

# Cold tolerance in the genus *Arabidopsis*

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**PREMISE:** Cold tolerance is an important factor limiting the geographic distribution and growing season for many plant species, yet few studies have examined variation in cold tolerance extensively within and among closely related species and compared that to their geographic distribution.

**METHODS:** This study examines cold tolerance within and among species in the genus *Arabidopsis*. We assessed cold tolerance by measuring electrolyte leakage from detached leaves in multiple populations of five *Arabidopsis* taxa. The temperature at which 50% of cells were lysed was considered the lethal temperature ( $LT_{50}$ ).

**RESULTS:** We found variability within and among taxa in cold tolerance. There was no significant within-species relationship between latitude and cold tolerance. However, the northern taxa, *A. kamchatica*, *A. lyrata* subsp. *petraea*, and *A. lyrata* subsp. *lyrata*, were more cold tolerant than *A. thaliana* and *A. halleri* subsp. *gemmifera* both before and after cold acclimation. Cold tolerance increased after cold acclimation (exposure to low, but nonfreezing temperatures) for all taxa, although the difference was not significant for *A. halleri* subsp. *gemmifera*. For all taxa except *A. lyrata* subsp. *lyrata*, the  $LT_{50}$  values for cold-acclimated plants were higher than the January mean daily minimum temperature ( $T_{min}$ ), indicating that if plants were not insulated by snow cover, they would not likely survive winter at the northern edge of their range.

**CONCLUSIONS:** *Arabidopsis lyrata* and *A. kamchatica* were far more cold tolerant than *A. thaliana*. These extremely cold-tolerant taxa are excellent candidates for studying both the molecular and ecological aspects of cold tolerance.

**KEY WORDS** *Arabidopsis lyrata*; *Arabidopsis thaliana*; *Arabidopsis halleri* subsp. *gemmifera*; *Arabidopsis kamchatica*; Brassicaceae; cline; cold tolerance; cold acclimation; electrolyte leakage;  $LT_{50}$ .

The low diversity of plant species in the far north may in part be due to the inherent difficulties of adapting to the stress of life at high latitudes. Overwintering structures of plants growing at high latitudes may experience extreme cold, and northern plants often experience a broad range of temperatures throughout the year. Cold tolerance increases after cold acclimation, which is induced by exposure to low but nonfreezing temperatures (Xin and Browse, 2000), and most species are more cold tolerant in the winter than during the growing season. In general, it is predicted that species living in the far north will be tolerant to more extreme cold and will exhibit greater cold acclimation.

Ecological niche modeling suggests that low temperatures, especially winter minimum temperatures (January  $T_{min}$ ), are one of the primary forces determining the geographic boundaries of many plant species (Pither, 2003; Hoffmann, 2005). One would expect that a species' physiological tolerance to cold and freezing temperatures

would be correlated with its geographic range, and there appears to be a strong relationship in insects (Addo-Bediako et al., 2000). In the few plant studies that have been conducted, it is not yet clear whether such a relationship exists (Hofmann et al., 2013; Hofmann and Bruelheide, 2015).

Physiological tolerance to cold also varies among populations within species (Le et al., 2008; Kreyling et al., 2012; Hofmann et al., 2015). In some species, the variation in cold tolerance is correlated with latitude or winter minimum temperatures ( $T_{min}$ ), forming a cline (Hannah et al., 2006; Zhen and Ungerer, 2008; Pagter et al., 2010; Kreyling et al., 2012; Zuther et al., 2012; Menon et al., 2015). The development of a cline depends on interactions between gene flow and selection (Endler, 1973; Slatkin, 1987). A latitudinal cline is expected if there is selection for increased cold tolerance in northern regions and selection against cold tolerance in southern regions, perhaps due to physiological costs of cold

tolerance. Further, the differentiating effects of selection must overcome the homogenizing effects of gene flow (Endler, 1973; Slatkin, 1973, 1987). If there are no costs to cold tolerance in the south, or ample gene flow, plants should exhibit the same high level of cold tolerance across the species geographic range (Xin and Browse, 2000). The fact that cold tolerance is generally inducible (cold acclimation), suggests that it likely has a physiological cost, or else plants would always have maximum cold tolerance. Some studies in *Arabidopsis* suggest that there is a cost of cold tolerance (Jackson et al., 2004; Vos and Willi, 2015), but others do not (Zhen et al., 2011; Vos and Willi, 2018). Although a cline in cold tolerance has been observed in some species, such as *A. thaliana* (Hannah et al., 2006; Zhen and Ungerer, 2008; Zuther et al., 2012), we do not necessarily expect to see this in other species which may have different levels of gene flow and selection.

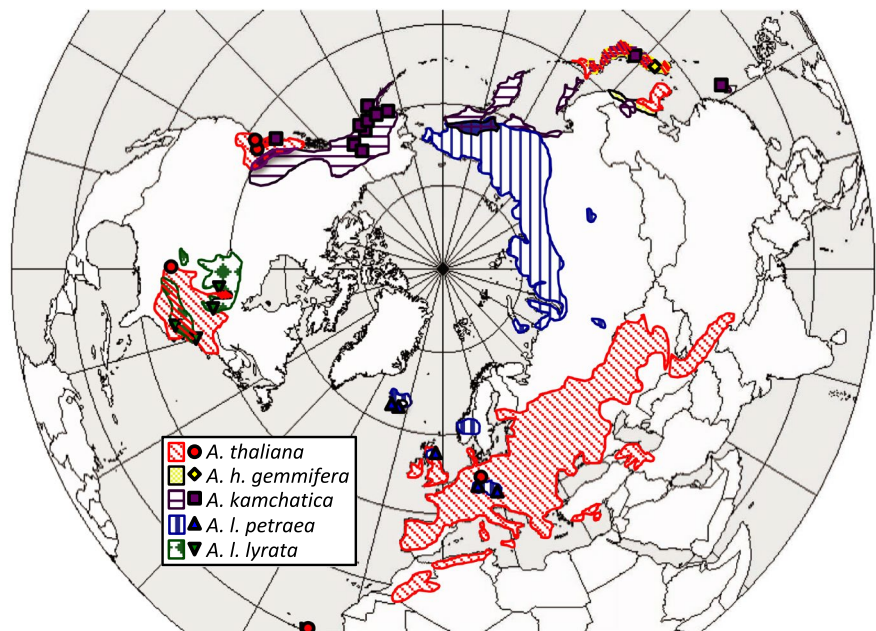
*Arabidopsis thaliana* is a model organism for understanding the molecular and physiological underpinnings of cold tolerance and cold acclimation in plants (Thomashow, 1999, 2010; Xin and Browse, 2000; Hannah et al., 2006; Horton et al., 2016). Many genes important for cold acclimation have been identified in *A. thaliana*, including INDUCER OF CBF EXPRESSION 1 (*ICE1*), and C-repeat binding factors (*CBF* genes), which regulate many downstream genes (Thomashow, 1999, 2010; Zhu et al., 2007). Cold exposure in *A. thaliana* causes extensive reconfiguration of the metabolome (Cook et al., 2004; Kaplan et al., 2004; Hannah et al., 2006), changes to the extracellular proteome (Takahashi et al., 2019), changes to cellular membranes (Uemura et al., 1995), the chloroplast membrane (Barnes et al., 2016) and the cell wall (Takahashi et al., 2019). Many of these important studies have spurred similar research in other species, especially crops and other economically important plants (Renaut et al., 2005; Uemura et al., 2006; Shi et al., 2018).

Studies of cold tolerance among members of the *Arabidopsis* genus have great potential to advance our understanding of the interplay among the ecology, evolution and genetics of cold tolerance. The genus is distributed throughout the northern hemisphere (Fig. 1). Ecological niche modeling shows that there are differences in the climate niches occupied by different *Arabidopsis* species (Hoffmann, 2005). *Arabidopsis thaliana* and *A. kamchatica* have the widest climatic amplitudes in the genus with *A. thaliana* growing in warmer, moister conditions and *A. kamchatica* growing in colder, drier conditions (Hoffmann, 2005). *Arabidopsis thaliana* has the widest geographic distribution in the genus (Fig. 1). It was introduced to both coasts of the United States from Europe and is found in climates ranging from the Mediterranean to the Arctic Circle in northern Europe (Hoffmann, 2005). *Arabidopsis halleri* subsp. *gemmifera* has a relatively small geographic distribution and occupies areas that are warmer and moister in summer than other taxa in the genus. *Arabidopsis lyrata* subsp. *lyrata* also occupies a small climate niche and does not occupy extremely cold areas. However, the other subspecies of the *A. lyrata* complex that we

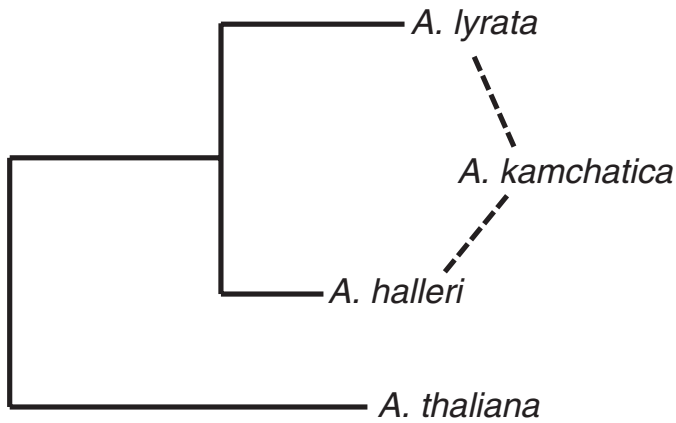
investigated, *A. lyrata* subsp. *petraea*, is found in some of the coldest regions occupied by any taxa in this genus, including northern and central Siberia (Hoffmann, 2005).

The evolutionary history of the genus *Arabidopsis* has been well studied (Novikova et al., 2016). It is thought that *A. lyrata* and *A. halleri* diverged from *A. thaliana* 4 to 7 million years ago (Ma) (Hohmann et al., 2015; Novikova et al., 2016). Subsequently, *A. lyrata* and *A. halleri* diverged from each other between 0.4 and 2 Ma, although there may have been more recent admixture in East Asia (Castric et al., 2008; Hohmann et al., 2015; Novikova et al., 2016). *Arabidopsis lyrata* can be separated into at least two subspecies: *A. lyrata* subsp. *lyrata* in North America and *A. lyrata* subsp. *petraea* in Iceland and Eurasia. North American *A. lyrata* subsp. *lyrata* (hereafter *A. l. lyrata*) is thought to have been derived from *A. lyrata* subsp. *petraea* (*A. l. petraea*) via a founder event ~260,000 years ago (190,000–310,000) (Wright et al., 2003; Mattila et al., 2017). *Arabidopsis halleri* also has multiple subspecies, with *A. halleri* subsp. *halleri* and *A. halleri* subsp. *ovirensis* in Europe, and *A. halleri* subsp. *gemmifera* (*A. h. gemmifera*) in the Russian Far East and East Asia. *Arabidopsis kamchatica* is an allotetraploid that originated ~64,000–145,000 years ago from hybridization of diploid *A. h. gemmifera* and *A. l. petraea* (Shimizu-Inatsugi et al., 2009; Paape et al., 2018). It is likely to have spread outward from Japan to eastern Russia, across the Bering land bridge to Alaska, and down the western edge of Canada (Shimizu-Inatsugi et al., 2009).

Our study investigates differences in cold tolerance and acclimation capacity within and among five *Arabidopsis* taxa. Our first goal was to determine whether *Arabidopsis* taxa differed in cold tolerance. We predicted that taxa with northern distributions, such as *A. kamchatica* and *A. l. petraea*, would be more cold tolerant than taxa like *A. thaliana* and *A. h. gemmifera*, that have more southern distributions (Fig. 2). The second goal of our study was to compare the cold tolerance of populations originating from different latitudes to determine whether there was a relationship between latitude and



**FIGURE 1.** Map of range distributions for *A. kamchatica*, *A. lyrata* subsp. *lyrata*, *A. lyrata* subsp. *petraea*, *A. thaliana*, and *A. halleri* subsp. *gemmifera*. Points represent collections used in this study. Map from Smolensk (2011). Species ranges from Hoffmann (2005).



**FIGURE 2.** Relationships among *Arabidopsis* species used. Neighbor joining tree (Jukes–Cantor correction) of diploid taxa was made using ITS accessions DQ528813.1, DQ528913.1, and DQ528880.1 from Koch and Matschinger (2007) in GenBank. Branch lengths for allotetraploid *A. kamchatica* are not representative of genetic distance.

cold tolerance within each species. We predicted that northern populations would be more cold tolerant than southern populations.

## MATERIALS AND METHODS

### Study species

We assessed freeze damage before and after cold acclimation in plants from five *Arabidopsis* taxa: *A. thaliana*, *A. kamchatica*,

*A. lyrata* subsp. *lyrata*, *A. l.* subsp. *petraea*, and *A. halleri* subsp. *gemmifera*. The species range of each taxa and collection localities used in this study are described in Table 1 and Fig. 2.

All taxa studied are perennials except *A. thaliana*. *Arabidopsis thaliana* can have either a summer annual or winter annual life history throughout most of its range (Griffith et al., 2004), but plants at the northern limit are primarily winter annuals due to a short growing season (Kuittinen et al., 1997a, b; Hoffmann, 2002). Perennials and winter annuals overwinter as rosettes and flower in the spring. Leaves are retained throughout winter and are likely to be important in storing resources needed for new growth and flowering soon after snowmelt because there is no obvious underground storage structure. For this reason, cold tolerance of leaves is likely a good proxy of overall cold tolerance for both actively growing plants and overwintering plants. Because rosettes are small and low to the ground, snow is likely to provide substantial insulation from extremely cold air temperatures in winter, and mid-winter snowmelt will expose plants to subsequent cold snaps.

### Electrolyte leakage assay

The measurement of electrolyte leakage in detached leaves is a common method to estimate freeze tolerance in plants (Hannah et al., 2006). When cell membranes are damaged due to cold exposure, the cell contents leak out and are measured after being dispersed in water. Electrolyte leakage is expressed in terms of relative conductivity, where conductivity is measured after exposure to cold temperatures (initial electrolyte leakage;  $EL_i$ ), then the total conductivity (total electrolyte leakage;  $EL_t$ ) is measured after the sample has been autoclaved to lyse the remaining cells (Prasil and Zamecnik, 1998). The relative leakage ( $EL_r$ ) is  $EL_i/EL_t$ , and the lethal temperature ( $LT_{50}$ ) is the temperature that causes 50% of electrolytes to leak from cells

**TABLE 1.** *Arabidopsis* taxa, locations of population tested for cold tolerance, and number of individuals per population. *A. thaliana* was obtained from The Arabidopsis Information Resource (TAIR).

Taxon	Source locality	Latitude	Longitude	Num plants	Collector/Source
<i>A. kamchatica</i>	Taiwan	24	121	2	Hsu, J.B.Beck
<i>A. kamchatica</i>	Lake Biwa, Japan	35.4444444	136.05	1	H.Marui
<i>A. kamchatica</i>	Strathcona Park, BC	49.82915	-125.8728	12	J.A.Steets, D.E.W
<i>A. kamchatica</i>	Grant Lagoon, Kodiak, AK	57.37	-154.65	1	C.Parker
<i>A. kamchatica</i>	Portage Glacier, AK	60.7916167	-148.90213	12	N.T., D.E.W.
<i>A. kamchatica</i>	Thompson Pass, AK	61.13	-145.73	1	N.T., D.E.W.
<i>A. kamchatica</i>	Exit Glacier, AK, USA	60.18795	-149.6312833	3	N.T., D.E.W.
<i>A. kamchatica</i>	Goodnews Bay, AK	59.1166667	-161.58333	1	C.Parker
<i>A. kamchatica</i>	Rainbow Ridge, AK	63.32	-145.64	1	N.T., D.E.W.
<i>A. kamchatica</i>	Ptarmigan Creek, AK	65.4529	-145.5075657	12	N.T., D.E.W
<i>A. lyrata</i> subsp. <i>lyrata</i>	Cedar Mountain, NC	36.426667	-79.93777778	1	R.Mauricio
<i>A. lyrata</i> subsp. <i>lyrata</i>	New York	40	-74	1	Mitchell-Olds
<i>A. lyrata</i> subsp. <i>lyrata</i>	Presque Isle, PA	42.14	-80.11	1	J.A.Steets
<i>A. lyrata</i> subsp. <i>lyrata</i>	Bailey's Harbor, WI	45.07036N	-87.1711	1	A.Breen
<i>A. lyrata</i> subsp. <i>petraea</i>	Plech, Germany	49.65	11.47	1	Mitchell-Olds
<i>A. lyrata</i> subsp. <i>petraea</i>	Exeter McNair, England	50.72	-3.53	1	Mitchell-Olds
<i>A. lyrata</i> subsp. <i>petraea</i>	Braemer, Scotland	57.01	-3.4	1	R.Ennos
<i>A. lyrata</i> subsp. <i>petraea</i>	Esja Mountain, Iceland	64.2	-21.7	1	M.Schierup
<i>A. lyrata</i> subsp. <i>petraea</i>	Reykjavik, Iceland	64° N	-21	1	M.Schierup
<i>A. thaliana</i> Cvi-0	Cape Verde Islands	15° N	-23	1	TAIR
<i>A. thaliana</i> Col-0	Landsberg, Germany	52° N	10	1	TAIR
<i>A. thaliana</i> Seattle-0	Seattle, WA	47° N	-122	1	TAIR
<i>A. thaliana</i> Van-0	Vancouver, BC	49° N	-123	1	TAIR
<i>A. thaliana</i> Ler-1	Landsberg, Germany	52° N	10	1	TAIR
<i>A. halleri</i> subsp. <i>gemmifera</i>	Fujita (Gifu), Japan	34.93	133.63	3	Fujita Corp.

(or 50% cell lysis), which is commonly used to estimate the temperature at which tissues die.

We measured electrolyte leakage and  $LT_{50}$  according to the methods of Armstrong et al. (2015), with a few modifications. Briefly, leaves were cooled at a rate of 4°C/h. We ensured that leaves froze, rather than supercooling, by nucleating with ice chips. Three replicate leaves per plant were removed at 6–7 different temperatures to assay the amount of cellular damage: 0, –2, –6, –10, –14, and –18°C for non-acclimated plants, and 0, –4, –11, –18, –25, –32, and –35°C for acclimated plants. Thus, each assay included 18–21 similarly-sized leaves per plant. While temperatures during the growing season are unlikely to be as low as –18°C, it is necessary to include colder temperatures to estimate the  $LT_{50}$ . Even colder temperatures were used for cold-acclimated plants because our preliminary studies showed that they suffered very little damage at the temperatures used for non-acclimated plants (Armstrong et al., 2015).

To minimize variation due to the hydration status of the leaves, we watered plants 24 h before the leakage assays. We assayed three replicate leaves per plant for each temperature. Since *A. thaliana* plants are small and did not have enough leaves, we treated leaves from each inbred accession as if all were from the same individual. Perennials were grown in the greenhouse for at least 1 yr before testing for electrolyte leakage from non-acclimated plants. After initial measurements of “non-acclimated” electrolyte leakage, all plants were cold acclimated at 4°C with 8 h of soft-white fluorescent light per day for 4 wk, then used for the “acclimated” electrolyte leakage trial.

### Data analyses

To estimate the point at which 50% of electrolyte leakage would occur ( $LT_{50}$ ), we fit the data to a sigmoidal curve in which the relative leakage  $EL_R$  at the temperature  $T$  follows a two-parameter logistic model:

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

where the inflection point,  $d$ , gives the  $LT_{50}$  estimate, and  $s$  is a scale parameter. With this method, the lower asymptote of the curve approaches an  $EL_R$  of 0 at temperatures that are too warm to cause damage to the leaf tissue, the upper asymptote approaches an  $EL_R$  of 1, corresponding to 100% cell lysis due to freeze damage.

The sigmoidal curve fitting method is a much more statistically rigorous method of estimating the  $LT_{50}$  than other methods because it utilizes all of the electrolyte leakage data. Some methods of reporting freeze damage such as simply assuming that  $LT_{50}$  is 50% of the maximum leakage obtained from measurements (Lipp et al., 1994; Loik and Redar, 2003) are based on a single point rather than including the electrolyte leakage data from multiple temperatures to estimate the  $LT_{50}$ , and a linear relationship between temperature and electrolyte leakage was assumed. Other studies reported only the percentage of electrolyte leakage at one or a few temperatures and did not attempt to estimate  $LT_{50}$  (Nunes and Smith, 2003; Hasdai et al., 2006). Estimates based on a single point are less accurate than the method we used. Most importantly, our method allows direct statistical comparisons of  $LT_{50}$  across multiple species.

Population structure within taxa and the sigmoidal nature of the data made it necessary for us to use a nonlinear mixed-effects model to analyze the electrolyte leakage data. We used the nlme package (Pinheiro and Bates, 2000) implemented in the R statistical environment (R Core Team, 2011). The dependent variable was the relative

electrolyte leakage ( $EL_R$ ). Our independent variables, taxon and acclimation treatment, were treated as fixed effects; and population, family, individual, and date of EL measurement were modeled as random effects. In this model, these independent variables were allowed to have linear effects on the two parameters of the logistic function. We first conducted a model selection (Faraway, 2006) to include the relevant random effects by the Akaike information criterion (AIC), using the method of Pinheiro and Bates (2000). To test the significance of fixed effects, i.e., whether acclimation and/or taxa influenced the shape of the electrolyte leakage response curve, we performed likelihood ratio tests to compare models with and without a fixed effect (including the interaction of taxon and acclimation effects). The test statistic reported for the likelihood ratio tests is  $D$ , where  $D$  is twice the difference in the log likelihoods of two models:  $D = -2 \ln(\text{Likelihood for a simpler model}) - \ln(\text{Likelihood for a model with more parameters})$ .

To estimate the confidence intervals of the estimated parameters for each taxon, we conducted nonparametric bootstrapping for 1000 iterations (Mooney and Duval, 1993; Efron and Tibshirani, 1998; Roberts and Fan, 2004), where each observation (the relative electrolyte leakage measurement) was the unit of resampling, and the total numbers of observations per species were constrained to match the data. Briefly, nonlinear mixed-effects models were fitted to each bootstrapped data set, and confidence intervals were calculated from the distribution of parameter estimates. We checked for normality and homogeneity of errors by inspections of plots of residuals against fitted values and quantile–quantile (QQ) plots. If the assumptions were violated, we attempted to fit an extended nonlinear mixed-effect model, where appropriate variance functions can be used to model heteroscedasticity of within group errors (Chapter 8: Pinheiro and Bates, 2000). The variance function we used was “varPower(fixed = 0.5, form = ~ fitted(.) - fitted.(.)^2)”. However, if this correction failed to converge due to added complexity, we were forced to use the original models without the correction.

For within-taxon comparisons, our methodology for estimating the  $LT_{50}$  for each population was identical to among-taxon comparisons except that the independent variables were family, individual, and date of electrolyte leakage measurement as the random effects and population as the fixed effect. For *A. h. gemmifera*, only one population was available for testing, so it was excluded from the population-level comparisons.

We used analysis of covariance to test whether acclimation treatment and the latitude of origin of the population influenced  $LT_{50}$ , as estimated for each population with the nonlinear mixed-effects models described above. The independent variables were latitude, taxon, and acclimation. We chose a model by AIC-based stepwise selection (Hastie and Pregibon, 1992; Venables and Ripley, 2002), implemented with step() function of R (R Core Team, 2011). Box–Cox transformation of the dependent variable was used to reduce non-normal distribution of residuals (Box and Cox, 1964; Fox and Weisberg, 2011). All statistical analysis was done in the R statistical environment (R Core Team, 2011).  $LT_{50}$  values for each population are provided in Appendix S2, and raw electrolyte leakage values are provided in Appendix S3.

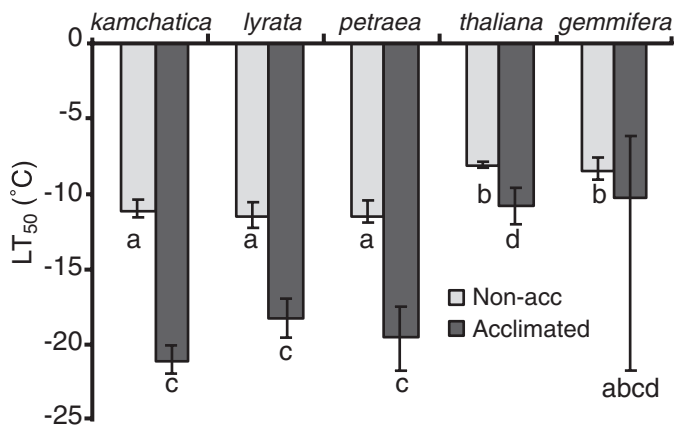
## RESULTS

### Variation among taxa

There was significant variation among taxa in cold tolerance, measured as the temperature of 50% cell lysis ( $LT_{50}$ ). This variation

was significant for both non-acclimated and acclimated plants (Fig. 3; non-acclimated:  $D = 10.138$ ,  $df = 11$ ,  $p = 0.0382$ ; acclimated:  $D = 9.534$ ,  $df = 12$ ,  $p = 0.049$ ). Non-acclimated *Arabidopsis* taxa had  $LT_{50}$  values ranging from  $-8.1^{\circ}$  to  $-11.1^{\circ}\text{C}$ . “Acclimated” values are lower, ranging from  $-10.2^{\circ}$  to  $-21.1^{\circ}\text{C}$  (Fig. 3). Interestingly, “cold-acclimated”  $LT_{50}$  values appear to be more variable among taxa than non-acclimated  $LT_{50}$ . Post hoc comparisons among taxa to determine which taxa are significantly different from each other are not well developed under this nlme statistical framework. Bootstrapped confidence intervals of  $LT_{50}$  estimates (Fig. 3) show that *A. kamchatica*, *A. l. lyrata*, and *A. l. petraea* were more cold tolerant than *A. thaliana*, both when non-acclimated and acclimated. It is difficult to judge whether acclimated *A. h. gemmifera* is different from other taxa due to extremely large confidence intervals, due in part to a small sample size. However, the best estimates of both non-acclimated and acclimated *A. h. gemmifera* are similar to those of *A. thaliana*.

Most of the *Arabidopsis* taxa showed evidence of acclimation in response to cold temperatures (Fig. 3). We measured each taxon’s capacity for cold acclimation as the difference between the non-acclimated and acclimated  $LT_{50}$  values. There were differences among taxa in the amount of cold acclimation, as evidenced by a significant interaction between taxa and acclimation ( $D = 11.629$ ,  $df = 26$ ,  $p < 0.02$ ). Therefore, we tested the effect of acclimation on  $LT_{50}$  by analyzing each taxon separately. All taxa except for *A. h. gemmifera* had a significant decrease in  $LT_{50}$  following exposure to cold temperatures, indicating that they do cold acclimate (*A. kamchatica*:  $D = 77.513$ ,  $df = 10$ ,  $p < 0.0001$ ; *A. l. lyrata*:  $D = 29.658$ ,  $df = 5$ ,  $p < 0.0001$ ; *A. l. petraea*:  $D = 145.867$ ,  $df = 7$ ,  $p < 0.0001$ ; *A. h. gemmifera*:  $D = 1.10$ ,  $df = 5$ ,  $p = 0.2942$ ; *A. thaliana*:  $D = 16.965$ ,  $df = 5$ ,  $p < 0.0001$ ). The lack of a significant acclimation effect in *A. h. gemmifera* is most likely due to the wide confidence interval for the estimate of acclimated  $LT_{50}$  rather than a complete lack of acclimation. Inspection of bootstrapped confidence intervals of  $LT_{50}$  suggests that the three most cold-tolerant taxa have much greater capacity for cold acclimation (*A. kamchatica*:  $-10.0^{\circ}\text{C}$ , *A. l. lyrata*:  $-6.7^{\circ}\text{C}$ , and



**FIGURE 3.** Mean lethal temperature ( $LT_{50}$ ) of each *Arabidopsis* species before (gray) and after (black) cold acclimation.  $LT_{50}$  values were estimated using a logistic curve fitted by nonlinear mixed modeling analysis on electrolyte leakage measurements. Means with the same letters were not significantly different. Error bars represent 95% confidence intervals around the means.

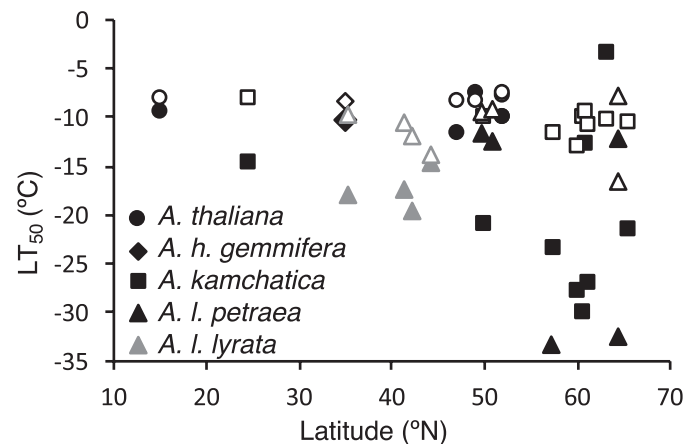
*A. l. petraea*:  $-8.0^{\circ}\text{C}$ ), whereas the other two taxa acclimated relatively little (*A. thaliana*:  $-2.8^{\circ}\text{C}$  and *A. h. gemmifera*:  $-1.7^{\circ}\text{C}$ ).

### Variation within taxa

To examine cold-tolerance variation within taxa and its relationship with latitude, we estimated  $LT_{50}$  values in multiple populations per taxon (only one population was used for *A. h. gemmifera*; Figs. 4 and 5). The AIC-based stepwise selection chose a model that included taxon and acclimation effects ( $F_{4,42} = 2.92$ ,  $p < 0.03$  for taxon effect and  $F_{1,42} = 15.59$ ,  $p < 0.001$  for acclimation effect), indicating that there were differences among taxa, and that acclimation influenced  $LT_{50}$ . However, latitude did not have a statistically significant effect on  $LT_{50}$  among populations. The relationship between  $LT_{50}$  and latitude was also not statistically different between the acclimation treatments. Despite the lack of within-taxon effects, there was a clear trend for more northern plants to have higher cold tolerance when all species are combined, but this is due to differences among taxa (Fig. 4).

In *A. kamchatica*, all populations had a high acclimation capacity except for the one at  $63^{\circ}\text{N}$  (Rainbow Ridge; Fig. 4; Appendix S1a, b). This population had very wide confidence intervals both before and after acclimation. The  $LT_{50}$  estimates for several populations had very wide confidence intervals, partially because several  $LT_{50}$  estimates were based on single plant assays. The Japanese *A. kamchatica* population was excluded from further analysis because of problems with  $LT_{50}$  estimation; non-acclimated and acclimated  $LT_{50}$  for this population were estimated to be  $-105.1^{\circ}\text{C}$  and  $-28.8^{\circ}\text{C}$ , respectively, and  $-105.1^{\circ}\text{C}$  is below the lowest temperature tested. Due to the nature of nonlinear mixed-effect models, estimation of an inflection point ( $LT_{50}$ ) that is outside of the measured range is highly unreliable.

*Arabidopsis l. petraea* from the far north had some of the lowest acclimated  $LT_{50}$  values (Fig. 4, Appendix S1f). Yet surprisingly, plants from two different northern populations, which are geographically very close (both in Iceland at  $\sim 65^{\circ}\text{N}$ ), showed very different acclimated  $LT_{50}$  values. The plant from the Reykjavik population had a high capacity for cold acclimation, whereas the plant from Esja Mountain had almost the same  $LT_{50}$  before and after acclimation. The nonlinear mixed-effect model did not converge with



**FIGURE 4.** Mean lethal temperature ( $LT_{50}$ ) of each population before (open symbols) and after acclimation (closed symbols) by latitude of origin.

the non-acclimated data for the population from Braemar, Scotland, so it was excluded from analysis.

## DISCUSSION

### Variation among taxa

A great deal is known about the molecular basis of cold tolerance and acclimation in *Arabidopsis thaliana* (Thomashow, 1999, 2010; Xin and Browse, 2000; Hannah et al., 2006; Horton et al., 2016). However, we showed that it is not nearly as cold tolerant as its close relatives, *A. lyrata* and *A. kamchatica*, for both non-acclimated, actively growing plants, and cold-acclimated plants. *Arabidopsis lyrata* and *A. kamchatica* also had a much greater capacity for cold acclimation. A distant *Arabidopsis* relative, *Eutrema salsugineum* (= *Thellungiella salsuginea* O.E.Schultz; Brassicaceae), has been described as exhibiting extreme cold tolerance. Its non-acclimated  $LT_{50}$  estimates range from  $-9$  to  $-13^{\circ}\text{C}$ , while acclimated  $LT_{50}$  values range from  $-17.4$  to  $-21.9^{\circ}\text{C}$ , (Griffith et al., 2007; Khanal et al., 2015). Thus, the cold tolerance and acclimation capacity levels of *A. lyrata* and *A. kamchatica* are similar to those of *E. salsugineum*. However, their closer relationship to *A. thaliana* makes the transfer of molecular tools much easier. *Arabidopsis lyrata* and *A. kamchatica* have the potential to provide a valuable complement to *A. thaliana* for cold tolerance research.

As we expected, *Arabidopsis* taxa with more northerly distributions, that experience colder winter temperatures (*A. lyrata* and *A. kamchatica*), to exhibit more cold tolerance and more acclimation capacity than the more southerly taxa (*A. thaliana* and *A. h. gemmifera*) (Figs. 3 and 4). Climatic range estimates suggest that *A. lyrata* and *A. kamchatica* occur in colder regions than *A. thaliana* and *A. h. gemmifera*. The January mean daily minimum temperature ( $T_{\min}$ ) in the coldest regions where *A. thaliana* occurs is  $-31^{\circ}\text{C}$ , and  $T_{\min}$  for *A. h. gemmifera* was  $-25^{\circ}\text{C}$ , whereas the lowest  $T_{\min}$  for *A. kamchatica* is  $-40^{\circ}\text{C}$  and  $T_{\min}$  for *A. lyrata* is  $-48^{\circ}\text{C}$  (Hoffmann, 2005).

When we separate the two *A. lyrata* subspecies (*A. l. lyrata* and *A. l. petraea*), the results are less congruent with climatic estimates. The European subspecies, *A. l. petraea*, has a much more northerly distribution and occupies much colder regions than the North American subspecies, *A. l. lyrata*; the lowest January  $T_{\min}$  for *A. l. petraea* is  $-48^{\circ}\text{C}$ , whereas  $T_{\min}$  for *A. l. lyrata* is only  $-15^{\circ}\text{C}$  (Hoffmann, 2005). However, there was little apparent difference between the subspecies; both taxa were very cold tolerant. The North American subspecies, *A. l. lyrata* is thought to be recently derived from *A. l. petraea*, via a founder event from Europe (Wright et al., 2003; Mattila et al., 2017). It is also likely that *A. l. lyrata* experienced a much colder climate during the last glacial period because much of its current range is within previously glaciated regions (Hoffmann, 2005). There may not have been sufficient time or selection pressures for cold tolerance to be lost in *A. l. lyrata*. Thus, the historical climate and biogeographic history are likely to explain the high cold tolerance of *A. l. lyrata* despite the relatively warm conditions in its current range. Further, the high cold tolerance of *A. l. lyrata* suggests that this taxon may not fully occupy its potential climate niche and that it may have the capacity to migrate into much colder regions.

It is notable that for all taxa except *A. l. lyrata*, the mean acclimated  $LT_{50}$  values are higher than the January  $T_{\min}$ , indicating that if plants were not insulated by snow cover, they would not likely survive winter at the northern edge of their range. Snow is a powerful insulator.

*Arabidopsis* species overwinter as low-lying rosettes. In the coldest climates, plants are insulated by snow cover throughout winter, greatly reducing the severity of cold that plants are exposed to. For instance, two winters of temperature measurements show that *A. kamchatica* in interior Alaska was never exposed to temperatures below  $-11^{\circ}\text{C}$  even when the air temperatures plummeted below  $-40^{\circ}\text{C}$  (Armstrong et al., 2015). However, in warmer regions, the snow frequently melts during winter, and rosettes are directly exposed to freezing air temperatures during subsequent cold snaps. This exposure may also be responsible for the retention of cold tolerance in *A. l. lyrata*. It also suggests that as winter temperatures in the far north increase and snow melting events become more common, plants may paradoxically require higher levels of cold tolerance.

### Variation within taxa

It is interesting that differences among taxa were related to latitude and climate, but within-taxon variation was not. The lack of a cline for cold tolerance in *A. thaliana* was especially surprising, as cold tolerance has previously been shown to be correlated with both latitude (Zhen and Ungerer, 2008) and January minimum temperatures ( $T_{\min}$ ) in this species (Hannah et al., 2006). Our failure to observe a cline may be due to the limited number of *A. thaliana* accessions in our study ( $N = 5$ ). Sample sizes in *A. lyrata* ( $N = 9$ ) and *A. kamchatica* ( $N = 46$ ) were larger, so the lack of a latitudinal cline is more likely to be real.

*Arabidopsis kamchatica* recently crossed the Bering land bridge from eastern Russia into Alaska and southward into western Canada, and there is little genetic differentiation among populations in North America (Shimizu-Inatsugi et al., 2009). Most of our *A. kamchatica* samples were from North America. These plants would have required high cold tolerance when crossing the Bering land bridge, and there may not have been sufficient time for loss of cold tolerance in plants that migrated southward, even if there was selection against cold tolerance. Consistent with selection for cold tolerance when *A. kamchatica* crossed the land bridge, the southernmost sample in our analysis, from Taiwan, was less cold tolerant than most North American samples. An investigation of *A. kamchatica* cold tolerance in Asian and Russian, where genetic diversity is higher (Shimizu-Inatsugi et al., 2009), would help clarify the evolution of cold tolerance in this species.

Other studies of *A. lyrata* have had mixed results. Like us, Davey et al. (2018) failed to find a correlation between cold tolerance and latitude in *A. l. petraea*. However, Wos and Willi (2015) did find that northern populations had greater cold tolerance than southern populations. Thus, although some measures of cold tolerance may reveal a latitudinal cline in *A. lyrata*, our results do not. Unlike populations of *A. thaliana* and *A. kamchatica*, *A. lyrata* populations are highly differentiated (Wright et al., 2003; Mattila et al., 2017), and they show strong local climate adaptation (Leinonen et al., 2009, 2011, 2013; Hämälä et al., 2018; Lucek et al., 2019). The counter-adaptive effects of gene flow or recent divergence are not a likely explanation for the lack of a latitudinal cline in *A. lyrata*. Perhaps there is no selection against cold tolerance in the south.

Some studies on both *A. thaliana* and *A. lyrata* suggest that there may be a cost of cold tolerance (Jackson et al., 2004; Wos and Willi, 2015), but conclusions depend on how costs are measured. Zhen et al. (2011) found no evidence of a cost in the absence of freezing stress, and Wos and Willi (2018) found that the ability to acclimate did not reduce fitness in any environment. Thus, there may or may not be selection against cold tolerance in regions that do

not experience cold winters. Alternatively, southern plants, may have less persistent winter snow cover, so that they experience just as much cold as northern plants, which are insulated by snow.

### Accuracy of freeze tolerance ( $LT_{50}$ ) estimates

Our study found lower  $LT_{50}$  values than some other studies in the same *Arabidopsis* species. We found slightly more freeze tolerance in *A. thaliana* (Hannah et al., 2006; Reyes-Diaz et al., 2006; Zuther et al., 2012), and considerably more freeze tolerance in *A. lyrata* (Davey et al., 2018). Our methodologies differed in a number of ways from Davey et al. (2018), primarily in how total electrolyte leakage ( $EL_T$ ) was estimated. Total electrolyte leakage ( $EL_T$ ) is intended to estimate EL when all cells have been lysed, so that  $LT_{50}$  represents the point when 50% of cells have been lysed. To estimate the reference conductivity corresponding to 100% lysing, Davey exposed leaves to  $-80^{\circ}\text{C}$  for 3 h, whereas we autoclaved leaves. Our preliminary tests found that autoclaving leaves gave a higher  $EL_T$  than freezing at  $-80^{\circ}\text{C}$ , indicating that freezing did not lyse all cells in these highly freeze-tolerant plants. Because our  $EL_T$  estimates were higher, more cell damage and lower temperatures were necessary to reach  $LT_{50}$ . Thus, we believe that our  $LT_{50}$  estimates are reliable although we need to be cautious with the comparison among different studies.

### CONCLUSIONS

We examined cold tolerance within and among *Arabidopsis* taxa. Taxa with northern distributions (*A. lyrata* and *A. kamchatica*) were generally more cold tolerant than those with southern distributions (*A. thaliana* and *A. halleri* subsp. *gemmifera*) both before and after acclimation and had a higher capacity to acclimate. Cold tolerance increased after cold acclimation (exposure to low, but non-freezing temperatures) for all taxa, although the difference was not significant for *A. h. gemmifera*. For all taxa except *A. l. lyrata*, acclimated  $LT_{50}$  values are higher than the January mean daily minimum temperature ( $T_{\min}$ ), suggesting that if plants were not insulated by snow cover, they would not likely survive winter at the northern edge of their range. *Arabidopsis lyrata* subsp. *lyrata*, on the other hand, has higher cold tolerance than is needed, even at the northern edge of its range. This subspecies was recently derived in North America by a founder event from European *A. lyrata* subsp. *petraea* (Wright et al., 2003; Mattila et al., 2017). It appears to have retained its ancestral high levels of cold tolerance and should have the capacity to expand northward. Since molecular tools are easy to transfer from *A. thaliana* to its relatives, these extremely cold-tolerant taxa (*A. lyrata* and *A. kamchatica*) are excellent candidates for studying both the molecular and ecological aspects of cold tolerance.

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### AUTHOR CONTRIBUTIONS

J.J.A. and D.E.W. designed the experiments, J.J.A. carried out the experiments, J.J.A. and N.T. analyzed the data, J.J.A., N.T., and D.E.W. wrote the paper.

### SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

**APPENDIX S1.** Relationships between latitude and population cold tolerance for each taxon.

**APPENDIX S2.** The estimated cold tolerance ( $LT_{50}$ ) values for each population.

**APPENDIX S3.** Raw electrolyte leakage (EL) data.

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