Dissection of Traits Relating to Flowering and Reproductive Frost Tolerance on Chromosome 2HL of Barley (*Hordeum vulgare* L.)

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Abstract

In Australia, cereal crops such as wheat and barley are planted in autumn with the majority of the growing season occurring over winter. This results in occasional exposure of cold sensitive reproduction organs of the florets to frost events (-2 to -4°C) that sporadically occur in winter and early spring. Direct frost damage to cereal reproductive tissues can cause up to 85% yield losses and is estimated to cause 10% reduction in long-term yield in Australia. Two loci (2H and 5H) controlling frost tolerance at the reproductive stage were identified in Amagi Nijo × WI2585 and Galleon × Haruna Nijo DH populations, with alleles inherited from the Japanese parents associated with tolerance. The 5H locus (*Fr-5H*) position is close to the *Triticeae* homoeoloci influencing vernalization response (*Vrn-1*) and vegetative frost tolerance (*Fr-1*), while no frost tolerance effects had previously been reported in the region of the 2H locus (*Fr-2H*) in cereals. In the current study, the 2H and 5H chromosome regions controlling frost tolerance were also found to control developmental traits (e.g. flowering time), suggesting that developmental effects could directly or indirectly determine frost tolerance at one or both loci. However, preliminary data suggest that none of the developmental traits were consistently associated with tolerance. Using rice-barley co-linearity, the flowering time effect on 2HL (we named *Flt-2L*) was delimited to a 1.3 cM genetic interval in barley where it co-segregated with flowering time, spike compactness, plant height and an *APETALA2*-like gene. The *AP2* gene represents a plausible candidate for *Flt-2L* because members of the *AP2* gene family have been shown to control flowering time in maize, rice and wheat. Further analysis showed that the 2H frost tolerance effect can be genetically separated from *Flt-2L* by recombination. Thus frost tolerance at this locus appears to be controlled by a tolerance *per se* mechanism and is not as a result of flowering time differences (frost escape). Therefore, tolerance is unlikely to be due to a pleiotropic effect of *Flt-2L*. Floret sterility levels obtained using a frost simulation chamber distinguished the parents and F_2 derived individuals carrying contrasting alleles at the 2H tolerance locus. The use of an ice nucleator facilitated uniform freezing on the surfaces of the spikes and leaves, and was used to demonstrate that the 2H effect likely depends on freezing and not chilling. Future activities will include using rice-barley co-linearity to isolate the gene(s) responsible for frost tolerance at the 2H and 5H loci. The emerging physical maps of barley and wheat and the genome sequence of *Brachypodium* will accelerate the positional cloning. Candidate genes will be functionally analyzed using both forward and reverse genetic approaches. Markers linked to the genes controlling tolerance will be given to breeders to assess the value of the tolerance alleles in the field.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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- Chen A, Brûlé-Babel A, Baumann U and Collins NC (2008) Structure-function analysis of the barley genome: the gene-rich region of chromosome 2HL. Funct Integr Genomics (In press)

- Chen A, Reinheimer JL, Brûlé-Babel A, Baumann U, Fincher GB and Collins NC (2009) Genes and traits associated with barley 2H and 5H chromosome regions controlling sensitivity of reproductive tissues to frost. Theor Appl Genet (Revised submission)

- Chen A, Baumann U, Fincher GB and Collins NC (2009) *Flt-2L*, a locus in barley (*Hordeum vulgare* L.) controlling flowering time, spike density and plant height. Funct Integr Genomics (In press)

- Chen A, Gusta LV, Brûlé-Babel A, Leach RC, Baumann U, Fincher GB and Collins NC (2009) Varietal and locus-specific frost tolerance in reproductive tissues of barley (*Hordeum vulgare* L.) detected using a frost simulation chamber. Theor Appl Genet (Revised submission)

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1 Introduction

1.1 The impact of frost on cereal crops

Exposure of cereal crops at or soon after anthesis to low temperature events (-2 to -6 °C) can lead to significant yield reductions and downgrading of grain quality. In Australia and other regions with a sub-tropical climate, drought in the summer restricts the main growing season to winter and spring when conditions are most favorable for growth during the day with occasional frost events occurring at night (Single 1985; Fujita et al. 1992; Woodruff 1992; Reinheimer et al. 2004). Frost events that occur during winter often damage the frost-sensitive reproduction organs of plants, inducing floret sterility or shriveled grains. Direct frost damage can cause up to 85% yield losses in localized regions and is estimated to be responsible for 10% reduction in long term average yield in Australia (Paulsen and Heyne 1983). The total economic impact of frost on wheat and barley production in southern Australia is estimated at 60 million per annum (Reinheimer et al. 2002). Agronomical measures such as soil delving have been employed in attempts to reduce frost damage (Rebbeck et al. 2007). Other strategies involve the use of multiple sowing dates and late maturing varieties to delay flowering time so that anthesis occurs during a period of lower frost risk. However, delayed sowing times can subject the plants to unfavourable conditions late in the season. Yield loss associated with late flowering was in the order of 6 to 16 % per week's delay in sowing and anthesis, respectively (McDonald et al. 1983). Therefore, cropping opportunities are limited by frost in winter/spring and drought in summer, highlighting the need to identify sources of enhanced frost tolerance in cereals. Even small increases (1 °C) in frost tolerance have the potential to deliver significant benefits to the grain industry (Reinheimer et al. 2002).

1.2 Low temperature tolerance at the reproductive stage of development

Despite various reports of genotypic variation for low temperature tolerance at the reproductive stage (LTR tolerance) in barley and wheat (Single and Marcellos 1974; Marcellos and Single 1984; Single 1985), locally adapted varieties or synthetics possessing useful levels of LTR tolerance are not yet identified (Maes et al. 2001; Fuller et al. 2007). Efforts to breed tolerant varieties have been hampered by the sporadic nature of frost in the field, uneven freezing of plant

tissues, and the variation in susceptibility of heads at different stages of development (Single 1988; Reinheimer et al. 2004; Fuller et al. 2007). Therefore, in order to formulate a reliable and reproducible screening method, steps must be taken to ensure the accurate marking of individual spikes, standardization of temperature profiles, and uniform ice nucleation and formation (Fuller et al. 2007). Using Amagi Nijo × WI2585 and Galleon × Haruna Nijo F_1 derived doubled haploid (DH) populations, Reinheimer et al. (2004) identified two LTR tolerance loci, on chromosome arms 5HL and 2HL of barley. The tolerance allele at each locus was derived from the Amagi Nijo and Haruna Nijo parents, bred by the Sapporo brewery in Japan. One QTL was mapped to a region overlapping with the *Vrn-H1*/*Fr-H1* loci for vernalization response and low-temperature tolerance at the vegetative stage (LTV tolerance) on the long arm of chromosome 5H. The other was mapped to the distal part of the long arm of chromosome 2H and was not co-incident with any major genes previously known to be involved in low temperature responses or development (Reinheimer et al. 2004). The 2H LTR tolerance could potentially be controlled by a gene that is not directly involved in a cold responsive pathway. The 5H LTR tolerance might be due to pleiotropic effects of one gene (e.g., *Fr-H1* or *Vrn-H1* which may be equivalent to *Fr-H1*) conferring frost tolerance at both reproductive and vegetative stages of growth. Alternatively, the 5H LTR effect may be due to novel mechanisms of frost tolerance *per se* or frost avoidance controlled by an unidentified gene that is closely linked to the *Vrn-H1*/*Fr-H1* locus.

1.3 The regulation of vernalization in cereals

Vernalization is a requirement for a period of low temperature (0 – 10 °C) for plants to move from a vegetative to reproductive phase of growth (Flood and Halloran 1984). Vernalization sensitive barley or wheat varieties are sown in late summer or autumn and are vernalized during winter, allowing the plants to flower in spring. In wheat, an AP1-like transcription factor which serves as a promoter of flowering, and its dominant repressor, a ZCCT (zinc-finger, CONSTANS, CONSTANS-like and TOC) domain containing transcription factor, are the determinants of the vernalization response at the *Vrn-1* and *Vrn-2* loci respectively (Yan et al. 2003; Yan et al. 2004). *Vrn-1* is epistatic to *Vrn-2* and is dominant for spring habit, whereas *Vrn-2* is dominant for winter growth habit. In barley, the *Vrn-H1* and *Vrn-H2* loci on the respective chromosome arms 5HL and 4HL control much of the natural variation for the vernalization requirement for floral initiation (Dubcovsky et al. 2005; Fu et al. 2005; Trevaskis et al. 2006; Cockram et al. 2007; Trevaskis et al. 2007). A homologue of the wheat *Vrn-1* gene (*HvMB5A*) is

a candidate gene for the *Vrn-H1* locus (Schmitz et al. 2000), whereas one or more of three tightly linked *ZCCT* genes located in a syntenous region to the wheat *Vrn-2* locus is the *Vrn-H2* gene (Dubcovsky et al. 2005). Under long days, 'winter' type plants flower late unless vernalized, whereas 'spring types' flower early with or without vernalization. *Vrn-H2* is a dominant repressor of *Vrn-H1*, such that only plants carrying either a dominant (*Vrn-H1*) spring allele at the *Vrn-H1* locus or two copies of the recessive (*vrn-H2*) spring allele at the *Vrn-H2* locus are spring type (Kóti et al. 2006). Large deletions (2.8 kb) in the first intron of the *Vrn-H1* gene and completely deleted *Vrn-H2* gene copies may represent functional polymorphisms determining vernalization sensitivity at the *Vrn-H1* and *Vrn-H2* loci, respectively (Dubcovsky et al. 2005; Fu et al. 2005; Cockram et al. 2007). Polymorphisms in the *Vrn-1* promoter did not show complete correlation with *Vrn-1* allele type and therefore are unlikely to be critical in determining vernalization requirement (von Zitzewitz et al. 2005).

1.4 Low temperature tolerance at the vegetative stage of development

The survival and performance of temperate crop species depends on their ability to tolerate some degree of freezing (Pearce and Fuller 2001). To distinguish frost tolerance at the vegetative stage of growth from that of reproductive tissues (LTR tolerance), we refer to it as low temperature tolerance in vegetative tissues (LTV tolerance). This is the capacity to withstand long periods of extremely cold temperatures at the vegetative phase of growth. Vegetative tissues are generally more tolerant to cold than reproductive issues because plants steadily lose most if not all LTV tolerance when reproductive development is initiated (Fuller et al. 2007). Plants have the ability to express enhanced level of LTV tolerance following exposure to low non-freezing temperatures (weeks) – a process known as cold acclimation (Thomashow 1999). LTV acclimation is cumulative and has an inverse relationship between temperature and duration of acclimation (Fowler et al. 1999). Furthermore, genes regulating plant development, such as the ones controlling vernalization and photoperiod sensitivity, can enhance LTV tolerance by delaying the transition to reproductive development. It has been shown that full expression of LTV tolerance occurs at the vegetative stage, whereas plants in the reproductive phase have a limited ability to cold acclimate (Fowler et al. 1996; Fowler et al. 2001; Mahfoozi et al. 2001; Mahfoozi et al. 2006). It was suggested that the point of vernalization saturation (when plants are completely vernalized) is a critical switch that leads to the down-regulation of LT tolerance genes in vernalization sensitive genotypes (Prášil et al. 2004; Limin and Fowler 2006). Similarly,

photoperiod sensitivity allows plants to maintain the potential for LTV tolerance for a longer period of time under short days compared with long day environments in genotypes where short days lead to delayed flowering (Mahfoozi et al. 2000; Mahfoozi et al. 2001). Therefore, vernalization and photoperiod growth effects can enhance LTV tolerance by delaying the transition from the vegetative to reproductive stage and allowing plants to accumulate the potential for LTV tolerance over a longer period of time.

1.5 Freezing induced plant damage

When a low enough temperature is reached, water molecules on the surface or inside of the plants come together to form a stable ice nucleus, either spontaneously or catalyzed by another substance (Pearce 2001). During natural frost events, moisture tends to condense onto plant surfaces, providing an opportunity for any heterogenous substances such as plant molecules, structures and debris to initiate ice nucleation by elevating the minimum freezing temperature (Pearce 2001). Consequently, in the field, grasses may freeze between -1.5 and -2.5°C (Pearce and Fuller 2001). However, plants can avoid damage in the absence of a nucleator when water on the plant surface remains in liquid form and supercools to a temperature below the freezing point. In comparison, supercooling effects allow wheat spikes to escape frost damage at temperatures as low as -15 °C (Fuller et al. 2007). During the freezing process, ice accumulates between cells and grows at the expense of water drawn from inside of the cells. This subjects the cells to dehydration, destabilizing and eventually rupturing the membranes of the cell (Pearce and Willison 1985; Pearce and Ashworth 1992). This type of damage inflicted on the membrane is detectable by measuring the leakage of electrolytes and other solutes (Stout et al. 1980; Zhang and Willison 1992). Freezing induced membrane damage is often regarded as the primary cause of frost injury in plants (Thomashow 1999). The key step in the de-stabilization of the membrane is a phase change from a lipid bi-layer structure to a non bi-layer, which is a direct effect of dehydration caused by ice accumulation (Pearce and Willison 1985; Steponkus et al. 1993). Disruption in membrane integrity can be caused by the production of reactive oxygen species (McKersie and Bowley 1998), denaturation of molecular chaperone proteins (Guy et al. 1998) and the fact that ice formed in the extracellular space can adhere to the cell wall to the membrane, causing cell rupture (Olien and Smith 1977).

1.6 Mechanisms of freezing tolerance in plants

A key function of cold acclimation is to stabilize membranes against freezing injury by preventing membrane damage from freeze-induced cellular dehydration (Thomashow 1999). Many mechanisms seem to be correlated with the acquisition of tolerance through membrane stabilization, but the most commonly documented one involves changes in the proportion of some lipids present in plant tissues such as hexagonal II phase lipids (Steponkus et al. 1993; Uemura and Steponkus 1997). However, accumulation of sucrose and other simple sugars in the apoplastic space may also contribute to cold acclimation. These molecules have been observed to protect membranes against freeze-induced damage *in vitro* (Strauss and Hauser 1986; Anchordoguy et al. 1987; Livingston and Henson 1998). Other mechanisms include the enhancement of anti-oxidative mechanisms (McKersie and Bowley 1998) and the induction of genes encoding molecular chaperones (Guy et al. 1998). At the reproductive stage, mechanisms of LTR tolerance have not yet been identified. In wheat, freezing tests performed on head tissues failed to detect tolerance once freezing had occurred (Single and Marcellos 1974; Single 1988). The freezing damage inflicted on the rachis and the reproduction organs of the florets is generally considered to be irreversible. Other studies showed that certain parts of plants, including the flag leaf, crowns and rachis nodes can either prevent freezing from occurring or serve as barriers to deter the spread of ice through the plant tissues (Marcellos and Single 1984; Fuller et al. 2007). Therefore, escape of frost damage, either by slowing the propagation of ice in plant tissues (Marcellos and Single 1984), or by supercooling in the absence of ice nucleating agents (Gusta et al. 2004; Fuller et al. 2007), may be factors that may potentially determine LTR tolerance.

1.7 Cold acclimation

Studies of cold and freezing tolerance at the molecular level have mainly been centred around cold acclimation (Pearce 1999; Thomashow 1999). A number of LT induced genes, referred to as cold regulated genes (*COR*) have been identified and characterized in plants (Thomashow 1999). Some *COR* genes are not only induced by frost, but also tend to be induced by drought, salinity and abscisic acid (ABA), suggesting a general role of these genes in the adaptation of plants to environmental changes (Thomashow 1999). A group of *COR* genes, referred to as dehydrins, are members of the late-embryogenesis abundant (*LEA*) gene family that have been shown to be responsive to freezing in plants (Borovskii et al. 2002; Allagulova et al. 2003). In wheat,

dehydrin families *wcs120*, *wcor410* and *wcs19* have been implicated in conferring LTV tolerance (Chauvin et al. 1993; Houde et al. 1995; Gray et al. 1997; Sarhan et al. 1997) and the protein expression levels of their barley homologues were positively correlated with the potential of genotypes to develop LTV tolerance (Houde et al. 1992; Fowler et al. 2001). The gene products of *wcs120* and *wcor410* accumulate in cells of the vascular transition zone, a region that is critical for the plant's tolerance to freezing induced dehydration (Danyluk et al. 1998). Thus, it was speculated that dehydrins are involved in the protection of the plasma membranes by replacing water and stabilizing membranes against freezing or dehydration. However as yet, no locus controlling natural variation in LTV tolerance has been mapped to any such *COR* gene.

1.8 Genes regulating LTV tolerance in cereals

In comparison to LTR tolerance, LTV tolerance in cereal crops has been relatively well studied genetically. LTV tolerance has mainly been reported to be controlled by two loci (*Fr-1* and *Fr-2*) located on Triticeae group 5 chromosomes (Tóth et al. 2003; Vágújfalvi et al. 2003; Francia et al. 2004; Skinner et al. 2006; Tondelli et al. 2006). Tolerance at *Fr-1* is closely linked to the *Vrn-1* locus and may or may not represent the *Vrn-1* gene, whereas tolerance at *Fr-2* appears to be controlled by clusters of C-repeat binding factor (CBF) transcription factor genes that reside at *Fr-2* in barley and wheat (Francia et al. 2007; Knox et al. 2008). *CBF* genes are cold-responsive and are believed to be involved in enhanced cold/freezing tolerance in plants (Shinozaki and Yamaguchi-Shinozaki 2000). The Arabidopsis CBF1 protein binds to the CRT (C-repeat)/DRE (Drought responsive element) DNA regulatory element present in the promoters of the *COR* genes and stimulates their expression in response to low temperature and drought (Thomashow et al. 1997; Jaglo-Ottosen et al. 1998). Over-expression of *CBF1* produces a systemic enhancement of both freezing and drought tolerance in Arabidopsis (Liu et al. 1998). Mutant analysis showed that a deletion in the promoter of the Arabidopsis *CBF2* gene was associated with a loss of freezing tolerance and the low expression of *CBF2* and several target genes (Alonso-Blanco et al. 2005). In wheat and barley, *Fr-2* is located ~20 cM proximal to *Fr-1*/*Vrn-1*, close to the centromere on chromosome arm 5HL (Vágújfalvi et al. 2000; Francia et al. 2004). In a segregating family, an intolerant/spring allele of *Vrn-H1*/*Fr-H1* was genetically correlated with reduced expression of *CBF* genes at the *Fr-H2* locus (Stockinger et al. 2007), suggesting that *Vrn-H1/Fr-H1* contributes to tolerance by controlling *CBF* expression levels at *Fr-H2*. Tolerant alleles at *Fr-1* are commonly reported to occur in coupling phase with winter *Vrn-1* alleles,

indicating that these two loci may represent the same gene (Limin and Fowler 2002; Francia et al. 2004; Stockinger et al. 2007).

1.9 Cereal genome co-linearity

The major cereal crop species diverged from a common ancestor 50 – 80 million years ago (MYA) (Gale and Devos 1998). The diploid genome sizes of these species now vary by more than 10 fold. Despite the substantial variation in genome size and chromosome number, gene order and content are largely conserved between these species (Gale and Devos 1998; Devos and Gale 2000). At 400 Mb, the rice genome is the smallest of the cultivated cereals. The complete sequence of the rice genome provides a useful reference for comparative genomic studies in the cereals (Goff et al. 2002). The well established conservation in gene content and order between rice and other cereal species can be exploited to generate gene derived markers targeted to a particular chromosome region for positional cloning (Perovic et al. 2004; Huang et al. 2006; Komatsuda et al. 2007; Schnurbusch et al. 2007; Sutton et al. 2007; Collins et al. 2008). Previous studies have found that chromosome rearrangements such as translocations, inversions and duplications can disrupt cereal genome co-linearity at the sub-chromosome level defined by genetic markers (Huang et al. 2006; Rossini et al. 2006; Stein et al. 2007) or at the sequence level (Dubcovsky et al. 2001; Brunner et al. 2003; Caldwell et al. 2004). Chromosome rearrangements of different sizes are believed to have occurred frequently during the divergence of the cereal genomes (Bennetzen 2000; Bennetzen and Ramakrishna 2002). At the centiMorgan level, chromosome rearrangements can often be missed due to the low resolution of genetic maps (Conley et al. 2004). The large numbers of these small rearrangements could complicate the use of co-linearity with a model species such as rice as a tool in positional cloning (Delseny 2004).

1.10 *Brachypodium*

Another model for grass functional genomics is *Brachypodium*, which has the smallest genome size (200 – 300 Mb) described to date in grasses (Draper et al. 2001). *Brachypodium* diverged from the *Triticeae* lineage 35 – 40 MYA (Bossolini et al. 2007), while the evolutionary split between rice and *Triticeae* occurred approximately 50 MYA (Paterson et al. 2004). At the level of gene sequences *Brachypodium* is also more similar to wheat and barley than to rice (Catalán and Olmstead 2000; Vogel et al. 2006; Faris et al. 2008). This makes *Brachypodium* a better

model for predicting gene number and content in the corresponding regions of barley and wheat than rice. An 8x coverage genome sequence of *Brachypodium distachyon* has been recently completed under the *Brachypodium* genome sequencing project (http://www.brachypodium.org/). *B. distachyon* has a diploid genome size of ~300 Mb (Draper et al. 2001). It has useful physiological properties (short life cycle, non-stringent growth requirements and efficient somatic embryogenesis) that make it a good model for transformation studies using genes isolated from cereal crops (Draper et al. 2001). The complete genome sequence of *B. distachyon* will be a useful tool in elucidating gene content in other major cereal crops, complementing rice as a grass genome model.

1.11 EST mining

Expressed sequence tags (ESTs) are short cDNA sequences derived from expressed genes. Typically, ESTs represent single sequencing passes of 200-700 bp obtained from cDNA clones. ESTs provide an understanding of the expressed portion of the genome of an organism (Varshney et al. 2006). A large number of ESTs have become available in cereal crops including wheat (1,051,300) and barley (478,734) (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html; March 2008). ESTs can be used for studying phylogeny of genes or gene families at the local or whole genome scales, to develop microarrays for expression studies, discovery of single nucleotide polymorphisms (SNPs) and development of markers for breeding and forward genetic studies (Varshney et al. 2006). In barley and wheat, large sets (>100,000) of EST sequences were assembled in to contigs, revealing about 30,000 unique genes (Ogihara et al. 2003; Lazo et al. 2004; Zhang et al. 2004). Thousands of EST-based unigenes have been mapped to genetic and physical maps in wheat and barley (Qi et al. 2004; Nasuda et al. 2005; Cho et al. 2006; Stein et al. 2007). This has provided a better understanding of co-linearity in *Triticeae* genomes as well as a tool to facilitate gene isolation in *Triticeae* species (Stein 2007). Redundancy and sequence artifacts present in the EST libraries still represent a major obstacle in distinguishing whether SNPs identified from EST sequence databases derive from variation between alleles (Kota et al. 2003).

1.12 SNP discovery

SNPs are the most abundant type of sequence variation encountered in low-copy transcribed genome regions (Picoult-Newberg et al. 1999). SNPs are especially useful for association studies because of their high frequency in the genome, and because they are genetically more stable than microsatellite markers (Kota et al. 2003). Thus SNPs are ideally suited for the generation of high-density genetic maps (Cho et al. 1999). SNPs can be used to generate cleaved amplified polymorphic sequence (CAPS) markers that are based on the restriction endonuclease digestion pattern of a DNA amplicon containing the SNP (Ilic et al. 2004). This robust type of marker can be useful for mapping and positional cloning in diploid species such as barley and rye (Perovic et al. 2004; Hackauf and Wehling 2005; Sutton et al. 2007; Collins et al. 2008). In hexaploid bread wheat, Restriction Fragment Length Polymorphisms (RFLPs) still remains the most practical option due to the low level of genic polymorphism and difficulties associated with generating sub-genome specific PCR markers in this species (Bryan et al. 1999; Schnurbusch et al. 2007). In hexaploid bread wheat, ESTs corresponding to homoeologous loci can be mapped to specific chromosome arms using nullisomic/tetrasomic wheat deletion lines (Mochida et al. 2003; Schnurbusch et al. 2007). Other SNP detection methods are based on oligonucleotide hybridization and ligation, primer extension, DNA sequencing, PCR primer mismatch, pyrosequencing or heteroduplex assays (Kota et al. 2003). SNPs can be identified *in silico* using SNP detection algorithms (Kota et al. 2003). SNPs can also be identified from differences in relative hybridization intensity of individual oligonucleotide gene probes on Affymetrix expression chips when hybridized to cDNA from different genotypes (Rostoks et al. 2005).

1.13 Yeast or bacterial artificial chromosome libraries

Important tools for physical mapping, genome structure analysis, and map-based cloning are yeast artificial chromosome (YAC) or bacterial artificial chromosome (BAC) vectors which can carry large inserts of genomic DNA fragments. YAC genomic libraries have been constructed for several plant species but their usefulness is limited by the high frequency of chimeric and unstable clones typically obtained using YAC vectors (Yu et al. 2000). BAC libraries are more stable than YAC libraries and have been developed from many plant species including Arabidopsis, rice, soybean, sorghum, sugar cane and barley (Yu et al. 2000). BAC libraries have been constructed for various barley cultivars such as Haruna Nijo and Morex (Yu et al. 2000; Saisho et al. 2007). These have good insert sizes (> 105 kb) and genome coverage (>6.3), theoretically allowing a >99% probability of recovering at least one clone containing a given

single copy sequence. An international project initiated by the international barley sequencing consortium (IBSC) has been underway to physically map the barley genome using Morex BAC clones (http://barleygenome.org). In wheat, the construction of physical maps to be used as a backbone for large scale marker-anchored genome sequencing has also been initiated for individual wheat chromosomes (Paux et al. 2006). A full genome physical map for the D-genome of *Aegilops tauschii* is also being constructed (http://wheatdb.ucdavis.edu:8080/wheatdb/). Thus, the genomes of wheat and barley may well be sequenced in large part within the next decade. Another useful tool for map-based cloning is the use of genomic libraries constructed using transformation competent artificial chromosome (TAC) vectors, which are also capable of carrying large inserts. TAC clones can be transformed directly into the plant genome via *Agrobacterium* (Hamilton et al. 1996; Liu et al. 1999). The ability to use TAC vectors directly in plant transformation can streamline map-based cloning of genes by eliminating the sub-cloning step normally required to transfer candidate genes to conventional transformation vectors (Qu et al. 2003). Large insert TAC libraries available in rice and wheat (Liu et al. 2000; Qu et al. 2003) represent valuable resources for the isolation of agronomically important genes from these species.

1.14 Summary and aims

Processes determining LT tolerance in plants are likely to be complex, and appear to incorporate plant developmental responses to environmental cues (photoperiod and vernalization). In vegetative tissues, various genes and their products have been identified that are cold responsive, but the contribution these genes might make to frost tolerance are largely unknown. Genes that potentially confer frost tolerance at the reproductive stage of growth have not been identified so far. In Victoria and South Australia, annual costs of frost damage in all cereal crops are estimated to be $9.2 m from direct yield losses, $22.5 m from indirect yield losses and $1.9 m from quality downgrading (Reinheimer et al. 2002). As frost is a random and sporadic event, this burden is unevenly spread over the barley community and often a relatively small number of farmers suffer very high economic loss in a particular season (Reinheimer et al. 2004). It has been estimated that an increase in tolerance of 1-2 °C would provide the added protection needed to withstand the majority of damaging frost events in southern Australia (Reinheimer et al. 2002). Therefore, a small improvement in the level of reproductive frost tolerance in barley has the potential to significantly reduce the economic impact of frost. The refinement of genomic regions controlling

LTV tolerance and the development of closer molecular markers would enable marker assisted selection for LTV tolerance in early generations of large breeding populations.

Genotypes differing for tolerance at the reproductive stage have been identified and QTLs mapped on barley chromosomes 2H and 5H using phenotypic data obtained from field experiments (Reinheimer et al. 2004). One of the overall objectives of this project was to make progress towards positionally cloning the gene controlling the LTR tolerance QTL on 2H. The isolated gene will provide opportunities to elucidate the molecular mechanisms governing LTV tolerance in cereals, may allow the engineering of frost tolerance by transformation, and should also provide a perfect molecular marker for selecting the tolerance allele in breeding programs. The cloned gene may also enable identification of alleles/homologues with superior performance from wild and domesticated grasses (e.g. by EcoTILLING (Slade et al. 2005; Slade and Knauf 2005; Mejlhede et al. 2006)). Another aim was to investigate morphological/physiological traits genetically associated with the 2H and 5H tolerance loci as potential factors contributing/relating to the tolerance observed at these loci.

1.15 Publication synopsis

The main body of the thesis is comprised of 4 submitted and/or published papers, describing the outcomes of this project in a logical manner.

The first experimental chapter (Chapter two) describes the mapping of 62 barley genes derived from their corresponding rice chromosome 4 orthologues to co-linear regions on the long arm of barley chromosome 2H. The comparison of gene order between rice and barley revealed frequent genome rearrangements in the form of interstitial inversions, disrupting the co-linearity at the megabase level. This targeted approach allowed us to characterize a gene-rich region at the distal end of 2HL, where the 2H LTR tolerance locus is located. The marker framework generated from this study provided tools for the studies in subsequent chapters.

In chapter three, genetic associations between various traits (cleistogamy, flowering time and dense spike) and chromosome regions controlling LTR tolerance were investigated. Significant flowering time effects were detected at both 2H and 5H LTR tolerance locus regions. Since the plant's sensitivity to frost differs at different growth stages, with maximum sensitivity expressed

at or just after anthesis, flowering time differences could potentially allow plants to escape rather than resist frost. Therefore the possibility remains that flowering time was the underlying basis for frost tolerance at both loci. Avoidance (as opposed to frost tolerance *per se*) is unlikely to be of net value to the grains industry, as early flowering could expose a crop to more or less frost damage depending when the frost event occurs. Therefore, it is important determine whether there is any genuine frost tolerance *per se* effects at these loci. Another possibility is that flowering time may affect genuine tolerance. Furthermore, other structural or biochemical traits apart from flowering may also offer potential tolerance mechanisms. Thus, we needed to examine whether any traits can be consistently associated with tolerance so that strategies for phenotyping/genotyping the loci effects can be formulated and used to isolate the genes controlling tolerance. In this study, flowering time effects were detected in the 2H and 5H chromosome regions controlling LTR tolerance. However, preliminary data revealed that none of them are consistently associated with tolerance, suggesting that tolerance is derived from a tolerance *per se* mechanism and is not a pleiotropic effect of flowering time (escape).

In chapter four, the flowering time effect in the region controlling LTR tolerance on chromosome 2HL was fine-mapped as a single Mendelian locus (*Flt-2L*) to a 1.3 cM genetic interval. *Flt-2L* affected not only flowering time, but also spike density and plant height. A *HvAP2* gene marker co-segregated with all three traits in the *Flt-2L* genetic interval. Members of the *AP2* (*APETALA2*) gene family have been shown to control flowering time in Arabidopsis and maize and domestication related traits (spike compactness, brittleness, plant height) at the domestication locus *Q* from wheat. Thus the *HvAP2* gene represents a plausible candidate for the gene controlling the developmental traits at the *Flt-2L* locus.

Chapter five goes on to describe the detection of LTR tolerance in barley genotypes using an artificial frost simulation chamber. Using optimized frost simulation profiles, genuine frost tolerance controlled by the chromosome 2H LTR tolerance QTL interval was detected and was separated by recombination from flowering time controlled by the *Flt-2L* locus.

Chapter 6 provides concluding remarks on future work, addressing different approaches that might be used to clone the 2H LTR tolerance and flowering time (*Flt-2L*) genes in barley. Reverse genetic studies such as RNAi gene silencing, over-expression and TILLING of the

tolerance genes may allow us to better understand the molecular mechanisms of LTR tolerance and perhaps flowering time in cereals.

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2 Structure-function analysis of the barley genome: the gene-rich region of chromosome 2HL

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Chen A (Candidate)

Statement of contribution

Designed and conducted the research, analyzed the data and drafted the manuscript

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Statement of contribution

Generated the PCR markers *ABC468*, *BCD355*, *MWG865*, *PSR540*, *CDO680*, *cMWG694*, *AO*, *CAR*, *CAL*, *TCA*, *HY1* and mapped them in both DH populations

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Baumann U

Statement of contribution

Contributed to the coordination of the study and contributed to the final manuscript

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Abstract

A major gene-rich region on the end of the long arm of Triticeae group 2 chromosomes exhibits high recombination frequencies, making it an attractive region for positional cloning. Traits known to be controlled by this region include chasmogamy/cleistogamy, frost tolerance at flowering, grain yield, head architecture and resistance to Fusarium head blight and rusts. To assist these cloning efforts, we constructed detailed genetic maps of barley chromosome 2H, including 61 PCR markers. Co-linearity with rice occurred in eight distinct blocks, including five blocks in the terminal gene-rich region. Alignment of rice sequences from the junctions of co-linear chromosome segments provided no evidence for the involvement of long (>2.5 kb) inverted repeats in generating inversions. However, re-use of some junction sequences in two or three separate evolutionary breakage/fusion events was implicated, suggesting the presence of fragile sites. Sequencing across 91 gene fragments totaling 107 kb from four barley genotypes revealed the highest SNP and InDel polymorphism levels in the terminal regions of the chromosome arms. The maps will assist in the isolation of genes from the chromosome 2L gene-rich region in barley and wheat by providing markers and accelerating the identification of the corresponding points in the rice genome sequence.

Key words: barley, sequence polymorphism, chromosome evolution, 2H

Introduction

The cultivated cereals last shared a common evolutionary ancestor up to 60 million years ago, and furthermore, the diploid genome sizes of these species now vary by more than 10-fold (Gale and Devos 1998). Nevertheless, the conservation in gene order and content observed between these species is strikingly high. At 400 Mb, the rice genome is the smallest of the cereal genomes, and its complete sequence has provided an important tool in facilitating efficient positional cloning of genes from cereals with large unsequenced genomes such as barley and wheat. Markers in the vicinity of a target locus for positional cloning in wheat or barley can be generated from homologues of rice genes in the corresponding interval of the rice sequence. In the diploid species barley and rye, the genes can be converted easily to robust and easy-to-use PCR markers such as cleaved amplified polymorphic sequence (CAPS) markers (Perovic et al. 2004; Hackauf and Wehling 2005; Sutton et al. 2007; Collins et al. 2008), while in hexaploid bread wheat, restriction fragment length polymorphisms (RFLPs) still appear to offer the most practical option, owing to the low level of genic polymorphism and difficulties posed by the three homoeologous genomes of wheat (Bryan et al. 1999; Schnurbusch et al. 2007).

Comparative mapping has provided a picture of the broad-scale relationships between the rice genome and the chromosomes of other grasses, where blocks of conservation are interrupted by breakpoints of evolutionary inversions and translocations (Gale and Devos 1998; Sorrells et al. 2003; Qi et al. 2004; Stein et al. 2007). However, more detailed mapping of specific chromosome regions during positional cloning has revealed additional small rearrangements relative to rice, such as short inversions, which were not identified by the lower resolution genome-wide analyses (Huang et al. 2006; Valárik et al. 2006; Bossolini et al. 2007). This indicates that the full suite of rearrangements distinguishing rice from the other cereal genomes is not yet known. Indeed,

comparisons of genomic sequences from specific chromosome regions have revealed that differences between the grasses can occur on a smaller scale, involving translocation, inversion, duplication or deletion of individual genes or small groups of genes (Dubcovsky et al. 2001; Ramakrishna et al. 2002; Brunner et al. 2003; Caldwell et al. 2004; Sutton et al. 2007). Unanticipated medium-scale chromosome rearrangements between rice and the target species can set back a positional cloning effort by guiding marker generation in the wrong direction. Therefore, the more of these rearrangements that become known, the more efficiently we can exploit the rice sequence for positional cloning in these species.

Gene distribution in the large wheat genome is non-random, with most genes being located in gene rich regions, which typically exhibit high levels of recombination and are located towards the ends of the chromosome arms (Erayman et al. 2004). One such region is the 2L1.0 chromosome segment located at the end of the long arm of wheat group 2 chromosomes, which comprises around 5% and 68% of the physical and genetic arm length, respectively, and contains 50% of the long arm genes (Dilbirligi et al. 2005). In wheat and barley, the 2L chromosome arms, and in particular the terminal regions, contain loci controlling a variety of traits of agronomic and commercial importance, including reproductive development, time to flowering, reproductive frost tolerance and disease resistance (Franckowiak 1996; Costa et al. 2001; Pillen et al. 2004; Reinheimer et al. 2004; Turuspekov et al. 2004; Dilbirligi et al. 2005; Li et al. 2005; Jafary et al. 2006, 2008; von Korff et al. 2006; Marcel et al. 2007a). The high concentration of genes and the high recombination frequency in the 2L1.0 chromosome region in barley and wheat make it an attractive region for map-based cloning of genes that confer these agronomically and commercially important traits.

In this study, the details of rice-barley co-linearity on the long arm of barley chromosome 2H, with special attention paid to the terminal 2L section corresponding to the 2L1.0 gene-rich region, have been defined, in order to provide a framework for the efficient positional cloning of genes from this region in wheat and barley.

Materials and methods

Barley lines and genetic mapping

Separate populations were used to map the middle of barley chromosome 2H and the distal region of 2HL. For mapping in the central region of 2H, 96 randomly selected F_2 plants from an Amagi Nijo × WI2585 cross were used. Recombinants identified from this family were intended to provide a resource for mapping the *Eps-2S* flowering time locus (Laurie et al. 1995) which is segregating in this cross (Reinheimer et al. 2004). An F_2 population instead of a doubled-haploid (DH) population was chosen for this purpose so that an F_3 progeny-testing approach to mapping a QTL as a discrete Mendelian locus (Sutton et al. 2007) could be employed for subsequent fine-mapping of *Eps-2S*. Mapping in the distal half of 2HL was performed using subsets of lines from Amagi Nijo × WI2585 and Galleon × Haruna Nijo populations of F_1-derived DHs. The two DH populations had previously been used to construct whole-genome molecular marker maps (Karakousis et al. 2003; Pallotta et al. 2003a). The subsets of DH lines used in the current study were those identified as being recombinant for the distal half of 2HL, based on the original molecular marker data set. From the original 139 Amagi Nijo × WI2585 DHs, the 58 lines that were recombinant in the region between markers *ksuD22* and *Bmag749* were used, and from the population of 112 Galleon × Haruna Nijo lines, the 54 lines that were recombinant for the region between *ksuD22* and *BCD339b* were used. Seeds of DH lines, F_2s and parents, plus the original DH population marker data, were kindly provided by Peter Langridge, Margaret Pallotta and Stewart Coventry (University of Adelaide).

For the DH lines, recombination fraction was calculated as the number of recombinants observed in the analyzed sub-population divided by the original population size (139 or 112), except for intervals lying outside of the *ksuD22-Bmag749* (Amagi Nijo × WI2585) or *ksuD22-*

HYP16 (Galleon × Haruna Nijo) regions, for which recombination fraction was taken as the number of observed recombinants divided by the sub-population size (58 or 54, respectively). The two calculation methods reflect the fact that the DH lines chosen for mapping were biased for recombination in the selected intervals but were a relatively unbiased sample for recombination outside of these intervals. For the F_2 population, recombination fraction was calculated as the number of recombinants observed divided by the number of meioses sampled (192). Recombination fraction was converted to genetic distance in cM using the Kosambi mapping function. Poor quality markers in the original DH population data sets, as indicated by a high proportion of missing data and the presence of singleton scores, were removed from the datasets used for final map construction. Markers were ordered by using calculated genetic distances and by placing each new marker in such a position as to minimize the number of double crossovers required to explain the order.

PCR markers

Genomic DNA of parental lines was extracted using the method of Rogowsky et al. (1991). DNA of lines from the mapping populations were extracted from freeze-dried leaf tissue in racks of 96 1.2 mL Collection Microtubes (Qiagen, Australia), following the procedure of Pallotta et al. (2003b). Each extraction was performed using a 3 cm long section of the second leaf, and pellets were re-suspended in 100 µL, typically yielding 10 ng/µl nucleic acid.

Broadly speaking, the central portion of Triticeae group 2 (including the centromere) corresponds to the whole of rice chromosome 7 (in inverted orientation) and the distal regions on the long and short arms correspond to opposite ends of chromosome 4, in the same orientation (Gale and Devos 1998; Conley et al. 2004; Stein et al. 2007). To generate markers on barley 2H, from the centromeric region to the end of the long arm, we targeted genes on the long arm of rice

chromosome 4, from 22 Mb to the end at 35.5 Mb, and on the long arm of rice chromosome 7, from 21 to 30 Mb. Genes from the 14-22 Mb region of rice 4L and from the 0-21 Mb region of rice chromosome 7 were not used for marker generation - these were predicted to locate to a segment of 2H around the centromere which exhibits very little recombination and therefore would have revealed little about the co-linearity. Genes located after the 30 Mb position on rice chromosome 7 were also not mapped because they were predicted to locate above *Ppd-H1* on the short arm of barley chromosome 2H, which contains no loci of immediate interest to our laboratory.

Sections of the rice genome sequence (10-20 kb) were used in BLASTn searches against barley ESTs at NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) or barley transcript assemblies at TIGR (http://tigrblast.tigr.org/euk-blast/plantta_blast.cgi; July 2007 version). Genes identifying barley ESTs from putative orthologues (>80% simlarity) and suitable intron-exon spacing (see below) were analyzed further. Barley EST sequences were used in BLASTn searches back on to the rice genome sequence at NCBI to determine gene copy number in rice and to verify that the closest match was on rice chromosome 4 or 7. ESTs with best matches on other rice chromosomes and/or revealing high copy number in rice were eliminated from the pipeline. Barley ESTs were also used to search for wheat ESTs (wESTs) that had been mapped using deletion lines (http://wheat.pw.usda.gov/GG2/blast.shtml), and in most cases, those with matches to wheat ESTs mapped to chromosomes other than 2A, 2B or 2D were discarded. Of the sequences eventually used for primer design, 42 showed significant hits to wESTs and 33 of these had been mapped to group 2 chromosomes only (Supplemental Table 1). Finally, genomic sequences of selected rice genes were retrieved using the TIGR Rice Genome Browser (http://www.tigr.org/tigr-scripts/osa1_web/gbrowse/rice/) and used in BLASTn searches against barley ESTs at NCBI. Using the graphic output to identify the location and size of the introns, primers from barley exons were designed to amplify predicted genomic fragments of 1.0-1.5 kb

containing mostly intron. Occasionally, markers were based on genes matching two or more homologous mRNAs in barley. In these cases, primers specific for the 'correct' copy (with the closest match on rice chromosome 4 or 7) were designed from ClustalW (http://align.genome.jp/) alignments of the barley EST sequences.

We attempted to convert ten previously described RFLP markers to PCR markers (*ABC468*, *BCD292*, *BCD355*, *BCD453*, *BCD512*, *CDO36*, *CDO680*, *cMWG694*, *MWG865*, *PSR540*). The probe sequences were retrieved from GrainGenes (http://wheat.pw.usda.gov/GG2/index.shtml) and used to identify homologous genes on rice chromosome 4 or 7 by BLASTn searches at TIGR. For *MWG865*, identity to rice was not found and the primers were based directly on the probe sequence. For the remaining probes, primer design proceeded as described above.

For *MSU21*, *ABC165* and *MWG2200*, representing previously described sequence-tagged site (STS) markers, amplicons were obtained from the mapping parents using the published primers (Shin et al. 1990; Turuspekov et al. 2004). *ABC165* and *MWG2200* were found to be polymorphic using the previously described restriction enzymes and were not re-sequenced from our parents, whereas *MSU21* was re-sequenced and used to design a new CAPS marker. A marker fragment was also amplified from the cloned *Ppd-H1* photoperiod response gene (Turner et al. 2005) using primers located in exons 3 and 6 (Supplemental Table 1).

Primers were 25 nt in length and synthesized by Proligo (Sigma-Aldrich, Australia). Gene fragments used in the initial polymorphism screen were amplified using primers and conditions indicated in Supplemental Table 1. Each fragment was amplified and sequenced from each of the four parent lines. Amplifications were performed in 10 µL reactions containing 40-80 ng genomic DNA template, 0.25 µM each primer, 0.2 mM each dNTP, 0.3 U hot-start Immolase DNA polymerase (Bioline, Australia), 1 × reaction buffer and 0.5 mM supplemental $MgCl_2$ (total concentration 2.0 mM $MgCl_2$). PCR products were direct-purified using the QIAquick PCR

purification kit (Qiagen), or products were subjected to electrophoresis in 2.0 % agarose gels and purified from gel slices using the QIAquick Gel-Extraction kit (Qiagen). Purified fragments were sequenced from each direction using the BigDye v3.1 sequencing kit (Applied Biosystems, California). Parental sequences were aligned to identify polymorphisms using the ContigExpress program (Vector NTI 9.1.0 suite; Invitrogen, Australia).

Primers, PCR conditions, and methods of scoring marker polymorphisms are provided in Supplemental Table 1. Several markers (*KEFB*, *TRA* and *ZFP*) contained insertion-deletion (InDel) polymorphisms which were scored by using primers closely flanking the InDels and resolving the allelic PCR products of different size on agarose gels. Restriction sites covering polymorphic sites were identified using the NEBcutter V2.0 program (http://tools.neb.com/NEBcutter2/index.php) and used to design CAPS markers. PCR products were digested at the recommended temperature for 3-5 hr in reactions of 12 µL containing 10 µL unpurified PCR product, 1.0 mg/mL acetylated BSA, 1 × reaction buffer and 2 U restriction enzyme. To make some markers more reliable for routine scoring, new primers were designed internal to the original primers (Supplemental Table 1) to produce products that were smaller (150-500 bp) and thus more reproducibly amplified. Marker *CRP* was scored using the tetra-primer ARMS-PCR approach (Chiapparino et al. 2004) requiring four primers (Supplemental Table 1). Polymorphic sites in *NBP*, *OXO* and *HYP29* genes were assayed using a novel PCR-based allele detection method (Tabone and Hayden 2007). Marker fragments (8 µL of restriction digestion or PCR reaction) were subjected to electrophoresis on 1.5-3.0% agarose gels and visualized using ethidium bromide staining.

Inversion junction analysis

To investigate potential mechanisms of chromosome rearrangement in cereals, rice sequences at junctions of rice-barley co-linear chromosome segments were compared to identify regions of sequence similarity. The junction intervals were defined as the sequences lying between the outermost markers on adjacent blocks of barley-rice co-linearity. Pairs of intervals were selected as being potential precursors or products of the same inversion event according the evolutionary models in Supplemental Fig. 1. Sequences were compared using the LBDot program (Huang and Zhang (2004); Lynnon Corporation, Canada), using a window size of 18-19 bp and a maximum mismatch value of 1 for analysis of intervals A, B, C and D, and a window size of 22 and a mismatch value of 2 for analysis of intervals E and F.

Results

High-density genetic maps of chromosome 2H

Marker generation on barley chromosome 2H was performed using barley homologues of genes from the corresponding regions on rice chromosomes 4 and 7, and based on previously described RFLP and STS markers on 2H. Single PCR products were obtained from the barley parental DNAs using 93 primer pairs. Products obtained with 66 of the primer pairs contained polymorphisms and were used to generate PCR markers. These included the marker fragments *MWG2200* and *ABC165*, for which polymorphic restriction sites were identified without sequencing. With the exception of *DHD*, *WDP*, *BCD512* and *ZFP*, all fragments showing polymorphisms were mapped. Putative functions of the mapped genes were indicted by BLASTx searches (Supplemental Table 1).

The maps of the barley 2HL distal section, and the 2H mid section, aligned with the related chromosome regions in rice, are shown in Figs. 1 and 2, respectively. Inversion/translocation differences defined 8 blocks of co-linearity between barley chromosome 2H and sections of rice chromosomes 4 and 7. A number of individual markers did not conform to this overall pattern of conservation. Gene *LRP* with a homologue on rice 4L (Fig. 2) mapped to barley 7HL (not shown), which has no known relationship to rice chromosome 4. The previously known chromosome 2H marker *ABC165* showed sequence identity to genes on rice chromosomes 11 and 12 only. Genes *HYP29* and *HYP31* with homologues on rice 4L, and *HYP27* with a homologue on rice chromosome 7, mapped to positions on 2H that did not conform to any of the defined blocks of conservation (Figs. 1 and 2).

No polymorphisms were detected between the parents of the Amagi Nijo × WI2585 mapping cross for the genes *DHD*, *WDP*, *BCD512* and *ZFP*. Polymorphisms were detected in

these genes between Galleon and Haruna Nijo, and PCR markers were made which discriminated between the parental DNAs (not shown). However, in the Galleon × Haruna Nijo DH lines, these markers showed only the Haruna Nijo sized marker fragments (not shown). These four genes are located together on a relatively small interval of 215 kb in rice within chromosome segment 2 (Fig. 1). Hence, these discrepancies are probably due to heterogeneity in the corresponding barley chromosome 2HL segment, between the Galleon used to make the DH population and the Galleon used to make the parental DNA. Galleon was shown to be heterogeneous in a previous study on powdery mildew resistance (Hossain and Sparrow 1991). The *AMT* marker was the only other marker developed from the same 215 kb rice segment, and it segregated in the Galleon × Haruna Nijo DH lines, presumably because the two Galleon chromosome segments contained the same sequence at the *AMT* polymorphic site used for marker development.

The chromosome 2H centromere was located above *HYP26* and below the co-segregating markers *HLH* and *ABC468* (Fig. 2) by assigning these markers to the long and short chromosome arms, respectively. The *HLH* gene was homologous to wheat EST BE490521 that had been located to 2AS/2DS deletion bins in wheat (Conley et al. 2004). The *ABC468* marker sequence corresponded to EST Contig2155_at of the Affymetrix Barley1 GeneChip, located to 2HS by microarray analysis of wheat-barley ditelosomic addition line RNA (Bilgic et al. 2007). The *HYP26* gene corresponded to a Barley1 GeneChip contig located to 2HL (Contig6104_at), and was also homologous to the wheat EST BE405285 located to deletion bins on wheat 2AL and 2DL.

The *HYP16* gene, representing the most terminal point of gene co-linearity between the long arms of rice chromosome 4 and barley 2H (segment 1; Fig. 1), is located 23 kb from the end of the rice chromosome 4 sequence (AP008210.1), and is therefore likely to be located close to the 4L telomere. In barley, *HYP16* is also likely to be located close to the barley 2HL telomere, because *MWG2200* gene, mapped only 2.7-5.6 cM from *HYP16* (Fig. 1), represents the most

terminal 2HL RFLP marker on the whole genome map of Marcel et al. (2007a). These observations are consistent with the positions of the telomere on these barley/rice chromosome arms having been conserved.

Origin of rearrangements

Discontinuities in the barley-rice co-linearity are the result of chromosome rearrangements that took place after divergence from the common ancestor. If it is assumed that this process occurred by a sequence of single rearrangements each involving a minimum number of chromosome breakages, then these differences can be explained by four separate inversion events (Fig. 3). The displacement of chromosome segment 4 to the opposite sides of segment 3 in barley and rice can be explained by an inversion of segment 4 and another inversion encompassing both segment 3 and 4 (Fig. 3). The six rice regions at the junctions of rice-barley co-linear chromosome segments were delimited to intervals of 69 to 468 kb (A-F; Figs. 1 and 2). According to the model, chromosome intervals A, B, C or D represent products or precursors of multiple (2-3) chromosome breakage/fusion events (Supplemental Fig. 1).

Interchromosomal or intrachromatid recombination between non-allelic homologous sequences present in opposite orientation on a chromosome can give rise to inversion (Stankiewicz and Lupski 2002). Therefore, rice chromosome 4 sequences at the junctions of barley-rice co-linear chromosome sections were compared to identify regions of inverted-repeat similarity which potentially may have given rise to the inversions that interrupt rice/barley co-linearity. Sequences which were putative products or precursors of the same inversion event were compared, i.e., A, B, C and D in all possible combinations, and E with F. Comparisons involving intervals A, B, C and D (69-160 kb) revealed regions of inverted-repeat similarity (>94%), with the maximum length of similarity for each comparison ranging from 88 to 550 bp. For

comparison of E (413 kb) with F (468 kb), the longest stretch of inverted-repeat similarity (>91%) was 2.5 kb. The size of these inverted-repeat regions was no greater than that observed on average between randomly chosen rice sequences of comparable length.

Polymorphism

The 91 gene fragments sequenced from the four parents covered a total of about 107 kb per genotype and contained 557 single nucleotide substitutions (SNPs) and 57 insertion/deletions (InDels), providing an average of one polymorphism every 163.4 bp. Fragment sizes and sequence accession names are provided in Supplemental Table 1.

Amagi Nijo and Haruna Nijo, bred by the Sapporo brewing company in Japan, were the most closely related pair of genotypes, showing identical sequence variants (haplotypes) for 71 gene fragments. The two genotypes from Australia (Galleon and WI2585) showed similar levels of relatedness to one another and to the Japanese varieties, sharing 48 identical sequence haplotypes with one another and between 41 and 44 identical haplotypes in pairwise comparisons with the Japanese varieties. These observations are consistent with the pedigrees (Supplemental Fig. 2). Most of the founding parents of Haruna Nijo and Amagi Nijo are the same (Duckbill, Golden Melon, Hanna, Kitsuki Wase, Prior and Rokkaku Chevalier), whereas comparisons of the Australian varieties to one another and to the Japanese lines revealed only a few common parents (Archer, Binder, Gull and Prior) which contributed only a fraction of the parentage of these lines.

Fig. 4 shows the frequency of SNPs and InDel polymorphisms observed per kb for each gene along 2H. The genes *OXO* and *GLP* from chromosome segment 6 showed unique haplotypes for each of the four parent lines, while the remaining genes showed three or fewer haplotypes. Genes *HYP27* and *HYP20* representing the most distal genes on the short arm, showed the highest frequency of SNP polymorphism (40 and 117 /kb, respectively) and *HYP27*

also showed the highest frequency of InDel polymorphism (9.6 /kb). A consistently high level of haplotype diversity and SNP and InDel polymorphism was observed in the most distal part of 2HL, from the *AO* gene to the telomere (Fig. 4). Apart from this high level of polymorphism in the most distal regions of the chromosome arms, no other clear trend in polymorphism distribution along 2H was evident.

Discussion

The genetic maps as tools for gene cloning

The 61 PCR markers described here were used to make detailed genetic maps of barley chromosome 2H. Of these PCR markers, 11 represent previously described RFLP or STS loci, 49 are entirely new markers developed using gene co-linearity with rice, and one was derived from the *Ppd-H1* photoperiod response gene. The maps also incorporate SSR and RFLP markers previously mapped in the Amagi Nijo × WI2585 and Galleon × Haruna Nijo doubled haploid mapping populations. The previously described markers could be used to link to mapping studies of trait loci on chromosome 2H, enabling selection of markers/primers from the current study which are suitable for further fine mapping of these loci. Furthermore, the details of barley/rice co-linearity revealed here could be utilized to identify the specific intervals in the rice genome for targeted marker generation and/or candidate gene identification for the cloning of particular loci.

The section of the long arm of Triticeae group 2 chromosomes from the telomere to a point between markers *ksuD22* and *PSR540* represents the 2L1.0 gene-rich region (Erayman et al. 2004; Dilbirligi et al. 2005). Our comparative maps define 4 to 5 distinct blocks of rice co-linearity in this region (Fig. 1). These rearrangements can be considered reliable, because they were observed independently in the two mapping populations and by using co-dominant PCR markers which we found to be highly robust. In the comparative mapping study of Stein et al. (2007), these barley-rice rearrangements were not readily discernable, owing perhaps to inaccuracies associated with the use of a consensus barley genetic map, or because of the smaller number of markers that were available. Distal of *ksuD22*, Dilbirligi et al. (2005) identified no interruptions to the co-linearity between barley chromosome 2H and the corresponding parts of

wheat homoeologous group 2 chromosomes. Therefore, the rearrangements described here in barley relative to rice are also likely to exist in wheat and be relevant for cloning of genes from these wheat genome regions.

The *Cly1*/*Cly2* locus controlling open or closed florets (chasmogamy/cleistogamy) has been mapped on the long arm of chromosome 2H, 0-1.6 cM distal of the marker *MSU21* (Fig. 1; Turuspekov et al. 2004, 2005). Loci for several other traits have been shown to be associated with *Cly1*/*Cly2* or the nearby markers *HVM54* or *EBmac415* (Fig. 1), including QTL controlling levels of frost damage to reproductive tissues (Reinheimer et al. 2004), days to heading, grain yield, lodging, resistance to Fusarium head blight, and the length of the rachis, rachis internode and culm (Pillen et al. 2003, 2004; Turuspekov et al. 2004, 2005; Hori et al. 2005; Li et al. 2005; von Korff et al. 2006). The *Zeocriton-1* locus has been mapped 2.3 cM distal of *MSU21*, and shows a mutant phenotype associated with dwarfing and compact heads (Franckowiak et al. 1996; Costa et al. 2001). Several other loci controlling barley leaf stripe (*Rdg1*) and powdery mildrew (*Ml*(*La*)) disease resistances have been mapped apprixmately 6.5 and 20 cM distal of *MSU21* respectively (Giese et al. 1993; Thomsen 1997; Arru et al. 2002). Partial resistance to rust pathogens, typically representing a non-hypersensitive and durable form of resistance, has been reported to be controlled by several QTL on the long arm of barley chromosome 2H (Jafary et al. 2006, 2008; Marcel et al. 2007a). Several cold inducible *COR* genes including *cor14b* and *blt14* have also been mapped on chromosome 2HL (Francia et al. 2004). Other loci on barley chromosome 2H are known to determine waxiness, awn number, pigmentation, male fertility and the presence of ligules (Franckowiak et al. 1996).

On some regions of chromosome 2H, rice co-linearity has already been exploited for mapping/cloning several trait loci. A 10.7 cM region of 2H containing the *Sdw3* dwarfing locus was mapped using genes from the corresponding rice chromosome 7 interval (between positions 23.2 and 25.3 Mb), revealing no rearrangements (Gottwald et al. 2004). Likewise, the *Rphq2*

QTL locus controlling partial resistance to barley leaf rust has been fine mapped to a position distal on 2HL corresponding to the interval 35.1-35.4 Mb on rice chromosome 4 (Marcel et al. 2007b). Comparative mapping with rice led to the isolation of the *Vrs1* 2-row/6-row spike locus and the *Ppd-H1* photoperiod response locus (Fig. 2; Turner et al. 2005; Komatsuda et al. 2007). Good overall co-linearity was observed in the direct vicinity of both loci, although the rice homologues of *Vrs1* and an additional nearby sequence were displaced to apparently non co-linear positions on rice chromosome 7 (Pourkheirandish et al. 2007).

Chromosome rearrangements

The mechanisms of evolutionary chromosome rearrangements in plants have not been explored in any great detail. Potentially, chromosome rearrangements in plants can occur by homology dependent or independent repair of chromosome breaks (Pacher et al. 2007). Furthermore, recombination between regions of sequence similarity arranged in inverted orientation on a chromosome could give rise to an inversion (Stankiewicz and Lupski 2002). Two inverted repeats, one of 590 bp and 75% identity and another of 537 bp and 90% identity, have been identified as likely causes of separate inversions that had occurred during the evolution of *Arabidopsis thaliana* (Ziolkowski et al. 2003). In humans, large (10-400 kb) low-copy repeats of 95-97% identity are documented to cause a range of chromosome rearrangements, including inversions (Stankiewicz and Lupski 2002). Regions of sequence similarity of varying length have also been implicated in causing evolutionary chromosome rearrangements in mammal, yeast, and *Plasmodium* species (Cáceres et al. 1999; Dehal et al. 2001; Carlton et al. 2002; Kellis et al. 2003). The longest stretches of inverted-repeat similarity observed here between the junctions of rice-barley co-linearity (0.88-2.5 kb) were no more frequent than those observed between randomly chosen stretches of rice sequence of comparative length. Therefore, no evidence was

obtained for the involvement of large (>2.5 kb) sections of inverted-repeat similarity in causing evolutionary chromosome inversions in the cereals. However, it is possible that such repeats have caused such rearrangements in the ancestors of rice/barley and are no longer present in the *Oryza sativa* genome.

According to our model (Supplemental Fig. 1), evolutionary rearrangements responsible for the breaks in rice-barley co-linearity involved multiple (2-3) breakage/fusion events in intervals A, B, C or D, delimited to 69 to 160 kb in rice (Fig. 1), suggesting that some of these intervals in the ancestors of barley/rice were prone to chromosome breakage/fusion. Pevzner and Tesler (2003) also observed that junctions of inversions distinguishing the mouse and human genomes were non-randomly distributed due to the re-use of particular small chromosome intervals in multiple breakage/fusion events. In mammals and yeast, regions of recurrent chromosome breakage (fragile sites) have been localized to sequences thought to promote strand breakage by virtue of their ability to form DNA secondary structures, such as particular trinucleotide or AT-rich repeat stretches, and palindromic sequences (Aguilera and Gómez-González 2008). Assignment of any such small sequence as the cause of chromosome instability in the junctions characterized here will clearly require further delimitation of the junctions and perhaps also sequencing of the corresponding intervals in barley and barley/rice relatives.

Polymorphism distribution

Sequencing of 91 gene fragments totaling 107 kb identified 614 polymorphic sites, providing an opportunity to investigate the distribution of genic polymorphism along barley 2HL. While the number of genes assayed was considerable, our data would be partially biased due to the use of only four barley genotypes in the survey. The Japanese varieties Haruna Nijo and Amagi Nijo have highly related pedigrees and showed the highest number of shared marker haplotypes (Fig. 4

and Supplemental Fig. 2). Markers polymorphic between these Japanese varieties were localized to three chromosome regions (*HYP27-IRP*, *OXO-HYP6* and *TFP-PKP*; Fig. 4). The intervals between these polymorphic regions in these varieties may therefore be descendant from the same parents. Consequently, our data may provide slight underestimates of barley polymorphism for these intervals. A more accurate picture of polymorphism distribution on barley 2HL may be provided by sequencing the same gene fragments across a wider range of barley germplasm.

A positive correlation between recombination frequency and polymorphism level along the chromosomes has been reported in maize, Arabidopsis and diploid wheat relatives (Dvořák et al. 1998; Tenaillon et al. 2002; Dvořák and Akhunov 2005; Borevitz et al. 2007). This relationship may partly arise from the effect of selection acting against deleterious mutations (Hudson and Kaplan 1995), and in the case of single nucleotide substitutions and small InDels, deployment of error prone DNA polymerases during repair of double-stranded DNA breaks during recombination (Rattray and Strathern 2003; Gaut et al. 2007). As for most barley chromosomes, recombination on 2H is essentially confined to the terminal halves of the chromosome arms. Accordingly, the interval from *MWG865* to the centromere (Fig. 2) represents more than half physical length of the 2HL arm but only a tenth of the genetic length (Künzel et al. 2000). Markers from the low-recombination *MWG865*-centromere region (*HYP24* to *IRP*) generally showed lower polymorphism levels than those from other regions, while markers with the greatest levels of SNP and InDel polymorphism were found at the most distal regions of 2HL (*AO*-telomere) and 2HS (markers *HYP20* and *HYP27*, located 37.9 and 43.8 cM from the centromere, respectively) (Figs. 1, 2 and 4). These broad patterns are consistent with the positive relationships between recombination and polymorphism expected from other studies. Elsewhere on 2HL, there were groups of genes with generally high polymorphism (e.g. *ZFP-DGK*, *TCA-OXO* and *MWG865-HYP23*; Fig. 4), as well as groups of genes with no polymorphism whose locations were inferred from the rice gene positions (e.g. *HYP4-GLN*, *HYP32-CTR*; Fig. 4).

Previous studies showed local recombination frequencies to vary widely along the recombinogenic regions of cereal chromosomes (Sidhu and Gill 2004). Further work would be required to establish whether any such local variations in recombination frequency correspond to the patterns of observed polymorphism on chromosome 2H.

Acknowledgements

This work was funded by the GRDC, ARC, the University of Adelaide and the South Australian government. We thank Paul Gooding for technical assistance, Kazahiro Sato for pedigree information, Tania Tabone and Matthew Hayden for assistance in developing the marker assays for *NBP*, *OXO* and *HYP29*, Peter Langridge, Margaret Pallotta and Stewart Coventry for seeds and marker data, and Geoffrey Fincher for critical reading of the manuscript.

Figure legends

Fig. 1.

Distal section of the long arm of barley chromosome 2H, aligned to the related section of the long arm of rice chromosome 4. The barley genetic maps were made using two doubled haploid populations. Lines connect orthologous genes in rice and barley. Markers in bold represent previously described RFLP, STS and microsatellite markers – those with an asterisk were scored as PCR markers. All other markers that are in non-bold type and linked to rice are PCR markers. Intervals interrupted by parallel lines are not drawn to scale. Sections of rice/barley co-linearity are indicated by numbers (1-5) and shading. Junctions between co-linear sections are marked (A-D), together with their sizes in rice. Nucleotide locations on rice chromosome 4 refer to GenBank entry AP008210.1.

Fig. 2.

Central section of barley chromosome 2H aligned to related intervals on rice chromosomes 4 and 7. The barley map was constructed using an Amagi Nijo × WI2585 F_2 population. The lower end joins the maps in Fig. 1. Markers inferred to be located on the short or long chromosome arms are designated (S) and (L), respectively. The location of *Vrs1* 0 to 1.6 cM proximal to *MWG865* is based on previous information (Komatsuda and Tanno 2004; Komatsuda et al. 2007). Nucleotide locations on rice chromosome 4 refer to GenBank entry AP008213.1. See Fig. 1 caption for additional details.

Fig. 3.

Model of chromosome rearrangements leading to the present-day patterns of co-linearity between barley chromosome 2H and rice chromosome 4. Inversions involving chromosome segments

defined in Figs. 1 and 2 (arrows) are shown. Without additional information from evolultionary intermediates, the sequential order of these inversions, and whether an inversion occurred in the barley or rice lineage, can not be known.

Fig. 4.

Frequency of SNP and InDel polymorphisms in genes located along barley chromosome 2H. Genes are arranged according to their determined order in barley or predicted order in barley based on rice co-linearity. Sections of rice-barley co-linearity (1-8) are indicated. Symbol '■' indicates genes that showed sequence differences between closely related cultivars Amagi Nijo and Haruna Nijo. Markers *THIO*, *HYP35*, *HYP33*, *MAN*, *PPT* and *ABC165* were not included, as these were not mapped in barley, and their locations in barley could not be predicted from rice because they were located at the junctions of co-linearity blocks.

Supplemental Fig. 1.

Rearrangements of chromosome segments 2, 3 and 4 involve re-use of junction intervals A, B, C or D in multiple chromosome breakage/fusion events. The six possible scenarios in regard to the chronological sequence of inversions involving chromosome segments 2, 3 and 4 are illustrated. For each scenario, the number of inversions that each junction sequence is involved in is indicated in the table below.

Supplemental Fig. 2.

Pedigrees of barley mapping parents Amagi Nijo, Haruna Nijo, WI2585 and Galleon. Dotted lines indicate derivation by selection alone. The extent of backcrossing is not shown.

Tables and figures

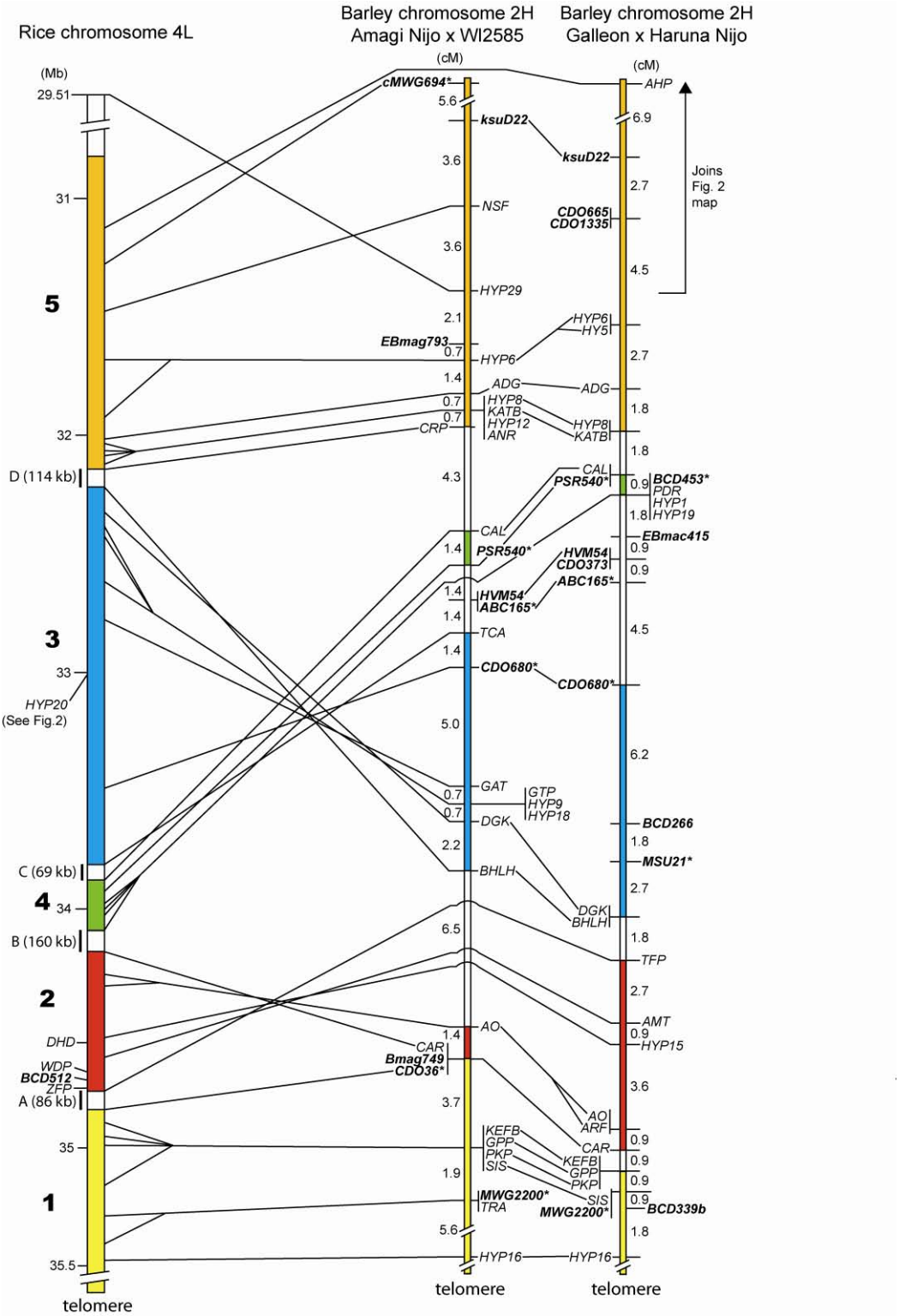

Fig. 1.

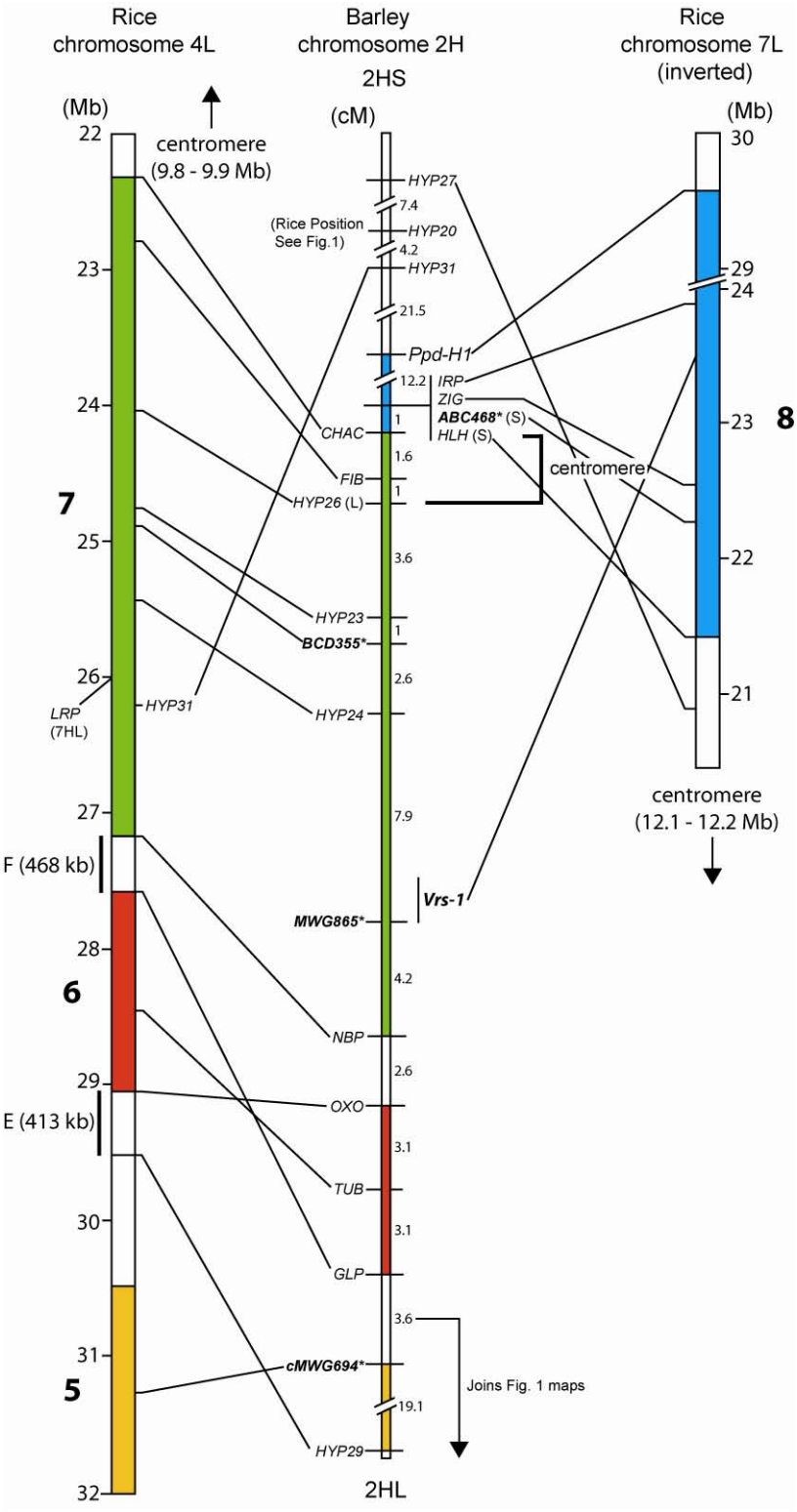
Rice
chromosome 4L
Barley
chromosome 2H
2HS
Rice
chromosome 7L
(inverted)
(Mb)
(cM)
(Mb)
centromere
(9.8 - 9.9 Mb)
(Rice Position
See Fig.1)
HYP27
7.4
HYP20
4.2
HYP31
21.5
Ppd-H1
12.2
IRP
ZIG
ABC468* (S)
HLH (S)
CHAC
1
1.6
FIB
HYP26 (L)
1
3.6
HYP23
1
BCD355*
2.6
HYP24
HYP31
LRP
(7HL)
7.9
Vrs-1
MWG865*
4.2
NBP
2.6
OXO
3.1
TUB
3.1
GLP
3.6
cMWG694*
19.1
HYP29
2HL
Joins Fig. 1 maps
centromere
centromere
(12.1 - 12.2 Mb)
F (468 kb)
E (413 kb)
7
6
5
8
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Fig. 2.

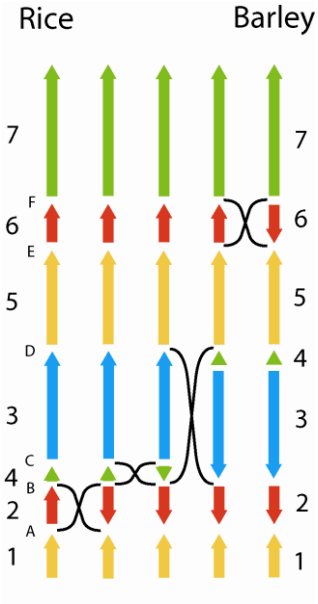
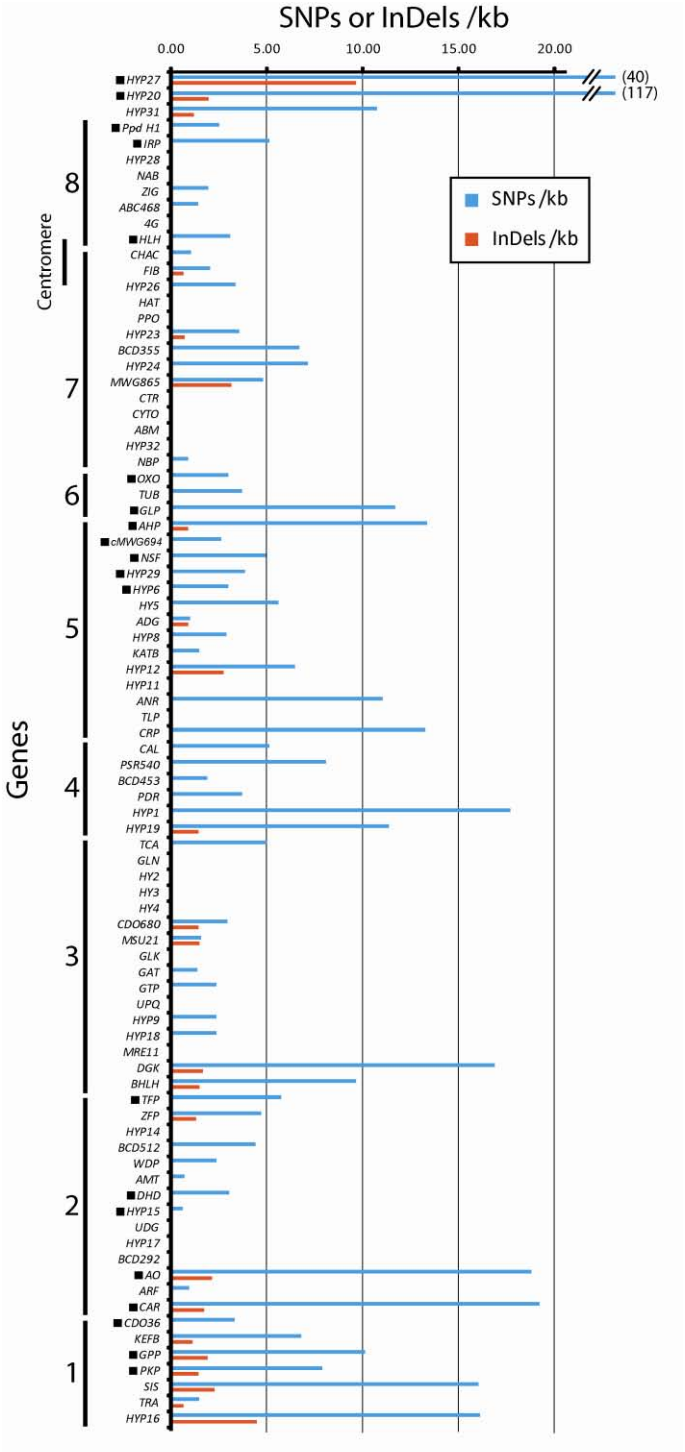


Fig. 3.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2
3
4
5
6
7
8
HYP27
HYP20
HYP31
Ppd H1
IRP
HYP28
NAB
ZIG
ABC468
4G
HLH
CHAC
FIB
HYP26
HAT
PPO
HYP23
BCD355
HYP24
MWG865
CTR
CYTO
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PDR
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HYP19
TCA
GLN
HY2
HY3
HY4
CDO680
MSU21
GLK
GAT
GTP
UPO
HYP9
HYP18
MRE11
DGK
BHLH
TFP
ZFP
HYP14
BCD512
WDP
AMT
DHD
HYP15
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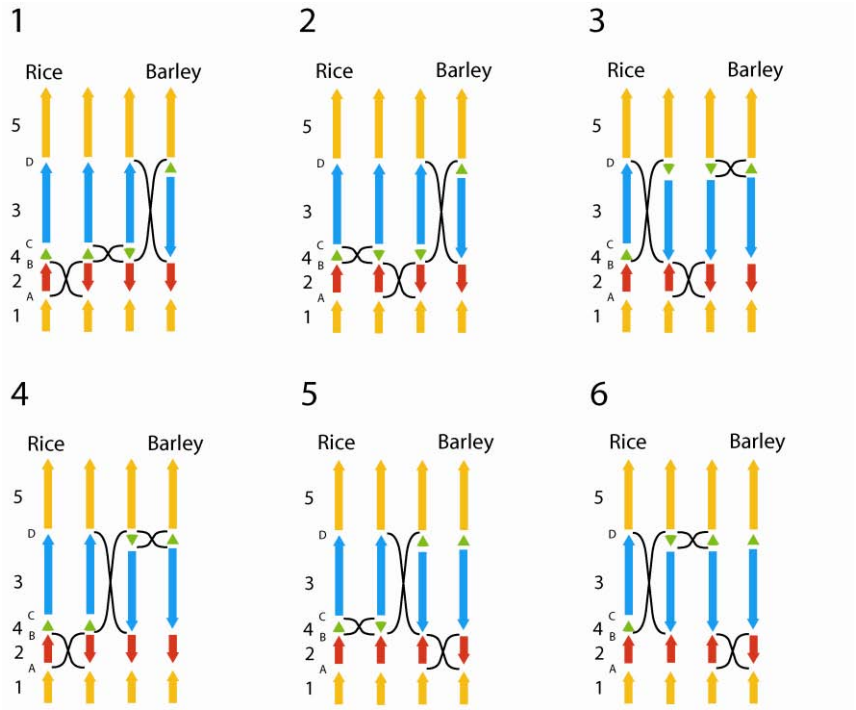
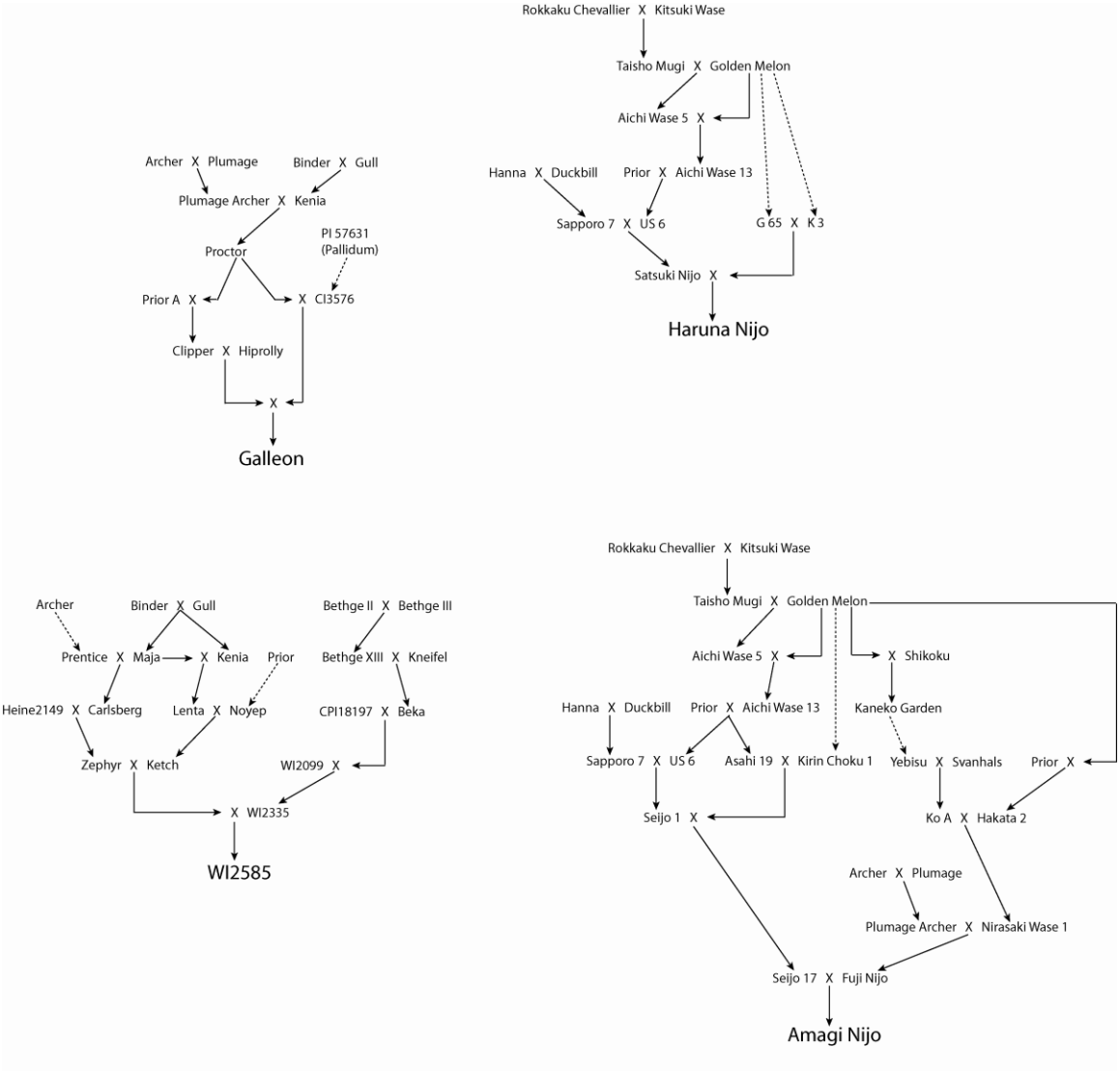
HYP16

Fig. 4.



Scenario	No. rearrangements A	B	C	D
1	3	3	2	1
2	2	3	3	1
3	1	3	1	3
4	3	3	1	2
5	1	3	3	2
6	1	3	1	3

Supplemental Fig. 1.



Supplemental Fig. 2.

Supplemental Table 1. Primers, PCR conditions, restriction enzymes and sequences relating to polymorphism screen and markers.

Markers[a]	Marker Primers (5' to 3')	Original Primers(5' to 3')[b]	Sequence Length (bp)	PCR cycle[c]	Scoring method[d,f]	GenBank Accessions	Putative function of gene product (E-value)	wBDTs (E-value)[i]
HYP27	F-HYP27-3 GTGCAATAGTGCAGCCTACATC R-HYP27-4 GTTTGTTTGTTGCCATAGC	F-HYP27-1 GTCTAGATCAATCAATGAACC R-HYP27-2 GCACAAAATTATCCGGATACAC	334[1382]	15[1]	*BznDIII*(AN/WI) *BclI*(G/HN)	AN[EU282561] HN[EU282701]	Thylakoid lumen 15.8 kDa protein (4E-71)	No significant hit
HYP20	no marker made	F-HYP20-3 TGGTTTCACTGGAGGTCAT R-HYP20-4 GAGATGATGTTGTGTACGTAGG	559	15	no marker made	AN[EU699425] HN[EU699426] WI,G[EU699424]	Zinc finger (ubiquitin-hydrolase) domain-containing protein (2E-43)	No significant hit
	F-HYP20-5 CTTCTGGCTAAGAAACTGTAC R-HYP20-6 CCAATGTTGCACAATGAAGATG		467	15	*BsrI*(G/HN) *AluI*(AN/WI)	AN[EU699428] HN[EU699429] WI,G[EU699427]		
HYP32	F-HYP32-1 GGATGGCTGGAGCTTTGCCA R-HYP32-3 CAGCTTCAAAAACACTGCAGG	F-HYP32-1 GGATGGCTGGAGCTTTGCCA R-HYP32-2 GCGTGTATTAGATGATACAG	221[844]	3[3]	*BseIII*	G,WI[EU282564] HN,AN[EU282562]	LCD1 (Lower Cell Density 1) protein (6E-116)	BS442655; 2AS, 2BS, 2DS (1E-8)
Ppd-H1	F-PPD-H1-3 GGTTTCTTTTGGTTTCTGGC R-PPD-H1-4 GGATAAACTTGAATCAACTGTTG	F-PPD-H1-1 CGATACGTAAGAACGAGCTTAAG R-PPD-H1-2 CAGCAGCTCGAACAATTGGCTC	274[1235]	15[10]	*TaqaI*	AN[EU282744] HN[EU282745] WI,G[EU282746]	Pseudo-response regulator PPD-H1 (0)	BS442811; 4AS, 4BL, 4DL (5E-11)
IRF	F-IRF-3 CCATAGCACCACCTACAGAAGC R-IRF-4 CTTGTGTCAACCTTCCTCTG	F-IRF-1 GATCCTTGTTCAGCTTCAGAGC R-IRF-2 CTTTCTGACCAAAGGATCTG	322[1382]	15[10^{B}]	*AflII*	AN[EU282566] HN[EU282708] WI,G[EU282709]	Putative Interferon-related developmental regulator (7E-57)	No significant hit
HYP28	F-HYP28-1 GCAGATGCAGTGCACTGATC R-HYP28-2 GAAGTACTAGCATCCTCCTCAGTG		1308	1	No SNPs	All[EU699411]	Unknown protein (0)	No significant hit
NAB	F-NAB-1 GACTGCCACCAGATCGATTTCC R-NAB-2 GTATAACTTCGGTGCTGAAGG		1696	11^{9}	No SNPs	All[EU699417]	Zinc finger AN1 and C2H2 domain-containing stress-associated protein 16 (OsSAP16) (4E-75)	BM138155; 2BS, 2DS (1E-155)
ZIG	F-ZIG-3 GGAGAGAGTAATCCATACATTG R-ZIG-4 CATTGGCTAGTGCATGAAGC	F-ZIG-1 GTGTTCTGGAATTCATAGAAGCC R-ZIG-2 CAATAGCCATTTGCTTTTC	284[1073]	3^{B}[1^{B}]	*TspE509I*	G,WI(EU282577) HN,AN[EU282574]	ARF GAP-like zinc finger-containing protein (5E-74)	No significant hit
ABC468	F-468-6 CCAAGGAAGCAGACTAACTAGTG R-468-7 CATAACTATTGGTACACGTGG	F-468-1 GGCTGTAGCTGAAGTTTCCCTTACC R-468-2 AGCATTTACAGTACAGTTCTACAAG	214[762]	3[8]	*SpeI*	WI,G[EU282588] HN,AN[EU282586]	Chloroplast inositol phosphatase-like protein (5E-135)	No significant hit
4G	F-4G-1 GCTATCACTCTTGGACCACAAG R-4G-2 CAACTCTTCAATACACAATGC		885	1^{9}	No SNPs	All[EU699397]	Translation initiation factor eIF4G (8E-91)	No significant hit
HLH	F-HLH-3 GTCAATACATTCTTATCCGCG R-HLH-2 GCTAGGTTGAGCACCAGTTTCTGATC	F-HLH-1 CCGCAGGAGGCTTCTACTG R-HLH-2 GCTAGGTTGAGCACCAGTTTCTGATC	191[1327]	15[1]	*BsiI*	WI[EU282668] G(KU282540) HN,AN[EU282666]	bHLH transcription factor (2E-93)	BS490521; 2AS, 2DS (0)
THIO	F-THIO-1 CATCTGTAATTGTTGACAGGAG R-THIO-2 CCAGTAGGTACTAAATTCCGATC		1440	16	No SNPs	All[EU699420]	Thioredoxin family-like protein (0)	BS444378; 2AS, 2BS, 2DS, 4DS (0)
HYP35	F-HYP35-1 GTACCTGATGAAATCAAAGG R-HYP35-2 CCAGCCAGAAATGGTGAGATTG		1418	10	No SNPs	All[EU699414]	Putative transmembrane protein (4E-29)	BE637702; 2AL, 2DL (4E-79)
CHAC	F-CHAC-3 CTGTCTGCTTATCTTGCTCCCTTG R-CHAC-4 GGACTGCGTTAGGATGGTGAATC	F-CHAC-1 CTCGTCGGCTTCGTCAGGGACTAC R-CHAC-2 CATATTGCTTCTCTCTTACTTC	299[1044]	15[1]	*BsrI*	WI,G(EU282632) HN,AN(EU282630)	ChaC-like family protein (5E-56)	No significant hit
FIB	F-FIB-4 AGTCATGCAATGCAGAGGTAG R-FIB-5 GTGACCGATGTCTGCATAGG	F-FIB-1 CAGAACTTCTGTGAGGATGTATC R-FIB-2 GAACGTGATGTTGCTTGGACC	123[1542]	15[1]	*XmaI*(AN/WI)	WI[EU282649] HN,AN,G[EU282646]	HN1-like protein isoform 1 (5E-89)	No significant hit
HYP26	F-HYP26-1 GTTGAACATGTTTAGCAGTCC R-HYP26-3 GTCACCATGGCATGTGAGATC	F-HYP26-1 GTTGAACATGTTTAGCAGTCC R-HYP26-2 CTCTAAGTGAGGTGGTGTTTATG	229[908]	15[1]	*DdeI*(AN/WI)	WI[EU282700] HN,AN,G[EU282697]	Alkaline alpha galactosidase (2E-147)	BE405285; 2AL, 2BL, 2DL (2E-65)
BRT	F-HAT-1 CTTTGATCGATGCTCCTTACG R-HAT-2 GAAATGCAGAGATGTCAGAAC		955	1	No SNPs	All[EU699404]	Histone acetyltransferase HAT2 (1E-101)	No significant hit
PPO	F-PPO-1 CTTTGTGCTAGACTTTCTTCC R-PPO-2 CCTTCTTAAATGCTGTTACCATG		804	1	No SNPs	All[EU699418]	Protoporphyrinogen IX oxidase (4E-120)	BE406962; 2AL, 2BL, 2DL (8E-48)
HYP23	F-HYP23-5 GGCAAACCTTTTTGTCCTTGC R-HYP23-2 CTTTGCGGTGTAGATGGATG	F-HYP23-1 GAAGAATCTGCAACCTTCTCC R-HYP23-2 CTTTGCGGTGTAGATGGATG	171[1448]	3[11^{B}]	*BsrI*	WI,G(EU282559) HN,AN(EU282557)	DNA binding protein (6E-30)	No significant hit
BCD353	F-335-3 GAGAAGGCAGATCCGTTGATG R-335-4 GGCAATGCCTATGTGATTG	F-335-1 GGCTACAGCATTGAAGACGTTCGAC R-335-2 GAGCTCCTGCTCCACGATGTTGTTG	292[1209]	3[8]	*StuI*	G(KU282580) WI(KU282581) HN,AN(KU282578)	S-adenosylmethionine decarboxylase (0)	BE494102; 5AL, 5BL, 5DL (1E-21)
HYP24	F-HYP24-1 CCTCCTGATGGATTCGCTCTC R-HYP24-2 GCACTTGCCAAATCAAGCC		284	1	*XpnI*	WI,G(EU282695) HN,AN(EU282693)	Unknown protein (7E-72)	No significant hit
MWG865	F-865-3 CCTAGTTTATCTCATCCAGTATC R-865-4 CACTTAATCTGTTCCAATACACGC	F-865-1 GGTTGTTGCCCTGTTGCTG R-865-2 GACAGTAACATTTCGGAATC	305[635]	3[8]	*SacII*(AN/WI)	WI[EU282608] HN,AN,G[EU282605]	No significant hit	No significant hit
CTR	F-CTR-1 GATTTCTGGTCAATTACTATATG R-CTR-2 GAAGCATCAAAATTGACTCTC		1152	1	No SNPs	All[EU699400]	Transporter-related protein (1E-37)	No significant hit
CYTO	F-CYTO-1 CAAGAACCTCATCTTCAACG R-CYTO-2 CAATCAGCAGCACGTAGCTC		1631	1^{9}	No SNPs	All[EU699401]	Putative cytochrome (5E-86)	No significant hit
ABM	F-ABM-1 CACTTCTCTTAGACTTTGG R-ABM-2 ATATGCGTGTGAGCTTTGAATCCCG		813	1	No SNPs	All[EU699398]	Kinesin-like protein 1 (5E-9)	No significant hit
HYP32	F-HYP32-1 CTCCAGATCGCATCAAATCC R-HYP32-2 GCACATCGAAGGTAAAGTTC		1320	1	No SNPs	All[EU699412]	Kinesin-like protein 15 (6E-160)	No significant hit
NBP	F-NBP-3 CAGCCCAAGGCAGCGTTAGA R-NBP-4 AACATGCCGGAACATCCACAA F-NBP-5 CGCCGGAATCGTCACTGCAT F-NBP-6 CGCCGGAATCGTCACTCGAC R-NBP-7 GCCTTGGACGATCCTCCCAGAA	F-NBP-1 CTTCAACCAACTCGATGTTCCTG R-NBP-2 CTTAGATTATGATGTTGGTGGTAGC	~ [1239]	17[1]	novel allele detection method	WI,G(EU282729) HN,AN(EU282727)	Water-stress protein (4E-122)	BF293021; 2AL, 2BL, 2DL (9E-4)
HYP33	F-HYP33-1 CCAGCAAATATCCATGTTGAAG R-HYP33-2 GCACTGACTTCATATACTTTC		578	16	No SNPs	All[EU699413]	Unknown protein (0)	No significant hit
OXO	F-OXO-3 CAGATCCGCTGCCATGCAAT R-OXO-4 GCGAGAACGGGGGTAGTCTTCA F-OXO-5 GCGAACTCTTCTTAAAGGATCATACCATATATT F-OXO-6 GCGAACTCTTCTTAAAGGATCATACCATATTAC R-OXO-7 CGGCAAGGCTTTTGCGTTTTC	F-OXO-1 CATCAGATCCGCTGCCATGCAATTC R-OXO-2 CTTCTGAAGGTACCATCTATGAG	~ [694]	17[10^{B}]	novel allele detection method	WI[EU282569] HN[EU282568] G(KU282735) AN[EU282567]	Putative 3-oxo-5-alpha-steroid 4-dehydrogenase (1E-57)	BE444293; 2AL (0)
TUB	F-TUB-3 GTTAGCAGCTTCACAATGAAG R-TUB-4 GTATCACATGAAGACCTCTGC	F-TUB-1 GCATTATGCAAGAAGTTCTG R-TUB-2 GTAATGACAATATTCACTGG	285[826]	15[1]	*BseIII*	G,WI[EU282762] HN,AN[EU282760]	Tubulin binding protein (5E-42)	No significant hit
GLP	F-GLP-3 CGAATTGTGTGTAAGTTTTGG R-GLP-4 CTTTCAAACCGCAAATTGAAC	F-GLP-1 CCTTTTCTTGCTAGGCTTCGTAT R-GLP-2 CCGTGTCAAAATATCAGA	314[1031]	15[1]	*AvaII*	WI[EU282657] G(KU282655) AN[EU282654] HN[EU282656]	Growth regulator like protein (5E-93)	No significant hit
MAN	F-MAN-1 GGAGATCCTAAGTATCAACTAAAG R-MAN-2 CCTGAATCCAGACCTTGAGC		1705	10	No SNPs	All[EU699415]	Mannosyl-oligosaccharide 1,2-alpha-mannosidase (0)	BE404909; 2AL, 2DL (0)
PPT	F-PPT-1 GATGCCATATACTGGCCAGCCTTTG R-PPT-2 TCAGAACCCAATACAAACTAACATC		1226	1	No SNPs	All[EU699419]	Putative potassium transporter (2E-143)	No significant hit
AHP	F-AHP-3 CTTCGGGACGGTGGAGCAGGTCTAC R-AHP-2 GAGTTCCAATGATCCATTCTGAATC	F-AHP-1 CCGAGATCCACCTCGACGGCGGCTC R-AHP-2 GAGTTCCAATGATCCATTCTGAATC	443[1200]	3^{B}[1^{B}]	*NruI*(G/HN)	G(KU282523) HN(KU282524) WI,AN(EU282525)	QUARTET 3 protein (4E-112)	BS500324; 2AL, 2BL, 2DL (1E-156)
cBM0694	F-694-7 CTGAGCGAAAGTGGGAGATTGAGAC R-694-6 CGGTGACTTCCATCATCTTCACATC	F-694-3 CTTACAGGGTCTTGATGTCATTGTG R-694-4 CATCTGAATGCCAAGGTCACAGCAC	500[1198]	3^{B}[1^{B}]	*StuI*(AN/WI)	AN[EU282601] HN,WI,G[EU282602]	PAZ domain-containing protein (1E-35)	BF145606; 2AL, 2BL, 2DL (2E-4)
NSF	F-NSF-4 CGACCGGAGAAGCTCATGCCAAG R-NSF-5 GGCAAGTCGTCTGAATCCTAC	F-NSF-4 CGACCGGAGAAGCTCATGCCAAG R-NSF-3 CATCTCTTGTCCTTTCGAAGCCTTC	368[1282]	3[1]	*AvaII*	WI[EU282734] HN[EU282733] AN,G (EU282731)	GRNAP (Gamma-Soluble NSF Attachment Protein) transporter protein (2E-109)	BE444599; 2AL, 2BL, 2DL (0)
HYP29	F-HYP29A-3 GTGGGCAAGCGAACCATCCT R-HYP29A-4 TCCTATGCAGAGCAGAACTTCAAACC F-HYP29A-5 GCGGCACATCATGTGTATTTGTTC F-HYP29A-6 GCGGGCACATCATGTGTATTTGTTT R-HYP29A-7 GCGACATACCCCTGACACAGCAC	F-HYP29A-1 TGGGCAAGCGAACCATCCTCTC R-HYP29A-2 GAACTTCAAACCTCTGAACATC	~ [799]	17[10]	novel allele detection method	WI,HN(EU282706) AN,G(EU282704)	Unknown protein (7E-95)	No significant hit
HYP6	F-HYP6-3 GGTCAGTCAGAACACAGGATG R-HYP6-4 GCTCGTCCAAGAGATGCTCC	F-HYP6-1 CAACAGGATGTTGAGGCCTTTGTTG R-HYP6-2 AGCCTACCAAAATGTAAGCCATATC	370[1028]	1	*XmaI*	WI[EU282672] HN[EU282671] AN,G(EU282669)	Unknown protein (1E-149)	BE443833; 2AL, 2BL, 2DL (0)
HY5	F-HY5-1 TGATGAACAAGCTCTTCCAGCGCTG R-HY5-2 CTACTACCGATGAGAGTTCCAGAAC		1600	1^{9}	*AseI*(G/HN)	G(KU282544) WI,HN,AN(EU282541)	Putative binding protein (1E-64)	No significant hit
ADG	F-ADG-3 GTTGTCAATACCTGTTGTTC R-ADG-2 GATTCATCCACCAGTTTAGAATG	F-ADG-1 CAGCAAGCATCTGGACTCCTGATGG R-ADG-2 GATTCATCCACCAGTTTAGAATG	(310)1138	1	*XbaI*	WI,G(EU282611) HN,AN(EU282609)	Transposase-related protein b-gary (0)	BM134387; 2AL, 2BL, 2DL (0)
HYP8	F-HYP8-3 CATGTCACAGTGAGCCTTCTGTC R-HYP8-4 CATAGCTTATGGGGCATTGCAGAAC		1067	1	*BssHII*(AN/WI)	WI[EU282676] HN,AN,G[EU282673]	PER22 (PEROXIN 22) (5E-65)	BE445431; 2AL, 2BL, 2DL (0)
KATB	F-KATB-5 CACTTATAGTTTGCACAATC R-KATB-4 CCTCAATTGCAGTCACATCAG	F-KATB-1 GAAGAGTTGAAAGCGGCAAACTTG R-KATB-3 GGCTTCTTCCTGACCATTAG	533[1400]	4^{B}[1^{B}]	*XmnI*	WI,G(EU282713) HN,AN(EU282711)	Kinesin-like protein (0)	No significant hit
HYP12	F-HYP12-1 GATGTTGAAGGTCATAGCAAGGAC R-HYP12-2 GGTCTTGGAGTAGATCCTCATTC		1131	1	*BstF5I*	WI[EU282680] G(KU282679) HN,AN(EU282677)	SDA1 family protein (1E-70)	No significant hit
HYP11	F-HYP11-1 CCATCTTAGTCGACATCTCCTAAC R-HYP11-2 GGATCAGATCCATCAGGAGTCCAG		561	1	No SNPs	All[EU699408]	Transposase-related protein b-gary (3E-129)	No significant hit
ANR	F-ANR-3 CTTTATCAGCCAGTGTGCCAATC R-ANR-4 GTTCGACGACGTCAGGATCACG		934	1^{9}	*BstXI*(AN/WI)	WI[EU282616] HN,AN,G[EU282613]	Anthocyanidin reductase (5E-91)	BE423717; 4BS, 4DS (7E-51)
TLP	F-TLP-1 CTGACGATGCCATGCGCGTCTTG R-TLP-2 CCGACACGCCAAACACTGCAAAC		703	1^{9}	No SNPs	All[EU699421]	Organic amion transporter-like protein (1E-128)	No significant hit
CRP	F1-CRPa-1 AGAACTCCCACCTCAAAGACCTGC F2-CRPa-2 GGCTGCGACTACGTCTTCCTCTTC R1-CRPa-3 AACATGTTCACCGGAGCGTCA R2-CRPa-4 AATGTTAGAGCCGACGCTTGAACA	F-CRP-1 CTCTCGTCAAGATGCTGCTGGAGAAG R-CRP-2 CTTCACGCACGACCTCATCACGTTGAG	211[800]WI 141[800]AN,HN,G	18[1]	tetra-primer ARMS-PCR	WI(EU282637) AN,HN,G(EU282634)	Anthocyanidin reductase (7E-82)	BF473679; 2DL (2E-17)
CAL	F-CAL-6 GTAGCATCAGTTTCAGAAGC R-CAL-7 CCGAGATGTGATATCCGTCC	F-CAL-3 CCTGCATCTAGAGAAGCCAGATGAG R-CAL-4 TTAGTGTCTCTTCCACATCATCAGG	416[1635]	8[11^{B}]	*AvaII*	WI[EU282536] G(KU282533) HN,AN(EU282534)	Calmodulin binding protein IQ-DOMAIN 32 (4E-40)	BE500307; 2BL, 2DL (1E-149)
BE8540	F-540-3 CTAGGCTTGACTCTTGGCTCGC R-540-4 GCATCTGCAAATATGTCACC	F-540-1 CAAAATCACCATGAGTAAGTGCACC R-540-2 AGCGACAATGCCTGCCTGGTTATGG	451[700]	2	*Sau96I*	WI[EU282597] G(KU282594) HN,AN[EU282595]	Auxin response factor 11 (1E-60)	No significant hit
BG453	F-453-3 CGTCCAAGTCCTTCTTCGAC R-453-4 CCGTTGTTGAACACGTACAG		589	2^{B}	*AfeI*(G/HN)	WI,AN,HN(EU282582) G(KU282583)	Extracellular invertase (0)	BF428883; 2AL, 2BL, 2DL (4E-90)
PDR	F-PDR-1 CCATTCAGCTTCGAAGGACGCAAAC R-PDR-2 CCAAGCTTGTCACAATCTG		1259	1^{9}	*AvaII*(G/HN)	WI,AN,HN(EU282737) G(KU282736)	Plastid division protein Fts2 (3E-148)	No significant hit

Supplemental Table 1.

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3 Genes and traits associated with chromosome 2H and 5H regions controlling sensitivity of reproductive tissues to frost in barley

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Statement of authorship

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Statement of contribution

Designed the experiment, conducted the research, analyzed the data and drafted the manuscript

I hereby certify that the statement of contribution is accurate

Signed..

Reinheimer J

Statement of contribution

Developed markers *CK881* and *AJ927*, provided the data for ESM figure 2 to the final manuscript

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Brûlé-Babel A

Statement of contribution

Developed markers *WG644*, *SNF*, *NUC* and contributed to the final manuscript

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Baumann U

Statement of contribution

Participated in the supervision of the work and contributed to the final manuscript

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Pallotta M

Statement of contribution

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Statement of contribution

Participated in the supervision of the work, contributed to the final manuscript

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Statement of contribution

Coordinated the development of the work, contributed to the final manuscript and acted as the corresponding author

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Abstract

Frost at flowering can cause significant damage to cereal crops. QTL for low temperature tolerance in reproductive tissues (LTR tolerance) were previously described on barley 2HL and 5HL chromosome arms. With the aim of identifying potential LTR tolerance mechanisms, barley Amagi Nijo × WI2585 and Haruna Nijo × Galleon populations were examined for flowering time and spike morphology traits associated with the LTR tolerance loci. In spring-type progeny of both crosses, winter alleles at the *Vrn-H1* vernalization response locus on 5H were linked in coupling with LTR tolerance and were unexpectedly associated with earlier flowering. In contrast, tolerance on 2HL was coupled with late flowering alleles at a locus we named *Flt-2L*. Both chromosome regions influenced chasmogamy/cleistogamy (open/closed florets), although tolerance was associated with cleistogamy at the 2HL locus and chasmogamy at the 5HL locus. LTR tolerance controlled by both loci was accompanied by shorter spikes, which were due to fewer florets per spike on 5HL, but shorter rachis internodes on 2HL. The *Eps-2S* locus also segregated in both crosses and influenced spike length and flowering time but not LTR tolerance. Thus, none of the traits was consistently correlated with LTR tolerance, suggesting that the tolerance may be due to some other visible trait or an intrinsic (biochemical) property. Winter alleles at the *Vrn-H1* locus and short rachis internodes may be of potential use in barley breeding, as markers for selection of LTR tolerance at 5HL and 2HL loci, respectively.

Introduction

Frost at anthesis or early grain fill can damage cereal crops by causing floret sterility or shriveled grains. Frost damage to reproductive tissues is most limiting in areas experiencing subtropical/Mediterranean climates such as in Australia, West Asia and North Africa, where cereals mature during winter when conditions are most favorable for growth but occasional night time frost events in the order of -1 to -5 °C occur (Single 1985; Fujita et al. 1992; Reinheimer et al. 2004). In these areas, the choice of sowing date is constrained both by the higher probability of frost early in the season and by the hot and dry conditions that typically limit growth late in the season. In temperate regions, mid to late spring freezes may also cause spike damage to winter wheat crops which flower during spring and summer (Livingston and Swinbank 1950; Paulsen and Heyne 1983; Cromey et al. 1998; Whaley et al. 2004). Agronomic practices, such as clay delving or optimization of sowing time, can be employed to reduce frost damage at flowering (Rebbeck et al. 2007). While there is also a need to breed tolerant varieties, efforts to do so have been hampered by the sporadic nature of frost in the field, uneven freezing of plant tissues, and the variation in susceptibility of spikes at different stages of development. Despite various reports of genotypic variation for low temperature tolerance at the reproductive stage (LTR tolerance) in barley and wheat (Single and Marcellos 1974; Marcellos and Single 1984; Single 1985), locally adapted varieties possessing useful levels of LTR tolerance are not yet available (Fuller et al. 2007).

Reinheimer et al. (2004) described LTR tolerance loci on chromosome arms 5HL and 2HL of barley. Only the 5HL locus was detected in an Arapiles × Franklin population where it was found to control levels of frost induced sterility and frost induced grain damage. Both loci were detected in Amagi Nijo × WI2585 and Galleon × Haruna Nijo populations where LTR tolerance was measured by the level of frost induced sterility, expressed as the percentage of sterile florets on selected spikes. The tolerance allele at each locus was derived from the Amagi Nijo and Haruna Nijo parents, which are closely related cultivars (Chen et al. 2008) bred by the Sapporo brewery in Japan.

Compared to LTR tolerance, low temperature tolerance in vegetative tissues (LTV tolerance) has been relatively well characterized. LTV tolerance has been reported to be mainly controlled by

two loci (*Fr-1* and *Fr-2*) located on Triticeae group 5 chromosomes (Tóth et al. 2003; Vágújfalvi et al. 2003; Francia et al. 2004; Skinner et al. 2006; Tondelli et al. 2006). Tolerance at *Fr-2* appears to be controlled by clusters of C-repeat binding factor (CBF) transcription factor genes that reside at this locus in barley and wheat (Francia et al. 2007; Knox et al. 2008), whereas *Fr-1* is closely linked to the *Vrn-1* vernalization response locus. The LTR tolerance locus on chromosome 5H is also in the vicinity of *Vrn-H1*/*Fr-H1* (Reinheimer et al. 2004). LTV tolerance QTL have been detected on group 2 chromosomes of wheat and barley (Tuberosa et al. 1997; Båga et al. 2007), however it is difficult to compare the position of these loci with that of the 2H LTR tolerance locus due to a lack of common genetic markers.

Given that the loci on barley chromosomes 5H and 2H are the only Triticeae LTR loci so far described, there is a need to characterize these loci further in order to explore the basis for LTR tolerance in cereals and to ensure effective utilization of these tolerance sources in breeding programs. Genes that alter the rate of crop development may potentially impact frost damage to reproductive tissues, either by facilitating escape (Reinheimer et al. 2004), or by extending the period that plants have available to accumulate cold tolerance potential (Limin and Fowler 2006). While the *Vrn-H1* locus linked to the 5H LTR tolerance locus affects flowering time, no major developmental effect was found to be linked with the 2H LTR tolerance locus (Reinheimer et al. 2004). However, a locus controlling a compressed rachis (*Cr*) phenotype has been shown to segregate in the 2H frost tolerance QTL region in the Galleon × Haruna Nijo population (Karakousis et al. 2003). The degree to which barley florets extrude their anthers due to the open/closed nature of the palea and lemma is also controlled by a locus in the vicinity of the 2H LTR tolerance QTL (Turuspekov et al. 2004). Most 2-row barley varieties from Japan have closed (cleistogamous) florets, whereas most varieties from elsewhere have open (chasmogamous) florets (Kuraichi et al. 1994), suggesting that the two mapping crosses with Japanese parents may segregate for this trait. Wheat spikes remain relatively resistant to freezing until they emerge from the boot and become exposed to extrinsic ice nucleation factors (Single 1985). Therefore, it is conceivable that the length of the spike or cleistogamy/chasmogamy may segregate at the 2H locus and influence LTR tolerance by altering the degree to which the spike or internal flower structures are exposed at the time of frosting.

In the current study we explored the basis for the LTR tolerance encoded by the 2H and 5H QTL by performing a more detailed analysis of flowering time and spike traits segregating in the

Amagi Nijo × WI2585 and Galleon × Haruna Nijo crosses. These analyses were assisted by the availability of PCR-based genetic markers we previously developed along the 2HL chromosome arm (Chen et al. 2008) and knowledge derived from the recent cloning and characterization of underlying genes for several major developmental loci in barley.

Materials and methods

Molecular markers

DNA extraction and general procedures for developing and scoring PCR-based markers were as described by Chen et al. (2008). Except for the markers described below, all of the markers were previously described by Chen et al. (2008).

PCR assays were used to detect *Vrn-H1* intron-1 variants, as described by Fu et al. (2005). In these assays, primer pair Intr1/H/F1 plus Intr1/H/R1 produces a 474 bp product specifically from spring *Vrn-H1* alleles carrying the 5.2 kb intron-1 deletion (cv. Morex-type), and primer pair Intr1/H/F3 plus Intr1/H/R3 produces a 403 bp product specifically from non-deleted winter *vrn-H1* alleles. The presence or absence of the *ZCCT-Hc* gene at the *Vrn-H2* locus was assayed by PCR using the primers HvZCCT.HcF and HvZCCT.HcR, as described by von Zitzewitz et al. (2005). A positive control was provided by amplification from the nearby *SNF2* gene, which is not affected by the *Vrn-H2* deletions, using the primers Snf2.F and Snf2.R (von Zitzewitz et al. 2005).

Polymorphism at 'site 23' of the *Ppd-H1* gene was assayed using a *Bst*UI cleaved amplified polymorphic sequence (CAPS) marker, as described by Turner et al. (2005), except that that amplification was performed in the presence of v/v 5% DMSO and using the newly designed primers: Ppd-F (5′-AATGGTGGATCGGCAGGAGGCACTG-3′) and Ppd-R (5′-GTGGCGGGAGGTTATCTCTCCACGG-3′). This marker produces an undigested product of 488 bp from *ppd-H1* alleles and digestion products of 428 bp plus 60 bp from *Ppd-H1* alleles.

CAPS markers close to the *Vrn-H1* and *Vrn-H2* vernalization response loci were developed for both the Amagi Nijo × WI2585 and Haruna Nijo × Galleon crosses. Details of these markers are provided in Supplemental Table 1. Marker *WG644* was originally defined by a wheat genomic RFLP probe and derives from a putative ABC transporter gene. The *WG644* primers were based on the sequence of the BAC clone BAC 635P2 (AY013246) from the *Vrn-H1* region in barley (Dubcovsky et al. 2001). Co-linearity between the Triticeae *Vrn-1* chromosome region and the terminal part of rice chromosome arm 3L (Yan et al. 2003; Stein et al. 2007) was exploited to generate markers *CK881* and *AJ927*, which were based on barley orthologues of genes located

approximately 600 kb above and 790 kb below the *Vrn1* orthologue on rice chromosome 3, respectively. The *SNF* and *NUC* markers were developed from barley homologues of the *SNF2P* and *NUCELLIN* genes, which are closely linked to *Vrn-A^{m}2* in *T. monococcum* (Yan et al. 2004). The primers were based on the sequence of BAC clone 615K1 (AY485643) from the corresponding region in barley.

Plant material

Barley cvs. Haruna Nijo and Galleon were crossed to make an F_2 family of 96 individuals, which was used to test for marker-trait associations on chromosome 2H and in the vicinity of the *Vrn-H1* and *Vrn-H2* loci. An F_4 family derived from the same cross (34-2-73) was used for further evaluation of flowering time controlled by *Flt-2L*. The 34-2-73 F_3 parent was selected using molecular markers, and was shown to be homozygous for Galleon alleles for markers closely flanking *Vrn-H1*, homozygous Galleon for a segment of 2H carrying the *Eps-2S* locus, and heterozygous in the region containing *Flt-2L* on the same chromosome (Fig. 1). Five markers that spanned the *Flt-2L* chromosome region (Fig. 1), were used to screen 96 individuals from the 34-2-73 family. The 82 F_4 seedlings determined to be non-recombinant for the *ADG-MSU21* marker interval containing *Flt-2L* were grown on for phenotypic analysis.

Amagi Nijo × WI2585 F_2 seeds were obtained from a previous study (Chen et al. 2008) and used to produce an F_2 derived F_4 recombinant inbred family. This F_4 family was screened with molecular markers, identifying two individuals (103-1 and 110-4) which were homozygous for WI2585 alleles in the *Vrn-H1* locus region and heterozygous for markers located across a large section of chromosome arm 2HL. An F_6 family derived from plant 103-1 by two more rounds of self-pollination was screened with markers located across 2HL, identifying one individual (103-1-2-114) that was homozygous for the WI2585 marker alleles in the *Eps-2S* region but heterozygous for the *Flt-2L* region on 2HL (Fig. 1). Similarly, an F_5 offspring of plant 110-4 was identified (110-4-34) that was heterozygous across the *Eps-2S* region but homozygous for Amagi Nijo marker alleles in the *Flt-2L* region (Fig. 1). Plants 103-1-2-114 and 110-4-34 were allowed to self-pollinate to produce families (F_7 and F_6) for further evaluation of *Flt-2L* and *Eps-2S* phenotypes, respectively. Five markers that span the *Flt-2L* locus (Fig. 1), were used to screen around 80 plants from the 103-1-2-114 F_7 family. A random selection of 32 individuals which were non-recombinant for the region spanned by these markers were used in the phenotypic

analysis. For family 110-4-34, 48 randomly selected F_6 individuals were scored for eight markers located on the segregating chromosome 2H segment (Fig. 1) and all were used for the phenotype analysis, with the *Eps-2S* locus genotype being inferred using the linked *IRP* marker.

The Amagi Nijo × WI2585 F_1-derived doubled-haploid population of 139 lines has been previously described (Pallotta et al. 2003).

Growth conditions and phenotyping

The Haruna Nijo × Galleon F_2s were grown in a greenhouse in Adelaide during January-February when day length was 13-14.5 hours and average day/night temperatures in the greenhouse were approximately 28°C/22°C. Families 34-2-73, 103-1-2-114 and 110-4-34 were grown in a greenhouse during March-April when the day length was 11-13 hours and average day/night temperatures in the greenhouse were 26°C/20°C. The Amagi Nijo × WI2585 F_1-derived doubled-haploid population was grown in a greenhouse from early June to mid October when day length was 10-13 hrs and average day/night temperatures in the greenhouse were 22°C/16°C.

Flowering time was defined as the date at which the awns first became visible on the primary tiller. Chasmogamy was assessed by estimating the percentage of anthers in a spike that had extruded from the florets just after anthesis. Length of rachis internodes and whole spikes were recorded when spikes began to dry. Total spike length was measured as the distance between the uppermost and lowermost fertile rachis nodes, whereas rachis internode length was obtained by dividing spike length by the number of fertile nodes on a spike.

Statistical analysis

QTL mapping in the Amagi Nijo × WI2585 doubled haploid population was performed using Map Manager QTX version 0.30 software (Manly et al. 2001). Other associations between marker alleles and trait scores were tested by one-way analysis of variance (ANOVA) in GenStat (6^{th} edition). The proportion of phenotypic variation attributed to a particular locus was calculated as the sum of squares for that locus divided by the sum of squares for the whole dataset, multiplied by 100%.

Results

Parental alleles at *Vrn-H1, Vrn-H2* and *Ppd-H1* loci

Genotypes of the mapping parents for the *Vrn-H1* and *Vrn-H2* vernalization response loci were determined by assaying polymorphisms within the cloned genes (Table 1). WI2585 and Galleon had the 5.2 kb deletion within intron-1 of the barley *Vrn-H1* gene (= *BM5A* gene) whereas Amagi Nijo and Haruna Nijo carried undeleted intron-1 versions of *Vrn-H1* (Table 1). These sequence features are associated with spring and winter alleles of *Vrn-H1*, respectively (Fu et al. 2005; Cockram et al. 2007). Galleon was found to possess the *ZCCT-Hc* gene at the *Vrn-H2* locus, whereas the other three parents were found to carry a deletion of *ZCCT-Hc*. The presence and absence of the *ZCCT* genes at the *Vrn-H2* locus is associated with winter and spring alleles of *Vrn-H2*, respectively (Dubcovsky et al. 2005; Cockram et al. 2007). Therefore, the genotypes were determined to be *Vrn-H1 Vrn-H2* for Galleon, *Vrn-H1 vrn-H2* for WI2585 and *vrn-H1 vrn-H2* for Haruna Nijo and Amagi Nijo (Table 1). Genotype *vrn-H1 Vrn-H2* confers winter growth habit, whereas all other genotypes are spring type (Kóti et al. 2006). The deduced *Vrn* locus genotypes were therefore consistent with the spring growth habit of all four parental lines and a report that Galleon × Haruna Nijo F_1-derived doubled-haploid family segregates for winter/spring habit (Reinheimer et al. 2004).

The *Ppd-H1* locus is a major factor determining flowering time under long days. A single nucleotide polymorphism in the *Ppd-H1* gene has been identified as being the likely determinant of the functional difference between sensitive (*Ppd-H1*) and insensitive (*ppd-H1*) alleles (Turner et al. 2005). Using a CAPS marker, all four parents were shown to possess the G nucleotide at this position (Table 1), which is associated with functional *Ppd-H1* photoperiod sensitive alleles. The result for Haruna Nijo was consistent with the previous report that stated that this variety carries a *Ppd-H1* allele (Turner et al. 2005).

Marker trait analysis: Haruna Nijo × Galleon derived families

A total of 96 randomly selected F_2 individuals derived from a Haruna Nijo × Galleon cross were scored for molecular marker to test for marker-trait associations. In the *Vrn-H1* region, markers

CK881, *WG644* and *AJ92* mapped to a region of 15.5 cM, in the same linear order as their corresponding orthologues on rice chromosome 3 (Fig. 1). The central marker, *WG644*, was expected to be very tightly linked to *Vrn-H1* because it mapped only 0.11 cM distal of *Vrn-A^{m}1* in *T. monococcum* (Yan et al. 2003). In the *Vrn-H2* region, the *SNF* and *NUC* marker genes mapped 1.6 cM apart (Fig. 1). In *T. monococcum*, these genes map 0.02 and 0.30 cM from *Vrn-A^{m}2*, respectively (Yan et al. 2004). The Haruna Nijo × Galleon F_2 population was also scored for 12 chromosome 2H markers covering most of the genetic length of the chromosome (Fig. 1), one of which was based on the *Ppd-H1* gene. The order in which these markers mapped on 2H was the same as that obtained previously (Chen et al. 2008).

The Haruna Nijo × Galleon F_2 population was grown under long days (14 hrs.) and in the absence of vernalization - conditions that allow cereal *Vrn1* and *Vrn2* loci to have a major influence on flowering time (Dubcovsky et al. 2006; Trevaskis et al. 2006). As expected from the deduced *Vrn* locus genotypes of the parents, the Haruna Nijo × Galleon F_2 population segregated for winter/spring growth habit. A total of 77 plants flowered within 33 days (spring types), whereas the remaining 19 plants remained vegetative for the duration of the experiment (39 days; winter types). According to the co-dominant markers tightly linked to *Vrn-H1* and *Vrn-H2* (*WG644* and *SNF*, respectively), all plants that remained vegetative (winter types) were homozygous for the *vrn-H1* winter allele and had either one or two copies of the *Vrn-H2* winter allele, whereas all spring types carried a dominant spring *Vrn-H1* allele or were homozygous for the recessive *vrn-H2* spring allele. Therefore, winter/spring growth habit was determined by segregation at *Vrn-H1* and *Vrn-H2* in this cross, and in a manner which was consistent with the previously described dominance and epistatic interactions between alleles of these two loci (Kóti et al. 2006).

Marker-trait associations were investigated in the 77 spring-type individuals from the Haruna Nijo × Galleon F_2 population (Fig. 2). Date of awn emergence was found to be influenced by the *Vrn-H2* chromosome region and by the *Vrn-H1* locus interval between markers *CK881* and *AJ927* (Fig. 2a). Earlier flowering was associated with the *vrn-H1* winter allele and *vrn-H2* spring allele, both from Haruna Nijo. Flowering time effects were located to two distinct regions on chromosome 2H, with the Galleon alleles associated with early flowering (Fig. 2a). Although *Ppd-H1* effects should be observable under 14 hours day length (Laurie et al. 1994), the peak of the earliness effect near the centromere was located below the *Ppd-H1* gene marker (Fig. 2a), indicating that this effect did not derive from *Ppd-H1*. This is also consistent with the fact that

Haruna Nijo and Galleon both possessed a *Ppd-H1* sequence variant associated with photoperiod sensitive *Ppd-H1* alleles (Table 1). The flowering time effect near the centromere of 2H corresponds to the location of the previously defined *Eps-2S* earliness *per se* locus (Laurie et al. 1995) and was assumed to be the same. The other region influencing flowering time on chromosome 2H was located on the long arm, between markers *ADG* and *DGK* (Fig. 2a). We call this locus *Flowering time-2L* (*Flt-2L*). The *Flt-2L* effect centered around the microsatellite locus *HVM54*, which was the marker Reinheimer et al. (2004) reported to be most highly associated with the frost tolerance QTL on 2HL. Plants homozygous for contrasting alleles at *Vrn-H1*, *Vrn-H2*, *Eps-2S* and *Flt-2L* differed in their average flowering times by 5, 2, 2 and 1.5 days, respectively (Fig. 2a). Among the 77 spring types, the four loci together accounted for 72% of the variation in flowering time, with *Vrn-H1*, *Vrn-H2*, *Eps-2S* and *Flt-2L* accounting for 32, 12, 14 and 14% of the variation, respectively (Table 2).

To characterize *Vrn-H1*- and *Vrn-H2*- linked flowering time effects in spring types in greater detail, we compared the flowering times of the seven spring genotypic classes with respect to the *Vrn-H1* and *Vrn-H2* loci (Fig. 3). Among the spring types, the winter allele at *Vrn-H1* (*vrn-H1* allele from Haruna Nijo) was associated with significantly ($p<0.05$) earlier flowering, both in the *Vrn-H2* heterozygotes (*Vrn-H2*/*vrn-H2* genotypes) and in its absence (*vrn-H2*/*vrn-H2* genotypes). The flowering time effect appeared to be slightly greater in genotypes lacking *Vrn-H2* than in those containing *Vrn-H2* (3.5 to 6 days versus 2 days), indicating a possible interaction between the two chromosome regions.

Chasmogamy was quantitatively scored for each of the 77 spring-type Haruna Nijo × Galleon F_2 plants by measuring the percentage of anthers extruded after anthesis. A significant genetic effect was detected in the *Vrn-H1* region, as well as on the long arm of chromosome 2H, peaking at the *CDO680* and *MSU21* marker loci (Fig. 2b). Chasmogamy was associated with Haruna Nijo alleles in the *Vrn-H1* region and Galleon alleles on 2HL. The 2HL locus controlled approximately twice the amount of variation as the *Vrn-H1* region (Table 2). This locus on 2HL probably corresponds to the *Cly* locus which had previously been mapped to 2HL, 0 to 1.6 cM distal of *MSU21* (Turuspekov et al. 2004, 2005).

Rachis internode length, total spike length and floret number per spike were also measured in the 77 spring-type individuals from the Haruna Nijo × Galleon F_2 population. An effect on rachis

internode length was observed only on 2HL, peaking in the same marker interval as the *Flt-2L* flowering time effect (Fig. 2c). This region accounted for 91% of the variation in the trait (Table 2). Plants homozygous for Haruna Nijo marker alleles in this region had rachis internodes which were 2.2 mm shorter on average than individuals homozygous for the Galleon alleles. Variation in total spike length was influenced by chromosome regions containing *Eps-2S*, *Flt-2L* and *Vrn-H1* loci (Fig. 2d) but the number of florets per spike was influenced by the *Eps-2S* and *Vrn-H1* chromosome regions only (Fig. 2e). Reduced spike length and fewer florets per spike was associated with the early alleles at *Eps-2S* and *Vrn-H1* (from Galleon and Haruna Nijo, respectively), whereas a reduced spike length was associated with the late flowering (Haruna Nijo) allele at *Flt-2L*. The extent to which the different chromosome regions controlled the spike morphology traits are indicated in Table 2.

The Haruna Nijo × Galleon F_4 family 34-2-73 homozygous for *Eps-2S* and *Vrn-H1* but segregating for the *Flt-2L* chromosome region (Fig. 1) was used for further characterization of flowering time controlled by *Flt-2L*. Individuals homozygous for the Galleon *Flt-2L* allele flowered significantly earlier (by an average of 3.5 days) than those homozygous for the Haruna Nijo allele (Fig. 4a), confirming the presence of a *Flt-2L* flowering time effect independent of *Eps-2S*. *Flt-2L* heterozygotes exhibited a heading date between that of the two homozygous classes (Fig. 4a), suggesting an incompletely dominant mode of inheritance.

Characterization of *Vrn-H1*-linked, *Eps-2S* and *Flt-2L* flowering time effects in the Amagi Nijo × WI2585 cross

In the Amagi Nijo × WI2585 F_1-derived doubled-haploid population, QTL for development (plant height and decimal growth stage of the primary tiller) were detected in the vicinity of the LTR QTL on the long arm of chromosome 5HL (Reinheimer et al. 2004). To characterize this developmental effect in terms of flowering time, the doubled-haploid population of 139 lines was grown in a greenhouse under natural lighting during winter and early spring and scored for heading date. A QTL accounting for 59% of the variation in heading date was detected in this chromosome region. The RFLP marker *MWG514*, located under the peak of the previously described developmental QTL on 5HL, was most closely associated with the effect. On average, lines carrying the Amagi Nijo allele flowered 4.7 weeks before those carrying the WI2585 marker alleles (Fig. S2). Although the major variation for flowering time in the Amagi Nijo ×

WI2585 doubled-haploid population had been proposed to be due to vernalization requirement (Reinheimer et al. 2004), this has since been found to be untrue (J. Reinheimer, unpublished data). All Amagi Nijo × WI2585 F_1-derived doubled haploids were spring type because both parents carried *vrn-H2* spring alleles, and no flowering time variation was observed at the *Vrn-H2* locus.

To investigate the effects of chromosome 2H loci *Flt-2L* and *Eps-2S* on flowering time in the Amagi Nijo × WI2585 cross, we used families 103-1-2-114 and 110-4-34, which were homozygous or segregating for different segments of chromosome 2H, and homozygous for WI2585 marker alleles in the *Vrn-H1* region on chromosome 5H (Fig. 1). In the 110-4-34 F_6 family which was homozygous for the *Flt-2L* region and the *Ppd-H1* locus, a flowering time effect on chromosome 2H peaked at the *IRP* marker locus (Fig. S1). This was consistent with the previous report of *Eps-2S* segregation in an Amagi Nijo × WI2585 cross (Reinheimer et al. 2004). The association plot (Fig. S1) indicated that *Eps-2S* was located between the *HYP26* marker and the *Ppd-H1* gene. In the 103-1-2-114 F_7 family homozygous for the *Eps-2S* region, a flowering time effect was observed in the *Flt-2L* interval (not shown) as defined using the Haruna Nijo × Galleon F_2 population, indicating that *Flt-2L* was also segregating in the Amagi Nijo × WI2585 cross and was distinct from *Eps-2S*. Flowering time at both *Eps-2S* and *Flt-2L* was expressed in an incompletely dominant manner, with the WI2585 allele conferring earliness (Figs. 4b and 4c).

Discussion

The current and previous (Reinheimer et al. 2004) studies have together identified flowering time effects linked to both the 5H and 2H LTR tolerance loci. In the field grown Galleon × Haruna Nijo and Amagi Nijo × WI2585 doubled haploid populations used for detection of these LTR tolerance QTL, Reinheimer et al. (2004) detected no flowering time effects at *Flt-2L*, located in the vicinity of the 2H QTL. *Flt-2L* may have gone undetected in the previous study due to its relatively small effect compared to other heading time loci segregating in these crosses (Figs. 2b, 4b and 4c) and/or because the conditions in the field may not have favored its expression. Future experiments will examine these loci under different conditions. The LTR tolerance QTL were unlikely to have arisen by escape, as they were detected using sterility data collected from spikes which had been at a narrow developmental stage at the time of the frost event (Reinheimer et al. 2004). Therefore, it seems likely that the QTL on chromosomes 2H and 5H control genuine LTR tolerance, despite their associations with heading time.

Freezing tolerance at developmental stages prior to flowering (LTV tolerance) has been relatively well studied in cereals, and offers insights in to how development may affect the expression of genuine tolerance. LTV tolerance in cereals increases after a period of exposure to cold but non-freezing temperatures ('cold-acclimation'). The potential to cold-acclimate accumulates during the vegetative phase and may diminish after the transition of the apical meristem to a reproductive mode of development (Prášil et al. 2004; Limin and Fowler 2006). Furthermore, LTV tolerance controlled by the *Fr-1* locus has been reported to occur tightly linked in coupling phase with winter *vrn-1* alleles (Hayes et al. 1993; Galiba et al. 1995; Francia et al. 2004). Consequently, it has been proposed that *Fr-1* LTV tolerance may be a direct result of the ability of *vrn-1* winter alleles to delay the transition to flowering and thereby increase the opportunity to acquire acclimation potential (Limin and Fowler, 2006). A similar mechanism may account for observations that environmental cues that delay flowering initiation also increase the potential of cereals to cold-acclimate (Limin and Fowler 2006; Mahfoozi et al. 2006; Limin et al. 2007). On the other hand, the *Fr-2* LTV tolerance locus has not been associated with any difference in flowering time, and therefore appears to operate independent of development.

LTR tolerance was associated with late flowering at the 2H locus. However, although LTR tolerance at the 5H locus was associated with *vrn-H1* winter alleles in both populations, these

winter alleles were associated with early flowering in both in the Amagi Nijo × WI2585 mapping population which was completely spring type, and in spring lines in the Haruna Nijo × Galleon population which was segregating for winter/spring growth habit. Re-examination of the Haruna Nijo × Galleon field data of Reinheimer et al. (2004) revealed significantly lower frost induced sterility associated with the winter *vrn-H1* allele in both spring-spring (p= 0.11) and spring-winter (p=0.04) comparisons (Fig. S3). Hence, LTR tolerance was not exclusively associated with late flowering, suggesting that mechanisms of LTR tolerance differ from those proposed to control LTV tolerance involving an extended vegetative growth phase. In support of this idea, wheat ears have been reported to be capable of expressing a cold-acclimation response at an early stage of development, but not at a later stage, following emergence from the boot (Single 1966; Single and Marcellos 1974; Fuller et al. 2007), suggesting that residual LTV (acclimatable) tolerance mechanisms in cereals may be completely lost by the time anthesis occurs.

In the determination of winter/spring growth habit by the *Vrn-H1* and *Vrn-H2* loci, only plants that are homozygous for the *vrn-H1* winter allele and which carry one or more *Vrn-H2* winter alleles are winter type (Kóti et al. 2006; Fig. 3). Under long days and in the absence of vernalization, winter types flower later than spring types (by up to 3 months). In this context, winter *vrn-H1* alleles confer late flowering, and are dependent on the presence of winter *Vrn-H2* alleles for this action. The association of *vrn-H1* winter alleles with earlier flowering in spring progeny of the Amagi Nijo × WI2585 and Haruna Nijo × Galleon crosses, either in the presence or absence of *Vrn-H2* winter alleles (Figs. 3 and S2), contrasts with the control of winter/spring growth habit by alleles of *Vrn-H1*, in relation to both the direction of the effect and the dependence on *Vrn-H2*. Thus, our study either reveals an allelic variant of *Vrn-H1* gene promoting early flowering in spring backgrounds, or another flowering time gene closely linked to *Vrn-H1*. The flowering time effects linked to *Vrn-H1* in spring progeny of the Galleon × Haruna Nijo and Amagi Nijo × WI2585 crosses differed in magnitude (4.7 weeks versus 1.5 to 5.5 days, respectively). However, the effect in the Amagi Nijo × WI2585 cross was smaller in other experiments (4.9 to 11.4 days; unpublished data), indicating a possible dependency on growth conditions.

A candidate for another flowering time gene closely linked to *Vrn-H1* is *PhyC* (encoding Phytochrome C). This gene has been mapped 0.5 cM distal of *Vrn-H1* in barley (Szűcs et al. 2006), and has a homologue that controls light dependent development (including flowering

time) in Arabidopsis (Franklin et al. 2003; Monte et al. 2003). Alternatively, the flowering time variation observed in spring lines may be due to the *Vrn-H1* gene itself. In cereals, *Vrn-1* expression is essential for flowering and its expression level can positively influence the rate of flowering (Loukoianov et al. 2005; Shitsukawa et al. 2007). In winter genotypes (*vrn-1*/*vrn-1*; *Vrn-2*/-), expression of *vrn-1* winter alleles is kept low early in development due to suppression by *Vrn-2*, which occurs through a regulatory domain which is present in *vrn-1* winter alleles but absent from *Vrn-1* spring alleles (Yan et al. 2003; Trevaskis et al. 2006). However, in genotypes also containing spring *Vrn-1* gene copies, *vrn-1* winter alleles may be transcribed at high levels early in development due to the ability of *Vrn-1* spring allele to suppress *Vrn-2* expression (Loukoianov et al. 2005; Trevaskis et al. 2006). Therefore, both alleles of *Vrn-H1* present in the *Vrn-H1*/*vrn-H1* heterozygous Haruna Nijo × Galleon F_2 plants may have been transcribed at high levels, independent of *Vrn-H2* genotype. Similarly, the *vrn-H1* winter allele should have expressed at high levels in the Amagi Nijo × WI2585 DH lines due to the absence of *Vrn-H2* in these lines. The earliness associated winter *vrn-H1* alleles from Haruna Nijo and Amagi Nijo may arise from a higher expression of this allele than the Galleon and WI2585 spring *Vrn-H1* alleles (e.g. due to a more active promoter), once repression by *Vrn-H2* is eliminated. In the Haruna Nijo × Galleon F_2 population, *Vrn-H1*/*vrn-H1* genotypes lacking *Vrn-H2* (*vrn-H2*/*vrn-H2* genotypes) flowered significantly earlier than those containing a functional *Vrn-H2* copy (by 2 days; Fig. 3) suggesting that *Vrn-H2* may have still partially limited expression of the Haruna Nijo *vrn-H1* allele in the *Vrn-H1*/*vrn-H1* heterozygotes. Further work could be carried out to test these possibilities. This could include further fine mapping, expression analysis of *Vrn1* (*HvBM5A*) and *Vrn2* (*ZCCT*) alleles from the various gentoypes by northern blot or qRT-PCR, and plant transformation with various *Vrn1* or *Vrn2* alleles or promoter-reporter constructs.

Variation for the chasmogamy/cleistogamy (open/closed floret) trait in barley is controlled by a major locus (*Cly*) that has been mapped 0 to 1.6 cM distal of the marker *MSU21* on chromosome 2HL (Turuspekov et al. 2004, 2005). Chasmogamy determined by this locus is characterized by a swelling of the lodicules, which pushes the palea and the lemma apart and allows the anthers to extrude from the florets at anthesis (Honda et al. 2005). A large proportion (36.9%) of the segregation for anther extrusion in the spring type Haruna Nijo × Galleon F_2 plants mapped at a position corresponding to *Cly*. However, we also found some (21.8%) variation for this trait to be controlled by a locus in the *Vrn-H1* region on chromosome 5H (Fig. 2b), indicating that chasmogamy/cleistogamy may be a pleiotropic effect of the *Vrn-H1* locus. Closed florets

(cleistogamy) were associated with frost tolerance alleles at *Flt-2L* but frost susceptibility at *Vrn-H1*. Furthermore, there was no noticeable anther extrusion from any of the Amagi Nijo × WI2585 derived lines or the parents of this cross (not shown). Therefore, cleistogamy appeared to be an unlikely underlying mechanism for the LTR tolerance controlled by the 5H and 2H loci.

In the Haruna Nijo × Galleon F_2 population, early flowering alleles at *Flt-2L* were associated with longer spikes as a result of longer rachis internodes, whereas early flowering alleles at *Eps-2S* and *Vrn-H1* were associated with shorter spikes because there were fewer florets (internodes) per spike (Fig. 2c, 2d and 2e), suggesting that the *Flt-2L* locus controls plant architecture in a different way to the *Eps-2S* and the *Vrn-H1* chromosome regions. At *Flt-2L*, the compact rachis allele from Haruna Nijo was the allele associated with cleistogamy. Cleistogamy from Japanese genotypes was found in two other crosses to be tightly linked in coupling with short rachis internodes (Honda et al. 2003; Turuspekov et al. 2005). However, while the Amagi Nijo × WI2585 cross segregated for rachis internode length at *Flt-2L* (data now shown), it did not segregate for chasmogamy/cleistogamy, suggesting that a compact spike may not be a pleiotropic effect of the *Cly* locus and that it could be encoded for by a separate closely linked gene. A locus controlling rachis internode length has been observed in this chromosome region before (Sameri et al. 2006), in the Galleon × Haruna Nijo doubled-haploid population (*Cr* locus; Karakousis et al. 2006) and in another population for which Haruna Nijo was a parent (Hori et al. 2005). The locus may be the same as *Zeocriton-1* (*Zeo-1*), which has a dense spike mutant phenotype (Lundqvist and Lundqvist 1998) and a map location 2.3 cM distal of the marker *MSU21* (Costa et al. 2001). A heading time QTL has also been observed in barley near the marker *HVM54* (von Korff et al. 2006). In wheat, spikes become much more prone to freezing once they emerge from the boot and become exposed to external nucleating factors (Single 1985). Therefore, traits such as spike length could conceivably impact on LTR tolerance if they alter when spikes emerge from the boot. At both the 2H and 5H loci, tolerance was associated with a shorter spike (Fig. 2d). However, *Eps-2S* influences spike length almost to the same degree as these other loci (Figure 2d; Table 2), yet has not been identified as a LTR tolerance locus, indicating that spike length *per se* is unlikely to be an underlying mechanism for LTR tolerance.

The failure to link any trait consistently with LTR tolerance suggests that the tolerance derives from a developmental trait not yet examined, or from an internal characteristic (e.g. difference in intrinsic ice nucleation). The knowledge of the traits segregating in the Haruna Nijo × Galleon

and Amagi Nijo × WI2585 crosses is allowing us to develop genetic materials more suited to fine-mapping and evaluation of the LTR tolerance loci. We have also developed protocols to detect LTR tolerance using a frost simulation chamber. Papers describing these additional studies are in preparation. This work is part of a broader effort aimed at the eventual cloning of the genes controlling LTR tolerance and elucidation of the mechanisms underlying the tolerance.

Since LTR tolerance on chromosome 2H is tightly linked to a locus controlling major variation for rachis internode length in both of the crosses studied, it should be possible to use short rachis internode length to select for the 2H LTR tolerance allele in breeding populations. Likewise, segregation for a vernalization requirement and/or flowering time in spring backgrounds might allow identification of families carrying the LTR tolerance allele on 5H in spring barley breeding programs.

Acknowledgement

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Figure legends

Fig. 1.

Genotypes of families used in the analyses, for chromosome 2H, and for *Vrn-H1* and *Vrn-H2* regions on 5HL and 4HL chromosome arms, respectively. Markers joined by dotted lines were used to determine the genotype of the parent plant of each family, while black boxes indicate markers used to test for marker-trait associations in segregating progeny families. Shading indicates chromosome regions of different genotype. AN, WI, HN, G = homozygous Amagi Nijo, WI2585, Haruna Nijo and Galleon, respectively; Seg = segregating. Alleles of *Ppd-H1* refer to the *Ppd-H1* PCR marker, which was based on a polymorphism present at a site distinct from the putative *Ppd-H1* functional polymorphic site (the latter having the same sequence across all four parents). The maps in the center are consensus maps made using data obtained from all families. The two LTR tolerance loci (*Fr*) had previously been mapped by Reinheimer et al. (2004) in Galleon × Haruna Nijo and Amagi Nijo × WI2585 F_1-derived doubled haploid populations, and were located relative to the illustrated CAPS markers by scoring CAPS markers in the two original QTL mapping populations. The 2H centromere position was defined previously (Chen et al. 2008). *Ppd-H1* was scored using a marker derived from the cloned gene (Turner et al. 2005). *Vrn-H1* and *Vrn-H2* positions are inferred from their locations relative to markers in *T. monococcum* (Yan et al. 2003; Yan et al. 2004). *Eps-2S* and *Flt-2L* flowering time loci were mapped in the current study.

Fig. 2.

Associations of markers with flowering time, anther extrusion, rachis internode length, total spike length and floret number per spike, in 77 spring type Haruna Nijo × Galleon F_2 plants. Plots show trait values (means +/- S.E.) for individuals homozygous for alternate alleles for molecular markers located along chromosome 2H and in the vicinity of the *Vrn-H1* and *Vrn-H2* loci on 5HL and 4HL. Thin dotted lines indicate probabilities of association (1 – p-value) as determined by one-way ANOVA, with the 0.95% confidence level indicated by a thick vertical dotted line. Maps to the left indicate locus positions applying to all figures. The interval between markers *SNF* and *NUC* is widened for clarity.

Fig. 3.

Flowering time (means +/- S.E.) of non-vernalized Haruna Nijo × Galleon F_2 plants carrying different allele combinations at *Vrn-H1* and *Vrn-H2* loci. Genotypes at the *Vrn-H1* and *Vrn-H2* loci were deduced using the *WG644* and *SNF* markers, respectively. The winter genotype combinations (*vrn-H1*/*vrn-H1*; *Vrn-H2*/-) did not flower for the duration of the experiment. Values that were not significantly different ($p>0.05$) are marked with the same letter code. Numbers of plants of each genotype (n) are indicated below the chart.

Fig. 4.

Control of flowering time by *Eps-2S* and *Flt-2L* loci on chromosome 2H, in F_4, F_6 and F_7 families. Days to awn emergence (means +/- S.E.) is shown for different genotypes in each family. *Flt-2L* but not *Eps-2S* was segregating in families 34-2-73 (a) and 103-1-2-114 (c), whereas *Eps-2S* but not *Flt-2L* was segregating in family 110-4-34 (b). Locus genotypes were defined using closely linked markers. In each chart, values that were not significantly different ($p>0.05$) are indicated by the same letter code. Numbers of plants of each genotype (n) are indicated below the charts.

Tables and figures

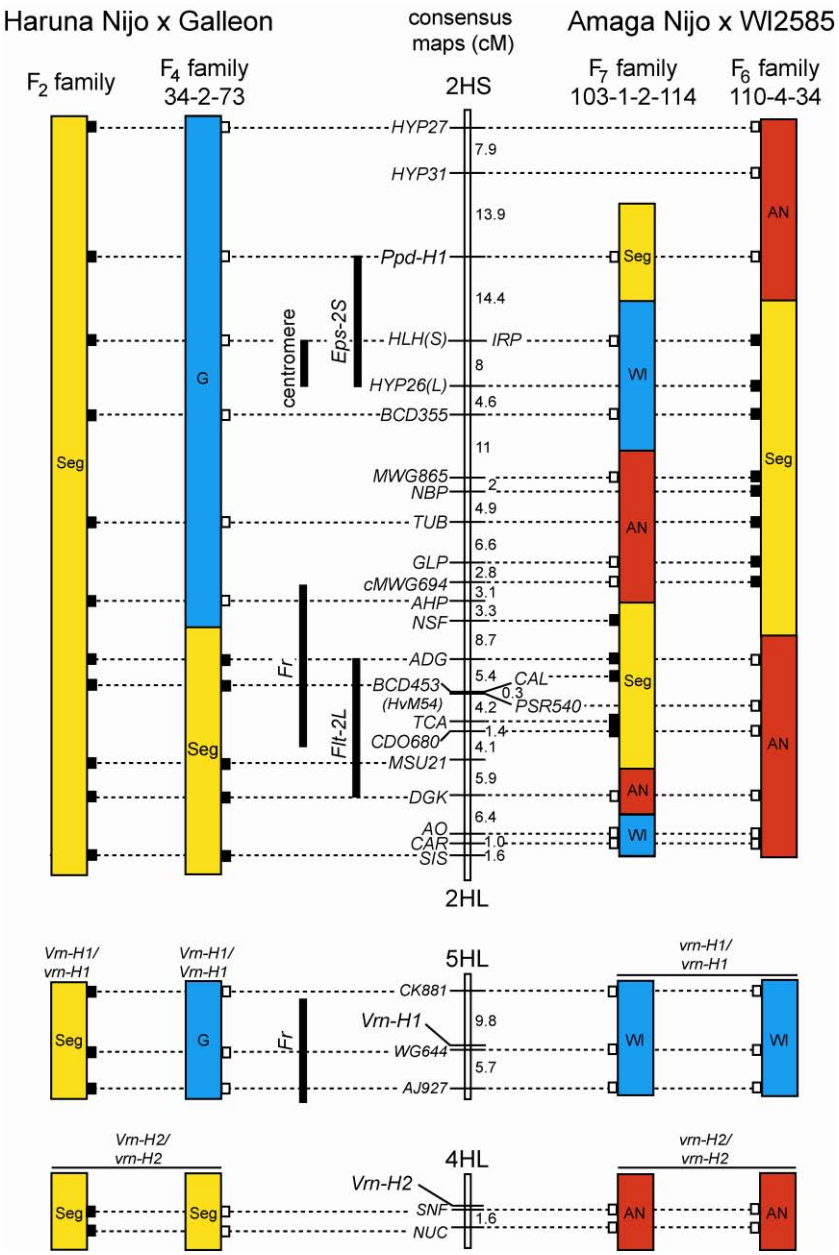


Fig. 1.

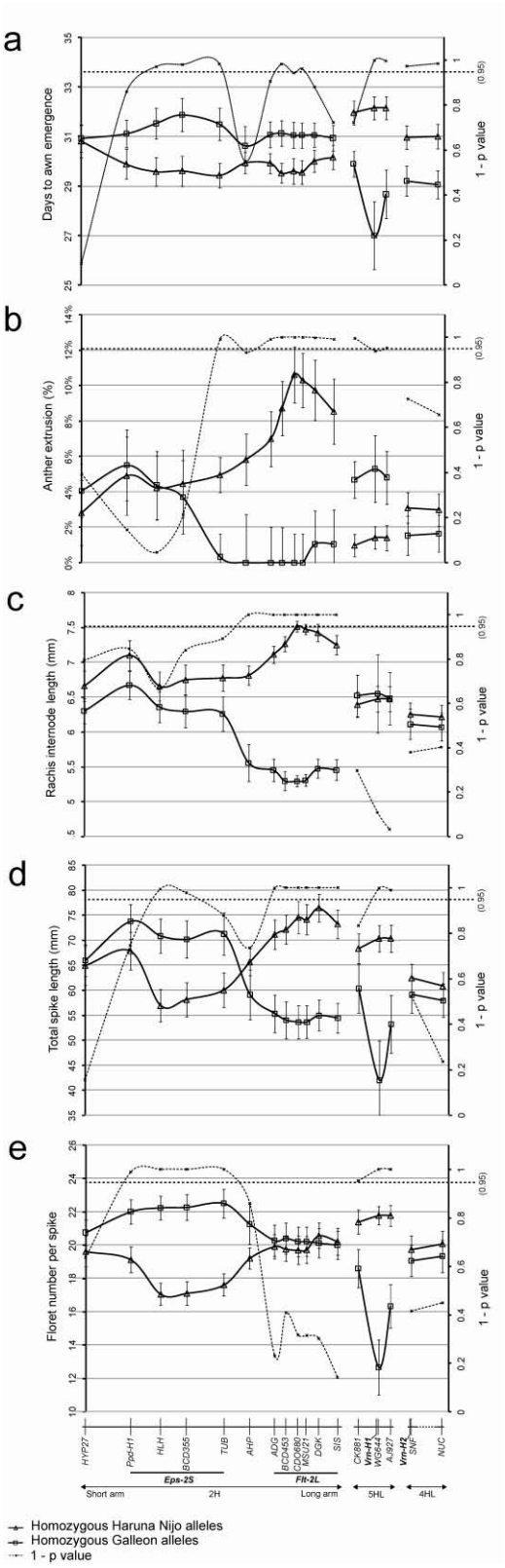

a
Days to awn emergence
1 - p value
(0.95)
b
Anther extrusion (%)
1 - p value
(0.95)
c
Rachis internode length (mm)
1 - p value
(0.95)
d
Total spike length (mm)
1 - p value
(0.95)
e
Floret number per spike
1 - p value
(0.95)
HVP27
Ppd-H1
HLH
BCD355
TUB
AHP
ADG
BCD453
CDO680
MSU21
DGK
SIS
CK881
Vrn-H1
WG644
AJ927
Vrn-H2
SNP
NUC
Eps-2S
Flt-2L
Short arm
2H
Long arm
5HL
4HL
Homozygous Haruna Nijo alleles
Homozygous Galleon alleles
1 - p value

Fig. 2.

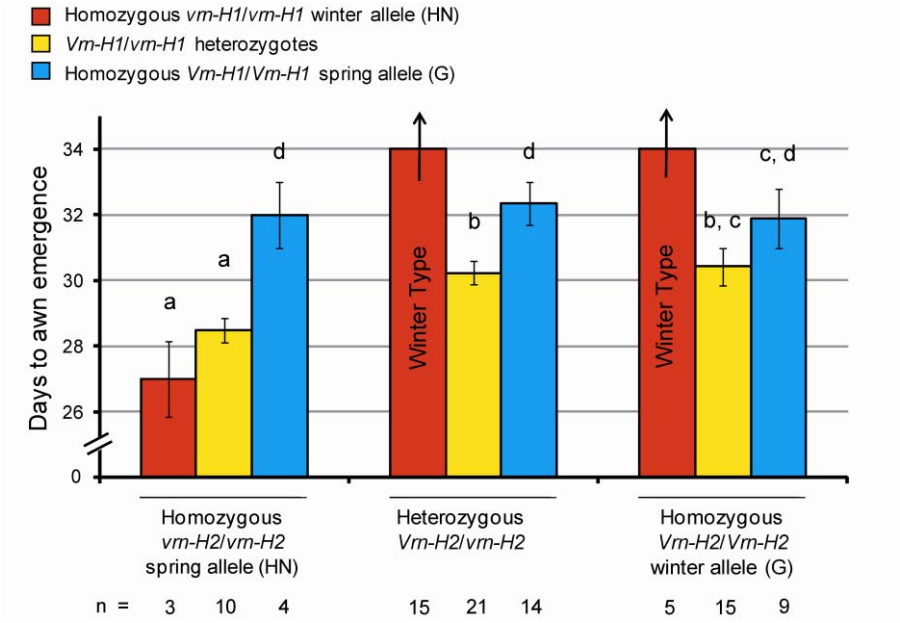

Homozygous vm-H1/vm-H1 winter allele (HN)
Vm-H1/vm-H1 heterozygotes
Homozygous Vm-H1/Vm-H1 spring allele (G)
Days to awn emergence
34
32
30
28
26
0
a
a
d
Winter Type
b
d
Winter Type
b, c
c, d
Homozygous
vm-H2/vm-H2
spring allele (HN)
Heterozygous
Vm-H2/vm-H2
Homozygous
Vm-H2/Vm-H2
winter allele (G)
n = 3 10 4 15 21 14 5 15 9

Fig. 3.

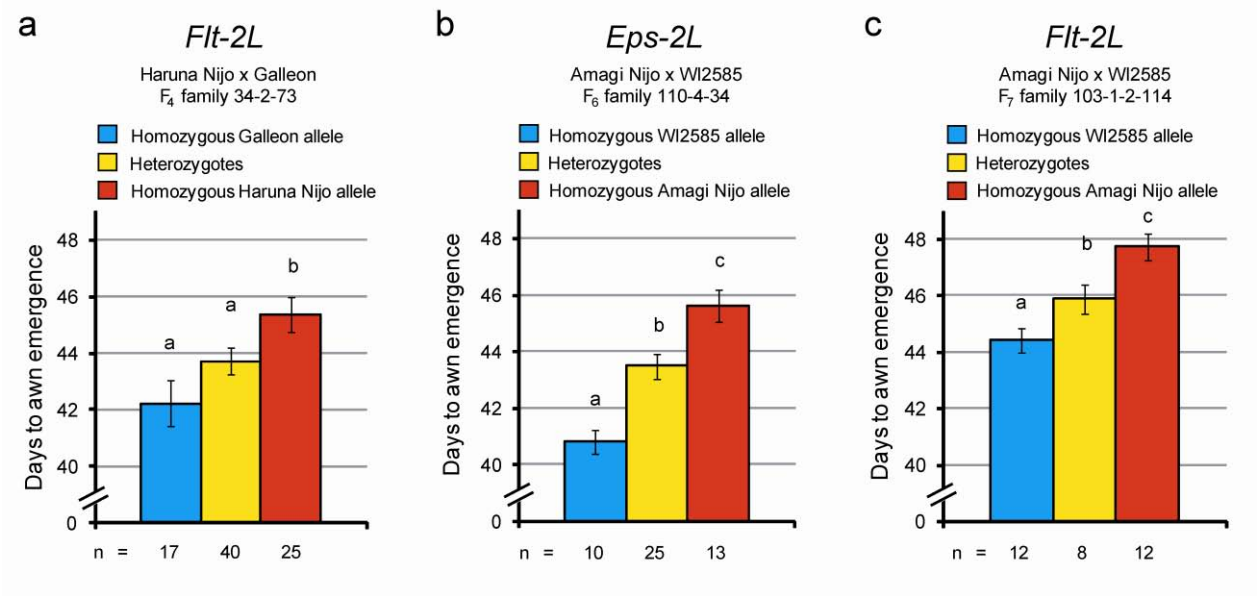

a
Flt-2L
Haruna Nijo x Galleon
F4 family 34-2-73
Homozygous Galleon allele
Heterozygotes
Homozygous Haruna Nijo allele
Days to awn emergence
48
46
44
42
40
0
a
a
b
n = 17 40 25
b
Eps-2L
Amagi Nijo x WI2585
F6 family 110-4-34
Homozygous WI2585 allele
Heterozygotes
Homozygous Amagi Nijo allele
Days to awn emergence
48
46
44
42
40
0
a
b
c
n = 10 25 13
c
Flt-2L
Amagi Nijo x WI2585
F7 family 103-1-2-114
Homozygous WI2585 allele
Heterozygotes
Homozygous Amagi Nijo allele
Days to awn emergence
48
46
44
42
40
0
a
b
c
n = 12 8 12

Fig. 4.

Table 1 Status of mapping parents for major developmental loci *Vrn-H1*, *Vrn-H2* and *Ppd-H1*

Genotype	*Vrn-H1* locus		*Vrn-H1* locus		*Ppd-H1* locus		Growth habit
	BM5A gene intron-1 variant	allele	*ZCCT.Hc* gene present or absent	allele	G or T at site 23 in *PPR* gene	allele	
Galleon	5.2 kb deletion	*Vrn-H1*	present	*Vrn-H2*	G	*Ppd-H1*	Spring; photoperiod sensitive
Haruna Nijo	non-deleted	*vrn-H1*	absent	*vrn-H2*	G	*Ppd-H1*	Spring; photoperiod sensitive
WI2585	5.2 kb deletion	*Vrn-H1*	absent	*vrn-H2*	G	*Ppd-H1*	Spring; photoperiod sensitive
Amagi Nijo	non-deleted	*vrn-H1*	absent	*vrn-H2*	G	*Ppd-H1*	Spring; photoperiod sensitive

Table 2 Percent variation in flowering time and head traits controlled by individual loci segregating in the 77 spring type plants from the Haruna Nijo × Galleon F_2 population

	Eps-2S[a]	*Flt-2L*[b]	*Vrn-H1*[c]	*Vrn-H2*[d]
Awn emergence	13.5%	14.2%	31.5%	12.2%
Chasmogamy	n.s.	36.9%	21.8%	n.s.
Rachis internode length	n.s.	91.8%	n.s.	n.s.
Total spike length	20.3%	35.7%	29.3%	n.s.
Floret number per spike	45.4%	n.s.	49.4%	n.s.

[a, b, c, d] genotypes inferred using markers *HLH, MSU21, WG644, NUC*, respectively; only homozygotes used.

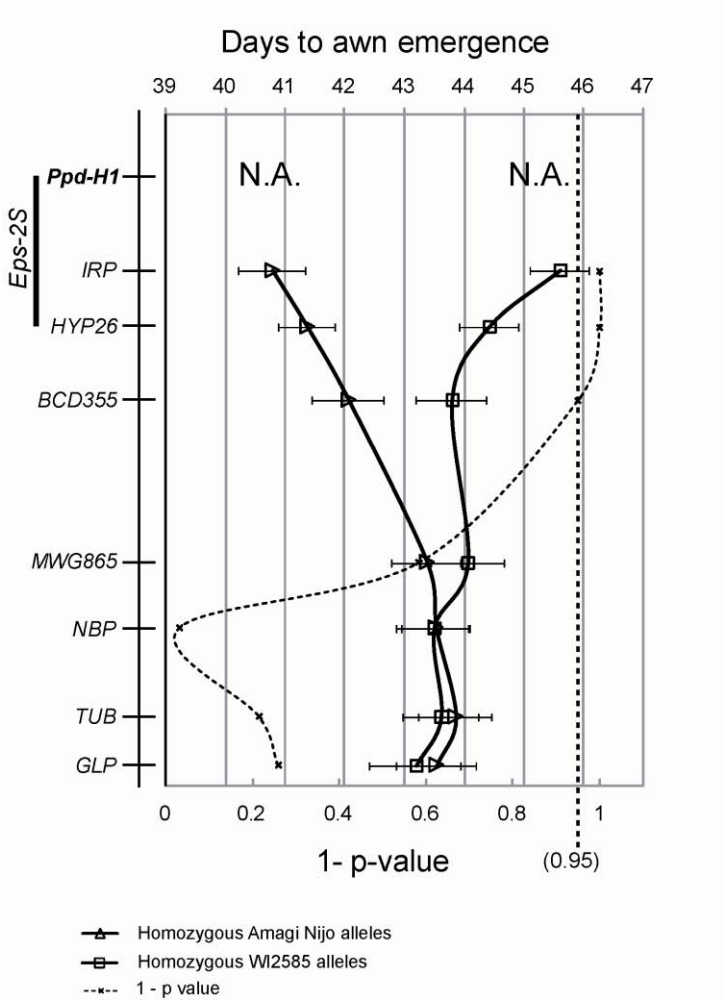
n.s. = no significant association found.

ESM Table 1 Primers, sequences and PCR conditions for markers linked to the *Vrn-H1* and *Vrn-H2* loci

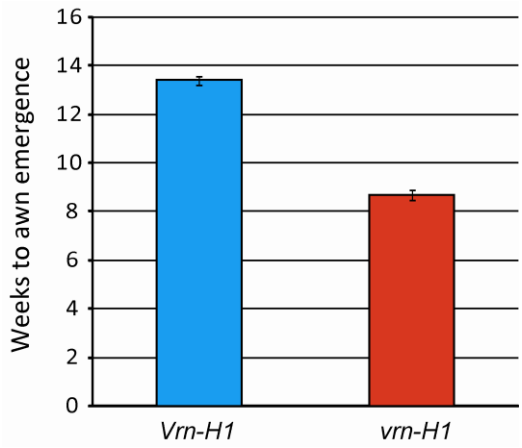
Marker Names	Marker Primers (5' to 3')	Original Primers(5' to 3')[a]	Sequence length (bp)[b]	PCR cycle[c,d]	Restriction Enzymes	Genbank Accession
CK881	F-CK881-3-CACCGTATTCATATTCATCCTCCAC R-CK881-4-CAGATGGTATCCATACTGTAGTCCG	F-CK881-1-CATCAAGTAAGTGAAGTTCCCTATAG R-CK881-2-GAATGAAAGGCAAGCACGAAGAAGC	461 [1350]	2 [4[d]]	*Hin*cII	WI (EU835503) G (EU835500) AN, HN (EU835501, EU835502)
WG644	F-WG644-5-ACACCATTTACATGCTTTGCACCTC R-WG644-6-CAAAGAACTTAATGGATGTGTCCCC	F-WG644-3-TATTATCTTTTGCTGCAGATATCAG R-WG644-4-CAAGACTTTCCCCAAGCACGAGACC	229 [927]	2 [3]	*Msp*I	AN, HN (EU835510) WI, G (EU835511)
AJ927	F-AJ927-3-CATGTACTCTAGATAACCATGCTTG R-AJ927-2-CTTCACCTTGCGCCGATGGCCGAAG	F-AJ927-1-GGATCAGTACCGACAAGACGGCATTC R-AJ927-2-CTTCACCTTGCGCCGATGGCCGAAG	570 [900]	2 [4[d]]	*Hae*III	AN (EU835497) G (EU835498) WI, HN (EU835499)
SNF	F-SNF-5-CCAACACCTTCAGAGCCAC R-SNF-6-TTACCGAACTCCGACAACCAG	F-SNF-3-CCAGCTCGACGGCGTCCACTGGCTC R-SNF-4-AAGTGCATTAACGCCCATAGTTCAG	307 [1153]	1 [4]	*Sau*96I	G (EU835508) AN, HN, WI (EU835509)
NUC	F-NUC-5-GTTCATTCCAAGATGGTTCTG R-NUC-6-GTAGTCCTCTAGTGCAATC	F-NUC-3-TCTTATCGGCTGCTTGTCAATGAAC R-NUC-4-GATCATCGGTCTCCTCCTGCTCCTG	388 [1200]	1 [4]	*Ban*I	WI (EU835504) G (EU835505) AN, HN (EU835506, EU835507)

All PCR programs are preceded by a 95ºC incubation for 7 mins, and followed by an incubation at 68ºC for 10 mins.
Programs:
1) 45 cycles of 10 s @ 94ºC, 30 s @ 55ºC, 50 s @ 68ºC.
2) 45 cycles of 10 s @ 94ºC, 30 s @ 60ºC, 50 s @ 68ºC.
3) 40 cycles of 10 s @ 94ºC, 30 s @ 60ºC, 1m 20 s @ 68ºC.
4) 35 cycles of 10 s @ 94ºC, 30 s @ 60ºC, 2m @ 68ºC.

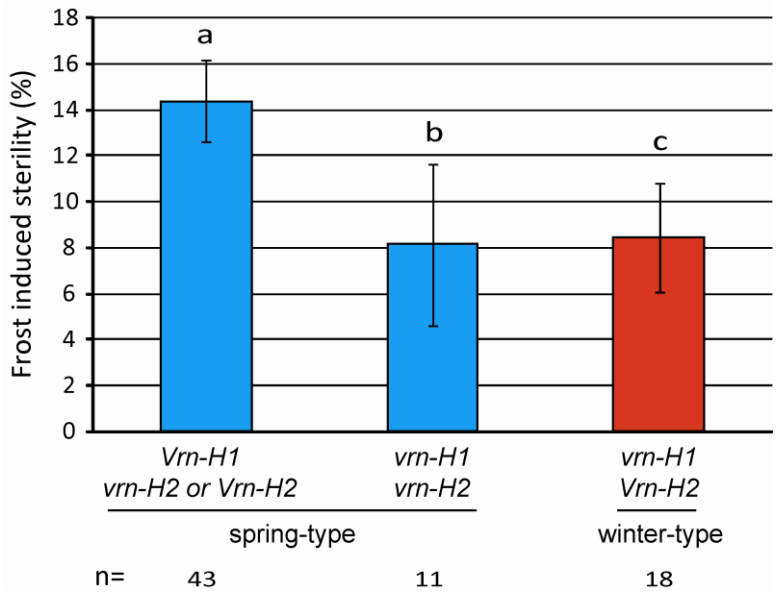
a Primers originally used in polymorphism screen.
b Fragments used in original polymorphism screen in square brackets
c PCR cycle for original primers in square brackets.
d PCR reactions contained 5% v/v DMSO.


Days to awn emergence
39
40
41
42
43
44
45
46
47
Ppd-H1
Eps-2S
IRP
HYP26
BCD355
MWG865
NBP
TUB
GLP
N.A.
N.A.
0
0.2
0.4
0.6
0.8
1
1- p-value
(0.95)
Homozygous Amagi Nijo alleles
Homozygous WI2585 alleles
1 - p value

ESM Fig. 1.



ESM Fig. 2.



ESM Fig. 3.

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4 *Flt-2L*, a locus in barley controlling flowering time, spike density and plant height.

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Statement of contribution

Designed the experiment, conducted the research, analyzed the data, drafted the manuscript, acted as a corresponding author

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Participated in the supervision of the work and contributed to the final manuscript

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Abstract

Flowering time represents an important adaptive trait for temperate cereal crops, and may also impact on frost damage in cereal reproductive tissues by enabling escape or by influencing accumulation of genuine tolerance. The *Flt-2L* flowering time QTL on the distal end of barley chromosome arm 2HL overlaps with QTL for rachis internode length and reproductive frost damage. *Flt-2L* was also found to be associated with plant height. By combining marker analysis with phenotyping in progeny families of selected Amagi Nijo × WI2585 F_6 recombinants, we were able to map quantitative flowering time, rachis internode length and plant height effects on 2HL as discrete Mendelian traits. The three developmental characters showed co-dominant modes of expression and perfectly co-segregated with one another in a 1.3 cM marker interval, indicating control by the same gene or closely linked genes. Twelve genes were identified in the related intervals in the rice and *Brachypodium distachyon* genomes. The *HvAP2* gene co-segregated with *Flt-2L* and represents a plausible candidate for *Flt-2L*, since it is highly similar to the wheat domestication gene *Q* which has similar developmental effects. These data will contribute to isolation of the *Flt-2L* gene(s) and help establish the basis of the frost damage QTL.

Key words

Hordeum vulgare L. – Co-linearity – Flowering time – Spike density – Plant height – Fine-mapping

Introduction

Variation for flowering time has been crucial in enabling the temperate cereals wheat and barley to be cultivated well beyond the range of their wild progenitors (Snape et al. 2001; Laurie et al. 2004; Cockram et al. 2007). To obtain optimal yields in its growth environment, a variety should make maximal use of favorable growth conditions available during the season. However, flowering should be late enough to avoid frequent exposure of the sensitive reproductive organs to freezing temperatures early in the season, but not so late as to expose the crop to damaging drought conditions and high temperatures during anthesis and grain fill (Tewolde et al. 2006; Rebbeck et al. 2007). Genes controlling major variation in flowering time in cereals orchestrate developmental responses to cold (vernalization), photoperiod and temperature (Cockram et al. 2007). Other loci which appear to control heading time independently of any environmental stimulus have been identified, and are referred to as earliness *per se* loci (Cockram et al. 2007).

Studies of the model dicotyledon species *Arabidopsis thaliana* has produced our most sophisticated view of flowering time control in a plant, involving complex interactions between various genes and regulatory pathways (reviewed by Bäurle and Dean, 2006). Recently, a number of genes responsible for major variation in the flowering response of cultivated cereals to vernalization or photoperiod have been cloned, revealing similarities to genes and processes controlling flowering time in *Arabidopsis* (Cockram et al. 2007; Trevaskis et al. 2007; Xue et al. 2008). Major flowering time genes cloned in wheat and barley include *Vrn1* and *Vrn2*, encoding an APETALA1-like MADS box transcription factor and a CCT-domain/B-box protein, respectively (Yan et al. 2003; Yan et al. 2004; Fu et al. 2005; von Zitzewitz et al. 2005), *Vrn3* encoding a FLOWERING LOCUS T -like protein (Yan et al. 2006), and *Ppd-H1* encoding a pseudo-response regulator (Turner et al. 2005). The *Vgt1* flowering time gene from maize encodes an AP2/ERF transcription factor closely related to *Arabidopsis* APETALA2, and has not been reported to mediate any vernalization or photoperiod response (Vlădutu et al. 1999; Salvi et al. 2007). Genetic analysis showed that the *Eps-A^{m}1* locus in *Triticum monococcum* controls heading time in a manner which was strongly dependent on growth temperature (Bullrich et al. 2002; Appendino and Slafer 2003).

In cereal crops, frost at heading time can cause floret sterility and quality downgrading of the grain, resulting in significant losses (Single 1985). Efforts to improve the frost tolerance of

cereals at the flowering stage have been largely unsuccessful, owing to various difficulties of working with this trait (Fuller et al. 2007). Variation in heading time represents one such hindrance, because spikes at different growth stages differ in their frost susceptibility, requiring the use of multiple seeding dates and scoring of heads which were at specific developmental stages at the time of frost exposure (Single 1985; Reinheimer et al. 2004). Studies of cold tolerance in vegetative tissues suggests that flowering time may also influence genuine frost tolerance, by altering the degree to which the plant can cold-acclimate (Limin and Fowler 2006; Mahfoozi et al. 2006; Limin et al. 2007). Two QTL controlling frost damage at flowering were identified in barley, on chromosome 5H, close to the vernalization locus *Vrn-H1*, and distal on the long arm of chromosome 2H (Reinheimer et al. 2004). In the Galleon × Haruna Nijo and Amagi Nijo × WI2585 mapping populations, the QTL were genetically associated with flowering time variation (Reinheimer et al. 2004; Chen et al. 2009c), raising the possibility that these QTL were the result of escape or genuine frost tolerance controlled by the heading time loci themselves.

We named the flowering time QTL on 2HL *Flowering time -2L* (*Flt-2L*), and found that it overlapped with a QTL for rachis internode length (Chen et al. 2009c). In the current study, we perform a more detailed analysis of the phenotypes, revealing that this region also controls plant height. Furthermore, we employ a strategy that allows mapping of these three phenotypes as single locus (Mendelian) factors in order to investigate the linkage between them. This information is assisting us in investigations to determine the true nature of the frost damage QTL on 2HL, and additionally, represents a step towards isolating the developmental genes at *Flt-2L* by a map-based cloning approach.

Materials and methods

Plant Material

In the Amagi Nijo × WI2585 cross, flowering time segregates at the *Eps-2S* locus positioned near the centromere of chromosome 2H, the *Flt-2L* locus distal on the long arm of chromosome 2H, and a locus linked to *Vrn-H1* on 5HL (Reinheimer et al. 2004; Chen et al. 2009c). By using molecular marker selection and several rounds of inbreeding, we produced a *Flt-2L* mapping population in which *Eps-2S* and *Vrn-H1* were not segregating and control of flowering time by other loci was minimized. An Amagi Nijo × WI2585 F_2 derived F_4 recombinant inbred family was genotyped for the three flowering time loci using molecular markers described by Chen et al. (2009a, 2009c). The F_4 plant 103-1 was selected as being heterozygous for the *Flt-2L* region and homozygous for WI2585 alleles at *Eps-2S* and *Vrn-H1*. Three F_5 offspring of plant 103-1 (103-1-2, 103-1-3 and 103-1-5), determined using molecular markers to be heterozygous across the *Flt-2L* region, were used to produce three F_6 families which were combined to make the *Flt-2L* mapping population of 192 individuals. Phenotyping was performed on F_7 progeny of selected F_6 recombinants.

Molecular Markers

Genomic DNA for PCR and Southern analyses was extracted from leaf tissue using the method of Rogowsky et al. (1991). New CAPS markers were generated using the approaches and methods previously described in Chen et al. (2009a). Other PCR markers and conditions for their use were described by Chen et al. (2009a).

RFLP probe preparation and Southern analysis was performed using procedures described by Collins et al. (2008). Fragments of the *480* and *570* genes were amplified from barley genomic DNA using primer pairs indicated in ESM Table 1, cloned into *Escherichia coli*, and re-amplified from *E. coli* cells to provide templates for making RFLP probes by random priming. Individuals homozygous for contrasting alleles in the *Flt-2L* chromosome region were marker-selected from two segregating Amagi Nijo × WI2585 derived F_6 families and used to make bulk DNA samples (10 plants per bulk) for use in screens for *Flt-2L* linked RFLPs with the restriction enzymes *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I.

Mapping and phenotyping

The Amagi Nijo × WI2585 F_6 mapping population was scored for multiple molecular markers in the *Flt-2L* region and a genetic map constructed as previously described (Chen et al. 2009a). Recombinants were allowed to self-pollinate and the resulting seeds (F_7; 60-90 per family) sown into shallow trays. Seedlings in each family were screened using one or more molecular markers located on the segregating chromosome section and within 10 cM of the recombination point, in order to follow the inheritance of recombinant and non-recombinant chromosomes from the parent. Ten randomly selected plants from each F_7 family were also scored for a molecular marker located on the other (homozygous) side of the recombination point. The non-segregating status of this marker, combined with the fact that the markers on the other side were segregating, was used to confirm that the parent was originally scored correctly as a recombinant. At the 3-4 leaf stage, 16 homozygous recombinant and 16 homozygous non recombinant progeny of each family were transplanted into 20 cm pots for phenotyping. Contrasting genotypes were arranged in alternating fashion around the pot, with 4 plants (two of each type) per pot. For some families, 12 randomly selected individuals carrying both parental chromosome types ('heterozygotes') were also transplanted into 20 cm pots for evaluation. These heterozygotes were used to establish the degree of dominance of the phenotypes controlled by the *Flt-2L* locus, in families that were ultimately found to segregate for *Flt-2L*. Plants resulting from additional recombination in the F_7 generation were discarded. The selected plants were phenotyped for heading time, rachis internode length and plant height. For each progeny family, the mean phenotype scores for individuals of each chromosome type were compared using the analysis of variance (ANOVA) function at the 95% confidence level using Genstat v.10 (VSN international Ltd, U.K.). The presence or absence of significant phenotypic differences between individuals of contrasting chromosome types was taken to indicate that the recombinant parent was heterozygous or homozygous for the trait locus, respectively. For each family homozygous for the trait locus, the allele present at this trait locus was deduced from the molecular marker genotype of the recombinant.

Phenotyping was performed on progeny of 39 F_6 plants that were recombinant in the *Flt-2L* region. To test the effect of environmental factors on the expression of *Flt-2L*, and partly also as a result of a shortage of growth chamber space, these families were assessed under three different

sets of growth conditions. This did not impact on the determination of *Flt-2L* genotype of the recombinants, as the genotyping was based on a strategy of comparing sib plants within families (see below), and the *Flt-2L* phenotypes were found to be expressed under all conditions. Growth chambers were used to grow 15 of the families, with 20°C/14°C day/night temperatures and 400 Watt incandescent light bulbs placed 2 meters above the pots. Seven of these chamber-grown families were grown under a 10/14 hours day/night cycle for 8 weeks, followed by 12/12 hours day/night cycle for the rest of the growing period ('growth regime 1'), and were scored only for flowering time. The remaining 8 chamber-grown families were grown under a 12/12 hours day/night regime for the entire time ('growth regime 2') and were scored for both flowering time and rachis internode length.

The remaining 24 phenotyped families were grown in a greenhouse in mid February to early March 2007, when the day/night temperatures averaged 28°C/20°C and day length was 12.5-13.2 hours ('growth regime 3'). These families were sown in three groups of 8 families, on dates that were one week apart. All 24 greenhouse-grown families were scored for both flowering time and rachis internode length, while 8 were also scored for plant height. In selected greenhouse grown families, rachis internode length and plant height were also scored in F_7 'heterozygotes' that carried both parental chromosomes.

Awn emergence date for each plant was defined as the date on which the tips of the awns first emerged from the boot on the primary tiller. Plant height and rachis internode length were scored on primary tillers when they had stopped elongating, just prior to drying. Plant height was taken as the distance from the tiller base to the first fertile node on the spike. Rachis internode length was calculated as the distance from the lowermost to the uppermost floret node of a spike, divided by the total number of internodes.

Comparative genome analysis and gene identification

The region of rice chromosome 4 (TIGR assembly release 5.0) corresponding to the *Flt-2L* interval in barley was identified by barley-rice co-linearity established in a previous study (Chen et al. 2009a). The corresponding interval in the partially completed *Brachypodium distachyon* sequence was identified by BLASTn searches with marker sequences at http://www.brachypodium.org. The relevant *B. distachyon* sequence super contig (contig 0) was

downloaded from the same site and analyzed for gene content using the RiceGAAS online automatic annotation tool (http://ricegaas.dna.affrc.go.jp/usr/). RiceGAAS was also used to access sequences of TIGR locus models from the rice interval. These were used in BLAST searches at NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), TAIR (http://www.arabidopsis.org/Blast/) and the *Brachypodium* site to detect similarities to ESTs, proteins, recognized protein domains and to other parts of the *B. distachyon* and rice genomes.

Results

Fine-mapping of the *Flt-2L* locus

The markers *NSF* and *DGK* had been shown to flank the previously identified *Flt-2L* QTL (Chen et al. 2009c). Eleven markers from this region were used to score the 192 Amagi Nijo × WI2585 F_6 plants, resulting in the genetic map shown in Fig. 1. The marker order agreed completely with a previous map (Chen et al. 2009a). Initially, 15 lines resulting from recombination throughout this region were genotyped for the *Flt-2L* locus by characterizing F_7 progeny individuals in a growth chamber, both for molecular markers and time required to reach the awn emergence stage. This positioned *Flt-2L* within the 7.6 cM *AGA-GAT* marker interval (Fig. 1), with one *Flt-2L* –genotyped recombinant separating the trait locus from each of the *AGA* and *GAT* markers located on either side of *Flt-2L*.

To further delimit the *Flt-2L* interval, 24 additional F_6 *AGA-GAT* recombinants from the same population of 192 individuals were genotyped for the *Flt-2L* locus by scoring progeny for markers and days to awn emergence. New markers in the *AGA-GAT* interval were also generated by exploiting the conservation in gene order with the corresponding region in rice. The barley *AGA-GAT* interval resides within one of the previously identified blocks of co-linearity between barley chromosome 2H and rice chromosome 4 (nt. 32.51 Mb to 32.95 Mb of rice chromosome 4; TIGR assembly 5.0), with the rice chromosome segment arranged in inverted orientation relative to barley (Chen et al. 2009a). Barley homologues of the genes *DUF*, *HvAP2* and *HYP36* from the rice interval were used to generate PCR-based CAPS markers. When PCR products from the barley genes *480* and *570* were sequenced from the parents, no polymorphisms were identified. These fragments were subsequently used as RFLP probes and identified polymorphisms that could be mapped (ESM Table 1). The new markers were scored in all 26 *AGA-GAT* recombinants. All 5 markers located to this interval, at positions that were co-linear with rice (Fig. 1). Use of all the combined phenotype and marker data located *Flt-2L* to the 1.3 cM interval between genes *DUF* and *480*, with two and three recombinants separating the trait locus from the proximal and distal marker, respectively (Fig. 1). *Flt-2L* perfectly co-segregated with the marker genes *HvAP2* and *570* (Fig. 1).

Flowering time data for the five most critical recombinants (for the interval *DUF* and *480*) are included in Fig. 2. Critical F_6 recombinants 2-94, 2-114, 3-10 and 3-30 were scored as heterozyogous for *Flt-2L*, because significant differences in flowering time were observed between F_7 progeny homozygous for recombinant and non-recombinant chromosomes, with the WI2585 marker allele being associated with earliness. The other critical recombinant (3-67) was scored as being homozygous for the WI2585 allele at *Flt-2L*, because no significant difference in flowering time was observed between sibling progeny of contrasting marker genotype, and because the chromosome segment on the homozygous side of the recombination point carried WI2585 marker alleles.

Rachis internode length, plant height, and the impact of growth conditions on flowering time

Recombinant families had been phenotyped under three different day length and temperature regimes, enabling potential effects of growth conditions on *Flt-2L* expression to be examined. Differences in heading times between plants homozygous for contrasting *Flt-2L* allele types averaged 7.4 and 7.1 days respectively, under regimes 1 and 2, and was reduced to 2.7 days under regime 3. Overall development was more rapid with increasing day length and temperature (Fig. 2), with plants carrying the Amagi Nijo and WI2585 alleles of *Flt-2L* flowering after an average of 101.4 and 94.0 days, 74.6 and 67.5 days, and 51.5 and 48.8 days, under regimes 1, 2 and 3, respectively. Plants grown under regime 3 were also much smaller and had fewer tillers than those grown under the other conditions (not shown).

The locus in the *Flt-2L* region controlling rachis internode length was mapped by phenotyping progeny of 32 of the recombinants, including all of the recombinants identified for the *AGA-GAT* interval. Rachis internode length data for the five most critical recombinants (for the interval *DUF* and *480*) are included in Fig. 3. This locus perfectly co-segregated with the flowering time locus. Rachis internodes of individuals of contrasting homozygous *Flt-2L* genotype differed by an average of 1.5 mm, with the late flowering allele from the Amagi Nijo parent being associated with a more compact spike. Rachis internode length of *Flt-2L* heterozygotes was between that of the homozygotes, indicating an incompletely dominant mode of expression (Fig. 3). At least under the conditions examined (growth regimes 2 and 3), rachis internode length appeared

unaffected by day length or temperature (ESM Fig. 1). The number of florets per spike did not appear to be affected by *Flt-2L* locus genotype or growth conditions (ESM Fig. 2).

Segregation of plant height in the Amagi Nijo × WI2585 F_6 mapping population was also found to be associated with *Flt-2L* locus, with short stature being associated with the late/compact allele. A total of eight F_6 recombinants for the *AGA-GAT* interval were scored for the plant height locus, including one for each of the marker intervals located immediately to either side of *Flt-2L* (recombinants 3-30 and 3-67; Fig. 4). No recombination was observed between the loci controlling plant height, rachis internode length or flowering time (Fig. 1), consistent with the idea that these traits are controlled by the same gene. Siblings carrying the WI2585 allele at *Flt-2L* grew on average 8 cm (10%) taller than their counterparts carrying the Amagi Nijo allele (Fig. 4). Average height of *Flt-2L* heterozygotes was generally between that of the two homozygous genotype classes, indicating an incompletely dominant mode of expression (Fig. 4). The effect of temperature and day length on plant height was not investigated, as this trait was scored only in plants grown under one set of conditions (growth regime 3).

Gene content in the corresponding rice and *B. distachyon* intervals

The genes *480* and *DUF*, representing the marker genes most closely flanking *Flt-2L* in barley, define an interval of 118 kb on a related section of rice chromosome 4. This rice interval also showed gene co-linearity with a part of the *B. distachyon* genome (Fig. 5). Co-linearity between rice and *B. distachyon* occurred in two blocks, with the segment carrying the rice genes *Os04g55480*, *Os04g55490* and *Os04g55500* being located at the opposite side of the other section, and aligning in a different orientation, in rice relative to *B. distachyon*. The order of genes mapped in barley (*480* - *AP2*/*570* - *DUF*; Fig. 5) is consistent with these two gene blocks in barley being arranged in the way they are in rice.

In the TIGR Osa1 Genome Annotation Database (Yuan et al. 2005; accessed at http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/), 17 genes (*Os04g55480* to *Os04g55640*) were predicated in the rice *Flt-2L* interval (including the two flanking marker genes). It appeared that seven of the genes (*Os04g55530*, *-540*, *-550*, *-580*, *-600* and *-610* and *-630*), were unlikely to represent 'genuine' genes. *Os04g55530* and *-540* showed similarity to retro-element encoded proteins and to highly repeated sequences in the rice genome. *Os04g55550*, *-580* and *-630*

showed no significant similarity to any other proteins or ESTs from either rice or other species, and similarities to *Os04g55600* and *-610* were limited to rice ESTs. Furthermore, none of these seven sequences showed any similarity to the *B. distachyon* genomic sequence.

The remaining 10 predicted genes from the rice *Flt-2L* interval had homologues in the corresponding portion of the *B. distachyon* genome (Fig. 5), and except for *Os04g55490*, all of these genes showed similarity to proteins in *Arabidopsis thaliana* and other species (Table 1). *Os04g55490* corresponds to a full-length cDNA sequence (clone 002-141-C11; Kikuchi et al. 2003), was low copy in rice, and showed similarity to sorghum ESTs. The corresponding region in *B. distachyon* contains two genes without homologues in the corresponding rice interval. Their closest homolgoues in rice are located on chromosome 10 (*Os10g32730*) and elsewhere on chromosome 4 (*Os04g55440*; Table 1). Therefore, rice and *B. distachyon* served as complementary tools to predict the presence of 12 genes in the *Flt-2L* interval. The details of these genes, including predicted products, protein motifs present within the predicted products, the most similar *Arabidopsis* proteins, and copy number in the rice genome, are presented in Table 1. These twelve genes represent a set from which a candidate for *Flt-2L* may be sought. The flanking marker genes *480* and *DUF* are included in this group, to allow for the possibility that recombination occurred between a polymorphism used for mapping and a functional polymorphism.

Efforts were made to delimit the *Flt-2L* interval further by mapping barley homologues of genes from the rice *480-DUF* interval, however these attempts were unsuccessful. No barley ESTs corresponding to genes *Os04g55490* or *Os04g55590* were available. For the remaining genes in the interval, barley ESTs representing putative orthologues were identified. Primers were made to putative barley orthologues of genes *Os04g55500* and *Os04g55520* but these failed to amplify PCR products from barley. A fragment amplified from a putative barley *Os04g55510* orthologue revealed no polymorphism, either by sequencing or when the fragment was used as an RFLP probe. Marker generation from *Os04g55620* was not attempted.

Discussion

Flt-2L was previously defined by flowering time and rachis internode length QTLs delimited to a 21.3 cM marker interval (Chen et al. 2008c). In the current study, we identify plant height as a third phenotype controlled by the *Flt2L* region, and use a strategy that enabled the inheritance of these traits to be followed as single locus (Mendelian) factors, allowing all three to be mapped to a single point within a 1.3 cM marker interval. This information has enabled us to conduct a subsequent study to address whether the QTL for frost damage at the flowering stage detected on chromosome 2HL could be the result of escape or genuine frost tolerance arising from flowering time variation encoded by *Flt-2L* (Chen et al. 2009b). The precise map location of *Flt-2L* allowed selection of an Amagi Nijo × WI2585 F_6 recombinant that was homozygous for *Flt-2L* yet heterozygous for an adjacent chromosome segment immediately proximal of *Flt-2L*. The progeny family segregated for frost induced sterility associated with the segregating chromosome segment, suggesting that the frost tolerance and flowering time effects could be genetically separated (Chen et al. 2009b). Families such as this one will be ideal for evaluating and fine mapping of the tolerance locus (for eventual cloning), because siblings carrying alternative alleles at the tolerance locus will be matched for flowering time. Fine mapping of the *Flt-2L* locus also represents a step towards isolating the *Flt-2L* gene(s) by a positional cloning approach, which in turn should shed light on processes which control flowering time, plant height and rachis internode length in cereals.

Control of flowering time, rachis internode length and culm length on 2HL were expressed in an incompletely dominant manner, and no recombination between the underlying loci was detected, consistent with these three traits being controlled by the same gene or separate but closely linked (<1 cM) genes. We refer to these phenotype effects together under one locus name (*Flt-2L*). Conceivably, a common process influencing organ elongation, manifested by the rachis length and culm length phenotypes, may have also been directly responsible for *Flt-2L* effects on flowering time, as measured by the date of awn emergence, i.e., by affecting the elongation rate of the spikes/awns/peduncle relative to that of the flag leaf. Future studies could investigate the source of the *Flt-2L* flowering time effect, in relation to the above hypothesis and the additional possibility that the locus may control the timing of floral meristem initiation.

Flowering time and rachis internode length effects of *Flt-2L* have now been detected in Haruna Nijo × Galleon and Amagi Nijo × WI2585 crosses, with the late-flowering and dense-spike allele(s) being derived from Haruna Nijo and Amaji Nijo. These two varieties, bred by the Sapporo company in Japan, have closely related pedigrees and also share a high proportion of identical sequence haplotypes for markers located in this part of chromosome arm 2HL (Chen et al. 2009a). Thus, Haruna Nijo and Amagi Nijo may share common *Flt-2L* allele(s) for compact spikes and late flowering.

Others have reported loci for rachis internode length in this region of chromosome arm 2HL, with dense spike alleles originating from Japanese parents. QTL for spike density were identified in Mikamo Golden × Harrington, Misato Golden × Satsuki Nijo and Azumamugi × Kanto Nakate Gold crosses (Turuspekov et al. 2005; Sameri et al. 2006). These crosses also segregated for a locus in the same chromosome region which determines chasmogamy/cleistogamy (open/closed floret types), with cleistogamy being associated with dense spikes (Turuspekov et al. 2004; Turuspekov et al. 2005). The chasmogamy/cleistogamy locus has been mapped close (<10 cM) to *Flt-2L* in the Galleon × Haruna Nijo cross (Chen et al. 2009c). In a population of 6,000 Misato Golden × Satsuki Nijo BC_4F_4 plants, no recombination was detected between the loci controlling chasmogamy/cleistogamy and spike density (Honda et al. 2003). However, while cleistogamy was associated with *Flt-2L* in the Haruna Nijo × Galleon cross, we were unable to detect segregation of this trait in the Amagi Nijo × WI2585 cross (Chen et al. 2009c), which we know segregates for spike density and other traits at *Flt-2L* (this study). Hori et al. (2005) detected QTL for culm length and rachis internode length in this chromosome region in a cross between Haruna Nijo and an accession of wild barley (*Hordeum vulgare* ssp. *spontaneum*). As in the current study, increased spike compactness was associated with reduced plant height. However, in the mapping populations examined by Samari et al. (2006) and Turuspekov et al. (2005), no effect of this chromosome region on plant height was detected. The *zeocriton* 1 (*zeo1*) locus is defined by an X-ray induced mutant that exhibits extreme dwarfing and very compact heads (Lundqvist and Lundqvist 1998). The *zeo1* locus was mapped 2.3 cM distal of the marker *MSU21* (Costa et al. 2001), which is in the vicinity of *Flt-2L* (Chen et al. 2009c), raising the possibility that the *zeo1* mutant represents an extreme allele of *Flt-2L* conferring compact-spikes and reduced plant height. In summary, there appears to be conflicting evidence as to whether the chasmogamy/cleistogamy, spike density and plant height traits are encoded by the same or

different genes in the *Flt-2L* region of 2HL. Further work, such as mutant analysis, fine mapping, and/or isolation of the underlying genes, will be required to resolve this issue.

The late flowering at *Flt-2L* was associated with a short plant height and compact rachis internodes, but not with any difference in the number of fertile nodes per spike (ESM Fig. 1 and 2; Chen et al. 2009c). A compact spike, without any effect on seed number per spike, also accompanies the reduced plant height conferred by many semidwarf genes in barley (Dahleen et al. 2005). Delayed flowering is also associated with reduced plant height at other dwarfing loci, e.g. *denso* locus in barley (Laurie et al. 1995). In contrast, late flowering alleles at other loci on barley chromosomes 2H (*Eps-2S*) and 5H (linked to *Vrn-H1*) are associated with longer heads and more fertile internodes per spike (Chen et al. 2009c), highlighting that various flowering time loci can affect development in fundamentally different ways.

Individuals flowered around 40 days earlier under conditions of increased temperature and day length, regardless of whether they carried the early or late flowering *Flt-2L* allele (Fig. 2). This is consistent with the observation that higher temperatures generally hasten cereal development (e.g., van Beem et al. 2005). A proportion of the differences may also be attributable to the photo sensitive *Ppd-H1* alleles present in both of the parents (Chen et al. 2009c), which confer early flowering under long days (Laurie et al. 1994). Compared to the growth conditions, segregation at the *Flt-2L* locus determined relatively small differences in flowering time. These differences were reduced under regime 3 relative to regime 1 (average 2.7 and 7.4 days, respectively), perhaps because the shorter overall lifespan of the plants under regime 3 allowed less time for flowering time differences to accumulate between plants of contrasting *Flt-2L* genotypes.

We combined several strategies to ensure that the relatively subtle phenotypes of the *Flt-2L* locus could be followed as discrete Mendelian traits. The four generations of self crossing used to derive the three F_5 parents of the mapping population would have eliminated some of the background heterozygosity. Molecular markers were also used to ensure the F_5 parents were homozygous for the *Vrn-H1* and *Eps-2S* loci, which were known to influence flowering time in this cross. PCR markers were also used to test for correlations between phenotypes and the inheritance of segregating recombinant or non-recombinant chromosomes, in order to distinguish variation determined by *Flt-2L* from that arising from unlinked loci. At least for the flowering time trait, the comparison of sib lines was shown to be necessary to determine the *Flt-2L*

genotype of F_6 recombinants, as variation between the F_7 families was similar to that determined by *Flt-2L* genotype (Fig. 2). Potentially, the sib-comparison approach offers advantages in accuracy and time over alternative methods for mapping QTL as discrete loci that depend on backcrossing to produce mapping populations in which the major source of phenotypic variation derives from the target locus (Salvi and Tuberosa 2005).

HvAP2 was one of two genes found to co-segregate with *Flt-2L* (Fig. 5). In *Arabidopsis*, *APETALA2* (*AP2*) is the gene with most similarity to the rice orthologue of *HvAP2* (81 % protein identity in the region of overlap; next highest match 78%). *Arabidopsis* AP2, a member of the AP2/ERF group of transcription factors, is required for stem cell maintenance in the shoot, the specification of floral organs and normal seed development (Jofuku et al. 1994), but does not influence flowering time unless mis-expressed (Aukerman and Sakai 2003; Chen 2004). However, the cloned gene *Q* from wheat chromosome 5A (=*WAP2*; Simons et al. 2006), representing a major domestication locus, is highly similar to the rice *AP2* gene (70% protein identity; 80% nucleotide identity over 637 bp), and controls a phenotype resembling that of *Flt-2L*. The *Q* domestication allele confers a compact spike, reduced plant height, free threshing grains and a fragile rachis (Simons et al. 2006), and is associated with delayed ear emergence (Kato et al. 1999). The rice *AP2* gene is also very similar (50-66% protein identity) to *Arabidopsis TOE1* (*AtRAP2.7*) and *TOE2*, and to maize *VGT1* (*ZmRap2.7*) and *GLOSSY15,* which have also been shown to influence flowering time (Lauter et al. 2005; Salvi et al. 2007). Similar to all the above mentioned genes from wheat, *Arabiodpsis* and maize, the rice gene encodes a member of the small subclass of AP2/ERF proteins that contain dual AP2 DNA binding domains, and the coding sequence contains the conserved binding site of the regulatory microRNA *miR172* (Aukerman and Sakai 2003; Salvi et al. 2007; and data not shown). While the levels of similarities to *VGT1*, *GLOSSY15* or *Q* were high, maize and rice contain other uncharacterized homologues with higher similarity to rice *AP2* (data not shown). Nevertheless, the close similarity of *HvAP2* to genes known to affect flowering time, spike compactness and plant height, together with the perfect co-segregation between *HvAP2* and *Flt-2L*, seem to make *HvAP2* a plausible candidate for *Flt-2L*.

The other genes shown/predicted to map to the *Flt-2L* region have weaker links to plant development, or no obvious link at all. The product of *Os04g55590* is related to the *Arabidopsis* WUSCHEL homeobox transcription factor. WUSCHEL and a related protein serve to maintain

stem cells in the root and shoot (Mayer et al. 1998; Sarkar et al. 2007), but a biological function has not yet been ascribed to *Os04g55590* or its closest *Arabidopsis* homologue (=*OsWOX4* and *AtWOX4*, respectively; Nardmann et al. 2007). The product of the rice gene *Os04g55620* (RLK Osi001608.1) belongs to the large family of receptor-like kinase (RLK) proteins, which impact on a diverse range of plant traits (Shiu et al. 2004), including general growth (Shpak et al. 2004). However, Osi001608.1 belongs to subgroup LRR-III, which has no ascribed functions relating to overall development (Shiu et al. 2004; Morillo and Tax 2006). *Os04g55440* encodes a putative LRR-receptor protein and is not closely related to any gene of known function. Gene *Os04g55520* encodes a member of the ERF family of AP2/ERF transcription factors that serve a range of diverse biological roles. However *Os04g55520* (OsERF#008) belongs to ERF subgroup IIa, for which no function has yet been determined (Nakano et al. 2006). In *Arabidopsis*, *At4g21470* is the gene with the highest protein similarity to the *Os10g32730* (*Hyd*) gene. This *Arabidopsis* protein has dual riboflavin kinase and FMN hydrolase activities that function in flavin biosynthesis (Sandoval and Roje 2005). Gene *Os04g55550* shows similarity to yeast *Eme1*, encoding a component of an endonuclease complex required for resolving Holliday junctions during meiosis (Boddy et al. 2001). The *Os04g55480* product is similar throughout its length to the human BRCA1-associated protein 2 (BRAP2) which binds nuclear localization signals in other proteins, affecting their movement from the cytoplasm into the nucleus (Asada et al. 2004). Products of *Os04g55480* and *Os04g55510* each contain a RING motif. This domain can facilitate protein-protein interactions and occurs in factors involved in ubiquitination and protein turnover. *Os04g55640* shows very distant similarity to pancreatic ribonuclease. *Os04g55490* and *Os04g55570* show no significant similarity to any characterized protein. Future fine mapping and functional studies should be able to determine which (if any) of the gene(s) from the rice and *B. distachyon* intervals control the phenotypes at *Flt-2L*.

Acknowledgements

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Figure legends

Fig. 1.

Genetic map of the barley *Flt-2L* region on the long arm of barley chromosome 2H, aligned with the corresponding part of the rice genomic sequence from the long arm of chromosome 4. Lines connect orthologous genes in barley and rice. The rice map is presented in inverted orientation (centromere towards the bottom) so that it will align with barley. Mb positions on the TIGR v 5.0 rice chromosome 4 sequence assembly are indicated. Recombinant numbers with no asterisks, one asterisk (*) and two asterisks (**) indicate recombinants that were scored for flowering time only, flowering time and rachis internode length, and all three traits (flowering time, rachis internode length and plant height), respectively.

Fig. 2.

Days to awn emergence of F_7 progeny of 15 F_6 plants recombinant for the *Flt-2L* region. Each set of columns represents means and standard errors for F_7 sibling plants determined to be homozygous for alternate alleles of a molecular marker located on the heterozygous (segregating) side of the recombination site. Except for 3-67, the sibling classes in all families differed significantly ($p<0.05$), indicating that the F_6 parents were heterozygous for *Flt-2L*. (*) Sibling classes in family 3-67 were not significantly different, indicating that the parent plant was *Flt-2L* homozygous. Families were grown under 3 different growth regimes: regime 1: 10/14 hrs day/night for the first 8 weeks followed by 12/12 hrs day/night cycle for the remainder of the growing period; 20/14°C day/night; growth chamber; regime 2: 12/12 hrs; 20/14°C day/night; growth chamber; regime 3: 12.5 – 13.2 hrs day length; 28/20°C day/night; greenhouse. Families 2-94, 2-114, 3-10, 3-30 and 3-67 represent the five critical recombination events (from the interval *DUF* and *480*), but otherwise five *Flt-2L* segregating families grown from each of the growth regimes were randomly selected for representation in the figure.

Fig. 3.

Rachis internode length (means and standard error) of F_7 progeny of eight F_6 plants recombinant for the *Flt-2L* region. Within each family, individuals were determined to be homozygous Amagi Nijo, heterozygous, or homozygous WI2585 for a molecular marker from the heterozygous (segregating) side of the recombination site. Families were determined to be segregating for the *Flt-2L* locus (2-94 to 3-30), or homozygous for the WI2585 (3-67 and 2-106) or Amagi Nijo (2-

65 and 2-117) alleles of *Flt-2L*. Plants were all assessed under growth regime 3. The five most critical recombinants are shown (2-94, 2-114, 3-10, 3-30 and 3-67), but otherwise the families were randomly selected for representation in the figure.

Fig. 4.

Plant height (mean and standard errors) of F_7 progeny of eight F_6 plants recombinant for the *Flt-2L* region. Using a molecular marker, sibling plants were classified into three genotypic classes with respect to the *Flt-2L* chromosome region, as outlined in Fig. 3. The first six families were segregating for the *Flt-2L* locus while the remaining two were homozygous for the Amagi Nijo (3-15) or WI2585 (3-67) allele of *Flt-2L*. Plants were all assessed under growth regime 3. These eight families represent the only families scored for plant height.

Fig. 5.

Gene co-linearity between the *Flt-2L* interval in barley and the corresponding intervals in rice and *B. distachyon*. Arrows indicate location and orientation of genes. Gene identifiers correspond to the last three digits of the TIGR rice locus designations listed in Table 1 (prefix *Os04g55*, except for *HYD*/*Os10g32730*). Physical maps of rice and *B. distachyon* are drawn to the same scale. Nucleotide positions in the rice chromosome 4 sequence (TIGR rice assembly 3.0) and in contig 0 of the *B. distachyon* 4× JGI sequence assembly (http://www.brachypodium.org) are indicated at the ends of the intervals.

Tables and figures

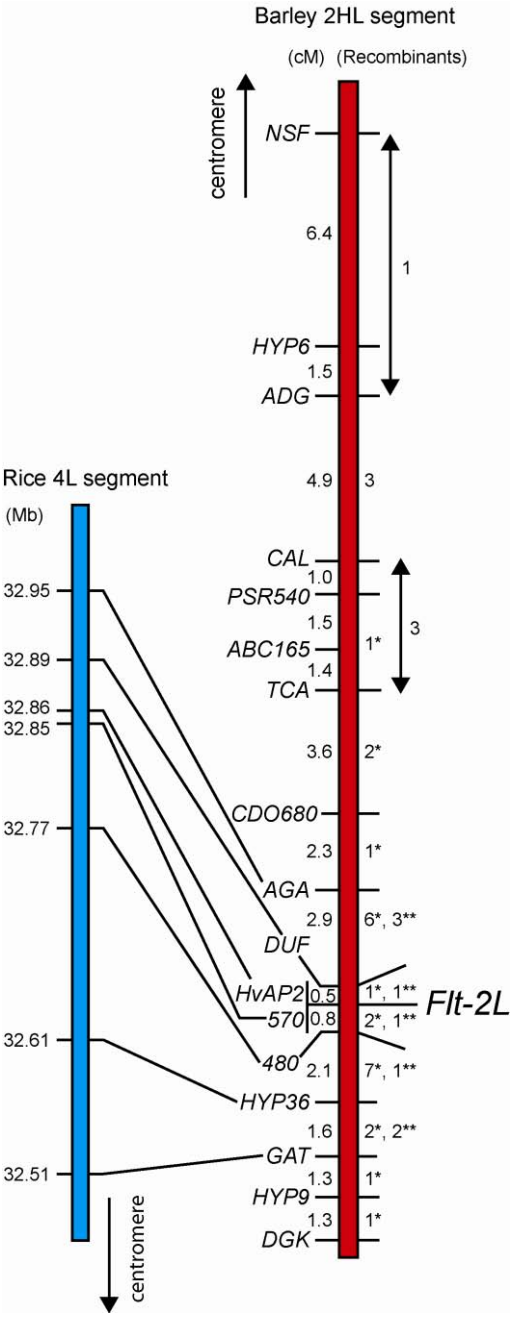


Fig. 1.

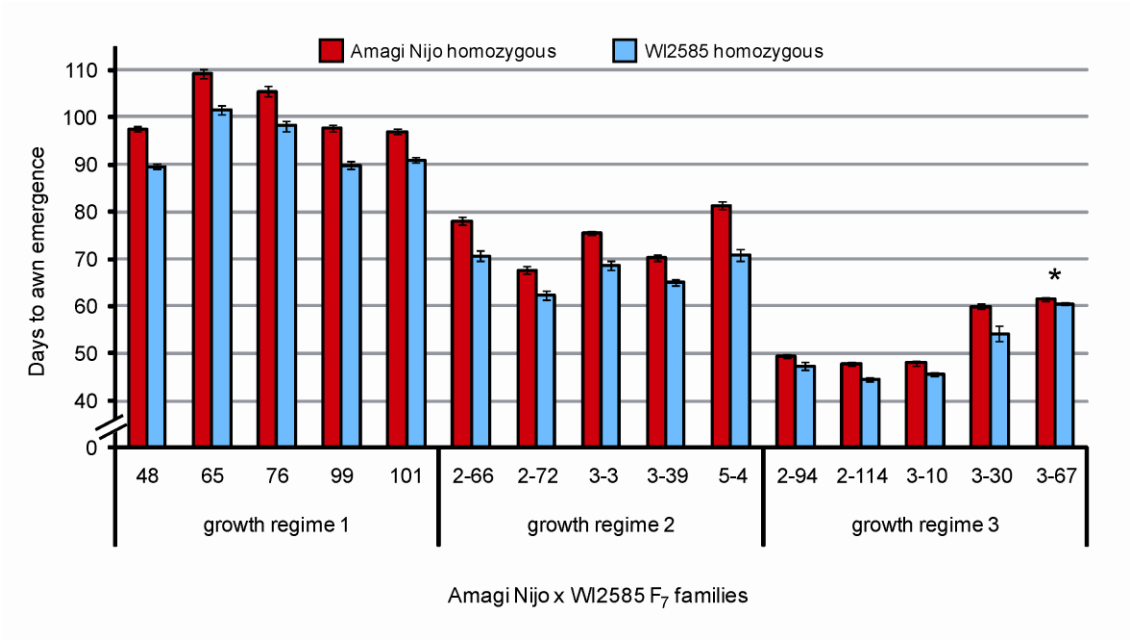

Amagi Nijo homozygous
WI2585 homozygous
Days to awn emergence
110
100
90
80
70
60
50
40
0
*
48
65
76
99
101
2-66
2-72
3-3
3-39
5-4
2-94
2-114
3-10
3-30
3-67
growth regime 1
growth regime 2
growth regime 3
Amagi Nijo x WI2585 F7 families

Fig. 2.

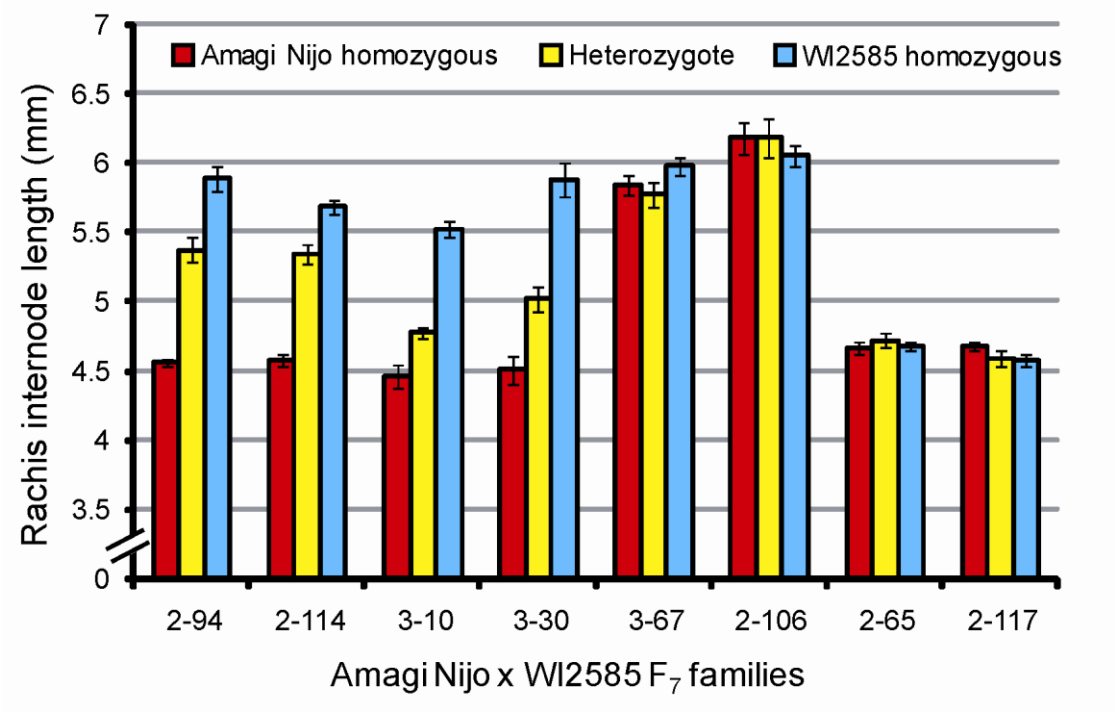

Amagi Nijo homozygous
Heterozygote
WI2585 homozygous
Rachis internode length (mm)
7
6.5
6
5.5
5
4.5
4
3.5
0
2-94
2-114
3-10
3-30
3-67
2-106
2-65
2-117
Amagi Nijo x WI2585 F7 families

Fig. 3.

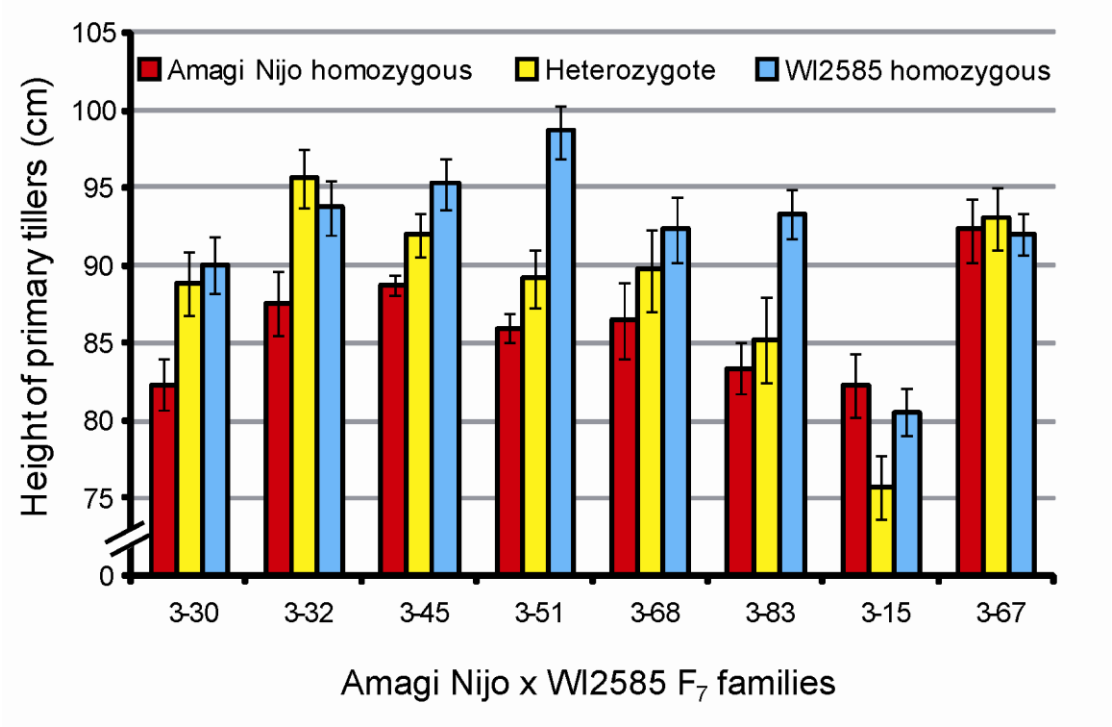

105
100
95
90
85
80
75
0
Height of primary tillers (cm)
Amagi Nijo homozygous
Heterozygote
WI2585 homozygous
3-30
3-32
3-45
3-51
3-68
3-83
3-15
3-67
Amagi Nijo x WI2585 F7 families

Fig. 4.

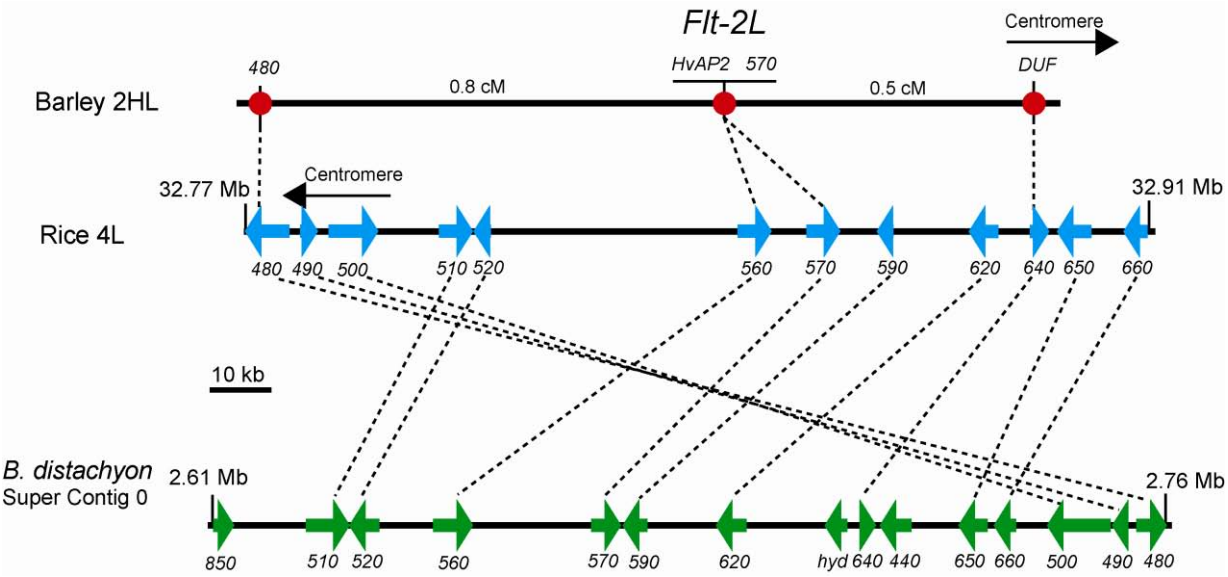
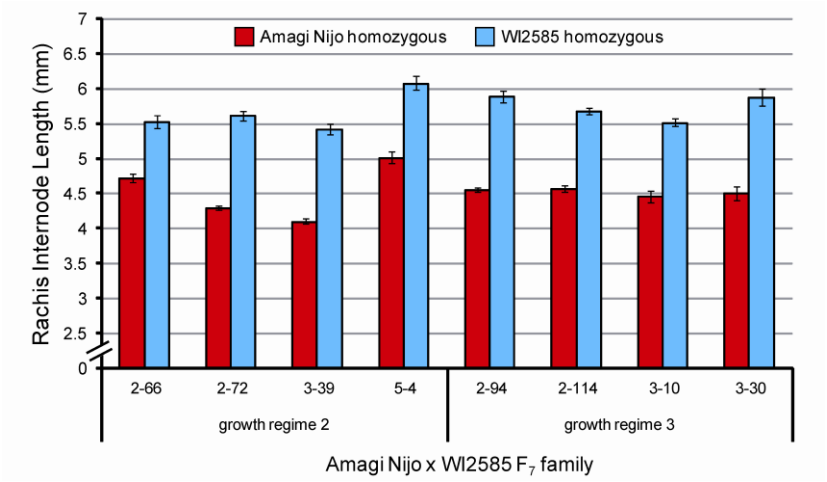

Flt-2L
Centromere
480
HvAP2 570
DUF
Barley 2HL
0.8 cM
0.5 cM
32.77 Mb
Centromere
32.91 Mb
Rice 4L
480 490 500 510 520 560 570 590 620 640 650 660
10 kb
B. distachyon
Super Contig 0
2.61 Mb
2.76 Mb
850 510 520 560 570 590 620 hyd 640 440 650 660 500 490 480

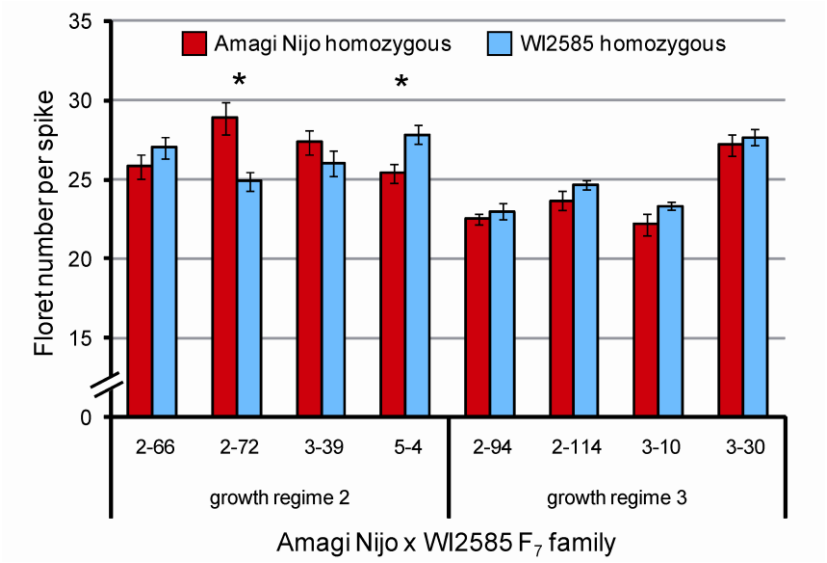
Fig. 5.

Rice TIGR locus (marker name)	Rice TIGR Annotation	conserved protein domains (BLASTp; NCBI; E-value)[a]	Top Arabidopsis BLASTp hit (BLASTp; TAIR)
Os04g55480 (*480*)	BRCA1-associated protein	1) pfam02148; zf-UBP, Zn-finger in ubiquitin hydrolases; $5e^{-20}$ 2) pfam07576; BRAP2, BRCA1-associated protein 2; $5e^{-19}$ 3) pfam 05565; Sipho Gp157 0.003 4) cd00162; RING finger; 0.004	*At2g42160*; Zinc finger (ubiquitin-hydrolase) domain-containing protein; $1e^{-110}$
Os04g55490	Unknown protein	No significant hits	No significant hits
Os04g55500	Unknown protein	pfam09434; Crossover junction endonuclease, EME1; $8e^{-55}$	*At2g21800*; Similar to unknown protein; $1e^{-77}$
Os04g55510	Zinc finger, C3HC4 type family protein	cd00162; RING domain; $9e^{-09}$	*At4g34040*; Zinc finger (C3HC4-type RING finger) family protein; $3e^{-45}$
Os04g55520	Dehydration responsive element binding protein	cd00018; DNA binding domain AP2; $2e^{-17}$	*At2g23340*; AP2 domain containing transcription factor; $1e^{-29}$
Os04g55560 (*HvAP2*)	AP2 domain containing protein	smart00380; DNA binding domain AP2. Domain 1; $2e^{-07}$ Domain 2; $1e^{-11}$	*At4g36920*; AP2 (APETALA 2) transcription factor; $4e^{-72}$
Os04g55570 (*570*)	Unknown protein	pfam05911; DUF869, plant protein of unknown function; $4e^{-41}$	*At1g19835*; DUF869 protein of unknown function; $1e^{-52}$
Os04g55590	WUSCHEL related homeobox 4	pfam00046; Homeobox domain; $4e^{-07}$	*At1g46480*; Homeobox-leucine zipper transcription factor family protein; $2e^{-28}$
Os04g55620	Putative protein kinase	cd00180; S_TKc; Serine/Threonine protein kinases, catalytic domain; $3e^{-30}$	*At5g43020*; Leucine-rich repeat transmembrane protein kinase; $1e^{-89}$
Os10g32730 (*Hyd*)	Haloacid dehalogenase-like hydrolase	1) COG0637; Hydrolase; $6e^{-33}$ 2) pfam01687; Flavokinase; $3e^{-32}$	*At4g21470*; Riboflavin kinase/FMN hydrolase; $3e^{-58}$
Os04g55640 (*DUF*)	Plant-specific domain TIGR01627 family protein	pfam04669; DUF579, protein of unknown function; $1e^{-62}$	*At5g67210*; Nucleic acid binding / pancreatic ribonuclease; $5e^{-57}$
Os04g55440	Unknown protein	cd00116; Leucine-rich repeats (LRRs), ribonuclease inhibitor (RI)-like subfamily; $3e^{-07}$	*At1g74190*; Leucine-rich repeat family protein; $5e^{-90}$

Table 1. Genes present in rice and *B. distachyon Flt-2L* intervals



ESM Fig. 1.



ESM Fig. 2.

Marker names	Marker Primers (5' to 3')	Original Primers (5' to 3')[a]	Sequence length (bp)[b,c]	PCR cycle[d]	Restriction Enzymes	Genbank Accession[c]
480 (RFLP)	F-x480-1 AGAAAGTTGCTGAAGTGA R-x480-2 CTTAATGGAATATCTGAAGTC		556	2	*EcoR* I	WI, G, AN, HN (EU827507)
570 (RFLP)	F-x570-1 CGATATGTCATAGTTTCC R-x570-2 CCAAAGGTGGTCCTGAGTGC		454	1	*Xba* I	WI, G, AN, HN (EU827506)
AGA (PCR)	F-AGA-1 CTGATCAGTTCTATGGTGTCC R-AGA-3 CACTTAGTCGAGCATCAATAG	F-AGA-1 CTGATCAGTTCTATGGTGTCC R-AGA-2 GTGTTCTGAATAACAGAAGG	210 [808] WI 210 [797] AN, HN	3[6]	*Mse* I	WI (EU827508) AN, HN (EU827509)
DUF (PCR)	F-DUF-1 TCAGCCACGCTGATTGATTC R-DUF-6 CGAAGACGAGGAGGTTGCAG	F-DUF-1 TCAGCCACGCTGATTGATTC R-DUF-2 CAGGTACTTGACGTAGAACTC	386 [455]	4[1]	*BsrB* I	WI, G (EU827510) AN, HN (EU827511)
HvAP2 (PCR)	F-HvAP2-5 GGCAGCGGCACTAATGAGAATC R-HvAP2-2 GGCTCGAACTCCTCGGCGTA	F-HvAP2-1 TGCGGCAAGCAGGTCTATC R-HvAP2-2 GGCTCGAACTCCTCGGCGTA	270 [937]	3[5]	*BsoB* I	WI (EU827515) AN, HN, G (EU827516)
HYP36 (PCR)	F-HYP36-3 CCAAGTGCCATCAGTTGACA R-HYP36-4 CAGTCCCAGAGAATGGTAAT	F-HYP36-1 GAAGGAAGAATCTGATAGCAAC R-HYP36-2 GCATATTGACTGGATATGTG	295 [623]	3[1]	*Tsp509* I	WI (EU827512) G (EU827513) AN, HN (EU827514)

[a] Primers originally used in polymorphism screen.

[b] Fragments used in original polymorphism screen in square brackets

[c] AN = Amagi Nijo; HN = Haruna Nijo; WI = WI2585; G = Galleon.

[d] PCR cycle for original primers in square brackets. All PCR programs are preceded by a 95ºC incubation for 7 mins, and followed by an incubation at 68ºC for 10 mins.

Programs:

1) 40 cycles of 10 s @ 94ºC, 30 s @ 55ºC, 1 m 30 s @ 68ºC.
2) 45 cycles of 10 s @ 94ºC, 30 s @ 50ºC, 50 s @ 68ºC.
3) 45 cycles of 10 s @ 94ºC, 30 s @ 60ºC, 50 s @ 68ºC.
4) 45 cycles of 10 s @ 94ºC, 30 s @ 62ºC, 50 s @ 68ºC.
5) 40 cycles of 10 s @ 94ºC, 30 s @ 60ºC, 1 m 30 s @ 68ºC.
6) 35 cycles of 10 s @ 94ºC, 30 s @ 55ºC, 2 m @ 68ºC.

[e] PCR reactions contained 5% v/v DMSO.

ESM Table 1.

Primers, PCR conditions, restriction enzymes and sequences relating to polymorphism screen and markers.

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5 Varietal and locus-specific frost tolerance in reproductive tissues of barley (*Hordeum vulgare* L.) detected using a frost simulation chamber

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Statement of authorship

Characterization of a frost tolerance locus on chromosome 2H of barley (*Hordeum vulgare* L.)

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Chen A (Candidate)

Statement of contribution

Designed the parental genotype screen (Figure 3 and 4) in conjunction with Gusta LV and Leach R, carried out the experiments in Figure 3 and 4, designed and performed the experiments for testing progeny families (Figure 5 – 7), analyzed the data and drafted the manuscript

I hereby certify that the statement of contribution is accurate

Signed. *Date..*

Gusta LV

Statement of contribution

In conjunction with Leach R and Chen A, designed the parental genotype screen (Figure 3 and 4), participated in the experiments leading to Figure 3 and 4, contributed to the experimental design for testing progeny families (Figure 5 – 7) and contributed to the final manuscript

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Brûlé-Babel A

Statement of contribution

Formulated the initial frost simulation profile (Figure 1A), designed and carried out the parental screen (Figure 2) and contributed to the final manuscript

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Leach R

Statement of contribution

In conjunction with Gusta LV and Chen A, designed the parental genotype screen (Figure 3 and 4), participated in the experiments leading to Figure 3 and 4, and contributed to the final manuscript

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Baumann U

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Participated in the supervision of the work and contributed to the final manuscript

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Geoffrey GB

Statement of contribution

Participated in the supervision of the work, contributed to the final manuscript, acted as the corresponding author

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Abstract

Exposure of flowering cereal crops to frost can cause sterility and grain damage, resulting in significant losses. However, efforts to breed for improved low temperature tolerance in reproductive tissues (LTR tolerance) has been hampered by the variable nature of natural frost events and the confounding effects of heading time on frost induced damage in these tissues. Here, we establish conditions for detection of LTR tolerance in barley under reproducible simulated frost conditions in a custom-built frost chamber. An ice nucleator spray was used to minimize potential effects arising from variation in naturally occurring extrinsic nucleation factors. Barley genotypes differing in their field tolerance could be distinguished. Additionally, an LTR tolerance QTL on the long arm of barley chromosome 2H could be detected in segregating families. In a recombinant family, the QTL was shown to be separable from the effects of the nearby flowering time locus *Flt-2L*. At a minimum temperature of -3.5 °C for 2 h, detection of the LTR tolerance locus was dependent on the presence of the nucleator spray, suggesting that the tolerance relates to freezing rather than chilling, and that it is not the result of plant-encoded variation in ice-nucleating properties of the tiller surface.

Key-words: flowering time; frost-induced sterility; frost tolerance; genetic mapping; ice nucleation

Introduction

In Australia, cereals such as wheat and barley are planted in autumn, with the majority of the growing season occurring in late winter and early spring. In these areas, growth later in the season is often limited by low water availability and higher temperatures. The growing season is characterized by average day time temperatures of around 18 °C, which are ideal for growth. However, temperatures during the flowering stage can occasionally fall to sub-zero levels at night, which can damage the tender reproductive tissues. The frost events usually occur under clear night skies, when more heat is radiated away from the crop canopy than is received, typically resulting in minimum temperatures of -2 °C to -5 °C. When these events occur around anthesis, they can result in sterile florets that fail to set grain, leading to yield reductions. Frost can also damage the developing grains, resulting in quality downgrading. Direct yield penalties due to frost of 10 % are common, but particular regions can suffer losses in excess of 85 % in a season (Paulsen and Heyne 1983).

Agronomic practices can be employed to reduce the impact of frost on cereal crops. Delving, which involves bringing clay to the surface using specialized cultivation equipment, can improve the heat holding capacity of sandy soils (Rebbeck et al. 2007). Delayed sowing times and late flowering varieties are used to reduce the chances of flowering occurring during periods of high frost risk early in the season (Gomez-Macpherson and Richards 1995). However, these strategies can restrict yield by shifting grain fill into a time of greater heat/water stress (Woodruff 1992). Therefore, frost impacts the grains industry, by directly or indirectly limiting the size or quality of the harvest, and by increasing the cost of farm management.

Efforts over the last few decades to improve the tolerance of Australian cereal varieties to low temperatures in reproductive tissues (LTR tolerance) have achieved little success (Single 1985; Fuller et al. 2007). Progress in field-based screens for LTR tolerance is hampered by the spatial variability and unpredictable timing and severity of natural frost events (Single 1988). The deployment of frost protection covers is also required to provide unfrosted controls. Screening in refrigerated chambers can also be challenging, due to the difficulty of simulating the temperature and humidity gradients that occur during frost events in the field, and because of the need to achieve uniform ice nucleation (Fuller et al. 2007). When assessing LTR tolerance, differences in flowering time between genotypes need to be taken into account, since reproductive tissues at different developmental stages differ in their degree of frost sensitivity. This requires the use of

multiple seeding dates and/or frost exposures, and the scoring of pre-tagged heads which were at a narrow developmental stage at the time of frost exposure (Reinheimer et al. 2004).

Despite these challenges, Reinheimer et al. (2004) identified several lines with potential LTR tolerance in the field, including the Japanese barley cultivars Amagi Nijo and Haruna Nijo. In Amagi Nijo × WI2585 and Galleon × Haruna Nijo mapping populations, quantitative trait loci (QTLs) for frost-induced floret sterility (FIS) were identified on the long arms of chromosomes 2H and 5H, with the tolerance alleles deriving from the Japanese parents (Reinheimer et al. 2004). While the 5HL FIS QTL showed a similar chromosomal location to the *Fr-1* vegetative LT tolerance locus of wheat and barley, no other LT tolerance loci had been reported in the vicinity of the 2HL FIS QTL (Reinheimer et al. 2004). Low FIS at the 2HL QTL was associated with late flowering, reduced plant height and shorter rachis internodes controlled by closely linked *Flt-2L* locus (Chen et al. 2009a, 2009c). These three developmental effects co-segregated with one another in the same 1.3 cM marker interval on chromosome 2H, indicating that they were encoded by the same gene or closely linked genes (Chen et al. 2009a).

In the current study, a panel of barley genotypes known to differ in their frost sensitivity in the field was used to explore conditions for effectively screening for LTR tolerance in a frost simulation chamber. Using two Amagi Nijo × WI2585 F_5 families, we address whether the 2H FIS QTL can be detected under the adopted frost simulation conditions, and whether this locus is genetically separable from the nearby *Flt-2L* locus.

Materials and methods

Plant growth and scoring of frost-induced sterility

Plants were grown in a greenhouse or a growth room in 20 cm plastic pots containing SARDI Cocopeat mix. In the plants to be frosted, samples of spikes were dissected, revealing that pollen shed (anthesis) occurred during growth stages 47 – 50, as defined by Zadoks et al. (1974). Tillers of this stage were tagged immediately prior to frosting. Approximately 3 – 4 weeks later when most of the tagged heads had reached the milk stage, the tagged heads were scored for frost induced sterility by determining the percentage of florets that had failed to set seed.

Frost simulation

A frost simulation chamber commissioned by the Australian Genome Research Facility (AGRF) was used. Pots were fitted into the temperature controlled wells which were maintained at about 10 °C and the plants subjected to a pre-programmed temperature regime to simulate an overnight frost event. An overhanging sensor probe that served as a thermostat for maintaining the pre-programmed temperatures was located at spike height near the middle of the plants. Other probes were located at various positions within the frost chamber to monitor temperatures. In some experiments, the ice nucleating agent SNOMAX® (York Snow, Victor, NY, via a local skifield), in which the active ingredient is a protein from *Pseudomonas syringae* strain 31a (Skirvin et al. 2000; Mazur et al. 2005; Missous et al. 2007; http://www.annecy.us/all-about-snomax/), was sprayed onto the plants as a 2 g / l solution, 1 - 2 h before the chamber reached the minimum temperature. After the frost treatment, plants were moved back to the greenhouse or growth room and maintained under the same growth conditions as they were grown under before the frost treatment.

Tests to explore frost conditions

The five spring barley genotypes Keel, Schooner, WI2585, Sloop and Amagi Nijo, possessing different levels of reported frost sensitivity in the field (Reinheimer et al. 2004), were used for initial testing of frost simulation conditions. Seed of these lines were sown on multiple seeding dates in the greenhouse during the Australian summer (January, 2005), where the photoperiod

was approximately 13.5 h and the average day / night time temperatures were 28 °C / 22 °C. These genotypes were subjected to four different frost simulation programs, using 16 plants of each genotype per experiment. Between 17 and 30 heads were scored for frost induced sterility per genotype and treatment. Temperature profiles differed only for the severity and duration of the minimum temperatures, which were -3.5 °C for 1 h, -5.5 °C for 1 h, -5.5 °C for 2 h and -5.5 °C for 4 h. After beginning at 20 °C, the temperature at spike height was reduced to 3.5 °C at a rate of 5 °C / h and then further reduced at a rate of 1 °C / h until the specified minimum temperature was reached. The chamber was held at that temperature for the specified duration and subsequently raised to 3.5 °C at 2 °C / h. Finally, the temperature was raised to 20 °C at 5 °C / h. The ice-nucleating bacteria (SNOMAX®) were not used in these experiments.

Another experiment was carried out using the four spring type parents of the Amagi Nijo × WI2585 and Galleon × Haruna Nijo crosses that had been used to identify the FIS QTLs on chromosomes 2H and 5H (Reinheimer et al. 2004). The plants were sown in a greenhouse during December 2006 where growth conditions were similar to those of the first experiment. To allow for differences in flowering times between the genotypes, multiple seeding dates were used to provide plants that were at a similar developmental stage at the time of frosting. Plants were grown 4 per 20 cm pot. Sixty plants of each genotype were subjected to a frost program in which the temperature began at 18 °C and decreased overnight at a rate of approximately 1 °C / h until the temperature at spike height was -2 °C. The temperature was held at -2 °C for 2 h. The temperature was then lowered in successive steps, 1 °C at a time down to -6 °C, using a 15 min ramping time and 1 h 15 min holding time at each temperature. Replicate groups of plants (12 plants per genotype) were removed from the frost chamber at the end of the -2, -3, -4, -5 and -6 °C steps. SNOMAX® was sprayed as a 2 g / l solution onto the surface of the plants 2 h before the chamber reached -2 °C. Twelve plants per genotype were left in the greenhouse and also sprayed with SNOMAX® at the same time in order to provide unfrosted controls. Plants were visually inspected and photographed 2 weeks after the frost treatment. Between 15 and 30 heads were scored for frost induced sterility per genotype and treatment.

Electrolyte conductance (EC) assays

In the experiment with the four mapping parents, a sample comprised of three spikes or leaves was collected from different plants of each genotype, immediately after each set of plants were

taken out of the frost chamber. Spikes were from tillers at late booting to the stage when one-half of the florets had emerged from the boot (Zadocks 49 – 55). Spikes were removed from the flag leaf sheath and the awns cut off. For the leaf samples, blades (lamina) were taken from the leaves directly below the spike, not including the flag leaf. Each sample was shaken at 220 rpm for 16 h in 30 mL water on an orbital mixer (OM11, Ratek, Australia) at room temperature prior to taking the initial EC reading using a portable conductivity meter (model TPS-LC81, TPS Ltd, Australia). Without changing the solution, the samples were then completely frozen at -80 °C for 5 h and shaken for another 16 h before taking a second reading. Percent electrolyte leakage was calculated as the EC value taken immediately after the frost treatment, divided by the EC after complete freezing, multiplied by 100%.

Segregating families

WI2585 is an Australian feed quality breeder's line (Pallotta et al. 2003), and Amagi Nijo is a Japanese malting cultivar bred by Sapporo Breweries. The Amagi Nijo × WI2585 cross segregated for FIS QTL on chromosome arms 2HL and 5HL, with no genetic interaction detected between the two loci (Reinheimer et al. 2004). The tolerance alleles were from Amagi Nijo and the locus on 5HL is in the vicinity of the *Vrn-H1* vernalization locus. By assaying polymorphisms associated with winter or spring alleles of the cloned *Vrn-H1* and *Vrn-H2* vernalization genes (Fu et al. 2005; von Zitzewitz et al. 2005), Chen et al. (2009c) concluded that Amagi Nijo and WI2585 had the genotypes *vrn-H1 vrn-H2* and *Vrn-H1 vrn-H2*, respectively. Consistent with the genetic determination of growth habit by these loci (Kóti et al. 2006), the progeny of these two lines are all spring type. Nevertheless, the *Vrn-H1* chromosome region controls flowering time in this cross, with the Amagi Nijo (winter; LTR tolerance associated) allele unexpectedly being associated with earlier flowering (Reinheimer et al. 2004; Chen et al. 2009c). Flowering time in this cross is also influenced by the *Eps-2S* and *Flt-2L* loci, positioned on chromosome 2H, near the centromere and distal on the long arm, respectively, with the WI2585 alleles conferring earliness (Reinheimer et al. 2004; Chen et al. 2009c). Molecular markers closely linked to *Vrn-H1*, *Eps-2S*, and *Flt-2L*, and across the distal half of chromosome arm 2HL spanning the FIS QTL, have been developed for the Amagi Nijo × WI2585 cross (Chen et al. 2009a, 2009b, 2009c). We used this information and the markers to select two families suitable for evaluation of the 2HL FIS QTL, in which variation for FIS and flowering time controlled by other loci was minimized. This selection scheme is illustrated in ESM Fig. 1, and

the chromosome genotypes of the parents of these two families are illustrated in Fig. 5. Briefly, an Amagi Nijo × WI2585 F_2-derived F_4 recombinant-inbred family was screened with molecular markers to select an individual (103-1) which was heterozygous for the *Flt-2L* and FIS QTL regions on 2HL, yet homozygous for WI2585 alleles at *Eps-2S* and *Vrn-H1*. Plant 103-1 was used to produce F_4-derived F_6 families by another two rounds of self pollination and individuals from these F_6 families screened with 16 markers located across the distal region of 2HL containing *Flt-2L* and the FIS QTL (marked by dotted lines in Fig. 5). F_6 family 103-1-5 (Family 1) was found to be segregating (with alleles in coupling) across this entire 2HL chromosome region. 103-1-5 F_6 individuals homozygous for WI2585 and Amagi Nijo marker alleles across the whole chromosome segment (three and four plants, respectively) were identified and allowed to self to produce F_7 plants for use in frost experiments. F_6 individual 103-1-2-117 was also derived from a family segregating (with alleles in coupling) across the entire 2HL segment, but had itself resulted from a recombination event between the markers *AGA* and *DUF*, such that it was homozygous for the WI2585 *Flt-2L* allele but heterozygous for the chromosome segment immediately proximal of it representing the putative FIS QTL region. F_7 progeny of 103-1-2-117 (Family 2) were screened with four markers from the segregating 2HL chromosome segment to select individuals homozygous for recombinant and non-recombinant chromosomes (14 plants of each type). These 28 plants were allowed to self to produce F_8 plants for use in frost experiments. The initial F_2 seeds of the Amagi Nijo × WI2585 cross were kindly supplied by Margaret Pallotta. Primers and conditions used for scoring molecular markers were those previously described (Chen et al. 2009a, 2009b, 2009c).

Growth conditions and frost treatments for the analysis of segregating families

Plants to be frosted from Families 1 and 2 were reared in a growth room under a day length of 12 h. Day / night temperatures of 20 °C / 14 °C were used for the first 4 weeks. Temperatures were then decreased over the next 4 weeks to 18 °C / 10 °C, and subsequently maintained at this setting. At flowering, each of the families were subjected to two different types of frost treatments – one involving a minimum temperature of -3.5 ^{o}C with application of the ice nucleator spray, and the other using a minimum temperature of -5.0 ^{o}C without the ice nucleator. Except for variation in the minimum temperature, these frost programs were the same as those used to test the four mapping parents. Per family and frost treatment type, 90 plants of each genotype were frost treated and 54 plants of each genotype were used as unfrosted controls.

Because Family 1 segregated for the *Flt-2L* locus, plants of contrasting 2H genotype (planted in separate pots) flowered at different times and needed to be frost treated on separate days (two different frost regimes × two flowering times = four frost runs). In contrast, because Family 2 was homozygous for *Flt-2L*, plants of contrasting genotype had the same average flowering time, enabling these to be planted in the same pots (2 of each type in alternating fashion per pot) and frost treated at the same time (2 frost runs). Non-frosted controls accompanying the -3.5 ^{o}C frost treatments were kept in the growth room and sprayed with the nucleator solution at the same time as their frosted counterparts. Plants frosted at -5 ^{o}C and their non-frosted controls were not sprayed at all (e.g. with water), as water droplets themselves can also promote freezing.

Statistical analysis

Statistical analyses were performed using analysis of variance (ANOVA) in the statistical software GenStat version 10 (VSN international Ltd, U.K.). The confidence limit was set to 95%.

Results

Frost simulation

Frost simulation profiles were based on natural frost events observed in the field (J. Reinheimer and N. Long, personal communication), in which temperature at spike height gradually decreased throughout the night and leveled out somewhat before rapidly increasing after sunrise. The 21st July 2002 frost event at Loxton which enabled detection of both the 5HLand 2HL FIS QTL by Reinheimer et al. (2004) is represented in ESM Fig. 2. During the running of a basic simulation program (Fig. 1a), the actual temperatures at spike height closely followed the set temperatures and oscillated by less than 0.5 °C around the set temperature during the coldest stage of the program. The variant of this program used for testing the four mapping parents at successively lower temperatures is illustrated in Fig. 1b. During this experiment, temperatures fluctuated by up to several degrees when the door was open to take samples, but then rapidly stabilized. Therefore, the chamber was capable of accurately producing the programmed frost simulation temperature profile over a broad range of temperatures.

Initial frost tests using a panel of five barley genotypes

In previous evaluations of LTR tolerance of barley genotypes in the field (Reinheimer et al. 2004) cv. Amagi Nijo was consistently tolerant, while cvs. Schooner, Keel and Sloop and the line WI2585 ranged from moderately intolerant to highly intolerant. These five barley genotypes were used in initial tests with four different frost simulation programs (Fig. 2). Overall genotype-sterility effects were significant for the programs in which a minimum temperature of -5.5 °C was held for 1 or 2 h. These regimes allowed discrimination of genotypes regarded as tolerant and intolerant, e.g., more damage sustained to intolerant WI2585 than tolerant Amagi Nijo, with the best discrimination achieved with the -5.5 °C / 2 h program (Fig. 2). The other treatments resulted in either more or less sterility, and failed to rank the genotypes according to their expected tolerance levels. The plants subjected to a minimum temperature of -5.5 °C for 4 h also suffered significant levels of damage to leaf and stem tissue (not shown), indicating that this treatment was too severe.

Frost tests using the four mapping parents

In the second set of experiments, conditions for screening frost-induced sterility were explored further using the parents of the Amagi Nijo × WI2585 and Galleon × Haruna Nijo mapping crosses and the ice nucleating agent. The -2 °C / 2 h cold treatment did not increase levels of sterility above that of the non-frosted control plants (Fig. 3). Visual inspection of these plants as they were being withdrawn from the chamber revealed very little freezing of the nucleator solution on the surfaces of the plants, consistent with the fact that the reported ice nucleation temperature of the ice nucleator (-2.6 °C; Missous et al. 2007) is below -2 °C. Thus, the -2 °C / 2 h cold treatment may not have caused freezing in the plant tissues. However, the -3 °C / 1.25 h treatment gave significant overall genotype-sterility effects and differentiated the tolerant and intolerant genotypes (Fig. 3). The frost intolerant lines WI2585 and Galleon showed floret sterility levels of 90 % or more, while the frost tolerant genotypes Haruna Nijo and Amagi Nijo exhibited sterility levels of 20 % and 50 %, respectively. The nucleator solution appeared uniformly frozen on the surfaces of these plants. While visual inspection revealed only minor damage to vegetative tissues in plants subjected to the -2 °C / 2 h or -3 °C / 1.25 h treatments, lower minimum temperatures (-4, -5 and -6 °C for 1.25 h) resulted in extensive damage to all aerial parts of the plants, so that frost induced sterility could not be scored (Fig. 4a). Lack of re-growth also indicated that the crowns of these plants had been killed.

Electrolyte conductance assays

The EC data revealed marked increases in damage to leaf tissues at temperatures of -3 °C or lower (Fig. 4c). Maximal levels of electrolyte leakage (80-90 %) were observed in leaves of all 4 cultivars at -4 °C (Fig. 4c), which also corresponds to the temperature at which the plants were completely killed (Fig. 4a). The electrolyte leakage data suggested that the spikes were slightly less prone to tissue damage than the leaves, because they showed increasing levels of damage from -3 °C down to -5 °C (Fig. 4c), whereas leaf damage increased from -2 °C to -4 °C (Fig. 4b).

Detection of tolerance controlled by the chromosome 2H LTR tolerance locus in Amagi Nijo × WI2585 derived segregating families

Two Amagi Nijo × WI2585 derived families were assessed for frost induced sterility, to evaluate the effect of the chromosome 2H FIS QTL under the adopted frost simulation conditions, and to see whether this QTL could be genetically separated from the closely linked *Flt-2L* flowering time locus. Family 1 was segregating for a relatively large segment of 2H containing both the FIS QTL and *Flt-2L*, whereas Family 2 was homozygous for the *Flt-2L* locus but segregating for the chromosome 2H segment immediately proximal of it (Fig. 5). In Family 2, contrasting genotypes for the 2H chromosome segment carrying the 2H FIS QTL were at similar developmental stages at the time of frosting (Fig. 6), confirming that this family was not segregating for *Flt-2L*. In Family 1 (Fig. 7a), sibling lines homozygous for contrasting alleles in the putative FIS QTL region showed significant ($p<0.05$) differences in sterility after frost treatment, with the allele from the 'tolerant' parent Amagi Nijo lowering sterility levels by an average of 32%. This effect was observed in the plants sprayed with the ice nucleator but not those that were not sprayed, despite the fact that the former were subjected to a less severe minimum temperature (-3.5 ^{o}C versus -5.0 ^{o}C, respectively; Fig. 7a). This suggested that the 2H FIS locus phenotype could be detected under these conditions, and that the effect of this locus is dependent on freezing rather than chilling. Likewise, in Family 2, frosted sibling lines carrying Amagi Nijo marker alleles for the chromosome segment proximal of *Flt-2L* showed significantly lower sterility (by 20%; $p<0.05$) after frosting than those carrying the WI2585 alleles, but only with the -3.5 ^{o}C plus nucleator frost treatment (Fig. 7b). This suggested that the FIS effect was independent of the *Flt-2L* locus. Overall sterility levels were consistently greater in Family 2 than in Family 1, in the unfrosted controls as well as the frost treated plants. During growth prior to frosting, the plants from Family 2 were relatively crowded and were partially etiolated, which we believe accounts for the higher levels of sterility observed in this family.

Discussion

Cold damage to plant tissues arises from alterations in plant membrane structure, as well as physical disruption and dehydration of cells caused by the growth of ice crystals (Pearce 2001; Gusta et al. 2003, 2004). However, water in plant tissues can remain in the liquid state at sub-zero temperatures as low as -12 ^{o}C, in a phenomenon known as supercooling (Huang et al. 2002). The initiation of freezing in biological systems is a complex process (Ashworth and Kieft 1995; Gusta et al. 2003, 2004). High resolution infrared video thermography has shown that freezing in herbaceous plants is initiated primarily on the external surfaces of plant organs and then spreads rapidly through the internal tissues and penetrates into the interior of cells (Pearce and Fuller 2001; Huang et al. 2002; Wisniewski et al. 2002; Gusta et al. 2004). Propagation of ice into internal tissues occurs through open stomata, leaf lesions or through the leaf cuticle (Gusta et al. 2003). A range of extrinsic or intrinsic agents, including bacteria, biological molecules and various forms of debris, serve as natural ice nucleators (Pearce 2001). Wheat spikes located within the boot typically supercool and become more prone to freezing once exposed to the air (Single 1985, 1988; Fuller et al. 2007). Structures which may act as barriers to ice propagation into the reproductive tissues include the inner cuticle of the boot, nodes of the stem, and the palea and lemma (Single and Marcellos 1974; Marcellos and Single 1984; Single 1985, 1988; Gusta et al. 2003; Fuller et al. 2007).

In frost simulation tests performed on barley plants raised in a greenhouse during the Adelaide summer, exposure of the plants to an ice nucleator spray (active ingredient from the bacterium *Pseudomonas syringe*) resulted in a dramatic increase in the induced sterility levels. For example, genotypes Amagi Nijo and WI2585 showed much higher sterility after -3 °C for 1.25 h with the nucleator than after -3.5 °C for 1 h without the nucleator (51 and 90% versus 13 and 12%, respectively; Fig. 2, 3). These data indicate that nucleating sites on the tiller surfaces of these glasshouse grown plants were limiting, and confirm that barley is capable of super-cooling to some level. These findings are also consistent with additional experiments in which detached leaves from the greenhouse were shown to freeze at lower temperatures than leaves collected

from the field in Adelaide (-6 versus -3 °C, respectively; our unpublished data), suggesting that natural nucleators in the greenhouse might be absent or less abundant than in the field.

The sharp increase in EC observed with frosted leaves and spikes at -3 °C and -4 °C, respectively (Fig. 4) is indicative of membrane damage caused by ice crystal growth and subsequent leakage of cellular contents. Spikes assayed by EC were largely enclosed by the flag leaf sheath at the time of frosting. This may have provided partial protection from freezing, potentially accounting for the lower temperature threshold for freezing damage observed in the spikes. In comparison, spread of ice crystals into the leaf tissues appears to have been rapid, because the temperature at which the leaves first showed damage (-3 °C) was close to the ice nucleation temperature of -2.6 °C ascribed to the nucleating agent (Missous et al. 2007). Overall, these EC data support the notion that freezing of the spike tissue occurred around the temperatures used to reveal varietal or gene specific LTR tolerance (-3.0 to 3.5 °C with nucleator spray).

In supercooled plants, organs typically undergo sporadic rapid freezing from discrete sites of nucleation. Within cereal spikes, frost induced floret sterility can vary greatly, occurring in individual florets or groups of florets as a result of propagation of ice from discrete sites (Single and Marcellos 1974; Single 1985; Gusta et al. 2003). Accordingly, it is considered advisable to use ice nucleation spray in plant freezing experiments to reduce the frequencies of non-frozen escapes (Fuller et al. 2007). As illustrated by our experiences (Fig. 2, 3, 4), for frost simulations to produce meaningful FIS data, they must induce some level of sterility but not be so severe that they result in complete sterility or tiller death. Use of a nucleator spray is therefore likely to be of value in achieving reproducible levels of freezing under a given temperature regime, when levels of natural nucleators may be low or variable. An additional requirement for reliable frost testing is a simulation chamber capable of accurately producing the programmed temperature profiles, particularly at the lowest temperatures. Monitoring of the AGRF chamber indicated that it met this requirement (Fig. 1). Using the ice nucleator and the AGRF frost simulation chamber, we were able to define a set of conditions (minimum temperature -3.0 to 3.5 °C for 1.25 to 2 h; Fig. 3, 7) that were both capable of effectively distinguishing between several tolerant and intolerant barley genotypes and able to detect differences in sterility associated with the FIS QTL on chromosome 2H. Further tests will be required to determine whether these conditions are suitable for evaluation of LTR tolerance in other cereal germplasm, including wheat.

In a process referred to as cold-acclimation, vegetative cereal tissues require a period of low but non-freezing temperatures before they can express maximum levels of freezing tolerance. This ability to acclimate steadily decreases after the double-ridge stage which marks the transition from the vegetative to reproductive stage of development (Prášil et al. 2004; Limin and Fowler 2006). A few studies suggest that wheat spikes are capable of some acclimation early in their development, but not later on, after their emergence from the boot (Single 1966; Single and Marcellos 1974; Fuller et al. 2007). In case the expression of LTR tolerance encoded by the barley chromosome 2H locus was acclimation dependent, segregating families used to assess the 2H putative LTR tolerance locus were pre-grown in a growth chamber under cool temperatures which would normally be experienced during winter by a cereal crop prior to flowering in southern Australia. Levels of induced sterility were generally lower in these plants than in the genotypes pre-grown in summer glasshouse conditions (Fig. 3), at least in comparisons with Family 1 (Fig. 7a), suggesting that the cooler conditions may have increased background tolerance levels. However, it should be noted that the growth conditions had a major effect on development, resulting in major delay in flowering time under the cooler conditions (~ 120 versus ~ 40 days). Further work will be required to verify whether cool pre-growth conditions alter background tolerance levels and whether they are necessary for expression of LTR tolerance encoded by the 2H locus.

Flowering time can influence levels of frost induced sterility by enabling escape. However, this factor which can potentially confound the measurement of genuine LTR tolerance can be accounted for to some extent by scoring heads that were at a specific developmental stage at the time of frosting (Reinheimer et al. 2004). This was the scoring strategy that was adopted in the current study. The potential to express vegetative (acclimatable) cold tolerance increases throughout the vegetative growth phase before it begins to decline at the transition to reproductive development (Prášil et al. 2004; Limin and Fowler 2006). Therefore, if vegetative cold tolerance can be residually expressed in heads, flowering time genes that alter the duration of vegetative growth might influence genuine LTR tolerance. Low FIS at the chromosome 2H QTL is associated with late flowering at a nearby locus we named *Flt-2L* (Chen et al. 2009a, 2009c). However in Family 2, which was not segregating for *Flt-2L* (Fig. 5) or for any difference in flowering time linked to *Flt-2L* (Fig. 6), FIS differences was found to be associated with the segregating chromosome segment immediately proximal of *Flt-2L* (Fig. 7b). Also, the reliance on the ice nucleator for detection of the 2H locus controlling FIS at -3.5 °C / 2 h (Fig. 7) indicates

that the phenotype of this locus was a result of freezing and not chilling. Therefore, based on these observations, it would appear that the chromosome 2H locus confers genuine frost tolerance which is independent of the *Flt-2L* locus or any direct/indirect effects related to flowering time. Assuming that an excess of nucleator was applied, it also seems unlikely that the locus confers tolerance by influencing the effectiveness of nucleation sites produced by the plant on the surface of the boot.

The 2H LTR tolerance locus has a relatively subtle effect, conferring differences in FIS levels of 15% (Reinheimer et al. 2004) to 20% (Family 2, Fig. 7b). Use of especially designed families such as Family 2 has particular advantages for the study of the small LTR tolerance effects encoded by this locus. Because the closely linked *Flt-2L* locus was homozygous in this family, plants carrying alternate alleles at the LTR tolerance locus flowered at the same time, could be grown in a paired arrangement in the same pots, and could be treated in the same frost run. This strategy minimizes variation in FIS that might otherwise result from spatial variation in pre-growth conditions, frost chamber temperatures or application of the ice nucleator. The family was also the product of several generations of inbreeding, and was selected as being homozygous for a chromosome 5H segment also known to control flowering time and FIS in the Amagi Nijo × WI2585 cross and the *Eps-2S* locus on chromosome 2H (Reinheimer et al. 2004; Chen et al. 2009c), which would have helped reduce background variation in FIS and flowering time resulting from segregation at other loci. Comparisons of marker-selected sib lines homozygous for each of the two segregating 2H chromosome versions further enabled us to distinguish variation in FIS encoded by the 2H locus from that controlled by other loci. Families such as this one will prove useful for fine mapping and physiological characterization of the 2H LTR tolerance locus. Given that this locus is only one of two LTR tolerance loci thus far reported, its further characterization and eventual isolation could provide important insights for the understanding of LTR tolerance mechanisms in cereals.

Acknowledgement

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assistance in the maintenance of growth rooms and greenhouses, and Joan Vickers from the University of Southern Queensland for helpful discussions and for provision of SNOMAX.

Figure legends

Fig. 1.

Set and actual temperatures at spike height in a custom designed chamber during the running of two frost simulation programs (**a, b)**. The spikes in the actual temperature during the stepwise lowering of the temperature in **b** are the result of opening the door for observation and removal of plants.

Fig. 2.

Spike sterility levels (means and S.E.) of barley lines following four different frost treatments (level and duration of minimum temperatures indicated). No ice nucleating agent was sprayed on the plants.

Fig. 3.

Spike sterility levels (means and S.E.) of parents of barley mapping crosses after exposure to different frost simulation regimes, with ice nucleator spray applied. The level and duration of minimum temperatures are indicated. Treatments of -4 to -6 °C were also used, but these killed the plants and are therefore not represented in the figure.

Fig. 4.

Frost-induced damage of Amagi Nijo, Haruna Nijo, Galleon and WI2585 after treatment with the ice nucleating agent and minimum temperatures of -2 to -6 °C. **a)** Increasing damage to Amagi Nijo plants upon exposure to lower temperatures. Stem damage was visible on plants treated at -3 °C, while plants subjected to the -4 to -6 °C treatments were completely killed. **b, c)** Degree of electrolyte leakage from leaves and spikes of plants from same frost run as the plants illustrated in **a**.

Fig. 5.

Genotypes of chromosomes segregating in two Amagi Nijo × WI2585 families used to assess the effects of the 2H FIS QTL. The segment of the 2HL chromosome arm containing the FIS QTL and *Flt-2L* locus is represented by the molecular marker map on the left, constructed by Chen et al. (2009b, 2009c). The *Flt-2L* location was determined by Chen et al. (2009a), while the interval of the FIS QTL was the one defined by Reinheimer et al. (2004). Genotypes were determined using the molecular markers which are joined to the map by dotted lines. Family 1 was segregating for both the FIS QTL and *Flt-2L* whereas Family 2 was segregating for the FIS QTL only and was homozygous for *Flt-2L*.

Fig. 6.

Developmental stages of all tillers on frosted plants from Family 2, recorded at the time of frosting. Stages were as defined by Zadoks et al. (1974): early booting: 41 – 45; boot swollen or just split and with awns only slightly visible: 47 – 50; tillers with spikes half or fully emerged out of the boot: 53 – 60. Anthesis occurred during stages 47 – 50.

Fig. 7.

FIS (mean and S.E.) of lines from Family 1 (**a**) and 2 (**b**). Lines homozygous for contrasting alleles (Amagi Nijo or WI2585) in the 2H chromosome segment carrying the FIS QTL locus were arranged either in separate pots (**a**) or in an alternating fashion in the same pots (**b**). Lines from Family 2 are all homozygous for the same (Amagi Nijo) allele of *Flt-2L*. Two types of frost treatments were used. One used a minimum temperature of -3.5 ^{o}C and the ice nucleator spray, while the other used -5.0 ^{o}C without the ice nucleator.

ESM Fig. 1.

Derivation of lines used in frost experiments. Each arrow represents one generation of selfing. Chromosome genotypes are illustrated by coloured lines, for the distal 2HL region containing

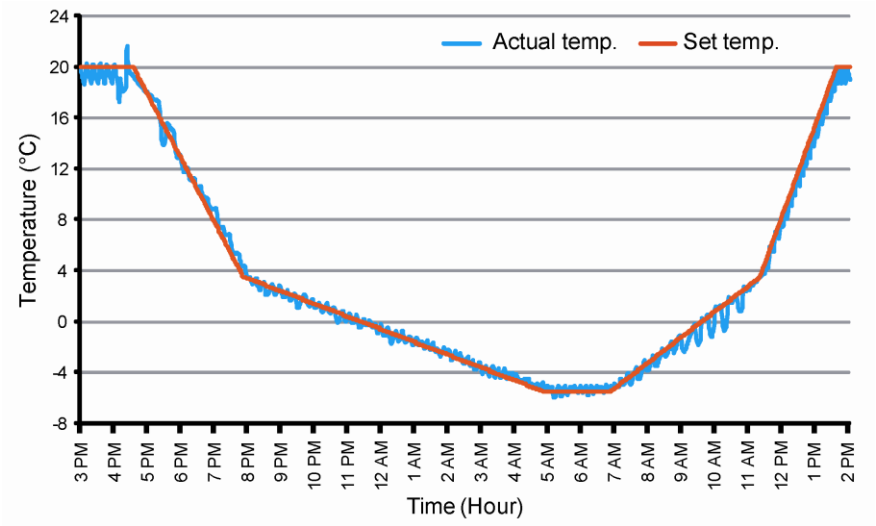
Flt-2L and a FIS QTL, the centromeric chromosome 2H region containing *Eps-2S*, and the 5HL region containing *Vrn-H1* and QTLs for FIS and flowering time.

ESM Fig. 2.

Natural frost event on 21st of July 2002 at Loxton, South Australia, which revealed both barley FIS QTL in the study of Reinheimer et al. (2004). Temperatures were recorded at 1.5 m above the soil surface in a sheltered box with airflow (Stevenson screen) (Air Temp) and by using two thermocouples suspended in the air at average spike height (80 cm) within frosted plots (Canopy Probe 1, Canopy Probe 2). The temperature profile is typical for a cereal crop experiencing a radiant frost event, as reported by others (Marcellos and Single 1975), whereby the top of the crop reaches lower temperatures than the air above due to radiant heat loss from the plants to the cloudless night sky. Data were kindly provided by Jason Reinheimer.

Tables and figures

A



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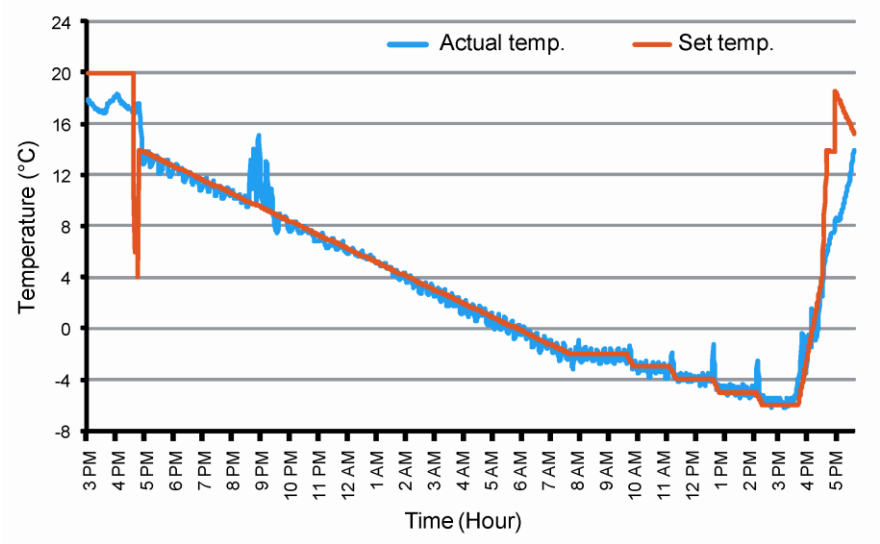

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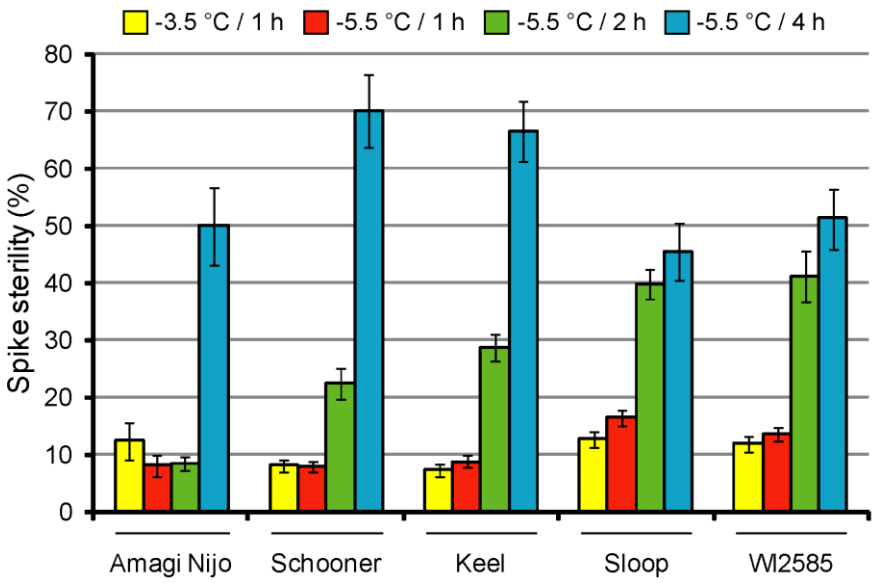

-3.5 °C / 1 h
-5.5 °C / 1 h
-5.5 °C / 2 h
-5.5 °C / 4 h
Spike sterility (%)
80
70
60
50
40
30
20
10
0
Amagi Nijo
Schooner
Keel
Sloop
WI2585

Fig. 2.

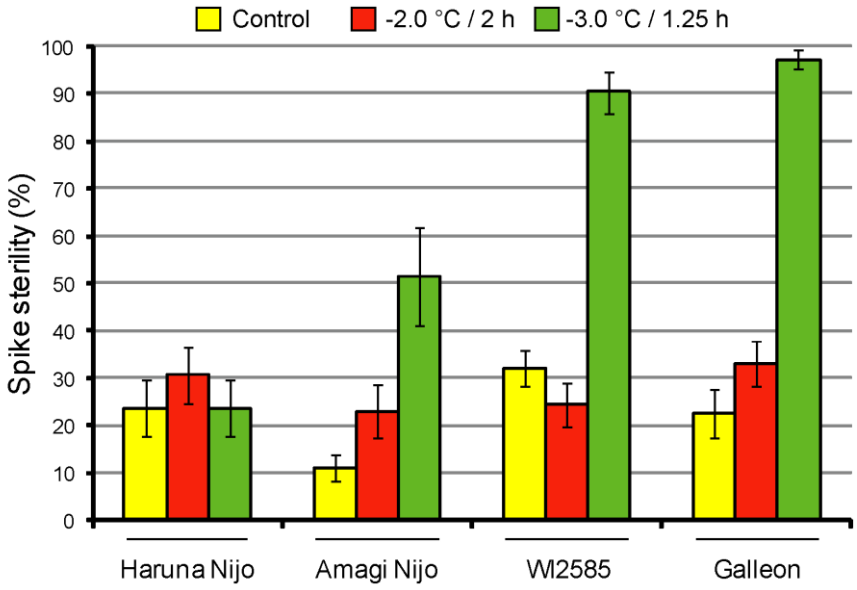
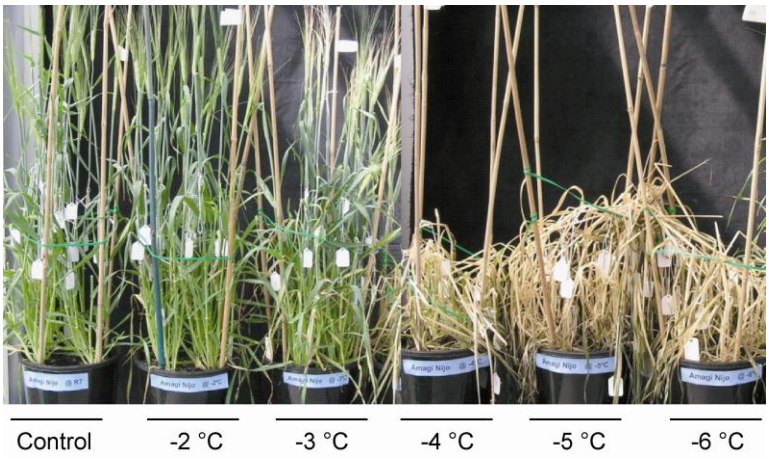

Control
-2.0 °C / 2 h
-3.0 °C / 1.25 h
Spike sterility (%)
100
90
80
70
60
50
40
30
20
10
0
Haruna Nijo
Amagi Nijo
WI2585
Galleon

Fig. 3.

A


Control
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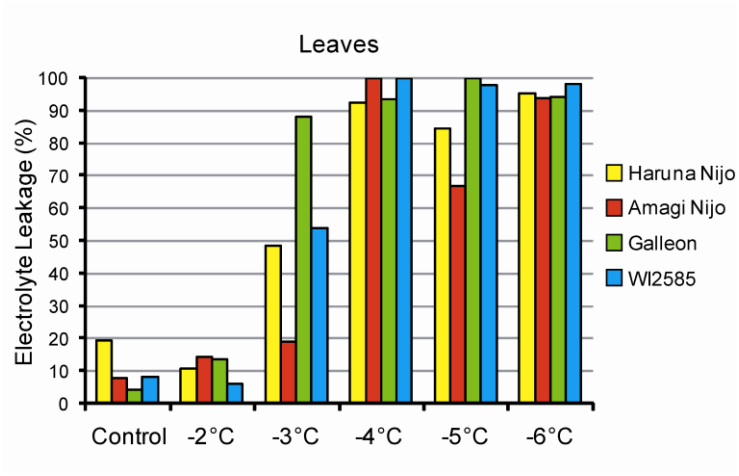
-3 °C
-4 °C
-5 °C
-6 °C

B


Leaves
Electrolyte Leakage (%)
100
90
80
70
60
50
40
30
20
10
0
Control
-2°C
-3°C
-4°C
-5°C
-6°C
Haruna Nijo
Amagi Nijo
Galleon
WI2585

C

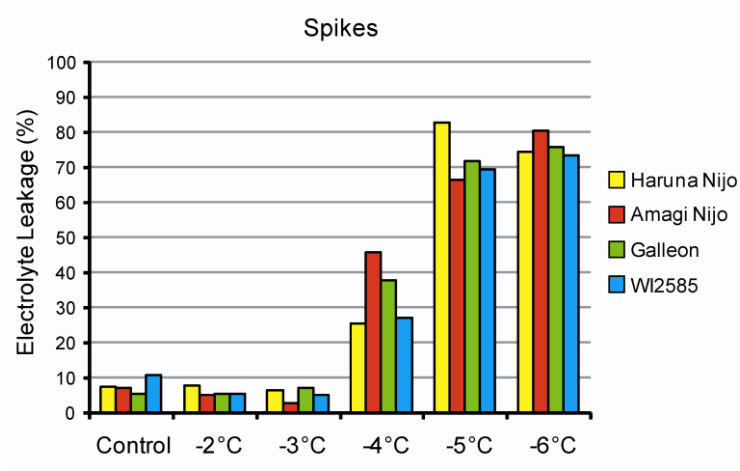

Spikes
Electrolyte Leakage (%)
100
90
80
70
60
50
40
30
20
10
0
Control
-2°C
-3°C
-4°C
-5°C
-6°C
Haruna Nijo
Amagi Nijo
Galleon
WI2585

Fig. 4.

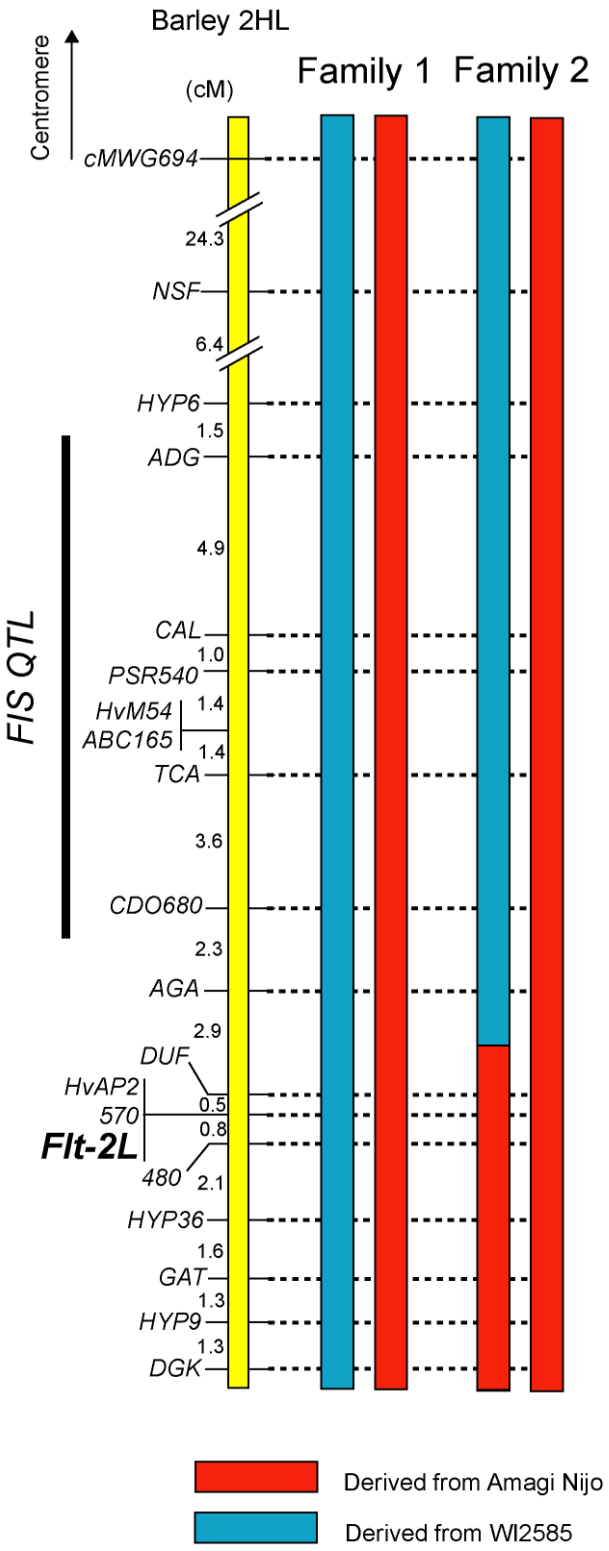

Barley 2HL
Family 1 Family 2
Centromere
(cM)
cMWG694
24.3
NSF
6.4
HYP6
1.5
ADG
4.9
CAL
1.0
PSR540
HvM54
1.4
ABC165
1.4
TCA
3.6
CDO680
2.3
AGA
2.9
DUF
HvAP2
570
0.5
0.8
Flt-2L
480
2.1
HYP36
1.6
GAT
1.3
HYP9
1.3
DGK
FIS QTL
Derived from Amagi Nijo
Derived from WI2585

Fig. 5.

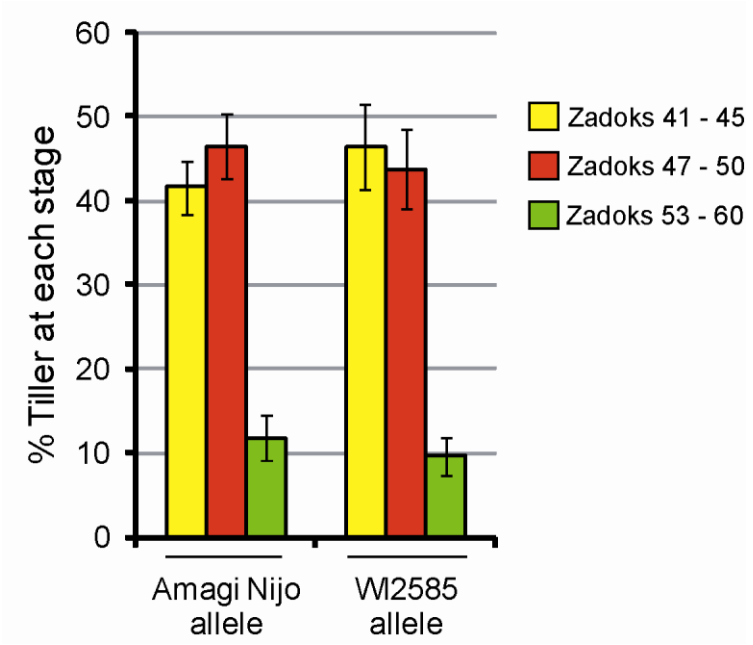
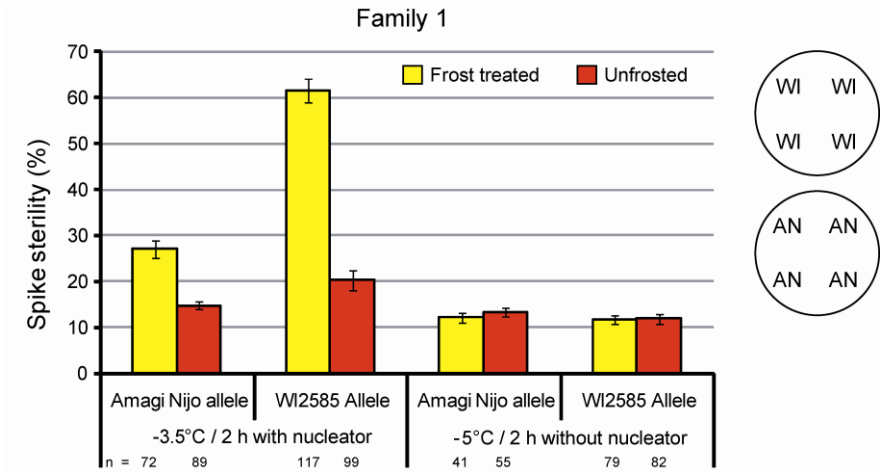

60
50
40
30
20
10
0
% Tiller at each stage
Zadoks 41 - 45
Zadoks 47 - 50
Zadoks 53 - 60
Amagi Nijo allele
W2585 allele

Fig. 6.

A


Family 1
Frost treated
Unfrosted
Spike sterility (%)
70
60
50
40
30
20
10
0
WI WI
WI WI
AN AN
AN AN
Amagi Nijo allele
WI2585 Allele
Amagi Nijo allele
WI2585 Allele
-3.5°C / 2 h with nucleator
-5°C / 2 h without nucleator
n = 72 89 117 99 41 55 79 82

B

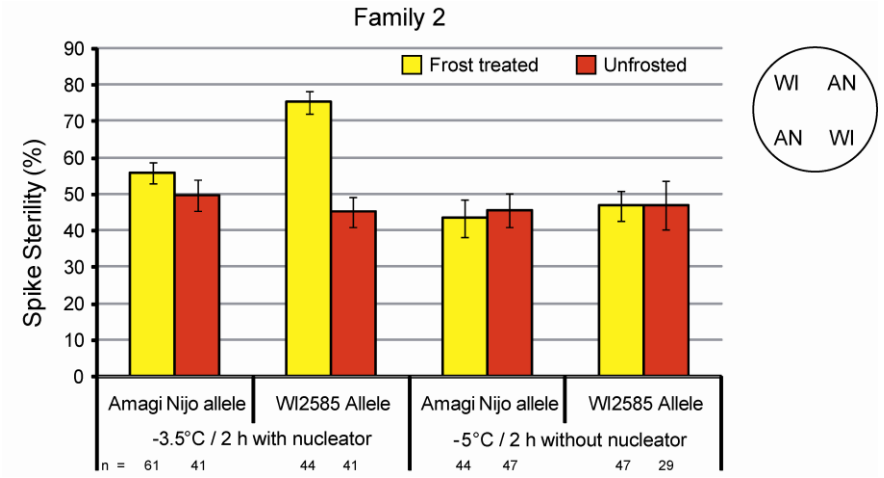
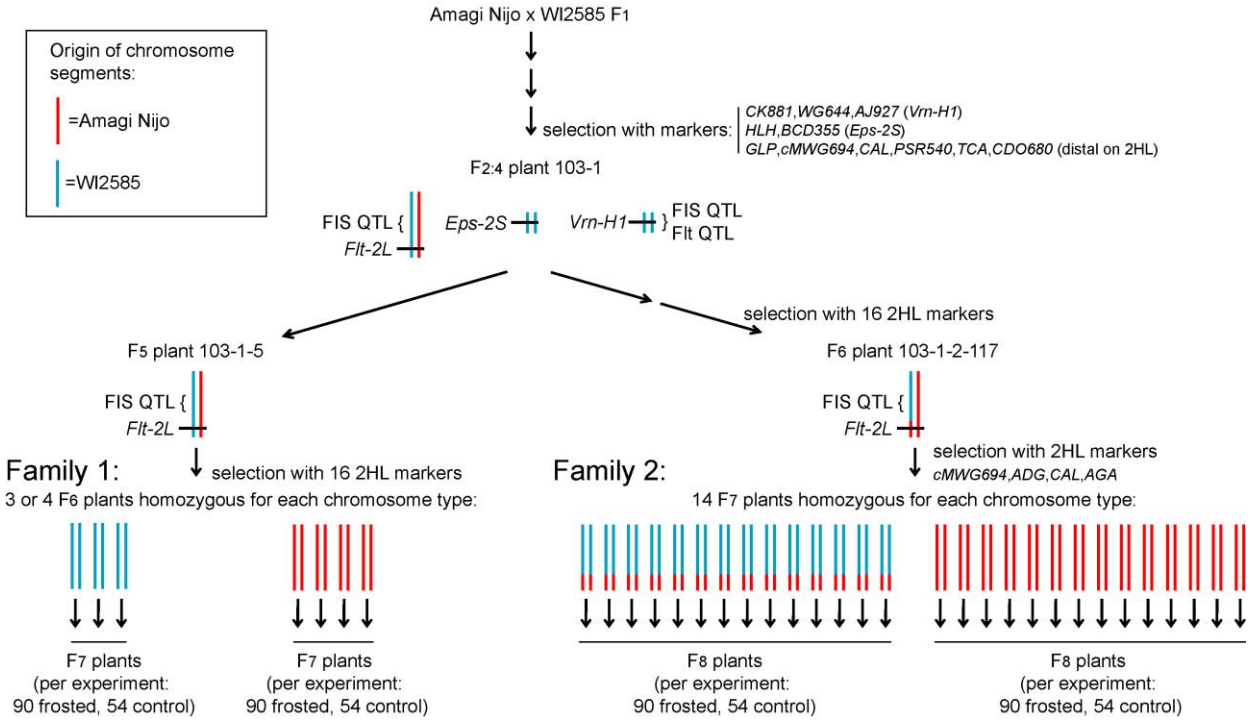
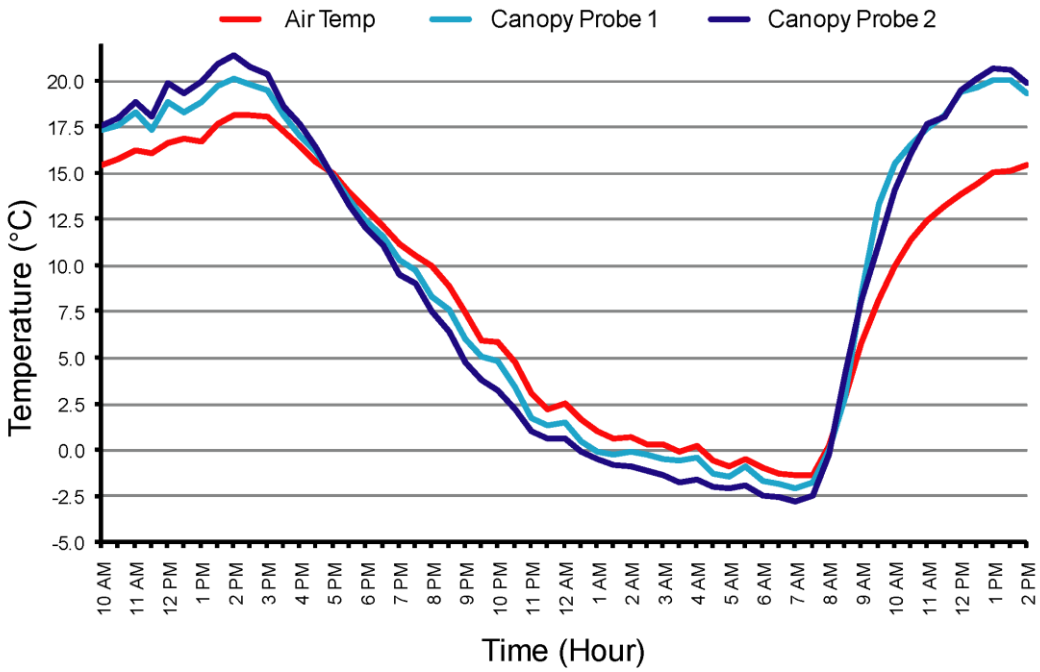

Family 2
Frost treated
Unfrosted
Spike Sterility (%)
90
80
70
60
50
40
30
20
10
0
WI AN
AN WI
Amagi Nijo allele
WI2585 Allele
Amagi Nijo allele
WI2585 Allele
-3.5°C / 2 h with nucleator
-5°C / 2 h without nucleator
n = 61 41 44 41 44 47 47 29

Fig. 7.


Origin of chromosome segments:
=Amagi Nijo
=WI2585
Amagi Nijo x WI2585 F1
selection with markers:
CK881,WG644,AJ927 (Vrn-H1)
HLH,BCD355 (Eps-2S)
GLP,cMWG694,CAL,PSR540,TCA,CDO680 (distal on 2HL)
F2:4 plant 103-1
FIS QTL {
Flt-2L
Eps-2S
Vrn-H1
} FIS QTL
Flt QTL
selection with 16 2HL markers
F5 plant 103-1-5
FIS QTL {
Flt-2L
F6 plant 103-1-2-117
FIS QTL {
Flt-2L
Family 1:
selection with 16 2HL markers
3 or 4 F6 plants homozygous for each chromosome type:
Family 2:
selection with 2HL markers
cMWG694,ADG,CAL,AGA
14 F7 plants homozygous for each chromosome type:
F7 plants
(per experiment:
90 frosted, 54 control)
F7 plants
(per experiment:
90 frosted, 54 control)
F8 plants
(per experiment:
90 frosted, 54 control)
F8 plants
(per experiment:
90 frosted, 54 control)

ESM Fig. 1.


Air Temp
Canopy Probe 1
Canopy Probe 2
Temperature (°C)
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
-2.5
-5.0
10 AM
11 AM
12 PM
1 PM
2 PM
3 PM
4 PM
5 PM
6 PM
7 PM
8 PM
9 PM
10 PM
11 PM
12 AM
1 AM
2 AM
3 AM
4 AM
5 AM
6 AM
7 AM
8 AM
9 AM
10 AM
11 AM
12 PM
1 PM
2 PM
Time (Hour)

ESM Fig. 2.

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6 Conclusion

6.1 Project summary

Efforts to determine the underlying basis of LTR tolerance in cereals have been promising so far. The dissection of LTR tolerance in barley using a forward genetics approach has revealed new insights into the regulation of LTR tolerance. The findings can be summarized as follows.

- Significant flowering time effects were segregating in the 2H and 5H chromosome regions controlling LTR tolerance.
- The *Flt-2L* locus (2HL) affected not only flowering time, but also plant height and spike compactness, possibly indicating pleitropic effects controlled by one gene. A homologue of the Arabidopsis *APETALA2* gene is the best candidate for *Flt-2L* based on the observation that *AP2* orthologues control flowering time in wheat and maize.
- Segregation of flowering time was also detected in the 5H LTR chromosome region carrying the *Vrn-1*/*Fr-1* loci. The extent of the effect varied between 7 to 25 days depending on the growth conditions used. In both Haruna Nijo × Galleon and Amagi Nijo × WI2585 crosses where vernalization segregated in the former but not latter, early flowering was consistently associated with the winter *vrn-H1* alleles derived from the Japanese parents. This suggests that early flowering at anthesis is functionally independent of the vernalization requirement. The effect contrasts with the vernalization requirement which normally associates winter *vrn-1* alleles with late flowering. Therefore, early flowering conferred by the winter *vrn-H1* alleles could be due to the ability of the *Vrn-1* gene to influence flowering time after floral initiation or a gene closely linked to *Vrn-1*.
- In addition to flowering time, the 5H *Vrn-1* chromosome region also influenced rachis length and floret number per spike, whereas *Flt-2L* affected rachis elongation but not floret number per spike, highlighting different ways in which these flowering time genes affect development.
- At the 5H locus, tolerance was associated with alleles derived from the Japanese parents. The tolerance seemed to be expressed in both winter and spring types from the Galleon ×

Haruna Nijo cross that segregates for vernalization, suggesting that expression of LTR tolerance at this locus is functionally independent of the determination of winter/spring growth habit by the *Vrn-1* locus.

- Flowering time effects can be accounted for by tagging spikes at the same developmental stage. At the 2H locus, LTR tolerance is unlikely to be determined by flowering time *per se*. On the other hand, possibility remains that an indirect effect of flowering time in altering some physiological or biochemical (intrinsic) properties of the plants can lead to tolerance at the 5H locus.
- Expression of LTR tolerance was detectable only when a narrow range of minimum temperature/duration was used. Uniformity of freezing can be improved by using an ice nucleating agent. In comparison, a lower minimum temperature without the nucleator did not detect the 2H effect, suggesting that the 2H tolerance gene provides enhanced tolerance to freezing rather than chilling. Furthermore, the 2H LTR tolerance can be genetically separated from *Flt-2L* by recombination.
- Different temperature (28 /22 °C vs 18 /10 °C; day /night) and photoperiod (11 – 12 vs >13 h) regimes can accelerate or delay flowering time (40 vs 120 days to awn emergence) and can also affect plant's ability to accumulate cold potential for acclimation at the vegetative stage. It still waits to be seen whether the low temperature pre-growth conditions (18 / 10 °C; day /night) have any impact on the expression of LTR tolerance.

The effects at these loci will need to be validated in different field environments (northern vs. southern Australia). Reliable and reproducible assay methods for detecting LTR tolerance will aid the positional cloning of genes controlling LTR tolerance in barley. Ultimately, the incorporation of 2H and 5H LTR tolerance alleles into breeding programs will deliver benefits to the farmers and the industry. This should enable the breeding of locally adapted barley varieties with frost tolerance. However, these loci should be vigorously examined using both genetic and physiological approaches to further understand the molecular mechanism controlling tolerance at these loci. Future work should aim to confirm and validate frost tolerance *per se* on 2H and 5H in different environments. The generation of improved map locations and closer markers (1 – 5 cM) will allow the breeders to exploit these LTR tolerance alleles in breeding programs.

6.2 Genetic resources for future work

An F_6 Amagi Nijo × WI2585 population consisting of approximately 2000 F_7 families (50-100 seeds per family) derived from a single F_4 line (103-1) was developed in 2006 (Chen, unpublished) and is available for high resolution genetic mapping of LTR tolerance and *Flt-2L* on 2HL. The F_4 progenitor line 103-1 was heterozygous for the chromosome segment covering the LTR and *Flt-2L* loci and homozygous for WI2585 marker alleles at the other developmental loci, namely *Eps-2S* and *Vrn-H1* that are known to be segregating in this cross. A marker screen was also performed at the F_5 generation and only individuals heterozygous for all marker defined loci in the 2H LTR region were selected for propagation in the next generation. Two generations of self crossing used to derive the F_6 mapping population would have reduced some of the background heterozygosity. A similar approach has been used to dissect the chromosome region controlling 5H LTR tolerance (Reinheimer, unpublished) but a large population has not yet been made. On the other hand, tolerance effects can now be validated using a newly developed Haruna Nijo × Galleon F_2 population (Chen et al. 2009).

A population of lines containing chromosome segments of a wild barley (*Hordeum spontaneum*) introgressed in to a Haruna Nijo genetic background has been made (Hori et al. 2005). Recombinant chromosome substitution lines (RCSLs; BC_3F_2) for both 2H and 5H QTL regions are available and can be used to independently verify the LTR tolerance QTL effects.

The Haruna Nijo BAC library obtained from Kazuhiro Sato's lab (Saisho et al. 2007) will enable recovery and sequencing of the genomic regions containing the frost tolerance genes, once the locus intervals have been significantly narrowed down by further marker development and phenotyping. The *Brachypodium* BAC library will provide an alternative source for gene prediction. Its advantages of smaller genome size and closer relationship to cereals than rice provide a fast effective way in pulling out BAC clones in the frost interval for sequencing. Candidate genes from the corresponding interval of *Brachypodium distachyon* can be retrieved using the complete genome sequence of *Brachypodium distachyon* to identify genes that are absent in rice.

6.3 Population development for field based screening

Genuine tolerance effects detected from our F_2 derived populations will need to be validated using other populations in the field. The strategy of marker-assisted backcross introgression

(Pillen et al. 2003) can be used to introduce the 2H and 5H frost-tolerance alleles into the genetic background of a locally adapted cultivar. Such advanced backcross strategies have been used to identify QTLs for agronomic traits in barley (Matus et al. 2003; Pillen et al. 2003; 2004; von Korff et al. 2004; 2006). Near-isogenic lines (NILs) for the 2H and 5H LTR tolerance loci have been developed (Reinheimer, unpublished). The donor line for backcross introgression was selected from the Galleon × Haruna Nijo DH population. It possessed tolerance marker alleles of Haruna Nijo at the 2H and 5H QTL regions and was mostly homozygous for Galleon in the genetic background. This line was backcrossed twice to WI3806 (Mundah/Keel/Barque; a well-adapted breeder's line) to derive BC_2F_1 plants which were used as donors for the production of 300 DH lines. SSR and DArT marker genotyping identified 66 DHs carrying introgression segments from the tolerant Haruna Nijo parent at either or both QTL regions. Some of these lines are currently being evaluated in the field for both frost tolerance and broad agronomic traits (Reinheimer, unpublished). Preliminary field work showed that lines introgressed with the 2H and 5H tolerance alleles flowered very early, indicating that flowering time effects at these loci can be quite severe under certain environments (Reinheimer, personal communication). Field testing of Amagi Nijo × WI2585 F_7 recombinants in the 2H LTR and *Flt-2L* interval revealed similar maturity effects that interfered with the scoring of FIS on plants after having been exposed to natural frost events (Shannon 2007). Therefore, flowering time effects from these regions must be removed in order for LTR tolerance to be assessed reliably. Possible attempts to produce a frost tolerant variety are some way off.

6.4 LTR tolerance: a phenotypic challenge

Positional cloning of LTR tolerance in barley has proved to be a challenge, mostly due difficulties in establishing reliable methods for phenotyping. Other abiotic stress traits in cereals including boron and/or aluminum tolerance can be easily characterized at seedling stages of growth using phenotypic variation in ion/metabolite accumulation, dry weight, root length and leaf symptoms (Schnurbusch et al. 2007; Sutton et al. 2007; Collins et al. 2008). In some cases, a gene controlling a trait might be involved in a known biochemical pathway so that direct proof-of-function on convincing candidates is possible without further phenotypic analysis and high resolution mapping. Using rice-barley co-linearity, Burton et al. (2006) demonstrated that a cluster of cellulose synthase-like genes from rice was linked to a major QTL for (1,3;1,4)-β-D-

Glucans in barley (Burton et al. 2006). Since (1,3;1,4)-β-D-Glucans are present in the walls of grasses, and are absent in walls of dicotyledons or most other monocotyledonous plants, direct functional complementation was confirmed by the presence of (1,3;1,4)-β-D-Glucans in the cell wall of transgenic *Arabidopsis thaliana* plants carrying the cellulose synthase-like genes. However, no such biochemical trait is known to control the LTR tolerance encoded by the 2H and 5H loci.

6.5 Metabolomics: an alternative approach to phenotype LTR tolerance

The relative floret sterility level is a measure of tolerance but direct biochemical and/or physiological changes leading to enhanced tolerance can provide a more efficient method for the detection of LTR tolerance. Changes in the profile of metabolites can often be used to define the biochemical phenotype of a cell or tissue (Sumner et al. 2003). A metabolomics approach could be potentially employed to investigate the changes in metabolite pools of plants differing in frost tolerance. Analysis could include metabolic pathways in primary metabolism, such as carbohydrates, organic acids and lipids/fatty acids. A mass spectroscopy facility is available on the Waite campus and could be used for metabolomic studies. Anther tissues from frost tolerant and susceptible lines exposed to a simulated frost event could be collected for analysis. Differences in the metabolic profiles, such as the accumulation of sucrose in the tolerant line, for example, may allow us to identify an easily measurable biochemical component of the tolerance mechanism. This could be used to more effectively narrow down the frost interval. Osmolyte accumulation is often associated with cold acclimation and winter hardness. For example, carbohydrates act as cryoprotectors that provide the osmotic buffer to stabilize cells during freezing caused dehydration. However, not all targeted genes could be detected via a metabolite. If the target gene produces a protein that interacts with membrane bi-layers in order to stabilize membranes against freezing, it could be difficult for mass spectroscopy to detect, depending on the type of MS analysis performed.

6.6 Chilling induced male sterility in cereals

Cold acclimation and freezing conditions can induce changes in the sugar content of plants at the vegetative stage of growth (Savitch et al. 2000; Livingston et al. 2005; Livingston et al. 2006;

Hincha et al. 2007; Valluru et al. 2008), leading to enhanced freezing tolerance (Livingston and Henson 1998; Uemura and Steponkus 2003; Klotke et al. 2004). Plants at the reproductive stage are generally more sensitive to low temperature than those at the vegetative stage. In rice and sorghum, low temperature exposure at the young microspore (YM) stage of development (double-ridge formation) can lead to chilling-induced pollen sterility (Oliver et al. 2005; Mamun et al. 2006; Wood et al. 2006). This stage coincides with the process of pollen cell formation, the deposition of pollen cell wall, synthesis of locular fluid and the delivery of nutrients to pollen grains via tapetum cells (Oliver et al. 2005; Mamun et al. 2006). It was shown that cold stress blocks the mobilization of sugars to the YM, leading to abortion of pollen development and sterility (Oliver et al. 2005; Oliver et al. 2007). Furthermore, tolerance can be positively correlated with the differential expression of well wall bound invertase genes induced by cold (Oliver et al. 2005). Homologues of these invertases from rice can be mapped in barley and the ones mapped close to the 2H and 5H LTR QTL regions can be analyzed further for head tissue specific expression in response to cold.

6.7 *BCD453*: a cell wall bound invertase from barley

In our study, the gene marker *BCD453* was derived from a rice orthologue representing a putative apoplastic invertase. Analysis of its EST sequence using the 'Signal P' program (BF258913, BF256656; http://www.cbs.dtu.dk/services/SignalP/) revealed that the BCD453 protein carries a signal peptide of 25 amino acids (Probability = 0.994) at the N-terminus that may play a role in the movement of the protein from golgi to the extracellular space via the default secretory pathway of endoplasmic reticulum (Chen, unpublished). EST sequence alignment showed that *BCD453* shared a 95% DNA sequence similarity to an extracellular invertase gene *exin1* isolated from *Triticum monoccocum* (Greenshields et al. 2004). The rice invertase gene *LOC_Os04g56920* used to derive the gene marker *BCD453*, shared 79% DNA similarity to another rice invertase gene *LOC_Os04g56930* located <5 kb downstream from it at nucleotide position 33.72 MB on rice chromosome 4, indicating a likely tandem duplicated gene copy. Their nucleotide position and sequence revealed that they are cell wall bound invertases *OsCIN6* and *OsCIN7* analyzed for phylogeny and gene expression from a previous study (Ji et al. 2005).

BCD453 presents a potential candidate for the 2H locus due to its close proximity (1 – 5 cM) to the 2H LTR tolerance QTL and likely roles of cold responsive invertases in the chilling tolerance from rice. Expression analysis was carried out to examine *BCD453* activity in response to cold treatment. Using the 'tolerant' Haruna Nijo (HN) and 'intolerant' Golden Promise (GP) parents, leaf and spike tissue samples were collected at different time points during a frost simulation experiment. Subsequent qRT-PCR analysis showed that *BCD453* transcript level was consistently higher in both leaves and spikes of HN, compared to GP (Chen, unpublished). At the same time, transcript levels were significantly higher in spikes than leaves of both parents. In the HN leaves and spikes, *BCD453* transcript level increased with decreasing temperature. In contrast, the transcript level of GP was slightly decreased in response to increasing temperatures, suggesting that this gene is differentially expressed in different genotypes. The overall expression data suggests that *BCD453* is a low temperature responsive gene. Future work should examine the transcript levels of *BCD453* in progeny families segregating for 2H LTR tolerance. Given that sugar compounds can act as osmoprotectants in plants (Gusta et al. 2004), gas chromatography-mass spectrometry (GC-MS) can be used to measure the relative changes in sugar content of plant leaf, spike and stem. Concentrations of other compounds of interest including prolines and betaines should also be measured. Significant correlations between the accumulation of a specific metabolite and the 2H LTR chromosome genotypes can be exploited and used to develop a quantitative detection method for validating the tolerance effect controlled by this locus.

6.8 Identification of candidate genes using integrated approaches

It is difficult to perform candidate gene analysis without having narrowed down the number of candidates to a manageable number. Whole genome transcript profiling experiments have identified genes responding to salt, drought and cold stress in barley and other plant species (Kawasaki et al. 2001; Seki et al. 2001; Fowler and Thomashow 2002; Kreps et al. 2002; Ozturk et al. 2002; Ueda et al. 2004; Walia et al. 2006). Transcript profiles allowed large sets of genes (thousands) that are differentially expressed under abiotic stress to be genetically mapped onto individual barley chromosomes, overlaid with abiotic stress QTLs (Rostoks et al. 2005a; Rostoks et al. 2005b). Similarly, barley homologs of Arabidopsis genes regulating low temperature tolerance can be mapped directly onto consensus genetic maps showing overlapping cold stress related QTLs across all chromosomes (Skinner et al. 2006; Tondelli et al. 2006). Thus, the data

generated from these integrated approaches can help to identify potential candidates for the 2H and 5H LTR tolerance. Although cold stress profiling on whole barley genome scale using reproductive organs has not been performed so far, it is still feasible to use some of the publically available EST data, transcript profiles targeting other stresses or reproductive development and homologs that have a known or predicted function in other model species, to identify candidate genes in our target region.

6.9 TILLING

Once candidate genes are identified, reverse genetic approaches such as targeting induced local lesions in genomes (TILLING) can be used to examine the effect of various mutations in the genes. TILLING is a non-transgenic approach that is applicable to most plant and animal genomes, regardless of its ploidy level or genome size (Comai and Henikoff 2006; Barkley and Wang 2008). TILLING searches the genomes of mutagenized plants for mutations in chosen genes, typically single base-pair substitutions or small insertions and deletions (Comai and Henikoff 2006; Barkley and Wang 2008). Mutations can be silent, miss-sense, non-sense or regulatory (splice sites), allowing the detection of phenotypic changes caused by the various mutations in a gene. Large ethyl methane sulfonate (EMS) or sodium azide induced mutant populations from barley are available (Caldwell et al. 2004; Talame et al. 2008) and can be used to identify mutants at targeted genes using TILLING. Therefore, TILLING provides a fast and effective method to determine the function without the use of transgenic plants.

6.10 Eco-TILLING

The cloned genes can be used to mine genotypes for naturally superior alleles of LTR tolerance at both loci. One approach is to use Eco-TILLING, a variant of TILLING. Eco-TILLING is cost effective for SNP discovery across a large number of germplasms due to the fact that same haplotype from different germplasms is only sequenced once, removing the need for large scale sequencing (Comai et al. 2004). Eco-TILLING approaches to identify and manipulate allelic variants of genes controlling agronomically important traits from wheat and barley have shown potential as tools for crop improvement (Slade et al. 2005; Slade and Knauf 2005; Mejlhede et al. 2006). The alleles identified from germplasm collections should be introduced into locally

adapted varieties so that its relative value in a target environment can be tested (Varshney et al. 2006). Ultimately, the introduction of superior alleles of LTR tolerance genes into breeding programs may deliver benefits to the farmers and the grain industry.

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