 5).

Phenotyping of $F_{4:5}$ populations in the frost chamber

There was a significant ($P<0.001$) F_5 family effect on FIS within the P-114-4 F_5 population. Of the developmental stages assayed for FIS data collection (booting to post anthesis), none were significant when added as a fixed effect to the REML analysis using the whole FIS data set ($P>0.05$). Therefore, developmental stage was not considered in the subsequent analysis. The allele at each marker locus for each plant was fitted singly to determine the effect of each locus on FIS (single marker regression). There was no significant effect of CK881, TC09, TLK or *Vrn*-H1 on FIS. There was a significant effect of PAGP ($P<0.05$), CKAS and AJ927 ($P<0.01$) on FIS. To delimit the FIS locus, the average effect of each allele at each locus was determined. CKAS was the locus with the highest association with FIS, with the WI2585 allele associated with an average FIS difference of 23.9% (Figure 5). The numbers of F_5 progeny and data points collected for each F_4 recombinant are listed in Table 3.

Within the P-122-2 population, there was no significant association detected between any of the markers alleles and FIS. Due to the spread of maturities and timing of the frost treatment, only the earlier maturing individuals were phenotyped for FIS in this population. This resulted in marker alleles at the 5H locus being highly skewed, so that they were derived from Amagi Nijo on average 85% of the time across the assayed marker loci. This included the alleles at CKAS and AJ927 that were distal to *vrn*-H1. The unbalanced nature of the dataset meant that the statistical power for identifying significant FIS effects from contrasting alleles were greatly reduced. Flowering time data was captured on the whole P-122-2 population with alleles at all marker loci represented more evenly which greatly improved the statistical power to identify significant maturity effects of contrasting alleles in this genomic region.

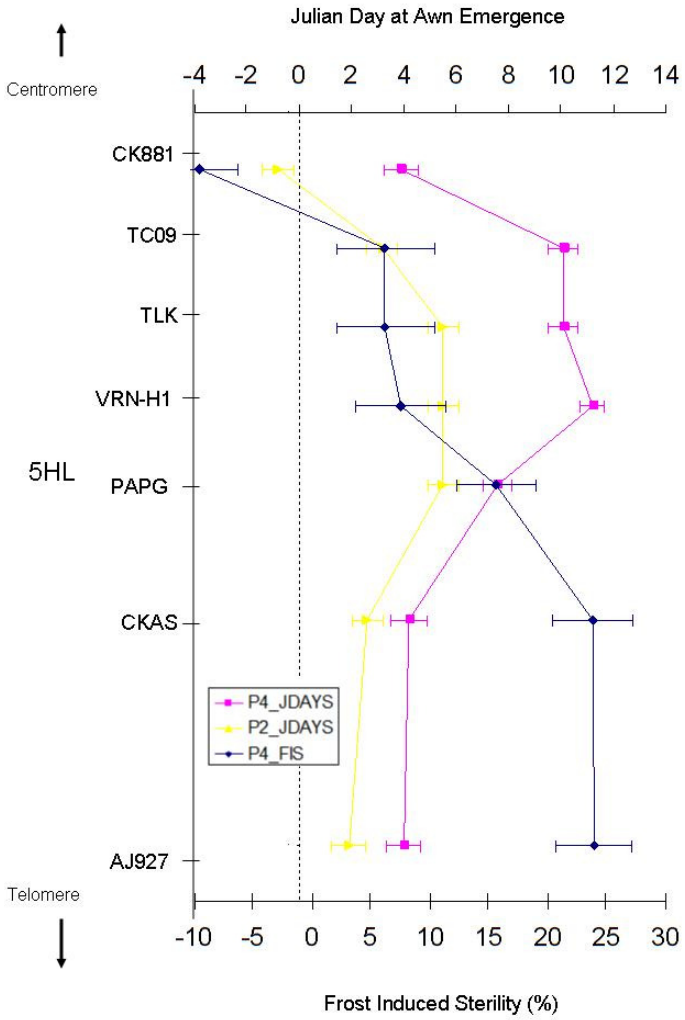

Figure 5. Association of marker loci in the 5H RFT QTL region with RFT and days to heading. The frost tolerance effect is shown only for the P-114-4 population (P4_FIS), and is expressed as the average Frost Induced Sterility (FIS) of individuals containing the WI2585 allele minus that of individuals carrying the Amagi Nijo allele. The flowering time effect is shown for both P-114-4 (P4_JDAY) and P-122-2 (P2_JDAY) populations and is expressed as Julian Days at flowering for individuals containing the WI2585 allele minus that of individuals carrying the Amagi Nijo allele. Error bars indicate the average standard error of the mean for each data point. FIS data were not presented for P-122-2 as no significant marker trait association was observed for any of the CAPS markers.

Table 3. The number of recombinant families (REC FAMILIES) and recombinant (REC) and non recombinant (NREC) individuals for each marker interval. The number of data points (DP) collected on Julian day at heading (JDAY) and frost induced sterility score (FIS) for each of the recombination classes for the progeny derived from the P-122-2 and P-144-4 F_4 families (F_5 plants) are also shown.

F4 PLANT	F5 PROGENY	CK881-TC09	TC09-TLK	TLK-VRNH1	VRNH1-PAPG	PAPG-CKAS	CKAS-AJ927
P-112-2	REC FAMILIES	4	2	1	0	1	1
P-112-2	REC INIVIDUALS	23	17	6	0	9	4
P-112-2	NREC INIVIDUALS	29	11	12	0	8	4
P-112-2	REC JDAY DP	23	17	6	0	9	4
P-112-2	NREC JDAY DP	29	11	12	0	8	4
P-112-2	REC FIS DP	12	5	7	0	7	0
P-112-2	NREC FIS DP	12	8	11	0	3	0
P-144-4	REC FAMILIES	3	1	1	1	2	2
P-144-4	REC INIVIDUALS	25	5	4	9	4	5
P-144-4	NREC INIVIDUALS	10	4	4	3	2	8
P-144-4	REC JDAY DP	25	5	4	9	4	5
P-144-4	NREC JDAY DP	10	4	4	3	2	8
P-144-4	REC FIS DP	54	7	4	24	17	8
P-144-4	NREC FIS DP	18	7	8	4	6	20

Confirming the separation of vrn-H1 and RFT-5HL

A third population was developed from the F_5 plant P-122-2-68 to confirm the separation of *vrn*-H1 and the RFT locus on chromosome 5HL. Homozygous recombinants and homozygous non recombinants were identified from the P-122-2-68 F_5 family by using the markers PAGP, CKAS and AJ927. 7 recombinant and 9 non recombinant F_5 individuals from this family were phenotyped using the AGRF frost chamber as previously described. Compared to the previous experiments, a high level of FIS was experienced by tillers at all stages in this experiment (Figure 6). There was no significant relationship between any of the segregating markers and time to awn emergence ($P>0.05$). Tillers at a range of maturity stages on each plant were scored for FIS, and a significant correlation ($P<0.001$) between tiller stage and FIS was observed. There was also a genotypic effect observed on FIS ($P<0.001$), with plants carrying the WI2585 allele at PAPG having higher FIS within each tiller maturity class (PAGP-AJ927 were co segregating in this family). This trend was not always significant for each maturity group (Figure 6). The growth stage where the highest number of data points were obtained was half head emergence/pre anthesis (Z55). This growth stage showed the largest significant difference in RFT of individuals containing contrasting PAPG alleles (Figure 6).

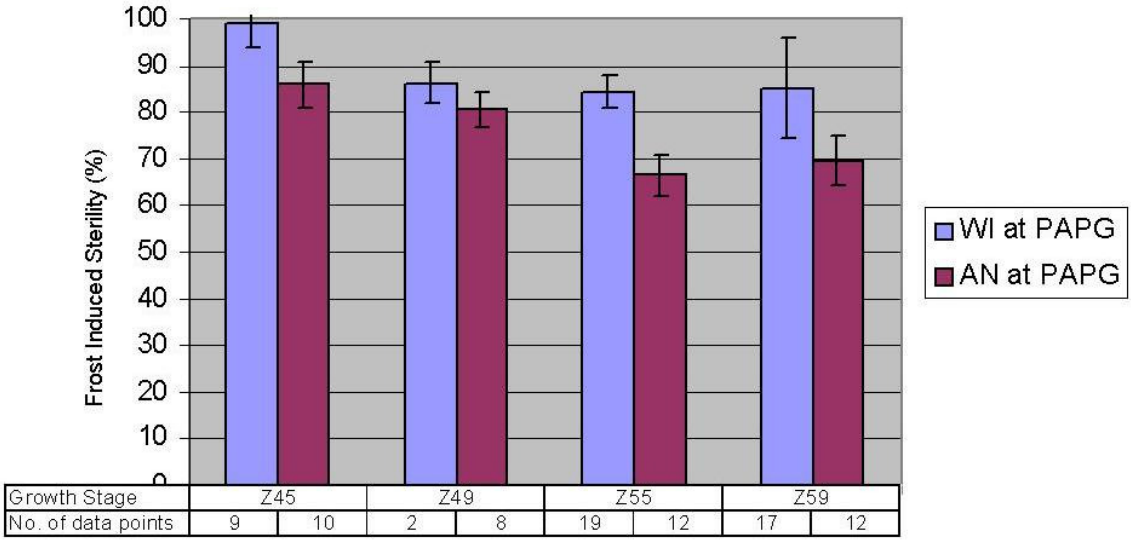

Figure 6. Mean and standard error of FIS of individuals homozygous for alternate alleles at the PAPG marker locus in family P-122-2-68. Family P-122-2-68 is segregating for the chromosome region PAPG-AJ927 (markers co-segregating) but homozygous for the nearby region containing *Vrn-H1* (see Figure 4 for marker locations). The number of heads sampled is shown. Data are grouped according to developmental stages of the scored tillers (Zadoks *et al.*, 1974).

Discussion

The coliniarity of the RFT 5HL genomic region with rice chromosome 3 has been reported to be highly conserved (Stein *et al.*, 2007). All genes that were used for marker development in the current study were found to be in the same order in the two species. The initial RFT QTL of chromosome 5HL spanned a considerable genetic distance in both Amagi Nijo×WI2585 and Galleon×Haruna Nijo DH populations, partly because of low marker densities. However, the development of CAPS markers increased the marker density in this region. Adding these markers to the DH mapping populations did not refine the QTL locations (not shown). This may have been due to limited FIS data captured on individuals displaying recombination in the 5HL RFT QTL. The CAPS markers that were developed were robust and could easily distinguish homozygotes from heterozygotes. The

choice of restriction enzyme was partly based on cost. This would be an important consideration if these markers were to be used for MAS in breeding programs, at least until the adoption of cheaper and higher throughput marker SNP detection systems.

The double haploid mapping populations initially used to identify the 5H RFT locus in barley were segregating for a second RFT locus on chromosome 2HL and other developmental loci such as *eps*2 on chromosome 2HS. Specific strategies were used to remove confounding effects of maturity in the initial DH mapping study (Chapter 2). However, selecting heads at the same developmental stage using the tagging method (Chapter 2) may not eliminate all error, as it is difficult to characterise the maturity of the anther and stigma without dissecting each floret. A small difference in anther and stigma developmental stages that is not accounted for in the tagging system has not been discounted as a potential underlying cause of differences in frost sensitivity. In the current chapter, an additional approach was used to remove the effects of the flowering time loci known to segregate in this cross. A strategy to 'fix' the major developmental and RFT loci was employed so the effects of the 5H locus could be more accurately determined. The P-122-2-68 progeny were not segregating for any detectable difference in maturity based on timing of head emergence. Repeating the analysis by characterising anther and stigma maturity in the progeny of the P-122-2-68 family may reveal whether the 5H RFT locus is associated with any small floral maturity differences. Fixing major maturity loci should have reduced variation in phenology between individuals and therefore variation in RFT. Unlinked loci that perhaps contribute to variation in RFT in a more minor way are also more likely to be fixed or randomised using the sibling-comparison method employed here. Using populations that are F_4 derived increases the probability of other confounding loci being in a homozygous state compared to an F_2 or F_3 derived sib analysis. More generations of selfing mean a higher proportion of alleles are in a homozygous state. The identification of individuals with recombination within the critical RFT region for phenotyping also

ensured frost data was only collected on lines that would contribute to narrowing the RFT QTL. Although this strategy appeared to be successful in minimising the effects of other non linked loci, the association of *vrn*-H1 and earliness was significant in the first 2 families studied, as illustrated by the average difference of 11.2 days in flowering time between the two *vrn*-H1 alleles in the P-114-4 population.

The winter *vrn*-H1 allele was associated with an earlier flowering phenotype which contrasts with previous reports finding an association between the winter *vrn*-H1 and a later flowering time (Francia *et al.*, 2004, Fu *et al.*, 2005, Karsai *et al.*, 1997, Koti *et al.*, 2006, Laurie *et al.*, 1995b, Szucs *et al.*, 2007, Trevaskis *et al.*, 2006). Szucs *et al.* (2007) studied the flowering time of two F_2 populations segregating for *vrn*-H1 and *vrn*-H2. When comparing groups that were homozygous for the spring *vrn*-H2 allele, there was no significant difference in flowering time between progeny carrying either the homozygous winter or spring allele at *vrn*-H1 from the Dicktoo × OWB-D cross. The winter allele at *vrn*-H1 had a significant effect on delayed flowering in progeny from the Dicktoo × Calicuchima cross. Tranquilli and Dubcovsky (2000) studied the effect of *vrn*-A^m1 and *vrn*-A^m2 in a population from a cross between to *T. monococcum* lines DV92 and G2528. The winter *vrn*-A^m1 allele was associated with significantly delaying flowering when comparing the flowering time of progeny having the spring *Vrn*-A^m1 and spring *vrn*-A^m2 genotype with the progeny having the winter *vrn*-A^m1 and spring *vrn*-A^m2 genotype. Data obtained from the Amajo Nijo and Haruna Nijo derived populations found an association between the winter *vrn*-H1 allele and significantly earlier maturity in genotypes carrying the spring *vrn*-H2 allele. To our knowledge, earliness associated with a winter *vrn*-H1 allele appears to have only been found in germplasm derived from these two Japanese lines (Figure 5 and Chapter 6).

This unexpected observation has a number of potential explanations: 1. The earliness at this locus is controlled by a linked but separate earliness gene which is revealed only in spring types where the stronger winter/spring flowering time effect determined by *vrn*-H1 does not occur due to the absence of *Vrn*-H2. 2. The Japanese source of winter *vrn*-H1 is a novel allele. In the absence of a winter *vrn*-H2 allele, the *vrn*-H1 winter allele confers an earliness effect independent of the epistatic interaction with the winter *vrn*-H2 allele. A major QTL for maturity was observed in the Amagi Nijo × WI2585 doubled haploid population at the *vrn*-H1 locus (Pallotta *et al.*, 2003). This result supports point 2 as the spring *vrn*-H2 allele was present in both parents (Chapter 5, Table 2) and not segregating in this population. 3. The 'winter' *vrn*-H1 allele, as identified by a completely linked marker, is not a true winter allele. This is unlikely as both Amagi Nijo and Haruna Nijo were determined to have the winter *vrn*-H1 allele by using a diagnostic marker. Also, crossing either Haruna Nijo or Amagi Nijo with genotypes carrying a winter *vrn*-H2 allele produce progeny with winter growth habit (J. Eglinton, personal communication, 2007).

Several models of flowering time gene interactions have been proposed for the cereals (Shimada *et al.*, 2009, Trevaskis *et al.*, 2007, Yan *et al.*, 2003, Pidal *et al.*, 2009). In all of these models, spring *vrn*2 alleles have either a direct or indirect dominant repressor effect on the expression of *vrn*1 winter alleles. The recessive *vrn*2 'spring' allele is viewed as non functional because it is associated with deletions of the putative gene and does not have any repressive effect on the winter *vrn*1 allele. In this model, in the absence of *Vrn*2, winter/spring alleles of *Vrn*1 have no differential effect on flowering time. There has been no report of the wild type winter allele at *vrn*1 being more efficient in promoting flowering then the spring allele that has a deletion in intron 1. Therefore these models do not explain the observation in this chapter where the winter allele of *vrn*-H1 in the presence of the spring *vrn*-H2 allele is associated with earliness and supports the hypothesis that the Japanese source of the winter allele is novel.

The influence of *vrn*-H1 or closely associated flowering time loci impacted on the ability to capture FIS data. No significant marker-RFT trait association was found for any marker in the critical RFT region in the P-122-2 population. However, this may have been because the data generated for this population was largely restricted to lines that were of an early maturity type because the frost treatment was applied too early to capture a balanced data set in a population and hence would have lowered the statistical power of the experiment. A population segregating for flowering time controlled by a locus in the target region needs to be carefully managed to ensure the frequency of alleles will not be too skewed. The P-114-4 population was scored at a more appropriate time, ensuring that alleles in the target RFT QTL region were represented more evenly in the data set.

Results (Figures 5 and 6) indicated that the RFT locus was closely linked but separate to *vrn*-H1 and/or other maturity loci located in this region, and situated distal of this maturity locus/loci. This was confirmed through analysis of an F_4 derived F_5 family that was fixed for the spring allele at *Vrn*-H1 but had a recombination event close to this locus to the distal side. Individuals from this family carrying Amagi Nijo or WI2585 alleles distal of *vrn*-H1 showed significant differences in FIS but not in maturity.

Results from the initial QTL mapping study (Chapter 2) using the DH population were unable to confidently situate the RFT locus in relation to *vrn*-H1. The diagnostic marker *vrn*-H1 was added to both of the Haruna Nijo × Galleon and Amagi Nijo × WI2585 linkage maps after the initial mapping study outlined in Chapter 2. *Vrn*-H1 was situated within the QTL for FIS in both populations (data not shown) although the most significant FIS QTL identified in the Amagi Nijo×WI2585 DH population did appear to be distal to the maturity QTL mapped in this population (Chapter 2)

Francia *et al.* (2004) reported a QTL for barley Vegetative Frost Tolerance (VFT) 20cM proximal to the *vrn*-H1 locus, designated *Fr*-H2. A similar locus has been reported in the syntenous position on wheat chromosomes 5A (Baga *et al.*, 2007), 5B (Toth *et al.*, 2003) and 5D (Sutka, 2001). The proximal location to *vrn*1 suggests that the *Fr*-H2 locus is distinct from the locus detected in this study, which was located distal to *vrn*-H1. The location of a frost tolerance QTL distal of *vrn*-H1 in barley has not been previously reported. In wheat Galiba (1995) reported a VFT locus close to but distal to *vrn*1 on chromosome 5A. However, the authors have since published data to suggest that this locus was in fact proximal to *vrn*-A1 (Snape *et al.*, 2001). In barley, *vrn*-H1 has not been reported to be genetically separable from the most closely associated VFT locus, *Fr*-H1 (Francia *et al.*, 2004, Hayes *et al.*, 1993). Therefore the 5H RFT locus appears to have a novel location when considering all previous literature in wheat and barley frost research.

The separation of *vrn*-H1 from the RFT locus has significant implications for spring wheat breeders. In barley, a spring allele at the *vrn*-H2 locus confers a spring phenotype regardless of the allele at *vrn*-H1. However spring alleles at *vrn*2 for all three loci in hexaploid wheat have not been identified (J. Dubcovsky, personal communication, 2004). Therefore true spring types would be difficult to obtain while selecting for frost tolerance associated with winter allele at all of the 3 *vrn*1 loci (*vrn*-A1, *vrn*-B1 and *vrn*-D1). The results from barley suggest it may be possible to use recombination screening to separate the winter vernalisation responsive alleles from *Fr1* frost tolerance alleles in wheat, enabling creation of chromosomes carrying a *Fr1* frost tolerance allele and spring *Vrn1* allele.

This study has further delimited the 5HL RFT locus. The separation of the 5H RFT gene from the winter *vrn*-H1 allele should allow spring barley breeders to select for RFT without the constraint of having to select for the spring allele at *vrn*-H2 on chromosome 4HL. For a cross segregating for one gene of interest, 50% of the progeny are expected to carry the

favourable allele. Every additional gene selected effectively reduces the population size by 50% (assuming no linkage). Removing the requirement for the specific 2 gene combination of the spring *Vrn*-H2 allele and winter *vrn*-H1 allele to achieve RFT spring types enables a higher proportion of RFT progeny to be obtained from the same population size. This can increase the chances of combining RFT with other economically important genes controlling important traits such as yield and quality.

Recombinants identified in this study could prove useful in breeding by providing parental material for crossing. Initial crossing and selection for RFT associated with the Japanese derived winter allele of *vrn*-H1 in a spring background (spring *vrn*-H2 allele) has produced progeny with a maturity type very early for main stream barley production (J. Eglinton, personal communication, 2008). Decoupling of the winter *vrn*-H1 allele from the RFT allele would remove this maturity constraint on RFT breeding. Markers found to be most closely associated with the 5H RFT locus (figure 5) should provide more effective selection of this tolerance than markers closer to *vrn*-H1. The genetic characterisation of this locus using the fine mapping approach has provided more evidence that the mechanism of tolerance is not related to flowering time processes controlled by *vrn*-H1 or a closely linked gene.

Chapter 5. A strategy to rapidly introgress frost tolerance genes into adapted barley germplasm

Introduction

Progress in reducing the impact of spring frost on crop production in Australia has been slow. Plant breeders and researchers have used a range of genetic and crop management techniques to attempt to reduce the impact of frost on grain yield and quality (Marcellos, 1988). Researchers have tried to identify variation for frost tolerance at the heading stages in cereals with limited success (Single, 1988). The main strategy used to reduce the incidence of frost damage to cereal crops has been to sow later to prevent the crop from flowering during the peak frost risk periods in early spring. This strategy has seen varieties flower later than optimal for maximum yield potential in the absence of frost due to the increased incidence of moisture and heat stress during the flowering and grain fill stages. This factor has cost farmers more than the potential frost damage that would be experienced without use of this frost avoidance strategy (Single, 1988).

The identification of genetic variation for Reproductive Frost Tolerance (RFT) in barley (Chapter 2) has provided an opportunity for plant breeders to improve this trait in Australian adapted cultivars. Incorporation of a new trait from a relatively poorly adapted donor parent has traditionally been attempted using a backcross breeding method. This approach has been most successful when incorporating a single major gene with dominant or partial dominant effect into elite germplam. The purpose of backcrossing is to recover the phenotype of the recurrent parent and substitute the undesirable allele(s) with the desirable allele(s) from the donor parent (Sleper and Poehlman, 2006). Backcrossing is a conservative breeding approach as it assumes that the donor parent will not contribute any desirable characteristics other than the target trait. Therefore it has been traditional standard

practice within inbreeding species to complete up to 6 backcrosses when incorporating genes from poorly adapted donor germplasm (Allard, 1999).

Field based selection for a quantitative trait with only moderate heritability requires replication. Therefore the availability of sufficient seed quantities to enable replication needs to be considered when designing a backcross breeding strategy to improve RFT. The unreliable nature of frost events in field based screening for RFT means that data may not be obtained every year. A breeding strategy for RFT has to allow for the fact that selection for RFT may not be possible every year using the field based screening method.

An example of part of a traditional backcross breeding strategy to incorporate RFT into a recurrent parent is outlined in Figure 1. The recurrent parent would be selected based on a profile of adaptation, quality and disease resistance that best suits production in the target environment. As RFT is a quantitative trait requiring selection under field conditions a high quantity of seed is required to enable replication. Therefore a 'cycle' backcrossing approach would most effectively be employed which focuses on deriving lines early in the cycle and then multiplying seed for phenotyping (Figure 1). The example backcrossing program assumes an availability of summer nurseries to increase the number of generations and crosses that can be undertaken per year. After 2 cycles of crossing and selection, the identification of a BC_3F_2 derived F_5 with a RFT phenotype would have taken 6 years from the first cross. It is likely that another cycle would be needed to advance to at least BC_5, considering 'standard' backcrossing is completed to BC_6 (Allard, 1999). This would take an additional 3 years, taking the total time from first cross to $BC_5F_{2:4}$ to 9 years.

The lines at the BC_5F_2 derived F_4 stage could then be multiplied and used to assess the performance for other economically important traits relative to the recurrent parent. Line(s)

that approach the agronomic and quality performance of the recurrent parent and having increased RFT would then be considered for release to growers after several years of testing.

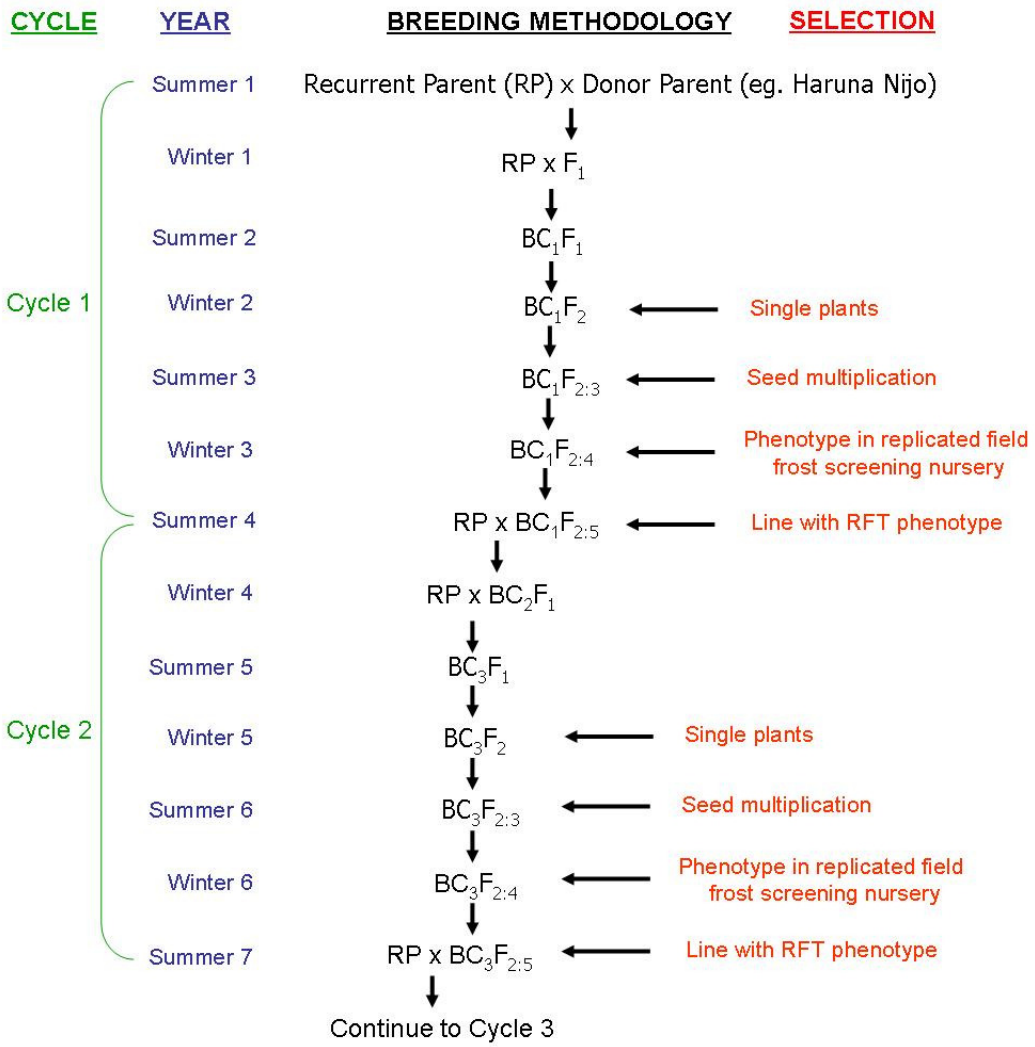

Figure 1. An example of a typical breeding strategy to incorporate a trait such as RFT into an adapted Recurrent Parent (RP) from an unadapted target trait donor. The 'Cycle' refers to the time taken to complete one round of crossing and phenotypic selection. 'Year' refers to the year from the first cross and assumes 2 generations can be grown per year. 'Breeding Methodology' refers to the BackCross (BC) number and filial (F_n) number for each of the generations of breeding. 'Selection' refers to the type of selection imposed on each generation in the breeding program.

A quicker crossing and selection strategy for RFT breeding can now be employed due to the identification of genetic loci for RFT in barley (Chapter 2). Several selection methods have been developed for RFT and could potentially be deployed in a breeding system to develop genotypes with superior RFT. These include:

- Field based phenotypic screening based on natural frost events (Chapter 2).
- Screening in a frost simulation chamber (Chapter 3).
- Use of molecular markers to select for frost tolerance alleles (Chapter 2)

The potential impact that varieties with improved RFT would have on barley production in Australia would be large. Therefore a breeding strategy that enables rapid introgression of this trait into a commercially relevant genetic background is required. Limitations on resources also needs to be considered when designing a breeding strategy of this nature. The lines carrying the RFT trait were Japanese varieties poorly adapted to most Australian barley production environments (Karakousis *et al.*, 2003a, Pallotta *et al.*, 2003). The poor adaptation of these donor lines necessitates a breeding strategy to specifically introgress small segments of donor genes into recurrent parent germplasm. Undesirable alleles/genes linked to desirable genes/alleles from donor germplasm can impact on the adaptation of the derived germplasm and limit the commercial value of the lines (Hospital, 2001).

The research reported in this chapter aims to develop and execute a breeding strategy to enable the most efficient and timely introgression of RFT into Australian adapted barley germplasm. The target will be a genetic background that is relevant to barley production areas of southern Australia that experience frequent damaging spring frost events. All available technologies that can aid in this breeding strategy are employed to ensure the highest probability of success in the shortest period of time. This breeding strategy will produce lines to formally validate the commercial value of the RFT identified in Chapter 2

and may potentially result in the commercial release of a new barley variety with improved frost tolerance.

Materials and Methods

Selection of a recurrent parent for the target environment

The selection of the recurrent parent was based on adaptation to areas of highest frost risk and where the economic impact of frost was greatest. Weather data from the Australian Bureau of Meteorology was used to determine the agro-ecological barley production zones where spring frost risk was the highest. The economic impact of frost in different production environments was determined primarily on the frost frequency and severity, but the flexibility in the farming systems to manage frost risk was also taken in to account.

The agronomic characteristics required to maintain a high level of production in this environment were considered after the target environment was identified. Phenotypic data from varieties and advanced breeders lines were sourced from the University of Adelaide barley breeding program to identify a suitable recurrent parent for crossing.

Selection of the reproductive frost tolerant donor parent

The Japanese varieties Amagi Nijo and Haruna Nijo were identified as the best source of donor alleles. These lines had both been well characterised for their increased level of RFT and showed the most consistent performance for RFT over multiple years and frost events. Doubled haploid populations derived from crosses between these lines and Australian adapted lines had been used to map the RFT loci in Chapter 2. From these populations, DH lines were identified which phenotypically displayed the RFT trait, had the tolerance alleles for both major RFT loci identified in Chapter 2, were visually agronomically superior to the

original Japanese parent and contained a high proportion of genetic background from the Australian adapted parent.

MAS of 2 reproductive frost tolerance loci

MAS was conducted using publicly available SSR markers at several stages of the breeding scheme. SSR markers Bmag222, GMS61 and GBM1438 were used for the 5H RFT QTL and HVM54 and Ebmac684 were used for the 2H RFT QTL. These 2H and 5H markers span a genetic region of approximately 10cM and 15cM, respectively, containing the whole RFT QTL regions (Chapter 2). The selected RFT regions covered a relatively large genetic distance as MAS was completed prior to the fine mapping study (Chapter 3).

DArT analysis to analyse introgression segments and recovery of the recurrent parent genetic background

Thirty four BC_1F_1-derived doubled haploids were genotyped with 686 Diversity Array Technology (DArT) markers (Triticarte Pty Ltd, ACT). The four parents used in the breeding program were subjected to the same DArT analysis. These were Galleon, Haruna Nijo, the RFT donor parent from the Galleon × Haruna Nijo DH mapping population, and the recurrent parent. The data was used to generate graphical genotypes of the derived material and determine the size of introgression segments carrying the two RFT loci. The view of the graphical genotypes were generated using the program GGT:Graphical GenoTyping (2002).

Results

Selection of a recurrent parent for the target environment

Identifying cereal production areas that experience frequent spring frost events in Australia was aided by temperature data from the Bureau of Meteorology. The minimum temperature from September 1 to October 30 in 2002 was one of the data sets used for identifying barley production regions at high risk (Figure 2). 2002 was a year which saw widespread frost events across Australia during the flowering period of cereals. The areas of moderate to high frost risk in Australia include the central wheat belt of WA, the mid north, Mallee and south east of SA, the Mallee and Wimmera of Victoria, south central NSW and small parts of northern NSW and southern Qld. These areas span the majority of Australia's different agroecological zones (Williams *et al.*, 2002). Therefore, a recurrent parent with broad adaptation was sought. Another consideration when determining the impact of frost on different production zones can be the potential for management practices to reduce frost risk. The high frost risk areas of northern Australia have a summer dominant rainfall. Moisture is stored in deep soils resulting in relatively reliable water supply to the crop at the end of the growing season. This enables more flexibility with sowing time, allowing flowering after the period of highest frost risk. In contrast, southern Australia and Western Australia has a winter dominant rainfall pattern. Ideally, cereals are sown after the first autumn rains to enable optimum establishment and early growth. As many of the soils in these regions are shallow with minimal water holding capacity the window of opportunity for seeding is very narrow. Manipulation of flowering time is therefore more difficult in these environments.

The Mallee of SA and Victoria were chosen as the primary target environments for the RFT breeding program. These regions were selected due to the prevalence of frost damage, the greater seeding time constraint and the highest concentration of barley production in

Australia (Figure 3). A successful variety in this environment would need to possess the following features: spring growth habit with early to mid maturity, good early vigour for weed competitiveness and groundcover on light sandy soils, resistance to cereal cyst nematode due to a scarcity of break crop management options, an acceptable foliar disease resistance profile, grain size stability, a moderately tall to tall plant type to maintain harvest height under drought stress, and grain yield comparable to current commercial varieties.

Figure 2. Lowest minimum temperature recorded in the months of September and October 2002 (peak flowering time of cereals) in Stevensons screens (covered screen located 1.2m above ground). Source: Australian Bureau of Meteorology (www.bom.gov.au). The areas of barley production are outlined in red.

Figure 3. Average Australian barley production per square kilometre. The South Australian and Victorian Mallee regions are circled in red. Source: The Australian Bureau of Agricultural and Resource Economics (ABARE), 2009.

The development of a malting variety can extend the time to release by 2-3 years compared to a feed variety. The commercialisation of malting varieties requires at least two years of commercial scale malting and brewing trials that is not required for feed varieties. Malting quality is under complex genetic control, requiring the accumulation of a number of genes to obtain a variety with a desirable malting quality profile. Therefore, the scheme aimed to produce a feed variety to reduce the complexity of the breeding strategy and to potentially shorten the time to delivery of a frost tolerant variety.

The University of Adelaide breeders line WI3806 was selected as the recurrent parent for crossing. WI3806 was desirable due to its broad adaptation across different Australian

growing regions and high relative yields in the environments in southern Australia that are frequently exposed to frost (Table 1). This line was derived from a cross between Mundah and Keel followed by a top cross with Barque. These three parents were the leading feed barley varieties grown in southern Australia at the time as illustrated in Table 1. WI3806 had a consistently higher grain yield across all environments tested when compared to the current commercial varieties grown in southern Australia (Table 1). The physical grain characteristics of WI3806 were also consistently good, with the retention above 2.5mm screen, thousand grain weight, and screenings below 2.2mm all in the desirable range compared to commercial checks. The hectolitre weight was average compared to the other varieties tested.

Introgression from diverse germplasm should also attempt to minimise the degree of segregation. Populations that segregate for many traits reduce the chance of identifying genotypes with the desirable gene combinations, especially when the population size is restricted by the cost of DH production. Haruna Nijo is genetically distinct from typical Australian varieties, as estimated by Nei's diversity index (Eglinton, 2003). To minimise segregation in the breeding population it was desirable to select a recurrent parent derived from Galleon as it is an Australian variety and a parent of the Galleon × Haruna Nijo DH line selected as the RFT donor. Barque contributes 50% to the pedigree of WI3806 and is derived from a Galleon × Triumph cross, but based on its phenotype and whole genome profiling, significantly more than 50% of its genome derives from Galleon (J. Eglinton, Personal Communication, 2009) Therefore, use of WI3806 as the other parent in the breeding program should minimise the background segregation in the breeding populations due to its high degree of Galleon parentage.

Table 1. A summary of the yield (MET analysis) and mean physical grain quality of lines tested at Minnipa (SA), Pt Wakefield (SA), Salmon Gums (WA), Ouyen (Vic), Condobolin (NSW) and Tara (Qld) from 1999-2004 (Coventry and Eglinton, 2005). %>2.5mm= percentage of grain retained above a 2.5mm screen, KG/HL= kilograms per hectolitre, TGW= Thousand Grain Weight, %<2.2mm= percentage of grain below 2.2mm screen, YLD%SCH= yield as a percentage of the barley variety 'Schooner'. Green indicates that the level of the trait is desirable. Yellow indicates the level of the trait is acceptable. Red indicates that the level of the trait is unacceptable. This interpretation is based on selection decisions that would be made on germplasm in the University of Adelaide barley breeding program (J. Eglinton, personal communication, 2007).

Name	%>2.5mm	KG/HL	TGW	%<2.2mm	YLD%SCH
Barque	62.4	66.7	41.4	10.7	100.4
Keel	68.5	68.3	39.6	7.7	107.4
Maritime	70.9	69.2	41.0	6.1	92.0
Mundah	64.8	67.7	44.3	6.3	108.8
Schooner	54.3	71.0	35.2	12.7	100.0
Sloop	55.6	69.8	36.1	11.4	92.8
Fleet	62.3	67.0	41.9	7.9	105.7
WI3806	72.6	66.9	42.6	6.6	114.5

*Developing the WI3806*2/GH-129 population*

The donor of RFT was chosen from the Galleon × Haruna Nijo F_1-derived DH population. Detailed RFT scores had been collected for most of the lines in this population in the Loxton field frost screening nursery (Chapter 2). The Galleon × Haruna Nijo DH population had also been genotyped for molecular markers throughout the genome (Chapter 2). The genotypic data enabled the selection of line GH-129 carrying tolerance alleles at the 2 RFT loci and a high proportion of the locally adapted parent, Galleon, in the genomic

background. Approximately 60% of the GH-129 marker alleles are derived from Galleon so a single backcross to WI3806 would result in progeny with an expected mean of approximately 10% Haruna Nijo (assuming no selection).

The RFT breeding methodology is outlined in Figure 4. The first cross between WI3806 and GH-129 was made in February 2003. The cross between the F_1 and WI3806 was completed in September 2003. 90 WI3806*2/GH-129 BC_1F_1 seeds were planted in February 2004 and selected by MAS for heterozygosity at the two RFT loci. SSR markers HVM54 and Ebmac684 were used to select the 2H RFT QTL and SSR markers Bmag222, GMS1 and GBM1438 were used to select the 5H RFT QTL. The 14 BC_1F_1 individuals with a desirable genotype were used as DH donor plants. 307 putative doubled haploid plants were produced by the isolated microspore method (Hoekstra *et al.*, 1992) and screened by MAS using the same SSR markers, identifying 66 individuals carrying the tolerance alleles at both target loci.

From 307 BC_1F_1-derived putative DHs it was expected that 77 individuals would be carrying both RFT alleles. Only 66 such individuals were identified, and based on a chi squared test, this number was not significantly different from expected ($P<0.05$).

Of these 66 individuals, 37 were confirmed as doubled haploids (i.e. produced seed), and 29 were haploids (i.e. sterile, not producing seed). The 37 fertile plants were grown to increase seed quantity over the summer of 2004/05 and notes were taken on growth habit. Lines of winter growth habit were dug up, placed in pots, and grown in a growth room to enable seed production. Of the 34 lines that produced seed in this generation, 19 displayed a spring growth habit and 15 displayed a winter growth habit.

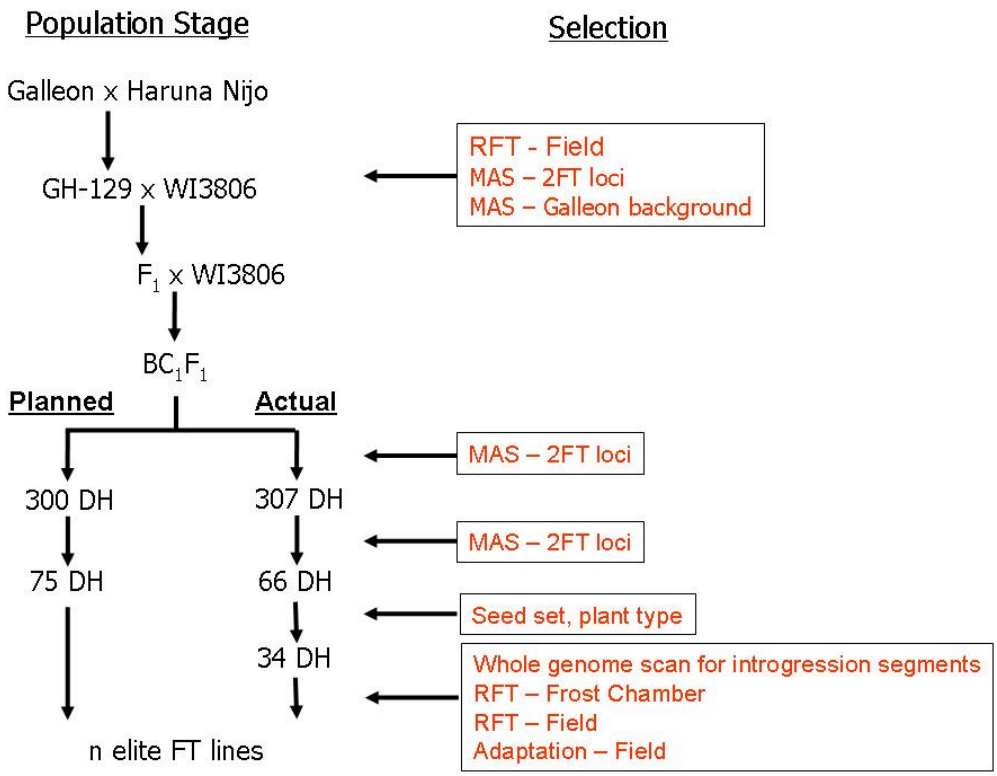

Figure 4. Schematic diagram of population development and selection of lines with reproductive frost tolerance. 'Planned' refers to the expected numbers of lines generated from the planned selection process. 'Actual' refers to the actual number of lines obtained. Selection was done by Marker Assisted Selection (MAS) of the 2 reproductive frost tolerance loci (2FT loci) and by Reproductive Frost Tolerance (RFT) phenotyping.

The winter allele at *vrn*-H1 is closely linked in coupling with the tolerance allele at the RFT locus which was selected for via MAS and was therefore expected to be in all of the marker-selected DH lines. GH-129 was known to have the spring allele at *Vrn*-H2, and the recurrent parent WI3806 must have been carrying a winter allele at *vrn*-H2, based on the fact that the population segregated for growth habit. This was subsequently confirmed by genotyping with the diagnostic marker for *vrn*-H2 (Table 2). As this was a BC_1F_1-derived DH population and no selection on *vrn*-H2 was performed via MAS, it was expected that the winter *vrn*-H2 allele would be inherited in 75% of the surviving DH lines and therefore 75% of DH lines would be of winter growth habit. When these plants were grown in the

summer nursery, which can discriminate easily between winter and spring growth habit, it was determined that the Winter:Spring ratio was closer to a 1:1 ($P<0.05$).

Table 2. Alleles at *vrn*-H1 (5HL) and *vrn*-H2 (4HL) loci in parents used for RFT breeding. Alleles were determined by diagnostic markers from (Fu *et al.*, 2005, Yan *et al.*, 2004b). 'S'= Spring allele and 'W'=Winter allele

NAME	*vrn*-H1	*vrn*-H2
GH-129	W	S
GALLEON	S	W
HARUNA NIJO	W	S
WI3806	S	W

DArT analysis of introgression segments and recovery of recurrent parent genetic background

DArT marker genotypes were produced for each of the 34 lines and the four parents. As predicted from pedigree data, polymorphism between the two parent lines was low (36%) compared to polymorphism between WI3806 and Haruna Nijo (56%). The DArT genotyping results confirmed that segregation for the background recurrent parent genome was low. The marker data were subjected to quality checks prior to producing graphical genotypes. This included removing monomorphic markers and markers that were giving obviously false data points, as judged from the occurrence of apparent double recombination events with flanking markers located within 1 cM. As DArT markers can only identify two alleles per marker, it was not possible to determine the origin of marker alleles in the BC_1F_1-derived DH lines precisely i.e. as being from WI3806, Galleon or Haruna Nijo. Consequently, for visualizing the introgression segments, alleles were simply classified as either WI3806/Galleon type or from Haruna Nijo, when polymorphism allowed this (i.e. Haruna Nijo showed a unique allele type).

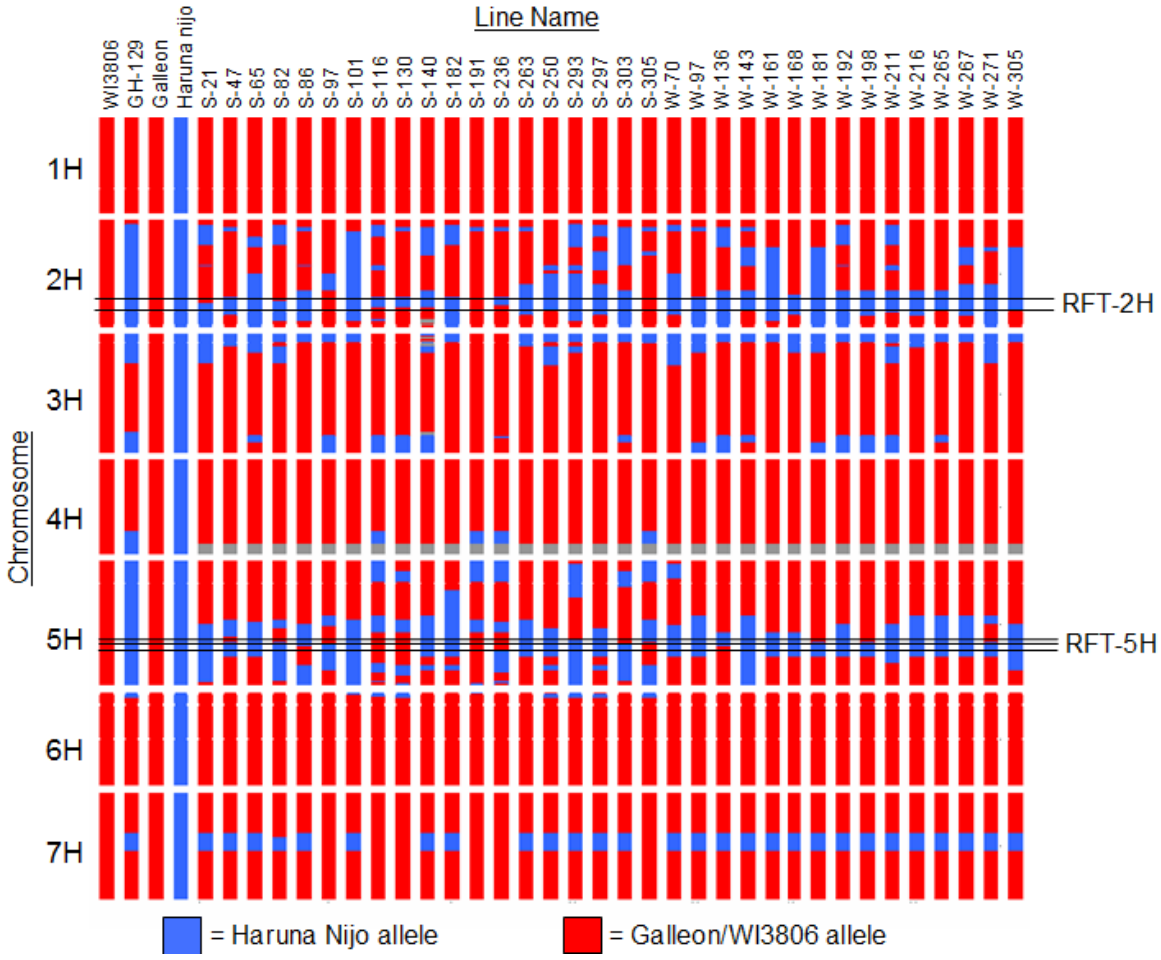

Figure 5. Graphical genotypes of parents and lines from the WI3806*2/GH-129 population. Colour denotes the origin of chromosome segments. Grey bars indicate missing data. Black lines indicate the predicted location of SSRs used to select the 2H and 5H RFT QTL. 'S'=line with Spring growth habit and 'W'= line with Winter growth habit.

The donor parent GH-129 had 44.6% of the RFT parent Haruna Nijo in its genetic background and carried virtually the whole 2H and 5H chromosomes intact from Haruna Nijo. The WI3806*2/GH-129 BC_1F_1-derived DH individuals carried an average of 16.3%

of the Haruna Nijo genome, with a range of 8.9% to 25.9% that was normally distributed around the mean (Figure 6). The expected percentage of donor genome retained if no selection was applied was 11.15%. The fact that MAS was applied to retain the tolerance alleles at both RFT loci may account for the higher than expected degree of donor genome retention.

Genotyping with SSR markers was used to select at the 2H and 5H RFT loci in several generations (Figure 4). DArT analysis suggested that several BC_1F_1-derived DH lines did not contain all/part of the RFT locus intervals from Haruna Nijo (Figure 5).

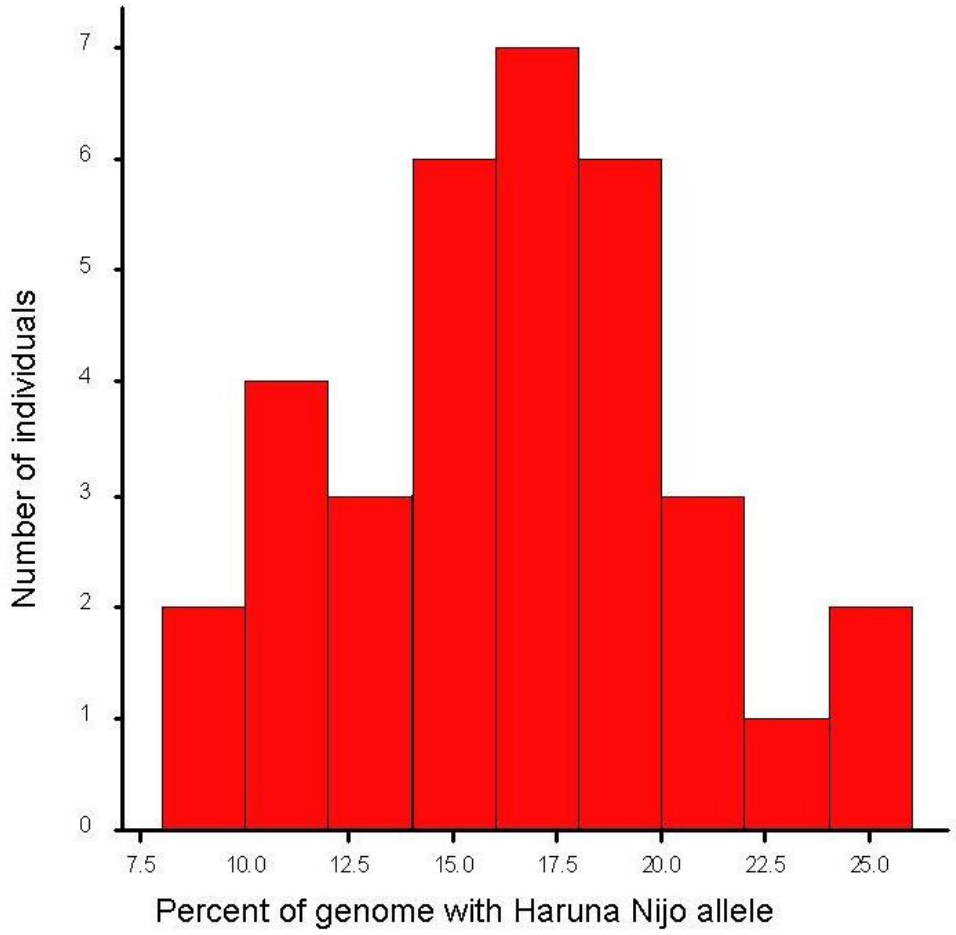

Figure 6. Histogram of the proportion of donor genome retained in lines from the WI3806*2/GH-129 population.

Discussion

The fast track breeding strategy detailed in this chapter was able to produce fixed lines from the first cross within 2 years. This is a much faster process than traditional backcrossing strategies. The example in Figure 1 illustrates that a traditional backcross breeding using a BC_5 approach would extend this period to at least 9 years, even with the relatively new practice of running summer nurseries to grow 2 generations per year. The conservative BC_5 breeding strategy was historically employed to reduce the potential of introducing deleterious genes linked to the donor gene(s) of interest (Allard, 1999). Without selection, it is expected that the proportion of donor germplasm remaining at the BC_5 stage would average 1.56%. With selection, the proportion of donor genome retained would be higher due to linkage to the gene(s) being selected. Stam and Zven (1981) attempted to estimate the amount of donor genome that would be retained after backcrossing in an inbreeding species. To apply this method to the backcrossing approach in Figure 1, two assumptions have been made: 1. The average chromosome length is 150cM. This figure is derived from viewing a consensus map of barley (Hearnden *et al.*, 2007). 2. All of the phenotypically selected RFT would be controlled by the two known RFT loci. For a chromosome of 150cM containing a single gene selected in a backcrossing program, the proportion of donor genome retained in BC_5 would be 30% (Stam and Zeven, 1981). For a chromosome that does not contain a gene that is being selected, the proportion of donor genome in BC_5 would be 1.56%. Therefore, the total percentage of donor genome retained at BC_5 if 2 genes were selected on 2 separate chromosomes would be (30*2/7)+(1.56*5/7) = 9.7% of the total genome.

The average proportion of donor genome retained after the fast track breeding program outlined in this chapter was 16.3% which is comparable to the value expected from the traditional backcross breeding method outlined in Figure 1 (9.7%). Therefore, the use of the

fast track breeding program provides a similar outcome to the historical BC_5 breeding program (Figure 5), but in much less time.

The breeding program outlined in this study had significant advantages over a breeding strategy using a historical backcrossing approach. The example traditional breeding program outlined in Figure 1 assumed that data generated from 1 year of field based frost screening would be adequate to confidently identify lines carrying the RFT alleles from the donor. This would probably not be the case as multiple data sets are often needed to discriminate between tolerant and intolerant lines. Phenotypic assessment across multiple years would further increase the time from cross to release in the example BC_5 breeding program.

The use of multiple donor BC_1F_1 plants increased the probability that a range of donor introgression segment sizes would be obtained in the derived DH lines. The use of MAS also enable a larger population size to be maintained than would have been the case with no selection because the F_1s used as donors for DH production were all determined to carry tolerance alleles at both RFT loci.

The isolated microspore technique for DH production in barley typically produces approximately 20% haploids (i.e. failure of the doubling process via colchicine) although up to 50% has been observed (P. Davies, personal communication, 2007). Of the 66 marker-selected plants arising from the doubled haploid production program, 23 (44%) failed to set seed and were therefore probably haploid. This frequency of haploids was higher than expected, but within the range occasionally observed (P. Davies, personal communication, 2007).

There was also some discrepancy between the SSR data and the DArT data. Five lines identified to be carrying the 5H RFT allele, and three lines identified as carrying the 2H RFT allele, using the linked SSR markers, were subsequently shown by the DArTs to be carrying the intolerance alleles. The position of DArT markers have been derived from a linkage map from a cross using different barley genotypes to those in this study (Hearnden *et al.*, 2007). Inferring the position of DArT markers in the WI3806*2/GH-129 population may not be accurate as discrepancies between linkage maps have previously been reported (Karakousis *et al.*, 2003b). Errors when genotyping with SSR markers or DArT markers may have contributed to the disagreement between some of the genotyping data.

28 of the 34 selected WI3806*2/GH-129 DH lines contained a common chromosome 7H segment from Haruna Nijo (via GH-129) that had not been selected by MAS (Figure 5). The original SSR, AFLP and RFLP genotypic data for the DH donor line GH-129 suggested that the entire 7H chromosome was Galleon derived. This suggests that markers have either been assigned to the wrong chromosomes or that the DArT markers assayed a region of 7H which was not covered by the original markers. The 7H segment in the selected WI3806*2/GH-129 DH lines is represented by 8 DArT markers clustering at 2 loci approximately 5cM apart. The SSR, AFLP and RFLP for the whole Galleon × Haruna Nijo DH populations showed no distortion in this genomic region on 7H. However, it is possible that a gene from WI3806 in this region had a negative influence on DH regeneration. The majority of evidence supports the explanation that an error in DArT genotyping for markers at this locus contributed to the common donor alleles displayed at this unselected locus in Figure 4.

Lines with winter growth habit were observed in progeny from this cross. Subsequent genotyping using the perfect markers for *vrn*-H1 (Yan *et al.*, 2003) and *vrn*-H2 (Yan *et al.*, 2004b) showed that the frost tolerance donor GH-129 carried the winter allele at *vrn*-H1 and spring allele at *vrn*-H2 (Table 2). The recurrent parent had the spring allele at *Vrn*-H1 and

the winter allele at *Vrn*-H2 (Table 2). This resulted in a spring growth habit in both parental lines, but segregation for winter/spring growth habit in the breeding population. Plants that carry winter alleles at both *vrn* loci have a winter growth habit (ie. a strong vernalisation response) (Koti *et al.*, 2006), while all other combinations produce a spring type or facultative growth habit (Takahashi and Yasuda, 1971, Koti *et al.*, 2006).

A more efficient strategy would have been to use MAS to select the spring *vrn*-H2 allele as well as the 2 RFT alleles in the BC_1F_1s used for DH production. The resultant population would have had a higher frequency of spring *vrn*-H2 alleles (1:1 winter:spring) and therefore a larger number of spring type DH lines. An alternative strategy would have been to use a recurrent parent carrying a spring *vrn*-H2 allele. This would have ensured that all the progeny were spring type. It was not possible to use this strategy in the described study, as diagnostic markers for *vrn*-H2 were not available at the time (and it was not recognised that WI3806 carried the winter vrn-H2 allele).

The use of widely flanking markers to introgress an imprecisely mapped locus can have implications for population sizes and the confidence in retaining the gene(s) of interest. Although the genomic regions containing the 5H and 2H RFT QTL were mapped in multiple populations including some common markers (Chapter 2) these were delimited to relatively large marker intervals of 15cM and 10cM respectively. Selection of the genomic regions with confidence therefore necessitated selection across relatively large chromosome intervals. Introgressing a large QTL region can introduce undesirable genes linked to target loci. Selecting a large segment increases the chances of selecting for unfavourable genes/alleles linked to the target genes. An example of this is the incorporation of boron tolerance from the landrace 'Sahara' into an Australian adapted background, which reduced visual symptoms of boron toxicity but did not increase yield. This observation, coupled with further genotyping around the boron tolerance locus showed that a significant yield

penalty is likely to be associated with genes linked to boron tolerance (McDonald *et al.*, 2009)

Graphical genotyping can accelerate the elimination of unwanted donor genome segments. When Jefferies (2000) used whole genome genotyping on BC_1 derived individuals, a recurrent parent genome content similar to that normally obtained with BC_3 derived lines was obtained. Jefferies (2000) concluded that it was possible to avoid several cycles of backcrossing by selecting for the recurrent parent genome using molecular markers. In the current study, selecting for 2 intervals totalling around 25cM, out of a total of 1189cM, meant that a perfect scenario would be a 2% retention of the donor genome. The line with the lowest amount of donor genome was BX03S;198DMW-168 with 10.5% of the donor genome retained. This is similar to the average of 9.7% expected if using the much slower BC_5 backcrossing approach.

The study successfully generated lines combining the Japanese derived frost tolerance alleles with an elite Australian genetic background. The breeding strategy incorporated the use of multiple cycles of marker assisted selection, doubled haploid production and off season multiplication to develop fixed lines with the targeted molecular ideotype in the shortest possible time. Subsequent whole genome analysis provided a detailed characterisation of the resultant fixed lines. These lines form a foundation for the formal assessment of the RFT loci and the potential commercial release of a RFT variety suitable for Australian barley production.

Chapter 6. Evaluation of the RFT barley breeding strategy

Introduction

The previous chapters reported on approaches for the identification, mapping and development of a breeding strategy for the introgression of frost tolerance into an Australian adapted barley background. The aim of the breeding strategy was to introgress small chromosome segments carrying the identified Reproductive Frost Tolerance (RFT) alleles into an adapted genetic background in the shortest possible time. The performance of the 2 RFT loci in commercially relevant genetic backgrounds in a barley breeding program is as yet unknown. However, the lines generated in Chapter 5 can now be used to validate the effectiveness of these 2 RFT loci in a genetic background that has direct commercial potential. If the effectiveness of the 2HL and 5HL RFT loci can be validated in the WI3806 genetic background it will provide plant breeders with confidence to use these alleles in plant improvement. This could provide the final step from experimental and theoretical applications for this new source of variation into a working plant breeding application.

The RFT loci were identified in germplasm derived from Japan, and the adaptation of these lines to Australian conditions is poor, as shown by evaluations under Australian production environments (Karakousis *et al.*, 2003a). This prompted the backcross strategy with a particular focus on introgression segment quantification, with the view that smaller introgression segments and higher recurrent parent retention will confer better adapted germplasm. The validity of such an approach has been presented by several authors (Ribaut *et al.*, 2002, Frisch and Melchinger, 2005). How successful this approach was in retaining the adaptation of the recurrent parent in the WI3806*2/GH-129 lines will be evaluated in this chapter.

The effectiveness of the new trait is not the only consideration when integrating a new source of genetic variation in breeding. Deleterious genes linked to the gene of interest can have negative effects on adaptation and/or quality (Frisch and Melchinger, 2005). Assessing the overall effects of the chromosome regions carrying the target loci will inform the best strategy for using the donor material. For example, if an association can be found between Haruna Nijo genome retention and decreased yield, then it could be concluded that the RFT genes or genes closely linked to them reduce yield. If such an association is observed, the population size or number of backcrosses may need to be increased to rid the lines of these undesirable genes.

The 2 major experimental aims of this chapter are:

1. To assess RFT in the WI3806*2/GH-129 derived lines and compare their RFT phenotype with those of the parents. This will be done using both a frost simulation chamber and field based screening.

2. To evaluate the yield of the WI3806*2/GH-129 derived lines in the absence of frost at flowering to determine if the yield and adaptation of the recurrent parent can be recovered despite selection for relatively large donor introgression segments. This will be completed by measuring grain yield across a range of barley production environments in southern Australia.

Materials and Methods

*RFT of selected WI3806*2/GH-129 derived lines in a frost chamber*

The Australian Genome Research Facility (AGRF) frost chamber was used for phenotyping selected lines from the WI3806*2/GH-129 population as well as parents GH-129 and WI3806. The limited space in the AGRF frost chamber restricted the number of lines that could be examined so a subset of lines were randomly selected for phenotyping. Each

genotype was planted in multiple pots over 4 seeding dates to allow for maturity differences. All plants were grown outside during the 'normal' crop production period so that the day length and temperature conditions would be the same as those experienced by a commercial crop. When at least one of the seeding times of every line had reached head emergence, in particular stage 3 as outlined below, individual tillers were tagged according to their developmental stage so this could be taken in to account when analysing the Frost Induced Sterility (FIS) data. Tillers were categorized in to four developmental stages (Zadoks *et al.*, 1974):

Stage 1: Booting/pre anthesis (Z45)

Stage 2: Awns emerging/pre anthesis/anthers green (Z49)

Stage 3: Head emerging/pre anthesis/anthers yellow (Z55)

Stage 4: Head fully emerged/post anthesis (Z59 to Z69)

Plants were placed in the frost chamber in a randomised complete block design as illustrated in Figure 1. The coordinate (range and row) was recorded for each pot. Buffer pots, containing plants that were not used for data capture, were placed either side of the tester lines to reduce temperature variation within the chamber. Plants were exposed to a 24 hour treatment that simulated a single radiative frost event identical to that described in Chapter 4. After the frost treatment, plants were returned to the field and grown to physiological maturity. A second replicate of test lines were treated on the following day. Each spike was scored for the level of FIS by counting the number of sterile florets and expressing this as a percentage of the total number of florets on that spike.

Sterile florets/Total florets*100 = Frost Induced Sterility %

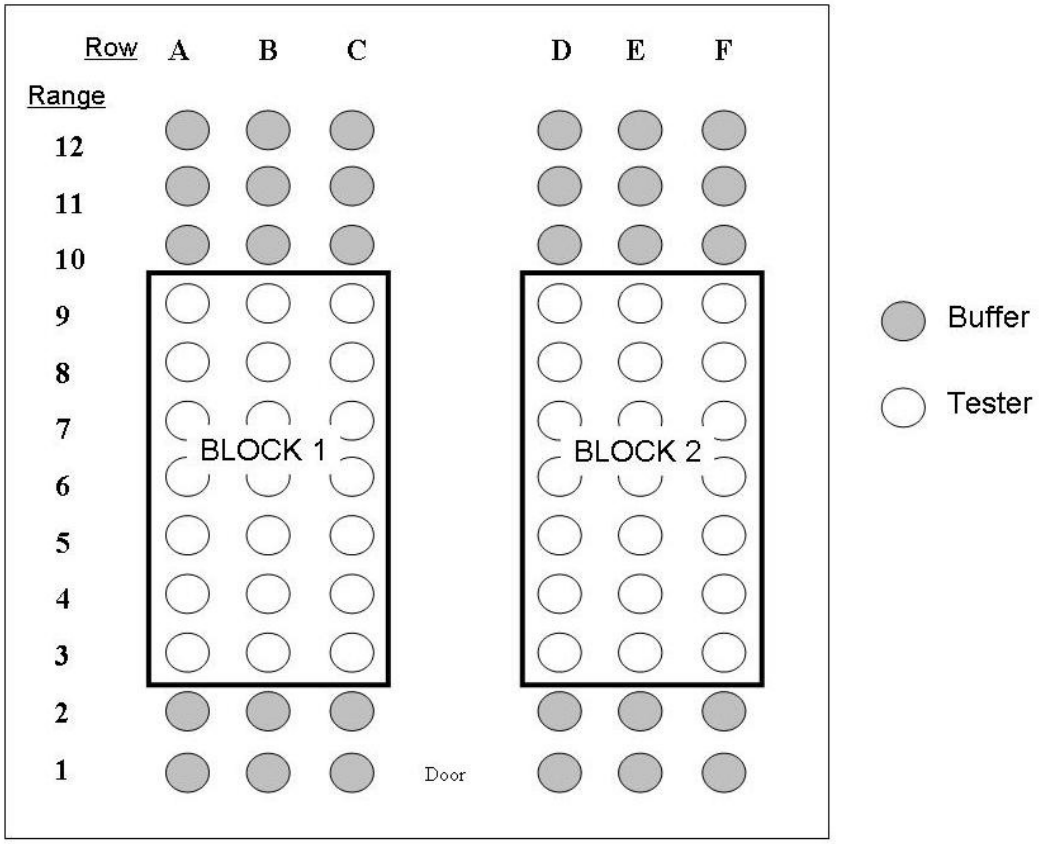

Figure 1. A schematic diagram of the layout of the 2 frost chamber experiments conducted in the AGRF frost simulation chamber in 2006. 'Tester' refers to the location of pots with plants for which FIS data was collected. 'Buffer' refers to pots with plants that did not have FIS data collected from them.

FIS data were spatially analysed using REML (Genstat© Version 8) to account for spatial temperature variation within the chamber. Maturity scores were included in the analysis as a covariate to remove effects from small differences in developmental stage. Data from experiment 1 and experiment 2 were analysed individually and also in combination, fitting the experiment as a covariate to increase statistical power. Means and standard errors were obtained to identify significant differences.

Field evaluation of RFT

Space for RFT phenotyping in the field was not as limited as for in the AGRF frost chamber. Therefore, a wide range of lines from the WI3806*2/GH-129 population, parents and long term checks were sown at the Loxton frost screening nursery in 2007 (Refer to Figures 5 and 6 for specific genotypes). These lines carried various allelic combinations for the two RFT loci to test the effects of the tolerance alleles both alone and in combination. The trial was planted in a randomised complete block design with 2 replicates per seeding time with 5 different seeding times. Each entry was grown in a 3 row, 1.5 meter plot. The 5 seeding dates for these lines were 30/03/07, 13/04/07, 20/04/07, 27/04/07 and 04/05/07. Multiple seeding times were used to allow for maturity differences between lines to be accounted for later in the season. Seeding times were strategically conducted earlier than 'normal' in a commercial scenario as to enable plants to reach a frost susceptible stage during the period of highest frost risk. So that the early seeding times could precede the opening season rains, irrigation was used to support establishment and early growth.

Data loggers were placed at the top of the plant canopy to record temperatures experienced by the spikes. When the temperature reached -2^{o}C, a phone text message alarm would notify that a frost event had occurred. Within 2 days after a frost event, each tiller of each plant across all seeding times were assessed for developmental stage. Those tillers that were at the target developmental stage (head emerging/pre anthesis/anthers yellow, Z55) were tagged with a coloured tape gun and scored later for FIS. It is worth noting that, although multiple seeding times were used, it was not possible to collect data from some lines due to very early or very late phenology. Two weeks after a frost event, spikes from tagged tillers were collected, dried and then scored for the level of FIS. Data collected were analysed by REML (Genstat© Version 8) to account for temperature variation that may have occurred during the frost event and spatial trends in FIS within the data set.

*Agronomic performance of selected WI3806*2/GH-129 derived lines*

Selected lines derived from the WI3806*2/GH-129 population were planted in yield trials over the 2007 growing season. The seed availability of each line was variable, so not all lines could be evaluated at all trial sites. A standard set of check varieties were located at each trial site to allow calculation of relative yield and therefore comparisons between sites. Yield trials were at Geranium, Cook Plains, Peebinga, Pinnaroo and Lameroo in South Australia. Trials were arranged in a randomised complete block design with 3 blocks. Yield data were analysed by REML (Genstat© Version 8), fitting significant fixed and/or random spatial effects into the model to reduce error before producing means.

Data loggers were located at a number of the yield trial sites to detect frost events. As the maturity of the lines varied significantly, direct FIS comparisons could not be made between lines in this set of trials. FIS data were collected from sites that were affected by frost so they could be statistically removed from the analysis of grain yield.

Visual observations were made on each line at each site during the growing season. Data was collected on the phenology of the lines using a visual rating system. Each line was visually categorised into the following classes; very early maturity, early maturity, mid maturity, late maturity and very late maturity.

Results

*Reproductive frost tolerance of selected WI3806*2/GH-129 introgression lines using a frost chamber*

The highest proportion of barley heads were tagged at the head emerging/pre anthesis/anthers yellow (Z55) developmental stage. This was also the developmental stage

used in the field based screening to originally identify the 5H and 2H RFT QTL. Therefore, only data collected from heads at this stage were used in the analysis. Barley lines exposed to both frost events simulated in the frost chamber displayed a range of FIS, from 0% to over 80% (Figures 2 and 3). Data collected from experiment 1 were analysed using REML (Genstat© Version 8), revealing a significant genotype effect on FIS ($P<0.001$). Also, a significant linear range.row effect ($P<0.001$) was observed to influence FIS in the chamber. Once this was taken into account predicted genotype means and standard errors were generated and are displayed in Figure 2.

In the second frost simulation, a significant genotype effect ($P<0.001$) and a linear range.row effect ($P<0.001$) was also observed. Means and standard errors are displayed in Figure 3. In both experiments, the donor line GH-129 showed significantly lower FIS compared to the recipient line WI3806. Means revealed a similar ranking of the WI3806*2/GH-129 derived lines across the two experiments.

The data from the 1st and 2nd frost chamber experiments were combined and analysed using REML (Genstat© Version 8). The genotype effect of FIS was not different between the two experiments. This indicated that experimental conditions were effectively replicated between the two experiments. There was, however, a significant range.row effect ($P<0.05$) and a significant genotype effect ($P<0.001$) shown by the combined data set.

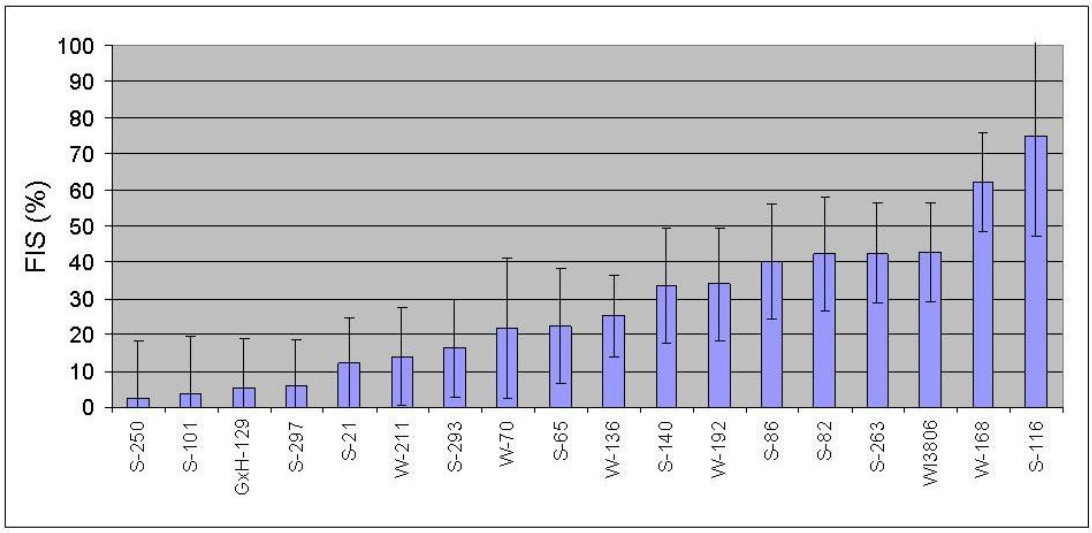

Figure 2. Mean FIS and standard error for lines from the WI3806*2/GH-129 population and parents, obtained from frost chamber experiment 1.

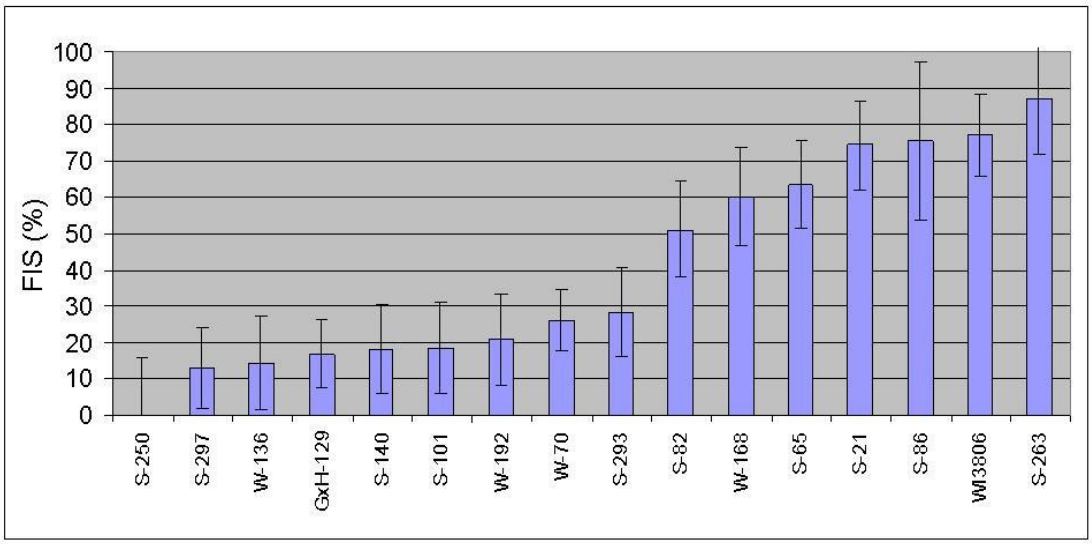

Figure 3. Mean FIS and standard errors for lines from the WI3806*2/GH-129 population and parents, obtained from frost chamber experiment 2.

The range.row effect on FIS in both experiments indicated that there was a common trend occurring in the frost simulation chamber. Temperature sensor data showed that a temperature gradient was occurring from the warmer front left to the cooler back right of the chamber. During the coldest stage of the simulation program, the gradient across the chamber was as great as 1.5^{o}C. Although this gradient was having an effect on FIS

independent of the genotype effect, the trend was consistent enough to be largely removed by the statistical analysis, indicated by the highly significant range.row effect (P<0.001).

The means and standard errors of the combined frost chamber data sets are displayed in Figure 4. Of the 16 lines from the WI3806*2/GH-129 cross, 11 had significantly lower FIS than the recurrent parent WI3806. Based on the graphical genotypes displayed in Figure 4, two of the lines that had FIS means that were not significantly lower than WI3806, S-116 and S-86, may not have been carrying the tolerance allele at the 5HL RFT locus. Although there were three lines that had the 2 RFT loci that were not significantly better for FIS than the recurrent parent, a t-test revealed that the group of lines carrying both of the donor RFT loci had significantly lower FIS than the group of lines that did not (P<0.01).

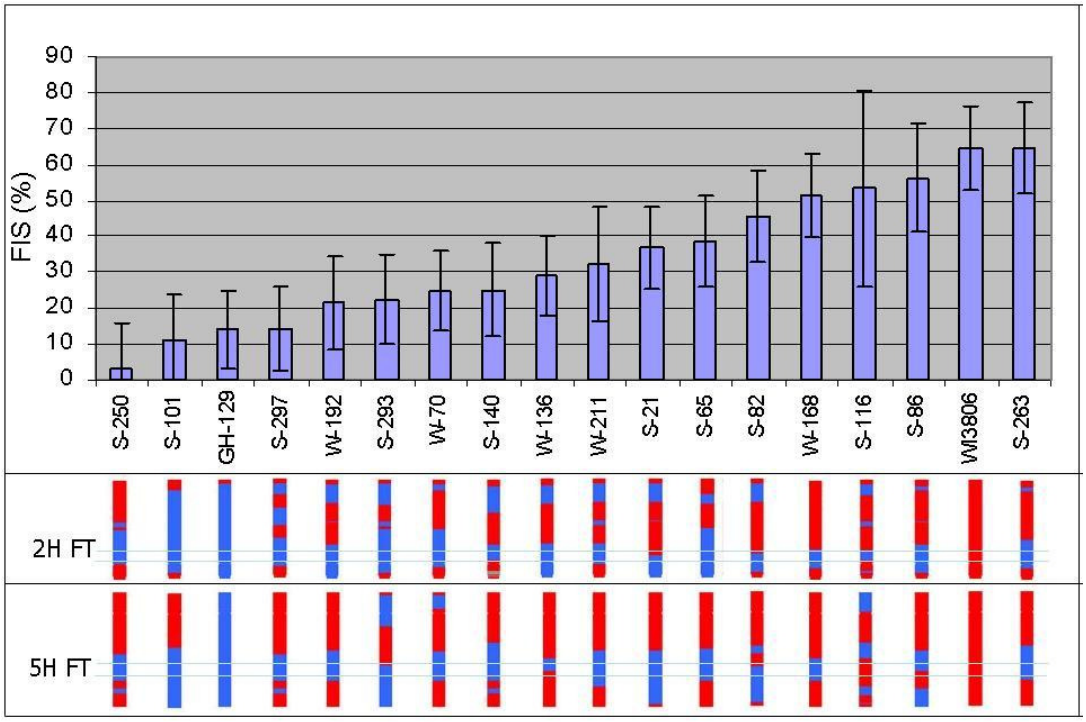

Figure 4. Mean FIS and standard errors from frost chamber experiments 1 and 2 compared to chromosome 2H and 5H genotypic profiles. Green lines indicate the boundaries of the RFT QTL (Chapter 2). Red bars correspond to intolerant parent genomes (Galleon or WI3806) and blue bars correspond to the donor genome of Haruna Nijo.

Field based RFT screening

During 2007 RFT screening at Loxton SA, 2 major groups of frost events occurred. These caused FIS to the lines from the WI3806*2/GH-129 population in addition to the parents and other well characterised barley lines. The first grouping of frost events occurred from the 19th-22nd of July when the minimum temperatures reached -4.5 °C, -3.7 °C, -3.3 °C and -5.1 °C, respectively. The second occurred on the 14th and 15th of August when the minimum temperatures reached -3.2 °C and -3.5 °C, respectively. A higher mean level of FIS was observed from the July event compared to August event.

The July frost events produced FIS in the approximate range of 18% to 75% (Figure 5). A significant genotype effect ($P<0.001$) on FIS was revealed. The RFT Japanese lines Haruna Nijo and Amagi Nijo and RFT donor line GH-129 recorded a significantly lower FIS compared to the Australian barley lines WI2585 and Schooner as well as the long term susceptible check ICARDA#70. However, the donor line GH-129 and the two RFT Japanese lines did not show significantly less FIS than the recurrent parent WI3806 or the Australian line Galleon. This was not consistent with results from previous analyses reported in Chapter 2. The majority of lines tested from the WI3806*2/GH-129 population exhibited lower FIS means compared to the Australian barley line WI2585. The July frost event revealed no significant effect of the RFT 2H, 5H or 2H+5H alleles on decreasing FIS ($P>0.05$).

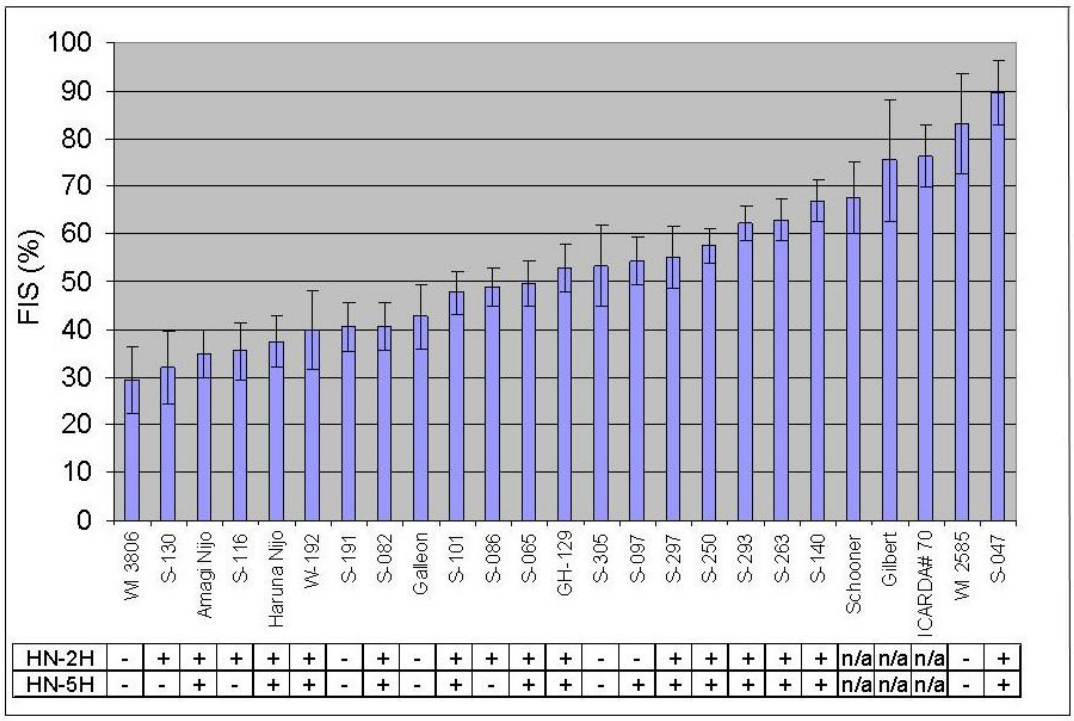

Figure 5. Mean FIS and standard errors of genotypes exposed to the July 2007 frost events at Loxton, SA. The presence or absence of the Haruna Nijo (donor) allele at the two RFT loci are shown in the table below each genotype.

FIS from the August frost event was on average lower that that observed from the July frost event. The analysis of data from the August frost event revealed a significant genotype effect on FIS ($P<0.01$). The RFT lines Amagi Nijo, Haruna Nijo and the RFT donor line GH-129 all had significantly lower FIS compared to ICARDA#70 and WI2585. This result is consistent with the July 07 frost event and previous reports in Chapter 2. The recurrent parent of the WI3806*2/GH-129 population displayed significantly lower FIS compared to the donor line GH-129 and lower (although not significantly) FIS compared to the Japanese RFT lines Haruna Nijo and Amagi Nijo. This lower FIS observed in the recurrent parent WI3806 is consistent between the 2 field frost events but inconsistent with the frost chamber experiments. As for the July frost event, no significant ($P>0.05$) donor 2H, 5H and 2H+5H RFT allele effects on FIS were observed.

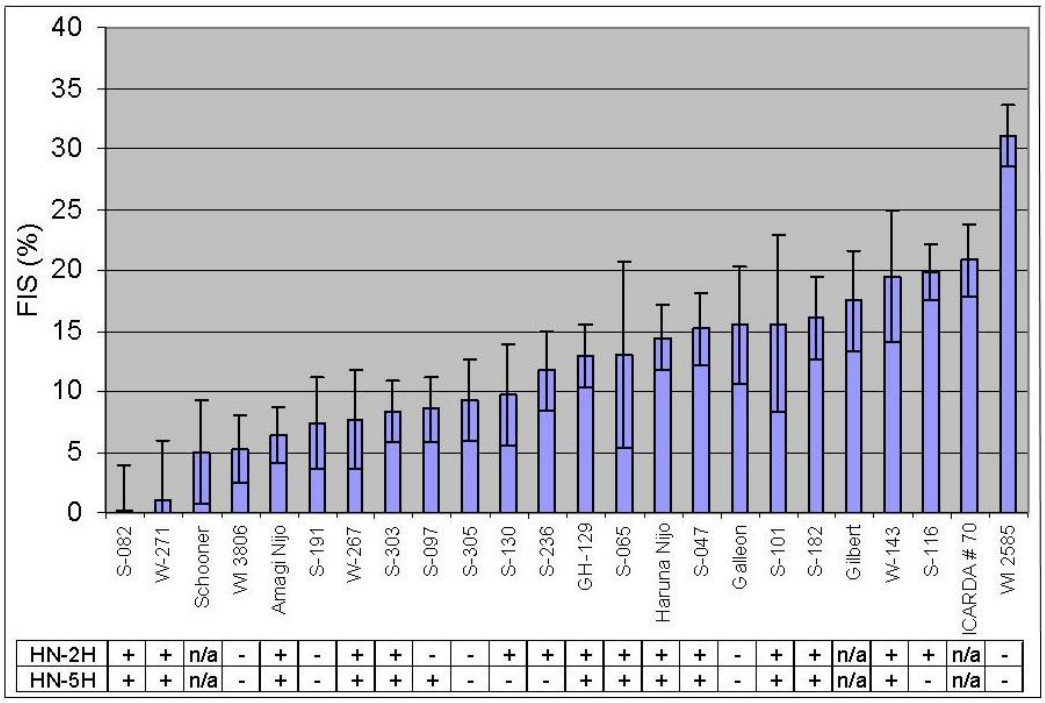

Figure 6. Mean FIS and standard errors of genotypes exposed to the August 2007 frost event at Loxton, SA. The presence or absence of the Haruna Nijo (donor) allele at the two RFT loci can be seen in the table below.

*Agronomic performance of selected lines from the WI3806*2/GH-129 population*

Mean yield ranged from 424 kg/ha at Peebinga to 1970 kg/ha at Cook Plains in 2007. Frost damage was recorded in some lines from the Geranium and Cook Plains trial sites. As the germplasm had a range of maturity types, and there was only one seeding time, a direct comparison between genotypes could not be made for frost tolerance. Therefore the FIS scores were fitted to the statistical model and the mean effect removed from the predicted means for grain yield. As the range in maturity was large, some head loss of early genotypes was recorded at Geranium. The loss of barley heads onto the ground, and therefore yield, can occur when barley plants become over ripe and stems become brittle. This effect was also removed from predicted means by fitting the scores into the analysis. Each site was analysed individually, fitting the FIS and head loss terms as well as spatial terms to remove any spatial trends in the trials affecting yield. A significant genotype effect

on grain yield (P<0.001) was observed at all 5 sites. To obtain an across site predicted mean for each genotype, all data was analysed together fitting terms to allow for spatial effects within sites as well as across sites. The following terms had a significant fixed effect on grain yield and were therefore added to the model when analysing the combined data set. 'Site', 'Site.FIS', 'Site.row' and 'Site.range'. A significant genotype effect was observed (P<0.001) from this analysis, with predicted means and standard errors displayed in Figure 7.

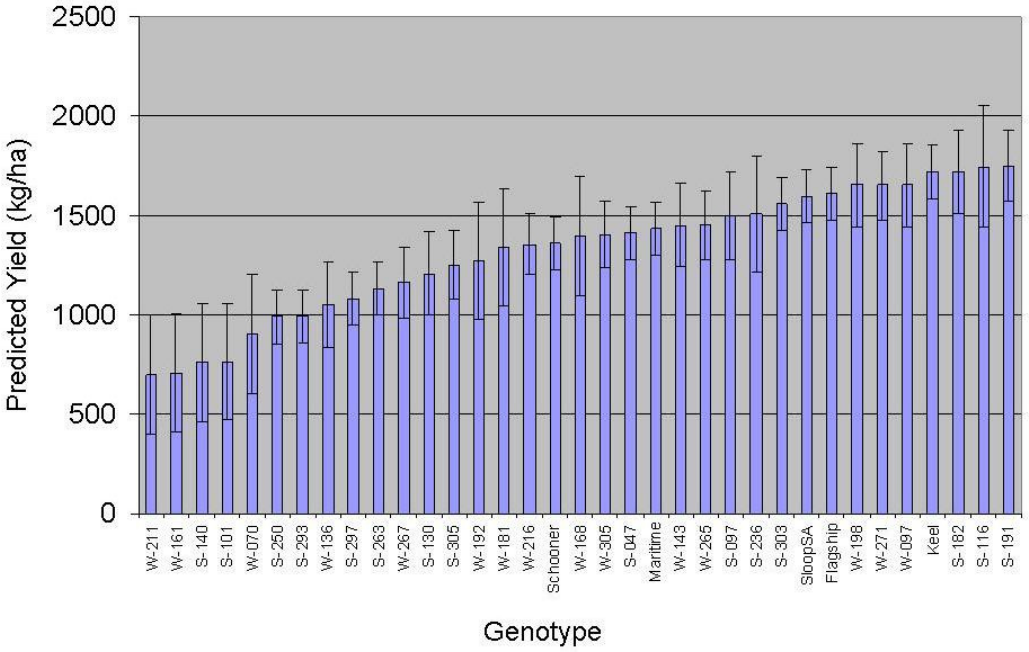

Figure 7. Predicted mean yield and standard error of progeny from the WI3806*2/GH-129 population and commercial checks across 5 sites in the South Australian Mallee in 2007.

Within the check varieties the current benchmark commercial barley variety for yield, Keel, performed the best. The older barley variety Schooner performed the worst. This is consistent with long term data obtained over a range of environments in the SA Mallee (Wheeler, 2007). Approximately 46% of the lines derived from the WI3806*2/GH-129 population performed better than the barley variety Schooner. Schooner represents the

minimum yield requirement for retention in South Australian barley breeding programs (J. Eglinton, personal communication, 2007).

Relationship of donor/recurrent parent on performance

DArT genotypic profile was used to calculate the proportion of the Haruna Nijo genome carried by each line. Each DArT marker has a centimorgan location on the chromosome determined by previous genetic mapping (Hearnden *et al.*, 2007). The percentage of the Haruna Nijo (donor) genome was determined by dividing the number of centimorgans covered by Haruna Nijo alleles by the total genetic distance of the genome-wide map, and multiplying by 100.

Ie. Number of cM with Haruna Nijo alleles/Total cM of genome*100

A regression analysis was performed between the percentage of Haruna Nijo in each individual and the predicted mean yield across the 5 sites where grain yield evaluation took place in 2007. A significant ($P<0.05$) relationship was observed between these two parameters, with the higher proportion of donor genome being associated with lower mean yield (Figure 8).

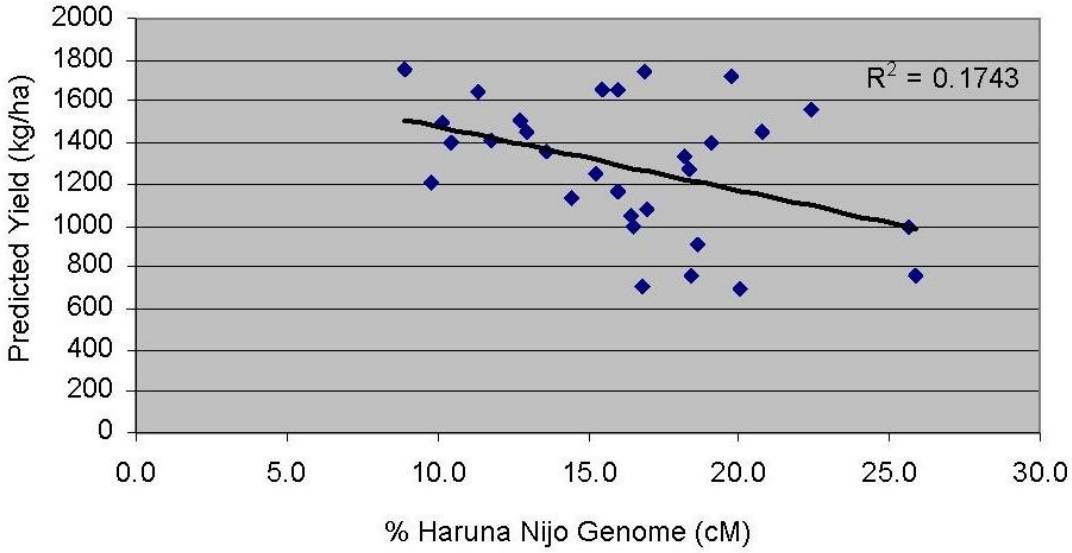

Figure 8. The proportion of the RFT donor genome (Haruna Nijo derived) versus predicted yield of lines from the WI3806*2/GH-129 population across 5 sites in the South Australian Mallee in 2007.

Time to maturity was segregating in this population and so an investigation into the relationship between predicted yield and maturity was conducted. An analysis of variance revealed that there was a significant association between maturity class and predicted yield ($P<0.001$). The Very Early (VE) maturity class exhibited a significantly lower mean yield compared to the other classes (Figure 9).

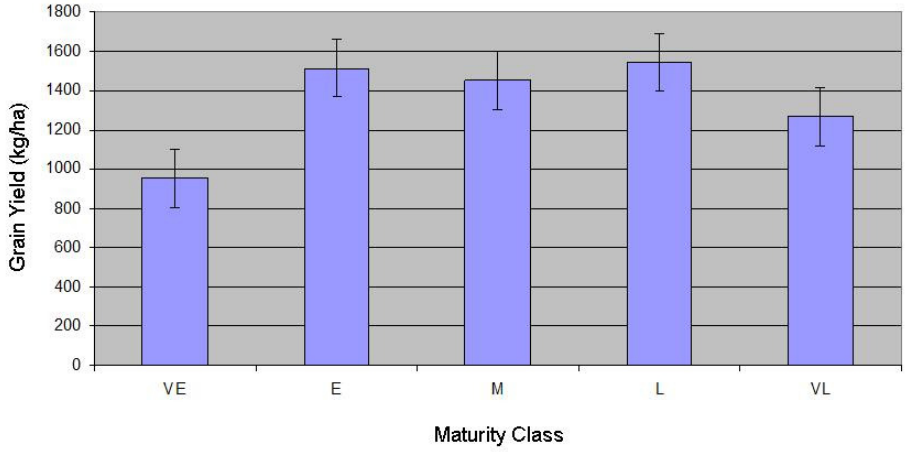

Figure 9. Mean grain yield of lines in different maturity classes from the WI3806*2/GH-129 population. VE = Very Early maturity, E = Early maturity, M = Mid maturity, L = Late maturity, VL = Very Late maturity.

The Very Early (VE) lines were excluded from the data set and the analysis of variance was re run on the subset of data. No significant maturity effect was observed on grain yield ($P>0.05$). This data set was used to re run the regression analysis between the percentage of Haruna Nijo donor genome and yield. There was no significant association between Haruna Nijo donor genome and yield when using this data set ($P>0.05$).

Discussion

*Validation of RFT in the WI3806*2/GH-129 population*

The frost chamber experiments were able to confirm that lines carrying the RFT loci from the donor parent were, in most cases, displaying less frost damage compared to the recurrent parent. This result is consistent with the findings in Chapter 2 where this source of tolerance was genetically mapped to 2 loci in 2 mapping populations derived from crosses between the Japanese lines and frost susceptible Australian genotypes. It also confirms that

the AGRF frost simulation chamber can be used to effectively discriminate between lines carrying tolerance or intolerance alleles at the two RFT loci.

The field result in 2007 was not consistent with the previous field based screening studies or the AGRF frost simulation chamber experiments. On both occasions, the recurrent parent WI3806 displayed frost tolerance that was similar to or better than the RFT donor source. Lines from the WI3806*2/GH-129 population did display tolerance that was better than current commercial barley varieties. In order to assess the effects of the RFT alleles alone and in combination, all 4 combinations of alleles at the 2 RFT loci were tested in germplasm from the WI3806*2/GH-129 population. No significant RFT effect was observed.

Several factors may potentially explain why the RFT loci had no detectable effects on RFT in the WI3806*2/GH-129 population:

1. WI3806 has a level of frost tolerance that is equal to or better than the donor parent Haruna Nijo. WI3806 was not tested extensively for its level of RFT prior to using it as a recurrent parent in the RFT breeding strategy (Chapter 5). WI3806 parents Mundah, Keel and Barque as well as WI3806 sister line cv.Fleet have all been screened more extensively in the field. All have shown less RFT than the Japanese lines Haruna Nijo and Amagi Nijo, although on some occasion Keel has shown levels of FIS comparable to the Japanese lines (data not shown). It is therefore possible that WI3806 has inherited a level of RFT from Keel that was effective under the particular conditions experience in the frost events at Loxton in 2007. If this was the case, the tolerance was not expressed in the experiments conducted in the AGRF frost simulation chamber.

2. The conditions experienced during the 2007 frost events at Loxton were not conducive to the expression of the Japanese source of RFT. The frost events that caused damage at Loxton in July 2007 was a result of 4 consecutive frost events with the lowest temperature

of -5.1 ^{o}C recorded on the 4th day. This minimum temperature was very low and only paralleled by one previous event that occurred in August 2005 at Loxton where the minimum temperature reached -5.5 ^{o}C. For this event, Japanese RFT germplasm and Australian derived germplasm both showed high levels of FIS, and were not significantly different (data not shown).

3. The extreme variation in phenology within the relatively small validation population confounded the accurate assessment of RFT. Comparing data from different genotypes with large differences in maturity means that data is collected from different seeding time blocks. The physical distance between different seeding times means that spatial effects maybe larger than that experienced within a single seeding time block. The small numbers of lines within different seeding times means that it is difficult to remove these spatial effects accurately.

Temperature data from the target production environment was used to characterise a typical frost event during the normal flowering time in commercial barley crops. Data was obtained from the Australian Bureau of Metrology. Minimum daily temperatures data from 1957-2006 for the Loxton weather station were used to determine frost occurrence and severity. The following criteria were used to define the frost events during this period:

i. A frost event occurs when a minimum temperature below 1^{o}C is recorded at 1.2 meters in a Stevenson screen.

ii. The frost risk period for commercial barley production is between the 1st of September until the 15th of October. This is the period where most commercial barley crops are at a stage between awn emergence and early grainfill.

iii. The difference between recorded temperature at a 1.2m Stevenson screen and the temperature experienced by the crop is -2.2 ^{o}C (http://www.bom.gov.au/climate/map/frost/what-is-frost.shtml).

Using the above criteria it was observed that 38 frost events occurred during the peak reproductive frost risk period between 1957 and 2006. Of the 38 frost events recorded, 6 were classed as multiple day frost events. This meant that the temperature dropped below 1^{o}C on 2 or more consecutive days. 32 frost events were classed as single day discrete events.

The temperature that the plant experienced during these 38 events can be estimated. The difference between recorded minimum temperature and that experienced at plant is approximately -2.2 ^{o}C as quoted by the Australian Bureau of Meteorology. Of the 38 events it is estimated that 2 events reached a temperature below -4^{o}C at crop canopy height, with the coldest temperature experienced estimated to be -4.7 ^{o}C in September 1995. 3 events were estimated to be between -3 ^{o}C and -4 ^{o}C. 11 events were estimated to be between -2 ^{o}C and -3 ^{o}C. 22 events were estimated to be between -1 ^{o}C and -2 ^{o}C.

The data for FIS collected from the field was from multiple frost events in 2007 but previous field data detecting expression of the Japanese source of RFT was predominantly derived from single day frost events. Therefore, this tolerance source may not be effective under conditions of consecutive days of sub zero temperatures. This was one of the differences between conditions experienced in the frost simulation chamber and the field based screening using the same germplasm. In this region, 6 out of the 38 frost events from the past 50 years have involved multiple consecutive days of frost. The commercial implications are quite minimal if this source of tolerance is found to be ineffective when exposed to multiple consecutive frost events.

The minimum temperature where the Japanese source of RFT is effective was investigated further. Data were collated from all previous frost events at Loxton where FIS was recorded to provide a better appreciation for the range of temperatures the Japanese level of RFT has

over established southern Australian commercial barley varieties. To represent the tolerant 'FT barley' category, the mean FIS of both Haruna Nijo and Amagi Nijo were averaged for each frost event. To represent to intolerant 'barley' category, the commercial varieties Schooner and Sloop were used, with mean FIS averaged for each event. It is worth noting that over several seasons and frost events the lines within each group have performed similarly in terms of their level of frost damage. Average FIS scores were plotted against the minimum temperature recorded during the frost event where the FIS scores were taken.

A linear trend was seen for both the RFT barley and Australian barley genotypes between -1 ^{o}C and -4.6 ^{o}C (Figure 10). A single data point at -5.5 ^{o}C was available for both genotype classes, and indicates that the linear trend may not hold for RFT barley at a temperature of -5.5 ^{o}C. Including the -5.5 ^{o}C data points in the correlation meant that the linear trend was weak between RFT and minimum temperature for 'FT BARLEY' class of data. Therefore the graph was redrawn with the trend line only including data points from -1 ^{o}C and -4.6 ^{o}C. The linear relationship was much more robust using data in this temperature range. The temperature at which the Japanese tolerance may no longer effective can not be defined between -4.6 ^{o}C and -5.5 ^{o}C from this data set. It is however possible that at -5.1 ^{o}C, as experienced in the July 07 frost event at Loxton, the Japanese level of tolerance may not be effective.

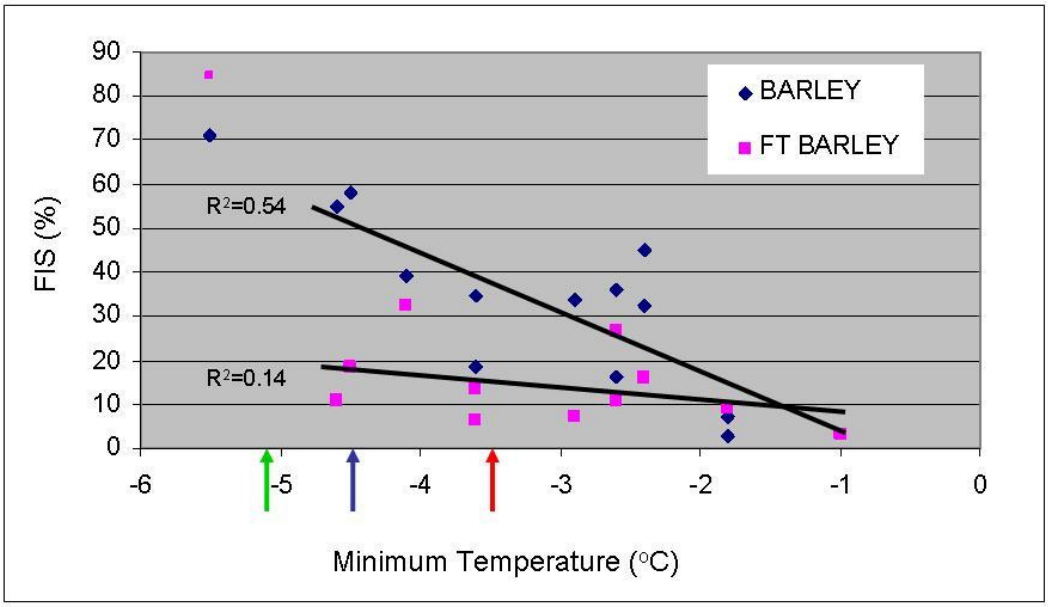

Figure 10. The relationship between minimum temperature and FIS for 'Barley' (Schooner and Sloop FIS means averaged) and 'FT Barley' (Amagi Nijo and Haruna Nijo FIS means averaged) from field based frost screening at Loxton, SA. Green arrow indicates the minimum temperature recorded for the July 2007 frost event at Loxton. Red arrow indicates the minimum temperature recorded for the August 2007 frost event at Loxton. Blue arrow indicates the minimum temperature recorded in the AGRF frost simulation chamber for both experiments in 2006. Trend lines represent data from frost events between -1 ^{o}C and -4.6 ^{o}C.

Data collected from the Loxton weather station over the past 50 years indicated that only 1 event was estimated to reach a temperature below -4.5 ^{o}C. This event recorded a temperature at Stevenson screen of -2.5 ^{o}C which would have been an estimated minimum temperature of -4.7 ^{o}C at crop canopy. The remaining 37 events all recorded temperatures that would have been above -4.5 ^{o}C at the plant canopy height. If the Japanese source of RFT is not effective at temperatures below -4.5 ^{o}C this barley germplasm would still be useful as temperatures very rarely fall below this temperature in this region.

Although a relationship between FIS and minimum temperature can be seen, this does not appear to explain all of the variation in FIS for the RFT and non RFT germplasm. Other factors such as rate of cooling prior to a frost event, rate of warming post a frost event, duration of time below a certain temperature and the number of consecutive days of minimum temperature may all play a role in determining the amount of damage incurred by a barley spike. The temperature leading up to a frost event also cannot be discounted. Temperature acclimation is a major driving force behind frost tolerance at the vegetative stage, with temperature of induction and rate of acclimation differing between tolerant and intolerant genotypes (Kalberer *et al.*, 2006, Fowler and Limin, 2004). Even though the literature suggests that acclimation is reduced post the vegetative to reproductive transition, a residual amount of acclimation at the reproductive stages of development cannot be discounted.

The frost event in August was milder than the event recorded in July 2007 at Loxton and inflicted a lower mean level of FIS. Although a significant genotype effect on FIS was observed, the low level of difference in FIS between the best and worst performer meant that not many genotypes were significantly different from each other. Data displayed in Figure 10 illustrated that at the milder end of the temperature range, frost events do not discriminate between tolerant and intolerant germplasm as well. This may have been a reason behind the difficulties in finding an effect of the Japanese derived sources of RFT in this experiment.

The frost simulations were able to reproduce a frost event with a minimum temperature of -4.5 ^{o}C. Data illustrated in Figure 10 shows that from previous field experiments -4.5 ^{o}C provides the highest level of discrimination between tolerant and intolerant germplasm. This may have helped discriminate between lines carrying the 2H and 5H RFT alleles and the recurrent parent WI3806.

*Agronomic performance of selected progeny of the WI3806*2/GH-129 population*

The progeny of the WI3806*2/GH-129 population were yield tested in the target frost prone environment in the Mallee region of SA. Almost half of the lines tested in these yield trials yielded above the commercial variety 'Schooner' and would therefore be competitive with other commercial varieties grown in this region. Three of the lines exhibited grain yield above 'Keel' which has shown consistent yield superiority in this production environment. If the yield of these could be validated across seasons in this production zone, they would be commercially attractive due to yield potential alone independent of potential benefits from increased RFT.

The significant relationship between decreased proportion of donor genome and increased yield did not hold once maturity effects were taken into account. There were lines from this cross that displayed high grain yield and did not have the lowest proportion of Haruna Nijo genetic background. An example is the line S-182 having 19.8% of the donor line genome but still recording grain yield above Keel and being in the top 3 lines for this series of trials. This percentage of donor genome is above the average for the selected individuals from the WI3806*2/GH-129 population (Chapter 5, Figure 5) and much higher than the line with the least percentage of donor genome (8.9%). The poor relationship between the proportion of donor genome and reduced yield observed may have been due to specific combination of donor and recurrent parent genes that were producing lines with superior adaptation in this production environment. Genes that can have a large effect on yield in this environment are those controlling maturity. Lines from the WI3806*2/GH-129 population did display maturity that were earlier and later than the parents of the population. Early maturity in this environment is often associated with higher yield, as it reduces exposure to drought stress which occurs mainly at the end of the growing season (Coventry *et al.*, 2003). Although

extreme early maturity has been associated with a yield penalty in this environment (S. Coventry, personal communication, 2009). The earliest genotypes from this population were significantly lower yielding than lines in the later maturity classes.

Implications for barley breeding

The research reported in this chapter has suggested that the amount of donor genome retained in progeny from Haruna Nijo does not necessarily directly relate to lower grain yield. Major effects on phenology tended to have a larger effect of the grain yield performance of the progeny of the WI3806*2/GH-129 cross. The breeding strategy employed was able to identify a number of lines derived from the WI3806*2/GH-129 cross that, based on 1 year's data from 5 sites, were commercially competitive for yield as well as carrying the 2 RFT loci identified in Chapter 2.

The validation of the 2 RFT loci in the WI3806*2/GH-129 population was not clearly resolved in this chapter. The AGRF frost chamber experiments using a minimum temperature of -4.5^{o}C did indicate that individuals carrying the 2 RFT loci had an improved level of RFT, comparable to the donor line GH-129. The field based experiments did not produce data that supported the chamber experiments or previous work conducted in the field using the Japanese source of RFT. Frost events in the field at Loxton in 2007 may not have been within the conditions that this source of tolerance can easily be discriminated from intolerant germplasm.

If the Japanese source of RFT is only effective at temperatures above -4.6 ^{o}C, breeders will need to assess whether this level of protection would be economically beneficial for farmers in their breeding target zone. In southern Australia, temperatures during spring, when commercial crops are at the sensitive flowering stage, rarely fall below -4.5 ^{o}C at canopy

height. Temperatures in northern Australia during late winter/early spring, when cereal crops are at the sensitive flowering stages of development, can fall well below this temperature. A -6.5 ^{o}C frost event Toowoomba, Queensland, in 2004 produced FIS levels in Haruna Nijo and Amagi Nijo that were not significantly lower than those of commercial checks (Fredericks *et al.*, 2005). Therefore, germplasm targeted for the northern region may not benefit from incorporation of the Japanese source of RFT.

Research presented in this chapter has given an insight into what environments this type of tolerance may be useful. More validation work will need to be completed on this and other breeding populations to further define the frost conditions in which this material is most effective. The breeding strategy employed in chapter 5 has, however, been successful in providing a framework for breeders to design an integrated breeding program for manipulating this source of RFT.

Chapter 7. General Discussion

The genetic basis of RFT in barley and wheat

The outcomes of this thesis have direct implications for improved cultivar development by plant breeders. The identification of genetic variation for Reproductive Frost Tolerance (RFT) in barley had not been reported prior to the research conducted in this thesis. Therefore, screening methods had to be developed for the initial attempt to identify barley germplasm with an increased level of RFT. Experimental design, screening methodology and analysis approaches that accounted for spatial variation in temperature and confounding effects of differential phenology were expected to be key factors. The resulting field based screening method effectively accounted for a significant proportion of the confounding effects on observed FIS. Compared to typical plant breeding field testing, a high residual error and moderate heritability was observed in these trials. Although the field based screening method was labour intensive a relatively large number of genotypes were phenotyped using this system. The identification of an increased level of RFT in the Japanese lines Haruna Nijo and Amagi Nijo provided a potential new source of genetic variation for this trait in barley.

Frost tolerance had been previously identified at the vegetative stages of development in barley (Hayes *et al.*, 1993). The research conducted in this thesis was, however, the first report of tolerance to frost recorded at the heading stages of development (Chapter 2). Research in Australia focussing on the RFT of wheat had been conducted in the northern Australian wheat belt for many decades with no reports of an increase in tolerance of a magnitude that would be useful to farmers (Fletcher, 1988, Marcellos and Single, 1976, Marcellos, 1988, Single, 1988). The limited success reported by previous wheat researchers leads to the assumption that finding any source of genetic variation for RFT in barley was unlikely. The identification of barley genotypes with improved RFT were not only

significant for the Australian barley industry, but suggested targeted evaluation of diverse wheat germplasm using appropriate methods could potentially identify genetic variation with a magnitude of relevance to Australian wheat production.

The field based screening system was able to identify RFT genotypes and also provided conditions for genetic analysis of the tolerance. The application of this field screening method to routine breeding would have been limited as the cost and effort to screen the numbers of individual genotypes on the scale needed for commercial breeding would be large and not practical.

Mapping the genetic location of RFT in populations derived from crosses with Haruna Nijo and Amagi Nijo aimed to understand the genetic basis of this tolerance. Also, if genes/loci with large effects on RFT could be identified, direct genetic selection could be employed in breeding programs rather than relying on the high cost and moderate heritability of the phenotypic selection. The identification of two loci, segregating in both populations in which the Japanese lines served as the RFT parent, indicated that the genetic basis of tolerance in Haruna Nijo and Amagi Nijo was similar.

One of the loci that had a major effect on FIS was located on chromosome 5HL. The genomic region where the QTL was identified is known to harbour the major vernalisation responsive locus *vrn*-H1 and a frost tolerance locus reported to be effective at the vegetative stages of development (Hayes *et al.*, 1993). The homologous loci to *Vrn*-H1 in wheat, (*vrn*-A1, *vrn*-B1 and *vrn*-D1) have also been reported to be associated with Vegetative Frost Tolerance (VFT) and also linked in coupling to the winter *vrn*1 allele (Koemel *et al.* (2004).

The winter *vrn*1 allele/RFT association in barley gave rise to the investigation of a similar association in wheat. Germplasm used in this study was developed using 2 isolines that

were differing at the 3 *vrn*1 loci. Creating a population from wheat isolines reduced the potential for other genetic effect to confound the experiments to quantify the effects of these vernalisation loci on RFT. Although the *vrn*-A1 and *vrn*-B1 winter alleles were associated with increased VFT, no association could be found between any of the 3 vernalisation response loci and RFT. Genotyping of current Australian varieties using diagnostic markers for each of the group 5 vernalisation response loci revealed that many Australian varieties had winter alleles at one or more *vrn*1 loci. Phenotyping a set of Australian varieties that differed at the 3 vernalisation loci was also consistent with the findings of the previous experiment indicating that these loci have no measurable effect on RFT. It was concluded that the group 5 vernalisation response genes were unlikely to provide a source of RFT in wheat.

An attempt to fine map the 5H RFT locus in barley was undertaken as the defined genomic region in barley was large in both Japanese derived populations (approximately 10cM). The barley 5H RFT QTL spanned the *vrn*-H1 vernalisation locus and was in coupling with the winter allele. Genetic separation of the winter *vrn*-H1 allele and frost tolerance would support the development of spring germplasm without the restriction of the *vrn*1 winter allele, which confers winter growth habit in the presence of winter alleles at the *Vrn*2 locus. Fine mapping of the 5HL RFT locus revealed that it resided distal to the *vrn*-H1 locus. No previous reports have shown that *Vrn*-H1 and *Fr*-H1 were genetically separate prior to this at either the vegetative or reproductive stages of development (some studies have suggested a separation but subsequent analysis has indicated the separation of *Vrn*1 and *Fr*2 only). A second frost tolerance locus has been reported on chromosome 5HL in barley that controls tolerance at the vegetative growth stages of development. This locus, designated *Fr*-H2 is located proximal to *vrn*-H1 and therefore proximal to the locus identified in this thesis (Francia *et al.*, 2004).

A strategy was devised to efficiently introgress RFT alleles at the 2 loci identified in Chapter 2 into an Australian adapted barley. The breeding strategy was devised after the mapping study (Chapter 2) and before the fine mapping study (Chapter 4). The strategy utilised many of the available technologies to select lines carrying the RFT trait as well as retaining the adaptation of the recurrent parent. This strategy was successful in developing lines with the 2 RFT alleles and a high proportion of the recurrent parent genome retained within 2 years.

Frost chamber experiments confirmed the Haruna Nijo alleles at the 2 RFT loci had a positive effect on RFT at a minimum temperature of -4.5^{o}C in the backcross lines. Field based experiments did not show an association between the 2 RFT loci and RFT at minimum temperatures of -3.5 ^{o}C with 2 consecutive frost events and -5.1 ^{o}C with 4 consecutive frost events. The range of frost conditions, especially the minimum temperature and number of consecutive frost events at which the Japanese source of RFT was effective were further investigated. Analysis of all data recorded from Loxton since 2001 and plotting FIS versus minimum temperature suggested that the Japanese source of RFT may only be effective to a minimum temperature somewhere between -4.6 ^{o}C and -5.5 ^{o}C (Chapter 6, Figure 10). This has implications for the regions in which germplasm maybe suited. As the majority of frost events in southern Australia occur at canopy temperatures above -4.6 ^{o}C then this germplasm still has a high probability of being useful in the target environment but may not be useful in the northern Australian barley production environments where temperatures can be frequently lower than -4.6 ^{o}C (Fredericks *et al.*, 2005).

Review of experimental approach and future work in RFT of barley and wheat

The screening methodology for the identification of RFT in barley focused on screening diverse germplasm in an environment that has frequent frost events. For a trait such as RFT, where small differences in developmental stage can have large confounding effects on identifying true tolerance, a system which allowed for maturity differences needed to be employed. Multiple seeding times enabled early and late developing genotypes to be compared, independent of maturity effects. This meant that when comparing early and late genotypes, they were spatially separated as they were planted at different times with a machine seeder. Although advanced statistical models were used to attempt to remove any spatial effect on the phenotype, this factor increased the error of the experiment. Also, it can not be completely discounted that a 'late' genotype which has taken significantly longer to develop to anthesis, may have been exposed to a pre conditioning for longer, which may effect its level of tolerance to a frost event eg. cold acclimation. This phenomenon has been reported at the vegetative growth stages in cereals (Fowler, 1996, Fowler and Limin, 2004) but has yet to be confirmed to be associated with RFT.

The fine mapping study conducted in Chapter 4 produced an isogenic set of lines that were only differing for the 5H RFT locus, and fixed for all other known developmental loci, including the closely linked *vrn*-H1. There were no observed differences in maturity within this germplasm set with the alternative alleles at the 5H RFT locus showing differences in RFT after being exposed to a frost event in the AGRF frost chamber. This result does somewhat discount any effect of maturity on the expression of the 5HL RFT locus.

Field screening methods developed attempted to generate reliable data on test lines while representing actual frost conditions. To increase the chances of exposing germplasm at the reproductive stages of development to a radiative frost event, earlier than normal seeding

times were conducted so lines could develop to the target phenological stage in the 'peak' frost risk period. Pre and post frost event conditions are not exactly the same during the frost events used to screen germplasm at Loxton and those experienced under a 'normal' frost event during spring. Lines grown in the Loxton frost screening nursery are planted up to 2 months earlier than standard practice in the commercial barley production. The differences in growing conditions may have an effect of the RFT phenotype observed. As the mechanism of tolerances is still unknown, differences in pre and post frost conditions may have an effect on how and to what level the RFT phenotype is expressed. This is supported by the inability to discriminate between tolerant and intolerant genotypes using plants that are grown over summer (data not shown). Summer grown plants are exposed to temperatures and photoperiod that are dissimilar to those grown under normal commercial production conditions. Further investigation into the mechanism of tolerance will hopefully provide further insight into what specific conditions this tolerance is best expressed.

Research was focused on RFT at a limited number of developmental stages of wheat and barley. Sampling all possible mechanisms of tolerance over the course of the reproductive lifecycle may not have occurred. Anthesis is considered as the most susceptible stage of development, and is the stage at which most damage from frost occurs in southern Australia (White, 2000). If a severe frost event occurs at other stages of development, such as during grainfill or prior to head emergence, significant economic damage can also occur to the crop although less frequently. The experiments described in Chapter 2 identified a QTL for grain damage coincident with the QTL found on chromosome 5HL for FIS. This suggested that the mechanism of tolerance may be effective at a range of developmental stages and this could be a topic of further investigation.

The frost tolerance study in wheat focussed on the vernalisation responsive genes on the group 5 chromosomes. This was conducted prior to the fine mapping study in barley which

was able to separate the RFT locus from *vrn*-H1. The vernalisation response genes did not appear to be directly useful for improving the RFT of wheat. Further characterisation and isolation of the genes that underlie the barley RFT loci may provide opportunity for allele mining in wheat. An additional frost tolerance locus designated *Fr*2 has been located on the group 5 chromosomes in both wheat (Baga *et al.*, 2007) and barley (Francia *et al.*, 2007). In both species this locus is separate from the vernalisation response genes and proximal to the RFT locus identified in this mapping study. This locus may provide some additional levels of tolerance above what has been described in Chapter 2.

Some improvements on the breeding strategy in chapter 5 could have been employed. The fast track breeding strategy described could have produced a larger number of individuals with the desired spring growth habit by genotyping the recurrent parent for *vrn*-H2 prior to crossing and selection. As the recurrent parent was of spring growth habit it was not anticipated that *vrn*-H2 would be segregating in this population. Therefore a higher proportion of winter/facultative types were produced which is undesirable in the majority of southern Australian barley producing environments. If selection for the spring allele from the donor line GH-129 was conducted via MAS on donors for DH production, a higher proportion of spring types would have been produced. As we are now able to separate the winter allele of *vrn*-H1 from the RFT locus on 5HL, it is not necessary to manipulate *vrn*-H2 to produce the specific combination of these two genes to obtain a spring growth habit.

Germplasm has now been developed that can be used to attempt to isolate the specific gene(s) underlying the RFT locus on 5HL. Isolating the gene(s) that control RFT on 5HL would enable a better understanding of what specific mechanism is controlling the RFT trait. It would also provide an opportunity to search for potentially superior alleles in other barley germplasm. If the gene(s) controlling the tolerance at this locus can be sequenced a search in diverse germplasm for sequence variation may lead to the identification of

genotypes with a better functioning allele. Engineering plants with versions of the gene(s) isolated that are acting in a superior fashion to the gene(s) isolated from the Japanese source may also increase the level of tolerance that is able to be achieved in barley or wheat. This could be achieved by manipulating the promoter of the isolated gene to enable an increased or constitutive expression of the tolerance gene(s). Consumer acceptance of genetically modified barley and wheat would be needed to make this commercially feasible. The isolation of the gene(s) underlying the 5H RFT locus in barley would also provide an opportunity to improve other plant species, particularity cereals, in their level of RFT by searching for homologs or engineering those plants with gene(s) based on this tolerance.

A major problem faced by this and future work in improving RFT of wheat and barley is the precision of the phenotyping. The amount of error associated with field and frost chamber experiments is high, reducing the ability to make significant genetic gain. This error also makes it difficult to discriminate between small differences in RFT, therefore it is unlikely that small incremental increases in RFT can be achieved using current phenotyping methods. Improvements in experimental design along with the production of targeted germplasm that isolate genetic regions of interest, fixing other confounding developmental or morphological regions that may increase the error of the phenotypic screen, are now increasing the precision in quantifying increased levels of RFT.

A proposed breeding strategy incorporating RFT genes identified from this and other mapping studies

The breeding strategy designed and employed in Chapter 5 was based on information about the RFT loci from Chapter 2. The initial mapping revealed two QTL, on chromosomes 2HL and 5HL, which were spanning relatively large genetic distances. The fine mapping study carried out in Chapter 4 has now provided molecular markers and a more defined genomic

region for the RFT locus on 5HL. In parallel to this PhD project another PhD student, Andrew Chen, has undertaken a study to fine map the 2HL RFT locus. Andrew refined the 2H RFT locus position, and as part of a fine mapping study, similar to what was conducted in chapter 4, developed closely linked molecular markers that can be used for breeding RFT barley varieties (Chen *et al.*, 2009a, Chen *et al.*, 2009b).

Chromosome 2HL and 5HL have been reported to carry loci important for disease resistance, malting quality and other physical quality traits (Fox *et al.*, 2003, Coventry *et al.*, 2003, Williams, 2003). With the marker-trait associations identified in these mapping studies it is now possible to develop a more targeted crossing and selection strategy to develop germplasm that carry not only the RFT genotype but a combination of desired loci on 2HL and 5HL where the RFT loci reside. Major loci that are located on the long arm of Chromosome 2H and 5H and are desirable when breeding for malting quality and disease resistant barley germplasm for southern Australia are illustrated in Figure 1 along with the location of the two RFT loci identified in this thesis.

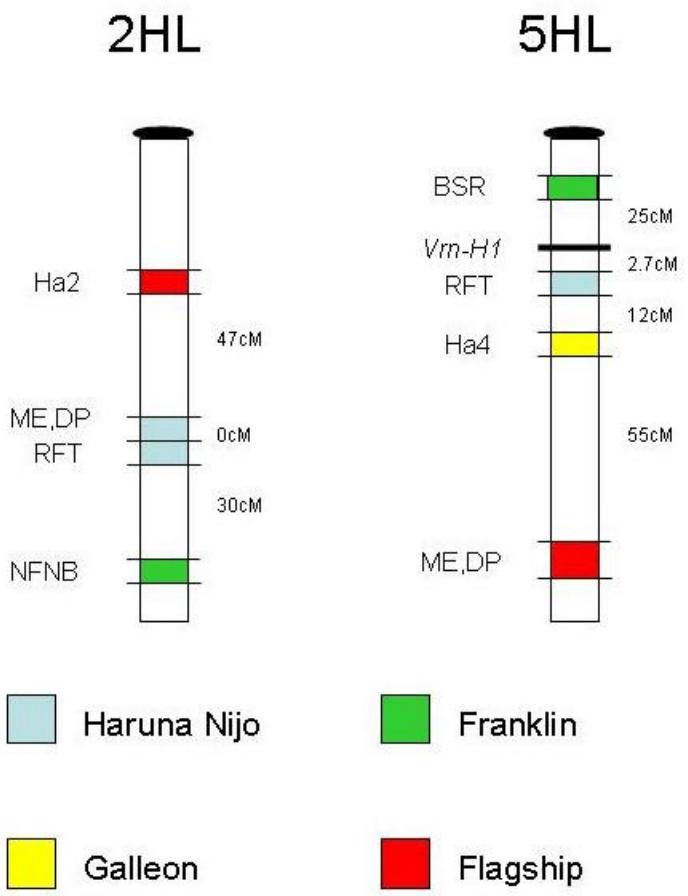

Figure 1. A schematic diagram of 2HL and 5HL chromosome arms indicating the location of desirable marker trait associations identified in Australian barley mapping populations. Approximate genetic distances between loci are based on mapping studies by Collins *et al.* (2003), Coventry *et al.* (2003), Park *et al.* (2003), Raman *et al.* (2003) and a barley consensus map by Karakousis *et al.* (2003b). Information on specific barley varieties carrying desirable alleles are from mapping studies above and from the University of Adelaide barley breeding program (S. Coventry, personal communication, 2008). Marker trait associations: Ha2 = Cereal cyst nematode resistance, ME = Malt Extract, DP = Diastatic Power, NFNB = Net Form Net Blotch, BSR = Barley Stripe Rust, Ha4 = Cereal cyst nematode resistance.

A proposed breeding strategy to combine these traits together into one genotype is displayed in Figure 2. Implementing the proposed strategy would provide barley breeders with

germplasm to use as parents for future crossing and breeding strategies. Note that this strategy focuses on the two chromosome arms where the RFT loci reside. None of the other genomic regions are considered in the breeding strategy.

A line from the Galleon × Haruna Nijo mapping population, GH-58, would be chosen as a donor of RFT and other loci. GH-58 carries the RFT alleles at the 2HL and 5HL loci (Chapter 2, Chapter 4), a desirable allele at a malt extract/diastatic power locus on 2HL from Haruna Nijo (Collins *et al.*, 2003) and the CCN resistance gene Ha4 on 5HL (Karakousis *et al.*, 2003a). GH-58 has been chosen over the previous donor as it carries the CCN resistance gene Ha4 whereas GH-129 does not. GH-129 was originally selected based on retention of recurrent parent genome, having a higher proportion of Galleon genetic background compared to GH-58. In yield trials grown in South Australia containing the Galleon × Haruna Nijo mapping population GH-58 was not significantly lower yielding than GH-129 (data not shown) and therefore is unlikely to show a significantly higher yield penalty that GH-129 when used as a donor of the RFT loci. The Australian variety Franklin carries a net form net blotch (NFNB) resistance gene on chromosome 2HL, a barley stripe rust resistance gene on chromosome 5HL (BSR) and the spring allele for *Vrn*-H1 on 5HL (Park *et al.*, 2003, Raman *et al.*, 2003). The Australian malting variety Flagship carries a CCN resistance gene Ha2 on chromosome 2HL and a desirable allele of a malt extract/diastatic power locus on 5HL (S. Coventry, personal communication, 2008).

The F_1 between GH-58 and Franklin would be top crossed with the cultivar Flagship. Flagship has been chosen as the top cross parent as it is the most widely adapted and has the best malting quality out of the three parents. Therefore a higher chance of retaining other desirable alleles from the progeny of the cross could be achieved although other specific desirable loci are not considered here. A very large number of top cross F_1s would have to be made to identify individuals having recombination between *vrn*-H1 and 5H-RFT as well

as the desirable combination of the NFNB allele from Franklin and 2H-RFT/ME,DP from GH-58. Producing 2000 top crossed F1s would be on the limit of what is practically feasible in a commercial breeding program but will be used in this hypothetical breeding strategy. An alternative strategy would be to use the individual with recombination between the RFT locus and *vrn*-H1 identified in Chapter 4 as the donor of RFT loci in this breeding strategy. It was decided against using this line as a donor of the 5H RFT allele because it did not carry the 2HL RFT allele and would not provide any other desirable loci on either the 2HL or 5HL chromosome arms. Therefore the DH line GH-58 from the Galleon × Haruna Nijo population was viewed as the most useful parent.

MAS would be undertaken on F2 plants, to maintain the desirable allele combinations from GH-58 and Franklin as well as at the Ha2 locus on 2HL and ME, DP loci on 5HL from Flagship. These alleles would be selected in either the homozygous or heterozygous state, as numbers needed to identify individual homozygous for all alleles at the F2 stage would be too large to practically screen via MAS (frequency approximately 1 in 3,333).

The F3 plants would be screened for homozygosity at all loci illustrated in Figure 1. The resulting lines would be available for further crossing by breeders, who could select these whole 2HL and 5HL chromosome arms using key markers linked to all loci in Figure 1.

Alternatives to this would be to produces smaller numbers of top crossed F1s, and run 2 parallel breeding and selection streams focusing on individual chromosome arms. The resulting desirable individuals, could then be intercrossed and F1s used as donors in a breeding program.

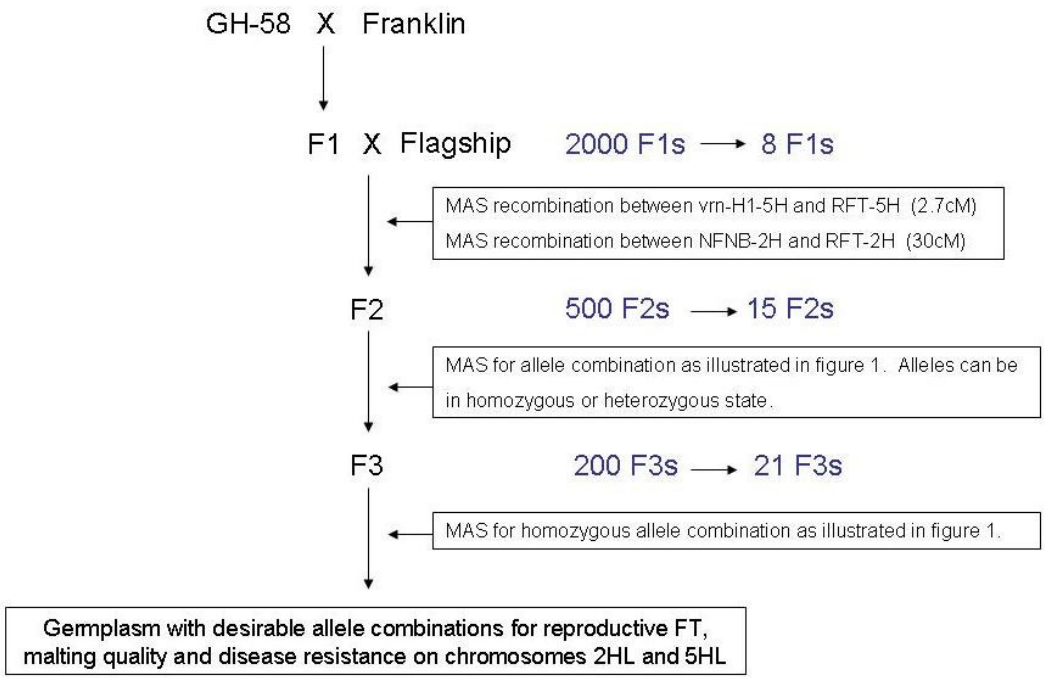

Figure 2. Proposed breeding strategy to combine all desirable alleles illustrated in Figure 1, into barley germplasm via MAS. Allele frequencies are based on the assumption that each marker trait association will be inherited as a single locus.

Conclusion

The research approach taken to improve RFT in a cereal crop of high commercial importance in Australia may be seen as a model for research into other similar traits of interest under multigenic control. A focus on the integration of the research with commercial breeding enabled the research outputs to be utilised by the breeding program in the shortest possible time. The systematic approach taken in this research program may pose as a framework for future research into similar traits for crop improvement. The main steps include:

1. Targeted collection of diverse germplasm from regions that experience frequent exposures to the stress of interest. Now formalised as FIGS http://www.figstraitmine.org

2. Development of a screening method to discriminate between tolerant and intolerant germplasm. Focusing on a method that is as close as possible to actual conditions experienced by the plant in commercial production. Throughput and reliability also need to be considered when developing the screening method.
3. Once genetic variation is identified, conducting a genetic analysis of the variation to identify genomic regions controlling the trait of interest.
4. Validation of the effectiveness of the genetic loci identified by incorporation into an adapted genetic background.
5. Fine mapping the locus to provide close molecular markers and germplasm that is less likely to be hindered by linkage of deleterious gene(s)/allele(s) associated with the desirable gene(s)/allele(s).
6. Development of the most efficient introgression strategy based on information gathered in the validation and fine mapping steps.

The research described in this thesis is an example of a process to improve a commercial crop for a trait that has no prior known genetic variation or screening methodology. This research has been focussed on supplying a variety with improved RFT to the farmer in the shortest possible time. The line of research attempted to integrate the outcomes from each chapter directly into a plant breeding context to contribute to variety development and delivery of an outcome to growers. This research approach meant that experiments were designed to deliver outcomes that could be directly applied to breeding.