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ORIGINAL PAPER

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

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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.

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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. 2009) 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 (Kurauchi 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. 2009) 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. (2009). Except for the markers described below, all of the markers were previously described by Chen et al. (2009).

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 22' 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′-AATGGTGGATCGGCAGGAGGCAC TG-3′) and Ppd-R (5′-GTGGCGGGAGGTTATCTCTCC ACGG-3′). This marker produces an undigested product of 488 bp from *ppd-H1* alleles and digestion products of 428 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). Colinearity 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. 2009) 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_2 s were grown in a greenhouse in Adelaide during January–February when day length was 13–14.5 h and average day/night temperatures in the greenhouse were approximately 28/22°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 h and average day/night temperatures in the greenhouse were 26/20°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 h and average day/night temperatures in the greenhouse were 22/16°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

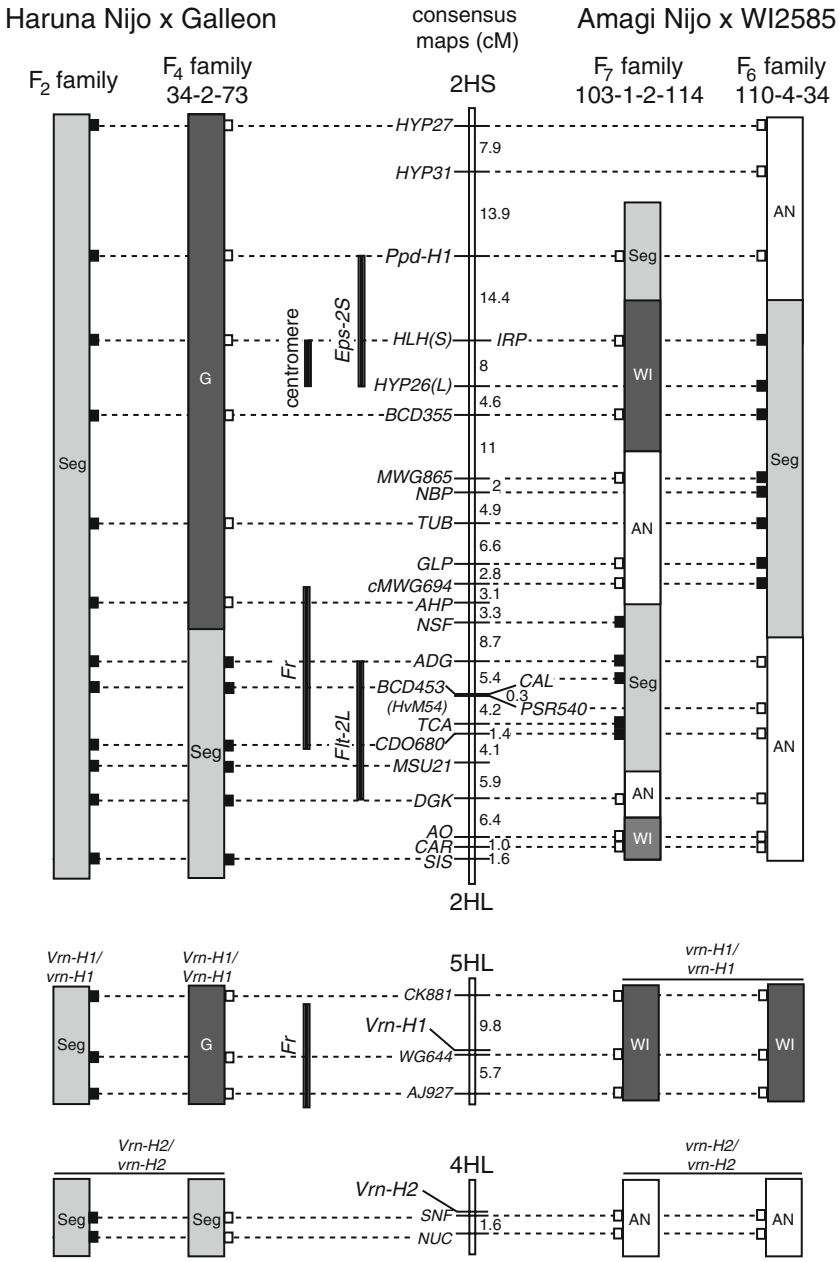

Fig. 1 Genotypes of families used in the analyses, for chromosome 2H, and for *Vrn-H1* and *Vrn-H2* regions on 5HL and 4HL chromosome arms, respectively. Markers joined by *dotted lines* were used to determine the genotype of the parent plant of each family, while *black boxes* indicate markers used 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. 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. 2009). *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

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 (6th 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

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 22 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

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 *AJ927* 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. 2009).

The Haruna Nijo × Galleon F_2 population was grown under long days (13–14.5 h) 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*

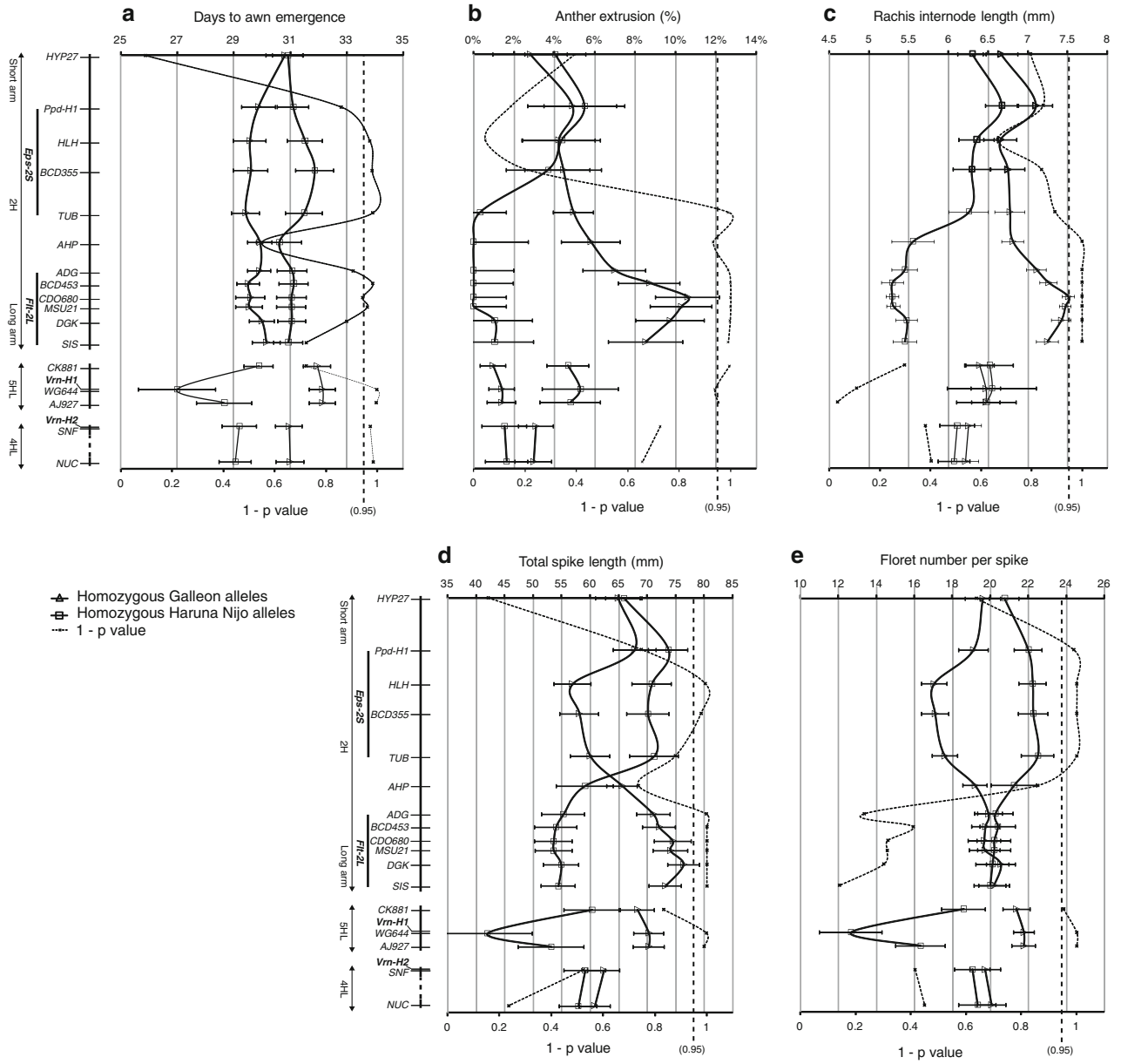

Fig. 2 Associations of markers with flowering time, anther extrusion, rachis internode length, total spike length and floret number per spike, in 77 spring type Haruna Nijo × Galleon F_2 plants. *Plots* show trait values (means ± SE) for individuals homozygous for alternate alleles for molecular markers 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

effects should be observable under 14 h 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). In 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 presence of the *Vrn-H2* gene (*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* (3.5–5 days vs. 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, 0–1.6 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.

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	NS	36.9%	21.8%	NS
Rachis internode length	NS	91.8%	NS	NS
Total spike length	20.3%	35.7%	29.3%	NS
Floret number per spike	45.4%	NS	49.4%	NS

Only homozygotes were used for inferring genotypes

NS no significant association found

[a] Genotypes inferred using marker *HLH*

[b] Genotypes inferred using marker *MSU21*

[c] Genotypes inferred using markers *WG644*

[d] Genotypes inferred using markers *NUC*

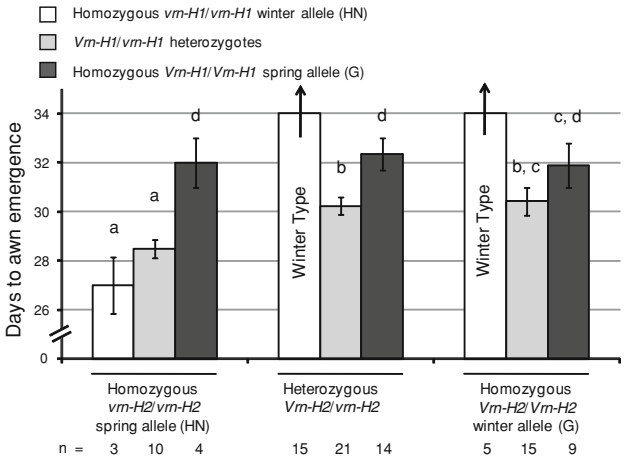

Fig. 3 Flowering time (means ± SE) of non-vernalized Haruna Nijo × Galleon F_2 plants carrying different allele combinations at *Vrn-H1* and *Vrn-H2* loci. Genotypes at the *Vrn-H1* and *Vrn-H2* loci were deduced using the *WG644* and *SNF* markers, respectively. The winter genotype combinations (*vrn-H1*/*vrn-H1*; *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

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 92% 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.

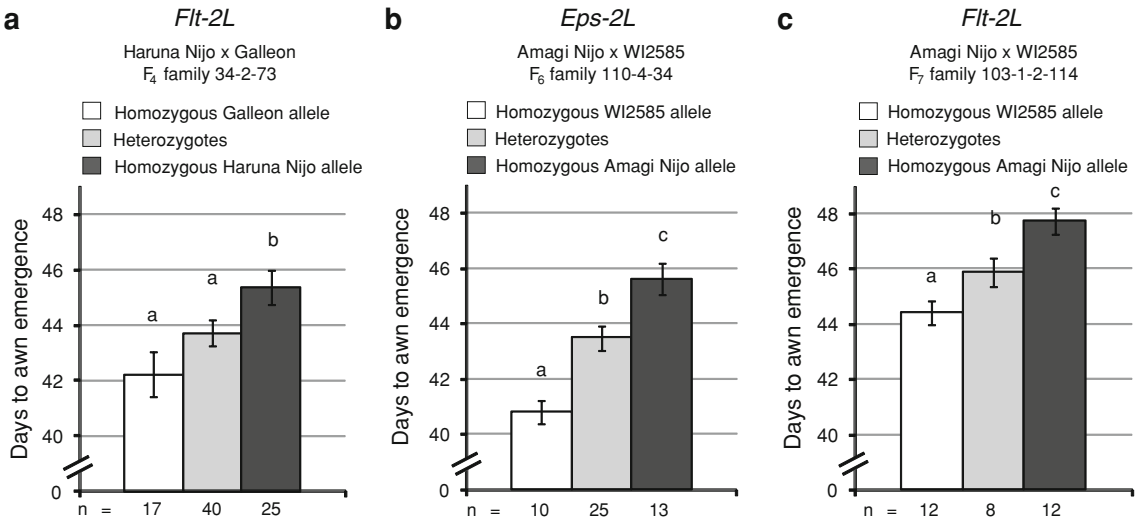

Fig. 4 Control of flowering time by *Eps-2S* and *Flt-2L* loci on chromosome 2H, in F_4, F_6 and F_7 families. Days to awn emergence (means ± S.E.) is shown for different genotypes in each family. *Flt-2L* but not *Eps-2S* was segregating in families 34-2-73 (**a**) and 103-1-2-114 (**c**), whereas *Eps-2S* 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

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 (Fig. 4b, c).

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 (Fig. 2a) and/or because the conditions in the field may not have favored its expression. 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). 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, 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*. This either reveals a novel activity of *Vrn-H1* alleles 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 (up to 5 days vs. 4.7 weeks, respectively). However, the effect in the Amagi Nijo × WI2585 cross was smaller in other experiments (4.9–11.4 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 proximal 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 experimentation (e.g. fine mapping or expression studies) will be required to explore these possibilities.

Variation for the chasmogamy/cleistogamy (open/closed floret) trait in barley is controlled by a major locus (*Cly*) that has been mapped 0–1.6 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–e), 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. 2003) 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 (Fig. 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.

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