Differential Cold Acclimation Ability of *Petunia* spp.

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Abstract. Freezing tolerance of many plant species increases after exposure to low, nonfreezing temperatures, a process termed cold acclimation. In some species, shortened photoperiods also bring about an increase in freezing tolerance. Within the plant family Solanaceae, species vary widely in cold acclimation ability. The objectives of this work were to examine the effects of low temperature and photoperiod on cold acclimation of *Petunia hybrida* (Hook.) Vilm. ‘Mitchell’ and to evaluate cold acclimation of several *Petunia* species by measuring freezing tolerance using an electrolyte leakage assay on leaf tissue discs. Temperature, but not photoperiod, influenced cold acclimation of *P. hybrida*. Whether grown under long days or short days, nonacclimated plants had an EL₅₀ value (temperature at which 50% of cellular electrolytes are lost) of $\approx -2^\circ\text{C}$. Plants acclimated by gradual cooling at temperatures of 15°C , 10°C , and 3°C for 7 days each reached an EL₅₀ of $\approx -5^\circ\text{C}$ regardless of photoperiod. Exposure to 3°C under short days for 1 or 3 weeks resulted in EL₅₀ temperatures of -3.9 and -4.9°C , respectively. Freezing tolerance of petunia species *P. exserta* Stehmann, *P. integrifolia* (Hook.) Schinz & Thell., *P. axillaris* (Lam.) Britton et al. (USDA accessions 28546 and 28548), and *P. hybrida* ‘Mitchell’ was similar before cold acclimation, but varied from -5°C for *P. exserta* to -8°C for *P. axillaris* (accession 28548) after cold acclimation. Our results demonstrate the cold acclimation ability of *Petunia* spp. and identify wild germplasm sources with potential usefulness for improving freezing tolerance of cultivated petunia.

Plants endure many biotic and abiotic stresses, including exposure to cold temperatures. Tremendous damage to plant cells can result from freezing temperatures, primarily to the cellular membranes (Thomashow, 1999). Many plants have adapted to life in cooler climates by developing mechanisms to tolerate low temperatures, but cold tolerance is a dynamic characteristic. When grown in warm temperatures, even cold-hardy plants have low freezing tolerance. Exposure to low nonfreezing temperatures brings about an increase in freezing tolerance through a process termed cold acclimation (Thomashow, 1999). Within the family Solanaceae, even within the genus *Solanum*, species vary considerably in their ability to cold-acclimate. The chilling-sensitive *S. lycopersicum* L. (tomato) and freezing-sensitive *S. tuberosum* L. (potato) do not cold acclimate (Chen and Li, 1980; Hsieh et al., 2002; Pino et al., 2007), whereas *S. commersonii* Dun. (a wild potato relative) is frost-tolerant and capable of acclimation (Chen and Li, 1980; Pino et al., 2008). *Petunia hybrida* does cold acclimate (Pennycooke et al., 2003; Yelenosky and Guy, 1989), whereas cold acclimation ability of other *Petunia* spp. is currently unknown.

Cold acclimation brings about changes in gene expression (Guy et al., 1985) and cellular metabolite profiles (Cook et al., 2004), resulting in survival at temperatures much lower than would be tolerated without acclimation. Stabilizing cellular membranes against dehydration resulting from extracellular ice formation is a critical component of cold acclimation. Electrolyte leakage assays provide a simple method to assess cellular membrane integrity of plants exposed to abiotic stresses, including low temperature (Pennycooke et al., 2003; Sukumaran and Weiser, 1972; Thapa et al., 2008). Typically, the temperature resulting in 50% of maximum electrical conductivity (EC; i.e., membrane injury) is reported (Palta et al., 1978).

In addition to exposure to low nonfreezing temperatures, shortened photoperiods induce cold acclimation of many woody plant species (Howell and Weiser, 1970; Li et al., 2002; Marian et al., 2004) and some herbaceous species such as *Hordeum vulgare* L. ‘Dicktoo’ (Fowler et al., 2001) and *Gaura coccinea* Pursh. (Pietsch et al., 2009). In *Betula pendula* Roth, both a shortened photoperiod (12 h) and low temperatures (4°C) result in acclimation, but low temperatures produce a stronger response, whereas simultaneous exposure to low temperatures and short days produces an additive response (Li et al., 2002). Freezing tolerance of *H. vulgare* ‘Dicktoo’ was greater for plants exposed to 4°C under an 8-h photoperiod compared with a 20-h photoperiod (Fowler et al., 2001). The effects of photoperiod and temperature on cold acclimation of *Petunia hybrida* are currently unknown.

Garden petunias (*P. hybrida*) are a very popular bedding plant, ranking first in sales among bedding plants in 2007 with a wholesale value of over \$111 million (U.S. Department of Agriculture–National Agricultural Statistics Service, 2008). Although *Petunia hybrida* does cold-acclimate, the acclimation only improves freezing tolerance by $\approx 2.5^\circ\text{C}$ (Pennycooke et al., 2003). Further improving freezing tolerance of petunia would benefit both consumers and commercial growers. The selection of bedding plants suited for growth in early spring and late fall, when cold night temperatures and frosts still limit the survival of most bedding plants, is limited. During the processes of domestication and breeding, the genetic diversity of a plant species tends to decrease dramatically (Tanksley and McCouch, 1997). Strong selection imposed by plant breeders, along with crossing between closely related individuals, leads to a narrowing of the genetic diversity in future generations. Using wild germplasm has proven to be an effective method for crop improvement. In the genus *Petunia*, interspecific crosses usually produce fertile progeny (Ando et al., 2001; Griesbach, 2007), making use of wild germplasm possible. Griesbach et al. (1999) determined that the red color of *P. exserta* results from genetic factors distinct from those in current red-colored *P. hybrida* cultivars. Interspecific hybridization using *P. exserta* is adding to the genetic diversity of *P. hybrida* (Griesbach et al., 1999).

The objectives of the current study are 1) to examine the effects of low temperature and photoperiod on cold acclimation of *P. hybrida*; and 2) to evaluate cold acclimation of *P. axillaris*, *P. integrifolia*, *P. exserta*, and *P. hybrida* ‘Mitchell’ in an effort to identify genetic material that may be useful in breeding more stress-tolerant cultivars.

Materials and Methods

Plant growth and cold acclimation conditions. Seeds of *P. hybrida* ‘Mitchell’ were surface-planted onto 288-cell plug trays filled with 70% peatmoss, 21% perlite, and 9% vermiculite (v/v) (Suremix; Michigan Grower Products Inc., Galesburg, MI). Trays were placed under intermittent overhead mist irrigation at 23°C until the development of two true leaves. Plants were then transferred to a greenhouse at 22°C with a 16-h photoperiod (ambient plus $50\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ supplemental high-pressure sodium lighting from 0600 to 2200 hr) for 3 weeks. Seedlings were then transplanted to 50-cell trays containing the same soilless media and moved to a controlled environment growth chamber at 22°C under a 16-h photoperiod [$115 \pm 7\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (mean $\pm$ SD) photosynthetically active radiation provided by fluorescent plus incandescent lighting] for 1 week. To determine the influence of photoperiod and temperature on cold acclimation, 50 plants were subjected to each of seven different treatments described in Table 1.

In a second experiment, *P. integrifolia*, *P. exserta* (kindly provided by Dr. Rob

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Table 1. Growth conditions [temperature, photoperiod, photosynthetic photon flux (PPF) and duration] of *P. hybrida* 'Mitchell' plants before electrolyte leakage assays.

Growth regimes	Temperature (°C)	Photoperiod (h)	PPF (μmol·m ⁻² ·s ⁻¹)	Duration (weeks)
Nonacclimated LD (NONLD)	22	16	115	6
Nonacclimated SD (NONS D)	22	16	115	4
	22	9	115	3
Cold acclimation 1 (CA1)	22	16	115	6
	3	9	30	1
Cold acclimation 2 (CA2)	22	16	115	4
	22	9	115	2
	3	9	30	1
Cold acclimation 3 (CA3)	22	16	115	5
	3	9	30	3
RampdownSD	22	16	115	5
	15	9	115	1
	10	9	115	1
	3	9	30	1
RampdownLD	22	16	115	5
	15	16	115	1
	10	16	115	1
	3	16	30	1

LD = long day; SD = short day.

Griesbach, USDA-ARS), two accessions of *P. axillaris* (USDA accessions 28546 and 28548; obtained from the USDA Ornamental Plant Germplasm Center, Columbus, OH), and *P. hybrida* 'Mitchell' were sown as described previously. When seedlings had developed two true leaves, plants were transferred to a greenhouse at 20 °C with a 16-h photoperiod (as described previously) for 4 weeks. Seedlings were then transplanted into 50-cell trays and moved to a controlled environment growth chamber at 22 °C with a 16-h photoperiod (115 ± 7 μmol·m⁻²·s⁻¹). After 2 weeks, plants of each species were divided into two groups: a nonacclimated group remaining at 22 °C for an additional week before testing and a cold-acclimated group. Cold acclimation was induced following a protocol previously described to be effective for *P. hybrida* (RampdownSD; Pennycooke et al., 2003). Plants were moved to 15 °C with a 9-h photoperiod (115 ± 7 μmol·m⁻²·s⁻¹) for 1 week followed by 10 °C with a 9-h photoperiod (115 ± 7 μmol·m⁻²·s⁻¹ light) for 1 week, and finally 3 °C with a 9-h photoperiod (30 ± 3 μmol·m⁻²·s⁻¹ light) for an additional week. Within each chamber, trays were rerandomized approximately every 3 d to minimize the effects of variable irradiance. The irradiance level at 3 °C was kept low to minimize the potential for photooxidative stress (Wise and Naylor, 1987). At the time of testing, nonacclimated plants were 7 weeks old and acclimated plants were 9 weeks old. However, plants in both groups had developed approximately the same number of nodes at the time of sampling.

Determination of freezing tolerance. Freezing tolerance of leaves was determined by the method of Sukumaran and Weiser (1972) with slight modifications. After temperature/photoperiod treatments, leaf discs from the upper portion of the plant were collected using a 6-mm cork borer and rinsed by floating on deionized water (dH₂O) with gentle stirring. The youngest leaves allowing a complete 6-mm diameter disc to be har-

vested without cutting into the midrib were sampled. Approximately 120 punches were taken from a population of 50 plants for each treatment. Three discs were transferred to each of 30 glass culture tubes per treatment and placed on ice. Tubes were placed in a -1 °C controlled-temperature antifreeze-filled bath (master bath) for 60 min. Three tubes of each treatment remained on ice as controls. After 60 min, a small amount of ice was added to each tube for extracellular ice nucleation. Tubes were plugged with foam and kept at -1 °C for an additional 60 min, after which three tubes of each treatment were moved to a second antifreeze bath at -1 °C, kept there for 40 min, and removed to ice. Meanwhile, the temperature of the master bath was lowered to -2 °C. After 20 min, three tubes of each treatment were moved from the master bath to another antifreeze bath at -2 °C for 40 min and then removed to ice (a total of 60 min at the test temperature). This process continued for all temperatures tested (-1 to -9 °C). Tubes were then put in racks on top of ice and placed at 2.5 °C to thaw slowly overnight. The next day, 6 mL of dH₂O was added to each tube followed by gentle shaking for 3 h at room temperature. The water was transferred to a new culture tube and EC (L1) was measured using a CON 110 conductivity meter (Oakton Instruments, Vernon Hills, IL). Plant discs remained in the original tube and were frozen to -80 °C overnight. The next day, the water from the previously measured tubes was poured back into the corresponding plant disc tube followed by shaking for 3 h at room temperature. Conductivity of the water was measured again to obtain the reading for maximum electrolyte leakage (L2). Percent of maximum electrolyte leakage at each test temperature was calculated as: percent leakage = (L1/L2) × 100.

Data analysis. Data were analyzed using Sigmaplot (SPSS Inc., Chicago, IL) and SAS (SAS Institute Inc., Cary, NC) software. A sigmoidal curve was fit to the leakage data for

each treatment according to the equation: $y = a1 + \{a2/[1 + \exp(a3 - a4 \times T)]\}$, where y is the average percent electrolyte leakage of the three tubes at each temperature (T) using the curve fitting function of Sigmaplot. The initial parameters were specified as $a1 = 0.1$, $a2 = 99.9$, $a3 = 0.1$, and $a4 = 0.1$ with constraints imposed such that $a1 > 0$ and $0 < a2 < 100$. The temperature at which 50% of maximal conductivity was reached (EL₅₀) was calculated by solving the equation $EL_{50} = \{a3 - \log[a2/(50 - a1)] - 1\}/a4$, where the parameters $a1$, $a2$, $a3$, and $a4$ are given by the equation of the fitted curve. At least two EL₅₀ values were calculated for each treatment from two separate replications of the experiment. Analysis of variance and mean separations using Fisher's least significant difference ($\alpha = 0.05$) were conducted on the EL₅₀ values using PROC GLM from SAS to compare treatment effects.

Results

Influence of environmental cues on cold acclimation. Temperature regime, but not photoperiod, influenced cold acclimation of *P. hybrida* 'Mitchell' (Fig. 1). When grown at 22 °C and 16-h long days (NONLD), *P. hybrida* 'Mitchell' EL₅₀ value was -1.9 °C. Exposing plants to 3 weeks of short days alone (NONS D) did not increase freezing tolerance and the EL₅₀ temperature remained at -2.0 °C. Gradual cooling, in which plants were grown under a 16-h photoperiod for 7 d each at temperatures of 15, 10, and 3 °C (RampdownLD) resulted in significant cold acclimation, because the EL₅₀ temperature decreased to -5.1 °C. Plants exposed to the same cooling regime under 9-h days (RampdownSD) exhibited similar cold acclimation as Rampdown LD with an EL₅₀ temperature of -5.0 °C.

Moving plants directly from long days at 22 °C to short days at 3 °C for just 1 week (CA1) increased freezing tolerance slightly (EL₅₀ = -3.9 °C) compared with NONLD and NONSD (Fig. 1). Increasing the time of exposure to 3 °C under short days from 1 to 3 weeks (CA3) further increased freezing tolerance (EL₅₀ = -4.9 °C) compared with the CA1 treatment. Exposing plants to short days at 22 °C for 2 weeks before exposure to 3 °C with short days for 1 week (CA2) resulted in an EL₅₀ value of -4.0 °C, similar to CA1 (Fig. 2). Exposure to 3 weeks of gradual cooling in both the RampdownSD and RampdownLD treatments resulted in the same degree of cold acclimation as 3 weeks at 3 °C (CA3; Fig. 1). However, the RampdownSD and RampdownLD treatments were more effective than the CA1 treatment with just 1 week at 3 °C.

Cold acclimation ability of Petunia species. All species tested had similar constitutive freezing tolerance (Fig. 2) with an EL₅₀ temperature of ≈ -2 °C without cold acclimation. Acclimated EL₅₀ temperatures varied across species. The most freezing-tolerant species was *P. axillaris* (USDA accession 28548) with an acclimated EL₅₀

Fig. 1. Freezing tolerance [EL₅₀ (temperature at which 50% of maximum electrolyte leakage occurred)] for *P. hybrida* 'Mitchell' after various cold acclimation regimes. NONLD = 22 °C, long day (LD; 16-h long days). NONSD = 22 °C, LD; then 3 weeks at 22 °C, short day (SD; 9-h short days). CA1 = 22 °C, LD; then 1 week at 3 °C, SD. CA2 = 22 °C, LD; then 2 weeks at 22 °C, SD; then 1 week at 3 °C, SD. CA3 = 22 °C, LD; then 3 weeks at 3 °C, SD. RampdownSD = 22 °C, LD; then 1 week at 15 °C, SD; then 1 week at 10 °C, SD; then 1 week at 3 °C, SD. RampdownLD = 22 °C, LD; then 1 week at 15 °C, LD; then 1 week at 10 °C, LD; then 1 week at 3 °C, LD. Bars with the same letter are not statistically different according Fisher's least significant difference ($\alpha = 0.05$). Error bars represent SD.

Fig. 2. Freezing tolerance [EL₅₀ (temperature at which 50% of maximum electrolyte leakage occurred)] for *Petunia* species nonacclimated [7 weeks at 22 °C long day (LD)] and acclimated [6 weeks at 22 °C LD, 1 week at 15 °C short days (SD), 1 week at 10 °C SD, and 1 week at 3 °C SD]. Bars depict EL₅₀ temperatures averaged over at least two replications for each species. Bars with the same letter are not statistically different according Fisher's least significant difference ($\alpha = 0.05$). Error bars represent SD.

temperature of -7.8 °C, whereas *P. exserta* was the least freezing-tolerant with an EL₅₀ temperature of -4.9 °C. In addition to the observed interspecific variation, there was also significant variation in the acclimated freezing tolerance between the two *P. axillaris* accessions. The acclimated EL₅₀ temperatures for *P. hybrida*, *P. axillaris* (USDA accession 28546), and *P. integrifolia* are -6.0, -6.1, and -6.6 °C, respectively. Although there was no statistical difference in the acclimated freezing tolerance among these three species, all were all significantly less freezing-tolerant than *P. axillaris* (accession 28548) and all except *P. axillaris*

(accession 28546) were more freezing-tolerant than *P. exserta*.

Discussion

Our results indicate that cool temperatures, but not reduced photoperiod, influence cold acclimation of *P. hybrida* 'Mitchell' (Fig. 1). Plants grown under long day conditions (NONLD) showed similar basal freezing tolerance levels as those exposed to a reduced photoperiod without a reduction in temperature (NONSD), indicating that in the absence of cooling, short days alone are insufficient to induce cold acclimation.

Petunia hybrida 'Mitchell' did cold-acclimate when exposed to 3 °C for 1 week along with a reduction in photoperiod from 16 h of light to 9 h (CA1), increasing in freezing tolerance by ≈ 2 °C. However, freezing tolerance did not increase further with the addition of 2 weeks of exposure to short days before cold treatment (CA2) (Fig. 1). In addition, subjecting plants to gradual cooling had the same effect on cold acclimation whether under long days (Rampdown LD) or short days (Rampdown SD) with an EL₅₀ value of ≈ -4.9 °C after either treatment. *Petunia hybrida* is a facultative long-day plant that exhibits a severe delay in flowering if the photoperiod is less than 14 h (Adams et al., 1998). Therefore, the 9-h photoperiod used as a short day in the current study should be sufficient to induce a short-day response. Because none of our photoperiod treatments influenced cold acclimation, it is unlikely that a different short photoperiod length would induce cold acclimation without a concurrent reduction in temperature.

The duration of exposure to 3 °C influenced cold acclimation (Fig. 1). Similar results were reported for *Solanum commersonii* in which freezing tolerance gradually increased with duration of exposure to 2 °C until a maximum was reached after 7 d (Pino et al., 2008). One week at 3 °C preceded by a week at 15 °C and a week at 10 °C (RampdownSD and RampdownLD) induced similar freezing tolerance as 3 weeks at 3 °C (CA3). In *Arabidopsis thaliana*, the level of CBF transcription is dependent on the magnitude of temperature change and the absolute temperature. With gradual cooling at a rate of 2 °C·h⁻¹, transcripts of the cold acclimation pathway transcription factor gene family CBF become detectable in *A. thaliana* at temperatures as high as 14 °C with further temperature reductions increasing transcript accumulation (Zarka et al., 2003). This may help to explain why the Rampdown treatments with only 1 week at 3 °C were more effective cold acclimation regimes than 1 week at 3 °C alone (CA1), and equally effective as CA3. The initial weeks at 15 and 10 °C during the RampdownSD and RampdownLD regimes may be sufficiently cool to induce cold-acclimation pathways in petunia, allowing plants to eventually reach the same level of freezing tolerance after the final week at 3 °C as was reached after 3 weeks at 3 °C (CA3).

We examined the freezing tolerance of several wild *Petunia* species to identify potential genetic sources of cold tolerance for use in future breeding. All of the tested species showed similar nonacclimated freezing tolerance with an EL₅₀ value near -2 °C (Fig. 2). These results are consistent those of Pennycooke et al. (2003) who reported an EL₅₀ value of ≈ -2.5 °C for nonacclimated *P. hybrida* 'Mitchell' using a slightly different electrolyte leakage assay procedure. The nonacclimated freezing tolerance of *P. hybrida* 'Mitchell' is similar to *S. lycopersicum* with an EL₅₀ temperature of -2 °C (Zhang et al., 2004) and *S. tuberosum* with an

EL₅₀ of -3 °C (Pino et al., 2007). The frost-tolerant species *S. commersonii* and *A. thaliana* ecotype RLD have nonacclimated EL₅₀ temperatures of -5 and -4 °C, respectively (Jaglo-Ottosen et al., 1998; Pino et al., 2008). These comparisons suggest that *Petunia* is relatively sensitive to freezing before cold acclimation.

The two *P. axillaris* accessions showed differential cold acclimation ability (Fig. 2). These accessions also exhibited morphological differences with accession 28548 having a much longer corolla tube and leaves than accession 28546 (data not shown). Although the exact collection sites of these two accessions are unknown, they are likely different ecotypes, and differential selection pressures may have resulted in increased cold acclimation ability for accession 28548. Freezing tolerance of the species *P. integrifolia*, *P. hybrida*, and *P. axillaris* (accession 28546) were not statistically different after acclimation with an EL₅₀ near -6 °C. *Petunia hybrida* is believed to be a complex hybrid of *P. integrifolia* and *P. axillaris* (Griesbach, 2007; Sink, 1984), and *P. hybrida* 'Mitchell' is a hybrid of *P. axillaris* × (*P. axillaris* × *P. hybrida* 'Rose du Ciel') (Griesbach, 2007). Therefore, it is not surprising that *P. hybrida* 'Mitchell' has cold tolerance characteristics similar to these two putative parental species.

As a point of comparison between petunia and other cold-acclimating herbaceous species, *S. commersonii* (Chen and Li, 1980; Pino et al., 2008) and *A. thaliana* ecotype RLD (Jaglo-Ottosen et al., 1998) both increase in freezing tolerance by ≈4 °C to reach acclimated EL₅₀ temperatures of -9 and -8 °C, respectively. Therefore, we conclude that *Petunia*, and especially *P. axillaris* (accession 28548) with an acclimated EL₅₀ temperature near -8 °C (a 5.8 °C increase over nonacclimated plants), has the ability to cold-acclimate by a significant amount for an herbaceous species, particularly within the family Solanaceae. However, this acclimation ability is modest compared with other herbaceous species, especially grasses such as rye (*Secale cereale* L. sbsp. *cereale*) and wheat (*Triticum aestivum* L.), which can cold-acclimate by 15 °C or more (Cloutier, 1983; Webb et al., 1994).

The results of these experiments indicate significant variation in the freezing tolerance of *Petunia* spp. after a period of cold acclimation that could be used to breed *Petunia* cultivars with improved freezing tolerance. Cold tolerance is a quantitative trait expected to be controlled by several genetic loci. Identification of quantitative trait loci underlying cold acclimation ability in *Petunia* would greatly aid implementation of marker-assisted selection to introgress cold tolerance traits from wild germplasm with superior cold ac-

climation ability such as *P. axillaris* 28548 into cultivated genotypes.

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