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In Vitro Freezing Tolerance in Relation to Winter Survival of Rapeseed Cultivars

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ABSTRACT

Winter survival is a complex trait dependent on a number of parameters, including the morphological and physiological characteristics of the plant, soil conditions, and weather fluctuations. Freezing tolerance of the plant is a major factor determining winter survival and can be assessed indirectly by measuring ion leakage from leaves exposed to freezing temperatures. This study was conducted to determine the relationship between in vitro freezing tolerance and winter survival in rapeseed. Ten rapeseed cultivars (*Brassica napus* and *B. rapa*) were subjected to an in vitro controlled freeze and assayed for ion leakage both before and after cold acclimation for three weeks. In addition, these same cultivars were fall-seeded at two field locations and their winter survival evaluated. Freezing tolerances determined by the in vitro freezing assay increased after acclimation, and cultivars differed significantly for freezing tolerance before and after acclimation. The increase in in vitro freezing tolerance of the cultivars after acclimation was correlated significantly with winter survival (r = 0.82–0.85, $P < 0.05$), and, therefore, this in vitro assay could be a useful predictor of field results. One exception to this correlation was the *B. napus* cultivar Santana, which had a large capacity for acclimation but did not survive in either field location. These results suggest that freezing tolerance is an important, but not the only, component of winter survival.

RAPESEED CULTIVARS , both *Brassica napus* and *B. rapa*, can be grown as biennials, spring annuals, or winter annuals. Areas where spring annuals can be produced economically are limited by high summer temperatures and pest problems (Raymer et al., 1990). In addition, seeding of frost-sensitive spring canola, which is rapeseed with low erucic acid and low glucosinolate contents, must be delayed in some areas to avoid spring frosts (Gusta and O'Connor, 1987). Late seeding can result in reduced yield, as well as delayed maturity, which increases the possibility of damage by fall frosts. In areas where winters are mild, spring varieties can be fall-seeded and grown as winter annuals (Raymer et al., 1990; Raymer, 1991; Auld et al., 1984). Winter canola varieties are grown in more northern climates and are desirable because of high yields (Mahler and Auld, 1987, 1988, 1989), but they must survive sufficiently cold weather to satisfy their vernalization requirements of between 6 to 8 wk of cold temperatures to initiate flowering (Raymer et al., 1990). Freezing tolerance is an important trait necessary for optimum seed yield of both winter and spring rapeseed varieties.

Winter survival is a complex trait that is dependent on many parameters. Freezing tolerance is the ability of a plant to survive subfreezing temperatures and is the major component of winter survival. Another important factor is acclimation ability, which is the ability of a plant to increase its freezing tolerance, or survive at lower temperatures, after a period of cold-temperature treatment. In addition, poor seedling establishment, soil compaction, waterlogging, damage by diseases and pests, and lack of snow cover are known to have negative effects on winter survival (Thompson and Hughes, 1986). Several plant morphological characteristics also are associated with freezing tolerance of rapeseed cultivars, including a prostrate rosette of leaves and cessation of growth at temperatures <2°C (Kacperska, 1984).

There are several reports on the effects of freezing on the physiology of *Brassica* plants, but these studies do not indicate whether any of these changes are required for freezing tolerance. For example, exposure of *B. napus* to freezing temperatures induced accumulation of reducing sugars in leaves (Krause et al., 1982; Kacperska, 1984), altered the phospholipid composition of leaves, activated phospholipase-D (Sikorska and Kacperska, 1982) changed protein synthesis patterns in seedlings (Meza-Basso et al., 1986), induced seed pigment synthesis (Johnson-Flanagan et al., 1991), inhibited seed photosynthesis (Hodgins et al., 1989), and altered seed chlorophyllase and peroxidase activities (Johnson-Flanagan, 1989).

Field survival is the usual method for evaluating winter survival and freezing tolerance, but results are often inconclusive due to either complete death or complete survival of all genotypes depending on the severity of the particular winter season (Fowler and Gusta, 1979). In addition, results of field tests are affected by a number of uncontrollable factors, such as temperature fluctuations, snowfall, and field variability. Therefore, it would be useful to have an indirect predictive test with which to screen genotypes for winter survival.

A number of tests have been developed to predict freezing tolerance. These usually involve controlled freezing followed by observations on plant recovery, which is often a long process that is destructive and requires large numbers of plants (Brule-Babel and Fowler, 1989). Plant-tissue water content also has been used to screen for freezing tolerance in winter cereals (Brule-Babel and Fowler, 1989). Water content of fully acclimated plants was found to correlate with field evaluation, but the technique was not able to detect small differences between cultivars without excessive replication.

Alternatively, the dielectric properties of plant tissues can be used as an indicator of cold survival. The technique involves measurement of the proportion of bound

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Abbreviations: ANOVA, analysis of variance; GCIRC, Groupe Consultatif International de Recherche sur le Colza; RFT, relative freezing tolerance.

water on structural proteins in the plant's terminal meristem. This proportion was found to increase during the hardening of plans (Thompson and Hughes, 1986). The Swedish *B. napus* cultivar Brink was most cold resistant by this method, which was in good agreement with the field observations (Mladek et al., 1984). This method is neither rapid nor simple and does not measure a physiological parameter which is associated with freezing stress.

Another method of determining freezing tolerance of plants is based on measuring leakage of ions from plant cells after a freezing stress (Palta et al., 1977). Since the first perturbation observed after a freezing stress is a 2.5-fold increase in ion leakage (Steffen et al., 1989), this leakage can be used as an indicator of freezing damage to tissues, specifically the loss in the integrity of cellular membranes. This method has been used to study freeze-thaw injury and recovery in onion bulbs (*Allium cepa* L.; Arora and Palta, 1991), potato leaves (tuber-bearing *Solanum* spp.; Stone et al., 1991; Palta and Li, 1980); and alfalfa cultivars (*Medicago sativa* L.; Sulc et al., 1991). The advantages of this method are that it is simple, repeatable, fairly rapid, and imposes a realistic freeze–thaw stress on intact tissues (Steffen et al., 1989). This study was conducted to determine whether in vitro assessment of freezing tolerance based on ion leakage was related to winter survival of field-grown rapeseed.

MATERIALS AND METHODS

Plant materials

Both spring and winter rapeseed cultivars of *B. napus* and *B. rapa* (syn. *B. campestris*) were used. These included four spring-type *B. napus* cultivars (Stellar, Marnoo, Hanna, and 353/86, which is a breeder's line from Ameri-Can Seeds, Memphis, TN, recently released as the cultivar D123) and four winter-type *B. napus* cultivars (Major, Lirabon, Santana, and Ceres). One spring-type *B. rapa* cultivar, R500, and one winter type, Per, were also included. Seeds for most of the cultivars were obtained from M. Sovero (Ameri-Can Seeds). Seeds for Stellar were obtained from R. Scarth (University of Manitoba, Winnipeg, MB); seeds for Major, from A. Chevre (INRA, Le Rheu, France); and for R500, from L. Sernyk (Agrigenetics Co., Madison, WI).

In Vitro Freezing Assays

Two sets of eight plants of each cultivar were grown in a 1:2 mixture of Jiffy Mix (Jiffy Products of America, Inc., W. Chicago, IL) and soil mix (1 soil:2 sand:3 peat), under 14-h daylength (150 μmol photon m^{-2} s^{-1}), at 26 to 27 °C, and watered daily with half-strength Hoagland's solution for 5 wk. One group of plants was assayed for freezing tolerance at this time and are referred to as *nonacclimated plants*. The second set of plants of each cultivar were incubated for an additional 3 wk at 4/2 °C, day/night temperature, 14-h daylength (25 μmol photon m^{-2} s^{-1}) to become the group of *acclimated plants*.

Leaf tissue was subjected to a freeze–thaw stress, using procedures similar to Steffen et al. (1989). Leaves of similar size and approximate developmental age (1–2 leaves per plant) were excised from six different plants for each cultivar. After the midribs were removed, each leaf half was placed in a test tube (20 by 200 mm) and subjected to a controlled freeze in a glycol bath from the initial temperature of 0 °C. For nonacclimated plants, the bath temperature was lowered 0.5 °C every 30 min from 0 to −7 °C. For acclimated plants, the bath temperature was lowered 0.5 °C every 30 min until −3 °C, then lowered 1 °C every 30 min until −16 °C. Ice nucleation was initiated

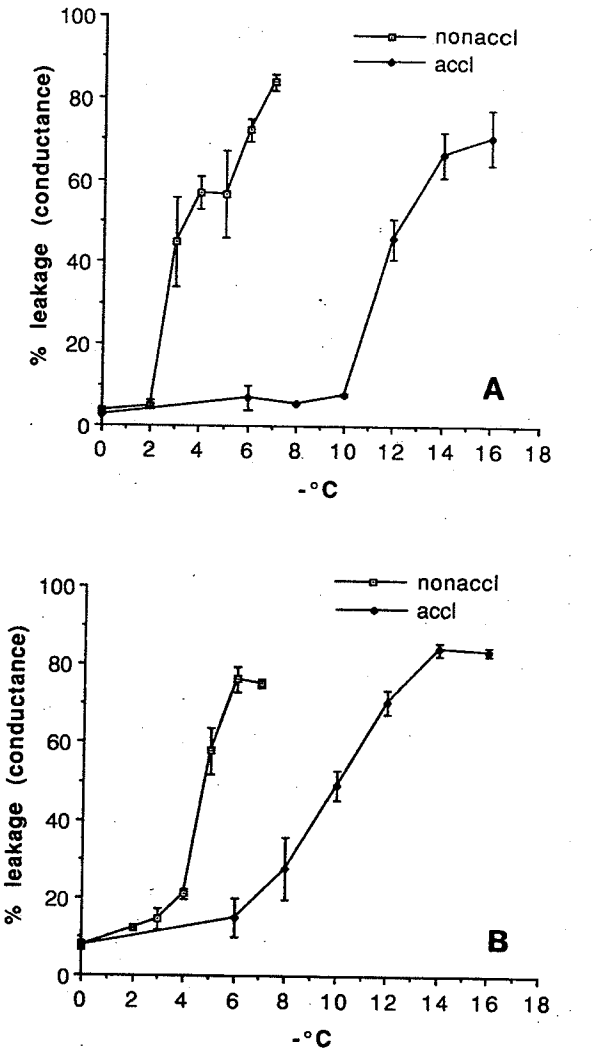

Fig. 1. Relationship between temperature and % ion leakage from leaves of nonacclimated (nonaccl) and acclimated (accl) plants of two winter-type rapeseed cultivars, *B. napus* Ceres (A) and *B. rapa* Per (B) for one experiment. Vertical bars; SE for 3 subsamples at each temperature.

at −1 °C in both cases by placing a small piece of ice in each tube with the isolated leaf half. Subsamples of three tubes per cultivar were removed, beginning at a temperature of −2 °C, at specified intervals (every 1 °C for nonacclimated and every 2 °C for acclimated plants). The control treatment consisted of three replicates per cultivar, kept on ice at 0 °C. All samples were thawed slowly on ice overnight. Thawed leaves were then cut into 5-mm strips; wetted with 20 mL of distilled water in the same test tubes in which they were frozen; degassed; and shaken at 200 rpm for 3 h.

Freezing damage was assessed by monitoring ion leakage from thawed leaf samples with a YSI conductance meter (Yellow Springs, Yellow Springs, OH). The total conductivity of the leaf leachate was determined by autoclaving the samples for 15 min at 121 °C. This total ion content was used to calculate the percent ion leakage at each temperature: ion leakage at specific temperature/total ion content = % ion leakage. Minimum ion leakage was determined by measuring the ion leakage from the controls held at 0 °C. A freezing curve was constructed for each cultivar by plotting temperature vs. % ion leakage (mean of three subsamples). The relative freezing tolerance (RFT) for each cultivar was calculated from its respective freezing curve by determining the temperature where 50%

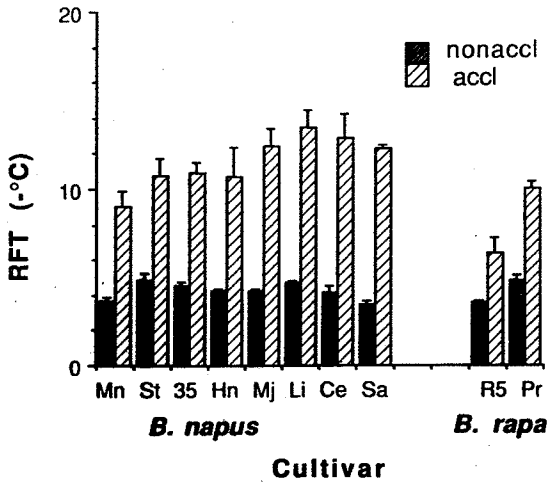

Fig. 2. Relative freezing tolerances (RFT) for nonacclimated (nonaccl) and acclimated (accl) plants of eight *B. napus* and two *B. rapa* cultivars. Mn = Marnoo, St = Stellar, 35 = 353/86, Hn = Hanna, Mj = Major, Li = Lirabon, Ce = Ceres, Sa = Santana, R5 = R500, Pr = Per. Vertical bars: SE for 3 replicated experiments.

of total leakage occurred, according to the procedure used by Sutinen et al. (1992). Specifically, 50% of total ion leakage was calculated for each cultivar as (maximum % leakage − minimum % leakage)/2 + minimum % leakage. The RFT was determined from the freezing curve as the temperature at which 50% ion leakage occurred. The acclimation ability of each cultivar was calculated as the absolute value of the difference in RFT between nonacclimated and acclimated plants. This entire experiment was repeated three times.

Field Trials

Seeds were planted on 7 Sept. 1990 at two different locations in Wisconsin, the Arlington and the Spooner agricultural research stations. Each plot consisted of 60 seeds planted in a row 1.5 m long; rows were spaced 0.76 m apart. Three replicate plots of each cultivar were planted in a randomized complete-block design.

The number of live plants per plot was counted at each location in the fall of 1990 (28 Sept. at Arlington, 6 Nov. at Spooner) and again in the spring of 1991 (17 Apr. at Arlington, 29 Apr. at Spooner). Percentage survival was recorded as (no. of plants per plot in spring/no. of plants per plot in fall) × 100.

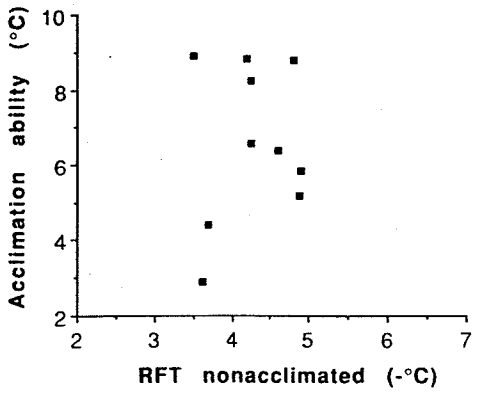

Fig. 3. Relationship between relative freezing tolerance of nonacclimated plants (RFT nonacclimated) and acclimation ability of eight *B. napus* and two *B. rapa* cultivars. Mn = Marnoo, St = Stellar, 35 = 353/86, Hn = Hanna, Mj = Major, Li = Lirabon, Ce = Ceres, Sa = Santana, R5 = R500, Pr = Per.

Statistical Analyses

A separate analysis of variance was performed on RFT values from the in vitro freezing assay for nonacclimated and acclimated plants, as well as on the percentage survival from the field trials. Factors in the model for the in vitro assays were cultivars, blocks (i.e. the three replicated experiments) and cultivar × block (used as the error term). Factors in the model for the field trial were location, block within location, cultivar, cultivar × location, and cultivar × block within location (used as the error term). Analyses were performed using the general linear model procedure of SAS. Minitab was used to calculate correlation coefficients of the 10 rapeseed cultivars for the variables RFT of nonacclimated plants, RFT of acclimated plants, acclimation ability, percentage survival at Arlington, and percentage survival at Spooner.

RESULTS

In Vitro Freezing Assays

The in vitro freezing assay was based on ion leakage as a measure of freezing damage. The relationship between temperature and percent ion leakage (freezing curves) of acclimated and nonacclimated plants for two of the winter *Brassica* cultivars, one *B. napus* and one *B. rapa* is shown in Fig. 1. The shape of these curves was similar for all the cultivars, and in each case acclimation shifted the entire freezing curve to lower temperatures, although the specific temperature range varied between cultivars. Acclimation increased the freezing tolerance (RFT) of Ceres from −3.8 to −11.5 °C (Fig. 1A) and of Per from −4.5 to −9.3 °C (Fig. 1B). Thus, the freezing tolerances of these two cultivars were more different from each other after acclimation than when nonacclimated.

The RFTs of nonacclimated and acclimated plants were determined for eight *B. napus* and two *B. rapa* cultivars (Fig. 2). The initial RFT (nonacclimated plants) of all the 10 cultivars was quite similar (≈ −5 °C); however, based on ANOVA, the RFT of the nonacclimated plants differed significantly between cultivars ($P < 0.01$), as did the RFT of the acclimated plants ($P < 0.01$). There was no significant correlation between acclimation ability and RFT of the 10 cultivars (Fig. 3).

The growth habit of the eight *B. napus* and two *B. rapa* cultivars, whether winter or spring type, was related to acclimation ability of the plant in vitro (Fig. 4A). All the spring types (Marnoo, Stellar, 353/86, Hanna, and R500) acclimated less than the winter types (Major, Lirabon, Ceres, Santana, and Per). Overall, the *B. rapa* cultivars acclimated less, but the winter-type Per did exhibit greater acclimation than the spring-type R500 (Fig. 4A).

Field Trials

Based on results from ANOVA, cultivar effects on winter survival were significant ($P < 0.001$) at the two locations. Locations and location × cultivar were not significant factors in the model. The percentage survival at the two locations is presented for the cultivars in order of increasing ability to acclimate (Fig. 4B). The winter survival generally parallels the acclimation ability (as

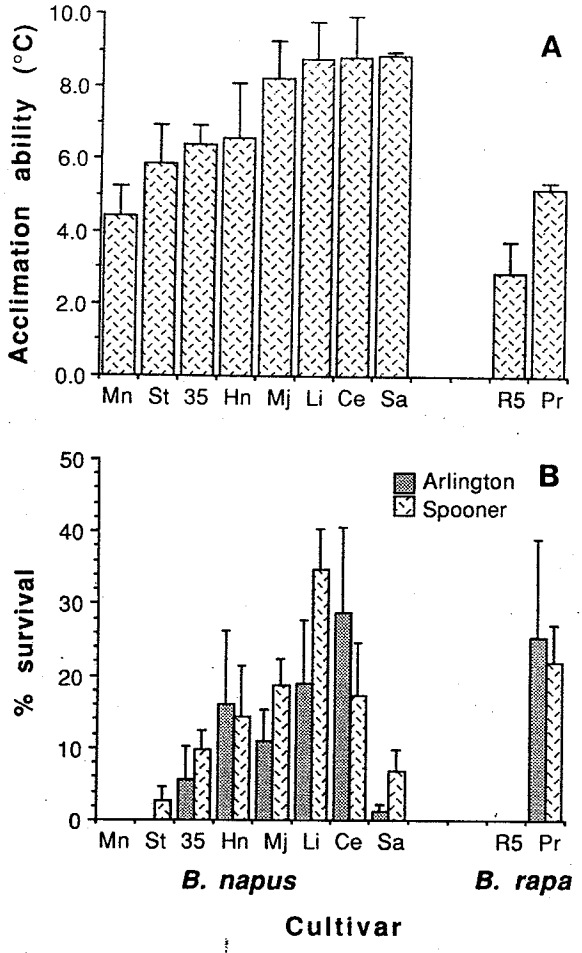

Fig. 4. Acclimation ability and winter field survival of eight *B. napus* and two *B. rapa* cultivars. A. Ability to acclimate, expressed as increase in freezing tolerance following acclimation B. Winter field survival at two locations (Arlington and Spooner, WI), expressed as percent survival of fall-seeded plants after the winter. Mn = Marnoo, St = Stellar, 35 = 353/86, Hn = Hanna, Mj = Major, Li = Lirabon, Ce = Ceres, Sa = Santana, R5 = R500, Pr = Per. Vertical bars: SE for 3 replicated experiments (A) or 3 blocks (B).

determined by the in vitro assay), except for Santana (Sa) which had a high capacity for acclimation but did not survive in either field location (Fig. 4A,B).

Correlation coefficients were calculated for the associations of winter survival at the two locations with RFT of acclimated and nonacclimated plants and with acclimation ability. When Santana was deleted from the comparisons, freezing tolerance of acclimated plants was correlated with winter survival ($r = 0.61$, $P < 0.1$ for Arlington; $r = 0.72$, $P < 0.05$ for Spooner), as was acclimation ability ($r = 0.61$, $P < 0.1$ for Arlington; $r = 0.68$, $P < 0.05$ for Spooner). When only seven *B. napus* cultivars were compared (omitting Santana), acclimation ability was more significantly correlated with winter survival ($r = 0.85$, $P < 0.05$ for Arlington; $r = 0.82$, $P < 0.05$ for Spooner), as was the freezing tolerance of acclimated plants ($r = 0.77$, $P < 0.05$ for Arlington; $r = 0.86$, $P < 0.05$ for Spooner). Freezing tolerance of nonacclimated plants was not correlated significantly with winter survival.

DISCUSSION

The main points to be drawn from the data presented are: (i) acclimation increases the freezing tolerance of both *B. napus* and *B. rapa* cultivars, (ii) there is no correlation between acclimation ability and nonacclimated freezing tolerances, (iii) ability to acclimate in vitro correlates with winter field survival, and (iv) acclimation ability is important for winter survival, but is not the only factor involved.

Cold treatment of *Brassica* plants for a few weeks increases their ability to survive freezing temperatures (Kacperska-Palacz, 1978; Li et al., 1989). Both *B. napus* and *B. rapa* cultivars tested could acclimate, as measured by ion leakage (Fig. 1), although there were differences in degree. The *B. rapa* cultivars were found to acclimate to a lesser extent than the *B. napus* cultivars; however, these results cannot be generalized to differences between the species, since differences observed could be due to variability between the cultivars tested and not to overall species differences.

Rapeseed cultivars are classified as winter or spring types based on their requirement for vernalization to flower (Andersson and Olsson, 1961; Downey et al., 1975), not on their level of winter survival. In this study, the spring *B. napus* cultivars did acclimate less and most had lower winter survival than the winter-type cultivars (Fig. 4); however, there was no clear distinction in acclimation ability or in winter survival between winter and spring cultivars. This suggests that the lack of a vernalization requirement may not preclude the development of freezing tolerance.

Winter field survival involves a number of plant characteristics, including freezing tolerance and acclimation ability (Thompson and Hughes, 1986). The in vitro freezing tolerance assay we used measured both initial freezing tolerance of leaves and the ability of the plant to acclimate to low temperatures. These two traits were not correlated (Fig. 3), suggesting that they are regulated independently. These results are in agreement with the lack of relationship observed for these two traits in potato species (Stone et al., 1991; Palta, 1992). Since the two traits were not correlated, it was important to determine which one could be used as an indicator of winter survival. Ability to acclimate (Fig. 4A) had the highest correlation with field survival (Fig. 4B). This assay, therefore, could be useful for predicting winter survival.

The fact that the *B. napus* cultivar Santana was able to acclimate in the in vitro assay, but did not survive the field trial, indicates that acclimation ability is important, but not always sufficient for winter survival. The Santana plants were very small when stand counts were taken in the early fall (3 to 4 wk after planting), so the initial rate of seedling growth was measured under controlled conditions to see if seedling growth could have been a factor in field survival differences. Santana seeds took twice as long (8 d) for the cotyledons to emerge than any of the other *B. napus* cultivars (3.3–4.8 d), so plants of this cultivar would have been smaller and at a younger developmental stage when the first frosts occurred. Planting date is known to have a dramatic effect on winter survival (Auld et al., 1984; Topinka et al., 1991); this result is attributed to the effect that planting date has on the size and morphology of plants as they enter the winter.

Although acclimation ability was correlated highly with field survival (Fig. 4A,B) other factors probably contributed to winter survival. Some factors known to be critical in *Brassica* winter survival have been identified previously. Good freezing tolerance in biennial rapeseed cultivars required that seedlings have 6 to 8 leaves and good crown development prior to the onset of severe weather (Auld et al., 1989), a root diameter between 5 and 16 mm, rosette formation, and lack of stem elongation (Kacperska-Palacz, 1978; Topinka et al., 1991). In addition, winter turnip rape (*B. rapa*) usually survives winter better than *B. napus* because the terminal growing point of turnip rape plants are almost below ground level, while the winter *B. napus* are aboveground (Olsson, 1960).

To study the genetic regulation of winter survival and freezing tolerance, we currently are using segregating populations of *B. rapa* and *B. napus* to map genes for these different cold responses. The fact that freezing tolerance of nonacclimated plants and ability to acclimate are not correlated with each other suggests that they measure distinct traits and most likely are regulated independently. In contrast, the correlation between winter survival and acclimation ability suggests that these two traits share some common regulatory mechanism. Analysis of our segregating population should let us determine whether different sets of genes control these cold responses and if any of these same genes are responsible for the variation observed in winter survival.

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