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**Mapping Quantitative Trait Loci in the Woody
Perennial Plant Genus Citrus**

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**הספריה המרכזית
למדעי החקלאות
בית דגן**

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- **Cooperation**, briefly explain whether synergistic, complementary or supportive.

The documentable results from this project may look somewhat one-sided on the US side. However, it should be noted that the Israeli part of the project turned out to be much more technically difficult. One of the things we learned as a result of this research is that it is much more difficult to map within the genus Citrus than it is when an intergeneric cross, as was analyzed in the US, is used. This is important information that will be useful in the future, but the immediate result is that Dr. Ben-Hayyim did extensive marker evaluation without being able to identify enough polymorphic marker loci to effectively map in her population. We could not have envisioned this disappointing result before the experiments were done. Dr. Hayyim's extensive knowledge of salt tolerance contributed greatly to the US part of this effort.

Mapping Quantitative Trait Loci in the Woody Perennial Plant Genus *Citrus*

As is true for all crops, production of *Citrus* fruit is limited by traits whose characteristics are the products of many genes (i.e. cold hardiness). In order to modify these traits by marker aided selection or molecular genetic techniques, it is first necessary to map the relevant genes. Mapping of quantitative trait loci (QTLs) in perennial plants has been extremely difficult, requiring large numbers of mature plants. Production of suitable mapping populations has been inhibited by aspects of reproductive biology (e.g. incompatibility, apomixis) and delayed by juvenility. New approaches promise to overcome some of these obstacles. The overall objective of this project was to determine whether QTLs for environmental stress tolerance could be effectively mapped in the perennial crop *Citrus*, using an extensive linkage map consisting of various types of molecular markers. Specific objectives were to: 1) Produce a highly saturated genetic linkage map of *Citrus* by continuing to place molecular markers of several types on the map. 2) Exploiting recently developed technology and already characterized parental types, determine whether QTLs governing cold acclimation can be mapped using very young seedling populations. 3) Determine whether the same strategy can be transferred to a different situation by mapping QTLs influencing Na^+ and Cl^- exclusion (likely components of salinity tolerance) in the already characterized cross and in new alternative crosses. 4) Construct a YAC library of the citrus genome for future mapping and cloning.

Achievements

1) Produce a highly saturated genetic linkage map of *Citrus* by continuing to place molecular markers of several types on the map.

Inter-simple sequence repeat markers were placed on the previously characterized *Citrus* x *Poncirus* backcross map. These 75 markers allowed further characterization of linkage relationships on this map.

In related collaborative work not directly supported by this grant, AFLP markers were developed on this map. There are now a total of 680 marker loci on this map.

Two genetic linkage maps were produced for a *Citrus grandis* x *Poncirus trifoliata* pseudo-testcross population using molecular markers, one for each parent.

The map for the citrus parent is the first map based solely upon markers derived from *Citrus* and not mixed with markers from *Poncirus*.

Sequence-based PCR markers including sequence-characterized amplified regions (SCAR), cleaved amplified polymorphic sequences (CAPS), and sequence tagged sites were designed for use in diverse citrus populations.

2) Exploiting recently developed technology and already characterized parental types, determine whether QTLs governing cold acclimation can be mapped using very young seedling populations.

An F₁ pseudo-testcross population of 500 seedlings was derived from heterozygous parents that display a wide disparity in cold tolerance, the very cold-sensitive pummelo, *Citrus grandis*, and the interfertile highly cold hardy citrus relative *Poncirus trifoliata*.

A highly accurate freezing chamber was designed.

As a result of freezing tests, potential QTLs for freezing tolerance were located on both the *Citrus* and *Poncirus* maps.

Several methods for detecting QTLs were evaluated and found to be in general agreement.

The mapping of QTL in this population indicates there is one major QTL or group of QTL located in *Poncirus* that has a large effect on the level of cold tolerance seen. In addition 3-5 or more QTL contribute a smaller effect on the level of tolerance.

3) Determine whether the same strategy can be transferred to a different situation by mapping QTLs influencing Na⁺ and Cl⁻ exclusion (likely components of salinity tolerance) in the already characterized cross and in new alternative crosses.

In the already characterized BC population:

A large data set that is still being analyzed was generated.

A total of 38 traits related to different tissue or whole plant Na⁺ and/or Cl⁻ accumulation were analyzed, resulting in the location of 73 potential QTLs with LOD scores >3.0 on the linkage map. More than half of these QTLs were associated with salinity.

Further analysis of these loci yielded the identification of 17 regions of the citrus genome of potential interest; 8 regions may contain QTLs of large effect and the remainder are worthy of further study.

Similar results were obtained when morphological traits and the accumulations of other minerals were analyzed.

In the Pommelo x Cleopatra population:

It was determined that Na and Cl accumulation are not correlated.

Likewise, Na and K accumulation were not correlated.

Cl content was not necessarily correlated with growth., although high Cl and Na content often was associated with retarded growth.

Progeny that were able to grow vigorously under salt stress were identified.

Progeny plants that potentially have more salt tolerance than Cleopatra were identified and are being field-tested.

Mapping efforts were stymied by the very low levels of polymorphism in molecular marker alleles in this cross, despite extensive analysis.

4) Construct a YAC library of the citrus genome for future mapping and cloning.

After evaluating the experiences of others attempting to construct large insert cDNA libraries, we decided to construct a BAC (bacterial artificial chromosome) library instead of trying to construct one in yeast; there are advantages in insert integrity and in clone maintenance.

Background

Citrus production is limited now, and threatened to a greater degree in the future, by the abiotic stresses caused by saline irrigation water and soils and/or low temperatures. Most researchers who study stress in plants specialize in only one stress. This is undesirable because 1) plants are typically exposed to more than one stress at a time; 2) germplasm lines used as gene sources may exhibit tolerance to more than one stress; and 3) it is generally accepted that tolerance to various stresses may be at least partly controlled by common physiological and molecular mechanisms. We have been interested for some time in the genetic and physiological responses of plants, and particularly *Citrus*, to stresses induced by salt and/or cold. We have two working hypotheses: 1) There are molecular responses in common upon exposure to high salinity or low temperature in *Citrus*, as have been found in a number of model systems. 2) Both of these traits are quantitative in nature, but it is not unreasonable to expect that some associated genes have relatively large phenotypic effects, and that it should be possible to map these genes. Locating quantitative trait loci (QTLs) positively influencing tolerance to these stresses on a relatively saturated linkage map of *Citrus* would provide breeders with the powerful option of map-based selection for these traits at a very young seedling stage thereby accelerating the rate of progress. It would also be the first step towards map-based cloning of these genes, using YAC libraries and chromosomal walking or landing methods.

In addition, it is very difficult to map QTLs in perennial plants. Analyses have been hampered by difficulties in producing appropriate mapping populations. Large plant size limits the size of populations that can be produced and maintained. It is also much more difficult to do the necessary experimental manipulations (e.g. cold-acclimation, freezing studies) with large plants. Long juvenile periods impede progeny testing for many important crop traits. Finally, replication can only be achieved by vegetative propagation, which requires large amounts of labor, greenhouse space, and long intervals of time. Therefore, a second consideration of this project was to ask: **How can mapping in perennial plants be made more efficient?** Possibilities included using new mapping programs with more robust statistical capabilities, developing new marker types, testing alternative mapping populations, designing more efficient assays, and using large populations but selective genotyping. We have tested all of these possibilities.

Methodologies and Materials

Mapping populations: Three mapping populations were used in these studies. In the US, two intergeneric populations were studied that involved extremely cold-sensitive, monoembryonic *Citrus grandis* and extremely cold hardy *Poncirus trifoliata*. A previously created population consisted of 60 BC₁ progeny from the cross *Citrus grandis* (L.) Osb. cv. Thong Dee x [*C. grandis* x *Poncirus trifoliata* (L.) Raf. cv. Pomeroy]. This will be referred to as the BC population and was used in studies on salt tolerance. To study cold tolerance, the same parents were crossed, but the initial hybrids from the cross (the 'F₁') were evaluated. This is referred to as a "pseudo-testcross" (PT population), and 500 progeny from this cross were used to study cold tolerance. The third population, constructed in Israel, was from an intragenetic cross of Pommelo x Cleopatra mandarin, to be referred to as the PC population. Seeds of the cross, Cleopatra and open pollinated Pommelo were collected in early November and were germinated the following February. At a size of about 10 cm, 500 seedlings of the cross, about 400 seedlings of Pommelo, and 200 seedling of Cleopatra were transferred in April to the greenhouse and grown in 3 liter pots containing sand soil, 3 plants per pot.

Molecular marker development: Genomic DNA was extracted from 60 progeny plants from the BC₁ population and 61 plants from the PT population for development of molecular markers. Primers to develop inter simple sequence repeat (ISSR) markers (Zietkiewicz et al. 1994) were purchased from the University of British Columbia (UBC Set#9), primers for RAPD development (Cai et al. 1994) were purchased from Operon Corporation, and primers for specific sequences were obtained from custom-primer agencies. PCR reactions, gel electrophoresis, and analyses of bands were carried out as described by Cai et al. (1994) with slight modifications. Methods were similar for the PC population, with primers obtained from Operon and the Horticulture Department in Geneva, NY used.

QTL studies: Salt tolerance Clonally propagated rooted shoots of parental plants [*P. trifoliata* (L.) Raf. cv. Pomeroy, *C. grandis* Osb. cv. Thong Dee, their F₁ (*C. grandis* X *P. trifoliata*)] and 48 BC progeny plants were maintained in a polyhouse where all of the experiments were conducted. One month prior to the initiation of the experiment, 12–18 month old rooted clones were each pruned to either two shoots in *C. grandis*, the F₁, and BC₁ progeny or three shoots in *P. trifoliata*,

and maintained in this manner throughout the 16 week long treatment. A total of eight clones for each parental type and four to 16 clones for each BC₁ progeny plant were used in the experiment. Some of the original progeny from the mapping population were eliminated from the analysis either because it was not possible to propagate adequate numbers of clones, or because the ramets varied so much in size and age that it was thought that averaging the measurements would not give a true estimate of mineral accumulation. Plants were irrigated with either tap water (control) or saline water (40 mM NaCl) as needed. At the end of the experiment, entire plants were harvested and separated into four tissues: leaves, stems, structural roots (≥ 2 mm in diameter), and fine roots (< 2 mm in diameter). All tissues were dried in an oven at 65° C. After dry weight measurements were recorded, mineral analyses were conducted individually on homogeneous subsamples of Wiley Mill processed tissues using standard procedures.

For the PC population, at the beginning of June, when plants were established, irrigation with salt 600 mg Cl was begun, and the level was elevated to 900 mg Cl in August. Irrigation water was supplemented with salt in a ratio of Na:Ca of 2:1. All the seedlings of the cross were irrigated with salt, while half of the Cleopatra and Pommelo seedling were irrigated with salt and the rest served as controls. Towards the rainy season, at the end of October, irrigation with salt was halted and the seedlings were analyzed for various parameters including ion analyses, size and DNA.

Freeze tests: Freeze testing was done on acclimated 18 month old seedlings following the procedures outlined by Yelenosky et al. (1993). The freeze tests were performed in a computer-controlled walk-in chamber especially designed to achieve low temperatures (Gaffney Engineering, Gainesville, Florida). Conetainer trays containing 20 seedlings were placed in insulated boxes molded to the shape of the conetainers, and the soil surface was covered with a 1-inch layer of vermiculite to prevent damage to the root system, since the root system of a citrus tree does not acclimate and is vulnerable to freezing temperatures. Five trays of 20 seedlings were tested simultaneously; until all 500 seedlings were tested. Preliminary tests determined that -9°C could be used to identify individuals especially susceptible to cold, and that -15°C could be used to identify the most tolerant individuals. Therefore the trees were first frozen at -9°C, allowed to regrow for 3 months, and then retested 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. The stem dieback data at both

temperatures was used for interval mapping and mean comparison testing with the mapping subpopulation. It was also used to identify individuals at the distributional extremes for BSA.

Statistical Analyses and Mapping: Standard methods were used for statistical analyses, analyses of molecular markers and map construction, and QTL mapping. maps were constructed using MapMaker and/or JoinMap version 2.0. To identify putative QTLs, analyses were performed using both interval mapping (Lander and Botstein, 1989) and composite interval mapping (Zeng, 1993, 1994) methods as implemented by the MAPMAKER/QTL version 1.1b (Lincoln et al., 1993) and QTL CARTOGRAPHER (Basten et al., 1994; Basten et al., 1998) computer programs, respectively.

BAC Library Preparation:

Preparation of protoplasts: Callus grown in liquid medium was washed and incubated in 0.7 M mannitol, 0.2% macerozyme, 0.3% cellulase and 0.1% driselase at 26° C overnight. Then it was filtered through NITEX 50 and 34 µm and spun at 500-700 rpm for 5 min. The pellet was washed 3 or 4 times with mannitol 0.7 M and resuspended at 2×10^8 in 5 ml of 1 M sorbitol, 0.1 M sodium citrate and 60 mM EDTA, pH 7.0.

DNA preparation: Protoplasts were mixed with an equal volume of 1% low melting point agarose and aliquots of 200 µl were put in a mold to let them solidify at 4° C for 10 min. The solidified plugs were transferred into 2-3 volumes of ESP (1% sarkosyl, 0.5 M EDTA, pH 9.3, 3 mg/ml pronase) and incubated 24 h at 50° C with slow agitation. Plugs were washed six times, one hour each, with TE on ice. The first three washes included 0.1 mM PMSF to inactivate pronase. Half a plug (100 µl) was first pre-incubated with 1X *Hind*III buffer, 100 µg/µl BSA, and 4 mM spermidine in a final volume of 200 µl on ice for 20 min. Then, 4 units of *Hind*III were added and the incubation continued on ice for an additional 20 min to allow the enzyme to diffuse into the plugs. For partial digestion, the mixture was incubated at 37°C in a water bath for 5 min and the reaction was stopped by the addition of 1/10 volume of 0.5 M EDTA, pH 8.0, on ice.

Size selection of the DNA: The plugs with partially restricted DNA were submitted to pulse field gel electrophoresis (PFGE). The plugs were loaded on a 1% low melting point agarose gel and run at 200 V with ramping switching time from 1 to 25 sec, for 24 h at 15° C. The band

corresponding to a size of 100-300 kb was cut out of the gel (Figure 1) and the agarose was digested by gelase. The clear solution was dialyzed against TE buffer overnight at 4° C.

Isolation and preparation of the vector: The vector pBeloBAC II was given to us by Dr. Rod Wing, and it contains the lacZ gene which was inserted into the vector pBeloBAC 108L. A single colony of *E. coli* DH10B harboring this vector was inoculated into 10 ml of LB medium and cultured for 5 h, whereupon the culture was transferred into 500 ml of LB medium. The inoculum was grown for about 16 h at 37° C until it reached an OD600 of 1.3-1.5. The plasmid was isolated with the PROMEGA wizard maxi kit. The plasmid pBeloBAC II was completely digested with *Hind*III for 3 h and then dephosphorylated (Figure 2). The ligation was carried out in a volume of approximately 100 µl at 16° C overnight with T4 DNA ligase. Before transformation, the ligation was dialyzed against TE.

Transformation: Transformation of competent *E. coli* DH10B cells was carried out by electroporation at the following settings: voltage, 1.8 kV; voltage booster resistance, 200 Ohms; capacitance, 25 mFD. Competent cells (40 µl) were mixed with 1 µl of ligation reaction for each electroporation. After electroporation, cells were transferred to 1 ml SOC medium (Maniatis) and incubated at 37° C for 1 h. The cells were spread on LB plates containing 12.5 µg/ml chloramphenicol. Plates were incubated at 37° C for 24 h. Colonies that grew on this medium were then replicate plated onto a medium containing chloramphenicol, X-gal and IPTG, 12.5, 40 and 0.072 µg/ml, respectively. White colonies possibly containing citrus DNA inserts were picked to a new LB plate for a second color screen. Colonies that were white for the second time were picked and grown in a liquid medium. The plasmids from these colonies were purified by alkali lysis. Preliminary results of poorly resolved agarose electrophoresis show that *Not*I digested plasmids of at least 3 colonies contain DNA inserts, but their real size is not clear yet.

Results

1) Produce a highly saturated genetic linkage map of *Citrus* by continuing to place molecular markers of several types on the map. Inter-Simple Sequence Repeat (ISSR) analysis was evaluated for its usefulness in generating markers to extend the genetic linkage map of *Citrus* using the BC population that had previously been mapped with RFLP, RAPD and isozyme markers. ISSR markers were obtained through the simple technique of PCR analysis,

using simple sequence repeat (SSR) primers. Optimization of reaction conditions was achieved for 50% of the SSR primers screened, and the primers amplified reproducible polymorphic bands in the parents and progeny of the BC population. Mendelian segregation of the polymorphic bands was demonstrated, with an insignificant number of skewed loci. Most of the SSR primers produced dominant loci; however co-dominance was observed with loci derived from three primers. A new genetic map was produced by combining the segregation data for the ISSR markers with that of the other markers. A total of 75 ISSR markers and 235 of the markers from the previous linkage map were placed on the final version of the map. The incorporation of ISSR markers into the genetic linkage map demonstrates that ISSR markers are suitable for genetic mapping in *Citrus*. The new map has an improved distribution of markers along the linkage groups with fewer gaps and marker order showed partial or complete conservation in the linkage groups.

Two genetic linkage maps were produced for the PT cross, one for each parent, using a random sub-population of 61 individuals. The final *Citrus* map consists of 105 markers with a total map distance of 655 cM and an average distance of 6.2 cM between markers. The *Poncirus* map consists of 88 markers with a total map distance of 587 cM and an average distance of 6.7 cM between markers.

Sequence-based PCR markers including sequence-characterized amplified regions (SCAR), cleaved amplified polymorphic sequences (CAPS), and sequence tagged sites were designed using information from the markers developed in these 2 maps. These markers were then used in initial efforts to join the mapping populations used in these studies.

2) Exploiting recently developed technology and already characterized parental types, determine whether QTLs governing cold acclimation can be mapped using very young seedling populations.

Previous attempts to collect accurate and repeatable freezing data were unsuccessful because there was too much variability in the various assays evaluated. Therefore, a highly accurate computer-controlled freezing chamber was designed. Freeze testing was done on all 500 acclimated 18 month old seedlings. The freeze test to -9°C produced a damage curve skewed towards individuals showing no stem dieback; only 71 of 403 (18%) of the trees tested had any stem dieback at this temperature. Freezing at -15°C produced damage in a more normal

distribution skewed toward those individuals showing > 95% damage. 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%. The mean stem dieback at -9°C for the cold-sensitive parent, *C. grandis*, was >95%, and the mean stem dieback at -15°C for the cold-resistant parent, *P. trifoliata* was <10%. No parental or F₁ seedlings were killed during the freeze tests as even those with 100% dieback had regrowth from below the stem level protected by vermiculite. The mean dieback in the mapping subpopulation at -9°C was 3.8%, and the mean dieback at -15°C was 56.6.

The 10 trees most cold damaged at -9°C selected for the freezing susceptible bulk had a mean of 34% stem dieback at this temperature. The ten trees most resistant to freezing at -15°C selected for the freezing resistant bulk had a mean stem dieback of 7.4% at this temperature. The cold-sensitive trees all had significant stem dieback at -15°C with an average of 69% dieback. Of the cold-tolerant bulk, only 1 tree showed any stem dieback at -9°C.

Interval mapping using the quantitative data and segregation data from the mapping subpopulation produced significant LOD scores using both MapMaker-QTL and MapQTL. With the markers originating from *C. grandis* (i.e. the band was present in *C. grandis* and not in *P. trifoliata*), two overlapping intervals correlated to damage at -9°C were identified on linkage group C1 with peak LOD scores of 2.25 and 2.45 using MapQTL and MapMaker-QTL, respectively. Another interval associated with damage at -15°C was located on linkage group C5 with a peak LOD score of 1.8 using MapQTL. With the markers originating from *P. trifoliata*, a QTL was located with MapQTL on linkage group P1 with a peak LOD score of 2.5 associated with damage at -15°C. On linkage group P5, three overlapping intervals associated with damage at -9°C were located with MapMaker-QTL with peak LOD scores of 3.25, 19.46, and 17.5, and two overlapping QTL intervals in the same region with LOD scores of 1.8 and 17.4 were identified using MapQTL.

Mean comparisons and one way ANOVAs produced similar results in that many of the markers showing significant association with quantitative data were identified with both methods. On the *Citrus* map, 21 markers with significantly different means for the marker present and absent classes were mapped with 6 associated with damage at the higher temperature and 15 with damage at the lower temperature. Eleven markers were located with a one-way ANOVA and 9 were the same markers located through mean comparisons. Four were associated

with damage at the higher temperature, while 7 were associated with damage at the lower temperature.

On the *Poncirus* map, 19 markers were identified using mean comparison analysis with 10 associated with damage at -9°C and 10 with damage at -15°C (1 was associated with both). Using a one-way ANOVA, 4 markers associated with damage at -9°C and 9 associated with damage at -15°C were identified. Eleven of these markers were located with both methods.

All of the intervals mapped were associated with clusters of markers identified through mean comparisons and one-way ANOVA. On the *Citrus* map there was also a cluster of markers with significantly different means between the marker present and marker absent classes on the end of linkage group C7 and on the *Poncirus* map a similar region was identified at the top of linkage group P3.

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, 8 markers were identified with significantly different means between the marker present and absent classes in the bulk populations and 5 were mapped. Five of these markers showed significantly different means in the bulk populations between the classes at both temperatures tested. Marker OPC02043 was located on the map near the interval identified on linkage group P1, and marker OPE07100 was mapped near the intervals placed on linkage group P5. Marker OPT12110 mapped to linkage group P9. Marker A01058 was located in the interval placed on the linkage group C1 and marker OPF05019 mapped on group C3.

3) Determine whether the same strategy can be transferred to a different situation by mapping QTLs influencing Na⁺ and Cl⁻ exclusion (likely components of salinity tolerance) in the already characterized cross and in new alternative crosses.

In the already characterized cross:

Prior to QTL studies in the BC population, physiological studies were done with the parents. The effects of salinization with 0, 30, 60, 90, and 120 mM sodium chloride (NaCl) on *Poncirus trifoliata* (L.) Raf. cv. Pomeroy were studied by means of stem elongation patterns, whole plant and tissue

mass production, and mineral nutrient accumulation. The 11 elements analyzed in leaf, stem, structural root and fine root tissues included Na, Cl, P, K, Ca, Mg, Mn, Fe, Cu, Al, and Zn. At the end of the 12-week experimental period, shoot length was reduced 30 to 80% in the 30 to 120 mM NaCl treatments. The linear relationship found between stem elongation and salt concentrations and different tissues and salt concentrations suggests that a 40-60 mM NaCl concentration is optimal to test *P. trifoliata* or its progeny for salt stress. Root production was found to be continuous and plants apparently used this process as an avoidance mechanism to remove excess ions and delay onset of ion accumulation in this tissue. This phenomenon, designated "Fine Root Turnover", is unique to *P. trifoliata* and may be used as a genetic resource to improve *Citrus* for salinity tolerance through intergeneric hybridization. Plants were able to delay accumulation of Na ions in leaves but not Cl ions, resulting in high Cl accumulation in leaves and accumulation of both ions in fine roots. The data suggested that, while Cl ions were more toxic in leaf tissues, Na ions were at least as toxic in fine root tissues. Among other nutrients, K was affected the most in response to salinity; decreasing within root tissues and increasing in leaf tissues with increased salinization. A similar phenomenon was observed for P levels in salinized tissues.

In another study, salt stress responses of *C. grandis* L. (Osborne), *P. trifoliata* (L.) Raf and their F₁ were investigated. Growth, growth rates, as well as leaf, stem, structural root (> 2mm diameter), fine root (≤ 2mm diameter) and whole plant dry masses were determined for the three genotypes tested in 0, 40 and 80 mM NaCl environments for 20 weeks. *P. trifoliata* and *C. grandis* plants were phenotypically distinct and their F₁ had features that were a combination of both parents. The different growth habits resulted in significant differences between the net growth and growth ratios ($[\text{net growth}/\text{initial growth}] \times 100$) of the three genotypes and between control and salinized plants within each genotype. The average growth and dry weights of nearly all tissues were reduced in salinized plants compared to those of control plants. The exceptions were the fine roots of *P. trifoliata* at both salinities and of the F₁ plants at 40 mM NaCl. The 40 mM NaCl treatment stimulated fine root production in *P. trifoliata* plants, significantly increasing dry weight by 30% compared to control plants. Average shoot dry weight reduction was greatest in *C. grandis* and least in *P. trifoliata* in the 40 mM NaCl treatment. While leaf tissues of *P. trifoliata* were the most sensitive to salinity, root tissues were the most sensitive in *C. grandis*. To avoid salt accumulation, *P. trifoliata* plants increased g/m

root dry mass while *C. grandis* plants increased g/m leaf mass. These traits appeared to be heritable, since the F_1 plants displayed responses intermediate to its parents leading to increased salinity tolerance. We suggest that not only ion content of leaf tissues but ion content and mass production of all tissues should be considered when the salinity tolerance of *Citrus* and related genera is characterized.

The effects of salinization with 40 mM sodium chloride on the BC progeny population [*C. grandis* x (F_1)] were investigated by analyses of mineral accumulation and morphological changes in the progeny and QTL mapping. This generated a huge data set that is still being analyzed. For example, a total of 38 traits related to different tissue or whole plant Na^+ and/or Cl^- accumulation was analyzed in the salinized and non-salinized BC progeny clones. First mapping analyses resulted in a total of 73 potential quantitative trait loci (PQTL) with LOD scores ≥ 3.0 located on the previously generated linkage map. Fifty-three percent of the mapped PQTLs were for traits associated with salinity. By considering LOD scores, map locations, and correlation analyses of the traits, it was possible to identify 17 regions of the citrus genome of interest; eight of them may contain genuine QTLs of large effect and nine regions are worthy of further study. Correlation analyses and locations of PQTLs indicated that many traits were controlled by fewer genes than the actual number of QTLs mapped for them e.g., 21 PQTLs mapped for Na^+ accumulation and Cl^-/Na^+ ratios were located in a cluster at the beginning of one linkage group (LG) while 10 PQTLs mapped for Cl^- accumulation and Cl^-/Na^+ ratio were located in a cluster at the beginning of another LG. Similar results were obtained when morphological traits and the accumulations of other minerals were analyzed. Four papers have been published thus far, 2 are submitted, and others are in preparation.

Analyses in the Cleopatra x Pummelo population

The choice of the two parents was based on one common parent, Pummelo, with the existing cross of *Poncirus trifoliata* x Pummelo described above, and a known salt-tolerant partner, Cleopatra mandarin. Pummelo was chosen because it is mono-embryonic, whereas *Poncirus* is not, and Cleopatra mandarin has been studied and found as a salt-tolerant cultivar, especially in excluding chloride ions under mild salt stress. Thus, a cross was done between these two species.

We analyzed 200 plants of the cross and about 10 of the parents for ion content and their size after the first period of irrigation. Because of the density of the plants in the greenhouse we

had to cut the plants to allow their growth during the second year. We repeated the ion analysis after the second year irrigation with salt, which lasted between May and October. The results presented in Table 1 (see Appendix) represent the data from the first year, but the ion analyses of leaves harvested after the second year of irrigation with salt were essentially the same.

The data presented in Table 1 show:

1. Accumulation of Na and Cl is not necessarily correlated. For example, plant 3a had high Na content and relatively low Cl content, whereas plant 82b had a high Cl content and relatively low Na content.
2. High Na content is not correlated with low K content, see plants 54a, 57b, 80a and 81c.
3. High Cl content is not correlated with size, i.e. growth. For example plants 5c, 75a, 81c, 82b, 84b and 88b.
4. In most cases retarded growth is correlated with both high Na and high Cl content, but plant 78a had low Na and high Cl content, whereas plant 54a had high Na and relatively low Cl content.
5. Plant height of several of the seedlings of the cross under salt stress was often smaller than that of both parents grown under stress, but some of the seedlings had a very high vigor even compared with the parents irrigated with control water.

RAPD markers

We have utilized DNA extracted from both parents, Cleopatra (C) and Pommelo (P), with random sequences of oligonucleotides for PCR products. We used primers obtained from Operon and primers obtained from the Horticulture department in Geneva, NY. We have analyzed more than 150 different primers in order to obtain consistent differences in the pattern of bands obtained with the two extracts of DNA. As presented in Table 1, only a very few primers showed a reproducible variation between the parents, although some primers showed differences in more than one band.

During the period of this proposal, we analyzed about 80 plants of the population of the cross, 50 of them were carried through all the primers that showed variability. The results we obtained are very poor. We tried to include SCAR markers in this study to improve the variability, but

none of the SCAR markers received from the lab in Florida showed reproducible differences between the parents.

Our conclusions from this part are:

1. Genetic variation between Cleopatra mandarin and Pommelo are not enough for this kind of study employing RAPD markers. It may be that this is true only for the pair of parents chosen for this study, but it may be that this type of study cannot be done with two citrus species and the use of a related species, such as *Poncirus*, as one parent is essential.
2. None of the primers we have used can point to any PCR product related to either plant growth rate, sodium or chloride ion accumulation, or loss of potassium.

Ion content in salt-treated cross of Cleopatra x Pommelo

The distribution of sodium, potassium and chloride ions in the seedling of the cross are presented in Figure 1 (Appendix). It is of interest that essentially 2 groups of plants have been obtained in respect to potassium content, each representing the content of the parents. Only a very few seedlings had a higher or lower potassium content. This observation is not valid when analyzing the population for either sodium or chloride content in the leaves. Sodium content of the parents is at the 2 extreme levels in the figure, whereas most plants had a lower sodium content than Pommelo, with values more close to the low content in Cleopatra. Chloride content of most plants was between the values of the parents, with a higher number of plants more close to Cleopatra than Pommelo. It is of interest that some plants had chloride content lower even than Cleopatra.

The data show again that sodium accumulation and loss of potassium may not be related, as mentioned before for individual plants. The most practical benefit of this cross is that it has a potential of producing plants with higher salt tolerance than Cleopatra and with a much better vigor. The selected plants have been transferred to the orchard, grafted, and they will soon be evaluated under irrigation with salt for potential new rootstocks.

4) Construct a YAC library of the citrus genome for future mapping and cloning.

We have obtained some white colonies with putative DNA inserts, but we need to run PFGE to verify the real size of these inserts. We can see on the PFGE figure that we do not have

good resolution above 260 kb. The DNA seems to be of good size, but we need to use better markers (also above 260 kb) and another running protocol to have better resolution between 100 and 500 kb.

Discussion

One of the things investigated in these studies was *selective genotyping*. This involves growing a large number of progeny, testing their phenotypes for the dependent variable(s) in question (in our case cold tolerance or Na^+ or Cl^- exclusion), but only genotyping those progeny plants whose phenotypes differ significantly from the mean. These extreme progeny are the most informative ones. By growing larger populations and assaying them for the dependent variable(s), but only genotyping the extreme progeny, a considerable savings in labor and expense can be achieved. Although there are some attendant disadvantages to this strategy, it has been successful in practice. Other similar basic strategies have been used or proposed to detect or fine map QTLs; e.g. *bulk segregant analysis (BSA)*. In these methods DNA samples from 2 phenotypic classes of individuals are pooled (bulk) separately and used to identify or map a target gene by screening the 2 bulked samples for marker loci that display polymorphism and so are linked to the gene of interest (unlinked markers should segregate at random in the bulked individuals). Although this procedure has been used primarily to map single genes of large effect, e.g. ones for disease resistance, it has also been used to target and add additional markers to sparsely populated areas of a linkage map and we have found in this research that the technique can be used to target QTLs if there are relatively few highly heritable loci involved in the trait of interest.

Scientific Implications

As we predicted, 2 kinds of information arose from this project. First, we obtained information on the biology of cold tolerance and ion exclusion in *Citrus*, which should, with further verification, be applicable to citrus production or to plant biology in general. These are the first genetic studies on these phenomena in citrus. Second, we gained knowledge about how best to map genes for quantitative traits in perennial plants. Since conventional breeding is so

difficult in *Citrus*, and in many other perennial plants, marker-assisted selection for genes involved in important quantitative traits would be of enormous benefit to breeders, and we have taken the first steps in identifying such markers. Most other laboratories where mapping research is being done are focusing on annual crop plants where the problems encountered are entirely different. Given the small genome size of *Citrus*, it should be possible with more fine mapping in the regions we have identified to develop useful markers, and, potentially to eventually clone genes involved in these processes. The other necessary component for transferring such genes, genetic transformation has been developed for *Citrus* in our lab.

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Publications Resulting from Support of This Project

Refereed Publications:

- Tozlu, I., Moore, G.A., and Guy, C.L. Regulation of Na⁺ and Cl⁻ accumulations by *Citrus grandis* (L.) Osb, *Poncirus trifoliata* (L.) Raf, and their F₁ under salinized and non-salinized environments. Submitted to Tree Physiology.
- Tozlu, I., Moore, G.A., and Guy, C.L. Effects of increasing NaCl concentration on some macro- and micro-element accumulation by *Citrus grandis* (L.) Osb, *Poncirus trifoliata* (L.) Raf, and their F₁. Submitted to Tree Physiology.
- Sankar, A.A., and G.A. Moore. Evaluation of inter-simple sequence repeat analysis for mapping in *Citrus* and extension of the genetic linkage map. In press, Theoretical and Applied Genetics.
- Tozlu I, GA Moore, and CL Guy. 2000. Effects of increasing NaCl concentration on stem elongation, dry mass production, and macro- and micro-nutrient accumulation by *Poncirus trifoliata*. Australian Journal of Plant Physiology 27:35-42.
- Tozlu I, GA Moore, and CL Guy. 2000. Regulation of growth and differential tissue dry mass accumulation by *Citrus grandis*, *Poncirus trifoliata*, and their F₁ under salinized and non-salinized environments. Australian Journal of Plant Physiology 27:35-42.
- Tozlu, I, CL Guy, and GA Moore. 1999. QTL analysis of morphological traits in an intergeneric BC₁ progeny of *Citrus* and *Poncirus* under saline and nonsaline environments. Genome 42:1020-1029.
- Tozlu, I, CL Guy, and GA Moore. 1999. QTL analysis of Na- and Cl-accumulation related traits in an intergeneric BC₁ progeny of *Citrus* and *Poncirus* under saline and nonsaline environments. Genome 42:692-705.

Non-referreed Publications and Presentations

- Gmitter FG, Z Deng, and GA Moore. 1998. Utilization of DNA markers in citrus breeding programs. Fruits 53:303-306.
- Guy C, D Haskell, Q-B Li, G Moore, D-Y Sung, I Tozlu, and C Zhang. 1998. Plant temperature stress: Comparative analysis of high and low temperature stress protein responses and prospects for genetic modification. Proceedings of the International Workshop on Breeding and Biotechnology for Environmental Stress in Rice, October 6-9, 1998, Sapporo, Japan.
- Moore GA, I Tozlu, CA Weber, and CL Guy. Mapping quantitative trait loci for salt tolerance and cold tolerance in *Citrus grandis* (L.) Osb. X *Poncirus trifoliata* (L.) Raf. hybrid

populations. To be published in Acta Hort., as the proceedings of the First International Symposium on Citrus Biotechnology.

Bowman, K., J. Chapparo, F. Gmitter, J. Grosser, G. Moore, and W. Sherman. 1997. Citrus Variety Improvement Corner: Breeding cold hardy citrus. Citrus Industry, November/December p. 21-23.

Moore, G.A., I. Tozlu, and C. Weber. 1997. Mapping citrus genes for stress tolerance. Citrus & Vegetable Proceedings, pp. 37-39.

Student Theses and Dissertations

Sahin-Cevik, M. 1999. QTL analysis of morphological traits in Citrus using RAPD markers. M.S. Thesis, University of Florida.

Sankar, A.A. 1999. Evaluation of inter-simple sequence repeat analysis for mapping in Citrus and extension of the genetic linkage map. M.S. Thesis, University of Florida.

Weber, C.A. 1999. Mapping cold tolerance quantitative trait loci (qtl) in a Citrus grandis x Poncirus trifoliata F1 pseudo- testcross population using molecular markers and evaluation of homology with a related population map. Ph.D. Dissertation, University of Florida. Publications are being prepared from this dissertation.

Abstracts

Weber, CA, Z Deng, F Gmitter, and GA Moore. Evaluation of sequence-based PCR markers in an F1 pseudotestcross and a BC1 hybrid citrus population. Presented at the ASHS 1999 Annual Meeting. HortScience 34:542.

Sankar, A., and G.A. Moore. 1998. ISSR analysis for mapping in *Citrus*. Plant & Animal Genome VI, San Diego, January 18-22 (Poster presentation by Sankar).

Tozlu, I., C.L. Guy, and G.A. Moore. 1998. QTL mapping of intergeneric (*Citrus* x *Poncirus*) BC₁ progeny under saline and non-saline environments. Plant & Animal Genome VI, San Diego, January 18-22 (Poster presentation by Tozlu).

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Tozlu, I., C.L. Guy, Q. Cai, and G.A. Moore. 1994. Growth and ion content of intergeneric BC₁ *Citrus* x *Poncirus* progeny in response to a 9 month salinization periods. HortScience 29:580 (Poster presentation by Tozlu, 1994 ASHS meeting, Nashville, TN. This poster won third prize in the graduate student competition.)