USE OF STOMATAL INDEX AS A MARKER TO SCREEN BACKCROSS POPULATIONS OF TWO WILD POTATO SPECIES SEGREGATING FOR FREEZING TOLERANCE

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Abstract

The purpose of this study was to investigate differences in stomatal index among backcross progeny of *Solanum commersonii* (freezing tolerant) and *Solanum cardiophyllum* (freezing sensitive) to assess the feasibility of using this trait as a marker for screening *Solanum* populations for freezing tolerance. Measurements were taken on three terminal fully-expanded leaflets per genotype by completing microscopic examination of epidermal impressions made in partially dissolved cellophane tape. Freezing tolerance was estimated in parallel studies on the same plant material.

Values of SI were significantly greater (Fisher T-test, 0.05) for the *S. cmm.* group (parent + backcross progeny) compared with the *S. cph.* group. Stomatal index of the F_1 was significantly greater than SI of *S. cph.* parents and similar to *S. cmm.* parents. Values of SI for both backcross progenies were greater than parental values. Non-acclimated relative freezing tolerance values were in the following order: *S. cmm.* parents > *S. cmm.* backcrosses > F_1 > *S. cph.* backcrosses > *S. cph.* parents. Stomatal index values followed a similar pattern with the exception *S. cmm.* backcross > *S. cmm.* parents. These data suggest: a) increased SI is inherited as a dominant trait, b) SI may be a useful screening marker in breeding programs interested in improving freezing tolerance.

Compendio

El propósito de este estudio fue investigar las diferencias de los índices de estomas entre la progenie de retrocruzamiento de *Solanum commersonii* (tolerante a las heladas) y *Solanum cardiophyllum* (sensible a las heladas) para determinar la factibilidad de utilizar este atributo como un marcador para evaluar y seleccionar poblaciones de *Solanum* para tolerancia a las heladas. Se efectuaron mediciones sobre tres foliolos terminales, completamente expandidos, por genotipo, efectuando exámenes microscópicos de impresiones epidérmicas hechas en cinta de celofán parcialmente disuelta. La tolerancia a las heladas fue estimada en estudios paralelos sobre el mismo material vegetal.

Los valores de IE (Indice de Estomas) fueron significativamente mayores (prueba T Fisher, 0.05) para el grupo de *S. commersonii* (progenitores + pro-

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genie de retrocruzamiento) en comparación con el grupo de *S. cardiophyllum.* El IE de la F_1, fue significativamente mayor que el de los progenitores de *S. cardiophyllum* y similar al de los progenitores de *S. commersonii.* Los valores de IE para ambas progenies de retrocruzamiento fueron mayores que los valores para los progenitores. Los valores no aclimatados de tolerancia relativa a las heladas guardaron el siguiente orden: progenitores *S. commersonii* > retrocruzamientos de *S. commersonii* > F_1 > retrocruzamientos de *S. cardiophyllum* > progenitores *S. cardiophyllum.* Los valores de IE siguieron un esquema similar con la excepción de retrocruzamientos de *S. commersonii* > progenitores *S. commersonii.* Estos datos sugieren: a) el incremento en el IE es heredado como una característica dominante, b) el IE puede ser un marcador muy útil de evaluación y selección en los programas de mejoramiento interesados en mejorar le tolerancia a las heladas.

Introduction

Occasional spring frost episodes may severely limit potato production in temperate regions and mountainous tropical and sub-tropical zones (7). Although a number of wild potato species are known to be frost tolerant (7, 12), efforts to improve the frost resistance of cultivated varieties have had limited success. Incorporation of freezing tolerance into commercially valuable potato cultivars has been difficult for several reasons, including a lack of simple and reliable screening markers (5, 7, 11, 13). Plants native to extreme environments typically possess specific morphological/anatomical features which presumably assist survival in sub-optimum conditions (1, 3). Freezing tolerant members of tuber-bearing *Solanum* species often show one or more of the following: rosette growth habit, modified leaf form, enhanced pigmentation, higher stomatal frequency/index on the upper leaf surface, and multiple, larger leaf palisade layers (11, 12, 13). Palta and Li (12) reported a positive relationship among multiple, thicker palisade layers, higher stomatal index values and freezing tolerance in twenty-four species of potato. In support of these results, Estrada (2) was able to identify multiple leaf palisade layers in frost-resistant germplasm. But, measuring the thickness and number of leaf palisade layers is a cumbersome process which requires preparing leaf cross-sections and completing careful microscopic evaluation. Screening for freezing tolerance in potato may not have taken complete advantage of other potentially useful morphological/anatomical markers. Stomatal index is measurable with a simple, rapid, and accurate technique. The purpose of the present study was to estimate the degree of association between stomatal index values and freezing tolerance in two potato species, *Solanum commersonii (cmm.)* and *Solanum cardiophyllum (cph.),* which differ in their cold tolerance and in an F_1 and backcross progenies of these two species. These progenies segregate for the two separate components of cold resistance, namely non-acclimated freezing tolerance and cold acclimation capacity (16).

Materials and Methods

Experimental Plant Material

The material used in the present study was generated from the original cross of *S. commersonii* (PI 243503) x *S. cardiophyllum* (PI 186548). A single F_1 individual was used as the maternal parent and backcrossed to parents to produce the backcross progeny investigated in this study. Previous work has shown these genotypes to segregate for both non-acclimated frost tolerance and the capacity to cold acclimate (16). In addition, data were collected from two individuals (clones) of each parental type.

Culture Conditions

Micropropagated plantlets were transferred to 8L pots containing Jiffy-Mix (JP, West Chicago, IL) and grown at the University of Wisconsin-Madison Biotron under non-acclimating and cold acclimating conditions. Non-acclimating conditions were 20/18 C light/dark, 14 hr photoperiod of 400 µmol photons m^2s^{-1}, and 70% RH. Plants were grown 7 weeks before sampling for measurement of non-acclimated relative freezing tolerance (non-acclimated RFT). The plants were transferred to cold acclimating conditions of 4/2 C light/dark and 14 hr photoperiod of 100 µmol photons m^2s^{-1} for an additional 14 days prior to sampling for measurement of acclimated relative freezing tolerance. Plants were watered automatically to excess four times daily with a 25% Hoagland solution.

Freezing Tolerance

The acclimated and non-acclimated RFT of the experimental plant material was estimated concominant with other measurements using the electrolyte leakage method (16). In brief, fully-expanded mature terminal leaflets were removed from plants and exposed to a simulated freeze-thaw stress. Three samples were removed at each temperature and the amount of ion leakage from thawed leaf tissue was measured by electrical conductance at decreasing temperatures and compared to the total ion leakage obtained from cooled and autoclaved leaf material. Thereafter, the percent ion leakage was plotted as a function of freezing temperature and the relative freezing tolerance (RFT) was determined from the midpoint of the maximum and minimum (control) ion leakage values obtained for each genotype. The acclimation capacity is expressed as acclimated RFT minus nonacclimated RFT (16).

Leaf Tissue Sampling for Anatomical/Morphological Measurements

The third or fourth leaf counting basipetally was removed from 3 separate plants for each genotype and used for observation of the upper surface of the terminal leaflet. The terminal leaflet of all leaves was fully-expanded. Each leaf was placed vertically in a glass jar (4 cm. ID x 10 cm

height) with the petiole submersed in 100 ml of tap water. The jars and leaves were then loosely enclosed in a clear plastic bag and placed in a growth chamber under the same conditions described above for non-acclimating growth conditions.

Leaves were taken from the growth chamber during the light period and tracings of each terminal leaflet were obtained for leaflet area measurement. Immediately thereafter, four discs were removed from each terminal leaflet with a cork borer (9 mm ID). A single leaf disc was taken from midway between the midvein and margin in the apical and basal one-third of the lamina on each side of the midvein. With the exact location of each disc known, it was possible to record data specifically for each disc (*e.g.*, apical/basal and left/right discs). When possible, areas with prominent primary and/or secondary veins were avoided for disc sampling since vascular rib tissue often made it difficult to obtain a high quality leaf surface impression. Permanent impressions of the adaxial leaf surface were made in cellophane tape affixed to glass slides and partially dissolved with acetonitrile (methyl cyanide). In this procedure, a section of cellophane tape was affixed to a glass slide and partially dissolved by allowing 4 drops of acetonitrile to stand on the tape section for 3-5 sec. After this time, the excess acetonitrile was removed by shaking, the leaf disc was immediately placed on the tape (adaxial side down) and pressed gently into the tape for 3 sec. The leaf disc was removed prior to solidification of the remaining tape. Areas of the impression which showed light shading typically provided the best quality views of the leaf surface during microscopic observation.

Measurement of Stomatal Density and Stomatal Index

Preliminary observation of the impressions revealed no consistent significant difference in cell types (epidermal, stomatal guard) and number between apical and basal leaflet regions. Therefore, data were collected from randomly chosen impressions based solely on the quality of the cellophane impression as determined by microscopic evaluation. But, all leaflets for each genotype and no fewer than two impressions (discs) per leaflet were included for observation. In total, 18 microscopic views (0.3 mm^2 each) distributed among the 3 leaflets were evaluated for each genotype.

A video-camera link between the microscope and a television allowed for projection of the leaf surface image and efficient cell typing and counting. Epidermal ground and stomatal guard cells were easily distinguished. Permanent records of cell type, location and number for each 0.3 mm^2 view of the adaxial leaf surface were obtained with marks made on thin transparent sheets of polyethylene held by static attraction to the television screen. Cell counts were taken with a hand-held counter. For counting purposes, stomata were defined as the guard cells-stomatal pore complex while epidermal ground cells were defined as all cells not part of a stomatal complex (guard cells). Stomatal index (SI) was calculated according to the for-

mula of McCauley and Evert (6): SI = [number of stomata/(number of stomata + number of epidermal ground cells)] x 100.

Results and Discussion

Values of stomatal and epidermal ground cell density (number of cells/ $0.1mm^2$), stomatal index (SI), and freezing tolerance parameters for the major and minor genotypic groups are shown in Table 1. Significant differences in the mean value of all traits studied were noted among the major genotype groups. For example, the mean stomatal density and SI value for the major *S. cmm.* group was significantly greater compared with both the F_1 and major *S. cph.* group although the latter two groups did not differ in the two traits (Table 1). Major genotype group epidermal ground and total cell density values showed *S. cmm.* > *S. cph.* > F_1 (Table 1).

Backcross population values were significantly greater than parental values of epidermal ground cell, stomatal complex, and total cell density on the adaxial leaf surface (Table 1, minor groups). The *S. cmm.* backcross progeny group had the highest SI value among all minor genotypic groups. Stomatal index was significantly higher in the *S. cmm.* parent group compared with the *S. cph.* parent group and the F_1 had values closer to but lower than the *S. cmm.* parent group. The mean SI value of *S. cmm.* backcross progenies was nearly double the SI value of the *S. cph.* backcross progenies and parents (Table 1, minor groups).

Our data from segregating populations suggest that SI values may assist in selecting potentially freezing tolerant germplasm in segregating families. Little statistical correlation between SI and NARFT was found for the individual genotypes studied (data not shown). However, the data in Table 1 show that high SI values are equally associated with greater acclimated and non-acclimated RFT in the major and minor genotypic groups. To our knowledge, this is the first report of such a relationship in segregating populations. Similar results have been obtained (12) but in different potato species so the potential genetic linkage of stomatal index and freezing tolerance remained untested to date. In general, data of this experiment support the contention of other authors that selection based on morphology/anatomy may facilitate improvement of freezing tolerance in cultivated potato (2, 7, 12). Although there may be no direct association between SI and freezing tolerance, it appears that SI may assist in identifying genotypic groups with superior freezing tolerance in large populations showing high variability in freezing tolerance. For example, the freezing tolerant parent *S. cmm.* and its backcross populations showed significantly greater stomatal density and SI values compared with both the F_1 and freezing sensitive *S. cph.* parent and backcross populations (Table 1). Measures of SI are accomplished with a simple, rapid, and relatively non-destructive procedure that would be easily integrated into a breeding scheme. However, this

TABLE 1.—*Cell type and number per 0.1mm² of adaxial surface of terminal leaflets and relative freezing tolerance comparisons of parents and backcross (BC) progeny of 2 wild potato species. Cells were typed and counted by direct microscopic observation of leaflet impressions made in partially digested cellophane tape.*

Genotype	Individuals Studied	N	Cell Type and Number per 0.1mm² Adaxial Leaf Surface: Stomatal Epidermal	Stomatal Complexes	Total	SI² (%)	Relative Freezing Tolerance: Non-Acclimated (–C)	Acclimated	Acclimation Capacity (C)
Major Groups									
S. cmm.									
parents + BCs	13	229	47.2 a[1]	4.7 a	51.9 a	8.7 a	4.3	8.0	3.7
F_1	1	18	26.4 c	1.8 b	28.2 c	6.2 b	2.7	4.0	1.3
S. cph.									
parents + BCs	7	126	41.9 b	2.6 b	44.5 b	5.4 b	2.1	2.9	0.8
Fisher L.S.D.			4.2	0.9	4.7	1.7			
Minor Groups									
S. cmm.									
parents	2	36	40.5 b	3.0 b	43.5 b	6.9 b	4.7	9.4	4.7
BCs	11	193	48.5 a	5.0 a	53.5 a	9.0 a	3.8	6.5	2.7
F_1	1	18	26.4 c	1.8 c	28.2 c	6.2 b	2.7	4.0	1.3
S. cph.									
parents	2	36	20.7 d	1.0 c	21.6 d	4.2 c	1.8	2.2	0.4
BCs	5	90	50.4 a	3.2 b	53.6 a	5.9 bc	2.3	3.5	1.2
Fisher L.S.D.			4.5	1.0	5.0	1.8			

[1]Means within the same column and genotype group (major, minor) and containing the same letter are not significantly different by the Fisher T-test ($\alpha = 0.05$).
[2]SI = stomatal index = [number of stomata/(number of stomata + number of epidermal ground cells)] x 100.

technique must be tested in a greater variety of material before SI is considered a reliable screening marker in varied populations.

The data of Table 1 suggest that inheritance of increased stomatal index may be partially dominant: SI of the F_1 was between the parental values. However, the backcross group SI value was greater than the parental SI value for both species (Table 1, minor groups). It was not possible to determine whether changes in SI depend primarily upon variations in epidermal ground cell or stomatal density. Although stomatal density and SI showed the same pattern of means separation in the major genotypic groups (Table 1), the ratio epi-

dermal ground cell : stomatal complex density was 10:1 and 16:1 in the major *S. cmm.* and *S. cph.* groups, respectively (data not shown). Similar ratios were noted among the minor genotypic groups (data not shown).

Multiple palisade layers and higher stomatal index values are often associated in freezing tolerant members of *Solanum* (2, 11, 12). These characters may provide specific physiological benefits in dynamic sub-optimum growth conditions, including periods of low temperature stress. For example, the ability of the plant to process and utilize captured light energy is substantially reduced at cold temperatures (11). The damaging effects of photooxidation due to excess light energy at low temperatures have been reported in several plant species (8, 9, 10, 14, 15). It is possible that light-dependent injury is reduced by greater internal shading provided by multiple palisade layers in freezing tolerant *Solanum.*

Thicker and multiple palisade layers have been found to be associated with higher SI on the upper leaf surface (12). Greater SI may facilitate gas exchange in leaves with thicker and multiple palisade layers. Three separate sources of resistance to CO_2 diffusion into a leaf have been identified: the boundary layer, stomata, and leaf intercellular spaces (17). Under most natural conditions, the main limitation to CO_2 flow into a leaf is thought to be stomatal resistance (17). In addition, the partial pressure of CO_2 declines rapidly with increasing altitude (350*u*bar at sea level, 295*u*bar at 150m) and may reduce plant carbon fixation. This is potentially significant given that Li *et al.* (4) reported for various genotypes of *S. acaule* a significant positive relationship between frost hardiness and the altitude of origin. It is possible that photosynthetic CO_2 fixation in cold tolerant *Solanum* growing at high altitudes may be enhanced by their possessing greater stomatal density and/or index values which would facilitate greater CO_2 exchange.

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