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Frost injury is one of the major factors limiting potato production in many parts of the world. It would be desirable to transfer the frost-tolerance traits from the hardy relatives to the cultivated potato, however, progress has been very slow. Since *Solanum commersonii* has been the major source of frost hardiness for improving the frost tolerance of the cultivated potato, we investigated the molecular basis of frost hardiness traits in this species.

Three segregating populations were derived from a cross between *Solanum commersonii* (cmm) and *S. cardiophyllum* (cph), two parental genotypes with contrasting frost hardiness and cold acclimation potential. There was considerable variation in both traits studied in all three populations (F_1, F_1 x cmm, and F_1 x cph). Frost hardiness and cold acclimation were not correlated and variation for both traits could be best explained by an additive-dominance model, with "additive" effects the most important. Broad sense heritability was moderate to high (0.45-0.85) indicating that these traits are highly heritable.

A pseudo-testcross F_1 population, consisting of 73 individuals segregating for both frost hardiness and cold acclimation traits, was used to generate either cmm or cph specific RAPD and AFLP markers. A 95-marker-map with 12 linkage groups was constructed from these data. The cumulative length of this cmm-specific map is 194.5 cM, covering approximately 20% of the potato genome, with an average of 2.05 cM between contiguous loci.

Interval mapping QTL analysis revealed one significant QTL associated with the cold acclimation trait. By regression analysis two markers that accounted for 22.30% of the variation in frost hardiness were detected, and five markers were associated with cold acclimation, and could explain 43.80 % of the phenotypic variance. These data will provide the foundation for future studies to dissect the complex genetic traits of frost tolerance in *Solanum* species.

Molecular Genetics of Frost Hardiness and Cold Acclimation in *Solanum* species

By

Roberto Valverde

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Approved:

Major professor, representing Horticulture

Head of Department of Horticulture

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request

Roberto Valverde, Author

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDIX TABLES

DEDICATION

To my parents Guillermo and Teresa, my brothers Fernando and Jorge, and my sister Grace for their love and unconditional support. And to my nephew Esteban for being such and inspiration.

MOLECULAR GENETICS OF FROST HARDINESS AND COLD ACCLIMATION IN *SOLANUM* SPECIES

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Potato is the world's fourth most important crop; however, it ranks first in yield per unit of land among the world's major food crops (F.A.O. Production Year Book, 1991). It is mainly grown in the temperate zone and in the highlands of the Andean Tropics of South America, where frosts are often the major factors in reducing the yield and quality of potato crops. For example, in the Andean countries alone, it is estimated that over 400,000 ha of potato fields are threatened by frost injury every year (Barrientos et al., 1994; Estrada et al., 1993; Van Eck, 1995a; Vega and Bamberg, 1995). The commonly cultivated potato, *Solanum tuberosum* L., possesses little frost hardiness, whereas many other tuber-bearing *Solanum* species are much hardier. It would be desirable to combine the higher yield potential of *S. tuberosum* with the frost hardiness traits from the hardy relatives. However, efforts to transfer frost hardiness genes from wild species to cultivated *S. tuberosum* by conventional breeding have been proven possible but are likely a lengthy process (Estrada, 1987; Estrada et al., 1993).

It appears that potato plants have two levels of frost hardiness: one when they are growing under warm temperatures, and one when they are growing under cool

temperatures between about 0 and 10°C (Chen and Li, 1980a). Individual *Solanum* genotypes have a characteristic frost hardiness before and after cold treatment. A better understanding of the genetics, biochemistry, and physiology of frost hardiness and cold acclimation certainly will facilitate the development of frost hardy potato genotypes.

In the past 20 years, considerable progress has been made in the area of potato frost hardiness research. In fact, *Solanum* species have become one of the few plant systems, which are under active research for the molecular aspects of cold acclimation (Lee and Chen, 1993). With the advances obtained in this area, many cold and ABA induced genes, whose expressions are closely associated with freezing tolerance, have been cloned in *Solanum* species (Baudo et al., 1996; Meza-Zepeda et al.; 1998; Rorat et al 1997, 1998; Zhu et al., 1993; 1995a,b; 1996). Since neither has the function of these genes been defined, nor their chromosomal location determined, research in both areas is justified.

The availability of a robust marker map is a prerequisite for QTL (quantitative trait locus) analysis. The identification of QTLs controlling the frost hardiness and cold acclimation traits will help us to establish the chromosomal location of cold-inducible genes and their association with QTLs controlling these traits. Molecular markers tightly linked with the traits of interest will be very useful in selecting frost hardy genotypes by marker-assisted selection in potato-breeding programs. This would certainly enhance the efficiency of breeding efforts to combine the higher yield potential of *S. tuberosum* with the frost hardiness traits from its hardy relatives. Furthermore, this information may lead to the isolation of genes controlling either frost hardiness or cold acclimation by map-

based cloning. The isolation of cold-hardiness genes will then be used for improving potato frost hardiness by genetic engineering.

The main objective of this thesis is to locate QTLs controlling frost tolerance and cold acclimation traits in *S. commersonii*, which is a frost hardy species capable of cold acclimation. Toward this goal, specific objectives are: 1) to study the inheritance of frost hardiness and cold acclimation potential in F_1 and backcross populations derived from a cross between *S. commersonii* and *S. cardiophyllum*; 2) to construct a DNA marker-based map of *Solanum commersonii*; 3) to locate QTLs controlling frost hardiness and cold acclimation potential.

Literature Review

Frost injury

Low-temperature stress, including freezing and chilling, is one of the primary factors that limit crop production around the world. In the U.S., freezing injury costs about $1 billion annually (White and Hass, 1975), and losses are much higher when freezing and chilling injuries are combined. Although efforts have been made to select better-adapted cultivars and improve cultural management, progress has been very slow because tolerance to low-temperature stress is a complex genetic trait. Despite considerable efforts, we are still far from completely understanding the cause of freezing and chilling injuries, and the mechanisms by which some plants are able to tolerate these stresses.

Often, the distinction between a good crop and a crop failure is determined by only a 2- to 3- °C difference in cold tolerance, or by a few days difference in the stage of crop development. Such subtle differences are highly amenable to research solutions. Furthermore, crop production is generally proportional to the length of the growing season, and yield can be considerably increased if planting is done earlier in the spring and harvest is later in the fall. Because of the low-temperature constraints, farmers are not always able to implement these unconventional practices because of the lack of hardier cultivars. Potato production could be increased 30% by planting just 5 to 10 days earlier in the growing regions of Northern America and Europe. These early potato plants would develop better root systems and, consequently, have better growth and higher yield. The same strategy could be applied to other crops such as maize, wheat, vegetables, etc.

Freezing injury is not limited to particular geographic regions; producers throughout the U.S. have suffered significant economic losses during the past decade. Lost revenues also influence the economic well being of rural communities, and crop losses affect product availability and consumer prices. Freezing injury also has a great impact on the stability of world food supplies, commodity prices, and the international trade balance. Thus, there are ample economic incentives to develop more freeze-tolerant plants.

There is a wide range of susceptibility to freezing injury among plant species. For example, the foliage of most annual crops and the developing blossoms of perennial species have little or no tolerance to ice formation. Injury occurs as soon as ice formation is initiated (at -1 to -3 °C), and is commonly observed after untimely spring frosts

(Ashworth, 1992; Chen et al., 1995, Levitt; 1980; Sakai and Larcher, 1987). Other species and tissue types can tolerate ice formation, but are damaged at lower freezing temperatures (Chen et al., 1995; Levitt, 1980; Sakai and Larcher, 1987).

Plants that survive sub-zero temperatures generally do so by tolerating ice formation within their tissues. Freezing is usually initiated at -2 to -3°C (Ashworth 1992; Ashworth and Kieft, 1995) when ice crystals first form within xylem vessels and intercellular spaces (Levitt, 1980). Growth of ice crystals is typically restricted to the apoplast; because the plasma membrane and cell wall prevent ice from propagating into cells and initiating the freezing of intracellular water. The presence of extracellular ice, however, establishes a water potential gradient within tissues. Because ice has a lower chemical potential than liquid water at the same temperature, cells progressively lose water to growing extracellular ice crystals as the temperature of the tissue declines (Guy, 1990; Levitt, 1980). Plant cells are then subjected to dehydration stress caused by the formation of extracellular ice crystals. Plants capable of surviving freezing are able to tolerate the formation of extracellular ice and the subsequent severe dehydration stress. Unfortunately, the mechanism for plant tolerance of freezing stress is not fully understood.

Cold acclimation in plants

The capacity of plants for cold acclimation in response to prevailing environmental conditions varies among plant genotypes. Woody perennials are often quite sensitive to freezing in summer while actively growing, but can survive to very low temperatures in midwinter (Burke et al., 1976; Chen et al., 1995; Levitt, 1980; Sakai and Larcher, 1987; Weiser, 1970). These plants respond to environmental cues such as shortening daylength, cooler temperatures, or frost episodes, and acclimate to increase their freeze tolerance.

Cold acclimation is a complex process during which biochemical, physiological, and morphological changes occur coincident with the acquisition of plant cold hardiness (Chen et al., 1995; Guy, 1990; Levitt, 1980). The most consistently reported changes include the accumulation of sugars and other potential cryoprotectants; altered membrane lipid composition; quantitative and qualitative changes in protein composition; changes in gene expression; and altered cell ultrastructure. The fact that these changes occur with changes in freeze tolerance, and that similar changes are observed in different species, provides circumstantial evidence that these changes play a role in increased freezing tolerance. However, direct evidence of the role of a particular change in survival at freezing temperatures is still lacking. Such information could be very useful for improving selection criteria in conventional breeding programs, as well as providing specific targets for genetic engineering.

Although the mechanisms by which plants sense and respond to environmental cues and subsequently cold acclimate are not fully understood, significant progress has

been made and some key components have been identified. First, many cold-regulated (COR) genes from various plant species have been isolated and characterized (Chen et al., 1995; Thomashaw, 1998). Second, steps involved in low-temperature signal transduction have been identified (Dhindsa et al., 1997; Ding and Pickard, 1993; Knight et al., 1991, 1996). Calcium acts as a second messenger in signal transduction and cold acclimation, apparently coupling to a protein phosphorylation cascade that regulates expression of some COR genes (Dhindsa et al., 1997; Mizoguchi et al., 1996; Monroy et al., 1993). Third, in *Arabidopsis*, COR genes are apparently regulated by a common transcription factor; increased expression of the *CBF1* transcriptional activator causes both constitutive expression of several cold-inducible genes and increased freeze tolerance (Jaglo-Ottosen et al., 1998). Fourth, plants also synthesize antifreeze proteins (AFPs) in response to low temperature (Griffith et al., 1997).

Frost hardiness and cold acclimation in Solanum species

In *Solanum* species each genotype is characterized by its frost hardiness level before and after cold acclimation. For the simplicity of discussion, we will call them "frost hardiness" and "cold acclimation potential", respectively. As the wild *Solanum* species are potential sources of frost hardiness genes, screening of many genotypes for frost hardiness has been conducted both in controlled environmental conditions (Li, 1977; Palta and Li, 1979; Chen and Li, 1980a; Hanneman and Bamberg, 1986), and under field conditions (Vega and Bamberg, 1995). From those screening efforts, many *Solanum* species exhibiting frost hardiness far superior to that of the cultivated species have been

identified. Based on frost hardiness and cold acclimation potentials, Chen and Li (1980a) were able to classify *Solanum* species into five groups: group 1, frost tolerant and able to cold harden; group 2, frost sensitive but able to cold harden; group 3, frost tolerant but unable to cold harden; group 4, frost sensitive and unable to cold harden; and group 5, chilling sensitive.

Recently, Vega and Bamberg (1995) conducted an extensive field screening of 2,635 accessions from 101 tuber-bearing *Solanum* species in Wisconsin. Their results confirmed that the hardiest species are *Solanum acaule, S. albicans, S. commersonii,* and *S. demissum.* In addition, they *identified* a previously unreported frost hardy species, *S. paucissectum.* In general, the field data agreed well with the laboratory screening results, indicating that the laboratory tests are reliable for evaluating frost hardiness of potato germplasm.

Palta and Li (1979) studied 24 tuber-bearing *Solanum* species, including seven cultivars of *S. tuberosum*, for anatomical and morphological differences that might be associated with frost hardiness. They found no consistent relationship between various gross morphological characteristics of the plants and frost hardiness. However, the number and thickness of palisade parenchyma layers and stomatal index on the upper leaf surface were clearly related to frost hardiness. They observed that all hardy species studied have two palisade layers, and all except three of the non-hardy accessions have one palisade layer. Also, these hardy species have three times greater stomatal index on the upper surface of the leaf than the non-hardy ones. The heritability of the number of palisade layers in relation to frost hardiness was also investigated in hybrids derived from crosses involving hardy x non-hardy species. They found among about 200 hybrids

examined, less than 2% were frost hardy and all had two distinct palisade layers. Thus, the two-palisade layer trait is heritable and it is closely associated with frost hardiness. Rajashekar et al. (1983) determined the heterogeneous ice nucleation characteristics of leaves of several tuber-bearing *Solanum* species. They also studied frost injury in supercooled leaves upon ice formation. It appeared that the leaves of *Solanum* did not contain ice nuclei active above -6.9°C. In the absence of supercooling, when ice nucleation is initiated at -1°C, the non-hardened and cold hardened leaves tolerated extracellular freezing between -3.5°C and -8.5°C, respectively. However, if the leaves were allowed to supercool and then ice formation was initiated, they were killed at the ice nucleation temperature. For example, *S. acaule* leaves can survive to -8.5°C if supercooling is prevented by initiation of ice formation at -1°C. However, if they were allowed to supercool first and then ice nucleation initiated, the leaves were killed at -2.6°C.

Since most plant cells do not contain intrinsic ice nuclei that are active close to 0 °C, and the degree of supercooling seems to have a significant effect on the frost hardiness of plant cells (Rajashekar et al., 1983), it would be desirable to introduce the ice nucleator into plant cells to prevent extensive supercooling. Theoretically, this would result in a less injurious equilibrium freezing (Chen et al., 1995). There are many species of ice nucleation active bacteria from which genes encoding ice nucleation proteins have been cloned (Orser et al., 1985). The first attempt to introduce bacterial ice nucleators into cells of *S. commersonii* was reported by Baertlein et al.(1992). They introduced an ice nucleation gene (*inaZ*) from *Pseudomonas syringae pv. syringae* into *S. commersonii* by *Agrobacterium*-mediated transformation. Transgenic *S. commersonii* plants showed

an increase in ice nucleation activity over un-transformed controls. The most active transformants contain over 100 ice nuclei per mg of tissue at -10.5°C. There is also a significant change in the ice nucleation temperature in the transgenic plants. The non-transformed plants supercooled to -12°C, whereas in the *inaZ* transformants ice nucleation occurred at -4°C. The successful introduction of a stable ice nucleation gene into *Solanum* species provided us with an opportunity to alter the freezing characteristics of plant cells and to alter their freezing tolerance.

During cold acclimation, many changes, which are associated with development of freezing tolerance, have been reported in *Solanum* species. These include cellular and ultrastructural (Palta and Li, 1978; Toivio-Kinnucan et al., 1980), biochemical (Barrientos et al., 1994; Chen and Li, 1980b; Palta et al., 1993), abscisic acid (ABA) (Chen et al., 1983), and molecular biological (see below) changes. However, the significance of such changes to freezing tolerance is not yet completely understood.

Genetics of potato frost hardiness

Traditionally, frost hardiness has been considered to be a complex trait with polygenic inheritance (Limin and Fowler, 1983; Thomashow, 1990). The complex genetic basis of frost hardiness in cereals has been studied through techniques of quantitative trait analysis (Eunnus et al., 1962). QTLs associated with frost hardiness in a population of doubled-haploid barley lines derived from the cross of a winter and a spring barley have been mapped (Hayes et al., 1993; Pan et al., 1994).

Recently, QTLs affecting cold hardiness traits have been also detected in wheat (Galiba et al., 1995), *Brassica* (Teutonico et al., 1995), and *Eucalyptus* (Byrne et al., 1997).

There is only limited information available on the inheritance of frost hardiness in *Solanum* species. The mode of inheritance of frost hardiness and cold acclimation potential was investigated in F_1 and backcross populations of *S. commersonii* and *S. cardiophyllum* (Stone et al., 1993). These two species have contrasting frost hardiness and cold acclimation potentials. *S. commersonii* is a frost hardy species and can be cold acclimated (group 1), whereas *S. cardiophyllum* is frost sensitive and can not be frost hardened (group 4). They found that frost hardiness and cold acclimation potential traits are under independent genetic control. Furthermore, the analysis of generation means indicated that all of the variance for both traits (frost hardiness and cold acclimation potentials) could be best explained by an additive-dominance model with both traits being partially recessive. From a relatively small population, they were able to recover the parental phenotypes and thus suggested that both traits are controlled by relatively few genes. The same populations were also used to determine the stomatal index (SI) to assess the feasibility of using this trait to indirectly select for freezing tolerance. They reported that high SI is inherited as a dominant trait; and SI may be useful in breeding programs for improving the freezing tolerance of potato (Kleinhenz et al., 1995).

Somatic hybrids between *S. commersonii* (2n=2x=24) and dihaploid *S. tuberosum* (2n=2x=24) were produced by protoplast fusion (Cardi et al., 1993a). The freezing tolerance of one of the tetraploid somatic hybrids (SH9A), a self-compatible and fully fertile clone, was characterized. For both frost hardiness and cold acclimation traits, the hybrid was intermediate between the two parental clones (Cardi et al., 1993b). These

results clearly demonstrated the feasibility of transferring frost hardiness and cold acclimation traits from hardy wild species to the cultivated potato species.

Molecular biology of cold acclimation in potato

Weiser (1970) proposed that there is an alteration in gene expression during cold acclimation. Since then, considerable evidence has suggested that gene expression is involved in plant cold acclimation. Guy et al. (1985) provided direct evidence that cold acclimation of spinach involves changes in gene expression. In the past few years, progress has been made in the characterization of molecular events associated with cold acclimation.

During cold acclimation, one frequently observed change is the accumulation of proteins (Chen and Li, 1980a; Guy, 1990; Johnson-Flanagan and Singh, 1988; Lee and Chen, 1993). From the literature, there is an increase in both the total protein content (Levitt, 1980) and in specific polypeptide species (Chen et al., 1995). In potato leaves, a high correlation between the increase in protein content and cold hardiness was observed (Chen and Li, 1980b). Furthermore, cycloheximide, a protein synthesis inhibitor, inhibited the development of cold hardiness in *S. commersonii* stem cultures if applied prior to the beginning of cold acclimation. Taken together, these results suggested that there is a need for *de novo* protein synthesis during cold acclimation. This hypothesis was well supported by the results of Costa et al. (1993) and Ryu and Li (1994a, 1994b, 1995).

Cold acclimation of *S. commersonii* induces the synthesis of many new polypeptides (Tseng and Li, 1990,1991). The majority of these cold-induced polypeptides

disappeared after one day of deacclimation, and the frost hardiness correspondingly decreased. Some of these cold-induced polypeptides were present in the early and middle phases of cold acclimation and were not present when plants reached their maximum hardiness. The transient expression of these polypeptides during cold acclimation may be indicative of the involvement of separate sets of proteins, which are required for the induction and maintenance of frost hardiness. Similar to the temporal pattern of cold induced protein synthesis, the development of freezing tolerance in *S. commersonii* is associated with the cold-induced expression of novel translatable RNAs (Tseng and Li, 1990, 1991). The close association between the appearance and disappearance of these cold-induced RNAs implied a direct role for gene expression during cold acclimation.

Lee et al. (1992) reported the induction of freezing tolerance by ABA and cold treatment in suspension cultured cells of *S. commersonii*. Both ABA at warm temperature (23°C) and cold treatment (4°C) increased freezing tolerance in cultured cells of *S. commersonii* to a level comparable to that of hardened plants of *S. commersonii*. ABA treatment and cold treatment resulted in changes in abundance of about 26 and 20 translatable RNAs, respectively.

A cDNA library was constructed using poly $(A)^+$ RNA isolated from *S. commersonii* cell cultures treated with ABA for two days (Zhu et al., 1993). By differential screening, they isolated 4 cDNAs (pA8, pA13, pA35, and pA81) of ABA responsive genes and studied their expression in response to various environmental stimuli (Zhu et al., 1995a). In cell cultures, RNA gel blot analysis indicated that the accumulation of transcripts corresponding to these cDNAs was transient in response to ABA (75 μM), and was ABA concentration dependent, as was the induction of freezing

tolerance. These ABA responsive genes were also cold responsive, and the accumulation of their corresponding transcript was temperature dependent, and associated with the induction of cold induced freezing tolerance. Cold induced accumulation of mRNAs corresponding to pA8, pA13, and pA81 was partially suppressed by fluridone (10 μM), and restored by exogenous ABA (75 μM) to the control level (cold treatment alone). These results suggest that ABA plays a crucial role in frost hardiness induction, presumably at least in part by the regulation of gene expression. Similar ABA and low temperature responses were observed in *in vitro* cultured *S. commersonii* plants (Zhu et al., 1993; 1995a). Southern blot analysis indicated that these ABA and cold responsive genes belong to multi-gene families (Zhu et al., 1993).

The polypeptides encoded by these cDNAs share high homology with tobacco osmotin (Zhu et al., 1993). For example, the deduced amino acid sequence of pA13 cDNA shares 89% identity with tobacco osmotin (Zhu et al., 1993). The 16-cysteine residues in osmotin are also found in the putative polypeptide encoded by pA13 (Zhu et al., 1993). Restriction site and nucleotide sequence analysis of these cDNAs indicated that they represent highly homologous but distinct mRNAs (Zhu et al., 1995a). In cell cultures of *S. commersonii*, RNA gel blot analysis, using gene specific probes, it was found that there were low levels of mRNAs in the un-treated controls indicating that the genes corresponding to the pA13, pA35, and pA81 cDNAs are constitutively transcribed (Zhu et al., 1995a). Treatment with ABA, NaCl, and low temperature resulted in an increased accumulation of transcripts hybridizing to pA13, pA35, and pA81 gene specific probes. Generally speaking, the accumulation patterns for these three mRNAs were very similar in response to ABA, NaCl, and low temperature treatments.

The accumulation patterns for these three mRNAs in *in vitro* grown plants were also examined (Zhu et al., 1995a). RNA gel blot analyses revealed that treatments with ABA, NaCl, salicylic acid, and wounding increased the level of transcripts hybridizing to the pA13 and pA81 probes, whereas the same treatments resulted in a less increase in the accumulation of mRNA hybridizing to the pA35 probe (Zhu et al., 1995a). In the soil-grown *S. commersonii* plants, there was an organ-specific accumulation of osmotin-like protein mRNAs. Constitutive accumulation of all three mRNAs in leaves, stem, roots, and flower buds and fully opened flowers of 2-month-old plants. High levels of mRNAs corresponding to pA13 and pA81 were found in the roots, and lower levels in the leaves. A similar level of transcript hybridizing to the pA35 probe was found in both stems and roots. Infection of *S. commersonii* plants with the potato late-blight fungus, *Phytophthora infestans*, caused a significant accumulation of all three osmotin-like protein mRNAs in the infected leaves.

To gain insight into the regulation of osmotin-like protein gene expression, a genomic clone which contains two osmotin-like protein genes corresponding to the cDNAs of pA13 and pA81 was isolated (Zhu et al., 1995b). The 5' flanking DNA sequence (-1078 to +35 of OSML13 and -1054 to +41 of OSML81) was then fused to the GUS reporter gene, and the chimeric gene fusions were introduced into *S. commersonii* plants via *Agrobacterium*-mediated transformation. The resulting transgenic plants were used to study the transcriptional activation of OSML13 and OSML81 promoters in response to a number of abiotic stresses and to fungal infection. Their results showed that both promoter regions are sufficient to impart the expression of the GUS reporter gene by ABA, NaCl, salicylic acid, wounding, low temperature

treatments, and by infection with *P. infestans.* The expression patterns of the GUS reporter gene are in agreement with the patterns of mRNA accumulation (Zhu et al., 1995b).

Transgenic potato (*S. commersonii)* plants constitutively expressing sense or antisense RNAs from chimeric gene constructs consisting of the cauliflower mosaic virus (CaMV) 35S promoter and pA13 cDNA were developed (Zhu et al., 1996). It was expected that sense transgenic plants would show an elevated level of osmotin-like proteins under un-induced conditions, and the antisense transformants would show a reduced level of osmotin-like proteins under conditions which normally induce the expression of osmotin-like proteins. This is one way to evaluate the roles of osmotin-like proteins in freezing tolerance and fungal resistance. Transgenic potato plants expressing high levels of osmotin-like proteins showed an increased tolerance to late-blight, but there was no significant increase in freezing tolerance (Zhu et al., 1996). However, antisense transgenic plants showed no effects on either fungal or freezing tolerance. These results thus do not indicate that pA13 osmotin-like protein is a major determinant of freezing tolerance (Zhu et al., 1996).

In order to learn more about the molecular differences between frost-tolerant and frost-sensitive species, Baudo et al. (1996), used the polymerase chain reaction (PCR) to amplify a DNA fragment of a low-temperature and ABA-responsive gene (*Scdhn1*) from genomic DNA of *S. commersonii.* They also identified a homologous gene (*Stdhn 1*) in *S. tuberosum* cv. Bintje. The DNA sequences of these two genes (*Scdhn* and *Stdhn 1*) share 96% homology, and showed very similar expression patterns under low temperature treatment. Their roles in cold acclimation are not clear currently.

Rorat et al. (1997, 1998) isolated 24 cDNA clones (*Ssci*) corresponding to the cold induced mRNAs from a *Solanum sogarandinum* cDNA library. *Solanum sogarandinum* is a frost hardy species with a capability for cold acclimation, similar to *S. commersonii*. Among these 24 cDNAs, *Ssci1*, *Ssci12*, *Ssci17*, and *Ssci20* showed high homology with genes encoding S-adenosyl-L-methionine decarboxylase (SAMDC), TAS14 protein (dehydrin), glucosyl transferase, and the PSBS (22kD) protein from photosystem II, respectively. They were the only ones that showed higher mRNA accumulation when *S. sogarandinum* was cold treated. Detailed Northern blot analysis revealed that the level of transcripts that hybridize only *Ssci17* and *Ssci20* cDNAs was closely correlated with cold acclimation (Rorat et al., 1998).

Recently, a cDNA clone encoding cyclophilin was isolated from *S. commersonii*. The corresponding gene was found to be constitutively expressed in leaves. Moreover, the level of mRNA increased when plants were exposed to abscisic acid, low temperature, drought, and wounding. Also, the gene was responsive to salicylic acid and pathogen challenge (Meza-Zepeda et al., 1998).

Construction of molecular marker maps in potato

Developing germplasm with increased freeze tolerance through breeding or genetic engineering would provide a long-term solution to the problem of freezing stress. However, breeding crops with increased cold hardiness has proven difficult, and improvements have been limited. The primary obstacle in conventional breeding is that freeze tolerance is a complex trait that is apparently affected by numerous genes (Gullord et al., 1975; Hummel et al., 1982; Law and Jenkins, 1970; Limin and Fowler, 1988; Rudolph and Nienstaedt, 1962; Sutka, 1981; Thomashaw, 1990). While crop improvement programs work toward developing more cold-hardy genotypes (Pellet, 1997), an alternative and complementary strategy could involve identifying genes that contribute to freeze tolerance, and, subsequently, transforming less hardy species (or genotypes) with such genes.

One strategy for identifying freeze-tolerance genes involves monitoring protein synthesis and gene expression during cold acclimation, then identifying genes whose transcription increases (Guy et al., 1985; Lee and Chen, 1993b; Palva and Heino, 1997; Thomashaw, 1993,1998). Numerous cDNAs corresponding to low temperature induced genes have been identified in a number of plant species (Guy et al., 1990, Guy et al.,1997; Lee and Chen , 1993; Palva and Heino, 1997; Thomashaw 1993, 1998). The function of the proteins encoded by these genes sometimes can be predicted from the open reading frames, but in most instances, protein function and role in freeze tolerance have not been deduced (Guy, 1990; Lee and Chen, 1993; Thomashaw 1993, 1998).

A second strategy for identifying genes that are important in freezing tolerance is through quantitative trait locus (QTL) mapping. Isolated genes that are expressed when freeze tolerance is increasing or maximal can be positioned on linkage maps (Cai et al., 1994; Hayes et al., 1993, 1997). When a gene is found to co-localize with a QTL for freeze tolerance, its importance is strengthened. Furthermore, establishing the position of QTLs for freeze tolerance can ultimately lead to map-based cloning of genes that greatly influence freezing tolerance.

Before recent advances in molecular marker technology, genetic maps were constructed mainly with morphological markers and isozymes. This was followed by the use of RFLPs (restriction fragment length polymorphisms). In potato, the first genome map was constructed with RFLPs in a population derived from *S. tuberosum* (2x) and *S. berthaultii*, and then backcrossed to *S. berthaultii* (Tanksley et al., 1992). With advancements in DNA marker technology, PCR-based markers have become more commonly used. Currently, the most commonly used markers are RAPDs (random amplified polymorphic DNA), AFLPs (amplified fragment length polymorphisms), and SSRs (simple sequence repeats)(Burow and Blake, 1998).

RAPD technology is based on the use of a single short oligonucleotide primer, which detects nucleotide sequence polymorphisms in the DNA template for PCR amplification. Since RAPD primers bind to many different loci, they are very useful to amplify random sequences from complex DNA templates. If the primer binds to the genomic DNA at two different sites on the DNA template, and if these priming sites are within an amplifiable distance of each other, a discrete DNA product is produced through thermocyclic amplification. The presence of each amplification product identifies

complete or partial nucleotide sequence homology, between the genomic DNA and the oligonucleotide primer, at each end of the amplified product. Because RAPD primers will direct the amplification of several discrete loci in the genome, RAPD technology provides an efficient way to screen for nucleotide sequence polymorphisms between genotypes (Burow and Blake, 1998; Waugh and Powell, 1992; Williams et al., 1990, 1995). RAPD markers are inherited as a dominant allele. Therefore, in highly heterozygous plant species, for example, informative dominant polymorphic markers are those that are heterozygous in one parent and null in the other (Marques et al., 1998). In potatoes this type of marker was used to construct a linkage map for the QTL analysis of tuber dormancy in a diploid *S. tuberosum* population (Freyre et al., 1994).

AFLPs are based on the selective PCR amplification of restriction fragments from totally digested genomic DNA (Vos et al., 1995). The AFLP process can be divided into three main steps. First, an initial phase, in which genomic DNA is digested with specific restriction enzymes (such as *EcoRI/MseI*) and oligonucleotide adapters (which consist of a core sequence and an enzyme-specific sequence) are ligated to those restriction fragments. Second, selective amplification of the restriction fragments. PCR amplification of the restriction fragments is achieved by utilizing the adapter and restriction site sequence as target sites for primer annealing. AFLP reactions generally employ two oligonucleotide primers, one corresponding to each end of the restriction fragment. AFLP primers consist of three parts, a core sequence, an enzyme specific sequence, and a selective extension. The selective amplification is accomplished by the use of primers that extend into the restriction fragments, amplifying only those fragments in which the primer extensions match the nucleotides flanking the restriction sites. And

third, is a gel separation of the amplified fragments. One of the main features of this methodology is that sets of restriction fragments can be visualized by PCR without previous knowledge of nucleotide sequence. The procedure can generate a large number of polymorphic bands. The number of fragments that can be analyzed is dependent on the resolution of the detection method (Burow and Blake, 1998; Vos et al., 1995). Like RAPDs, AFLPs are considered dominant markers (Marques et al., 1998). In potato several maps have been constructed with AFLPs (Meyer et al., 1998; Rouppe van der Voort et al., 1998 a and b; Van Eck et al, 1995b)

SSRs (simple sequence repeats), also called microsatellites, are becoming the preferred marker in animal and plant mapping. SSRs are short fragments of DNA, consisting of repeated nucleotide units, normally 1-8 nucleotides long. Microsatellites exhibit extensive site-specific length polymorphisms due to the high level of variation in the number of repeats. Thus, even closely related genotypes can be characterized with this type of molecular marker. This technology is based on PCR amplification using a unique pair of primers flanking the SSRs. Length polymorphisms at a particular SSR locus can be assayed on the basis of the differing electrophoretic mobilities of PCR products. Compared to other molecular markers, microsatellites have several advantages. They are co-dominant, very abundant, uniformly distributed in the genome, and a unique pair of primers defines each SSR locus (Bredemeijer et al., 1998; Burow and Blake, 1998; Giafranceschi et al., 1998). Recently, a map of *S. tuberosum* L. was constructed with SSRs (Milbourne et al., 1998).

QTL analysis in potato

As in other crops, many economically important traits in potato also show quantitative inheritance: segregating progeny show a continuous range of expression rather than the discrete classes characteristic of Mendelian, or qualitative, inheritance. Quantitative traits are usually affected by more than one gene and also by the environment (Paterson, 1998). The loci that are determinants of quantitative trait expression are termed Quantitative Trait Loci (QTL). QTLs are identified via statistical procedures that integrate genotypic (*i.e.* molecular markers) and phenotypic data. QTLs are assigned to chromosome locations based on the positions of the markers on linkage maps (Paterson, 1998).

QTL mapping has several major applications. First, it can be used to locate genes that are responsible for genetic variation in agriculturally important traits as a beginning point for using marker assisted selection in plant improvement. Second, to facilitate map-based cloning to isolate the genes responsible for specific genotypes. Third, to get a better understanding of evolutionary processes (Paterson, 1998).

QTL mapping has been used to study frost tolerance in barley (Hayes et al., 1993; Pan et al., 1994), wheat (Galiba et al., 1995), *Brassica* (Teutonico et al., 1995), and *Eucalyptus* (Byrne et al., 1997).

In potato, Ortiz and Peloquin (1992) reported QTLs for tuberization, flowering, pollen stainability and fruit set in a tetraploid progeny. They found one QTL for each of these traits and each QTL explained 12%, 19%, 73%, and 74% of the phenotypic variation for tuberization, flowering, pollen stainability, and fruit set, respectively. Van

den Berg et al. (1996a) performed a QTL analysis to re-examine potato tuberization using framework RFLP markers from the consensus potato map, and a population produced from reciprocal backcrosses of *S. tuberosum* and *S. berthaultii*. They found that a QTL on chromosome 5 explained 27% of the variance for tuberization. Moreover, additive effects of the QTLs were found to explain 53% of the phenotypic variation, which was increased to 60 % with the inclusion of epistatic effects.

QTLs for potato tuber dormancy were also examined in a diploid potato progeny of (*Solanum tuberosum* (2x) - *S. chacoense*) F_1 as a female parent and *S. phureja* as a male parent (Freyre et al., 1994). In this case, the QTL analysis was carried out in a map consisting of RFLPs and RAPDs distributed in 10 linkage groups. They found that 22 markers had a significant association with dormancy, and identified 6 QTLs localized on each of chromosomes 2, 3, 4, 5, 7, and 8. The QTL with the strongest effect on dormancy was found on chromosome 7. The multilocus model explained 57.5% of the phenotypic variation for dormancy (Freyre et al., 1994). Van den Berg et al. (1996b) also studied potato tuber dormancy using a population derived from reciprocal backcrosses of *S. tuberosum* and *S. berthaultii*. They detected QTLs on nine of the chromosomes; the QTL with the highest effect on tuber dormancy was found on chromosome 2. The map produced by backcross of the species mentioned above, was used by Simko et al. (1997) to conduct a QTL analysis for abscisic acid (ABA) content in tubers in relation to dormancy. QTLs related to this trait were found on chromosomes 2, 4, and 7. One of the QTLs was detected only as a main (single locus) effect, whereas the other two were found through two-locus interaction. Interactions between markers and QTLs explained 20 % of the phenotypic variation in ABA content. Comparing these results with the

findings of Van den Berg et al. (1996b), there is an interesting coincidence of QTLs for both traits on chromosome 7.

Recently, at least three papers have been published on QTL analysis in potato. Markers associated with quantitative resistance to late blight (*Phytophthora infestans*) were found to locate on chromosome 8 in an AFLP marker map using an F_1 population of tetraploid potatoes (Meyer et al., 1998). Resistance to cyst nematode species (*Globodera spp.*), was studied in a specific gene cluster on an AFLP marker map. The QTL analysis placed the *Grp1* locus (*Grp1* confers resistance to one population of *Globodera rostochiensis* and two different populations of *G. pallida*) on chromosome 5 (Rouppe van der Voort et al., 1998b).

Wild type *Solanum* species have been used extensively to introgress desirable genes into the cultivated *Solanum* species. However, some other undesirable traits may also be transferred along. One good example is the transfer of insect resistance and glycoalkaloid traits from *S. berthaultii* to *S. tuberosum*. Among these glycoalkaloids, which are toxic to humans, there are several categories depending on glycosidic grouping and steroid alkaloid skeleton. Since they are quantitatively inherited, QTL analysis in a progeny of a diploid reciprocal backcross from *S. tuberosum* and *S. berthaultii* was conducted based on an RFLP map (Yenko et al., 1998). QTLs on chromosomes 4, 6, and 12 explaining about 20% of the variation in solasodine were found. QTLs for the same glycoalkaloid were also found in the backcross to *tuberosum* on chromosomes 4, 8, and 11, however, in this second backcross those QTLs explained only 10% of the variation. Using the same populations they detected two QTLs, one on chromosome 1 and another on chromosome 4, for the accumulation of solanidine in the backcross to *tuberosum*. The

backcross to *berthaultii* also showed the same QTL on chromosome 1 (Yenko et al., 1998).

In conclusion, frost tolerance and cold acclimation are genetically complex traits with polygenic inheritance. Therefore, introgression through conventional breeding and/or somatic hybridization, although possible, is a slow process. It is evident that progress in the development of DNA-based marker technology, will contribute greatly to the identification of markers which are associated with quantitative trait loci. Marker-assisted selection may facilitate the identification of phenotypes with the trait of interest. Co-localization of QTLs associated with frost tolerance traits and cloned cold-inducible genes may help to determine the functional roles of these genes in frost hardiness and/or cold acclimation. Moreover, identification of QTLs may lead to map-based cloning of genes controlling those frost-tolerance-related traits. This will make it possible to improve the frost tolerance of cultivated potatoes by genetic transformation.

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CHAPTER 2

GENETIC ANALYSIS OF FROST TOLERANCE TRAITS IN TUBER-BEARING *SOLANUM* SPECIES

Abstract

The inheritance of frost hardiness and cold acclimation potential traits was studied in three segregating populations derived from a cross between *Solanum commersonii Dun.* PI 243503 (cmm) and *Solanum cardiophyllum* Lindl., PI 184762 (cph), two parental genotypes with contrasting frost hardiness and cold acclimation potential. The levels of frost hardiness and cold acclimation potential were expressed as the LT_{50}, the temperature at which 50% of the cells in leaf discs were killed, as measured by the ion leakage method, following a controlled freeze test. There was considerable variation in both frost hardiness and cold acclimation potential in all three segregating populations (F_1, F_1 x cmm, and F_1 x cph). Frost hardiness and cold acclimation potential were not correlated, suggesting that these two traits are under independent genetic control. The analysis of generation means indicated that the variation for both traits could be best explained by an additive-dominance model, with 'additive gene effects' the most important. Broad-sense heritability was 0.73 and 0.74 in the F_1 population, for frost hardiness and cold acclimation potential, respectively, and was 0.85 for both traits in the F_1 x cmm population, indicating that these two traits are highly heritable. Our results

suggest that it should be possible to incorporate the frost hardiness and cold acclimation traits from *S. commersonii* into cultivated potato species.

Introduction

Frosts are often the major factor limiting potato production in the temperate zone and in the highlands of the Andean Tropics of South America. In the Andean countries alone, it is estimated that over 400,000 ha of potatoes are threatened by frost injury every year (Barrientos et al., 1994; Estrada et al., 1993; Van Eck, 1995; Vega and Bamberg, 1995). The commonly cultivated potato, *Solanum tuberosum* L., possesses little frost hardiness, whereas many of the tuber-bearing *Solanum* species are much hardier (Chen and Li, 1980a). The superior horticultural potential of *S. tuberosum* should be combined with the frost hardiness traits from the hardy relatives.

As the wild *Solanum* species are potential sources of frost hardiness genes, screening of many genotypes for frost hardiness has been conducted both in controlled environmental conditions (Chen and Li, 1980a; Hanneman and Bamberg, 1986; Li, 1977; Palta and Li, 1979), and in field conditions (Vega and Bamberg, 1995). In these screening efforts, many *Solanum* species were identified that exhibited frost hardiness far superior to that of the cultivated species. Furthermore, potato plants have greater sensitivity to cold injury when grown under warm temperatures (>10°C), compared to when they are growing under cool temperatures (between about 0 and 10°C) (Chen and Li, 1980a). In this paper we define "frost hardiness" as the freezing tolerance of a plant

growing under warm conditions, and "cold acclimation potential" as the freezing tolerance of a plant after a period of cold hardening.

Among the frost hardy *Solanum* species that have been identified, *S. commersonii* Dun. (cmm) has been considered a good source of frost hardiness genes. Therefore, many attempts have been made to incorporate frost hardiness into the cultivated potato (Cardi et al., 1993a and b; Nyman and Waara, 1997). These studies have been done with only limited data on the inheritance of frost hardiness in *Solanum* species. One study investigated the inheritance of frost hardiness and cold acclimation potential in F_1 and backcross populations of *S. commersonii* (cmm) and *S. cardiophyllum* Lindl. (cph) (Stone et al., 1993). They found that frost hardiness and cold acclimation potential traits are under independent genetic control. Furthermore, the analysis of generation means indicated that all of the variance for frost tolerance and cold acclimation capacity could be best explained by an additive-dominance model with both traits being partially recessive. From a relatively small population, they were able to recover the parental phenotypes and thus suggested that both traits are controlled by relatively few genes. Because the study of Stone et al. (1993) was conducted on a very small number of genotypes (7 F_1s, 21 F_1 x cmm and 19 F_1 x cph), these results needed verification using larger populations.

With the goal of constructing a molecular marker linkage map and to locate quantitative trait loci (QTL) affecting frost hardiness and cold acclimation traits in *Solanum* species, we followed the procedures of Stone et al. (1993) to produce the F_1 and backcross populations from *S. commersonii* x *S. cardiophyllum*. In this communication, we present the phenotypic variation for frost hardiness and cold

acclimation potential, and we also re-examine the mode of inheritance of those two traits in these populations.

Materials and methods

Plant materials

Two diploid, tuber-bearing *Solanum* species were used for this study. These were *Solanum commersonii* Dun. (cmm), a frost tolerant species that possesses the ability to cold acclimate and *Solanum cardiophyllum* Lindl. (cph), a frost sensitive species which lacks the ability for cold acclimation (Chen and Li, 1980a). Twenty seeds of *S. commersonii* (PI 243503) and twenty seeds of *S. cardiophyllum* (PI 184762) were obtained from The Inter-Regional Potato Introduction Station in Sturgeon Bay, Wisconsin. They were germinated and grown in a soil mix of #8 pumice:peat moss:clay loam soil (v/v/v 2:1:1), and the resulting seedling clones were grown to maturity in the greenhouse. Controlled pollination under greenhouse conditions was conducted using cmm clone number 12 and cph clone number 12 to generate 73 F_1s (cmm x cph). Individual F_1 seedlings were clonally propagated by cuttings. The F_1 seedling clone number 34 was used to produce two backcross populations: 28 individuals of F_1 x cmm, and 85 individuals of F_1 x cph. The F_1 seeds were germinated in sand under mist conditions. Backcross seeds were sterilized, germinated, and propagated on Murashige and Skoog (1962) agar medium with no growth regulators. Plantlets propagated *in vitro* for each genotype, were transferred to 4" pots with a soil mix of #8 pumice:peat

moss:clay loam soil (v/v/v 2:1:1). Plants were kept on the mist bench in the greenhouse for two weeks, and were then transferred to 6" pots with the same substrate. Plants were grown in a 20° ± 2°C greenhouse with a 14-h photoperiod and light intensity of about 180 $\mu ES^{-1}m^{-2}$. All plants were fertilized every two months with Nutricote type 100 (Plant Products LTD, Brampton, Ontario, Canada), a controlled-release fertilizer.

Evaluation of frost tolerance

Frost hardiness and cold acclimation potential were measured for 6 cmm genotypes, 6 cph genotypes, and all individuals of the F_1 and backcross populations.

Ten-week-old plants of individual clones were divided into two groups: one group was used to measure frost tolerance without cold acclimation (for frost hardiness). The other group of plants was moved to a cold room (2 °C, 21.8 $\mu ES^{-1}m^{-2}$, 12-h photoperiod) for two weeks (for cold acclimation potential). Frost hardiness and cold acclimation potential were estimated by exposing leaf discs to a controlled freeze test (Sukumaran and Weiser, 1972). Briefly, fully expanded second leaves were excised and leaf discs were punched out, placed in test tubes (25 x150 mm), and incubated in a low temperature bath (model LT-50DD, NESLAB Instruments, Newington, NH). When the bath temperature reached -1 °C, ice nucleation was initiated by adding a small piece of ice to each tube. The samples were held at -1.5 °C for an additional 1 h., and then the temperature was lowered at a rate of 1 °C/h for non-acclimated samples, and at 2 °C/h for cold acclimated samples. Tubes were removed from the cooling bath at specific temperatures, and were thawed in a refrigerator overnight.

Freezing injury was estimated by the ion leakage method (Sukumaran and Weiser, 1972). Freezing tolerance was expressed as LT_{50}, defined as the temperature at which 50% of the cells were killed. Thawed leaf discs were transferred to individual vials containing 10 ml of deionized water at room temperature. Samples were vacuum infiltrated for 10 min at 25 in. Hg. Afterwards, samples were shaken for 1 h and conductivity was measured with a YSI model 35-conductance meter (Yellow Spring, OH, USA). Once the conductivity was measured, all samples were frozen in a -20 °C freezer for 24 h, thawed at room temperature, shaken for 1 h, and then the total conductivity for each sample measured. Percent ion leakage was calculated from triplicate samples, and the results were plotted as a function of freezing temperature. The experiment was repeated three times for all genotypes.

Estimation of genetic parameters and heritability

The means and variances of frost hardiness and cold acclimation potential were calculated for a group of seedlings of parental PIs, F_1, and 2 backcross generations. By using the LT_{50} of individual F_1, F_1 x cmm, and F_1 x cph populations, phenotypic correlation coefficients for both frost tolerance and cold acclimation potential were calculated, and their significance was evaluated by a t-test. The result from a Bartlett's test for variance homogeneity (Sokal and Rohlf, 1981) indicated that the population variances were homogeneous for the cold hardiness trait but not for cold acclimation potential. The Joint Scaling test of Mather and Jinks (1982) was used to estimate the genetic parameters: **m** which is the midpoint between parental means, **[d]** the additive

gene effects, and **[h]** the dominance gene effects. These genetic components were calculated through the Joint Scaling test, with weighted least squares (WLS), a multiple linear regression, using the reciprocals of the squared standard errors of each mean as weights (Mather and Jinks, 1982). The S-PLUS program for Windows was then used to resolve the information matrix produced. The squared root of the diagonal products of the inverted matrix was employed to calculate the standard error of the genetic parameters. With these genetic estimates, an expected mean was calculated for each generation. The same results were obtained by calculating those genetic parameters following the Beaver and Mojisdis (1988) least squared regression method. The goodness of fit for the additive-dominance model for frost hardiness was calculated as $X^2_{k-p}= \Sigma_{k-p}(O-E)^2 \times W$ (Mather and Jinks, 1982). Since the variance homogeneity test yielded different results for cold acclimation potential, the formula $X^2_{k-p}= (Y-CM)' SN^{-1}$ (Y-CM) was used for estimating the goodness of fit of the model, following the Beaver and Mojisdis (1988) notation and method. The degree of dominance of both traits for the three segregating generations was calculated as the h/d ratio, where -1 or +1 corresponds to complete dominance towards the least or most hardy parent, respectively, and 0 represents additive rather than dominance effects. In addition, the broad sense heritability (h^2) for the F_1, which is one of the sources of phenotypic variation in a pseudo-testcross, and backcross populations was calculated for both frost hardiness and cold acclimation potential according to Poehlman and Sleper (1994) as:

$$h^2 = \frac{\sigma^2 \text{(segregating population)} - \sigma^2 \text{(parental clones)}}{\sigma^2 \text{(segregating population)}}$$

Results

Frost hardiness and cold acclimation potential

As shown in Table 2.1, the means for frost hardiness of the parental genotypes were -5.7 ± 0.2 °C for cmm and -2.8 ± 0.1 °C for cph, while cold acclimation potential was -10.2 ± 0.1 °C and -3.5 ±0.2 °C for cmm and cph, respectively. In the F_1 population, the mean frost hardiness was -4.3 ±0.1 °C, which was exactly the mean of the two parental genotypes. Cold acclimation potential for the F_1 population was -6.4 ± 0.1 °C, which was very close to the mean of the two parental genotypes. When the backcross of F_1 x cph was evaluated, means of the whole population were -3.4 ± 0.1 °C and -5.0 ± 0.1 °C for frost hardiness and cold acclimation potential, respectively. Means of the backcross F_1 x cmm for the same traits were -4.8 ± 0.2 °C and -7.9± 0.3 °C, respectively.

The distribution of the frost hardiness (a) and cold acclimation potential (b) of the F_1 population is shown in Figure 2.1. The majority of the F_1 individuals fell between the two parental genotypes for both traits. In the backcross population of F_1 x cmm (Figure 2.1c, 2.1d), the distribution of both traits shifted to more negative values (*i.e.* hardier), whereas the backcross population of the F_1 x cph (Figure 2.1e, 2.1f) tended to shift to less negative values (*i.e.* more tender). Similar results were reported from backcross populations in blueberry (Arora et al., 1998). In addition, some transgressive segregants were observed in all segregating populations, with a higher number of them in the backcross populations (Figures 2.1c to 2.1f).

Table 2.1 Frost hardiness and cold acclimation potential mean calculated for *Solanum commersonii* and *S. cardiophyllum*, the F_1 between these two species and backcrosses to the parents.

Generation	N	Frost Hardiness (LT_{50}, °C)		Cold Acclimation Potential (LT_{50}, °C)	
		Observed Mean	Expected[a] Mean	Observed Mean	Expected[a] Mean
cmm	6	-5.7±0.2	-5.6	-10.2±0.1	-10.1
F_1 x cmm	28	-4.8±0.2	-4.9	-7.9±0.3	-8.7
F_1	73	-4.3±0.1	-4.2	-6.4±0.1	-7.3
F_1 x cph	85	-3.4±0.1	-3.4	-5.0±0.1	-5.5
cph	6	-2.8±0.1	-2.6	-3.5±0.2	-3.6
Goodness of fit		$P > 0.05$		$P > 0.05$	
R^2		0.999		0.991	

[a] Expected means were calculated by the weighted least squared regression (WLS). Values are presented with ± SEM

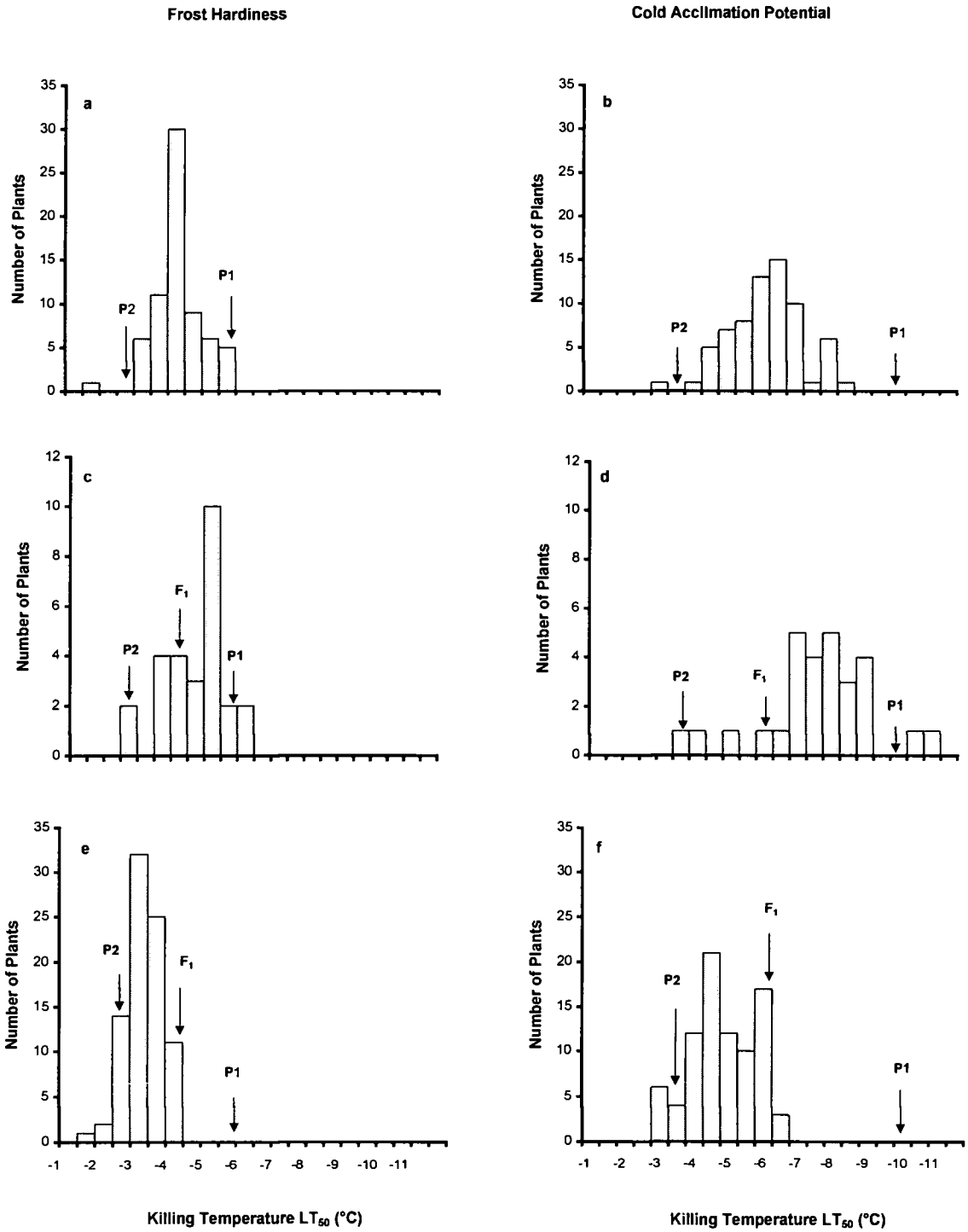

Figure 2.1. Distribution of progeny derived from a cross between *Solanum commersonii* (cmm, P1) and *S. cardiophyllum* (cph, P2) for frost hardiness (a, c, and d) and cold acclimation potential (b, d, and f). a & b : F_1 (cmm x cph); c & d : F_1 x cmm; and e & f. F_1 x cph.

By using the individual LT_{50}s from all segregating populations, a correlation analysis of frost hardiness vs. cold acclimation potentials gave rise to very low and non-significant r-values (Table 2.2).

Table 2.2 Correlation analysis between frost hardiness and cold acclimation potential in segregating populations of a cross between *Solanum commersonii* and *S. cardiophyllum*, and backcrosses of the F_1 to the parents.

Generation	r	N
F_1	0.06^{ns}	73
F_1 x cph	0.05^{ns}	85
F_1 x cmm	0.03^{ns}	28

^{ns} = Not significant at P=0.05

Genetic analysis of frost hardiness and cold acclimation potential traits

According to Stone et al. (1993), the additive-dominance model proposed by Cavalli (1952) could be used to explain the inheritance of frost hardiness and cold acclimation potential in *Solanum* species. The model includes an estimation of the mean, additive, and dominance genetic parameters (m, [d], and [h], respectively), calculated through the Joint Scaling test with weighted least squares (WLS) (Mather and Jinks, 1982). Based on these parameters (Table 2.3), the expected means for all populations in both traits were calculated (Table 2.1). The results indicated that both observed and expected means were not significantly different from each other. The significance of

these genetic parameters for each trait was analyzed by a t-test. We found non-significance for [h], which is the dominance component of the model. Since [h] was not significant for either trait, a verification of the usefulness of the additive-dominance model was performed using the data presented in Table 2.1. The goodness of fit for each trait, following the Beaver and Mosjidis (1988) and Mather and Jinks (1982) methodologies, indicated that the model was appropriate. Moreover, the very high values of R^2 calculated for both traits corroborated the appropriateness of the model to explain the variance in each trait. Also, as a way to verify the results in Table 2.3, the degree of dominance was calculated following Mather and Jinks (1982). The h/d ratios for each generation in each trait were very low (0.05-0.36) indicating that additive gene effects explain a large portion of the genetic variation.

Heritability

We calculated broad sense heritability using the variances of the two parental clones, as an estimate of the environmental variance, and the three individual segregating populations (F_1, F_1 x cmm, and F_1 x cph), as the source of variation. As shown in Table 2.4, the broad sense heritability values for frost hardiness were 0.73, 0.85, and 0.45 for F_1, F_1 x cmm, and F_1 x cph population, respectively; and was 0.74, 0.85, and 0.45 for cold acclimation potential for F_1, F_1 x cmm, and F_1 x cph population, respectively.

Table 2.3 Estimates of genetic parameters for frost hardiness and cold acclimation potential (LT_{50}, °C) from segregating populations from the cross of *Solanum commersonii* by *S. cardiophyllum*

	Components of stress tolerance	
Genetic Parameter	**Frost Hardiness**	**Cold Acclimation Potential**
m (mean value)	$4.1 \pm 0.1^{**}$	$6.8 \pm 0.1^{**}$
[d] (additive gene effects)	$1.5 \pm 0.1^{**}$	$3.2 \pm 0.1^{**}$
[h] (dominance gene effects)	0.1 ± 0.1^{ns}	-0.5 ± 0.2^{ns}

** = $P < 0.01$ ^{ns} = Not significant at $P = 0.05$

Table 2.4 Broad sense heritability of frost hardiness and cold acclimation potential for a cross between *Solanum commersonii* and *S. cardiophyllum*, and backcrosses of the F_1 to the parents.

	Broad Sense Heritability	
Generation	**Frost Hardiness**	**Cold Acclimation Potential**
F_1	0.73	0.74
F_1 x *cmm*	0.85	0.85
F_1 x cph	0.45	0.45

Discussion

There was a clear difference in LT_{50} between non-acclimated and acclimated plants of a particular genotype, and also among individual genotypes. The test was repeated three times, and the variation among runs was quite small (<13%). This small variation was also reflected in the very small individual standard errors. This is probably due to the sampling procedure we used and the controlled environment where our plant material was grown. As a result there was an increase in the precision of the genetic estimates ($R^2 = 0.99$) and the inferences made from the results obtained, suggesting that the methodology we used for assessing frost tolerance was reliable and reproducible. Moreover, our LT_{50} values from controlled freezing of leaf discs was also consistent with the frost hardiness data obtained from field grown plants where cmm is one of the five most hardy *Solanum* species, and cph is one of the most frost susceptible species (Vega and Bamberg, 1995).

The inheritance of non-acclimated freezing tolerance (equal to frost hardiness) and acclimation capacity (equal to cold acclimation potential minus frost hardiness) was previously reported (Stone et al., 1993). However, the populations used in this earlier study were relatively small (7 F_1s, 21 F_1 x cmm and 19 F_1 x cph). Our results support the conclusions that frost hardiness and cold acclimation potential are under independent genetic control, and relatively few genes control these traits. Nonetheless, there are also some differences between our results and those reported by Stone et al. (1993). First, there was significant variation in the F_1 generation for both traits in our study. This is not surprising since both parental clones used in this study are highly heterozygous. Second,

we found that the generation mean for the F_1 was almost at the midpoint of the two parents, whereas Stone et al., (1993) found that the F_1 mean was closer to the cph parent. Third, in the analysis of genetic parameters, our results indicated that additive gene effects could explain most of the phenotypic variation, whereas Stone et al. (1993) concluded that both traits were partially recessive.

One simple explanation for the difference could be the much larger population sizes used in this study. Besides, different cmm and cph clones may be used in both studies from the same PIs. Furthermore, in the case of cold acclimation potential, we used the LT_{50}s of cold acclimated plants. It may not be equivalent to the 'cold acclimation capacity', which is the net increase in frost hardiness after a fixed duration of cold conditioning. The difference in the terms used to express the relative level of frost tolerance after cold acclimation may also explain why our conclusions are different.

According to our results, there was a very low correlation between frost tolerance and cold acclimation potentials (Table 2.2). This suggests that different genes control the expression of these two traits. Similar results were obtained by Stone et al. (1993) in *Solanum* species, as well as by Teutonico et al. (1995) in oilseed *Brassica*.

An additive-dominance model adequately explains the gene action of frost hardiness and cold acclimation. Additive gene effects rather than dominance effects explain the majority of the phenotypic variation. Tibbits et al. (1991) also observed similar results in inter-specific hybrids of *Eucalyptus*, based on h/d ratios. Theoretically, an h/d = 0 indicates that the variance is totally due to additive gene effects, and an h/d = 1 indicates that the variance is totally due to dominant gene effects. In our research, all h/d ratios for both traits were < 0.37, indicating that frost hardiness and cold acclimation

capacity traits are inherited in a predominantly additive manner. Looking at the high broad-sense heritability values, it is possible to infer that the narrow-sense heritability values should also be high, further suggesting the additive inheritance of both traits. This conclusion also agrees with the results of Tibbits et al. (1991) for *Eucalyptus*. Moreover, these results also imply that both of these two traits are controlled by more than one gene.

The polygenic inheritance of frost hardiness has been reported in plant species including pine (Norell et al., 1986); and *Brassica* (Teutonico et al., 1995). Based on the observation that parental genotypes could be recovered from relatively small populations, Stone *et al.* (1993) concluded that only a few genes are controlling these two traits. Our results agree with theirs.

In the past decade, cmm has been the major source of frost hardiness genes for improving the frost tolerance of the cultivated potato (Nyman and Waara, 1997). Since the heritability values for the F_1 population are 0.73 and 0.74 for frost hardiness and cold acclimation potential, respectively, and 0.85 for either trait in the backcross of F_1 x cmm, reasonable advance in the introgression of both traits to the cultivated potatoes should be possible. Thus, information on the inheritance of frost hardiness traits will be useful for breeding efforts to transfer those traits to the cultivated potato. It will also serve as the foundation for further work on QTL analysis of frost hardiness-related traits. Based on our segregating population, a molecular marker linkage map has been constructed. The phenotypic data and the marker linkage map are being used for QTL analysis. Such information will be essential for advancing our understanding of the molecular mechanism

of cold acclimation and for developing a better breeding strategy for improving the frost tolerance of cultivated potato.

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CHAPTER 3

DETECTION OF QUANTITATIVE TRAIT LOCI INFLUENCING COLD HARDINESS TRAITS IN *SOLANUM* SPECIES

Abstract

Frost injury is one of the major factors limiting potato production in many parts of the world. It would be desirable to transfer frost-tolerance traits from hardy relatives to the cultivated potato. However, progress has been very slow. Since *Solanum commersonii* has been the major source of frost hardiness for improving the frost tolerance of the cultivated potato, we investigated the molecular basis of frost hardiness traits in this species. As a first step, we initiated map construction and quantitative trait locus (QTL) analysis of frost hardiness and cold acclimation. A pseudo-testcross F_1 population consisting of 73 individuals, segregating for both frost hardiness and cold acclimation, was derived from a cross between *Solanum commersonii* and *S. cardiophyllum*. A total of 420 RAPD (random amplified polymorphic DNA) primers and 64 AFLP (amplified fragment length polymorphism) *Eco*RI/ *Mse*I primer combinations were screened on both parents to generate a total of 953 polymorphisms. One hundred and seventy seven markers were *S. commersonii* specific and showed 1:1 segregation in the F_1 population. The linkage analysis conducted with Mapmaker software resulted in a 95-marker-map with 12 linkage groups, corresponding to the haploid number of this species. The cumulative length of this *S. commersonii*-specific map is 194.5 cM, with an

average of 2.05 cM between contiguous loci. Published maps of the potato (*S. tuberosum*) genome are 1120 cM, so clearly we have mapped only a fraction of this genome. QTL analysis using simple interval and simple composite interval mapping revealed only one significant QTL for cold acclimation. Multiple regression analysis identified two markers that accounts for 22.30% of the variation in frost hardiness and five markers that jointly accounted for 43.80% of the variance in cold acclimation potential.

Introduction

Potatoes are mainly grown in the temperate zone and in the highlands of the Andean Tropics of South America, where the yield and quality of the crop are often reduced by frosts (Barrientos et al., 1994; Estrada et al., 1993; Vega and Bamberg, 1995). The commonly cultivated potato, *Solanum tuberosum* L., has little frost hardiness, whereas many of the tuber-bearing *Solanum* species are much hardier (Chen and Li, 1980a). The wild *Solanum* species are potential sources of frost hardiness genes, so many genotypes have been screened for frost hardiness (Chen and Li, 1980a; Hanneman and Bamberg, 1986; Li, 1977; Palta and Li, 1979; Vega and Bamberg, 1995). From these efforts, many *Solanum* species have been identified that exhibit frost hardiness far superior to that of cultivated species (Chen and Li, 1980a; Hanneman and Bamberg, 1986; Li, 1977; Palta and Li, 1979; Vega and Bamberg, 1995). It was also found that some *Solanum* species, *e.g. S. acaule* Bitt. and *S. commersonii*, are capable of cold acclimation, but others, *e.g. S. cardiophyllum* and *S. tuberosum*, are not (Chen and Li,

1980a). It would be desirable to combine the high-yield potential of *S. tuberosum* with the frost-hardiness traits of hardy relatives. It is possible to transfer frost-hardiness genes from wild species to cultivated *S. tuberosum* by either conventional breeding (Estrada, 1993), or by somatic hybridization (Cardi et al., 1993a and b; Nyman and Waara, 1997) but the efforts would be a lengthy process. Unfortunately, there is little information available on the inheritance of frost hardiness in *Solanum* species.

The genetic basis of frost hardiness in many crops has been considered to be a complex trait with polygenic inheritance (Limin and Fowler, 1983; Thomashow, 1990). The complex genetic basis of frost hardiness has been studied through techniques of quantitative analysis (Eunnus et al., 1962). In *Solanum* species, the mode of inheritance of frost hardiness and cold acclimation potential was investigated in F_1 and backcross populations of *S. commersonii* and *S. cardiophyllum* (Stone et al., 1993). These two species have contrasting frost hardiness and cold acclimation capacity. *S. commersonii* is a frost hardy species and can be cold acclimated, whereas *S. cardiophyllum* is frost sensitive and can not be frost hardened. They found that frost hardiness and cold acclimation potential traits are under independent genetic control. Furthermore, the analysis of generation means indicated that all of the variance for both traits (frost hardiness and cold acclimation capacity) can be best explained by an additive-dominance model with both traits being partially recessive. From relatively small backcross populations, they were able to recover the parental phenotypes and thus suggested that both traits are controlled by relatively few genes.

With the long-term goal of elucidating the molecular basis of frost hardiness and cold acclimation in *Solanum* species, we followed the methodology reported by Stone et

al. (1993) to produce larger populations of the F_1 and backcross progenies from *S. commersonii* x *S. cardiophyllum* (Chapter 2). Our results agree with most of the conclusions reported by Stone et al. (1993). In addition, a moderate to high broad sense heritability was determined for both frost hardiness and cold acclimation traits (Chapter 2).

Quantitative trait locus (QTL) analysis is a useful tool for dissecting complex genetic traits, such as frost hardiness. QTLs affecting cold hardiness traits have been detected in barley (Hayes et al., 1993; Pan et al., 1994), wheat (Galiba et al., 1995), *Brassica* (Teutonico et al., 1995), and *Eucalyptus* (Byrne et al., 1997). In potato, a number of marker linkage maps have been constructed (Bradshaw et al., 1998; Freyre et al., 1994; Gebhardt et al., 1991; Jacobs et al., 1995; Meyer et al., 1998; Milbourne et al., 1998; Rouppe van der Voort et al., 1997 and 1998 a, b; Tanksley et al., 1992; Van der Berg et al., 1996a and b; Yenko et al., 1998). These maps have been used successfully to locate QTLs associated with resistance to cyst nematode species (Bradshaw et al., 1998; Rouppe van der Voort et al., 1998b;), tuber dormancy (Freyre et al., 1994; Van der Berg et al., 1996b), tuberization (Van der Berg et al., 1996a), resistance to late blight (Meyer et al., 1998), and abscisic acid content in relation to tuber dormancy (Simko et al., 1997). However, to our knowledge, a genomic map of *Solanum commersonii*, which has become a model plant for cold acclimation studies, is not available. Furthermore, no QTL analysis has been conducted for frost tolerance and/or cold acclimation in *Solanum* species.

The objectives of the present study were: 1) to initiate construction of a linkage map based on RAPD and AFLP markers; 2) to conduct QTL analysis to determine the associations between molecular markers and frost hardiness and cold acclimation traits.

Materials and methods

Plant material

Two diploid, tuber-bearing *Solanum* species were used for this study. These were *Solanum commersonii* Dun (**cmm**), a frost tolerant species that possesses the ability to cold acclimate and *Solanum cardiophyllum* Lindl. (**cph**), a frost sensitive species which lacks the ability for cold acclimation (Chen and Li, 1980). Twenty seeds of *S. commersonii* (PI 243503) and twenty seeds of *S. cardiophyllum* (PI 184762) were obtained from The Inter-Regional Potato Introduction Station in Sturgeon Bay, Wisconsin. They were germinated and grown in a soil mix of #8 pumice:peat moss:clay loam soil (v/v/v 2:1:1), and the resulting seedling clones were grown to maturity in the greenhouse. Controlled pollination under greenhouse conditions was conducted using cmm clone number 12 and cph clone number 12 to generate 73 F_1s (cmm x cph). In this particular case, the population is a pseudo-testcross design. The F_1 seeds were germinated in sand under mist conditions. Afterwards, individual seedlings were transferred to 4" pots with a soil mix of #8 pumice:peat moss:clay loam soil (v/v/v 2:1:1). Later, those seedlings were clonally propagated by cuttings using perlite as a substrate; cuttings were kept on the mist bench in the greenhouse for 3 weeks, and were then

transferred to 6" pots with the same type of substrate used in the 4"pots. Plants were grown in a 20° ± 2°C greenhouse with a 14-h photoperiod and light intensity of about 180 $\mu ES^{-1}m^{-2}$. All plants were fertilized every 2 months with Nutricote type 100 (Plant Products LTD, Brampton, Ontario, Canada), a controlled-release fertilizer.

DNA extraction

Genomic DNA was isolated from leaf tissues of individual plants using the Dellaporta (1983) method with minor modifications. Briefly, 150 µg of leaf tissue were ground with a hand-held pestle in a 1.5 ml Eppendorf microcentrifuge tube and gently homogenized in 0.5 ml of extraction buffer (EDTA 10 mM pH 8, NaCl 0.35 M, SDS 2%, Tris-HCl 50 mM pH 8, urea 7M, and water). Samples were kept on ice and 0.5 ml of phenol : chloroform (1:1) was added. Tubes were gently inverted for 5 min., and centrifuged at 4 °C for 3 min. at 14000 rpm. The aqueous phase was transferred to a new microcentrifuge tube and 0.5 ml of chloroform was added, which was shaken and centrifuged as described above. Once again the aqueous phase was transferred to a new microcentrifuge tube, where 1/10 volume of 3M sodium acetate and 2 volumes of cold absolute ethanol were added to precipitate the DNA. The tube was then centrifuged for 1 min at 14000 rpm. The pellet was re-suspended in 50 µl of TE buffer pH 8 and RNase was added to a final concentration of 1 µg/µl and then incubated at 65°C for 30 min. Samples were then subjected to an additional phenol:chloroform extraction, as described above. The pellet was washed with cold 70% ethanol and re-suspended in 50 µl of TE buffer. DNA quantity was estimated by spectrophotometry. This method yielded an

average of about 25 µg of DNA with a 260:280 ratio of 1.8 ± 0.1 from the 150 µg of leaf tissue.

RAPD amplification

Genomic DNA was amplified with *Taq* DNA polymerase (PROMEGA). A *Taq* polymerase PCR reaction solution (25 µl) containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.9 mM $MgCl_2$, 100µM each of dATP, dCTP, dGTP, and dTTP, 0.75 U *Taq*, 0.2 µM of random 10-mer primer (Operon Technologies; Alameda, Calif., and University of British Columbia (UBC)), and 20 ng of DNA template. DNA was amplified in a 96 well microtiter plate (Perkin Elmer, 9600 model), programmed for 45 cycles of 94°C for 50 sec, 37°C for 1 min and 15 sec, 54 °C for 20 sec, and 72°C for 1 min and 45 sec. This was followed by 1 cycle of final extension at 72°C for 14 min and 45 sec. PCR-products were subjected to electrophoresis on 2% agarose gels and amplified fragments were visualized by ethidium bromide staining. Black and white pictures were taken on an UV transilluminator using Polaroid 667 film.

RAPD screening

One hundred-twenty RAPD primers, kits A-G from Operon, and 300 UBC primers (Sets 100, 300, and 400) were screened with the parental genotypes (*Solanum commersonii* and *S. cardiophyllum*) (Table 3.1). Loci were scored as "-" for the absence and "+ "for the presence of a band. No primers on kit G from Operon or the 400 set from UBC showed polymorphisms for the parental genotypes, and therefore were

excluded from further analysis of the progeny. Molecular weights for the RAPD fragments were determined by comparison with a 1 kb ladder standard from Gibco BRL. For marker identification, the primer name followed by the fragment size in base pairs was used.

In a pseudo-testcross configuration, the expected ratio in an F_1 population for a dominant RAPD marker is 1:1, meaning that a band from only one of the parents will be present and the parent with the band is heterozygous. Nonetheless, in crops where selfing is not possible due to incompatibility, both parents are considered heterozygous, thus some 3:1 segregation may also be observed in a segregating population. Markers from the segregating population (F_1) were scored according to their parental origin and their segregation ratios. A Chi-square analysis test was performed to determine goodness-of-fit to the observed ratios at a probability of 0.05. Those markers segregating 1:1 and 3:1 according to the Chi-square test were considered informative, and PCR amplification was repeated two more times to verify their reproducibility.

AFLP amplification

Most of the reagents required were obtained from Perkin Elmer as a kit except for *Mse*I, *Eco*RI, T4 DNA Ligase, and Bovine serum albumin, which were purchased from Gibco/ BRL. The AFLP™ Plant mapping protocols provided by the manufacturer (PE Applied Biosystems, Foster City, CA) were followed. Briefly, 0.5 µg genomic DNA were digested with *Mse*I and *Eco*RI. Then adapters were ligated to the restriction fragments. Restriction-ligation target sequences were then subjected to a pre-selective

amplification, and PCR products visualized with ethidium bromide-UV light. Once amplification was confirmed, a selective amplification was performed using selected primers for *Mse*I and *Eco*RI. The *Eco*RI primers were fluorescence-labeled. Individual primer identification is as follows: (A) *Eco*RI-ACT, (B) *Eco*RI-AAC, (C) *Eco*RI-AAG, (D) *Eco*RI-ACC, (E) *Eco*RI-ACA, (F) *Eco*RI-AGG, (G) *Eco*RI-AGC, (H) *Eco*RI-ACG, (1) *Mse*I-CAA, (2) *Mse*I-CAC, (3) *Mse*I-CAG, (4) *Mse*I-CAT, (5) *Mse*I-CTA, (6) *Mse*I-CTC, (7) *Mse*I-CTG, (8) *Mse*I-CTT. Polyacrylamide gels were loaded with the selective amplified PCR products and electrophoresed using the ABI Prism 377 DNA sequencer. Then, the ABI Prism GeneScan analysis software, aided by the GeneScan-500 size standard, traced the separated fragments of each sample for further analysis. Finally, the output electropherograms from the GeneScan were categorized and final tables of polymorphic and segregating AFLPs were made using the Genotyper DNA fragment analysis software (AFLP™ Plant mapping protocol, PE Applied Biosystems, Foster City, CA).

AFLP screening

Sixty-four AFLP *Eco*RI/ *Mse*I primer combinations (A-H with 1-8) were evaluated with the parental genotypes (cmm and cph). Each band visualized with the GeneScan®2.1 was then evaluated with the Genotyper® using peak height and band size (bp). Each band was treated as a locus where "0" was used for an absent band and as "1" for a present band.

The segregation of individual AFLP fragment in the F_1 population was scored according to their parental origin as well as their segregation ratios. Segregating ratios were then tested for goodness-of-fit to a 1:1 ratio using a Chi-square test ($P \leq 0.05$).

Linkage map construction

RAPD and AFLP markers were analyzed using Mapmaker Version 2.0 (Lander et al., 1987). To assign linkage groups LOD scores of 3.0 to8.0 and recombination frequencies of 0.2 to 4.0 were evaluated. LOD scores lower or higher than 7.0 resulted in linkage group numbers far different from the actual haploid number of cmm (12 linkage groups). Thus, markers were grouped using a minimum LOD score of 7.0 and a maximum recombination frequency of 0.35. For all linkage groups, recombination values were transformed to centiMorgans (cM) using the Kosambi function. Then, map size estimates were calculated using the two point mapping function.

Quantitative trait loci analysis

QTL mapping analysis was conducted by applying interval-mapping procedures available in MQTL (Tinker et al., 1995). QTL analyses were carried out for the frost hardiness and cold acclimation traits, using the phenotype data for each F_1 individual. Since this is a pseudo-testcross mating configuration, the genetic analyses were conducted under a backcross model (Grattapaglia et al., 1995). Test statistics of 12.5 and 11.9 were used for frost hardiness and cold acclimation potential, respectively. A permutation test with 1000 repeats and a per-chromosome false positive rate of 5% were

used to determine QTL significance. Associations between informative markers and the two traits were evaluated through single and multiple regression, using the R-square, stepwise, and backward methods from the SAS regression model (SAS Inst., 1994)

Results

Marker analysis

A total of 420 RAPD primers were screened using the parental genotypes (Table 3.1), 845 polymorphic bands were identified: 434 bands were cmm specific, and 411 were cph specific. Two hundred primers producing the highest polymorphic band number between the two parents were selected for screening the F_1 progeny. We found that of these 200 primers only 66 primers yielded bands that were consistently reproducible in the F_1 population and resulted in a total of 384 markers (Table 3.1). Among those 384 markers, 145 (37.8%) showed a 1:1 segregation ratio, while the remaining 239 (61.2 %) deviated from the expected 1:1 ratio ($P \leq 0.05$). None of the RAPD markers showed 3:1 segregation. Among the 145 markers fitting 1:1 segregation ratios, 65 were cmm specific and 80 were cph specific (Table 3.1).

For developing AFLP markers, 64 *Eco*RI/ *Mse*I primer combinations were screened on the two parental genotypes (Table 3.1). From this screening, 10 *Eco*RI/ *Mse*I primer combinations showing the highest number of polymorphic bands between two parents were selected for screening the F_1 population. A total of 569 polymorphisms were detected using a peak height cut off threshold of 10 from the macro set of the GeneScan® software. Similar results were found with a 50 peak height cut-off threshold.

According to a Chi-square test, 262 out of these 569 markers showed 1:1 segregation (112 cmm-specific markers) or 3:1 segregation (56 cmm-specific markers) (Table 3.1). Table 3.2 shows the results of AFLP *Eco*RI/ *Mse*I primer combinations in the segregating F_1 population. Among these 262 markers, there were 112 (43%) cmm and 56 (21.4%) cph specific markers.

Table 3.1 Screening of RAPD and AFLP primers in parental genotypes (*S. commersonii* and *S. cardiophyllum*) and the F_1 progeny.

	Primers			**Fragments**[a]			
	Screened	**Selected**	**Screened**			**Segregating**	
1:1							
	cmm & cph	**cmm & cph**	**F_1**	**Total**	**Selected**	**cmm**	**cph**
RAPD	420	66	66	384	145[b]	65	80
AFLP	64	10	10[c]	569	262[d]	112	56

[a] Based on the F_1 [b] 1:1 segregation [c] primer combinations [d] 1:1 and 3:1 segregation

Table 3.2 Screening of AFLP primers in an F_1 population derived from *S. commersonii* x *S. cardiophyllum*.

Primer combination	cmm-specific			cph-specific		
	Total	1:1[a]	3:1[a]	Total	1:1[a]	3:1[a]
AAG/CTG (C7)	36	23	6	24	6	6
ACC/CTA (D5)	28	8	9	22	8	4
ACC/CTC (D6)	17	10	5	14	3	2
ACA/CAC (E2)	15	2	2	10	3	2
ACA/CAG (E3)	35	7	5	23	4	4
ACA/CTA (E5)	24	8	3	31	8	5
ACA/CTG (E7)	54	19	7	29	7	5
ACA/CTT (E8)	40	22	12	31	5	6
ACG/CAC (H2)	43	7	4	35	7	2
ACG/CTG (H7)	35	6	3	23	5	2
Total	327	112	56	242	56	38

[a] Segregation ratio

Construction of a linkage map.

Molecular markers showing 1:1 segregation were considered informative, and were used to construct a linkage map. A map consisting of 95 markers, with 12 linkage groups (which is the same as the haploid chromosome number of this *Solanum* species), was constructed with $\theta = 0.35$ and a LOD value of 7. Each linkage group was arbitrarily assigned a number from 1 to 12 (Figure 3.1) for easy identification. Only 5 (7.7%) of the RAPD markers, and 89 (79.5%) of the AFLP markers were included in this linkage map. Thus, the cumulative length of this cmm-specific map is 194.5 cM with an average of 2.05 cM between contiguous loci (Figure 3.1). A list of marker identification (name and size in bp) for all markers included in the map is given in Table 3.3.

Table 3.3 Identification and size of the molecular linked markers.

RAPDs	Size in bp	AFLPs	Size in bp	AFLPs	Size in bp
U3551	1840.00	D55	91.50	E730	230.79
U3274	560.00	D515	120.26	E732	239.67
U3961	400.00	D516	122.71	E733	249.83
OA021	800.00	D64	98.30	E734	250.97
OC061	1100.00	D65	126.75	E736	261.58
		D66	128.00	E745	318.89
AFLPs	Size in bp	D67	131.30	E747	350.16
		D68	132.75	E749	382.08
C71	53.00	D69	149.13	E750	384.70
C72	65.00	D611	184.90	E751	388.99
C73	70.65	D612	191.55	E82	64.85
C74	72.46	D616	370.60	E83	68.86
C75	74.58	E217	273.00	E85	88.50
C77	79.34	E310	124.11	E811	123.20
C710	92.52	E311	138.27	E812	124.20
C711	115.22	E321	241.66	E816	155.10
C712	118.49	E330	422.16	E817	156.89
C715	127.54	E334	461.47	E819	160.17
C716	135.43	E510	148.20	E820	170.32
C719	164.16	E512	164.30	E822	182.82
C720	172.10	E519	188.91	E823	191.18
C724	237.86	E521	192.49	E828	199.14
C728	263.84	E522	196.98	E829	213.00
C729	268.70	E524	231.00	E830	216.12
C732	285.51	E527	279.17	E831	224.24
C733	317.29	E74	74.60	E834	265.00
C734	325.23	E76	96.26	E835	268.83
C735	353.30	E714	132.25	E837	282.12
C736	370.19	E716	141.65	E839	335.57
C738	410.77	E720	167.56	H718	176.59
D52	51.83	E721	171.72	H724	235.31
D53	64.75	E726	191.03	H733	432.51

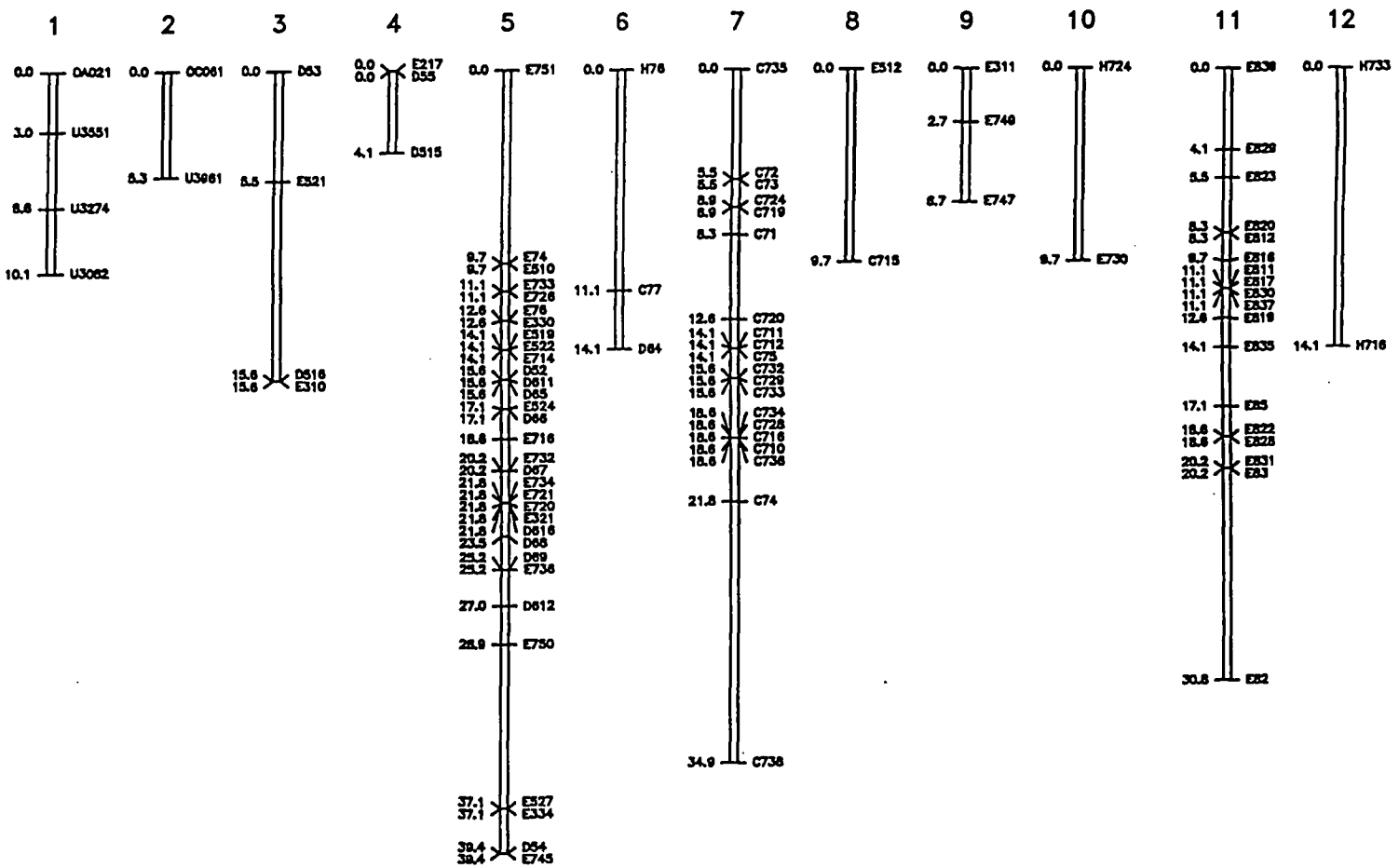

Figure 3.1 Genetic linkage map of *Solanum commersonii*. Twelve linkage groups with a total of 95 RAPD and AFLP markers was constructed using Mapmaker at LOD of 7.0 and $\theta = 0.35$. The total map distance contained within the 12 linkage groups is 194.6 cM.

Quantitative trait locus analysis

The interval mapping QTL analysis, detected only one significant peak for cold acclimation trait in the C711 and C733 interval in linkage group 7 (Figure 3.2 A). This peak has a test statistics of 12.6, which is higher than the 11.9 test statistics threshold. By single regression analysis using all (linked and un-linked) markers, 2 markers (D528 and C736), were found to be associated with frost hardiness (Table 3.4), with R^2 values of 12.91 and 11.33, respectively. The R^2 multilocus values accounted for 22.30% of the variability in frost hardiness ($P \leq 0.05$). The C736 marker is located in linkage group 7, between C732/C729/C733and C74 (Figures 3.1 and 3.2 B). Coincident results were found with the stepwise method (Table 3.4). When the cold acclimation trait was evaluated with single regression, 6 markers were found to be associated with this trait at P-values ≤ 0.09 (Table 3.5). Accordingly, a multiple regression analysis was performed using all 6 markers. Five out of 6 markers were found to be closely associated with cold acclimation and together could explain 43.8% of the total phenotypic variation. The stepwise method results agreed with those of the R-square method. Marker C712 is located in linkage group 7 whereas E311 is located in linkage group 9 (Figure 3.1), the other 3 markers are not linked.

Table 3.4 Association of molecular markers with QTLs for frost hardiness as revealed by the R-square and stepwise methods from single and multiple regression models

Marker	R^2 Single[a]	R^2 Multiple[b]	Stepwise Single	Stepwise Multiple	Sign[c]	Linkage group
D528	12.91	22.30	12.91	22.31	+	?
C736	11.33		9.40		-	7

[a] Single regression [b] Multiple regression
[c] + = positive allele from cph - = positive allele from cmm

Table 3.5. Association of molecular markers with QTLs for cold acclimation as revealed by the R-square and Stepwise methods from single and multiple regression models

Marker	R^2 Single	R^2 Multiple	Stepwise Single	Stepwise Multiple	Sign	Linkage group
C712	15.87	43.80	15.87	43.81	-	7
U3012	11.68		10.18		-	?
U1452	10.15		6.70		+	?
E731	13.13		5.40		-	?
E311	6.45		5.70		-	9

[a] Single regression [b] Multiple regression
[c] + = positive allele from cph - = positive allele from cmm

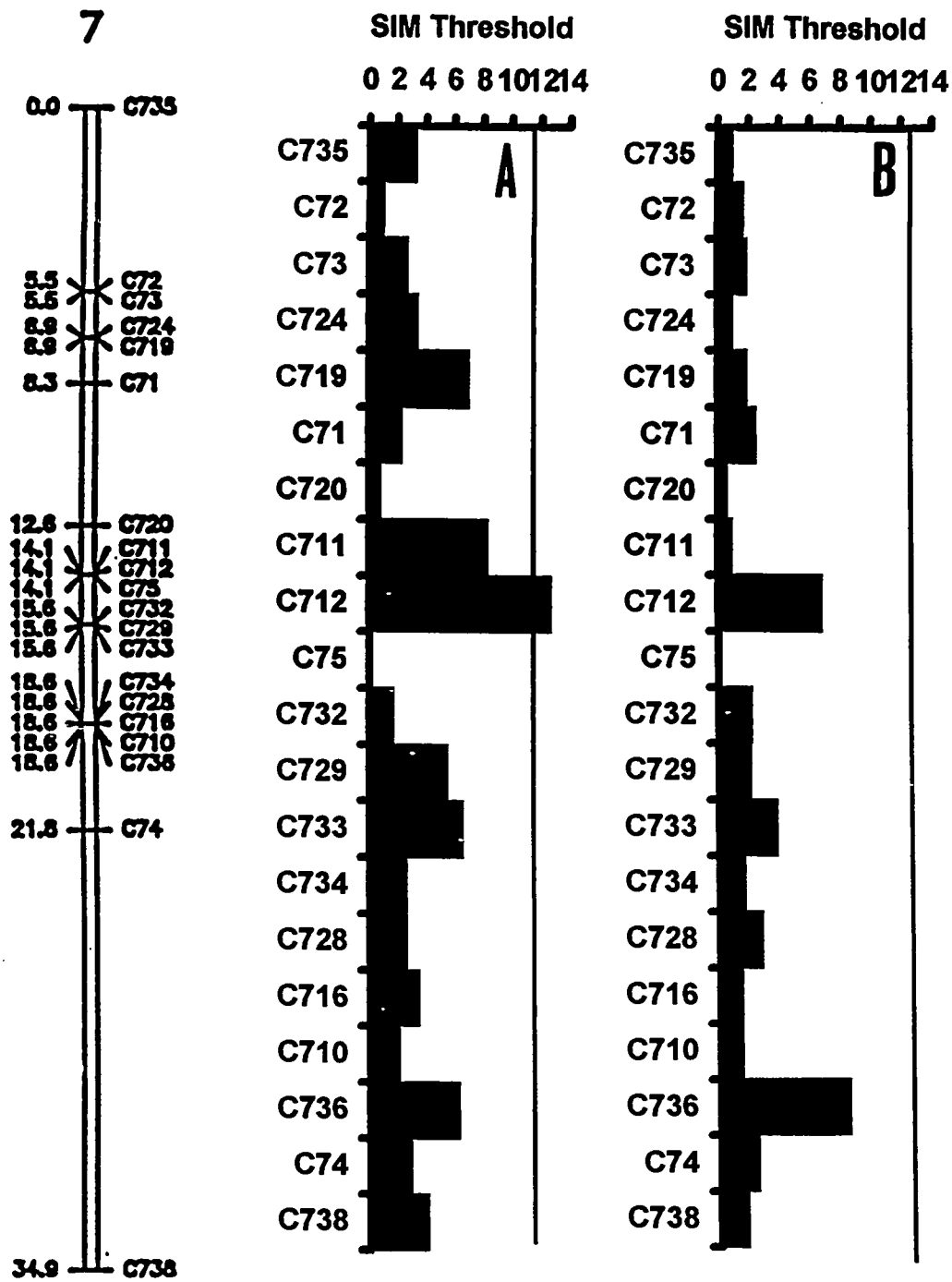

Figure 3.2 Linkage group activity for frost hardiness (B) and cold acclimation (A). The map for linkage group 7 is based on the segregation of alleles from the F_1 population. The significance levels (SIM Threshold) are presented as test statistics results from MQTL.

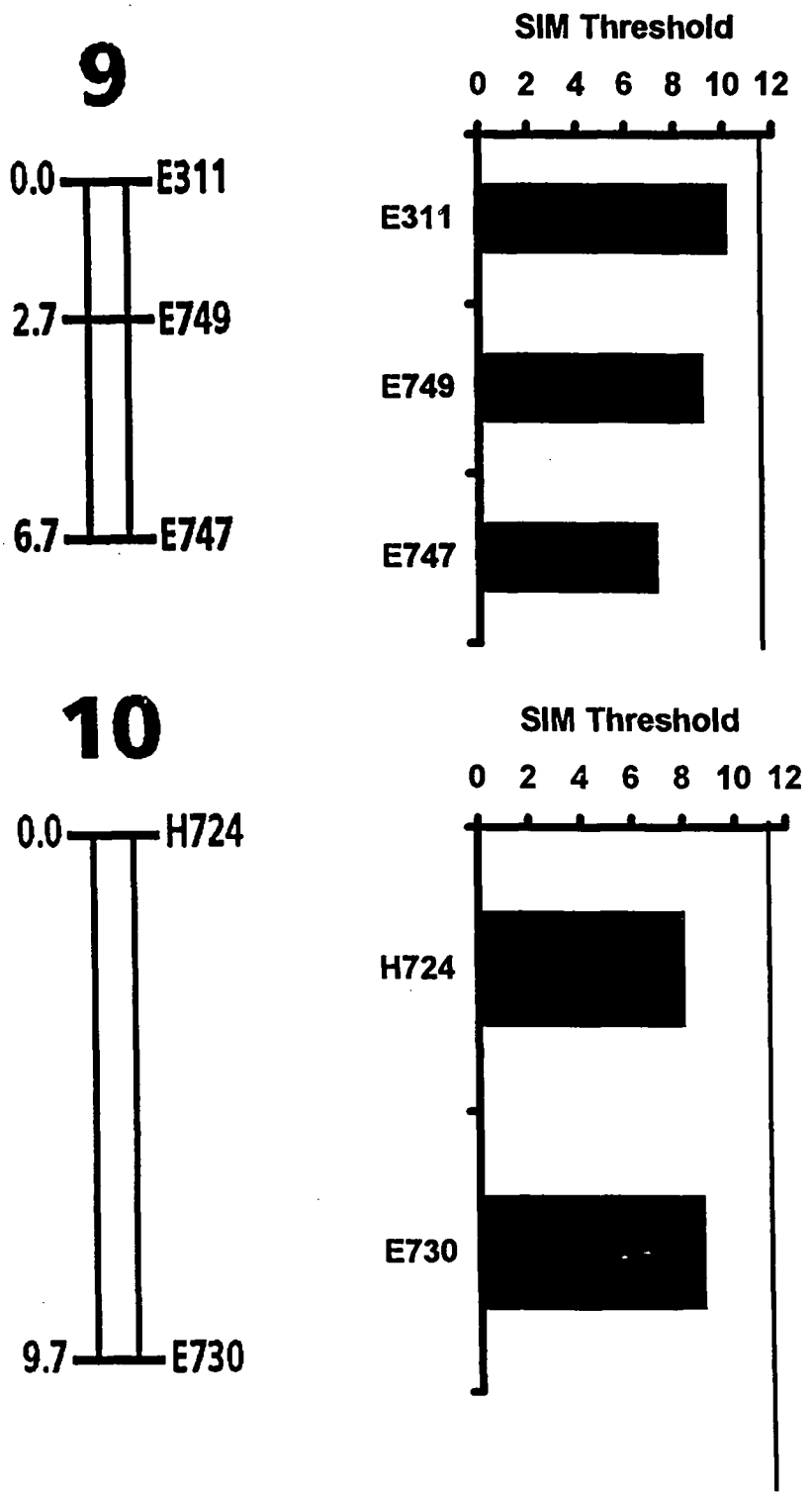

Figure 3.3 Linkage group activity for cold acclimation. Map for linkage group 9 and 10 are based on the segregation of alleles from the F_1 population. The significance levels (SIM Threshold) are presented as test statistics results from MQTL.

Discussion

In this study, only those RAPD and AFLP markers that segregated 1:1 in an F_1 population (cmm x cph) were used to construct a cmm-specific linkage map. Sixty percent of RAPD markers showed distorted segregation ratios (Table 3.1). This is probably one of the characteristics of the RAPD technology since distorted segregation ratios of 40% with RAPD markers were reported by Cai et al. (1994). In forest trees Krutovskii et al. (1998) mentioned distorted segregation ratios of 10-18% for RAPD markers. In genetic studies with diploid potatoes Gebhardt et al. (1991) reported distorted segregation ratios of 27%. One of the possible reasons why RAPDs, deviate from Mendelian expectations, is the lack of specific affinity between the primer and the genomic DNA during PCR amplification, which can produce false positive bands (Nozaki et al., 1997).

Considering the number of primers screened, the number of polymorphisms detected by AFLPs was much higher than the number detected by for RAPDs. Likewise, the number of informative markers was also much higher. Using RAPDs, Grattapaglia and Sedderoff (1994), reported the presence of a small number (less than 2%) of markers segregating 3:1. Similar results were also reported by Krutovskii et al. (1998). In our study, no RAPD markers were found to segregate in a 3:1 fashion. However, as shown in Table 3.3 one third of the informative AFLP markers segregated 3:1. A study by Bradshaw et al. (1998) in *Solanum tuberosum* subsp. *tuberosum*, a tetraploid potato, the proportion of RAPD markers showing 1:1, 3:1, and distorted segregation ratios were similar to our results with diploid potato species.

Segregation ratios for AFLPs markers were categorized into 3 different groups: 1:1, 3:1, and distorted segregation (fit neither 1:1 nor 3:1). The small number of AFLP markers segregating 1:1 in our study was disappointing. We do not know if these results are due to the nature of this particular cross combination, the product of the type of marker that we are using, or the type of analysis performed by the semi-automated screening and scoring system implemented by the GeneScan ®2.1 and Genotyper® software. With this software, band size differences at the level of 0.5 bp can be detected. Bradshaw et al. (1998) and Marques et al. (1998) using conventional autoradiographs, found the frequency of markers with 3:1 and distorted segregation ratios to be far lower than ours. Unfortunately, we do not have another comparative study with which results were analyzed with the same software so that we could verify our results.

From 73 F_1 individuals, we were able to construct a 95-marker linkage map consisting of 5 RAPD markers and 89 AFLP markers, representing 12 linkage groups of the maternal parent *Solanum commersonii*. The total length of the map is 194.6 cM, which represents a small fraction of the 1120 cM diploid potato map of reported by Jacobs et al. (1995). In relation to the small number of linked markers, a feasible explanation may be the use of false positive markers, although they segregated 1:1 according to the Chi-square results. The map has regions covered with dense markers, in contrast to the presence of some gaps and/or less dense areas. This might be due to specific variance in the levels or position of DNA polymorphisms, rates of recombination, deviation in copy number of specific genomic sequences (Rouppe van der Voort et al., 1997; Van Eck et al., 1995). Obviously, the small number of markers assigned to the linkage groups, the limited size of our population, and the use of only dominant markers

could be responsible for those gaps or less dense marker areas (Grattapaglia et al., 1995; Krutovskii et al., 1998; Marques et al., 1998; Meyer et al., 1998; Van Eck et al., 1995; Wang et al., 1997). On the other hand, most markers in the clusters were AFLPs, with the highest number in linkage groups 5, 7, and 11. Even though no statistical analysis was performed for this particular characteristic, clustering has been sufficiently documented for AFLP markers in potato (Rouppe van der Voort et al., 1997); RAPD markers in Douglas-Fir (Krutovskii et al., 1998); RFLP markers in tomato (Tanksley et al., 1992); RFLP markers in beans (Vallejos et al., 1992); RAPD markers in *Eucalyptus* (Grattapaglia and Sederof, 1994); RAPD markers and GATA microsatellites in BC populations derived from *Lycopersicum* species (Grandillo and Tanksley, 1996b); and RAPD markers in apple (Hemmat et al., 1994). Clustering of marker loci, from a biological point of view, might be the result of suppressed genetic recombination in heterochromatin around the centromeres and/or in telomeric regions (Grattapaglia and Sederoff, 1994; Rouppe van der Voort et al., 1997; Tanksley et al., 1992;).

RAPD and AFLP segregation data obtained by analysis of the F_1 progeny were used to carry out QTL analyses based on interval mapping, single and multiple regression. Only one QTL exceeding the significance threshold was detected with the interval mapping. More QTLs may not have been detected due to the incomplete genome coverage. Our population (n = 73) may not have been sufficient. Differences between alternative QTL alleles could affect the test statistics and consequently QTL detection. Finally, it is possible that many genes with small effects determine the target traits (Bradshaw et al., 1998; Grattapaglia et al., 1995; Meyer et al., 1998; Schafer-Pregl et al., 1996; Teutonico et al., 1995; Van Eck et al., 1995b). However, a genetic analysis of

frost hardiness traits in tuber-bearing *Solanum* species (Chapter 2) suggested that relatively few genes control these traits.

Using interval mapping and regression analyses, we were able to establish associations between the markers and at least one of the traits studied. With the interval mapping analysis one marker was found significantly associated with the cold acclimation trait (Figure 3.2 b). The same marker showed also the highest R^2 and stepwise values in both, single and multilocus analysis. Three out of the five markers in table 3.5 showed also coincident results when a backward analysis was performed (data not showed). Moreover, the robustness of the values presented in table 3.5 was corroborated by the trends observed in the interval mapping analysis (Figure 3.3). For linkage group 9 the trend is related to marker E311 and for linkage group 10 the trend is associated with marker H724, which was excluded by the stepwise analysis but showed up a significant response in the backward analysis. An association was also found between marker D528 and C736 with frost hardiness. These associations were also observed through the stepwise analysis (Table 3.4) and for marker C736 a significant response was obtained with the backward method (data not showed). The trend for the same marker in Figure 3.2 agreed with the results in Table 3.4. As shown in Tables 3.5 and 3.4, a number of unlinked markers were associated with the cold acclimation trait at P-value ≤ 0.09. We believe that by increasing the number of markers and the mapping population size, these unlinked loci will be assigned to linkage groups. Then, more stringent conditions could be applied to declare the significance, presence and location of these QTLs using interval mapping. At this moment, the tests performed showed that the models utilized in this research provide adequate estimates of QTLs associated with frost hardiness and cold

acclimation, under the pseudo-testcross-matting scheme. The proportion of phenotypic variation explained by single marker loci ranged from 6.45 % to 15.87% taking into account both traits. The multilocus model for cold acclimation (5 marker loci) accounted for 43.80 % of the variation in trait expression. In contrast, the marker associated with frost hardiness explained only 22.30% of the phenotypic variation. This suggests that different QTLs may determine the two traits. Although interval mapping increases accuracy and precision of QTL analysis, there are many research reports where regression and/or ANOVA analyses were used. The real issues are the power to detect QTLs and robustness of the procedures. In practice a t-test, an ANOVA F-test, or a regression test is often sufficient to declare QTL significance (Bradshaw et al., 1998; Byrne et al., 1997; Charcoset and Gallis 1996; Churchill and Goerge, 1998; Freyre et al., 1994; Grandillo and Tanksley 1996a; Meyer et al., 1998; Pan et al., 1994; Whittaker et al., 1996; Wu, 1998).

In conclusion, a partial linkage map of *Solanum commersonii* was constructed. Using multiple regression analysis, two markers were found to be associated with frost hardiness and five markers were associated with cold acclimation. Only one significant QTLs was detected using interval mapping. These data will provide a foundation for addressing the genetics of quantitative traits of frost tolerance in *Solanum* species.

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APPENDIX

Table A.1 LT_{50} (-°C) data for individual clones of *Solanum commersonii* PI 243503 (cmm) and *S. cardiophyllum* PI 184762 (cph). Each "Rep." represents an average of 3 leaf discs.

Plant identif.	Trait	Rep 1	Rep 2	Rep 3	**Average**	Std.
cmm 1	CA[a]	9.61	9.78	9.67	**9.69**	0.09
	FH[b]	5.86	5.76	5.83	**5.82**	0.05
cmm 4	CA	10.13	10.06	10.23	**10.14**	0.09
	FH	5.73	5.83	5.80	**5.79**	0.05
cmm 7	CA	10.46	10.8	10.52	**10.59**	0.18
	FH	5.40	5.90	5.62	**5.64**	0.25
cmm 8	CA	10.26	10.00	10.36	**10.21**	0.19
	FH	5.20	4.76	5.00	**4.99**	0.22
cmm 12	CA	10.33	10.63	10.20	**10.39**	0.22
(Parent 1)	FH	5.73	6.30	5.98	**6.00**	0.29
cmm 17	CA	10.06	10.17	10.31	**10.18**	0.13
	FH	5.88	5.97	6.48	**6.11**	0.32
cph 2	CA	2.86	2.96	2.81	**2.88**	0.08
	FH	2.60	2.73	2.62	**2.65**	0.07
cph 7	CA	3.13	3.06	3.06	**3.08**	0.04
	FH	2.91	2.86	2.52	**2.76**	0.21
cph 10	CA	3.93	3.56	3.31	**3.60**	0.31
	FH	2.40	2.33	2.48	**2.40**	0.08
cph 11	CA	3.46	3.21	3.67	**3.45**	0.23
	FH	3.13	3.06	3.33	**3.17**	0.14
cph 12	CA	3.86	3.75	3.88	**3.83**	0.07
(Parent 2)	FH	2.33	2.40	2.65	**2.46**	0.17
cph 13	CA	3.98	4.02	4.01	**4.00**	0.02
	FH	3.33	3.20	3.01	**3.18**	0.16

[a] Cold acclimation potential [b] Frost hardiness

Table A.2 LT_{50} (-°C) data for each individual of the F_1 progeny. Each "Rep." represents an average of 3 leaf discs.

Plant identif.	Trait	Rep 1	Rep 2	Rep 3	**Average**	Std.
cmm 12	CA[a]	10.50	10.42	10.00	**10.31**	0.27
Parent 1	FH[b]	5.32	6.42	6.70	**6.15**	0.73
cph 12	CA	3.00	3.25	2.90	**3.05**	0.18
Parent 2	FH	2.27	2.07	2.01	**2.12**	0.14
1	CA	5.62	5.75	5.68	**5.68**	0.07
	FH	4.02	3.46	3.94	**3.81**	0.30
2	CA	7.32	7.50	7.43	**7.42**	0.09
	FH	4.20	3.60	3.88	**3.89**	0.30
3	CA	6.45	6.75	6.53	**6.58**	0.16
	FH	4.30	4.26	4.28	**4.28**	0.02
5	CA	7.21	7.33	7.27	**7.27**	0.06
	FH	5.73	5.90	5.81	**5.81**	0.09
6	CA	4.72	4.83	4.77	**4.77**	0.06
	FH	3.51	3.70	3.62	**3.61**	0.10
7	CA	6.15	6.20	6.19	**6.18**	0.03
	FH	4.06	4.40	4.25	**4.24**	0.17
8	CA	5.63	5.56	5.60	**5.60**	0.04
	FH	4.95	4.73	4.81	**4.83**	0.11
9	CA	6.43	6.5	6.48	**6.47**	0.04
	FH	3.89	4.00	3.99	**3.96**	0.06
11	CA	8.04	8.16	8.09	**8.10**	0.06
	FH	4.37	4.50	4.44	**4.44**	0.07
12	CA	6.66	6.83	6.71	**6.73**	0.09
	FH	4.29	4.36	4.31	**4.32**	0.04
13	CA	8.13	8.67	8.40	**8.40**	0.27
	FH	5.20	5.53	5.33	**5.35**	0.17
14	CA	7.02	7.00	7.00	**7.01**	0.01
	FH	4.27	4.56	4.48	**4.44**	0.15

Table A.2 LT_{50} (-°C) data for each individual of the F_1 progeny. Each "Rep." represents an average of 3 leaf discs. [Continued]

Plant identif.	Trait	Rep 1	Rep 2	Rep 3	**Average**	Std.
15	CA	6.54	6.67	6.61	**6.61**	0.07
	FH	5.44	5.60	5.51	**5.52**	0.08
17	CA	8.15	8.30	8.22	**8.22**	0.08
	FH	4.09	4.17	4.11	**4.12**	0.04
19	CA	8.01	8.16	8.10	**8.09**	0.08
	FH	5.71	5.83	5.79	**5.78**	0.06
21	CA	5.80	5.83	5.81	**5.81**	0.02
	FH	3.92	3.77	3.88	**3.86**	0.08
22	CA	6.44	6.58	6.51	**6.51**	0.07
	FH	5.31	5.70	5.49	**5.50**	0.20
23	CA	6.41	6.58	6.49	**6.49**	0.09
	FH	4.27	4.50	4.38	**4.38**	0.12
24	CA	6.17	6.70	6.32	**6.40**	0.27
	FH	4.28	4.50	4.33	**4.37**	0.12
26	CA	6.06	6.33	6.21	**6.20**	0.14
	FH	4.48	4.70	4.66	**4.61**	0.12
28	CA	7.33	7.50	7.42	**7.42**	0.09
	FH	4.51	4.87	4.67	**4.68**	0.18
29	CA	6.00	6.17	6.11	**6.09**	0.09
	FH	4.35	4.27	4.30	**4.31**	0.04
30	CA	7.96	8.16	8.01	**8.04**	0.10
	FH	3.61	3.33	3.54	**3.49**	0.15
31	CA	6.52	6.02	6.81	**6.45**	0.40
	FH	4.10	4.48	4.41	**4.33**	0.20
32	CA	5.21	5.16	5.18	**5.18**	0.03
	FH	5.17	5.02	5.12	**5.10**	0.08

Table A.2 LT_{50} (-°C) data for each individual of the F_1 progeny. Each "Rep." represents an average of 3 leaf discs. [Continued]

Plant identif.	Trait	Rep 1	Rep 2	Rep 3	**Average**	Std.
34	CA	8.04	8.20	8.13	**8.12**	0.08
Parent BC	FH	4.11	4.10	4.90	**4.10**	0.46
35	CA	6.41	6.83	6.52	**6.59**	0.22
	FH	4.77	4.93	4.81	**4.84**	0.08
36	CA	6.08	6.17	6.09	**6.11**	0.05
	FH	3.98	4.04	4.00	**4.01**	0.03
37	CA	5.10	5.17	5.14	**5.14**	0.04
	FH	4.10	3.87	3.92	**3.96**	0.12
38	CA	4.74	4.83	4.79	**4.79**	0.05
	FH	4.46	4.32	4.38	**4.39**	0.07
39	CA	6.32	6.5	6.42	**6.41**	0.09
	FH	3.10	3.55	3.33	**3.33**	0.23
40	CA	6.74	6.83	6.80	**6.79**	0.05
	FH	4.18	4.07	4.12	**4.12**	0.06
42	CA	6.46	6.58	6.50	**6.51**	0.06
	FH	4.21	4.20	4.18	**4.20**	0.02
43	CA	5.97	6.20	6.15	**6.11**	0.12
	FH	5.11	5.30	5.21	**5.21**	0.10
44	CA	6.64	6.70	6.68	**6.67**	0.03
	FH	5.30	5.33	5.29	**5.31**	0.02
45	CA	6.08	6.17	6.12	**6.12**	0.05
	FH	4.10	4.00	4.13	**4.08**	0.07
46	CA	5.63	5.5	5.58	**5.57**	0.07
	FH	3.59	3.63	3.61	**3.61**	0.02
47	CA	7.07	7.08	7.10	**7.08**	0.02
	FH	4.10	3.60	3.98	**3.89**	0.26
49	CA	7.68	7.83	7.72	**7.74**	0.08

Table A.2 LT_{50} (-°C) data for each individual of the F_1 progeny. Each "Rep." represents an average of 3 leaf discs. [Continued]

Plant identif.	Trait	Rep 1	Rep 2	Rep 3	**Average**	Std.
	FH	6.00	6.00	5.94	**5.98**	0.03
50	CA	8.56	8.70	8.61	**8.62**	0.07
	FH	4.92	4.78	4.83	**4.84**	0.07
51	CA	6.02	6.10	6.00	**6.04**	0.05
	FH	5.35	5.47	5.41	**5.41**	0.06
52	CA	5.72	5.50	5.65	**5.62**	0.11
	FH	4.26	4.37	4.30	**4.31**	0.06
55	CA	6.71	6.83	6.77	**6.77**	0.06
	FH	4.03	4.13	4.09	**4.08**	0.05
57	CA	6.82	6.67	6.61	**6.70**	0.11
	FH	4.33	4.51	4.36	**4.40**	0.10
59	CA	6.32	6.01	5.97	**6.10**	0.19
	FH	4.02	4.30	4.31	**4.21**	0.16
60	CA	7.20	6.67	7.13	**7.00**	0.29
	FH	4.30	4.71	4.49	**4.50**	0.21
62	CA	4.71	4.75	4.69	**4.72**	0.03
	FH	4.06	4.13	4.11	**4.10**	0.04
63	CA	7.28	7.33	7.31	**7.31**	0.03
	FH	4.31	4.43	4.39	**4.38**	0.06
64	CA	6.55	6.83	6.66	**6.68**	0.14
	FH	4.40	4.47	4.41	**4.43**	0.04
65	CA	4.93	4.83	4.90	**4.89**	0.05
	FH	4.45	4.4	4.50	**4.45**	0.05
66	CA	3.41	3.33	3.39	**3.38**	0.04
	FH	1.92	1.73	1.74	**1.80**	0.11
67	CA	6.74	6.83	6.76	**6.78**	0.05
	FH	3.36	3.23	3.25	**3.28**	0.07

Table A.2 LT_{50} (-°C) data for each individual of the F_1 progeny. Each "Rep." represents an average of 3 leaf discs. [Continued]

Plant identif.	Trait	Rep 1	Rep 2	Rep 3	**Average**	Std.
70	CA	4.44	4.67	4.52	**4.54**	0.12
	FH	3.10	2.93	3.00	**3.01**	0.09
71	CA	6.80	6.83	6.79	**6.81**	0.02
	FH	4.56	4.7	4.63	**4.63**	0.07
72	CA	7.36	7.42	7.39	**7.39**	0.03
	FH	4.40	4.47	4.41	**4.43**	0.04
73	CA	5.94	6.00	6.00	**5.98**	0.03
	FH	4.39	4.48	4.44	**4.44**	0.05
74	CA	7.17	7.25	7.21	**7.21**	0.04
	FH	4.02	4.07	4.00	**4.03**	0.04
75	CA	7.21	7.20	7.25	**7.22**	0.03
	FH	4.43	4.53	4.51	**4.49**	0.05
76	CA	6.83	6.91	6.89	**6.88**	0.04
	FH	5.21	5.27	5.25	**5.24**	0.03
77	CA	4.41	4.58	4.39	**4.46**	0.10
	FH	3.27	3.13	3.20	**3.20**	0.07
78	CA	6.69	6.75	6.72	**6.72**	0.03
	FH	4.51	4.48	4.49	**4.49**	0.02
79	CA	5.42	5.50	5.51	**5.48**	0.05
	FH	3.42	3.30	3.39	**3.37**	0.06
80	CA	7.15	7.20	7.16	**7.17**	0.03
	FH	3.67	3.70	3.67	**3.68**	0.02
81	CA	7.52	7.40	6.98	**7.30**	0.28
	FH	4.21	4.49	4.29	**4.33**	0.14
82	CA	5.44	5.33	5.38	**5.38**	0.06
	FH	4.61	4.53	4.56	**4.57**	0.04

Table A.2 LT_{50} (-°C) data for each individual of the F_1 progeny. Each "Rep." represents an average of 3 leaf discs. [Continued]

Plant identif.	Trait	Rep 1	Rep 2	Rep 3	**Average**	Std.
83	CA	5.73	5.70	5.70	**5.71**	0.02
	FH	4.11	4.00	4.02	**4.04**	0.06
84	CA	6.41	6.50	6.47	**6.46**	0.05
	FH	4.58	4.60	4.60	**4.59**	0.01
85	CA	5.23	5.25	5.19	**5.22**	0.03
	FH	4.19	4.27	4.21	**4.22**	0.04
86	CA	6.17	6.2	6.19	**6.19**	0.02
	FH	4.00	4.07	4.01	**4.03**	0.04
87	CA	5.46	5.5	5.49	**5.48**	0.02
	FH	3.61	3.65	3.64	**3.63**	0.02
88	CA	5.20	5.33	5.26	**5.26**	0.07
	FH	3.80	3.75	3.77	**3.77**	0.03
89	CA	6.62	6.67	6.60	**6.63**	0.04
	FH	4.51	4.57	4.44	**4.51**	0.07
90	CA	6.00	6.00	5.97	**5.99**	0.02
	FH	4.10	4.33	4.22	**4.22**	0.12

[a] Cold acclimation potential [b] Frost hardiness

Table A. 3 LT_{50} (-°C) data for each individual of the backcross of F_1 x cmm Each "Rep." represents an average of 3 leaf discs.

Plant identif.	Trait	Rep 1	Rep 2	Rep 3	**Average**	Std.
cmm	CA[a]	10.12	10.18	10.21	**10.17**	0.05
Parent 1	FH[b]	5.72	6.10	6.12	**5.98**	0.23
cph	CA	3.10	3.21	3.17	**3.16**	0.06
Parent 2	FH	2.63	2.38	2.28	**2.43**	0.18
F1[c]	CA	7.45	7.99	8.20	**7.88**	0.39
# 34	FH	3.99	4.03	3.86	**3.96**	0.09
1	CA	8.33	8.53	8.41	**8.42**	0.10
	FH	5.38	5.44	5.00	**5.27**	0.24
2	CA	8.16	8.25	8.41	**8.27**	0.13
	FH	5.36	5.26	5.86	**5.49**	0.32
3	CA	7.93	7.71	7.87	**7.84**	0.11
	FH	4.76	4.82	4.91	**4.83**	0.08
4	CA	8.88	9.17	9.00	**9.02**	0.15
	FH	4.13	4.16	4.21	**4.17**	0.04
5	CA	7.93	7.85	8.00	**7.93**	0.08
	FH	4.76	4.81	4.79	**4.79**	0.03
6	CA	8.53	8.67	8.80	**8.67**	0.14
	FH	6.08	6.11	6.32	**6.17**	0.13
7	CA	3.97	4.00	4.20	**4.06**	0.13
	FH	2.63	2.56	2.60	**2.60**	0.04
8	CA	8.60	9.60	9.73	**9.31**	0.62
	FH	5.05	5.00	5.12	**5.06**	0.06
9	CA	9.20	9.35	9.27	**9.27**	0.08
	FH	3.66	3.74	3.68	**3.69**	0.04
10	CA	7.76	8.00	7.90	**7.89**	0.12
	FH	5.14	5.19	5.25	**5.19**	0.06
11	CA	4.00	4.31	3.6.0	**3.97**	0.36
	FH	2.96	2.68	2.68	**2.77**	0.16

Table A. 3 LT_{50} (-°C) data for each individual of the backcross of F_1 x cmm Each "Rep." represents an average of 3 leaf discs. [Continued]

Plant identif.	Trait	Rep 1	Rep 2	Rep 3	**Average**	Std.
12	CA	10.86	11.00	10.93	**10.93**	0.07
	FH	6.08	6.10	6.21	**6.13**	0.07
13	CA	8.27	8.36	8.33	**8.32**	0.05
	FH	3.78	3.70	3.94	**3.81**	0.12
14	CA	5.40	5.53	5.33	**5.42**	0.10
	FH	4.55	3.58	4.19	**4.11**	0.49
15	CA	7.07	7.21	7.00	**7.09**	0.11
	FH	4.50	4.52	4.64	**4.55**	0.08
16	CA	7.40	7.33	7.45	**7.39**	0.06
	FH	5.36	5.33	5.14	**5.28**	0.12
17	CA	7.46	8.43	8.40	**8.10**	0.55
	FH	5.00	5.12	5.22	**5.11**	0.11
18	CA	7.03	7.13	7.21	**7.12**	0.09
	FH	4.31	4.38	4.26	**4.32**	0.06
20	CA	7.07	7.13	7.00	**7.07**	0.07
	FH	3.56	3.68	3.71	**3.65**	0.08
21	CA	9.33	9.3	9.20	**9.28**	0.07
	FH	6.50	6.42	6.85	**6.59**	0.23
22	CA	8.59	8.8	8.73	**8.71**	0.11
	FH	5.17	5.17	5.05	**5.13**	0.07
23	CA	6.80	6.87	6.73	**6.80**	0.07
	FH	4.14	4.11	4.27	**4.17**	0.09
25	CA	7.87	7.93	7.53	**7.78**	0.22
	FH	5.42	5.28	5.36	**5.35**	0.07

Table A. 3 LT_{50} (-°C) data for each individual of the backcross of F_1 x cmm Each "Rep." represents an average of 3 leaf discs. [Continued]

Plant identif.	Trait	Rep 1	Rep 2	Rep 3	**Average**	Std.
26	CA	7.06	7.86	7.00	**7.31**	0.48
	FH	5.33	5.56	5.62	**5.50**	0.15
27	CA	8.73	8.53	9.20	**8.82**	0.34
	FH	4.95	5.00	5.15	**5.03**	0.10
29	CA	8.25	8.40	8.32	**8.32**	0.08
	FH	5.20	5.00	5.22	**5.14**	0.12
30	CA	6.33	6.27	6.58	**6.39**	0.16
	FH	3.62	3.94	3.58	**3.71**	0.20
31	CA	11.2	11.13	11.07	**11.13**	0.07
	FH	5.61	5.56	5.73	**5.63**	0.09

[a]Cold acclimation potential [b] Frost hardiness [c] Parent for backcross

Table A.4 LT_{50} (-°C) date for each individual of the backcross of F_1 x cph. Each "Rep." represents an average of 3 leaf discs.

Plant identif.		Rep 1	Rep 2	Rep 3	**Average**	Std.
cph	CA[a]	2.87	2.77	2.79	**2.81**	0.053
Parent 2	FH[b]	3.00	2.67	1.83	**2.50**	0.603
cmm	CA	10.12	10.31	10.32	**10.25**	0.113
Parent 1	FH	5.94	6.15	6.15	**6.08**	0.121
F 1[c]	CA	8.25	8.39	8.11	**8.25**	0.140
# 34	FH	3.84	3.89	4.03	**3.92**	0.098
1	CA	3.75	3.95	3.73	**3.81**	0.122
	FH	2.84	2.69	2.81	**2.78**	0.079
2	CA	5.56	5.43	5.51	**5.50**	0.066
	FH	2.89	2.78	2.85	**2.84**	0.056
3	CA	4.31	4.16	4.28	**4.25**	0.079
	FH	3.68	3.54	3.58	**3.60**	0.072
4	CA	4.87	4.75	4.81	**4.81**	0.060
	FH	2.81	3.45	3.91	**3.39**	0.552
5	CA	4.50	4.48	4.52	**4.50**	0.020
	FH	2.79	2.84	2.74	**2.79**	0.050
6	CA	6.31	6.14	6.30	**6.25**	0.095
	FH	3.87	3.71	3.79	**3.79**	0.080
11	CA	5.62	5.08	5.05	**5.25**	0.321
	FH	3.31	3.38	3.09	**3.26**	0.151
12	CA	4.00	3.79	3.82	**3.87**	0.114
	FH	3.18	3.09	3.21	**3.16**	0.062
13	CA	5.44	6.08	6.48	**6.00**	0.525
	FH	4.37	4.48	4.17	**4.34**	0.157
14	CA	4.94	4.77	4.90	**4.87**	0.089
	FH	3.60	3.33	3.81	**3.58**	0.241

Table A.4 LT_{50} (-°C) date for each individual of the backcross of F_1 x cph. Each "Rep." represents an average of 3 leaf discs. [Continued]

Plant identif.		Rep 1	Rep 2	Rep 3	**Average**	Std.
15	CA	4.44	4.29	4.38	**4.37**	0.075
	FH	3.59	3.64	3.42	**3.55**	0.115
16	CA	6.00	6.13	5.87	**6.00**	0.130
	FH	3.55	3.48	3.62	**3.55**	0.070
17	CA	5.06	5.19	5.14	**5.13**	0.066
	FH	4.05	4.15	3.95	**4.05**	0.100
18	CA	3.50	3.67	3.51	**3.56**	0.095
	FH	3.31	3.42	3.23	**3.32**	0.095
20	CA	6.19	6.27	6.11	**6.19**	0.080
	FH	3.00	2.94	2.73	**2.89**	0.142
21	CA	4.00	4.12	3.88	**4.00**	0.120
	FH	3.58	3.41	3.36	**3.45**	0.115
22	CA	4.69	4.48	4.51	**4.56**	0.114
	FH	3.53	3.47	3.59	**3.53**	0.060
23	CA	5.00	5.07	4.93	**5.00**	0.070
	FH	2.71	2.45	2.88	**2.68**	0.217
24	CA	4.81	4.95	4.67	**4.81**	0.140
	FH	3.42	3.54	3.15	**3.37**	0.200
25	CA	4.44	4.23	4.26	**4.31**	0.114
	FH	4.08	4.12	4.04	**4.08**	0.040
26	CA	5.75	5.62	5.88	**5.75**	0.130
	FH	2.81	2.73	2.89	**2.81**	0.080
28	CA	3.69	3.74	3.64	**3.69**	0.050
	FH	2.95	3.11	2.94	**3.00**	0.095
29	CA	6.00	6.10	5.90	**6.00**	0.100
	FH	3.26	3.37	3.15	**3.26**	0.110

Table A.4 LT_{50} (-°C) date for each individual of the backcross of F_1 x cph. Each "Rep." represents an average of 3 leaf discs. [Continued]

Plant identif.		Rep 1	Rep 2	Rep 3	**Average**	Std.
30	CA	4.25	4.17	4.33	**4.25**	0.080
	FH	3.50	3.33	3.58	**3.47**	0.128
31	CA	6.00	6.21	5.79	**6.00**	0.210
	FH	3.63	3.45	3.81	**3.63**	0.180
32	CA	5.69	5.55	5.65	**5.63**	0.072
	FH	3.63	3.58	3.83	**3.68**	0.132
34	CA	4.94	4.87	5.01	**4.94**	0.070
	FH	3.89	3.77	3.86	**3.84**	0.062
35	CA	6.75	6.54	6.57	**6.62**	0.114
	FH	3.79	3.61	3.82	**3.74**	0.114
36	CA	4.50	4.21	4.40	**4.37**	0.147
	FH	4.00	3.89	4.11	**4.00**	0.110
37	CA	4.69	4.48	4.51	**4.56**	0.114
	FH	3.31	3.48	3.14	**3.31**	0.170
38	CA	4.06	3.94	4.00	**4.00**	0.060
	FH	3.66	3.54	3.69	**3.63**	0.079
39	CA	4.62	4.45	4.61	**4.56**	0.095
	FH	3.47	3.36	3.52	**3.45**	0.082
40	CA	5.50	5.37	5.45	**5.44**	0.066
	FH	3.10	3.24	2.96	**3.10**	0.140
41	CA	6.19	6.01	6.16	**6.12**	0.096
	FH	3.37	3.45	3.20	**3.34**	0.128
42	CA	3.50	3.24	3.19	**3.31**	0.166
	FH	3.00	3.11	2.74	**2.95**	0.190
43	CA	4.87	4.62	4.76	**4.75**	0.125
	FH	3.10	2.93	3.12	**3.05**	0.104

Table A.4 LT_{50} (-°C) date for each individual of the backcross of F_1 x cph. Each "Rep." represents an average of 3 leaf discs. [Continued]

Plant identif.		Rep 1	Rep 2	Rep 3	**Average**	Std.
44	CA	3.19	3.21	2.99	**3.13**	0.122
	FH	2.79	2.66	2.83	**2.76**	0.089
45	CA	7.62	6.91	5.90	**6.81**	0.864
	FH	3.45	3.32	3.64	**3.47**	0.161
46	CA	4.75	4.74	4.37	**4.62**	0.217
	FH	3.00	2.93	3.07	**3.00**	0.070
47	CA	3.50	3.28	3.33	**3.37**	0.115
	FH	3.10	2.89	3.16	**3.05**	0.142
48	CA	4.31	4.11	4.15	**4.19**	0.106
	FH	4.00	3.91	4.09	**4.00**	0.090
49	CA	6.00	6.12	5.88	**6.00**	0.120
	FH	3.58	3.64	3.48	**3.57**	0.081
50	CA	5.56	6.18	7.19	**6.31**	0.823
	FH	4.21	4.09	4.42	**4.24**	0.167
52	CA	6.31	6.15	6.29	**6.25**	0.087
	FH	4.05	3.96	4.14	**4.05**	0.090
54	CA	5.50	5.29	5.32	**5.37**	0.114
	FH	3.00	2.93	3.07	**3.00**	0.070
55	CA	3.06	2.96	2.98	**3.00**	0.053
	FH	3.68	3.52	3.84	**3.68**	0.160
56	CA	4.94	4.76	4.73	**4.81**	0.114
	FH	3.50	3.41	3.65	**3.52**	0.121
57	CA	5.00	5.24	5.15	**5.13**	0.121
	FH	3.29	3.12	3.46	**3.29**	0.170
58	CA	6.50	6.18	6.25	**6.31**	0.168
	FH	3.74	3.65	3.83	**3.74**	0.090

Table A.4 LT_{50} (-°C) date for each individual of the backcross of F_1 x cph. Each "Rep." represents an average of 3 leaf discs. [Continued]

Plant identif.		Rep 1	Rep 2	Rep 3	**Average**	Std.
59	CA	4.16	**4.28**	3.95	**4.13**	0.167
	FH	**4.25**	3.42	3.40	**3.69**	0.485
60	CA	5.12	5.21	5.06	**5.13**	0.075
	FH	3.84	3.72	3.87	**3.81**	0.079
61	CA	4.81	4.62	4.82	**4.75**	0.113
	FH	2.50	2.41	2.50	**2.47**	0.052
62	CA	7.10	6.55	6.21	**6.62**	0.449
	FH	3.89	3.97	3.66	**3.84**	0.161
63	CA	4.94	4.74	4.93	**4.87**	0.113
	FH	3.73	3.59	3.72	**3.68**	0.078
64	CA	6.12	6.23	6.01	**6.12**	0.110
	FH	2.55	2.79	2.31	**2.55**	0.240
65	CA	6.00	6.45	5.55	**6.00**	0.450
	FH	3.00	3.12	2.88	**3.00**	0.120
66	CA	5.87	5.77	5.79	**5.81**	0.053
	FH	3.53	3.38	3.5	**3.47**	0.079
67	CA	5.50	5.39	5.22	**5.37**	0.141
	FH	4.18	4.07	4.29	**4.18**	0.110
68	CA	5.37	5.18	5.38	**5.31**	0.113
	FH	3.52	3.44	3.54	**3.50**	0.053
69	CA	5.62	5.38	5.68	**5.56**	0.159
	FH	2.87	2.64	3.10	**2.87**	0.230
71	CA	4.20	4.08	4.29	**4.19**	0.105
	FH	2.74	2.88	2.51	**2.71**	0.187
73	CA	5.25	5.11	5.21	**5.19**	0.072
	FH	3.74	3.86	3.62	**3.74**	0.120

Table A.4 LT_{50} (-°C) date for each individual of the backcross of F_1 x cph. Each "Rep." represents an average of 3 leaf discs. [Continued]

Plant identif.		Rep 1	Rep 2	Rep 3	**Average**	Std.
74	CA	5.40	5.72	5.77	**5.63**	0.201
	FH	3.58	3.71	3.75	**3.68**	0.089
76	CA	5.81	6.04	6.15	**6.00**	0.173
	FH	3.37	3.41	3.15	**3.31**	0.140
77	CA	5.62	5.48	5.58	**5.56**	0.072
	FH	3.81	3.62	3.94	**3.79**	0.161
78	CA	4.75	4.75	4.39	**4.63**	0.208
	FH	3.34	3.21	3.74	**3.43**	0.276
79	CA	4.87	4.73	4.83	**4.81**	0.072
	FH	3.21	3.27	3.06	**3.18**	0.108
80	CA	4.00	3.89	4.11	**4.00**	0.110
	FH	3.00	2.89	3.11	**3.00**	0.110
82	CA	4.62	4.69	4.55	**4.62**	0.070
	FH	3.16	3.20	3.03	**3.13**	0.089
83	CA	5.50	5.28	5.33	**5.37**	0.115
	FH	4.26	4.31	4.21	**4.26**	0.050
85	CA	3.50	3.36	3.46	**3.44**	0.072
	FH	2.18	2.01	2.20	**2.13**	0.104
87	CA	4.22	4.51	4.38	**4.37**	0.145
	FH	3.05	3.21	2.98	**3.08**	0.118
88	CA	4.81	4.97	4.83	**4.87**	0.087
	FH	2.89	3.01	2.71	**2.87**	0.151
89	CA	6.20	6.10	6.27	**6.19**	0.085
	FH	4.31	4.08	4.39	**4.26**	0.161
90	CA	4.56	4.38	4.74	**4.56**	0.180
	FH	3.21	3.13	3.38	**3.24**	0.128

Table A.4 LT_{50} (-°C) date for each individual of the backcross of F_1 x cph. Each "Rep." represents an average of 3 leaf discs. [Continued]

Plant identif.		Rep 1	Rep 2	Rep 3	Average	Std.
91	CA	5.88	6.12	6.00	**6.00**	0.120
	FH	3.00	2.94	3.30	**3.08**	0.193
92	CA	5.56	5.62	5.32	**5.50**	0.159
	FH	3.66	3.75	3.63	**3.68**	0.062
93	CA	5.62	5.37	5.51	**5.50**	0.125
	FH	3.00	3.15	2.85	**3.00**	0.150
94	CA	6.37	6.55	6.40	**6.44**	0.096
	FH	3.84	3.96	3.72	**3.84**	0.120
96	CA	5.00	5.15	5.03	**5.06**	0.079
	FH	3.66	3.01	2.72	**3.13**	0.481
97	CA	4.87	4.99	4.75	**4.87**	0.120
	FH	2.84	2.97	2.80	**2.87**	0.089
98	CA	4.87	4.67	4.89	**4.81**	0.122
	FH	1.47	1.66	2.15	**1.76**	0.351
99	CA	5.62	5.97	5.84	**5.81**	0.177
	FH	4.08	4.21	3.86	**4.05**	0.177
100	CA	4.87	4.68	4.88	**4.81**	0.113
	FH	2.71	2.84	2.49	**2.68**	0.177
101	CA	3.31	3.22	3.40	**3.31**	0.090
	FH	2.56	2.64	2.48	**2.56**	0.080

[a] Cold acclimation potential [b] Frost hardiness [c] Parent for the backcross