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ORIGINAL PAPER

Varietal and chromosome 2H locus-specific frost tolerance in reproductive tissues of barley (*Hordeum vulgare* L.) detected using a frost simulation chamber

Andrew Chen · Lawrence V. Gusta · Anita Brûlé-Babel · Richard Leach · Ute Baumann · Geoffrey B. Fincher · Nicholas C. Collins

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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 quantitative trait locus (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.

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A. Chen · U. Baumann · G. B. Fincher (✉) · N. C. Collins
Australian Centre for Plant Functional Genomics (ACPFG), School of Agriculture, Food and Wine, University of Adelaide, Glen Osmond, SA 5064, Australia
e-mail: geoff.fincher@adelaide.edu.au

L. V. Gusta
Department of Plant Sciences, University of Saskatchewan, Saskatoon, SK S7N 5A8, Canada

A. Brûlé-Babel
Department of Plant Science, Faculty of Agricultural and Food Sciences, University of Manitoba, Winnipeg, MB R3T 2N2, Canada

R. Leach
Plant and Food Science, School of Agriculture, Food and Wine, University of Adelaide, Glen Osmond, SA 5064, Australia

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 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, c). 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 45–50, 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 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 (Zadoks 49–54). 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 electrolyte conductance (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 allele (which is associated with LTR tolerance) 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). A genome-wide QTL analysis by Reinheimer et al. (2004) identified no other flowering time or FIS loci segregating in the Amagi Nijo × WI2585 cross. 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, b, c). 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, b, c).

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°C with application of the ice nucleator spray, and the other using a minimum temperature of −5.0°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°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°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, UK). 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 21 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 <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

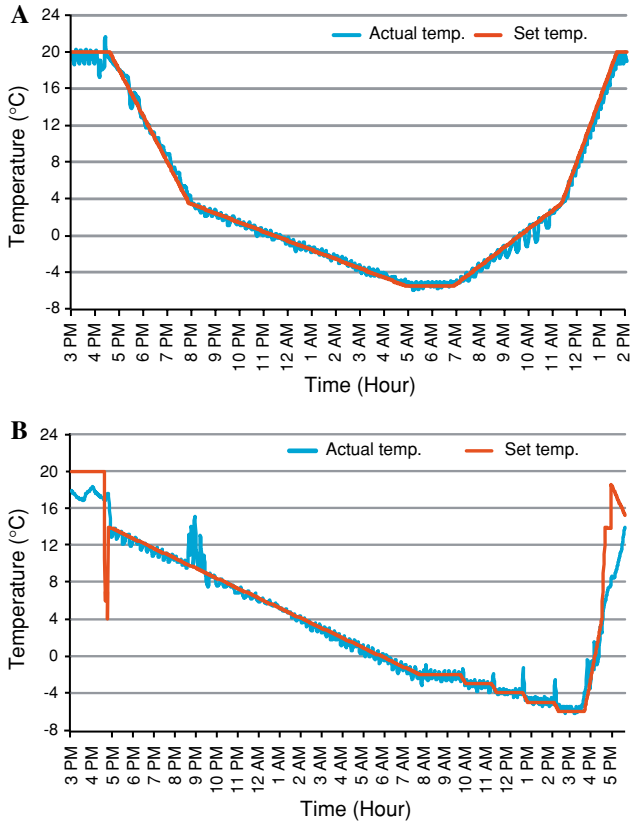

Fig. 1 Set and actual temperatures at spike height in a custom designed chamber during the running of two frost simulation programs (**a**, **b**). The spikes in the actual temperature during the stepwise lowering of the temperature in **b** are the result of opening the door for observation and removal of plants

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

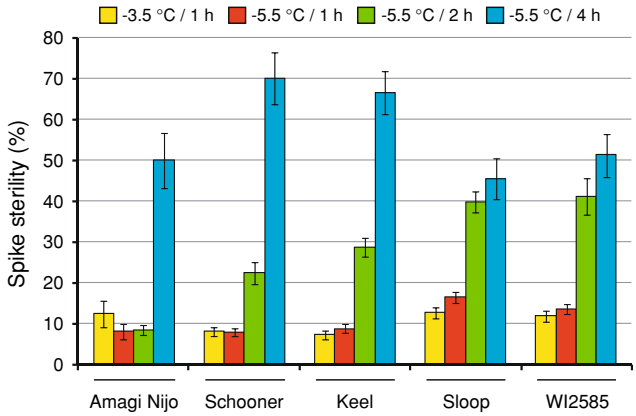

Fig. 2 Spike sterility levels (mean and SE) of *barley lines* following four different frost treatments (level and duration of minimum temperatures indicated). No ice nucleating agent was sprayed on the plants

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

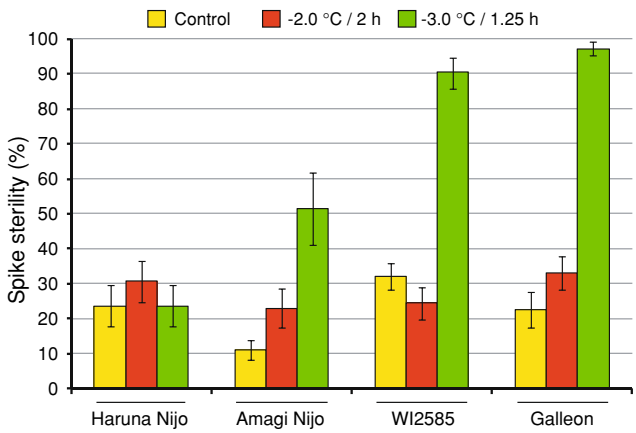

Fig. 3 Spike sterility levels (mean and SE) of parents of barley mapping crosses after exposure to different frost simulation regimes, with ice nucleator spray applied. The level and duration of minimum temperatures are indicated. Treatments of −4 to −6°C were also used, but these killed the plants and are therefore not represented in the figure

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.

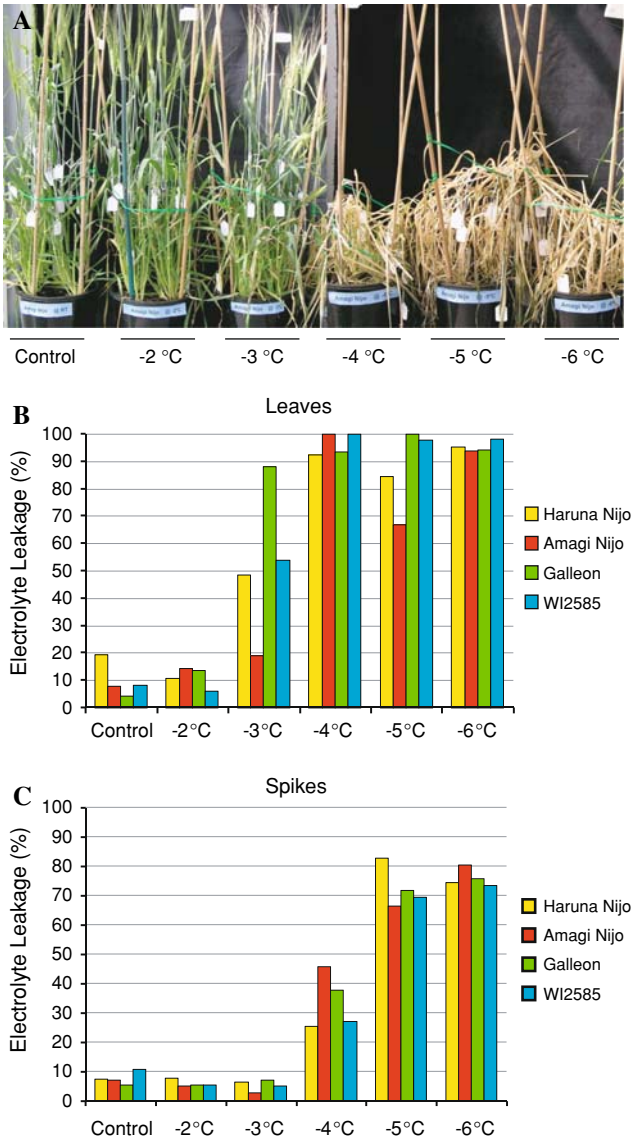

Fig. 4 Frost-induced damage of Amagi Nijo, Haruna Nijo, Galleon and WI2585 after treatment with the ice nucleating agent and minimum temperatures of −2 to −6°C. **a** Increasing damage to Amagi Nijo plants upon exposure to lower temperatures. Stem damage was visible on plants treated at −3°C, while plants 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**

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 four 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 to –4°C (Fig. 4b).

Detection of 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 vs. −5.0°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°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

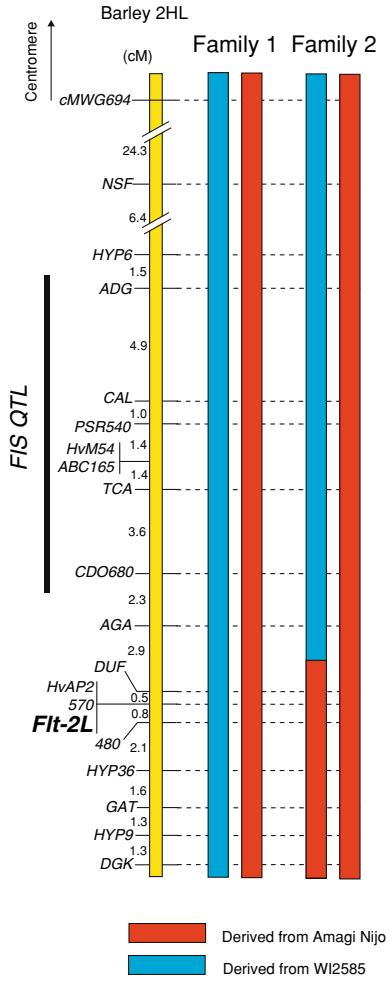

Fig. 5 Genotypes of chromosomes segregating in two Amagi Nijo × WI2585 families used to assess the effects of the 2H FIS QTL. The segment of the 2HL chromosome arm containing the FIS QTL and *Flt-2L* locus is represented by the molecular marker map on the *left*, constructed by Chen et al. (2009b, c). 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*

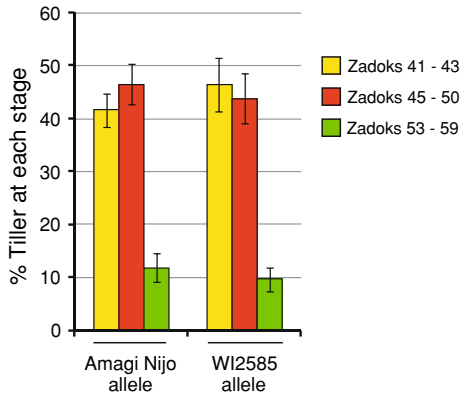

Fig. 6 Developmental stages of all tillers on frosted plants from Family 2, recorded at the time of frosting. Stages were as defined by Zadoks et al. (1974): early booting 41–43; boot swollen or just split, awns only slightly emerged and some spikelets sometimes visible 45–50; tillers with spikes a quarter to fully emerged from the boot 53–59. Anthesis occurred during stages 45–50

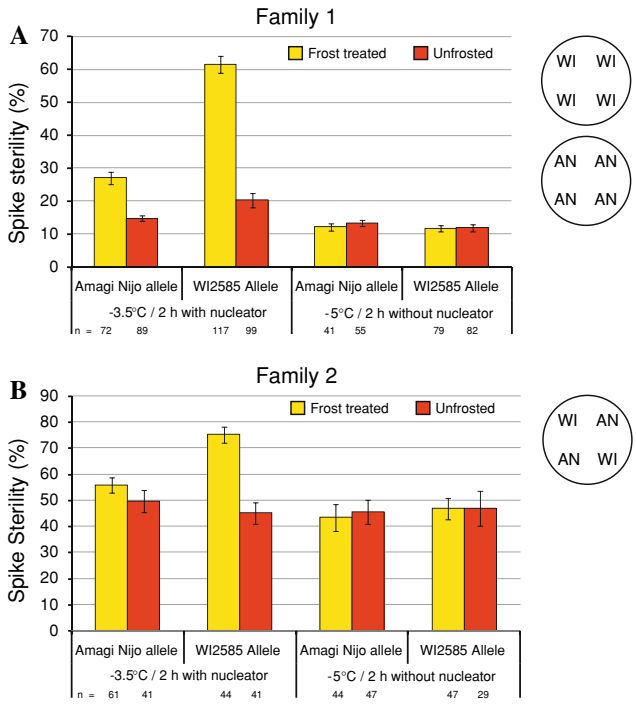

Fig. 7 FIS (mean and SE) of lines from Family 1 (**a**) and 2 (**b**). Lines homozygous for contrasting alleles (Amagi Nijo or WI2585) in the 2H chromosome segment carrying the FIS QTL locus were arranged either in separate pots (**a**) or in an alternating fashion in the same pots (**b**). Lines from 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°C and the ice nucleator spray, while the other used −5.0°C without the ice nucleator

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°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 *P. syringae*) 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 vs. 13 and 12%, respectively; Figs. 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 vs. −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 and −4°C, respectively (Fig. 4) is indicative of membrane damage caused by ice crystal growth and subsequent leakage of cellular contents. The flag leaf sheath protects enclosed spikes from exposure to external nucleation factors and provides considerable protection against freezing in the spike (Single and Marcellos 1974). The fact that spikes assayed by EC were largely enclosed by the flag leaf sheath at the time of frosting may therefore account 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, FIS 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 (Figs. 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 1.25–2 h; Figs. 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 vs. ~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, c). 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.

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