Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 7869–7873, August 1993
Genetics

Inheritance of freezing resistance in tuber-bearing *Solanum* species: Evidence for independent genetic control of nonacclimated freezing tolerance and cold acclimation capacity

(low-temperature response genes/generation means analysis/winter survival/frost injury/potato)

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Communicated by S. J. Peloquin, May 17, 1993

ABSTRACT **Frost or winter survival is regarded as a complex trait with polygenic inheritance. Two major components of this survival in crop plants are freezing tolerance in the nonacclimated state and cold acclimation capacity. To date researchers have not distinguished the two components as separate heritable traits. The mode of inheritance of these two traits was investigated in F_1 and backcross populations of two wild diploid potato species (*Solanum commersonii* and *Solanum cardiophyllum*) exhibiting extremes of freezing tolerance and acclimation capacity. Precise assessment of these two traits allowed distinction of small but significant differences among genotypes. The two traits were not correlated in segregating populations, suggesting independent genetic control. Analyses of generation means indicate that all of the variance for acclimation capacity and a major proportion of the variance for the nonacclimated freezing tolerance can be best explained by an additive-dominance model with both traits being partially recessive. Recovery of parental phenotypes in limited populations suggests that both traits are controlled by relatively few genes. To our knowledge this is the first study demonstrating independent genetic control of the two main traits associated with frost or winter survival. Our results show that it should be possible to incorporate these traits from wild germ plasm into cultivated crop plants by independent selection. These results help explain the lack of progress in improving winter survival through field selection. Furthermore, our study demonstrates relative simplicity of the inheritance of cold acclimation, thus providing avenues for understanding the link between biochemical and genetic aspects of low-temperature stress in crop plants.**

Freezing temperatures adversely affect plant productivity in many parts of the world (1). Traditional plant breeding methods for improvement of freezing resistance in crop plants by using field selection (frost or winter survival) have achieved only limited success (2–4). Much of the failure to achieve greater success has been attributed to lack of genetic diversity, lack of effective selection criteria (2), and limited or inconsistent information on genetic control of freezing resistance (3). Attempts to determine the mode of inheritance of freezing resistance have been made in numerous crop species, with the majority of studies focusing on overwintering cereal crops (2, 4). Most studies have concluded that freezing resistance is a very complex trait with polygenic inheritance (4). The major component of variability appears to be additive effects; however, there are conflicting reports of dominance of winter survival in cereals (5, 6).

Field selection for freezing tolerance has many inherent problems. Winter survival is a complex trait encompassing multiple facets of a plant's ability to survive biotic and abiotic stresses. Ideally, one hopes for a test winter that is severe enough to kill the most sensitive genotypes, cause various degrees of injury to intermediate genotypes, and cause no injury to the most resistant genotypes. However, such test winters are rare. Two major components of winter survival in crop plants are freezing tolerance in the nonacclimated state and cold acclimation ability. Freezing tolerance is the ability of a plant to survive extracellular ice, and cold acclimation capacity of a plant is the ability to increase its freezing tolerance (survive lower freezing temperatures) after a brief period of low-temperature exposure. To date, genetic studies of freezing resistance have not distinguished nonacclimated freezing tolerance and acclimation capacity as separate heritable traits. No attempt has been made to independently select for these two traits.

Solanum species are known to vary greatly in both nonacclimated freezing tolerance and acclimation capacity (7, 8), suggesting that the two traits may be genetically or physiologically distinct. Our study was aimed at resolving these two traits to gain a better understanding of the genetic mechanisms involved in freezing resistance. To do this, we used controlled acclimation conditions and a precise method of evaluation of freezing tolerance permitting distinction between small but significant differences between genotypes. This technique has been used successfully to estimate freezing tolerance of several species (8–12). We selected diploid tuber-bearing *Solanum* species, largely divergent in nonacclimated freezing tolerance and acclimation capacity, as parents. Generation means analyses of the parental, F_1, and backcross populations were used to fit a simple additive-dominance genetic model to the inheritance of the two traits.

MATERIALS AND METHODS

Plant Material. Seeds of *Solanum commersonii* (cmm) PI 243503 and *Solanum cardiophyllum* (cph) PI 184762 were obtained from the Inter-Regional Potato Introduction Station (Sturgeon Bay, WI). The F_1 (cmm × cph) and backcross (F_1 × cmm and F_1 × cph) generations were produced by using controlled pollination under greenhouse conditions. Three seedlings each of the cmm and cph parents, 7 seedlings of the F_1, and 21 and 19 seedlings from the cmm and cph backcrosses, respectively, were maintained in sterile culture on MS medium (13). Plantlets obtained from culture were potted in peat/vermiculite (1:1) in 8-liter pots and grown in a 3.7 × 2.6 m controlled environment room at the University of Wisconsin Biotron facility (Madison, WI). Nonacclimating

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Abbreviation: RFT, relative freezing tolerance.

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growth conditions were 20°/18°C light/dark temperatures, with a 14-hr photoperiod of 400 μmol of photons $m^{-2} \cdot s^{-1}$ from cool white fluorescent lamps. Relative humidity was maintained at 70%. Hoagland and Arnon nutrient solution I (14) modified by 5-fold increased zinc sulfate and addition of 85 μM potassium chloride was used at quarter-strength and delivered to excess automatically four times daily. To achieve cold acclimation, temperatures were lowered to 4°/2°C light/dark with a 14-hr photoperiod of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 14 days. These conditions were shown previously to result in full hardening in tuber-bearing *Solanum* species (15).

Freeze–Thaw Simulation. Freezing tolerance of the plant material was determined by a modification of the protocol of Steffen *et al.* (16). Fully expanded leaflets were excised and placed in covered culture tubes (25 × 200 mm) which were submerged in a glycol-containing controlled temperature cooling bath at 0°C. Controls were immediately put on ice. After 30 min, the temperature was lowered to −1.0°C and held for 30 min. Ice nucleation was then initiated by adding a small piece of ice to each tube, and samples were held at −1.0°C for an additional 1.5 hr. Then the temperature was lowered to −1.5°C and held for 1 hr. Further cooling below −2.0°C was at a rate of 0.5°C/30 min. Tubes were removed at predetermined temperatures and thawed on ice overnight prior to evaluation of injury.

Determination of Relative Freezing Tolerance (RFT). Freezing injury was assessed by measurement of ion leakage (11). Thawed leaflets were sliced into 5-mm strips prior to addition of 20 ml of deionized distilled water at room temperature. Samples were infiltrated for 5 min at 0.1 atmosphere (10 kPa) by using a vacuum pump and shaken for 1 hr, and conductivity was measured with a YSI model 32 conductance meter (Yellow Springs, OH). Total conductivity for each sample was determined after autoclaving at 121°C for 15 min. Percent ion leakage of averaged triplicates was plotted as a function of freezing temperature. RFT was determined from the midpoint of the maximum and minimum (control) ion leakage values obtained for each genotype (see Fig. 1). The absolute value of this temperature was defined as the RFT (see Fig. 1). Acclimation capacity (ΔRFT) was expressed as acclimated RFT minus nonacclimated RFT.

Statistical Analysis. Phenotypic correlation coefficients for nonacclimated RFT and acclimation capacity (ΔRFT) were determined from individual plants within the backcross populations, and significance was tested by means of the *t* distribution. The equality of the population variances of the traits examined was tested by Bartlett's test of homogeneity of variance (17). Since the population variances were determined to be homogeneous, a modification of the joint scaling test of Mather and Jinks (18) was used to determine the adequacy of an additive-dominance model and estimate the three genetic parameters: *m*, the estimated midpoint between parental means; [*d*], the additive genetic component; and [*h*], the dominance genetic component, using the notation of Mather and Jinks (18). The parental, F_1, and backcross means were analyzed by least-squares regression (19). The scale factor $\hat{\sigma}^2$ calculated as $\chi^2_{k-p}/(k-p)$, where *k* is the number of generation means and *p* is the number of parameters estimated, was used in the determination of standard errors of the estimated genetic parameters (20). The goodness of fit of the additive-dominance model was assessed by the statistic (19)

$$F = \hat{\sigma}^2/\hat{\sigma}_0^2, \quad \text{where } \hat{\sigma}_0^2 = \sum_{i=1}^{k}(n_i - 1)s_i^2 / \sum_{i=1}^{k}(n_i - 1).$$

Student *t* tests were performed to determine significance of the estimated genetic parameters. The adjusted squared correlation coefficients (R^2_{adj}) were used to judge the proportion of variance explained by the additive-dominance model. Weighted least-squares regression using the inverse of the variances of the generation means as weights (20) gave similar results. Also, an ordinary least-squares regression based on the individual observations rather than the generation means yielded the same regression parameter estimates (data not shown).

RESULTS

Screening Method. Determining ion leakage values at a range of freezing temperatures allowed us to distinguish between small differences in the RFT values among individuals and between the acclimated and nonacclimated states of individuals (Fig. 1). The two parents had extremely different nonacclimated RFT values and acclimation capacities. Plants derived from different seedlings within the two parental populations had uniform freezing tolerance and acclimation capacity (21). The cmm population had a mean (± SEM) nonacclimated RFT of 4.5 ± 0.2 and a mean acclimation capacity of 5.1 ± 0.5, while the cph population had a mean nonacclimated RFT of 1.6 ± 0.1 and a mean acclimation capacity of 0.6 ± 0.2 (Table 1). The mean nonacclimated RFT (2.4 ± 0.1) of the cmm × cph F_1 population was closer to that of the cph parent than to that of the cmm parent (Table 1; see also Figs. 3 and 4). Similarly, acclimation capacity of the F_1 population (1.5 ± 0.2) was closer to that of the cph parent. These results were reproducible, as the difference between RFTs determined from two replicated separate experiments on the same genotype was 7% of the mean on average.

Relationship. The relationship between the two individual components of cold resistance—nonacclimated freezing tolerance and acclimation capacity—was examined in the two segregating backcross populations. Correlation coefficients for nonacclimated RFT and acclimation capacity (ΔRFT) were determined for the two backcross populations (Fig. 2). The two traits were not significantly correlated; $r = -0.066$ and −0.317 in the F_1 × cph and F_1 × cmm progenies, respectively ($P > 0.2$ for both).

Genetic Analysis of Freezing Tolerance and Acclimation Capacity. The mean values for nonacclimated RFT and acclimation capacity of the five populations analyzed are shown in Table 1. Analyses of variance showed that there

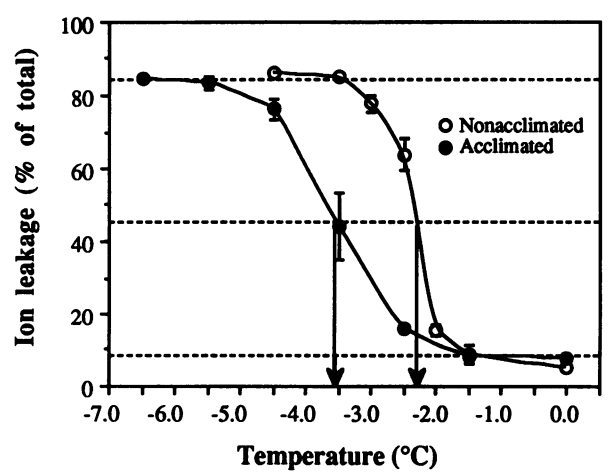

FIG. 1. Determination of RFT based on ion leakage measurements after freezing excised leaf tissue to specific temperatures: An example of data obtained from an individual in the F_1 population. Error bars represent SEM. The temperature corresponding to 50% ion leakage was taken (arrow) from the midpoint of the maximum and minimum ion leakage values obtained for each genotype as illustrated for the curves obtained for this F_1 in the nonacclimated and acclimated states. RFT was defined as the absolute value of the midpoint temperature.

Table 1. Means for the nonacclimated tolerance (RFT) and the acclimation capacity (ΔRFT) for the five generations analyzed

Generation	Nonacclimated tolerance (RFT) Observed mean	Nonacclimated tolerance (RFT) Expected mean	Acclimation capacity (ΔRFT) Observed mean	Acclimation capacity (ΔRFT) Expected mean
cph	1.6 ± 0.1	1.96	0.6 ± 0.2	0.44
F_1 × cph	2.3 ± 0.1	2.21	1.0 ± 0.1	1.03
F_1	2.4 ± 0.1	2.46	1.5 ± 0.1	1.61
F_1 × cmm	3.4 ± 0.1	3.42	3.6 ± 0.2	3.50
cmm	4.5 ± 0.2	4.37	5.1 ± 0.5	5.39
Goodness of fit				
$F = \hat{\sigma}^2/\hat{\sigma}_0^2$	4.020		0.513	
P	<0.025		>0.5	
R^2_{adj}	0.788		0.815	

The expected means are based on an additive-dominance model and estimated by least-squares regression. RFT was determined from the midpoint of the maximum and minimum (control) ion leakage values obtained from each genotype (Fig. 1). The absolute value of this temperature (°C) was defined as the RFT and acclimation capacity ΔRFT was expressed as acclimated RFT minus nonacclimated RFT. Values are given ±SEM.

were significant differences among the populations for the parameters examined (data not shown). The distributions of the nonacclimated RFT of the backcross progenies are shown in Fig. 3, while Fig. 4 shows the distributions of the acclimation capacity (ΔRFT). Within each population, backcross individuals were distributed between the parental values, with the center of the distributions shifted toward the recurrent parent (Figs. 3 and 4).

The generation means analysis of Beaver and Mosjidis (19) was used to test a simple additive-dominance model for genetic control of freezing tolerance and acclimation capacity. A model including mean (m), additive ([d]), and dominance ([h]) genetic components was used to calculate expected mean RFTs and acclimation capacity for each generation (Table 1) by least-squares regression. A goodness of fit test (Table 1) indicated that an additive-dominance model is adequate to explain acclimation capacity. Nonacclimated freezing tolerance, however, could not be explained simply by the additive and dominance parameters in the model. We did not test additional parameters for this trait. However, the R^2_{adj} (0.788) for fitting the additive-dominance model suggests that a large proportion of the variance in nonacclimated freezing tolerance can be explained by additive and dominance gene effects. The estimates of the genetic parameters in the model for nonacclimated freezing tolerance and acclimation capacity are shown in Table 2. Both additive gene effects and dominant gene effects were significant for these two traits except for the dominant gene effects ([h]) for nonacclimated RFT. The negative value for [h] indicates that RFT in the nonacclimated state and acclimation capacity are partially recessive.

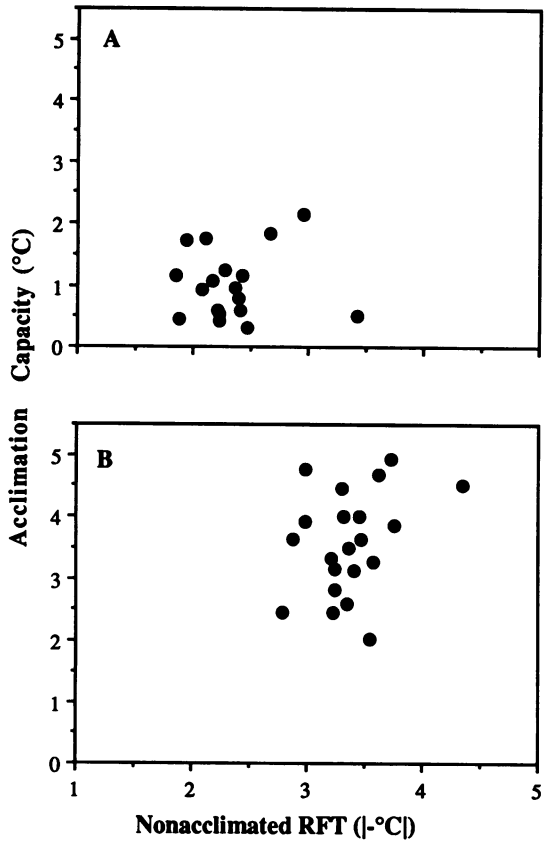

FIG. 2. Phenotypic correlation between nonacclimated RFT and acclimation capacity (ΔRFT) of individuals in segregating backcross populations. (*A*) F_1 × cph backcross progeny ($r = -0.066$, $P > 0.5$). (*B*) F_1 × cmm backcross progeny ($r = -0.317$, $P > 0.2$).

DISCUSSION

Populations derived from an interspecific cross between *S. cardiophyllum* and *S. commersonii* were found to be ideally suited for gaining insight into inheritance of freezing stress resistance. These species were chosen for this study because

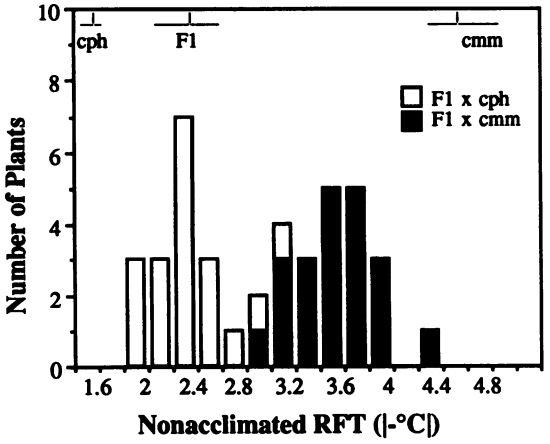

FIG. 3. Distribution of the nonacclimated RFTs of the backcross progenies. The range and mean RFT values for both parents (cmm and cph) and the F_1 (cmm × cph) are indicated at the top of the graph.

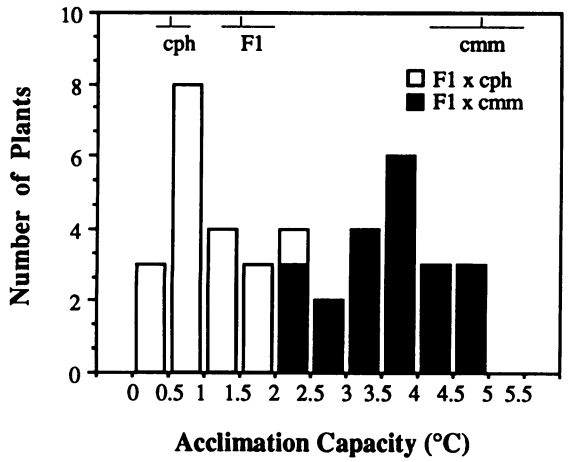

FIG. 4. Distribution of acclimation capacity (ΔRFT, the increase in RFT following acclimation) of the backcross progenies. The range and mean ΔRFT values for both parents (cmm and cph) and the F_1 (cmm × cph) are indicated at the top of the graph.

they exhibit the extremes of nonacclimated freezing tolerance and acclimation capacity among the nearly 120 tuber-bearing *Solanum* species maintained at the U.S. potato gene bank (Inter-Regional Potato Introduction Station, Sturgeon Bay, WI). Additionally, these widely divergent species were cross-compatible, allowing generation of F_1 and backcross populations necessary for genetic analysis.

Precise assessment of freezing tolerance and cold acclimation capacity allowed separation of these traits for each genotype examined. Ion leakage from excised leaflets subjected to a simulated freeze–thaw provided a precise test of relative freezing tolerance (Fig. 1) that has been demonstrated to correlate well with winter survival in *Brassica napus* (9) and freezing injury in *Solanum* (10, 11) and *Pinus* (12) species. Furthermore, the Biotron facility allowed exposure of all genotypes to identical environmental conditions for both nonacclimating growth and subsequent acclimation. Field assessment lacks the precision needed to distinguish genotypes for small but significant differences in freezing tolerance. This is because winter survival can be affected by many variations such as snow cover, water status, disease, midwinter thaw, and ice sheet formation and by the lowest minimum temperatures reached (22). Furthermore, environmental conditions preceding a spring or fall frost will have an impact on the state of acclimation, and thus freezing tolerance, of plant material at the time of frost.

We found that nonacclimated freezing tolerance and acclimation capacity were not correlated in segregating *Solanum* backcross populations (Fig. 2), suggesting independent genetic control for these traits. The relationship between these two individual traits of freezing resistance has not been investigated previously to our knowledge. Since nonacclimated freezing tolerance and acclimation capacity were inherited independently, they should be evaluated separately when selecting for freezing resistance. Field evaluations prohibit this distinction. Separation of these components may also facilitate the understanding of genetic control of freezing resistance in other crop species.

The evidence that unlinked genes control nonacclimated freezing tolerance and acclimation capacity is interesting. During the active growth period (nonacclimating conditions) plant survival depends upon the nonacclimated freezing tolerance. However, during cold acclimating conditions in fall, growth usually slows and the plant goes into dormancy. Thus it is not surprising that genetic controls for freezing tolerance are different in the nonacclimated and cold-acclimated states.

Table 2. Estimates and significance of genetic parameters from the joint scaling test for the two components of freezing stress resistance, RFT and ΔRFT

Genetic parameter	Components of freezing stress resistance: Nonacclimated freezing tolerance (RFT)	Acclimation capacity (ΔRFT)
m (mean value)	3.16 ± 0.21*	2.92 ± 0.15*
[*d*] (additive gene effects)	−1.21 ± 0.17*	−2.47 ± 0.12*
[*h*] (dominance gene effects)	−0.71 ± 0.38	−1.31 ± 0.26†

Values are given as mean ± SEM. *, Significance at $P = 0.01$; †, significance at $P = 0.05$.

Most studies on the genetic control of freezing resistance have implicated involvement of many genes with a complex mode of inheritance, although there are conflicting conclusions (23). For these *Solanum* populations, the results of the generation means analysis (Table 1) indicate that acclimation capacity can be explained by a simple additive-dominance genetic model. This model, however, was not completely adequate for nonacclimated RFT, although a major proportion of the variance could be explained by it. Physiological complexities that determine freezing tolerance during the active growth period in the nonacclimated state may be explained by other genic interactions.

Estimates obtained for the genetic parameters included in the model indicate nonacclimated freezing tolerance and acclimation capacity are partially recessive (Table 2). The fact that the F_1 was closer to the freezing-sensitive nonacclimating parent, *S. cardiophyllum* (Figs. 3 and 4), for these traits further supports this conclusion. Analogous to our findings in *Solanum* species, diallele analysis of winter wheat indicated that gene action was mostly additive, with frost sensitivity partially dominant (5). However, another study in winter wheat suggested freezing tolerance to be dominant (6). These apparent inconsistencies have been explained, in part, by differences in the methods used for evaluation (23).

The distributions of the backcross progenies (Figs. 3 and 4) support the conclusion that nonacclimated freezing tolerance and acclimation capacity are controlled by relatively few genes, since the backcross distributions show near recovery of parental phenotypes for nonacclimated freezing tolerance (Fig. 3) and full recovery for acclimation capacity (Fig. 4) in relatively small populations. By distinguishing between freezing tolerance and cold acclimation ability, we were able to show that cold acclimation in particular is genetically simple.

The genetic simplicity of cold acclimation demonstrated in these populations may be utilized to characterize the mechanism of acclimation to low temperature. These full-sib families with individuals segregating for the capacity to develop freezing tolerance upon exposure to low temperature can be used to confirm the importance of particular biochemical changes (24, 25) and induction of genes (26) that have been associated previously with cold acclimation in *Solanum* species. Differential screenings of cDNA libraries constructed from various acclimating species have resulted in the cloning of several genes that are induced by low temperature (27–32). Unfortunately, the role of these gene products in the cold acclimation process is still speculative. Further studies of these *Solanum* populations are necessary to clarify the molecular aspects of freezing resistance.

Freeze damage in potato has long been a significant concern, being first recorded by Cristóbal de Molina in 1573 in writings of Incan prayers (see ref. 33). Freezing temperatures limit potato production, especially in the Andean highlands of South America. Low temperatures of −2° to −4°C can reduce

yield about 30% (34). The materials we developed and the precise evaluation used have given us some important genetic and physiological insights into the nature of freezing resistance in potato. This information should be invaluable in the process of incorporating freezing resistance into future potato cultivars through classic breeding and molecular approaches.

This work was supported by a grant to J.P.P. and J.B.B. from the U.S. Department of Agriculture, National Research Initiative Competitive Grants Program (91-37100-6636). The College of Agricultural and Life Sciences, University of Wisconsin Hatch Project 142-2737 of J.P.P. provided a research assistantship to J.M.S.

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