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Mapping Freeze Tolerance Quantitative Trait Loci in a *Citrus grandis* x *Poncirus trifoliata* F_1 Pseudo-testcross Using Molecular Markers

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Abstract. Mapping quantitative trait loci (QTL) associated with freeze tolerance was accomplished using a *Citrus grandis* (L.) Osb. x *Poncirus trifoliata* (L.) Raf. F_1 pseudo-testcross population. A progeny population of 442 plants was acclimated and exposed to temperatures of –9 °C and –15 °C in two separate freeze tests. A subpopulation of 99 progeny was genotyped for random amplified polymorphic DNA (RAPD), cleaved amplified polymorphic sequence (CAPS), sequence characterized amplified region (SCAR), and sequence tagged site (STS) markers to produce a linkage map for each parent. Potential QTL were identified by interval mapping, and their validity was corroborated with results from means comparison (*t* test), one-way analysis of variance (F test), and bulked segregant analysis (BSA). Multiple analytical methods provided evidence supporting putative QTL and decreased the probability of missing significant QTL associated with freeze tolerance. QTL with a large effect on freeze tolerance were located on both the *Citrus* and *Poncirus* linkage maps. In addition, clusters of markers with significantly different means between marker present and absent classes indicating minor QTL that contribute smaller effects on the level of tolerance were found on the linkage maps of both species.

Freeze injury is thought to be the most important factor limiting citrus production in Florida (Yelenosky, 1978). The citrus industry in Florida has been forced ever farther south in recent decades due to unusually severe freezing events. The competition between agricultural and human land and water use has intensified with this migration. The majority of commercial citrus grown in the United States is subject to occasional damaging freezes (Soost and Cameron, 1975), but few scion cultivars with improved freeze resistance are in cultivation today. Progress has been made in protecting trees from freeze damage via mechanical means such as continuous overhead irrigation and/or air mixing during freezes and through barriers such as tree wraps. However, these techniques are limited by tree and orchard size and in some instances water supply. The most certain, efficient, and ultimately cost-effective solution to the freeze injury problem is the breeding of new freeze-hardy scion cultivars (Barrett, 1981) and rootstocks. The future of the citrus industry may rely on replanting in areas that are less desirable for human uses and which may be more susceptible to future freeze events. New freeze tolerant citrus cultivars will ease some of the competition for resources in the most desirable climate regions in Florida and in other *Citrus* growing regions.

Freeze hardiness in citrus is considered to be quantitatively inherited and thus controlled by the combined effects of numerous gene loci (quantitative trait loci: QTL) (Cai et al., 1995). Tree-fruit crops are generally not conducive to analysis of such traits due to long juvenility periods, large plant size, and reproductive irregularities (Monet, 1989). A molecular approach for improving a quantitative trait such as freeze tolerance in *Citrus* avoids these constraints. An appropriate experimental design can take advantage of the highly heterozygous nature of a crop like *Citrus* to analyze segregation in the first hybrid generation.

Molecular markers have made mapping genomes of diverse species possible via the generation of large numbers of segregating, phenotypically neutral markers. Markers based on the polymerase chain reaction (PCR) allow for the production of a genetic linkage map quickly and relatively inexpensively. In *Citrus*, random amplified polymorphic DNA (RAPD) primers have provided ample markers for mapping the genome due to the highly heterozygous nature of the genus and related species (Cai et al., 1994; Cristofani et al., 1999) and other PCR based markers have been developed that are useful for genetic mapping (Deng et al., 1997, 2000).

A two-way pseudo-testcross has been used to avoid long generation times in highly heterozygous tree species, including *Citrus*, by maximizing segregation of both marker loci and traits of interest in the F_1 population (Carlson et al., 1991; Cristofani et al., 1999; Grattapaglia and Sederoff, 1994; Hemmat et al., 1994). In this design, a map of each parent is constructed by grouping marker alleles originating from each parent for analysis. However, colinearity between the maps cannot be determined without an intermediary map or co-dominant markers present in both parents. Crossing over between parental genomes does not occur in the first generation thus suitable segregation for map alignment does not occur. Grattapaglia and Sederoff (1994) used the two-way pseudo-testcross population design with RAPD markers to construct maps for *Eucalyptus grandis* Hill ex Maiden and *E. urophylla* S.T. Blake, which were later used for QTL analysis of micropropagation response, stump sprouting ability, and rooting ability (Grattapaglia et al., 1995). A similar F_1 progeny of pine (*Pinus radiata* D. Don) was analyzed with RAPD markers for QTL

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controlling wood production (Emebiri et al., 1997). These studies demonstrate that, despite its limitations, this population design can be used for locating QTL in tree species.

QTL can be located by using various analytical methods, including interval mapping, bulked segregant analysis/evaluation of distributional extremes, marker genotype means comparisons (*t* test), and one-way analysis of variance (ANOVA) (F test) (Staub et al., 1996). Since the advent of molecular markers, interval mapping is the most common approach to mapping QTL. This approach takes advantage of information provided by linkage maps that have a relatively high level of genome saturation in the range of markers every 5 to 20 cM (Lander et al., 1987; Staub et al., 1996). A one-way ANOVA analyzes the variance among the individuals for each marker class to find associations between individual markers and mean trait values; significant associations imply a nearby QTL for a trait of interest.

Bulked segregant analysis (BSA) can be used to identify individual markers associated with a phenotype regardless of the level of map saturation; it is thus a rapid procedure for identifying genetic markers in a specific region of the genome (Michelmore et al., 1991). For the identification of QTL, a comparison of pooled DNA samples from individuals in the distributional extremes of a population segregating for a trait of interest allows for the identification of markers significantly associated with the trait. If the allele frequency or trait means between the marker allele classes at any molecular marker locus differ significantly between the two extreme subpopulations, it is inferred that a QTL influencing the character of interest is located near the marker. BSA and evaluation of distributional extremes in conjunction with interval mapping can be used to evaluate all segregating markers in a population for association with a quantitative trait of interest.

For this study, an F_1 pseudo-testcross population was derived from heterozygous parents that display a large disparity in freeze tolerance; the very freeze-sensitive pummelo, *Citrus grandis* (L.) Osb. and the interfertile, highly freeze hardy citrus relative, *Poncirus trifoliata* (L.) Raf.. In the *Citrus grandis* 'DPI6-4' × *Poncirus trifoliata* 'Rubideaux' pseudo-testcross, the pummelo parent, *C. grandis*, was not expected to contribute to increased freeze tolerance in the progeny. The trifoliate orange, *P. trifoliata*, is an apomictic line that should retain a high degree of heterozygosity at all loci, including those controlling freeze tolerance. Pummelo seedlings are significantly damaged at –9 °C (high temperature), while nucellar seedlings of the trifoliate orange parent display little damage at –15 °C (low temperature). Based on the heterozygosity observed in the progeny with molecular markers, it was expected that the F_1 pseudo-testcross population would show a wide range of variability for freeze tolerance. The objective was to identify QTL associated with freeze tolerance in these species, with the long-term goal of locating and directly manipulating genes responsible through marker assisted selection (MAS) or genetic modification technology.

Materials and methods

Seedlings from the *Citrus grandis* 'DPI6-4' × *Poncirus trifoliata* 'Rubideaux' pseudo-testcross were grown in 6.35 × 25.4 cm Deepots (Hummert International, Earth City, Mo.) for freeze testing. The tests were done when the plants were 18 months old following the procedures outlined by Yelenosky et al. (1993). The trees were first acclimated for 2 weeks with 21 °C day/10 °C night temperatures followed with 2 weeks of 15 °C day/4 °C night temperatures with a 12-h photoperiod for both regimes. After acclimation, the trees were trimmed of new, tender growth in preparation for freezing. The freeze tests were performed in a walk-in cooler modified for low temperatures and controlled by a precision temperature measurement and control system (GEC134S; Gaffney Engineering, Gainesville, Fla.). Deepot trays containing 20 seedlings were placed in insulated boxes molded to the shape of the Deepots. The soil surface was covered with a 2.5 cm layer of vermiculite to prevent damage to the root system. The freeze test began with a holding period of 2 h at 2 °C followed by temperature decrease of 1 °C/h to the desired low temperature. Ice crystals were nucleated in the trees by misting the plants with fine ice particles when the temperature reached –2 °C. When the test temperature was reached, the trees were held for 4 h. The temperature was then returned to 5 °C at 1 °C/h, following which the trees were moved to the greenhouse for eventual damage assessment.

Five trays of 20 seedlings were tested simultaneously in each of five tests (three trays in the fifth test). Each tree was first frozen at –9 °C and allowed to regrow for 3 months in the greenhouse. They were then tested at –15 °C. Stem dieback was measured approximately 1 month post freezing when new buds broke and began to grow from below damaged stem regions and was expressed as a percentage of total stem length. The stem dieback data at both temperatures were used for interval mapping and mean comparison testing.

A subset of 99 individuals from the population was used to produce PCR marker-based maps for interval mapping of QTL associated with freeze tolerance. These included 61 progeny selected at random and 38 progeny from the distributional extremes that were used in bulk segregant analysis. Genomic DNA was isolated as described by Durham et al. (1992) scaled for 1.5 mL microfuge tubes. RAPD primer kits (Operon Technologies Inc., Alameda, Calif.) were initially screened on 10 individuals chosen at random from the population to detect primer activity. PCR products were separated on 2% agarose gels with ethidium bromide staining. Primers that produced reproducible polymorphic products were used to amplify DNA from all 99 individuals. DNA amplification was done following the PCR conditions of Cai et al. (1994). In addition to RAPD markers, sequence-based markers developed for *Citrus* were analyzed for polymorphism in this population. These included sequence-characterized amplified regions (SCAR), cleaved-amplified polymorphic sequences (CAPS), and sequence-tagged sites (STS). Reactions using these markers were modified for use with separate forward and reverse primers with an annealing temperature of 55 °C. Those markers developed for this population are shown in Table 1. Additional primer pairs for SCAR, CAPS, and STS markers developed by Deng et al. (1997, 2000) were also screened for polymorphisms.

The stem dieback data were also used to identify 18 and 20 individuals each from the freeze-sensitive and freeze-tolerant distributional extremes, respectively, for BSA. Two bulk DNA samples from progeny at each extreme were prepared by mixing equal proportions of DNA from each individual in each bulk for RAPD analysis. One hundred RAPD primers were screened on the bulked DNA from the selected individuals to identify RAPD markers associated with the distributional extremes. Markers putatively polymorphic between the bulked samples, but monomorphic within the bulks, were then used to genotype the mapping sub-population for means comparisons and linkage mapping.

Polymorphic RAPD markers were grouped based on their parental origin and tested for goodness of fit to the hypothesized segregation ratio of 1:1 determined by chi-square analysis. Linkage analysis was done using JoinMap 1.3 (Stam, 1993) and interval mapping was done using MapMaker-QTL (Lander et al., 1987) and

Table 1. SCAR[z], CAPS[z], and STS[z] primers developed for use in mapping a *Citrus grandis* x *Poncirus trifoliata* F_1 population.

Marker name	Primer sequence (5' to 3')	Map location	Restriction enzyme (CAPS only)
SCA01057	CAGGCCCTTCTTATGTCATC		
	CAGGCCCTTCACCAAACACG	C1-49	
SCA01055b	CAGGCCCTTCACCAACACGAAC		
	CAGGCCCTTCTTATGTCATCAAAG	C1-62	
SCA01066	CAGGCCCTTCATGTTGAGAG		
	CAGGCCCTTCTCCGGGAAAG	C2-0	
CPG10084	AGGGCCGTCTATGGATTCATGC		
	AGGGCCGTCTCACTTGAAATTGG	C2-27	Rsa I
CPR08037	CCCGTTGCCTCTATAGATGTC		
	CCCGTTGCCTTGACCGCC	C2-60	Rsa I
SCG10085	AGGGCCGTCTATATGACGTGAG		
	AGGGCCGTCTCCAACTTCACATG	C3-62	
SCT12045	GGGTGTGTAGGTGCATAAA		
	GGGTGTGTAGCCTAAAGTC	C3-85	
SCC02047	GTGAGGCGTCAATTCAAAGAAG		
	GTGAGGCGTCCAATGACCTC	C6-0	
STSTHI	CTTCTAAGCAGCAGGGGAAGG		
	CCCATTGGCTGATGCCTCAAC	C8-0	
SCG06053	GTGCCTAACCCAGTGTCTGG		
	GTGCCTAACCTCACGAACACC	P1-0	
SCC05033	GATGACCGCCCCGTCCAAGAG		
	GATGACCGCCCTCGCTTCCTC	P3-8	
CPG06089	GTGCCTAACCACTACTGCAGAG		
	GTGCCTAACCTACCTTGCTAC	P3-40	Rsa I
SCC05020	GATGACCGCCAGGCGATAGC		
	GATGACCGCCCACAAAGCTG	P3-103	
SCC05037	GATGACCGCCAGGCGATAGC		
	GATGACCGCCCACAAAGCTG	P4-35	
SCG06074	GTGCCTAACCCCTGAGTACTTTTG		
	GTGCCTAACCTCTGGATGAATG	P5-84	
SCF05090	CCGAATTCCCAGACGTAAAGC		
	CCGAATTCCCATAAATTGATGTG	P5-111	
SCC02042	GTGAGGCGTCCTCGAACCAG		
	GTGAGGCGTCAACTCTAATCG	P5-135	

[z]SCAR and CAPS markers are denoted with SC and CP, respectively, the Operon primer kit letter and number it was derived from, and the DNA fragment size in 10× base pairs (i.e., SCA01057 = SCAR, Operon Kit A, Primer # 01, Fragment sized 570 base pairs). The STS marker was derived from a thiamine biosynthesis like gene sequence from citrus found in the Genebank EST database and is designated as STSTHI.

MapQTL (Van Ooijen and Maliepaard, 1996). For means comparison, a paired data *t* test was performed according to the procedures of Ott (1988) with a threshold *P* value ≤0.05 for significance. A one way ANOVA (F test) was performed by MapQTL to identify all markers with significant ($P \leq 0.05$) differences between allele classes (Van Ooijen and Maliepaard, 1996).

Results

Freeze tests. The freeze test to –9 °C (high temperature) produced a damage curve skewed towards individuals showing no stem dieback (Fig. 1) with only 71 of 403 (18%) trees tested having stem dieback at this temperature. Freezing at –15 °C (low temperature) produced damage in a more normal distribution but skewed toward those individuals showing >95% damage (Fig. 1). Of the trees tested at –15 °C, 49 of 442 (11%) showed >95% stem dieback, and only 30 of 442 (7%) had dieback of less than 20%. Mean dieback for the freeze-tolerant *P. trifoliata* seedlings was 0% and 8.9% at the high and low test temperature and for freeze-sensitive *C. grandis* seedlings was 96.2% and 100%, respectively. No

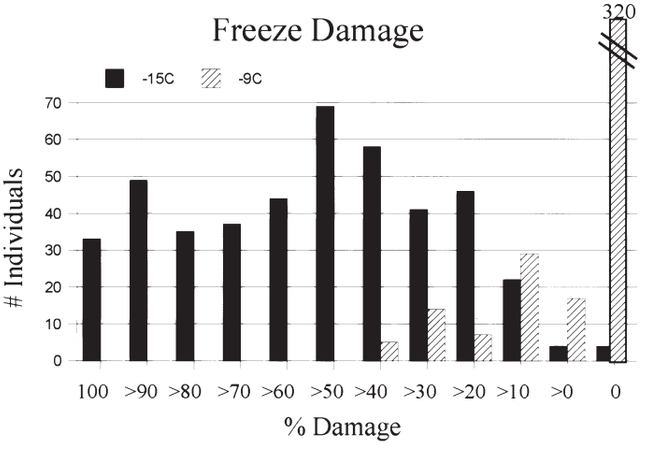

Fig. 1. Distribution of stem dieback at –9 °C and –15 °C in the pseudo-testcross population. Mean dieback for *P. trifoliata* was 0% and 8.9% and for *C. grandis* was 96.2% and 100% at the high and low test temperature, respectively.

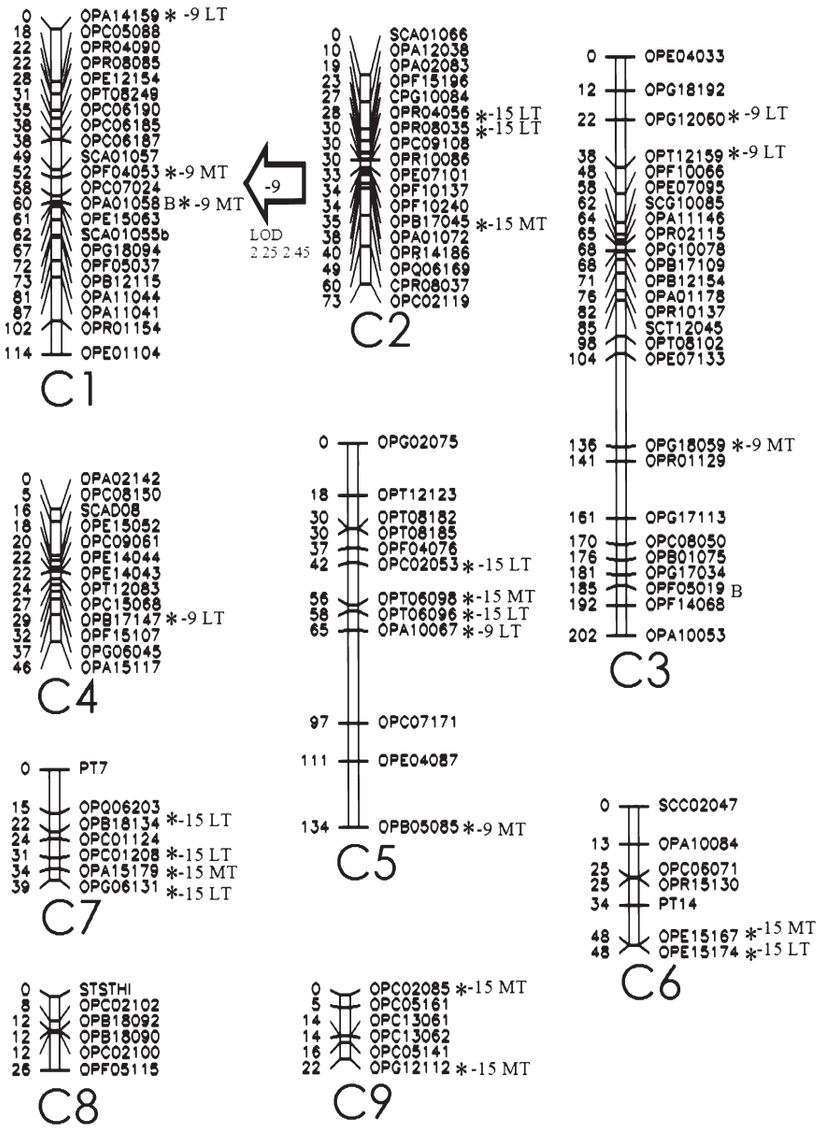

Fig. 2. Linkage map of *Citrus grandis* showing QTL identified with multiple methods. Areas identified by interval analysis are indicated with arrows and the temperature for which a LOD >2.0 was detected. Markers identified through bulked segregant analysis are identified with a **B**. Markers identified using mean comparison or ANOVA have an asterisk (*) and show the temperature at which the difference was found and whether presence of the marker indicated more tolerance (MT) or less tolerance (LT). RAPD, SCAR, and CAPS markers are denoted by the letters OP, SC, or CP followed by the original Operon primer kit letter and number (01-20) and marker size in 10x base pairs (i.e., OPA14159 = Operon Kit A, primer #14, marker size1590 base pairs). The STS markers are denoted by letters or numbers that are representative of their derivation or by previously published designations (Deng et al., 1997,2000). Numbers to the left of each group indicate map distance in cM.

parental or progeny seedlings were killed during the freeze tests as even those with 100% dieback had regrowth from the stem base that was protected by vermiculite.

The 18 trees most freeze damaged at -9 °C (high temperature) were selected for two freeze-susceptible bulks of 10 and 8 trees each and had mean stem dieback at this temperature of 41.0% and 32.3%, respectively. The 20 trees most resistant to freezing at –15 °C (low temperature) were selected for two freeze resistant bulks of 10 trees each and had a mean stem dieback at this temperature of 7.6% and 13.5%, respectively. The freeze-sensitive trees also had significant stem dieback at –15 °C with an average of 75.1% dieback. Of the freeze-tolerant bulks, only one tree showed any stem dieback at –9 °C (#17-35 had 14.6% dieback).

Linkage mapping. In total, 71 RAPD primers and 24 specific primer pairs that produced unambiguous polymorphisms were used to screen the mapping subpopulation. These primers yielded 283 polymorphic RAPD, SCAR, CAPS, and STS markers that could be screened for mapping either the *P. trifoliata* or the *C. grandis* genome. The segregation ratio of 29 of these markers had Chi-squared values that were significantly different from the predicted 1:1 ratio and were not used in the analysis. Core maps for the two parents were constructed using JoinMap 1.3 software (Stam, 1993) at a linkage group log of the odds (LOD) threshold of 3.0.

For the *Citrus* parental map, 132 polymorphic markers originating from the *Citrus* parent were analyzed. The core map developed for *Citrus* consisted of 13 linkage groups plus three marker pairs. Further analysis with JoinMap at a LOD of ≥2 coalesced the map into nine linkage groups which corresponded to the expected number of linkage groups based on the haploid chromosome number for *Citrus*, x = 9. The final *Citrus* map consisted of 117 markers on nine linkage groups with a total map distance of 704 cM and an average distance of 6.0 cM between markers (Fig. 2). The map is made up of 105 RAPD, 7 SCAR, 2 STS, and 3 CAPS markers. Significant linkages were not found for 15 markers.

To produce the *Poncirus* parental map, 122 markers originating from the *Poncirus* parent were analyzed in the same manner as the *Citrus* markers. The final *Poncirus* map consisted of 100 markers on nine linkage groups with a total map distance of 638 cM and an average distance of 6.4 cM between markers (Fig. 3). The map is made up of 89 RAPD, 8 SCAR, and 3 CAPS markers. Significant linkages were not found for 22 markers.

QTL mapping. Mean comparisons (*t* test) and one way ANOVA (F test) produced similar results with many markers showing significant differences ($P \leq 0.05$) in mean damage. On the *Citrus* map, 21 markers with a significant t statistic between the marker present and absent classes were mapped, with six associated with damage at the –9 °C (high temperature) and 15 with damage at –15 °C (low temperature) (Fig. 2). Additionally, a one-way ANOVA located 11 markers with significant F statistics including nine of the same markers identified with the *t* test (Fig. 2). Damage at the –9 °C was associated with four of these11 markers and seven of 11 markers were associated with damage at –15 °C (Fig. 2).

On the *Poncirus* map, 19 markers were identified using a *t* test with 10 associated with damage at –9 °C and 10 with damage at –15 °C (one was associated with both) (Fig. 3). Using a one-way ANOVA, four markers associated with damage at –9 °C and nine

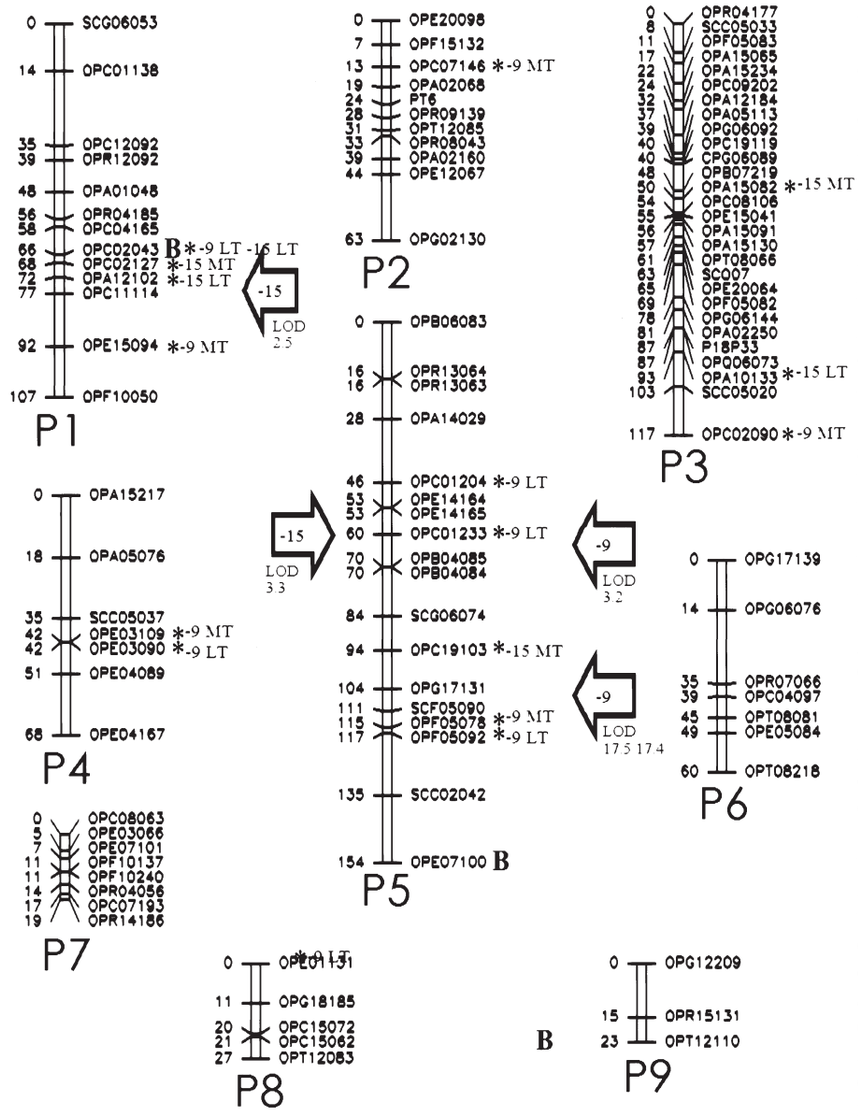

Fig. 3. Linkage map of *Poncirus trifoliata* showing QTL identified with multiple methods. Areas identified by interval analysis are indicated with arrows and the temperature for which a LOD >2.0 was detected. Markers identified through bulked segregant analysis are identified with a **B.** Markers identified using mean comparison or ANOVA have an asterisk (*) and show the temperature at which the difference was found and whether presence of the marker indicated more tolerance (MT) or less tolerance (LT). RAPD, SCAR, and CAPS markers are denoted by the letters OP, SC, or CP followed by the original Operon primer kit letter and number (01-20) and marker size in 10x base pairs (i.e., OPA14159 = Operon Kit A, primer #14, marker size1590 base pairs). Numbers to the left of each group indicate map distance in cM.

associated with damage at –15 °C were identified (Fig. 3). Of these markers, 11 were identified with both methods.

Interval mapping using the stem dieback and segregation data from the mapping sub-population produced LOD scores >2.0 using both MapMaker-QTL (Lander et al., 1987) and MapQTL (Van Ooijen and Maliepaard, 1996). In *C. grandis*, MapQTL and MapMaker-QTL identified the same QTL on linkage group C1 which correlated to damage at –9 °C with peak LOD scores of 2.45 and 2.25 at 52 and 58 cM, respectively. This QTL accounted for 14% of the observed variation (Fig. 2).

With the markers originating from *P. trifoliata,* a QTL associated with damage at –15 °C was located with MapQTL on linkage group P1 at 76 cM with a peak LOD score of 2.5, which explained 17.9% of the variation (Fig. 3). An additional QTL associated with damage at –15 °C was located with MapMaker-QTL on linkage group P5 with a peak LOD score of 3.3 at 60 cM, which explained 6.9% of the variation.

QTL associated with damage at –9 °C were located on group P5 with MapMaker-QTL with peak LOD scores of 3.3 and 17.5 at 62 cM and 107 cM, respectively. MapQTL also identified the QTL at107 cM with an LOD score of 17.4. These QTL accounted for 17.7% and 12.7% of the observed variation, respectively.

All of the QTL intervals mapped were associated with clusters of markers identified through mean comparisons (*t* test) and one-way ANOVA (F test) (Figs. 2 and 3). On the *Citrus* map there was also a cluster of markers with significantly different means between the marker present and marker absent classes in the middle of linkage group C5 and on the end of group C7 (Fig. 2). Another cluster was located on the *Poncirus* map at the top of linkage group P3 (Fig. 3).

With bulk segregant analysis, 11 primers identified putative markers associated with the distributional extremes. The individuals from each bulk population were genotyped for these 11 primers and 17 markers segregating between the groups were analyzed for differences in mean damage at both temperatures between the band present and absent classes. Of these, eight markers were identified with significantly different means between the marker present and absent classes in the bulk populations (Table 2) and five were mapped (Figs. 2 and 3). Marker OPC02043 was located at 66 cM on linkage group P1 near a putative QTL and individuals with this marker were significantly more susceptible to stem damage at both test temperatures. Marker OPE07100 was mapped at 154 cM on linkage group P5 but was not near a putative QTL (Fig. 3). Progeny with this marker had significantly more stem dieback at both test temperatures. Marker OPT12110 mapped to linkage group P9, which had no putative QTL (Fig. 3). Individuals with this marker had significantly less damage at –9 °C. Marker OPA01058 was located at 60 cM near the QTL on linkage group C1 and individuals with the marker were more freeze tolerant at both test temperatures (Fig. 2). Marker OPF05019 mapped to group C3 at 185 cM but was not near any QTL (Fig. 2). Progeny with this marker had significantly less stem dieback at both test temperatures.

Discussion

A large hurdle in evaluating the genetic components of QTL in tree crops is the time needed to produce successive generations such as F_2 or backcross populations for segregation analysis. Using the pseudo-testcross population design we were able to overcome this limitation and map QTL in the F_1 generation. The highly heterozygous parents used here produced a progeny population that

Table 2. RAPD markers identified using bulk segregant analysis showing significant mean stem dieback differences between the present and null classes.

Primer	Marker size (bases)	Mean dieback (%) –9 ºC[z]	Mean dieback (%) –15 ºC[z]	Linkage group	Parental origin
OPA01[y]	580	4.6/24.6**	19.5/51.3*	C1	*Citrus*
OPC02[y]	430	26.8/6.4**	57.1/20.0*	P1	*Poncirus*
OPE03	1100	8.3/22.1*	23.3/45.0NS	Unassigned	*Poncirus*
OPE07	1000	28.7/11.4**	63.1/28.0*	P5	*Poncirus*
OPE15	2036	26.3/8.6**	48.4/22.4NS	Unassigned	*Citrus*
OPE19	2100	26.0/8.9*	60.1/21.0**	Unassigned	*Citrus*
OPF05	190	6.3/20.0*	15.5/46.3*	C3	*Citrus*
OPT12	1100	10.5/25.8*	28.3/53.9NS	P9	*Poncirus*

[z]Marker band present/null.
[y]Also significantly different mean stem dieback between classes in the sub-populations used for mapping
NS,*,**Nonsignificant or significant at $P \leq 0.05$ or 0.01, respectively

segregated widely for freeze tolerance in the F_1 generation. Stem dieback data enabled us to identify QTL with relatively large effects on freeze tolerance ranging from 6.9% to 17.7% of the variation. A total of 30% and 25% of the variation at the high and low test-temperature was explained by two QTL each in the *Poncirus* genome, respectively.

Using more than one method of data analysis allowed us to identify putative QTL and confirm the presence of significant associations. This has been done in other crops such as in pea (*Pisum sativum* L.) in which QTL affecting seed weight were located using interval mapping, BSA, and selective genotyping (Timmerman-Vaughan et al., 1996), and maize (*Zea mays* L.) where analysis of regression coefficients and interval mapping were used in combination to locate QTL associated with resistance to stalk and ear rot (Pè et al., 1993). Additionally, William et al. (1997) employed BSA and one-way ANOVA to locate QTL associated with leaf rust resistance in bread wheat (*Triticum aestivum* L. em. Thell.), and in soybean (*Glycine max* L.) both one-way ANOVA and interval mapping were used in conjunction to investigate QTL controlling reproductive, morphological, and seed traits (Mansur et al., 1993).

For this population of *C. grandis* x *P. trifoliata,* the validity of QTL identified through interval analysis was corroborated with means comparison (*t* test), one-way ANOVA (F test), and BSA (distributional extremes analysis). The interval located on linkage group C1 was identified using two different QTL mapping programs and was associated with markers identified through means comparison, one-way ANOVA, and BSA (Fig. 2). The intervals located on linkage group P5 were identified using MapMakerQTL and MapQTL and also were associated with markers identified through all other methods (Fig. 3). The QTL interval on linkage group P1 was associated with markers identified using means comparison, one-way ANOVA, and BSA. In addition, three regions that were not identified through interval mapping were identified through means comparison and one-way ANOVA. Thus, by utilizing multiple analysis methods, it was possible to increase confidence in the putative QTL located and decrease the probability of missing significant QTL associated with freeze tolerance. However, multiple analysis methods did increase the likelihood that some of the markers with significantly different test statistics were due to chance. Isolated markers with significance may not identify valid QTL but cannot be ruled out without additional data.

In other hybrid populations of *Poncirus* with various *Citrus* species, the level of freeze tolerance remains high in some individuals in advanced populations even as the percentage of *Poncirus* parentage is reduced (Barrett, 1981). The relatively large effects seen by a few QTL in this population can explain such inheritance. Suitable markers associated with freeze tolerance should be obtainable for most hybrid *Citrus* populations for use in MAS to improve freeze tolerance in successive generations based on the information in this study.

Literature Cited

Barrett, H.C. 1981. Breeding cold-hardy citrus scion cultivars, p. 61–66 In: K. Matsumoto (ed.). Proc. Int. Soc. Citricult. Japan Chapter, Intl. Soc. Citricult., Shimizu, Japan.

Cai, Q., C.L. Guy, and G.A. Moore. 1994. Extension of the linkage map in Citrus using random amplified polymorphic DNA (RAPD) markers and RFLP mapping of cold-acclimation-responsive loci. Theor. Appl. Genet. 89:606–614.

Cai, Q., G.A. Moore, and C.L. Guy. 1995. An unusual Group 2 LEA gene family in citrus responsive to low temperature. Plant Mol. Biol. 29:11–23.

Carlson, J.E., L.K. Tulsieram, J.C. Glaubitz, V.W.K. Luk, C. Kauffeldt, and R. Rutledge. 1991. Segregation of random amplified DNA markers in F_1 progeny of conifers. Theor. Appl. Genet. 83:194–200.

Cristofani, M., M.A. Machado, and D. Grattapaglia. 1999. Genetic linkage maps of *Citrus sunki* Hort. ex. Tan. and *Poncirus trifoliata* (L.) Raf. and mapping of citrus tristeza virus resistance gene. Euphytica 109:25–32.

Deng Z., S. Huang, P. Ling, C. Chen, C. Yu, C.A. Weber, G.A. Moore, and F.G. Gmitter, Jr. 2000. Cloning and characterization of NBS-LRR class resistance-gene candidate sequences in citrus. Theor. Appl. Genet. 101:814–822.

Deng, Z., S. Huang, S. Xiao, and F.G. Gmitter, Jr. 1997. Development and characterization of SCAR markers linked to the citrus tristeza virus resistance gene from *Poncirus trifoliata*. Genome 40:697–704.

Durham, R.E., P.C. Liou, F.G. Gmitter, and G.A. Moore. 1992. Linkage of restriction fragment length polymorphisms and isozymes in *Citrus*. Theor. Appl. Genet. 84:39-48.

Emebiri, L.C., M.E. Devey, A.C. Matheson, and M.U. Slee. 1997. Linkage of RAPD markers to NESTUR, a stem growth index in radiata pine seedlings. Theor. Appl. Genet. 95:119–124.

Grattapaglia, D., F.L. Bertolucci, and R. Sederoff. 1995. Genetic mapping of QTLs controlling vegetative propagation in *Eucalyptus grandis* and *E. urophylla* using a pseudo-testcross strategy and RAPD markers. Theor. Appl. Genet. 90:933–947.

Grattapaglia, D., and R. Sederoff. 1994. Genetic linkage maps of *Eucalyptus grandis* an *Eucalyptus urophylla* using a pseudo-testcross: Mapping strategy and RAPD markers. Genetics 137:1121–1137.

Hemmat, M., N.F. Weeden, A.G. Manganaris, and D.M. Lawson. 1994. Molecular marker linkage map for apple. J. Hered. 85:4–11.

Lander, E.S., P. Green, J. Abrahamson, A. Barlow, M. Daly, S.E. Lincoln, and L. Newburg. 1987. Mapmaker: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181.

Mansur, L.M., K.G. Lark, J. Kross, and A. Oliveira. 1993. Interval mapping of quantitative trait loci for reproductive, morphological, and seed traits in soybean (*Glycine max* L.). Theor. Appl. Genet. 86:907–913.

Michelmore, R.W., I. Paran, and R.V. Kesseli. 1991. Identification of markers linked to disease-resistant genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. 88:9828–9832.

Monet, R. 1989. Peach genetics: past, present, and future. Acta Hort. 254:49–57.

Ott, L. 1988. An Introduction to Statistical Methods and Data Analysis. 3rd ed. Boston, Mass. PWS-Kent.

Pè, E.M., L. Gianfranceschi, G. Taramino, R. Tarchini, P. Angelini, M. Dani, and G. Binelli. 1993. Mapping quantitative trait loci (QTLs) for resistance to *Gibberella zeae* infection in maize. Mol. Gen. Genet. 241: 11–16.

Soost, R.K., and J.W. Cameron. 1975. Citrus, p. 507–540. In: J. Janick and J.N. Moore (eds.). Advances in fruit breeding. Purdue Univ., West Lafayette, Ind.

Stam, P. 1993. Construction of integrated genetic linkage maps by means of a new computer package: JoinMap. Plant J. 3:739–744.

Staub, J.E., F.C. Serquen, and M. Gupta. 1996. Genetic markers, map construction, and their application in plant breeding. HortScience 31: 729–741.

Timmerman-Vaughan, G.M., J.A. McCallum, T.J. Frew, N.F. Weeden, and A.C. Russell. 1996. Linkage mapping of quantitative trait loci controlling seed weight in pea (*Pisum sativum* L.). Theor. Appl. Genet. 93:431–439.

Van Ooijen, J.W. and C. Maliepaard. 1996. MapQTL version 3.0: software for the calculation of QTL positions on genetic maps. CPRO-DLO, Wageningen.

William, H.M., D. Hoisington. R.P. Singh, and D. González-de-León. 1997. Detection of quantitative trait loci associated with leaf rust resistance in bread wheat. Genome 40:253–260.

Yelenosky, G. 1978. Freeze survival of citrus trees in Florida, p. 297–312. In: P.H. Li and H. Sakai (eds.). Plant cold hardiness and freezing stress. Academic Press, New York.

Yelenosky, G., D. Hutchison, and H. Barrett. 1993. Freezing resistance of progeny from open-pollinated pummelo x trifoliate orange citrus hybrids. HortScience 28:1120–1121.