Marker-assisted Genetic Analysis of Non-acclimated Freezing Tolerance and Cold Acclimation Capacity in a Backcross *Solanum* Population

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ABSTRACT

Random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers were used to construct a partial genetic linkage map in a potato backcross population. The population, derived from two diploid wild *Solanum* species (frost tolerant, able to cold acclimate *S. commersonii;* frost sensitive, unable to cold acclimate *S. cardiophyllum*), was used to map quantitative trait loci (QTL) of non-acclimated relative freezing tolerance (NARFT) and cold acclimation capacity (CAC). Precise assessment of these traits allowed distinction of small but significant differences among 35 backcross genotypes. NARFT and CAC were not correlated in the segregating population, suggesting independent genetic control for these two major components of freezing tolerance. The linkage map spanned 479.4 cM and included 77 RAPD markers and two SSR markers, with 38 RAPD and 10 SSR unassigned markers. Two QTLs for NARFT were detected in two different linkage groups, accounting for 44.0% of the phenotypic variation for this trait. Two QTLs for CAC were detected, accounting for 24.9% of the phenotypic variation for this trait. QTLs for NARFT and CAC were detected at separate genomic regions, in support of the independent genetic control of these two traits. QTLs for NARFT and CAC were detected in a linkage group identified as part of chromosome V, suggesting that such chromosome constitutes a prime candidate for fine-mapping. Due to the relatively small progeny size evaluated in this study, additional QTLs for NARFT and CAC could have been involved but not identified. Therefore, the conclusions derived from this study should be considered preliminary.

RESUMEN

Marcadores de DNA polimórfico amplificado al azar (RAPD) y de secuencias simples repetidas (SSR) fueron utilizadas para construir un mapa parcial de ligamiento genético en una población de retro-cruza de papa. Esta población fue derivada de dos especies silvestres diploides, *S. commersonii* (resistente a heladas, capaz de acclimatarse a bajas temperaturas) y *S. cardiophyllum* (sensible a heladas, incapaz de aclimatarse a bajas temperaturas), y se utilizó para mapear loci de carácteres cuantitativos (QTL) para la tolerancia relativa a heladas sin aclimatación a bajas temperaturas (NARFT) y la capacidad de aclimatarse a bajas temperaturas (CAC). La determinación precisa de estos dos caracteres permitió encontrar diferencias pequeñas pero significativas entre los 35 genotipos de la retro-cruza. La correlación entre NARFT y CAC no fue significativa en la población segregante, sugieriendo que existe un control genético independiente para cada uno de estos dos

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ABBREVIATIONS: ACCRFT, acclimated relative freezing tolerance; BC, backcross; bp, base pair; CAC, cold acclimation capacity; GA, gibberellic acid; LG, linkage group; LOD, logarithm of the odds; NARFT, non-acclimated relative freezing tolerance; PAR, photosynthetically active radiation; r, recombinant fraction; RFT, relative freezing tolerance.

componentes mayores de la tolerancia a heladas. El tamaño del mapa fue de 479.4cM e incluyó 77 marcadores RAPD y dos marcadores SSR; con 38 marcadores RAPD y 10 marcadores SSR sin asignar. Para NARFT, dos QTLs fueron detectados en dos diferentes grupos de ligamiento representando 44.0% de la variación fenotípica observada en este carácter. Para CAC, dos QTLs fueron detectados, los cuales representaron 24.9% de la variación fenotípica observada en este carácter. Los QTLs para NARFT y CAC fueron detectados en diferentes regiones genómicas, confirmando el control genético independiente de estos dos caracteres. Los QTLs para NARFT y CAC fueron detectados en un grupo de ligamiento identificado como parte del cromosoma V, sugiriendo que dicho cromosoma podría ser un candidato principal para mapeo fino. Debido a que el tamaño de la población evaluada en este estudio fue relativamente pequeño, QTLs adicionales para NARFT y CAC podrían existir. En consecuencia, las conclusiones derivadas de este estudio deben ser consideradas como preliminares.

INTRODUCTION

Potatoes are mainly grown in the temperate zones and in the highlands of the Andean tropics of South America, where unpredictable frosts often reduce yield and quality. For example, it is estimated that in the Andean countries more than 400,000 ha of potato fields are threatened by frost injury every year (Estrada et al. 1993). One inherent problem is that most of the potato varieties cultivated worldwide are sensitive to low temperatures (cultivated species *S. tuberosum* is killed at –2 to –3 C; Li and Palta 1978). However, a screening of the United States potato collection for frost hardiness showed that many wild *Solanum* species exhibit frost tolerance far superior to that of the cultivated species (Vega and Bamberg 1995). This diversity provides an invaluable resource and an ideal model system for breeding to improve resistance to low temperatures and to study the mechanisms of freezing tolerance and cold acclimation.

Two major components of freezing stress resistance are freezing tolerance in the non-acclimated state (normal growing condition) and capacity to cold acclimate (increase in freezing tolerance upon exposure to chilling temperatures [Palta and Simon 1993]). *Solanum* species vary greatly in both (Chen and Li 1980; Vega and Bamberg 1995). Stone et al. (1993) demonstrated that freezing tolerance and ability to cold acclimate are under independent genetic control in potato. This was later confirmed in other plant species (Arora et al. 1998; Teutonico et al. 1995). These results have important implications for the improvement of cold tolerance of cultivated potatoes. For the successful improvement of frost hardiness, both components must be transferred to the cultivated potatoes (Palta and Simon 1993).

Over the years, limited success has been achieved using traditional plant-breeding methods to improve freezing stress resistance in crops (Marshall 1982; Palta and Simon 1993). Even those that have undergone extensive breeding, such as winter wheat, have not had significant improvements, and cultivars that were released more than 50 years ago remain among the most cold hardy today (Limin and Fowler 1991). Recent progress in plant breeding has focused on the use of molecular marker techniques to facilitate cloning and efficient introgression of favorable genes through marker-assisted selection (Dudley 1993; Lande and Thompson 1990). Genomic regions with significant effect on freezing tolerance have been detected in several crops: *Citrus* (Cai et al. 1994); *Brassica* (Kole et al. 2002; Teutonico et al. 1995); *Triticum* (wheat) (Sutka 1994); *Eucaliptus nitens* (Byrne et al. 1997), *Lycopersicon* (tomato) (Foolad et al. 1998), *Vaccinium* (blueberry) (Rowland et al. 1999), and *Oryza sativa* (rice) (Saito et al. 2001).

The objective of the present study was to provide genetic insights to potato cold hardiness by analyzing non-acclimated freezing tolerance (NARFT) and cold acclimation capacity (CAC) on a segregating population using molecular markers. A backcross population segregating for both traits was used to develop a random amplified polymorphic DNA (RAPD) linkage map. Two diploid tuber-bearing wild *Solanum* species that represent extremes of NARFT and CAC were used as parents, *S. commersonii* (frost tolerant and able to cold acclimate) and *S. cardiophyllum* (frost sensitive and unable to cold acclimate). Simple sequence repeat (SSR) loci of known chromosomal location (Milbourne et al. 1998) were included in the analysis. The resulting map and the phenotypic data of frost hardiness and cold acclimation capacity of the backcross population were used to identify quantitative trait loci (QTL).

MATERIALS AND METHODS

Plant Material

Seeds of *S. cardiophylum* (cph) PI 184762 and *S. commersonii* (cmm) PI 243503 were obtained from the Inter-Regional Potato Introduction Station (NRSP-6), Sturgeon Bay, WI, USA. One single seedling from each species was then propagated and used as parents; the pollen of *S. cardiophylum* was pollinated onto stigmas of *S. commersonii*. To develop the backcross population we used an F1 individual (clonal identity: F1-1) in which NARFT and CAC most resembled the sensitive parent cph. The F1 (cmm x cph) and BC1 backcross (F1-1 x cmm) populations were obtained using controlled pollinations as described by Stone et al. (1993). Nearly 2000 pollinations were performed under controlled conditions at the University of Wisconsin Biotron facility (Madison, WI, USA). Backcross to the sensitive parent failed to produce viable seeds. Therefore, the population evaluated in this study was derived from the backcross to the freezing tolerant acclimating parent. Clonal propagation of the backcross population was done as described by Vega et al. (2000). From more than 20 F1s and close to 100 backcross seeds harvested, we were able to successfully grow and evaluate 19 F1s and 35 backcross genotypes. Plantlets of both parents, their F1 progeny and BC1 backcross population obtained from stem culture were individually potted in 2.5-L plastic pots containing 1:1 peat:vermiculite (v/v) and grown in a controlled-environment room at the University of Wisconsin-Madison Biotron facility.

Growth conditions were 20 ± 1/18 ± 1 C day/night temperatures, with a 14-h photoperiod of 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR from cool-white fluorescent lamps. Relative humidity was 70 ± 5%. Plants were irrigated with half-strength Hoagland solution delivered automatically twice daily to ensure that plants would be watered in excess of the container capacity to prevent salt accumulation. Cold acclimation treatment was initiated 5 wk after potting. To achieve cold acclimation, the air temperatures were lowered to 4/2 C day/night with a 14-h photoperiod and 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR. Plants were kept under these conditions for 12 days. These conditions were previously determined to be ideal for cold acclimation of potato species (Steffen et al. 1989).

Determination of Relative Freezing Tolerance

Freezing tolerance of the plant material was determined by a modification of the protocol of Steffen et al. (1989). Fully expanded terminal leaflets were excised at the end of the dark period before the lights came on and placed in covered glass culture tubes. Glass tubes containing one leaflet each were submerged in a glycol-containing controlled temperature cooling bath (Forma Scientific, Model 2323, Marietta, OH, USA) held at 0 C. After 30 min the temperature in the cooling bath was lowered to –0.5 C, held for 30 min, and then lowered to –1.0 C. After 30 min at –1.0 C, ice nucleation was initiated by adding a small piece of ice to each tube. Samples were held at –1.0 C for another 30 min, then the temperature was lowered to –1.5 C and held for 1 h. Further cooling was at a rate of 0.5 C/30 min down to –10 C and 1.0 C/30 min below –10 C. Tubes containing the frozen leaflets were removed at predetermined temperatures (used to develop an ion leakage curve) and thawed on ice overnight prior to the evaluation of injury. Leaflets used as unfrozen controls were kept in an ice-filled cooler. Three leaflets were evaluated before and after cold acclimation at each temperature for the parental species, F1s, and backcross progeny.

Freezing injury was assessed by the measurement of ion leakage (Flint et al. 1967). The ion leakage was expressed as the ratio of electrolyte leakage from freeze-injured tissue to electrolyte leakage from autoclaved tissue. Thawed leaflets were sliced into strips before the addition of 25 mL of deionized, distilled water at approximately 22 C. Samples were infiltrated for 5 min at 10 kPa using a vacuum pump and then shaken for 1 h at 220 rpm on a gyratory shaker at room temperature. Electrical conductivity (R_1) was measured with an YSI model 32 conductance meter (Yellow Springs, OH, USA). Total conductivity (R_2) of each sample was measured following a 24 h cooling period after autoclaving at 121C for 15 min. Percentage mean ion leakage expressed as (R_1/R_2) x 100 of triplicates was plotted as a function of freezing temperature. Relative freezing tolerance (RFT) was determined from the midpoint of the maximum (autoclaved) and minimum (control) ion leakage values obtained for all the genotypes (average of the three leaflets) before and after cold acclimation as described by Stone et al. (1993). The absolute value of this temperature was defined as the RFT. CAC was assessed as acclimated relative freezing tolerance (ACCRFT) minus

NARFT. The distributions of the backcross progenies for the non-acclimated freezing tolerance and acclimation capacity were analyzed for deviations from normality using the Shapiro-Wilk Test (Shapiro and Wilk 1965). A correlation analysis was also performed for both traits.

DNA Isolation

DNA was isolated from two tissue-culture-propagated plantlets according to a procedure previously described by del Rio et al. (1997) in which potassium ethyl xanthogenate (PEX) served to liberate DNA. Extracted DNA was dissolved in Tris 10 mM, EDTA 1 mM (TE) 1X buffer (Promega, Madison, WI, USA) and stored at –20 C. DNA quantification was performed by fluorometry using TKO-100 Mini Fluorometer (Hoefer Scientific Supplies, San Francisco, CA).

RAPD/SSR Markers and PCR Amplification

The 10 bp oligonucleotide primers used in the RAPD assay were randomly selected from Operon Technologies (Alameda, CA, USA). PCR amplifications were performed in 15-µL reaction volumes containing 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.1 mM DTT, 50% glycerol, 1.0% Triton X-100 as reaction buffer, 25 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1 unit of Taq DNA polymerase (Promega, Madison, WI, USA), 15 ng genomic DNA template and 0.2 µM of a random primer. PCR amplification was performed in a Perkin Elmer Applied Biosystems Cetus DNA Thermal Cycler 9600 (Norwalk, CT, USA) programmed for one cycle of 94 C/4 min; three cycles of 94 C/15 sec, 35 C/15 sec, 72 C/75 sec; 40 cycles of 94 C/15 sec, 40 C/15 sec; one cycle of 72 C/7 min; and then 4 C soak.

Thirty-two primer pairs flanking SSR regions previously characterized in potato by Milbourne et al. (1998) were synthesized on a Perkin Elmer Applied Biosystems synthesizer (model 3948, Norwalk, CT, USA) at the DNA Synthesis and Sequencing Facility of the University of Wisconsin Biotechnology Center (Madison, WI, USA) and used for PCR amplification. PCR was performed in 15-µL reaction volumes containing the same reagents as specified for RAPD assays, but using 17 ng of genomic DNA and 0.6 µM of each primer of the pair. PCR was performed in the thermal cycler mentioned above programmed for one cycle of 94 C/1 min; three cycles of 94 C/15 sec, 45 C/20 sec, 72 C/1:15 min; 43 cycles of 94 C/15 sec, 50 C/15 sec, 72 C/1:15 min; one cycle of 72 C/7 min; and then 4 C soak.

RAPD and SSR products were fractionated by electrophoresis in 1.5% and 1.2% agarose gels, respectively, and visualized following ethidium bromide staining (gels were soaked in 0.5 µg EtBr/mL 1X TAE buffer). RAPD and SSR bands displaying polymorphism were scored as present (1) or absent (0). The approximate product lengths (within 50 bp) were estimated by visual comparison with a DNA fragment size marker (Promega, Madison, WI, USA). Each marker was then named by the letter (RAPD) and/or number (SSR) identifying the primer and the approximate length of the marker. This method was considered adequate to unambiguously name all markers.

Linkage Map Construction

Segregating bands were scored as present or absent. Observed segregation ratios were analyzed for deviation from the expected 1:1 Mendelian ratio using Chi-square test for goodness-of-fit ($P < 0.01$). The segregation analysis of 113 RAPD markers and 12 SSR markers was performed on data from 35 backcross genotypes. Linkage analysis was conducted using MAPMAKER Macintosh version 2.0 (Lander et al. 1987), and the mapping population was treated as an F_2 backcross population. Subsequent map construction followed established principles and recommendations (Keats et al. 1991) for framework mapping. After loading the data, loci were assigned to linkage groups based on a LOD score (LOD: logarithm of likelihood odds) >3 and a recombination fraction (r or theta) of 0.3. Map distances were calculated using the Kosambi function (Kosambi 1944). The final map distances were estimated using the MAP command with the error detection function engaged.

Identification of QTLs

The data were analyzed based on single-factor analysis of variance (ANOVA) for each pairwise combination of quantitative traits and marker loci. T-tests ($P < 0.05$) were used to determine if significant differences in trait expression were associated with differences in marker locus-genotypic classes (Edwards et al. 1987). Significant marker loci in the same linkage group were considered as one QTL if the distance between them was ≤50 cM (Paterson et al. 1991). When significant marker loci were linked, the marker locus having the greatest r^2 values was considered to be the most likely position of the QTL and used in further analysis. The marker loci significantly associated with trait variation based on the single-factor ANOVA were then entered into a stepwise multiple regression analysis in order to select the best set of markers and compute

the total phenotypic variation explained. The method of interval mapping was used for the localization of QTL and the estimation of their genetic effects (Lander and Botstein 1989). For this purpose, the computer program MAPMAKER/QTL version 1.9 (Lincoln and Lander 1989) was used with a LOD score for the QTL threshold set at 1.5. This LOD was chosen to ensure that any QTL with small, but significant, effects contributing to these polygenic traits were detected. Stepwise multiple regression was performed using marker loci associated with individual QTL as independent variables to determine the best multilocus model and the percentage of the phenotypic variation explained (Paterson et al. 1991). Marker loci were included in the final model if they were significant at $P \leq 0.05$. The p-value of 0.05 used for the detection of individual QTLs and for the stepwise regression analysis was relatively high. This p-value may increase the experimental Type I error rate; however, lower stringency of detection has been recommended as a way to reduce the probability of committing Type II errors (Edwards et al. 1992). In addition, significant molecular marker loci ($P \leq 0.05$) in the final model by the stepwise multiple regression analysis were tested for significant interactions by two-way ANOVA.

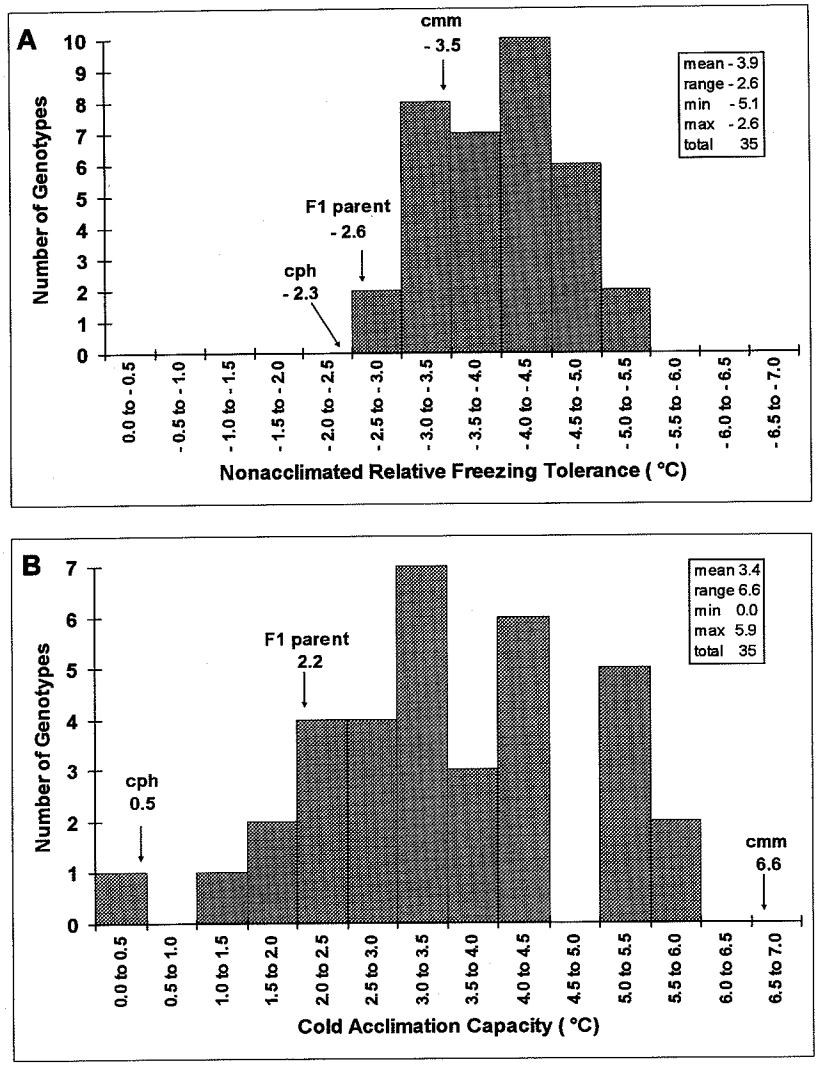

FIGURE 1.
Frequency distributions of (A) non-acclimated relative freezing tolerance (NARFT) and (B) cold acclimation capacity (CAC) of a backcross population derived from an interspecific cross of two diploid wild potato species, *S. commersonii* (cmm) frost tolerant and able to cold acclimate; and *S. cardiophyllum* (cph) frost sensitive and unable to cold acclimate. Backcross populations developed from F1 (cmm x cph) x cmm.

RESULTS

NARFT and CAC of the F1 and Segregating Backcross Population

The NARFT for the F1 (cmm x cph) population ranged between –2.4 and –3.3 C and the CAC ranged between 0.9 and 2.2 C; the means for NARFT and CAC of this population were –2.8 and 1.5 C, respectively (data not shown). One individual of this population, F1-1, with NARFT and CAC values close to that of the sensitive parent cph, was chosen to produce the backcross population.

The two parents of the backcross population showed extreme difference in their NARFT and CAC values. The F1 parent (clonal identity: F1-1) had a NARFT of –2.6 C and a CAC of 2.2 C and the *S. commersonii* parent had a NARFT of –3.5 C and a CAC of 6.6 C. For the backcross population, NARFT ranged between –2.6 and –5.1 C (Figure 1A) and CAC ranged between 0.0 and 5.9 C (Figure 1B). The means for NARFT and CAC of the backcross population were –3.9 and 3.4 C, respectively.

The relationship between NARFT and CAC was examined in the backcross population. The correlation analysis between

them was not significant ($P \geq 0.7611$), suggesting that NARFT and CAC in this backcross population are not correlated (Figure 2). The Shapiro-Wilk Test (Shapiro and Wilk 1965) confirmed that both traits showed normal distribution (NARFT: $P \geq 0.6295$, CAC: $P \geq 0.5831$).

Marker Segregation

From an initial screening of 142 RAPD primers, 45 primers were selected based on polymorphism between the parents (F1-1 and *S. commersonii*). These 45 primers generated 156 polymorphic bands, which were scored for the 35 backcross genotypes. Thus, an average of 3.5 polymorphic fragments was amplified per primer. Of the 156 RAPD markers, 113 (72%) fit the expected 1:1 ratio based on a chi-square goodness-of-fit test and were used for further analyses.

Based on the information published by Milbourne et al. (1998), 32 potato-specific SSR primer pairs were screened and 12 primer pairs were selected based on polymorphism between the parents. These 12 primer pairs generated 16 polymorphic fragments in the 35 backcross genotypes, 12 (75%) fit the expected 1:1 ratio based on a chi-square goodness-of-fit test and were used for further analyses.

Linkage Map Construction

From 125 markers (113 RAPD and 12 SSR), 53 markers (51 RAPD and one SSR) were grouped into 11 linkage groups (Table 1, Figure 3). Twenty-four markers (23 RAPD and one SSR) mapped to identical positions as other assigned markers (shown in parenthesis in Figure 3) and 15 markers were clustered within a short distance from other assigned markers (shown in brackets in Figure 3). Thirty-eight RAPD and ten SSR markers were unassigned. The number of markers per linkage group ranged from three to seven (Table 1). The average linkage distance between pairs of markers (marker density) among all linkage groups was 9.0 cM (Table 1). Two intervals located in linkage groups F and H were larger than 20 cM (21.9 and 29.5 cM, respectively). The linkage map reported here spans a total of 479.4 cM.

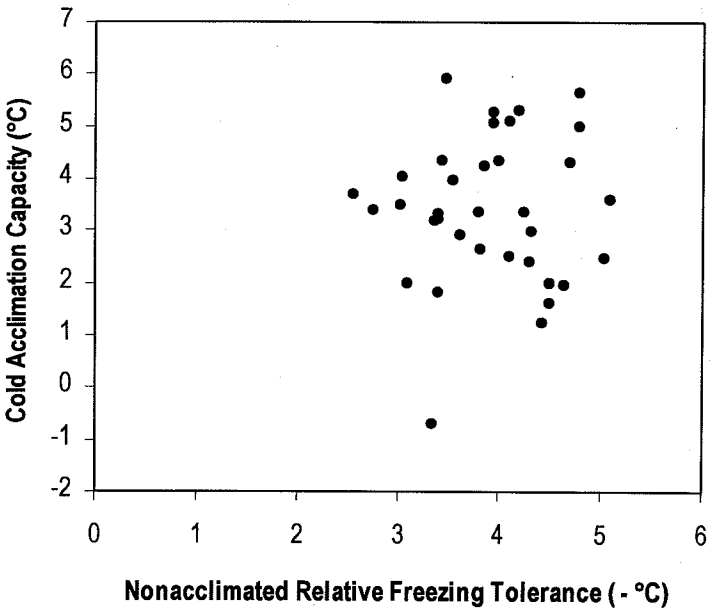

FIGURE 2.
Correlation between non-acclimated relative freezing tolerance (NARFT) and cold acclimation capacity (CAC) of a backcross population derived from an interspecific cross of two diploid wild potato species, *S. commersonii* (cmm) frost tolerant and able to cold acclimate; and *S. cardiophyllum* (cph) frost sensitive and unable to cold acclimate. Backcross populations developed from F1 (cmm x cph) x cmm. Each point represents a different genotype. NARFT and CAC correlation analysis was not significant ($P \geq 0.7611$).

QTL Analysis of NARFT and CAC

For NARFT, two QTLs were detected using interval mapping, one each in linkage groups B and D (Figure 3). Seven mapped markers (six RAPDs and one SSR) and one unmapped RAPD marker were significantly associated with NARFT based on single-factor ANOVA. Only two of these eight marker loci, however, were retained in the multiple regression model, with a cumulative r^2 of 44.0%. Marker $Y20_{2000}$, which mapped nearest to the most likely position of one of the QTLs identified by interval mapping, accounted for 24.7% of the variation for this trait (Table 2). Marker $E3_{450}$ mapped near the second QTL identified, accounting for 19.3% of the variation. No significant interaction was found between the two QTLs.

Two mapped RAPD markers were significantly associated with CAC based on single-factor ANOVA and were included in the multiple regression model, with a cumulative r^2 of 24.9%. Marker $Z20_{600}$, accounted for 13.6% of the variation for this trait and marker $A18_{550}$, accounted for 11.3% of the variation (Table 2). Interval mapping analysis detected two QTL, each linked to both of these markers (Figure 3.); however, both with only a LOD > 0.9. No significant interaction was found between the two QTLs.

TABLE 1—*Description of genetic linkage map based on 113 random amplified polymorphic DNA (RAPD) and 12 potato simple sequence repeat (SSR) markers, segregating in a backcross population derived from an interspecific cross of two diploid wild potato species,* S. commersonii *(cmm) frost tolerant and able to cold acclimate; and* S. cardiophyllum *(cph) frost sensitive and unable to cold acclimate. Backcross populations developed from F1(cmm x cph) x cmm.*

Linkage group	Number of markers	Map distance[1]	Marker density[2]
A	3	17.6	5.8
B	5	62.7	12.5
C	5	59.4	11.8
D	6	56.8	9.5
E	4	11.7	2.9
F	6	63.0	10.5
G	5	44.0	8.8
H	7	70.3	10.0
I	4	44.8	11.2
J	2	8.7	8.7
K	6	40.4	6.7
Total	53	479.4	9.0
Markers in same position of an assigned marker	24		
Unassigned RAPD markers	38		
Unassigned SSR markers	10		
Total molecular markers	125		

[1]Map distance (cM) based on mapping function described by Kosambi (1944).
[2]Map length (cM) divided by the number of markers.

TABLE 2—*Summary of the single-factor analysis of variance (ANOVA), interval mapping, and multiple regression analyses of molecular marker and phenotypic data for detecting quantitative trait loci (QTL) associated with either non-acclimated relative freezing tolerance (NARFT) or cold acclimation capacity (CAC) in a backcross population derived from an interspecific cross of two diploid wild potato species,* S. commersonii *(cmm) frost tolerant and able to cold acclimate; and* S. cardiophyllum *(cph) frost sensitive and unable to cold acclimate. Backcross populations developed from F1(cmm x cph) x cmm. Simple sequence repeat (SSR) marker is in bold.*

	Single-factor ANOVA	Interval mapping	Multiple regression		Linkage
[a]Location	[b]p > T	[c]LOD	[d]p > F	[e]r²	[f]group
Non-acclimated Relative Freezing Tolerance (NARFT)					
$E3_{450}$	0.0062	1.6	0.0068	19.3	B
$Y20_{2000}$	0.0096	1.8	0.0067	24.7	D
$A1_{450}$	0.0143	—	—	—	D
$E3_{400}$	0.0250	—	—	—	B
1031_{800}	0.0338	—	—	—	D
$Y1_{700}$	0.0397	—	—	—	H
$W19_{1000}$	0.0429	—	—	—	G
Cumulative r²				44.0	
Cold Acclimation Capacity (CAC)					
$Z20_{600}$	0.0290	1.1	0.0290	13.6	D
$A18_{550}$	0.0466	0.9	0.0406	11.3	B
Cumulative r²				24.9	

[a]Marker significantly associated with trait variation.
[b]Significance determined for T-tests based on single-factor ANOVA.
[c]LOD = logarithm of odds; value at most likely QTL location as determined by Mapmaker QTL.
[d]Significance levels determined for F-tests based on stepwise regression analysis of marker locus-trait associations for markers found to be significant by single-factor ANOVA ($P < 0.05$).
[e]Percentage of phenotypic variation explained, for significant ($P < 0.05$) marker locus-trait associations, based on stepwise regression analysis.
[f]Linkage group membership of markers significantly associated with trait variation for linkage groups as defined in Figure 3.

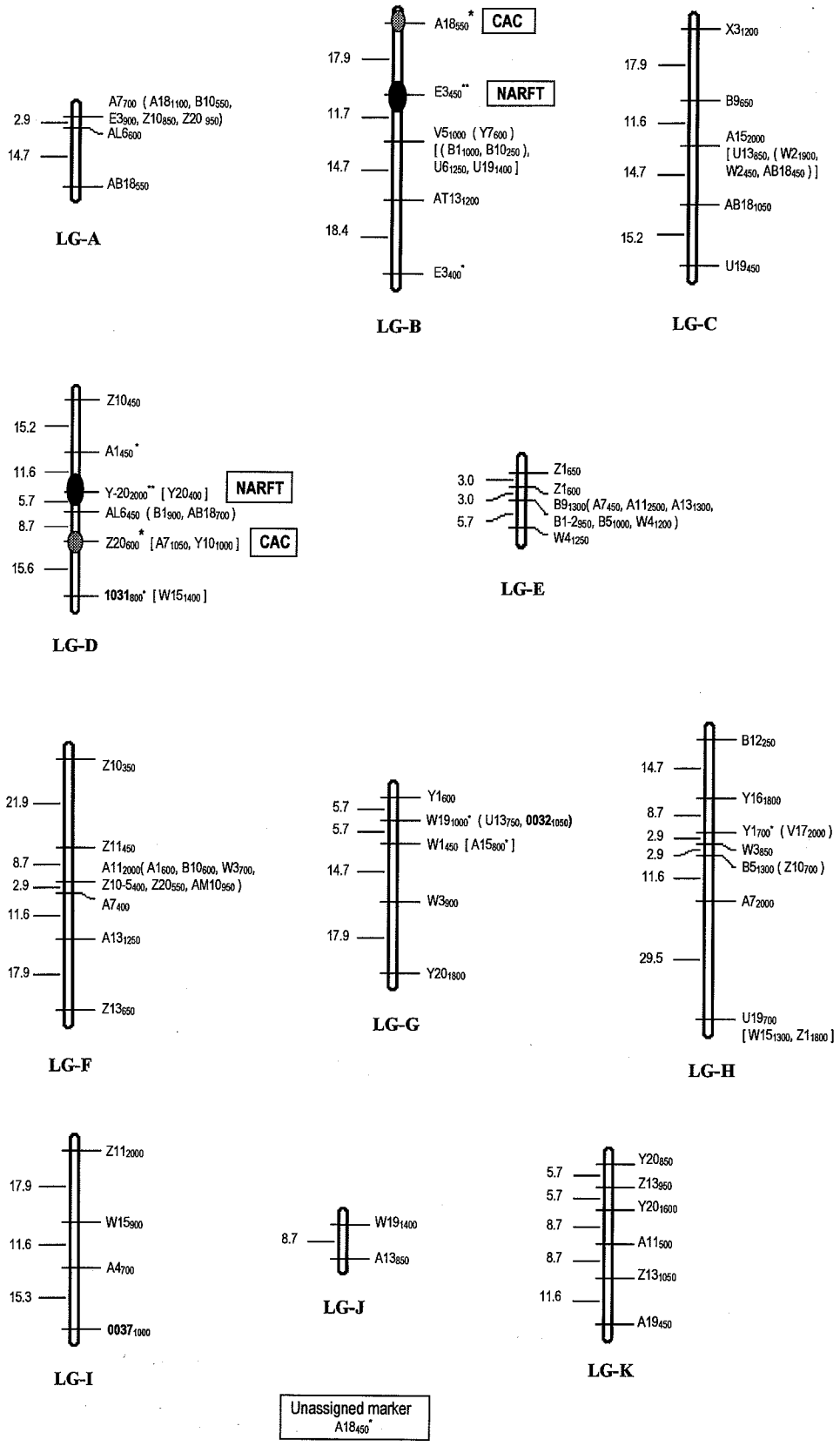

FIGURE 3.
Linkage map constructed using RAPD and SSR (in bold) markers. The marker names are given on the right and the length of each interval in cM is indicated on the left of each linkage group. Markers between ( ) are markers that mapped to identical positions of other assigned markers, markers between [ ] are markers that were clustered within short linkage distances. For each QTL detected by interval mapping, the most likely position of the QTL is indicated by an ellipse: non-acclimated relative freezing tolerance (NARFT) (black) (LOD>1.5) and acclimation capacity (CAC) (grey) (LOD >0.9). Markers significant ($P< 0.05$) in a multilocus stepwise regression analysis, using marker loci significantly associated with traits by single-factor ANOVA as independent variables, are indicated by boxes for NARFT and CAC. Unassigned markers that were significantly associated with trait variation are also identified. Significant markers associated with the traits determined by T-test on single-factor ANOVA are indicated: *, ** significant marker association with NARFT at $P<0.05$, 0.01, respectively; * significant marker association with CAC at $P<0.05$.

DISCUSSION

Part of the solution to problems of abiotic stress is the genetic modification of plants to grow and yield under unfavorable environmental conditions. Current strategies include QTL mapping, genetic engineering, and conventional breeding. For plant physiologists, molecular marker technology provides a new tool to study the mechanisms of stress response. For plant breeders, the most useful application of identified QTLs is to perform marker-assisted selection aimed at accumulating favorable alleles and breaking their possible linkage with undesirable loci. Our objective was to provide some insights to potato cold hardiness that would help both physiologists and plant breeders alike, by taking advantage of such technologies and by using unique plant material. Potatoes represent an ideal model system in part due to the following: (a) interspesific hybrids can be easily generated and used for developing segregating mapping populations (Ross and Rowe 1969), (b) production of 2n gametes (gametes with the sporophytic chromosome number) at a relatively high frequency in some wild tuber-bearing diploid *Solanum* species effectively and efficiently transmit this diversity to cultivated tetraploid potatoes (tetraploid hybrid populations can be generated from diploid x tetraploid crosses) (Jansky et al. 1990; Peloquin et al. 1989), and (c) potato transformation is relatively easy (i.e., *Agrobacterium tumefaciens*) (Shahin and Simpson 1986), thus, if freezing tolerance genes are identified and isolated, successful transformation with such genes would be feasible.

Populations derived from *S. cardiophyllum* and *S. commersonii* were previously found to be ideally suited for gaining insight into the genetics of freezing stress resistance in potato (Stone et al. 1993). These two diploid species exhibit the extremes of NARFT and CAC among the nearly 120 tuber-bearing *Solanum* species. The use of the Biotron facility allowed us to expose all genotypes to identical environmental conditions in both non-acclimating growth and cold acclimation conditions. The importance of detecting genotypic differences at the same level of stress in a population to map QTLs of physiological traits has been previously recognized (Nguyen et al. 1997). A precise assessment of NARFT and CAC by ion leakage, as reported earlier (Stone et al. 1993), allowed the separation of these traits for each backcross genotype examined. Freezing tolerance evaluated by this method has shown a highly significant positive correlation with the survival of the same *Solanum* species following a natural frost under field conditions (Chen et al. 1999).

No significant correlation was found between NARFT and CAC in the backcross population (Figure 2). These data suggest that these two traits are genetically distinct. Our results are in agreement with Stone et al. (1993), who reported that NARFT and CAC are under independent genetic control in potato. This has also been confirmed in other plant species (Arora et al. 1998; Kole, et al. 2002; Rowland et al. 1999; Teutonico et al. 1995).

Although 11 linkage groups are described here (Figure 3), the haploid chromosome number of *Solanum* is 12 (2n=24, for diploid potato species). Thus, one more linkage group remains to be detected. The linkage map reported here spans 479.4 cM in contrast to the 606 and 690 cM reported in published maps of potato using RFLP and RAPD markers (Bonierbale et al. 1988; Gebhardt et al. 1989; Hosaka 1999). Therefore, the linkage map reported here constitutes a partial map, and future research should concentrate efforts on increasing the number of individuals in the population as well as the number of markers. However, two QTLs for NARFT accounting for 44.0% of the phenotypic variation for this trait were identified (Table 2). The number of significant marker locus-trait associations for NARFT agrees with Teutonico et al. (1995), who found two unlinked regions associated with NARFT in a F2 population of *Brassica rapa*.

Previous studies have found at least two QTLs for CAC in *Eucalyptus* (Byrne et al. 1997), three in *Citrus* (Cai et al. 1994) and four in *B. rapa* (Teutonico et al. 1995). Our study found two RAPD markers significantly linked to CAC, which account for 24.9% of the phenotypic variation (Table 2). Thus, even though interval mapping failed to detect QTLs with a LOD above 1.5, we identified two QTLs with LOD less than 1.5 that were linked to such markers (Figure 2).

In addition to RAPD markers, at least one pair of SSR primers was included in the linkage analysis. The purpose of including these markers was to be able to assign the linkage groups to the potato chromosome where the SSR locus was mapped. However, only two out of the 12 SSR markers were mapped to a specific linkage group. One of the two SSR markers was mapped in linkage group D (1031_{800}). Thus, based on our results, we propose that linkage group D could represent part of chromosome V, since SSR marker 1031 has been previously mapped to chromosome V (Milbourne et al. 1998). The fact that this locus was found to be significantly associated

with NARFT based on single-factor ANOVA and that QTLs for NARFT and CAC were detected in this particular linkage group suggest that some of the genes for these two traits could be located in chromosome V. The second SSR marker was located in linkage group G (0032-1_{1050}), which could indicate that linkage group G is in chromosome XII; SSR marker 0032 has been mapped to that chromosome by Milbourne et al. (1998).

Our results are consistent with previous conclusions that freezing tolerance is a complex polygenic trait (Chen et al. 1999; Palta et al. 1997; Stushnoff et al. 1984) and that various components of hardiness (e.g., NARFT and CAC) are not necessarily controlled by the same genes (Palta and Simon 1993; Stone et al. 1993). The presence of individuals in the backcross population expressing similar NARFT and CAC as the parental genotypes suggests that freezing tolerance may be controlled by relatively few genes, thus, the few QTLs detected in the population. This is in agreement with previous studies done in potato (Stone et al. 1993), blueberry (Arora et al. 1998) and *Brassica* (Teutonico et al. 1995).

Previous studies done in our laboratory with *S. commersonii* and *S. cardiophyllum* and other wild potato species indicated that the expression of NARFT and CAC is largely recessive (Chen et al. 1999; Stone et al. 1993). Hypothetical recessive cold-hardiness alleles in *S. commersonii* could only be detected in a population segregating for homozygous recessive and heterozygous genotypes, which is accomplished by backcross to the hardy *S. commersonii* parent. One drawback associated to this type of gene action is that a RAPD marker will be associated with hardiness as a blank (absence of the band). RAPD bands of equal migration mobility are assumed to be genetically homologous, but a blank may be anything that is different. Thus, the usefulness of the QTLs detected here for marker-assisted selection in other populations depends on the homology of *S. commersonii* blanks with blanks from other hardy germplasm. Dominant and codominant alleles associated to hardiness from *S. commersonii* could have been detected from a segregating backcross to *S. cardiophyllum*, but attempts to make that backcross population were unsuccessful.

From close to 2,000 crosses made, we recovered close to 100 backcross seeds. Due to impaired germination or abnormal growth under *in vitro* and/or greenhouse conditions, however, only 35 genotypes were successfully included for further evaluations and analysis. Therefore it is important to note that sampling size could have been a limiting factor preventing the detection of additional QTLs for both NARFT and CAC. Nevertheless, the QTLs for NARFT and CAC identified in this study can be used as a starting point for future studies. For a QTL to be used successfully, fine mapping has to be conducted in the identified QTL region. From the results of this study we propose chromosome V in potato as a prime candidate for this purpose.

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