

COLD TOLERANCE IN *ARABIDOPSIS KAMCHATICA*¹

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- *Premise of the study:* Cold tolerance is a critically important factor determining how plants will be influenced by climate change, including changes in snowcover and extreme weather events. Although a great deal is known about cold tolerance in *Arabidopsis thaliana*, it is not highly cold tolerant. This study examined cold tolerance and its genetic diversity in an herbaceous subarctic relative, *Arabidopsis kamchatica*, which generally occurs in much colder climates.
- *Methods:* Thermal analysis and electrolyte leakage were used to estimate supercooling points and lethal temperatures (LT₅₀) in cold-acclimated and nonacclimated families from three populations of *A. kamchatica*.
- *Key results:* *Arabidopsis kamchatica* was highly cold tolerant, with a mean LT₅₀ of -10.8°C when actively growing, and -21.8°C when cold acclimated. It also was able to supercool to very low temperatures. Surprisingly, actively growing plants supercooled more than acclimated plants (-14.7 vs. -12.7°C). There was significant genetic variation for cold tolerance both within and among populations. However, both cold tolerance and genetic diversity were highest in the midlatitude population rather than in the far north, indicating that adaptations to climate change are most likely to arise in the center of the species range rather than at the edges.
- *Conclusions:* *Arabidopsis kamchatica* is highly cold tolerant throughout its range. It is far more freeze tolerant than *A. thaliana*, and supercooled to lower temperatures, suggesting that *A. kamchatica* provides a valuable complement to *A. thaliana* for cold tolerance research.

Key words: *Arabidopsis kamchatica*; Brassicaceae; climate change; cold acclimation; cold tolerance; electrolyte leakage; freeze avoidance; freeze tolerance; genetic variation; LT₅₀; supercooling.

Cold tolerance is a critical factor determining how plant populations will be influenced by climate change. Climate modeling scenarios predict that winters will be warmer in many regions

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(ACIA, 2005; Brown et al., 2008). This would initially suggest that less cold tolerance will be needed. However, in many cold regions, herbaceous plants that are typically covered by an insulative layer of snow throughout winter may be exposed to more severe cold either due to reduced snowfall or midwinter warming events that result in snowmelt, followed by normal winter cold (Shabbar and Bonsal, 2003; Beniston, 2005; Bokhorst et al., 2009, 2010). There have already been reductions to snow cover in many northern regions, and this trend is predicted to continue (Kreyling, 2010). Thus, winter warming in some parts of the far north may paradoxically require that herbaceous plants evolve greater cold tolerance. Furthermore, changes to atmospheric circulation are altering the geographic distribution of severe winter cold and causing extreme weather events (Cohen et al., 2014), which may require plants to become more cold tolerant. As the environment changes, populations must migrate, adapt, or respond plastically to persist (Aitken et al., 2008). Studies of cold tolerance and its genetic diversity in natural plant populations are needed to predict the potential for migration and adaptation in response to climate change.

Different species have different strategies for coping with freezing temperatures. Some taxa avoid freezing, while others have strategies to control and tolerate extracellular freezing without tissue damage (Sakai and Larcher, 1987; Reyes-Diaz et al., 2006; Jacobsen et al., 2007). Supercooling, the cooling of a liquid below its freezing point without freezing, is a common

mechanism of freeze avoidance (Reyes-Diaz et al., 2006; Hacker et al., 2011). In freeze avoidant plants, the temperature of tissue death is typically similar to the temperature at which ice forms in the tissue (Bravo et al., 2001; Reyes-Diaz et al., 2006). On the other hand, freeze tolerant plants can tolerate the formation of ice in extracellular spaces without tissue damage (Lipp et al., 1994). Thus plants can be considered freeze tolerant if the temperature that causes significant cellular damage (LT_{50}) is significantly below the temperature of tissue freezing (Lipp et al., 1994).

Strategies for dealing with freezing temperatures may differ seasonally. While almost all plants supercool to some extent, most herbaceous plants do not supercool to extremely low temperatures (Burke et al., 1976). It is thought that for many species, supercooling is not a stable long-term overwintering strategy, but is more useful for short-term freeze avoidance (Sakai and Larcher, 1987), such as surviving a cold snap during the growing season. Freezing of supercooled liquids is often rapid and uncontrolled, and more likely to damage tissues (Burke et al., 1976). Freeze tolerant plants often freeze at high subzero temperatures to better control ice proliferation and prevent intracellular ice formation, which is almost always lethal (Sakai and Larcher, 1987; Fujikawa et al., 1999; Guy, 2003). Thus, freeze tolerance with slow, controlled freezing is thought to be the main mechanism for winter plant survival when temperatures may be low for long periods of time (Sakai and Larcher, 1987; Puhakainen et al., 2004).

Arabidopsis thaliana (L.) Heynh. (Brassicaceae) has been widely used as a model species for understanding the genetics and physiology of cold tolerance and cold acclimation in plants (Gilmour et al., 1988; Thomashow, 1999; Xin and Browse, 2000; Hannah et al., 2006). However, there has been surprisingly little research on cold tolerance of *Arabidopsis* relatives (Davey et al., 2009), despite the many advances that have come from studying the ecological genetics of other traits such as mating systems, flowering time, and insect resistance in *Arabidopsis* relatives (Charlesworth et al., 2003; Comai et al., 2003; Mable et al., 2004; Riihimaki and Savolainen, 2004; Leinonen et al., 2009; Turner et al., 2010; Schmickl and Koch, 2011; Vergeer and Kunin, 2011). The herbaceous perennial, *Arabidopsis kamchatica* (Fisch. ex DC.) K. Shimizu & Kudoh, occurs in regions where the air temperatures regularly drop as low as -45°C . Because of its close relationship to the model organism, *A. kamchatica* provides an excellent opportunity to study cold acclimation and tolerance to extreme cold.

Arabidopsis kamchatica is a small perennial plant that overwinters under the snow as a rosette and flowers shortly after snowmelt. Although leaves are typically red and leathery after snowmelt, they survive winter and likely provide resources needed for spring growth and flowering because there is no bulb or other obvious storage organ. This species has an amphi-Berlingian distribution (Fig. 1), ranging from Taiwan, Japan, eastern Russia, Alaska, and western Canada. *Arabidopsis thaliana* lives throughout the United States, Europe, and Asia, but its North American range is more southerly than that of *A. kamchatica* (Fig. 1). *Arabidopsis thaliana* functions either as a summer annual that germinates and flowers in spring or a winter annual that overwinters as a rosette and flowers in spring (Griffith et al., 2004). Plants at the northern limit of the species range are primarily winter annuals due to a short growing season (Kuittinen et al., 1997a, b; Hoffmann, 2002). Both *Arabidopsis* species have a low-to-the-ground basal rosette and are found in disturbed areas such as roadsides, gravel pits, rocky slopes, and along creeks

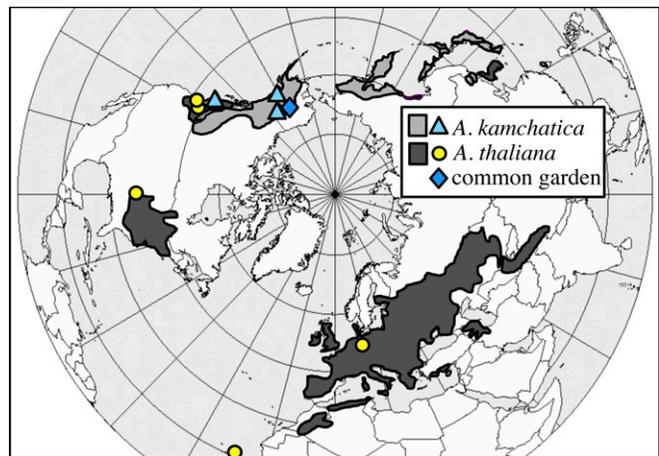


Fig. 1. Plants for studies of cold tolerance originated from three populations of *Arabidopsis kamchatica* (triangles: Strathcona Park, British Columbia, Canada; Portage Glacier, Alaska, USA; and Steese Highway, Alaska), and five populations of *A. thaliana* (circles). *A. kamchatica* from all three populations were grown in a common garden in Fairbanks, Alaska (rhombus). *A. kamchatica* and *A. thaliana* species distributions are shaded gray with stripes indicating where species distributions overlap (Hoffmann, 2005).

(Al-Shehbaz and O’Kane, 2002). Because herbaceous plants from northern regions often depend on snow cover to insulate overwintering structures against severe cold (Bertrand and Castonguay, 2003), short stature and snow cover are likely to be important contributing factors for winter survival.

The goal of this study was to investigate cold tolerance strategies of *Arabidopsis kamchatica* by measuring (1) leaf damage from freezing temperatures, (2) supercooling, (3) the capacity to cold acclimate, (4) levels of genetic variation for cold tolerance within and among populations, and (5) survival of populations from different latitudes at the northern edge of the species range. *Arabidopsis thaliana* was included in laboratory studies as a reference for comparison.

MATERIALS AND METHODS

Plant material—*Arabidopsis kamchatica*—In 2007, seeds were collected in the field from 60 plants (maternal families) from each of three populations representing the entire latitudinal range of the species in North America (Fig. 1). A total of 492 plants grown from these seeds were studied. Sample sizes for each assay are provided in Appendix S1A (see Supplemental Data with the online version of this article). The southern population was located at Elk River in Strathcona Park, Vancouver Island, British Columbia, Canada ($49^{\circ}49'74\text{ N}$, $125^{\circ}52'36\text{ W}$); the midlatitude population was at Portage Glacier, Alaska, USA ($60^{\circ}47'25\text{ N}$, $148^{\circ}54'06\text{ W}$); and the northern population was at Ptarmigan Creek, Alaska, USA ($65^{\circ}27'12\text{ N}$, $145^{\circ}30'27\text{ W}$). There are considerable differences in air temperatures among these locations (Fig. 2).

Arabidopsis thaliana—Seeds of five *A. thaliana* ecotypes from a wide latitudinal range (Fig. 1; ecotypes Col-0, Ler, Cvi, Seattle-0, Van-0) were obtained from The Arabidopsis Biological Resource Center at Ohio State University. Col-0, Ler, and Cvi were chosen because they have been widely studied for cold tolerance (Hannah et al., 2006; Reyes-Diaz et al., 2006; Zhen and Ungerer, 2008; Zuther et al., 2012), permitting comparison among laboratories. We assumed that each accession represents a self-fertilized lineage, and individuals within an accession are genetically homogeneous. Seeds were stratified at 4°C for 2 wk to induce germination.

Growth conditions for laboratory measurements of cold tolerance—Growth conditions are summarized in Appendix S1B. Seeds of *A. kamchatica*

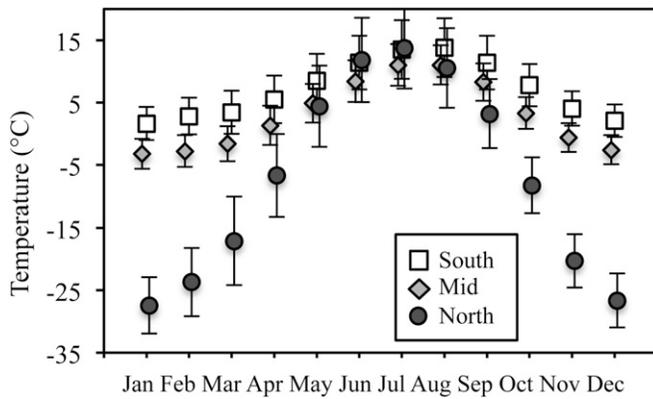


Fig. 2. Mean monthly air temperatures (°C) at the southern (Strathcona Park, British Columbia, Canada), midlatitude (Portage Glacier, Alaska, USA), and the northern (Ptarmigan Creek, Alaska) populations of *A. kamchatica*. Bars indicate mean maximum and minimum temperatures for each month. Air temperature data were obtained from the Global Climate Database (<http://www.worldclim.org/>) (Hijmans et al., 2005) using methods described by Steets et al. (2010).

were stratified at 4°C for 3 wk, then germinated in a 20° growth chamber with 24 h light. Electrolyte leakage (EL) assays were initiated when all plants were at least 45 d old (year 1 nonacclimated measurements). After nonacclimated measurements were complete, plants were cold acclimated for 10 wk at 4°C with 8 h of light per day and remeasured (year 1 acclimated measurements). After the year 1 measurements were complete, plants were moved to the greenhouse and allowed to reacclimate to summer conditions. After 5 mo, a hap-hazard sample of the plants was selected for year 2 measurements of EL, supercooling, and comparison with *A. thaliana*. Half of these plants were assayed immediately for nonacclimated EL and supercooling (year 2 acclimated measurements), while the other half was cold acclimated for 4 wk (year 2 acclimated measurements). A shorter acclimation period was used in year 2 because we were also testing *A. thaliana* and thought that 10 wk would be too long for *A. thaliana*. Seeds of *A. thaliana* were stratified at 4°C for 2 wk to induce germination, and plants were grown at 20°C with 24 h of light; EL was measured for 30-d-old plants. All plants were green and healthy when cold tolerance traits were measured, with no indications that the 4°C treatment was stressful. The location of plants in the growth chamber was randomized, so that differences among families or among populations would not be influenced by microenvironmental variation. Plants were watered 24 h before each laboratory assay, and assays were initiated at the same time each morning to reduce random variation.

Electrolyte leakage (EL) assay—Freeze damage was quantified by measuring electrolyte leakage (EL), which was used to estimate the temperature of 50% tissue death, LT_{50} . Freeze damage occurs when the cell's plasma membrane is damaged, allowing cellular contents, including electrolytes, to leak out (Sakai and Larcher, 1987). Fully expanded rosette leaves of similar age and size were collected from each plant and placed in the bottom of a 10 × 13 mm open-top glass tube, which was immersed in a NesLab programmable circulating ethanol bath (Portsmouth, New Hampshire, USA). Tubes were equilibrated at 0°C for 30 min and cooled at a rate of 1°C/h (nonacclimated leaves) or 4°C/h (acclimated leaves). More rapid cooling of acclimated leaves was necessary because acclimated leaves were damaged only at very low temperatures and the assay would take too long under the slower rate of cooling used for nonacclimated plants. Freezing was initiated by adding ice chips to the surface of each leaf at -2°C. Tubes were removed at various temperatures (Appendix S1C), and immediately placed in a 4°C refrigerator, where they were allowed to thaw for at least 16 h. Then 5.4 mL of deionized water was added, and tubes were shaken at room temperature for 1 h, and the initial electrolyte leakage was measured (EL_i). Total electrolyte content of the tissue (EL_t) was measured after autoclaving the sample (121°C for 20 min) and allowing it to shake at room temperature overnight to release all of the electrolytes. Electrolyte leakage was measured using an Oakton CON6 conductivity meter (Vernon Hills, Illinois, USA). The relative leakage (EL_R) was calculated as $100\% \times EL_i/EL_t$. Three replicate leaves per plant were tested at each temperature. These EL_R values

were used to estimate the temperature of 50% electrolyte leakage, LT_{50} . EL was measured for 14867 leaves of *A. kamchatica* and 201 for *A. thaliana*, with three replicate leaves per genotype for each temperature (Appendix S1A).

All statistical analyses were conducted in the R Statistical Environment (R Development Core Team, 2011), and our scripts are provided in Appendix S2A-E (see online Supplemental Data). To estimate the LT_{50} for each population, we used a logistic function to fit a sigmoidal curve to the relative electrolyte leakage values (EL_R). EL_R at temperature T is assumed to have the following relationship:

$$EL_R = 1 - 1 / (1 + \exp[-(T - d)/s]),$$

where the inflection point, d , gives the LT_{50} , and s is a scale parameter. With this method, the lower asymptote of the curve approaches an EL_R of 0, where there is no leaf damage, and the upper asymptote approaches an EL_R of 1, corresponding to 100% cell lysis due to freeze damage. Nonlinear mixed-effects models, implemented in an R package nlme (Pinheiro and Bates, 2000), were used to estimate the LT_{50} and to test for differences between species, among populations, and between acclimation treatments (further described in online Appendix S3). When the logistic function in the R statistical package (R Development Core Team, 2011) was used, the dependent variable was relative electrolyte leakage (EL_R), and the independent variables were population as a fixed effect and family and individual as random effects for the comparison among populations. For the second year EL measurement, the model did not contain the family random effect since we only used one individual per family. The independent variables were allowed to have linear effects on the two parameters (d and s). For between species comparisons of LT_{50} , our independent variables were species and acclimation treatment as fixed effects and population and individual as random effects. To determine whether there was genetic variation in LT_{50} within populations of *A. kamchatica* and to estimate heritability, we estimated the proportion of among family genetic variation in the LT_{50} using an intraclass correlation (ICC) approach (Appendix S1). Because multiple individuals per family were tested in year 1, but not year 2, only first year EL data were used for estimates of within-population genetic variation.

Supercooling assay—The supercooling point (temperature at which leaf freezing begins, also called the ice nucleation temperature) was determined by thermal analysis, which detects the exothermic release of heat as water freezes (Hekneby et al., 2006; Reyes-Diaz et al., 2006). Each leaf was placed in the bottom of a 10 × 13 mm glass tube with a PT-907 temperature probe (Pace Scientific, Mooresville, North Carolina, USA) secured against the surface of the leaf. The tubes were immersed in a NesLab programmable circulating bath, which was equilibrated at 0°C for 30 min and cooled at a rate of 4°C/h to -35°C. Leaf temperature was recorded every second using LogXR software with a programmable XR5-SE-M data logger (Pace Scientific). Three replicate leaves were tested for each plant.

We used a linear mixed-effects model to test for differences between species, among populations, or between acclimation treatments. We used an R package, lme4 (Bates, 2005). The dependent variable was the supercooling point. For comparisons between species, the fixed effects were species and cold acclimation treatment while the random effects were population, individual, and date of trial. For population level comparisons within a single species, the fixed effects were population and acclimation, while the random effects were individual and date of trial. We determined 95% confidence intervals by Markov chain Monte Carlo sampling implemented in an R package, gmodels. Hypothesis tests of the fixed effects were conducted with 5000 iterations of the parametric bootstrap approach based on the likelihood ratio statistics of Faraway (2006).

Common garden—To determine whether differences in cold tolerance among the three populations of *A. kamchatica* from different latitudes translated to survival differences in the field, we grew a total of 227 plants from all three populations in a common garden outside of the University of Alaska Fairbanks' Institute of Arctic Biology greenhouse, which is near the northern limit of the species range (Fig. 1; garden: 64°51' N, 147°50' W, 177 m above sea level; species range: 49°49' N to 65°42' N). Temperature was recorded with a Hobo U23 Pro v2 data logger (Cape Cod, Massachusetts, USA) placed in the garden at plant height (on the ground). Sample sizes for each population were determined by seed germination (Appendix S1A).

Seeds were planted in 15-cm pots in the greenhouse in mid-July 2008. Approximately 30 d after germination, seedlings were acclimated to outdoor conditions, and then pots were placed into holes in the ground at the end of August. Plants had full sun exposure as they would in many natural populations, but were given supplemental water as necessary until first snowfall. In late summer, there was widespread grasshopper herbivory, so the pots and plants were enclosed in bridal veil until the first snowfall. Plants remained snow covered and undisturbed throughout the winter, and survival was scored in early May 2009, approximately 2 wk after snow melt. Leaves from the previous season persisted with a reddish (anthocyanin), stressed appearance, and overwinter survival of each plant was determined by the presence of new green leaves. Pearson's χ^2 test was used to test for differences among populations in overwintering survival.

RESULTS

***Arabidopsis kamchatica* LT₅₀**—*Arabidopsis kamchatica* was highly cold tolerant, both before and after cold acclimation (Fig. 3; Appendix S1D). LT₅₀ estimates, measured by electrolyte leakage, ranged from -9.1°C to -13.3°C in nonacclimated populations and from -16.8°C to -28.3°C in acclimated populations. Plants were significantly more cold tolerant (had a lower LT₅₀) after being subjected to 4°C for either 10 wk (year 1) or 4 wk (year 2), indicating that *A. kamchatica* acclimates in response to cold, nonfreezing temperatures ($D = 64.98073$, $df = 1$, $P < 0.0001$).

Comparison of LT₅₀ estimates in year 1 and year 2 shows that plants were more cold tolerant in their second year (Fig. 3). Nonacclimated year 2 plants were tolerant to temperatures an average of 2.0°C lower than year 1 plants ($D = 85.15$, $df = 1$, $P < 0.001$). Year 2 acclimated plants were tolerant of temperatures an average of 4.8°C lower than year 1 plants, even with a shorter acclimation time (4 wk vs. 10 wk; Fig. 3). Although all populations were more cold tolerant in their second year of acclimation (PG: $D = 65.90$; PTC: $D = 88.68$; SP: $D = 13.027$, $df = 1$, all $P < 0.001$), the magnitude of the difference between years differed among populations, with the southern population having the smallest increase in cold hardiness (interaction between year and population: $D = 16.95$, $df = 1$, $P < 0.001$). The confidence intervals for year 2 estimates of LT₅₀ are wider than for year 1 (Fig. 3) due to a smaller sample size in year 2 and therefore a less precise estimate of parameters.

LT₅₀ variation within and among populations—Midlatitude *A. kamchatica* plants were consistently more tolerant of low temperatures than plants from the other populations (Fig. 3). In acclimated plants, LT₅₀ was significantly different among populations for both measurement years (year 1: $D = 93.80$, $df = 2$, $P < 0.0001$; year 2: $D = 12.28$, $df = 2$, $P = 0.0005$). In nonacclimated plants, LT₅₀ significantly differed among populations in the first year ($D = 66.16$, $df = 2$, $P < 0.0001$), and the variation among populations was nearly significant in the second year ($D = 5.33$, $df = 2$, $P = 0.07$). The decrease in significance was presumably due to the much smaller sample size in year 2. Statistical methods for post hoc comparisons of parameters in nonlinear mixed effects models are not well developed. However, pairwise comparisons of 95% confidence intervals show that the midlatitude plants consistently had the lowest LT₅₀ estimates; confidence intervals did not overlap with other populations in any of the treatments except between acclimated midlatitude and southern plants in year 2. In year 1, northern plants had a lower LT₅₀ than southern plants when nonacclimated, but surprisingly the relationship was reversed in acclimated plants. In year 2, confidence intervals for the northern

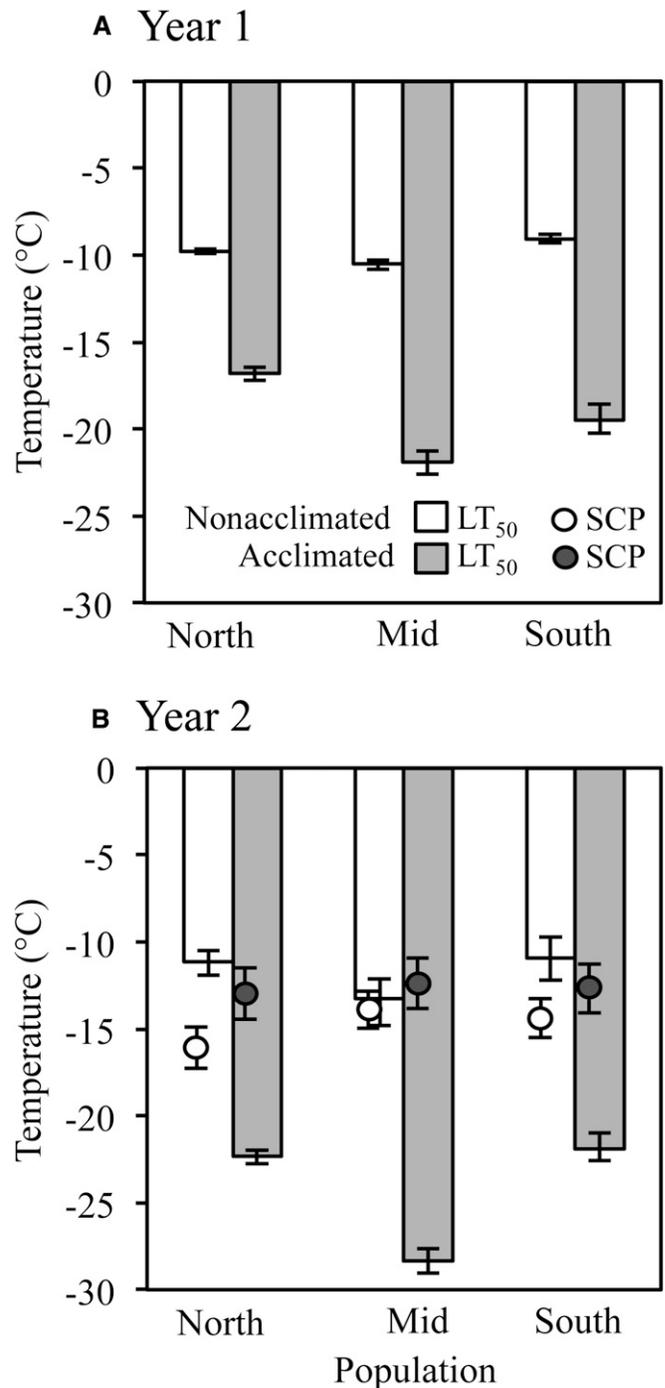


Fig. 3. Estimates of the lethal temperature (LT₅₀) for nonacclimated and acclimated plants of *Arabidopsis kamchatica* in (A) year 1 and (B) year 2. Year 2 includes supercooling points (SCP). Error bars represent 95% confidence intervals of the estimates. Note that direct comparisons of year 1 and year 2 plants are not appropriate as they differed in multiple variables: plant age, growing conditions, and acclimation time.

and southern populations overlapped for both nonacclimated and acclimated plants.

The significant differences among populations are likely to indicate genetic (or epigenetic) variation for cold tolerance among populations because all plants were grown in identical conditions and plant location was randomized to control for

possible microclimate variation (Clausen et al., 1940). There was also genetic variation for cold tolerance within some populations (Fig. 4). The midlatitude population showed high levels of intraclass correlation (ICC) among families in LT_{50} , both before and after cold acclimation (nonacclimated: $D = 19.75$, $P = 0.0001$; acclimated: $D = 17.43$, $df = 2$, $P = 0.0002$). Because ICC is proportional to heritability (Appendix S1), this indicates a high level of genetic variation for cold tolerance within this population. Similarly, the southern population had high levels of ICC, but the ICC was only statistically significant for nonacclimated plants (nonacclimated: $D = 14.48$, $df = 2$, $P = 0.0007$; acclimated: $D = 3.71$, $df = 2$, $P = 0.16$). On the contrary, ICC in the northern population was low and not significantly different from zero (nonacclimated: $D = 0.016$, $df = 2$, $P = 0.99$, acclimated: $D = 1.73$, $df = 2$, $P = 0.42$), suggesting that there is little genetic variation relative to the overall phenotypic variation. However, since the ICC estimates are accompanied by rather large 95% confidence intervals, it is possible that the northern population harbors some genetic variation.

Supercooling and comparison to LT_{50} for *A. kamchatica*—Surprisingly, supercooling was greater in nonacclimated leaves of *A. kamchatica* than in acclimated leaves. Only one exotherm per leaf was detected during thermal analysis, despite cooling to -35°C , suggesting that extracellular fluids froze at the supercooling point and there was no intracellular freezing (Sakai and Larcher, 1987). This is consistent with publications for *A. thaliana* (Vernon et al., 1999; Reyes-Diaz et al., 2006); however, it is possible that a small second exotherm was missed because our sensor was not sufficiently sensitive. Ice nucleation occurred between -13.9°C and -16.1°C for nonacclimated populations, and between -12.4°C and -12.9°C for acclimated populations (Fig. 3B; Appendix S1D). There was significant variation among populations ($D = 6.33$, $df = 2$, $P = 0.046$), and the northern population supercooled significantly more than

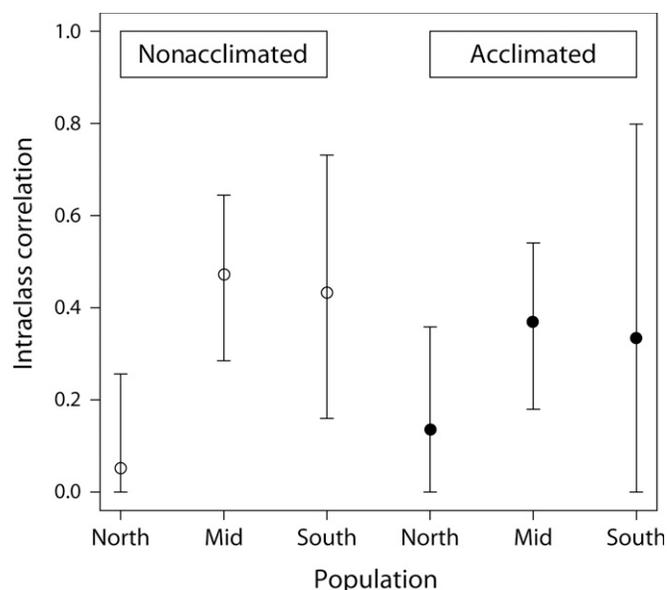


Fig. 4. Estimates of intraclass correlation (ICC) in lethal temperature (LT_{50}) among families for each *Arabidopsis kamchatica* population (year 1 plants). Open circles represent the estimated values; whiskers are the 95% confidence intervals from nonparametric bootstrapping. ICC is used as an index of within-population genetic variation which is proportional to heritability.

other populations when nonacclimated ($p < 0.05$, Tukey's posthoc test). However, the interaction between population and acclimation was not significant ($D = 2.75$, $df = 5$, $P = 0.25$), indicating that the effect of acclimation was similar across populations. In all populations, acclimated *A. kamchatica* plants supercooled less than nonacclimated plants (Fig. 3B; $D = 9.381$, $df = 1$, $P = 0.005$).

To determine whether plants were freeze tolerant or freeze avoidant, we compared the supercooling points with the LT_{50} estimates by inspecting 95% confidence intervals. Because the supercooling point and LT_{50} values are derived from very different types of data, which necessarily have a very different variance structure, this is more appropriate than a standard parametric statistical test (Payton et al., 2003). In nonacclimated *A. kamchatica*, the supercooling point was lower than (or the same as) the LT_{50} , but in acclimated leaves, the LT_{50} was much lower than the supercooling point (Figs. 3B, 5). This suggests that *A. kamchatica* is freeze tolerant when acclimated, but it may supercool to avoid freezing when nonacclimated.

Survival in the field—A common garden with individuals from the three *A. kamchatica* populations was planted at the northern limit of the species range to determine whether differences among *A. kamchatica* populations in laboratory measurements of cold tolerance translated to differences among populations in overwinter survival. Even though temperatures experienced by plants in the garden are likely to represent some of the coldest conditions this species naturally experiences, the survival rate was high in all populations (north: 89%; mid: 94%; south: 92%) and there was no significant difference among populations ($\chi^2 = 1.472$, $p = 0.479$, $df = 2$, ns). Temperatures experienced by plants under the snow were considerably warmer than air temperatures. Even though air temperatures were as low as -45°C , temperatures under the snow never dropped below -5°C in winter 2008–2009 (Fig. 6).

Comparison to *A. thaliana*—Electrolyte leakage tests show that *A. kamchatica* can withstand significantly lower temperatures than *A. thaliana* when the two species are tested under similar conditions (Fig. 5). For both nonacclimated and acclimated plants, the LT_{50} was considerably lower for *A. kamchatica* than

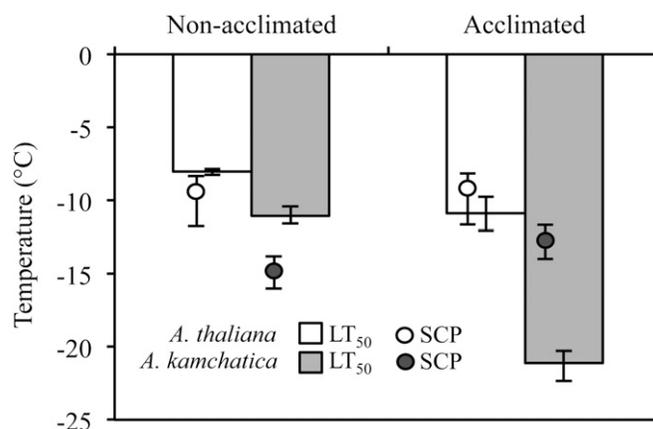


Fig. 5. Estimated lethal temperatures (LT_{50}) and supercooling points (SCP) for *Arabidopsis kamchatica* (year 2, all populations) and *A. thaliana* (all accessions). Values for individual *A. thaliana* accessions are in Appendix S1D. Error bars represent 95% confidence intervals of the estimates.

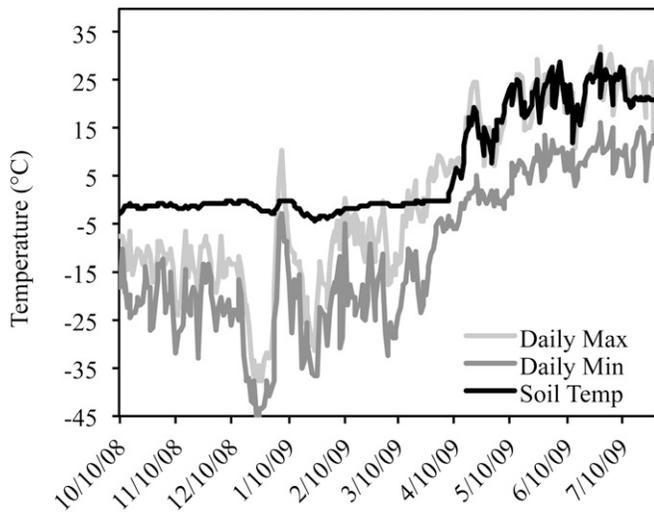


Fig. 6. Temperature experienced by common garden plants below the snow (black line) and maximum (light gray) and minimum air temperatures (dark gray) in Fairbanks, Alaska from October 2008 to July 2009. Air temperature data were obtained from the Alaska Climate Research Center (<http://climate.gi.alaska.edu/>).

A. thaliana (nonacclimated: $D = 8.77$, $df = 8$, $P = 0.003$; acclimated: $D = 16.92$, $df = 7$, $P < 0.001$). Further, *A. kamchatica* had a much greater capacity for cold acclimation than *A. thaliana*, which is indicated by a significant interaction effect (species by acclimation treatment effect on LT_{50} : $D = 55.2$, $df = 1$, $P < 0.0001$). In *A. kamchatica*, cold acclimation led to a 12.4°C mean decrease in LT_{50} , but only a 1.6°C mean decrease ($D = 16.96588$, $df = 1$, $P < 0.0001$) for the *A. thaliana* accessions that we measured (Fig. 5, Appendix S1D).

Supercooling points (ice nucleation temperatures) were also lower in *A. kamchatica* than *A. thaliana* (Fig. 5; Appendix S1D; $D = 13.75$, $df = 1$, $P = 0.002$), indicating that *A. kamchatica* supercools more. In *A. thaliana*, there was no significant difference in supercooling before and after acclimation. In both nonacclimated and acclimated *A. thaliana*, the LT_{50} and supercooling points were similar to each other, with overlapping 95% confidence intervals (Fig. 5), suggesting that *A. thaliana* may be able to avoid freezing through supercooling, but that plants may experience injury and leaf death at temperatures below the supercooling point. By contrast, the LT_{50} of acclimated *A. kamchatica* was well below the supercooling point.

DISCUSSION

***Arabidopsis kamchatica* is highly cold tolerant**—Few herbaceous plants can tolerate temperatures below -25°C (Wisniewski et al., 2003), yet the acclimated LT_{50} of *A. kamchatica* populations was quite low, ranging from -16.8°C to -28.3°C . This is comparable to cold tolerance in *Thellungiella salsuginea* O.E.Schultz (Brassicaceae), an *Arabidopsis* relative that has been described as exhibiting extreme cold tolerance (acclimated LT_{50} : $-18.5 \pm 0.2^{\circ}\text{C}$ [Griffith et al., 2007]). *Arabidopsis kamchatica* was capable of tolerating much lower temperatures than *A. thaliana* both before and after cold acclimation, and it had a much higher capacity for cold acclimation. While some previously measured accessions of *A. thaliana* can tolerate colder temperatures than the accessions we measured (Zuther et al.,

2012), *A. kamchatica* was more cold tolerant than even the most cold-tolerant *A. thaliana* (-12.1°C ; Zuther et al., 2012). There is considerable variation among laboratories in LT_{50} estimates of identical *A. thaliana* accessions (Uemura et al., 1995; Rohde et al., 2004; Hannah et al., 2006; Reyes-Diaz et al., 2006; Nagao et al., 2008; Mishra et al., 2011; Zuther et al., 2012) due to minor methodological, physiological, and environmental differences (Vernon et al., 1999; Reyes-Diaz et al., 2006; Thomashow, 2010; Catalá et al., 2011). Thus, it is difficult to compare results among laboratories. However, our LT_{50} estimates for acclimated *A. thaliana* were very similar to those of Zuther et al. (2012) for accessions in common and never more than 2°C lower. Two factors that may have lowered our LT_{50} estimates relative to other laboratories are evaporation from leaves and exposure to ethanol vapors in the cold bath (Vernon et al., 1999; Kato-Noguchi, 2008). Nonetheless, the 13.9°C difference between species in our study clearly indicates that *A. kamchatica* is more cold tolerant than *A. thaliana*.

Climatic range estimates suggest that *A. kamchatica* occurs in slightly colder regions than *A. thaliana*. The January mean daily minimum temperature (T_{\min}) in the coldest regions where *A. thaliana* occurs is -31°C , whereas the lowest T_{\min} for *A. kamchatica* is -40°C (Hoffmann, 2005). Thus, it is not unexpected that *A. kamchatica* would generally be more cold tolerant. With its higher acclimation capacity and cold tolerance, *A. kamchatica* provides a useful complement to cold tolerance research in *A. thaliana*.

Supercooling in *Arabidopsis kamchatica*—While freeze tolerant plants are predicted to limit extracellular supercooling to avoid damage from rapid, uncontrolled freezing of supercooled liquids (Sakai and Larcher, 1987), *A. kamchatica* leaves underwent supercooling between -12.4°C and -16.1°C , significantly colder than -7.0°C to -13.5°C for *A. thaliana*. Extracellular fluids of a few other freeze tolerant plants have been found to supercool to temperatures between -5°C and -16°C (Pukacki, 1987; Lipp et al., 1994; Bravo et al., 2001; Griffith et al., 2007; Sierra-Almeida et al., 2009; Sierra-Almeida et al., 2010), but both *Arabidopsis* taxa are among the lower estimates reported. Both taxa may avoid freezing during summer cold snaps by supercooling, but supercooling may be avoided in winter when snow or frost nucleation induces freezing in the leaves at temperatures above the supercooling point (Pearce, 2001), mitigating the hazards of freezing in supercooled liquids.

It is notable that the supercooling point significantly increased in acclimated *A. kamchatica* plants (i.e., acclimated plants froze at higher temperatures than acclimated plants). Most plants supercool to lower temperatures in response to acclimation (i.e., they freeze at lower temperatures; Pukacki, 1987; Gusta et al., 2004; Sierra-Almeida et al., 2009). However, *Thellungiella salsuginea*, another crucifer that lives in cold regions, also froze at higher temperatures after acclimation (Griffith et al., 2007). Reduced supercooling in acclimated plants could be due to a lack of selection on supercooling in winter when freezing is likely to be externally nucleated by snow or frost or to selection against supercooling in winter. Further studies of selection on supercooling temperatures in plants that naturally experience differences in external ice nucleation are likely to be very interesting.

The supercooling point is sensitive to the rate at which the temperature is decreased, with lowered supercooling points for rapidly cooled leaves. However, Bigg (1953) found only a 2.0°C difference in the supercooling point of 1 mm water droplets

cooled at very different rates: 3°C/h vs. 0.5°C/min. Our rate of cooling (4°C/h) is not uncommon in the natural habitat of *A. kamchatica* (D. Wolf, personal observations) and matches the cooling rate used for estimation of LT_{50} in acclimated plants. Further, our cooling rate was similar to other studies of plant leaves, including *A. thaliana* (7°C/h: Lipp et al., 1994; 2°C/h: Bravo et al., 2001; Reyes-Diaz et al., 2006; 3°C/h: Griffith et al., 2007), and our estimates of supercooling points were very similar to previous estimates for *A. thaliana* accessions in common (Reyes-Diaz et al., 2006). Estimates by Reyes-Diaz et al. (2006) are well within our 95% confidence intervals for both nonacclimated and acclimated plants. Thus, our estimated supercooling points appear to be as reliable as those of other laboratory studies.

Variation among *A. kamchatica* populations—Winter air temperatures are more extreme in the northern population of *A. kamchatica* (Fig. 2), and summer cold snaps are likely to be more common than in the other populations studied. Therefore, we expected that the northern population would have both the lowest supercooling point and LT_{50} . The northern population did have the lowest nonacclimated supercooling point (Fig. 3), suggesting that it has the greatest capacity to avoid freezing when subject to summer cold snaps. However, the midlatitude population actually had the lowest LT_{50} , both in nonacclimated and acclimated plants. This result was somewhat surprising, since latitudinal clines for cold tolerance have been seen in many taxa, including *A. thaliana* (Hurme et al., 1997; Li et al., 2002; Hannah et al., 2006; Zhen and Ungerer, 2008; Pagter et al., 2010; Mueller et al., 2011; Zuther et al., 2012; Menon, 2014), and there is likely to be a fitness cost to cold tolerance in warm environments (Jackson et al. [2004], but see Zhen et al. [2011]). It is possible that the midlatitude plants actually experience the most winter cold since the above-freezing air temperatures (Fig. 2) could allow the insulative layer of snow to melt, exposing plants to subsequent cold snaps. However, dataloggers within a kilometer of the midlatitude population confirm that winter temperatures at plant level only vary between 1°C and –1°C due to constant snow cover (A. Stephens, University of Alaska Fairbanks, personal communication). Likewise, the southern plants grow in mountainous regions that receive copious snowfall, which takes a long time to melt in spring (J. Armstrong, D. Wolf, personal observations), so winter temperatures experienced by the southern and midlatitude populations are likely similar. With a low of –5°C in 2008–2009 (Fig. 6) and –11°C in 2007–2008 (data not shown) under the snow in the Fairbanks common garden, winter temperatures at the northern populations are clearly colder than at the other populations.

The overall vigor of plants from different populations could have influenced the LT_{50} . Midlatitude plants in our common garden appeared to be the most vigorous: they were larger and produced more fruits than plants from the other populations (Armstrong, 2013) and had the lowest LT_{50} . Additionally, the northern population seems to harbor a relatively a small amount of quantitative genetic variation in LT_{50} . Thus, genetic variation overall may be lower in the northern population, which is at the edge of the species' North American range. This population may be more likely to suffer from expression of genetic load or be less adapted to its habitat due to source–sink dynamics (Lynch et al., 1995; Kirkpatrick and Barton, 1997; Bridle and Vines, 2007).

The lack of a latitudinal cline in the LT_{50} for *A. kamchatica* may also be explained by the biogeographic history of the species. *Arabidopsis kamchatica* appears to have recently

expanded from Asia, across the Bering Land Bridge and migrated southward to British Columbia (Shimizu-Inatsugi et al., 2009). Thus, southern populations in North America are likely to have been recently derived from highly cold-tolerant populations, and there may not have been sufficient time for the loss of freezing tolerance. Finally, three populations may have been too few to detect a latitudinal cline even if it does exist.

Genetic variation in *A. kamchatica*—There has been a great deal of interest in the genetic variation of cold tolerance in cultivated plants, and there is considerable genetic variation among cultivars (Keim and Gardner, 1984; Dörffling et al., 1997; Stefaniak, 2008). There is also genetic variation among accessions of *A. thaliana*, and that variation has been very useful in understanding the genetics of cold acclimation (Hannah et al., 2006; Zhen and Ungerer, 2008; Zuther et al., 2012). However, there have been few, if any, studies of within-population genetic variation for cold tolerance in natural plant populations (Guy, 1990). Work in *A. thaliana* is focused on differences among accessions from different populations. Here we show that in addition to genetic variation among populations, there is also significant genetic variation within some populations of *A. kamchatica*, evidenced by significant heritability (ICC) in LT_{50} . Natural selection is typically thought to act most effectively within populations. However, migration among populations is an important mechanism to introduce new genetic variation to populations. Thus, both within-population genetic variation and among-population genetic variation are critical factors in response to selection.

Considering the biogeographic history of *A. kamchatica* (Shimizu-Inatsugi et al., 2009), which would predict that the middle and southern populations would have the lower genetic variation, the low genetic variation for cold tolerance in the northern population is surprising. The discrepancy may be explained by source–sink dynamics; populations at the ecological extremes of the species range may be smaller and, therefore, have lower genetic variation than those at the center of the range (Lynch et al., 1995; Kirkpatrick and Barton, 1997; Bridle and Vines, 2007). Consistent with this prediction, we found the highest genetic variation in the midlatitude population near the center of the North American range of the species.

Climate change in the far north may paradoxically require that herbaceous plants evolve greater cold tolerance if insulation from snow cover is reduced (Shabbar and Bonsal, 2003; Beniston, 2005; Bokhorst et al., 2009, 2010; Kreyling, 2010). In response to climate change, populations may need to adapt, migrate northward, or face extirpation (Aitken et al., 2008). The low genetic variation in the northern population suggests that populations at the range limit may have a limited capacity to adapt to climate change. Low adaptive capacity could decrease fitness in the current range and limit the potential for northern populations to expand into the arctic as temperatures warm. Northern populations may be dependent on the introduction of genetic variation from the center of the species range to facilitate adaptation and range expansion.

Relevance of laboratory measurements to winter survival—Low LT_{50} estimates in *A. kamchatica* are consistent with the high winter survival seen in our common garden. Even though air temperatures were as low as –45°C at the northern limit of the species range, temperatures under the snow never dropped below –5°C in our common garden (Fig. 6; –11°C in 2008–2009). Acclimated LT_{50} estimates from all three populations were between

–16.8° to –28.3°C, considerably cooler than the lowest temperature experienced in our garden. The low LT₅₀ values are likely to reflect adaptation to more extreme cold experienced by plants during low snow years or during the spring and fall when plants may not be well insulated. Plants of *A. kamchatica* are not likely to survive winter at the northern range limit in the absence of snowcover.

Cold tolerance strategy—There are a number of different methods used to determine a plant's cold tolerance strategy (Reyes-Diaz et al., 2006; Griffith et al., 2007). A common method is to compare the temperature of leaf freezing (supercooling point) with the temperature of leaf death (LT₅₀). Plants are considered freeze tolerant if the LT₅₀ is below the supercooling point indicating that leaves can survive freezing, but freeze avoidant if the supercooling point is at or below the LT₅₀ (Reyes-Diaz et al., 2006; Jacobsen et al., 2007; Sierra-Almeida et al., 2009). However, it is important to recognize that freeze avoidance and freeze tolerance are not mutually exclusive strategies as is often suggested. Rather, a plant may employ different strategies in different environmental scenarios such as summer vs. winter, or they may supercool to avoid freezing when there is no external ice nucleation, but they may tolerate freezing when it is slow and externally nucleated (Gusta and Wisniewski, 2013).

There are a number of difficulties in classifying freeze tolerance strategies, which can be seen in studies of *A. thaliana*. We found that tissue death occurs at about the same temperature as tissue freezes in both acclimated and nonacclimated *A. thaliana* (Fig. 5). This finding is consistent with data by Reyes Diaz et al. (2006), who therefore suggested that *A. thaliana* has a freeze avoidant, rather than freeze tolerant strategy. Likewise, measurement of the ice formation temperature with thermocouples and subsequent survival showed that *A. thaliana* seedlings died if ice formed (Vernon et al., 1999), suggesting that *A. thaliana* is freeze avoidant rather than freeze tolerant. However, in most reports on cold tolerance in *A. thaliana*, it is considered to be freeze tolerant (Thomashow, 1999; Zuther et al., 2012). In the study of Vernon et al. (1999), ice formed at low temperatures due to a lack of external nucleation and rapid cooling (1°C/min). When cooling is rapid, plants are more susceptible to lethal intracellular ice formation, whereas slow, externally nucleated freezing is likely to be better controlled as cell water loss can maintain equilibrium with extracellular ice formation (Siminovitch et al., 1978; Gusta and Wisniewski, 2013). Indeed, when seedlings were externally nucleated and slowly frozen (2.5°C/h), they appeared to have survived freezing (Wanner and Junttila, 1999). Freezing was evidenced by the plants' water-soaked appearance and lack of turgidity. These plants lost their water-soaked appearance during the first day at 21°C (Wanner and Junttila, 1999). Thus, despite the similarity of the supercooling point and LT₅₀, it seems that *A. thaliana* is freeze tolerant, at least when freezing is slow and externally nucleated. When freezing is not externally nucleated, *A. thaliana* is also likely to avoid freezing by supercooling. The method of classifying the cold tolerance strategy will therefore influence a plant's classification.

Even with this caveat, we can safely conclude that *A. kamchatica* is freeze tolerant after acclimation because the LT₅₀ of acclimated *A. kamchatica* was far lower than the supercooling point. Nonacclimated *A. kamchatica* may or may not be freeze tolerant, because LT₅₀ and the supercooling point were similar to each other. However, nonacclimated supercooling points were quite low (–14°C or less), so during summer cold snaps, in the absence of external nucleation, *A. kamchatica* has the capacity

to avoid freezing by supercooling. We suggest that *A. kamchatica* may be freeze avoidant when nonacclimated, but switches to a freeze tolerant strategy when acclimated.

Conclusions—We found that *A. kamchatica* is highly cold tolerant both before and after cold acclimation. It is more cold tolerant than *A. thaliana* and is able to supercool to lower temperatures. When actively growing, *A. kamchatica* is able to avoid freezing by supercooling to very low temperatures (–14°C or below), but it becomes highly freeze tolerant when cold acclimated. *Arabidopsis kamchatica* at its northern range limit had the greatest ability to supercool when nonacclimated, perhaps due to a greater threat of cold snaps during the growing season. However, the midlatitude population of *A. kamchatica* population was tolerant of lower freezing temperatures. This population also had the highest genetic variation for freeze tolerance. Our results suggest that *A. kamchatica* has the potential to adapt to a changing climate; however, because the northern population had low genetic diversity, adaptation is more likely to arise in the center of the species' North American range than in the north, where climate change is the most extreme.

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