Theor Appl Genet (2004) 109: 839–846
DOI 10.1007/s00122-004-1686-9

ORIGINAL PAPER

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Cold hardiness of wheat near-isogenic lines differing in vernalization alleles

Received: 20 April 2003 / Accepted: 29 March 2004 / Published online: 27 May 2004

Abstract Four major genes in wheat (*Triticum aestivum* L.), with the dominant alleles designated *Vrn-A1, Vrn-B1, Vrn-D1*, and *Vrn4*, are known to have large effects on the vernalization response, but the effects on cold hardiness are ambiguous. Near-isogenic experimental lines (NILs) in a Triple Dirk (TD) genetic background with different vernalization alleles were evaluated for cold hardiness. Although TD is homozygous dominant for *Vrn-A1* (formerly *Vrn1)* and *Vrn-B1* (formerly *Vrn2)*, four of the lines are each homozygous dominant for a different vernalization gene, and one line is homozygous recessive for all four vernalization genes. Following establishment, the plants were initially acclimated for 6 weeks in a growth chamber and then stressed in a low temperature freezer from which they were removed over a range of temperatures as the chamber temperature was lowered 1.3°C h^{-1}. Temperatures resulting in no regrowth from 50% of the plants (LT_{50}) were determined by estimating the inflection point of the sigmoidal response curve by nonlinear regression. The LT_{50} values were −6.7°C for cv. TD, −6.6°C for the *Vrn-A1* and *Vrn4* lines, −8.1°C for the *Vrn-D1* (formerly *Vrn3*) line, −9.4°C for the *Vrn-B1* line, and −11.7°C for the homozygous recessive winter line. The LT_{50} of the true winter line was significantly lower than those of all the other lines. Significant differences were also observed between some, but not all, of the lines possessing dominant vernalization alleles. The presence of dominant vernalization alleles at one of the four loci studied significantly reduced cold hardiness following acclimation.

Communicated by D. Hoisington

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Introduction

A major objective in wheat improvement is to select for lines that minimize the effects of adverse environmental conditions, such as freeze damage during the vegetative phase. Cold hardiness is an important trait in cool-season cereals since it influences the area of adaptation and production (Fowler et al. 1977). The ability to survive or tolerate low temperatures is generally accomplished through cold acclimation or hardening (Levitt 1980). However, the maximum level of cold hardiness is not uniform among the winter cereals, nor is it uniform within a species (Fowler et al. 1977). In general, cultivars of rye can tolerate the coldest temperatures (approx. −30°C), followed by winter wheat (approx. −20°C), then barley (approx. −14°C), and finally oats (approx. −10°C) (Gusta and Fowler 1979).

Cold acclimation in winter wheat is controlled by an inducible genetic system dependent on temperatures below 10°C (for reviews, see Fowler et al. 1999; Thomashow 1999). A minimum of 6 weeks of exposure to acclimating temperatures is generally necessary to develop fully the hardiness potential (Gusta and Fowler 1979; Mahfoozi et al. 2001a, b). Typically in the field, winter cereals become hardened in the late fall, and they remain hardened through the winter months, then quickly deharden upon exposure to warm spring temperatures. However, even when fully acclimated, winter cereals do not necessarily maintain one cold hardiness level for the entire winter (Andrews et al. 1974; Pomeroy et al. 1975; Mahfoozi et al. 2001a, b). Plants rapidly lose hardiness upon exposure to warm

conditions, but the rate depends on the genotype and the length and intensity of the warm period.

Field trials are the ultimate tests of a cultivar's cold hardiness. However, these are often inconclusive due to either complete winterkill or complete survival in any one season (Gusta et al. 1982). Laboratory-based freeze tests provide an alternate method to estimate cold hardiness. Lethal temperatures (LT_{50}s) calculated from artificial freeze tests are highly correlated with field survival trials (Pomeroy and Fowler 1973; Gullord et al. 1975; Gusta and Fowler 1976; Anderson et al. 1993). The LT_{50} is defined as the lowest test temperature at which 50% of the plants survive freezing, as determined from the regrowth of leaves and roots. Genotypes can be separated with respect to levels of hardiness based on estimates of their LT_{50}s.

Another difficulty in studying freezing tolerance involves effects due to environmental confounding, particularly in comparisons of spring and winter wheat cultivars. Generally, spring cultivars are grown in warmer spring environments without vernalization, whereas winter wheat cultivars are grown during a cool season and require a period of cool temperatures to satisfy the vernalization requirement. The low temperatures required to fulfill the vernalization requirement in winter wheat overlap with the temperature range promoting hardiness, making it difficult to ascertain which system is causing the response (Laroche et al. 1992; Fowler et al. 1999; Streck et al. 2003). When given a vernalization treatment of 8 weeks or longer, spike emergence dates are expected to be uniform across spring or winter genotypes (for review, see Flood and Halloran 1986). However, when the different genotypes are grown under temperatures without cold treatment (no vernalization), they have distinctly different floral initiation responses.

Inheritance of cold hardiness in winter wheat is regarded as a quantitative trait controlled by many loci on several different chromosomes. Segregating progeny of crosses from winter wheat parents of differing cold hardiness typically exhibit a continuous range of hardiness between the parental extremes, and the freezing tolerance of hybrids typically is near the midpoint of the parents, indicating additive effects (Brule-Babel and Fowler 1988; Limin and Fowler 1993; Sutka 1994). Dominant and additive genetic effects have been found in crosses between spring and winter wheats (Sutka 1981, 1984; Brule-Babel and Fowler 1988; Limin and Fowler 1993; Askel 1994). The heritability of cold hardiness is estimated to be 63% to 70% (Limin and Fowler 1993).

Genes influencing cold hardiness have been identified on at least 15 of the 21 pairs of chromosomes in hexaploid wheat through the use of chromosome substitution lines (Law and Jenkins 1970; Cahalan and Law 1979; Sutka 1981, 1984, 1994; Roberts 1986, 1990). The homeologous group 5 chromosomes are most frequently implicated (Sutka and Kovacs 1985), and chromosomes 5A and 5D appear to carry major genes (Sutka and Snape 1989; Galiba et al. 1995; Storlie et al. 1998). Interestingly, the group 5 chromosomes are also most frequently implicated in the major vernalization responses in wheat.

The vernalization response in wheat is primarily controlled by *Vrn1* alleles located at homeologous loci on the long arm of the group 5 chromosomes (*Vrn-A1, Vrn-B1*, *Vrn-D1*) (McIntosh et al. 1998). Orthologous genes have been found in other cereals, including rye (*Vrn-R1*) (Plaschke et al. 1993), barley (*Vrn-H1*) (Laurie et al. 1995) and the diploid wheat *Triticum monococcum* (*Vrn-A^m 1*) (Dubcovsky et al. 1998). Yan et al. (2003) recently reported on the construction of detailed physical and genetic maps of the *Vrn-A^m 1* region in *T. monococcum* and found colinearity of this region to rice chromosome 3 and sorghum. A candidate gene for *Vrn1*, *AP1*, was identified, and alleles from genotypes with winter and spring growth habits were sequenced. Allelic variation between spring and winter wheats at *AP1* was found only in the promoter region. Other wheat vernalization genes include *Vrn-A^m 2* (Tranquilli and Dubcovsky 2000), *Vrn-B4* (formerly *Vrn5*) (Law and Wolf 1966), *Vrn4* (Pugsley 1972; Goncharov 2003), and *Vrn8* (Stelmakh and Avsenin 1996). In addition, two non-allelic genes, *$Vrn6^{sc}$* and *$Vrn7^{sc}$*, have been introgressed into hexaploid wheat from rye (Stelmakh and Avsenin 1996).

Much research has been devoted to defining the relationship between cold hardiness and vernalization. Cahalen and Law (1979) initially found that some cultivars carried the same recessive *vrn-A1* allele contributing to winter habit but differed significantly in cold hardiness. Sutka and Kovacs (1985), working with chromosome substitution lines, concluded that genes on chromosome 5A contributed to significant differences in levels of cold hardiness. Early literature was inconclusive on whether the relationship between cold hardiness and vernalization was pleiotropic or the result of close linkage. Brule-Babel and Fowler (1988) concluded that a lack of vernalization requirement, as indicated by spring growth habit, did not necessarily mean a lack of cold hardiness, suggesting that the two processes were unrelated; however, there was evidence of possible genetic linkage between cold hardiness and the vernalization requirement. Roberts (1990) investigated the linkage between the vernalization response and cold hardiness associated with chromosome arm 5AL and concluded that there were two, and probably more, loci on chromosome 5A affecting cold hardiness. One of the loci was suspected to be, or be closely linked to, *Vrn-A1*.

Recent research suggests that for the long arm of chromosome 5A, the two responses are the results of separate, but linked genes; *Vrn-A1* and two cold hardiness genes *Fr-A1* (formerly *Fr-1)* and *Fr-A2* (Galiba et al. 1995; Storlie et al. 1998; Sutka et al. 1999; Vaguifalvi et al. 2003). Limin and Fowler (2002) found, based on their analysis of reciprocal near-isogenic lines (NILs) for the interval surrounding the *Vrn-A1* locus from spring and winter wheats, that the acquisition of cold hardiness was also a function of the length of the vegetative stage of development. Cold hardiness genes have also been mapped to the colinear region of chromosome 5D that includes *Vrn-D1* (Snape et al. 1997).

The investigation reported here is the first characterization of cold hardiness for NILs representing four vernalization loci in wheat. The objective of the study was to determine the relationship between cold hardiness and the vernalization genes in a *Triticum aestivum* (L.) cv. Triple Dirk (TD) genetic background.

Materials and methods

Genetic stocks

Pugsley (1971, 1972, 1973) developed several NILs in which four different vernalization genes were isolated in the wheat TD genetic background (Table 1). Each of the NILs is also photoperiod-insensitive, thereby preventing daylength interaction on spike emergence. The NILs were developed through a minimum of three or four backcrosses (Zeven et al. 1986), with 94–97% of the TD genetic background being recovered. Five NILs were developed, each exhibiting different physiological behavior with respect to the vernalization response. Triple Dirk D (TDD) has dominant vernalization alleles for the *Vrn-A1* locus, Triple Dirk B (TDB) for the *Vrn-B1* locus, Triple Dirk E (TDE) for the *Vrn-D1* locus, and Triple Dirk F (TDF) for the *Vrn4* locus. Each of these genotypes behaves as spring wheat. Triple Dirk C (TDC) is homozygous recessive at the four vernalization loci and behaves as a true winter wheat. Seed of TD and the NILs were kindly provided by the Australian Cereals Collection.

Days to spike emergence

Without a vernalization period, true winter types experience an extreme delay in floral induction, whereas most spring types will develop normally without the necessity of a cool period. The number of days from plant emergence to spike emergence was recorded for each genotype grown without vernalization. Plant emergence was recorded as the first day the coleoptile was visible above the soil surface. Spike emergence was recorded as the number of days to the first fully emerged spike and was recorded for each line grown in a growth chamber (GC) [22/18°C (day/night) temperature regime and 12/12-h (day/night) photoperiod] and greenhouse (GH) [approx. 25°C constant and 14/10-h (day/night) photoperiod]. Conditions were somewhat variable depending on the outside climatic conditions, particularly for light intensity and temperature.

Growing conditions

Seeds of the six lines were imbibed in petri dishes in a Terraclor (Hummert Int, St. Louis, Mo.) solution (2.5 g l^{-1}) for 24 h at room temperature (25°C), after which the seeds were cold-shocked at 4°C for 24 h to overcome dormancy. Nonviable seeds were discarded, and the remaining seeds were planted in Cone-tainers (Stuewe & Sons, Corvallis, Ore.) containing Redi-earth (Hummert Int) growing medium. In each of the four replications in time, 21 cone-tainers were used for each line (seven temperatures with three subsamples), and three seeds were planted to each cone-tainer. The seedlings were grown for 10 days (two-leaf stage) at 22/18°C (day/night) and under a 12/12-h (day/night) photoperiod with light supplied at an intensity of 360 μmol m^{-2} s^{-1}. The plants were then transferred to an acclimation chamber for 6 weeks of growth at 8/2°C (day/night) under a 10/14-h (day/night) photoperiod. They were watered to field capacity twice per week with a dilute (1.25 g l^{-1}) Peter's nutrient solution (20-20-20; The Scotts Company, Marysville, Ohio) added every fourth watering. The stage of plant development was evaluated visually for all plants following this 6-week acclimation period to confirm uniformity of plant growth. A random sample of six plants from each line was also evaluated for developmental stage by dissection. Spikelet and floret development of the dissected plants were compared to growth development plates in the Cereal Development Guide (Kirby and Appleyard 1987) to confirm that none of the plants had advanced beyond the vegetative stage.

Freezing procedure

Cold hardiness was evaluated by subjecting the samples to freezing temperatures over time in a low temperature freezer (Gusta and Fowler 1977). Plants were subjected to the freeze test in the original cone-tainers in which they were being grown. The procedure of Anderson et al. (1993) was used for determining LT_{50}, modified slightly to allow for trimming of the wheat plants, adjustments in the cooling rate, and the bracketing of a larger temperature range.

Vegetative tissue was trimmed 1 day prior to plants being placed in the low-temperature freezer to allow them to be more easily maneuvered in the freezer. On the eve of the freeze test, the cone-tainers were watered to field capacity. On the morning of the freeze test, the plants were randomized by genotype, and thermocouples were inserted 2 cm into the growing media in each cone-tainer so that temperatures could be monitored on an individual cone-tainer basis (Anderson et al. 1993). Cone-tainers were fitted into predrilled slots in a 2-cm-thick removable aluminum plate. Plants were loaded into the low-temperature freezer (SureTemp CEC23, Rheem Scientific) and equilibrated overnight at −3°C. An ice chip was added to each cone-tainer as a nucleating source to prevent excessive supercooling.

The following morning the temperature in the freezer was lowered at the rate of 1.3°C h^{-1}, which was slightly slower than the 1.5°C h^{-1} described by Gusta and Fowler (1977). Plants were removed from the freezer at 1°C intervals rather than the 2°C intervals described by Gusta and Fowler (1977) in order to target a more narrow temperature range than that defined by preliminary estimates of LT_{50}. For each of the six lines a temperature range of at least 7°C bracketed the expected LT_{50}. For each genotype, three cone-tainers were removed from the freezer at 1°C intervals for each temperature in the targeted range. Temperatures were monitored using a data logger, and cone-tainers were removed based on individual thermocouple measurements. Thermocouple wires with detachable plugs (Omega Scientific) facilitated removal of the cone-

Table 1 Mean days to spike emergence without vernalization and LT_{50} for acclimated wheat cv.Triple Dirk (TD) and the TD NILs investigated

Line	Haploid genotype[a]	Spike emergence (days)		
		Growth chamber	Greenhouse	LT_{50} (°C)
TD	***Vrn-A1 Vrn-B1****vrn-D1 vrn4*	59.9	55.7	−6.7
TDD	***Vrn-A1****vrn-B1 vrn-D1 vrn4*	62.7	58.1	−6.6
TDE	*vrn-A1 vrn-B1****Vrn-D1****vrn4*	72.8	63.4	−8.1
TDB	*vrn-A1****Vrn-B1****vrn-D1 vrn4*	75.5	68.7	−9.4
TDF	*vrn-A1 vrn-B1 vrn-D1****Vrn4***	86.6	62.5	−6.6
TDC	*vrn-A1 vrn-B1 vrn-D1 vrn4*	165.7	128.9	−11.7
LSD (P<0.05)		9.5	14.1	1.1

[a]Dominant vernalization alleles for cv. TD and the TD NILs are in given in bold

tainers, since thermocouple junctions froze firmly to the growing media in the cone-tainer. Plants were thawed slowly over a 24-h period in an ice chest following their removal from the freezer.

The plants were allowed to grow in the GH and were scored for survival after 4–6 weeks. The LT_{50} was calculated by nonlinear regression (SAS Institute 1991). For each of the six NILs, four estimates of the LT_{50} were made by four independent freeze tests. These were treated as replications in time, and an analysis of variance was performed.

Results

Days to spike emergence

Days to spike emergence differed between GC and GH environments (Table 1). The mean number of days to spike emergence for genotypes grown in the GC was less variable than that for the respective genotypes grown in the GH. Mean dates varied with replications in time, particularly for the GH, and the differential responses made it difficult to distinguish genotypes on the basis of spike emergence data alone.

Homogeneity of variance for genotypic heading dates across all environments was assumed since only one experimental unit was available for each genotype-by-environment cell. In a combined ANOVA for both GH and GC environments, there were significant effects due to genotype-by-environment interaction (P=0.002). An ANOVA was then performed for each environment. In each analysis, effects due to genotype were highly significant (P<0.0001).

The mean days to spike emergence and critical values for multiple comparisons were calculated using the least significant difference (LSD) method (Table 1). There was a significant difference between the mean days to spike emergence between some, but not all, genotypes in the GC (Fig. 1). For the GH, there was a significant difference only between the true winter type (TDC) and all other lines.

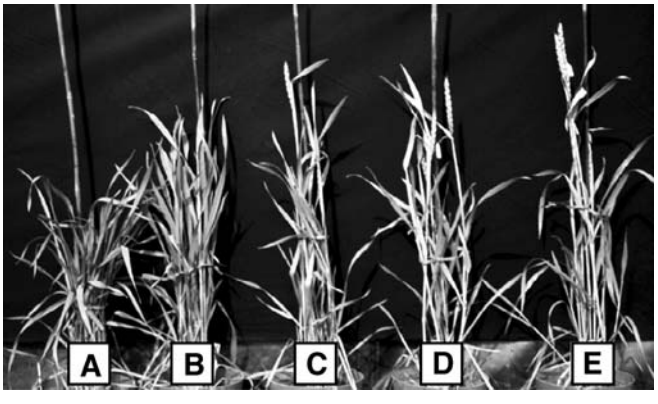

Fig. 1a–e Developmental stages of wheat cv. Triple Dirk (TD) NILs grown in a controlled environment chamber without vernalization at 60 days post-emergence. **a** TDC (recessive alleles at all loci), **b** TDF (dominant *Vrn4* allele), **c** TDB (dominant*Vrn-B1* allele), **d** TDE (dominant *Vrn-D1* allele), **e** TDD (dominant *Vrn-A1* allele)

Freeze analysis

The relationship between survival and exposure temperature was sigmoidal (Fig. 2). LT_{50}s estimated from the midpoint of the response curves indicated significant differences in cold hardiness across some, but not all, of the lines (Table 1). Since these lines should be genetically similar, except for the respective *Vrn* loci, the inference can be made that the differences in cold hardiness observed are due to the effect of the vernalization genes or closely linked genes.

Stage of development

No visual difference in the rate of growth was detected across lines for plants subjected to the freeze test following 6 weeks of acclimation. Visually, the plants appeared at the same level of maturity, with tillering beginning, but without evidence of reproductive growth. Additionally, random samples of apical meristems from each line were dissected and found to be in the late vegetative stage of maturity. However, it was apparent that stem elongation was beginning to occur among some plants of the TD cultivar (*Vrn-A1 Vrn-B1*). For a few, the shoot apex was beginning to elongate, signaling the beginning of the floral phase when spikelets are initiated (Kirby and Appleyard 1987). No primordia had begun to differentiate and no spikelets were visible. No plant in any line was identified with growth so advanced as to conclude that floral development had begun.

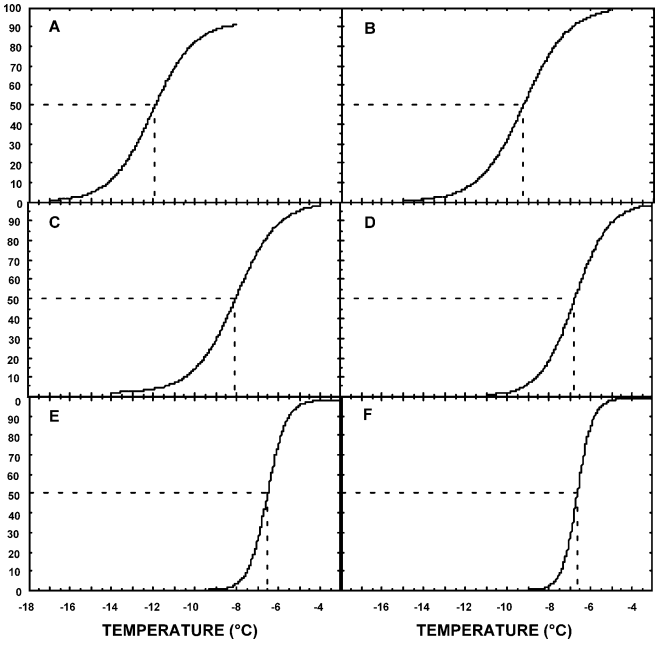

Fig. 2a–f The relationship between survival and exposure temperature for TD NILs differing in alleles for vernalization. **a** TDC (recessive alleles at all loci), **b** TDB (*Vrn-B1*), **c** TDE (*Vrn-D1*), **d** TD (*Vrn-A1 Vrn-B1*), **e** TDF (*Vrn4*), **f** TDD (*Vrn-A1*)

Discussion

Genetic stocks

There appeared to be some initial contamination of TDC (winter type) in the TDB (*Vrn-B1*) seed stocks in the preliminary heading date evaluation, as some plants failed to head within 100 days. These were discarded, and seeds for the TDB used in the freeze test were from plants with heading dates consistent with those reported by Flood and Halloran (1983, 1984). The days to spike emergence data were uniform within NILs for plants grown to increase the seed source for the freeze test, suggesting no further contamination.

Zeven et al. (1986) tested the TD lines to evaluate the extent of their similarity. Significant differences were found across NILs for some morphological traits, and some lines appeared to be a mixture of types. The limited number of backcrosses used in developing the NILs has probably not eliminated all linkage drag from the donors of the different vernalization alleles. The possibility also exists that the lines are not identical for all cold hardiness alleles, particularly for alleles closely linked to a vernalization gene.

Days to spike emergence

There was initial concern over the degree of variation within the true winter type (TDC). Occasionally, some plants would flower earlier than expected, and some later. Selections were made from some of the early heading plants and progeny grown from bagged and selfed heads. No segregation resulted. All were uniformly near the expected mean for the true winter type. Similarly, a late selection was isolated from TDC with the same response.

By evaluating the rankings of the mean number of days to spike emergence between plants grown in the GC and GH environments, we were able to identify changes in the rankings of some genotypes (TDB, TDE, TDF) across environments. TDF (*Vrn4*) appears to be the most environmentally sensitive, showing prominent changes in ranking between the GC and GH environments. It is possible that TDF, by chance, contains a minor photoperiod-sensitivity allele (*Ppd*) from the non-recurrent parent that accelerates development under the 14/10-h (day/night) photoperiod in the GH. Otherwise, the ranking is in general agreement with that of previous researchers (Gotoh 1976; Iwaki et al. 2000) who ranked the vernalization genes according to their strength in promoting heading dates: *Vrn-A1*>*Vrn-D1*>*Vrn4*>*Vrn-B1*.

There was a significant genotype-by-environment interaction when data from the GC and the GH were evaluated together. This may have been due to the differential response of genotypes resulting from photoperiod settings in the GH (14-h daylight) and the GC (12-h daylight) or to the somewhat variable temperature and light intensity, which were greater in the GH than in the GC. Variations in other environmental factors may also have obscured subtle differences in the number of days to spike emergence.

There were significant effects due to genotype when an ANOVA was performed for each environment. For both the GC and the GH, effects due to genotype were significant and effects due to replications in time were not. However, when the critical values for LSD were applied to the means, it was not possible to distinguish all genotypes by the mean number of days to spike emergence. The means of genotypes in the GC encompassed a wider range than those in the GH, with a smaller calculated LSD value, allowing classes to be separated more distinctly. The GH data were less discriminatory, and multiple comparisons on means of plants grown in the greenhouse failed to distinguish between any lines with a dominant vernalization gene.

Freeze analysis

The LT_{50}s of some of the lines studied were significantly different, suggesting that the vernalization genes or their associated linkage groups affect cold hardiness. The differences in cold hardiness in the freeze test do not appear to be due to differences in maturity, since all of the plants tested were at the same vegetative stage of development. TD (*Vrn-A1 Vrn-B1*), TDD (*Vrn-A1*), and TDF (*Vrn4*) all appear to have the same approximate levels of hardiness and differ from the other lines in this respect; TDE (*Vrn-D1*) was significantly hardier than these three lines, while TDB (*Vrn-B1*) was significantly hardier than TDE. TDC, the true winter line, was significantly hardier than all of the other lines.

If the NILs are truly isogenic, then all of them should have identical cold hardiness alleles at all shared loci. The differential response across the NILs implicates the vernalization genes and their associated linkage groups as affecting cold hardiness. However, there appears to be differences across the lines other than the vernalization genes (Zeven et al. 1986). There is a possibility of a cold hardiness gene(s), closely linked to a vernalization gene (s), whose linkage was not broken during the development of the NILs. This is of particular importance when considering the well-established linkage between *Vrn-A1* and frost tolerance loci *Fr-A1* and *Fr-A2* on chromosome 5A (Galiba et al. 1995; Storlie et al. 1998; Sutka et al. 1999; Vaguifalvi et al 2003). Storlie et al. (1998) found that two *Vrn-A1/Fr-A1* intervals, derived from non-hardy and cold-hardy winter wheat parents, respectively, explained 71% of the variation for cold hardiness between the winter wheats and 80–91% of the genetic variation for cold hardiness when they were backcrossed into spring wheat genetic backgrounds. In addition, Snape et al. (1997) mapped a colinear region containing *Vrn-D1* and a quantitative trait locus for cold tolerance on chromosome 5D.

The source of the vernalization alleles can often be determined by examining the pedigrees of the non-recurrent parents. For TDD, the contributor of the *Vrn-*

A1 allele was TD, but the contributor of the *vrn-B1* allele is unknown (Zeven et al. 1986). Presumably, TD also contributed *vrn-D1 and vrn4*. TDD (*Vrn-A1*) is not hardy and is not significantly different in hardiness from TD (recurrent parent). TDC (winter line) differs for at least two loci from TD and has a significant increase in hardiness. This increase in cold hardiness could be due to the effect of closely linked cold hardiness alleles, such as *Fr-A1* and *Fr-A2*.

TDB (*Vrn-B1*) is somewhat more intriguing. The pedigree confirms that cv. Winter Minflor was the contributor of *vrn-A1* for both TDB and TDC (Zeven et al. 1986), with *vrn-D1* and *vrn4* presumably coming from TD. One might expect that TDB would be similar in hardiness to either TD (which has the identical *Vrn-B1* allele) or to TDC (which presumably has all of the identical vernalization alleles except for *Vrn-B1*). However, TDB is intermediate in hardiness, being significantly hardier than TDD or TD, but not as hardy as TDC. The difference appears to be due to the *Vrn-B1* locus, or genes closely linked to it, although there is a possibility that the difference is due to effects of *vrn-D1* or *vrn4*, or genes closely linked to either, if the contributor of these alleles was actually Winter Minflor rather than TD.

Cultivar Loro contributed the *Vrn-D1*, *vrn-A1*, and *vrn-B1* alleles to TDE (Zeven et al. 1986). The *vrn4* allele presumably came from TD. TDE is hardier than the recurrent parent TD but less so than the winter type (TDC). Once again the *Vrn-D1* locus, or genes closely linked to it, appears to contribute to increased hardiness. Alternatively, the differences may be partially due to the effects of the recessive *vrn-A1* and *vrn-B1* alleles, or genes closely linked to them, that were contributed by Loro. No conclusion can be reached, as TDE was significantly different from TD and TDC.

The same inferences can be made for TDF (*Vrn4*), except that this line is not hardy. The *Vrn4* gene has been the subject of considerable controversy. Maystrenko (1980) and Stelmakh (1987) concluded that *Vrn4* was an allele to *Vrn-B1*, and it was so designated by McIntosh et al. (1998). Goncharov (2003) has since established that *Vrn4* is not allelic to *Vrn-B1* but is instead allelic to *Vrn8* (Stelmakh and Avsenin 1996). The chromosomal location of this gene is unknown, however chromosomes 5D and 7A have been eliminated as candidates (Gocharov 2003). It is interesting to note that the LT_{50} of TDF is not significantly different from those of TD or TDD. Cultivar Gabo contributed the *Vrn4, vrn-A1*, and *vrn-B1* alleles, and the *vrn-D1* allele presumably was contributed by TD (Pugsley 1973; Goncharov 2003). Only the *vrn-D1* allele is identical in both TDF and TD, suggesting that this allele does not independently contribute to increased cold hardiness. This differs from the results with TDE (*Vrn-D1*), which suggest that cold hardiness is associated with the dominant (*Vrn-D1)* allele or genes closes linked to it.

Does the presence of a dominant vernalization gene make a plant more susceptible to freeze damage? It appears that in all of the lines studied the presence of a dominant vernalization allele increased susceptibility to cold. This is in agreement with Limin and Fowler (2002) who found that the length of time to the vegetative/reproductive transition was a major factor in acquisition of cold hardiness. In all lines with *Vrn-A1*, the plants had low levels of cold hardiness, suggesting that the vernalization gene does not confer hardiness; rather, it may even cause susceptibility. However, the mere removal of the *Vrn-A1* allele, and any corresponding closely linked genes, does not have a uniform effect on hardiness. TDF remains sensitive, whereas TDC becomes hardy. TDB and TDE are intermediate and significantly different from other lines. This suggests that the differential level of hardiness may be associated with *Vrn-B1*, *Vrn-D1*, or the effects of closely linked cold hardiness genes.

Conclusion

An accurate assessment of the number of days to spike emergence can only be obtained from plants grown under strict environmental conditions, preferably in a GC rather than a GH environment. Spike emergence data used to characterize lines with different vernalization requirements were especially variable in the GH, probably due to subtle environmental differences contributing to phenotypic variation, thereby making it difficult to compare lines. In these lines, the *Vrn* alleles contributed differentially to number of days to spike emergence in the absence of vernalization, with *Vrn-A1* having the strongest response, followed by *Vrn-D1, Vrn-B1*, and *Vrn4*, although statistical differences were difficult to detect across environments. The true winter type (*vrn-A1vrn-B1vrn-D1vrn4*) was significantly later than all other lines.

There were significant differences in cold hardiness as measured by LT_{50} between some, but not all, of these NILs. Since these NILs should differ at relatively few loci, namely one of the four vernalization genes studied, and all are in a common TD genetic background, the implication is that the vernalization genes have an effect on cold hardiness. However, the possibility of effects due to closely linked genes cannot be excluded. The true winter type (TDC) was the most hardy in the group, suggesting that the absence of a dominant vernalization allele makes the plant more resistant to cold. There also appears to be an uneven contribution to hardiness by the different dominant vernalization genes, as evidenced by the LT_{50}s of TDB (*Vrn-B1* line) and TDE (*Vrn-D1* line), which were intermediate in hardiness but less hardy than TDC (winter line). This suggests that the *Vrn-B1* and *Vrn-D1* alleles should be used in spring types where increased cold hardiness is desired as they may have a particular utility for facultative wheat cultivars. These NILs will also be valuable in utilizing transcriptomics, proteomics, and metabolomics to dissect the complex interrelationship between vernalization and cold acclimation.

Acknowledgements This work was supported by the Oklahoma Wheat Research Foundation (to A.C.G) and the Oklahoma Agricultural Experiment Station (Project H1996). We thank the anonymous reviewers for their insights and suggestions.

References

Aksel R (1994) Quantitative-genetic analysis of reciprocal crosses between a winter and a spring cultivar of common wheat (*Triticum aestivum* L.). Theor Appl Genet 89:1032–1038

Anderson JA, Taliaferro CM, Martin DL (1993) Evaluating freeze tolerance of bermudagrass in a controlled environment. HortScience 28:955

Andrews CJ, Pomeroy MK, de la Roche IA (1974) Changes in cold hardiness of overwintering winter wheat. Can J Plant Sci 54:9–15

Brule-Babel AL, Fowler DB (1988) Genetic control of cold hardiness and vernalization requirement in winter wheat. Crop Sci 28:879–884

Cahalen C, Law CN (1979) The genetic control of cold resistance and vernalization requirement in wheat. Heredity 42:125–132

Dubcovsky J, Lijavetzky D, Appendino L, Tranquilli G (1998) Comparative RFLP mapping of *Triticum monococcum* genes controlling vernalization response. Theor Appl Genet 97:968–975

Flood RG, Halloran GM (1983) Developmental responses in tetraploid wheat (*Triticum turgidum*). Can J Bot 61:2539–2545

Flood RG, Halloran GM (1984) Temperature as a component of the expression of developmental responses in wheat. Euphytica 33:91–98

Flood RG, Halloran GM (1986) Genetics and physiology of vernalization response in wheat. Adv Agron 39:87–125

Fowler DB, Dvorak J, Gusta LV (1977) Comparative cold hardiness of several *Triticum* species and *Secale cereale* L. Crop Sci 17:941–943

Fowler DB, Limin AE, Ritchie JT (1999) Low temperature tolerance in cereals: model and genetic interpretation. Crop Sci 39:626–633

Galiba G, Quarrie SA, Sutka J, Morgounov A, Snape JW (1995) RFLP mapping of the vernalization (*Vrn-A1*) and frost resistance (*Fr1*) genes on chromosome 5A of wheat. Theor Appl Genet 90:1174–1179

Goncharov NP (2003) Genetics of growth habit (spring vs winter) in common wheat: confirmation of the existence of dominant gene *Vrn4*. Theor Appl Genet 107:768–772

Gotoh T (1976) Studies on varietal differences in vernalization requirement in wheat. Jpn J Breed 26:307–327

Gullord M, Olien CR, Everson EH (1975) Evaluation of freezing hardiness in winter wheat. Crop Sci 15:153–157

Gusta LV, Fowler DB (1976) Effects of temperature on dehardening and rehardening of winter cereals. Can J Plant Sci 56:673–678

Gusta LV, Fowler DB (1977) Factors affecting the cold survival of winter cereals. Can J Plant Sci 57:213–219

Gusta LV, Fowler DB (1979) Cold resistance and injury in winter cereals. In: Mussell H, Staples RC (eds) Stress physiology in crop plants. Wiley, New York, pp 159–178

Gusta LV, Fowler DB, Tyler NJ (1982) Factors influencing hardening and survival in winter wheat. In: Li PH, Sakai A (eds) Plant cold hardiness and freezing stress: mechanisms and crop implication, vol. 2. Academic, New York, pp 23–40

Iwaki K, Nakagawa K, Kuno H, Kato K (2000) Ecogeographical differentiation in east Asian wheat, revealed from the geographical variation of growth habit and *Vrn* genotype. Euphytica 111:137–143

Kirby EJM, Appleyard M (1987) Cereal development guide, 2nd edn. Arable Unit, National Agricultural Centre, Stoneleigh, Kenilworth, UK

Laroche A, Geng XM, Singh J (1992) Differentiation of freezing tolerance and vernalization responses in Cruciferae exposed to a low temperature. Plant Cell Environ 15:439–445

Laurie DA, Pratchett N, Bezant JH, Snape JW (1995) RFLP mapping of five major genes and eight quantitative trait loci controlling flowering time in a winter × spring barley *Hordeum vulgare* (L.) cross. Genome 38:575–585

Law CN, Jenkins G (1970) A genetic study of cold resistance in wheat. Genet Res 15:197–208

Law CN, Wolfe MS (1966) Location of genetic factors of mildew resistance and ear emergence time on chromosome 7B of wheat. Can J Genet Cytol 8:462–470

Levitt J (1980) Responses of plants to environmental stress. Section II. Chilling, freezing, and high temperature stresses, vol. 1, 2nd edn. Academic, New York, pp 67–344

Limin A, Fowler DB (1993) Inheritance of cold hardiness in *Triticum aestivum* × synthetic hexaploid wheat crosses. Plant Breed 110:103–108

Limin A, Fowler DB (2002) Developmental traits affecting low-temperature tolerance response in near-isogenic lines for the vernalization locus *Vrn-A1* in wheat (*Triticum aestivum* L. em Thell). Ann Bot 89:579–585

Mahfoozi S, Limin AE, Fowler DB (2001a) Influence of vernalization and photoperiod on cold hardiness in winter cereals. Crop Sci 41:1006–1011

Mahfoozi S, Limin AE, Fowler DB (2001b) Developmental regulation of low-temperature tolerance in winter wheat. Ann Bot 87:751–757

Maystrenko OI (1980) Cytogenetic study of the growth habit and ear-emergence time in wheat (*Triticum aestivum* L.). In: Altukhov YP (ed) Proc 14th Int Congr Genet, vol 1. Mir Publ, Moscow, pp 267–282

McIntosh RA, Hart GE, Devos KM, Gale MD, Roger WJ (1998) Catalogue of gene symbols for wheat. In: Sinkard AE (ed) Proc 9th Int Wheat Genet Symp, vol. 5. University of Saskatoon Extension Press, Saskatoon, Sask., pp 1–235

Plaschke J, Borner A, Xie DX, Koebner RMD, Schlegel R, Gale MD (1993) RFLP mapping of genes affecting plant height and growth habit in rye. Theor Appl Genet 85:1049–1054

Pomeroy MK, Fowler DB (1973) Use of lethal dose temperature estimates as indices of frost tolerance for wheat cold acclimation under natural and controlled environments. Can J Plant Sci 53:489–494

Pomeroy MK, Andrews CJ, Fedak G (1975) Cold hardiness and dehardening responses in winter wheat and winter barley. Can J Plant Sci 55:529–535

Pugsley AT (1971) A genetic analysis of spring–winter habit of growth in wheat. Aust J Agric Res 22:21–31

Pugsley AT (1972) Additional genes inhibiting winter habit in wheat. Euphytica 21:547–552

Pugsley AT (1973) Control of developmental patterns in wheat through breeding. In: Sears ER, Sears LMS (eds) Proc 4th Int Wheat Genet Symp . University of Missouri, Columbia, Mo., pp 857–859

Roberts DWA (1986) Chromosome in 'Cadet' and 'Rescue wheats carrying loci for cold hardiness and vernalization response. Can J Genet Cytol 28:991–997

Roberts DWA (1990) Identification of loci on chromosome 5A of wheat involved in control of cold hardiness, vernalization, leaf length, rosette growth habit, and height of hardened plants. Genome 33:247–259

SAS Institute (1991) SAS user's guide: statistics. SAS Institute, Cary, N.C.

Snape JW, Semikhodskii A, Fish L, Sarma RN, Quarrie SA, Galiba G, Sutka J (1997) Mapping frost tolerance loci in wheat and comparative mapping with other cereals. Acta Agron Hung 45:268–270

Stelmakh AF (1987) Growth habit in common wheat (*Triticum aestivum* L. em Thell.). Euphytica 89:513–519

Stelmakh AF, Avsenin VI (1996) Alien introgression of spring habit dominant genes into bread wheat genomes. Euphytica 89:65–68

Storlie EW, Allan RE, Walker-Simmons MK (1998) Effect of the *Vrn1-Fr1* interval on cold hardiness levels in near-isogenic wheat lines. Crop Sci 38:483–488

Streck NA, Weiss A, Baenziger PS (2003) A generalized vernalization response function in wheat. Agron J 95:155–159

Sutka J (1981) Genetic studies of frost resistance in wheat. Theor Appl Genet 59:145–152

Sutka J (1984) A ten-parental diallel analysis of frost resistance in winter wheat. Z Pflanzenzuecht 93:147–157

Sutka J (1994) Genetic control of frost tolerance in wheat (*Triticum aestivum* L.) Euphytica 77:277–282

Sutka J, Kovacs G (1985) Reciprocal monosomic analysis of frost resistance on chromosome 5A in wheat. Euphytica 34:367–370

Sutka J, Snape JW (1989) Location of a gene for frost resistance on chromosome 5A of wheat. Euphytica 42:41–44

Sutka J, Galiba G, Vaguifalvi A, Gill BS, Snape JW (1999) Physical mapping of the *Vrn-A1* and *Fr1* genes on chromosome 5A of wheat using deletion lines. Theor Appl Genet 99:199–202

Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory genes. Annu Rev Plant Physiol Plant Mol Biol 50:571–599

Tranquilli G, Dubcovsky J (2000) Epistatic interaction between vernalization genes *Vrn-A^{m}1* and *Vrn-A^{m}2* in diploid wheat. J Hered 91:304–306

Vaguifalvi A, Galiba G, Cattivelli L, Dubcovsky J (2003) The cold-regulated transcription activator *Cbf3* is linked to the frost-tolerant locus *Fr-A2* on wheat chromosome 5A. Mol Genet Genomics 269:60–67

Yan L, Loukoianov A, Tranquilli G, Helguera M, Fahima T, Dubcovsky J (2003) Positional cloning of the wheat vernalization gene VRN1. Proc Natl Acad Sci USA 100:6263–6268

Zeven AC, Waninge J, Colon LT (1986) The extent of similarity between near-isogenic lines of the Australian spring wheat variety Triple Dirk with their recurrent parent. Euphytica 35:381–393