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BARD

FINAL REPORT

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**Common Mechanisms of Response to the Stresses
of High Salinity and Low Temperature and Genetic
Mapping of Stress Tolerance Loci in *Citrus***

C. Guy, G. Ben-Hayyim, G. Moore, D. Holland, Y. Eshdat

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Bet Dagan, ISRAEL

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Common Mechanisms of Response to the Stresses of High Salinity and
Low Temperature and Genetic Mapping of Stress Tolerance Loci in Citrus

Investigators' Names

Charles Guy
Gozal Ben-Hayyim
Gloria Moore
Doron Holland
Yuval Eshdat

Investigators' Institutions

University of Florida
Volcani Centre
University of Florida
Volcani Centre
Volcani Centre

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Signature
Principal Investigator

Signature
Institution's Authorizing
Official SANDRA MANN
ASSISTANT DIRECTOR OF RESEARCH

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Final Progress Report for the BARD Project.

Listing of Accomplishments

During the funded interval we have accomplished the following objectives:

- Developed a reasonably high density linkage map for citrus
 - mapped the loci for two cold responsive genes that were cloned from *Poncirus*
 - mapped the loci for *csa*, the salt responsive gene for glutathione peroxidase, and *ccr* a circadian rhythm gene from citrus
 - identified loci that confer parental derived specific DNA methylation patterns in the *Citrus* X *Poncirus* cross
 - mapped 5 loci that determine shoot vigor
 - mapped 2 loci that influence leaf Na^+ accumulation patterns under non-saline conditions in the BC_1 population
 - mapped 3 loci that influence leaf Na^+ accumulation patterns during salt stress
 - mapped 2 loci that control leaf Cl^- accumulation patterns under non-saline conditions
 - mapped a locus that controls leaf Cl^- accumulation patterns during salt stress
- Screened the BC_1 population for growth reduction during salinization (controls and salinized), and cold tolerance
 - determined population variation for shoot/root ratio of Na^+ and Cl^-
 - determined levels for 12 inorganic nutrient elements in an effort to examine the influence of salinization on ion content with emphasis on foliar responses
 - collected data on ion distribution to reveal patterns of exclusion/sequestration/accumulation
 - analyzed relationships between ion content and growth
- Characterization of gene expression in response to salt or cold stress
 - cloned the gene for the salt responsive protein *csa*, identified it as glutathione peroxidase, determined the potential target substrate from enzymatic studies
 - cloned two other genes responsive to salt stress, one for the citrus homologue of a *Lea5*, and the other for an "oleosin" like gene
 - cold regulated (*cor*) genes belonging to five hybridization classes were isolated from *Poncirus*, two belonged to the group 2 *Lea* superfamily of stress proteins, the others show no significant homology to other known sequences
 - the expression of *csa* during cold acclimation was examined, and the expression of some of the *cor* genes were examined in response to salt stress
 - the influence of salinization on cold tolerance has been examined with seedling populations
 - conducted protein blot studies for expression of cold stress proteins during salt stress and vice versa

Executive Summary

The objectives that were outlined in our original proposal have largely been achieved or will be so by the end of the project in February 1995 with one exception; that of mapping cold tolerance loci based on the segregation of tolerance in the BC₁ progeny population. Briefly, our goals were to 1, construct a densely populated linkage map of the citrus genome; 2, map loci important in cold and/or salt stress tolerance; and 3, characterize the expression of genes responsive to cold and salt stress. As can be seen by the preceding listing of accomplishments, our original objectives A and B have been realized, objective C has been partially tested, objective D has been completed, and work on objectives E and F will be completed by the end of 1995. Although we have yet to map any loci that contribute to an ability of citrus to maintain growth when irrigated with saline water, our very encouraging results from the 1993 experiment provides us with considerable hope that 1994's much more comprehensive and better controlled experiment will yield the desired results once the data has been fully analyzed. Part of our optimism derives from the findings that loci for growth are closely linked with loci associated with foliar Cl⁻ and Na⁺ accumulation patterns under non-salinization conditions. In the 1994 experiment, if ion exclusion or sequestration traits are segregating in the population, the experimental design will permit their resolution.

Our fortunes with respect to cold tolerance is another situation. In three attempts to quantitatively characterize cold tolerance as an LT₅₀, the results have been too variable and the incremental differences between sensitive and tolerant too small to use for mapping. To adequately determine the LT₅₀ requires many plants, many more than we have been able to generate in the time and space available by making cuttings from small greenhouse-grown stock plants. As it has turned out, with citrus, to prepare enough plants needed to be successful in this objective would have required extensive facilities for both growing and testing hardiness which simply were not available at University of Florida. The large populations necessary to overcome the variability we encountered was unanticipated and unforeseeable at the project's outset. In spite of the setbacks, this project, when it is finally complete will be exceedingly successful.

COMMON MECHANISMS OF RESPONSE TO THE STRESSES OF HIGH SALINITY AND LOW TEMPERATURE AND GENETIC MAPPING OF STRESS TOLERANCE LOCI IN CITRUS

1.0 OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL

- A. Determine the effects of salinization and cold acclimation on the levels of salt or cold stress responsive proteins by protein blot analyses.
- B. Define the tissue distribution of the proteins during acclimation to high salt and low temperature by Western blot analyses.
- C. Determine if acclimation to one stress results in crossprotection against the other stress.
- D. Isolate cDNAs for genes induced by both salinization and low temperature.
- E. Map salt and cold tolerance loci in a segregating backcross progeny population between the a hardy deciduous salt sensitive *Poncirus trifoliata* and cold sensitive and more salt tolerant evergreen *Citrus grandis*.
- F. Determine the ion accumulation pattern of *Citrus grandis* and correlate the BC₁ population ion exclusion characteristics with salt tolerance and loci mapping.

2.0 REPORT NARRATIVE AND EVALUATION OF RESEARCH ACHIEVEMENTS

2.1 Antibody Studies

Three polyclonal antibodies raised against spinach CAP160, 85 and 79 and one monoclonal antibody reactive to cytosolic HSP70s were used in protein blot experiments to identify possible cross-reactive proteins responsive to salt stress. The anti-CAP160 antibodies did not cross-react with any citrus protein while antibodies for CAP85 and 79 did. However, no differential response was observed between the control and salt-treated callus. Antibodies to Cit-SAP were used to probe protein blots of spinach leaf extracts from plants acclimated to cold or desiccated by withholding water. One 66 kDa cross-reacting band was observed to be present in cold acclimated spinach leaf tissue, but it was absent in nonacclimated plants. The antibody was also used to examine the influence of cold acclimation on the expression of the protein in leaf tissue. Cold

acclimation did not appear to alter the abundance of the salt responsive protein. These experiments relate to objectives A and B.

2.2 Influence of Salinization on Freezing Tolerance

During the early stages of the project when we were in the process of vegetatively propagating the progeny of the BC₁ for freeze and salt stress tolerance, growth chamber studies were conducted with young seedlings of *Citrus grandis*, *Poncirus trifoliata*, and *Citrus sinensis*. Plants were salinized with 0, 40, 80, and 150 mM NaCl under acclimating and nonacclimating conditions for two months. Under acclimating conditions, 40 and 80 mM treatment of *Citrus grandis* increased freezing tolerance relative to nonsalinized plants. Salinized *Citrus sinensis* and *Poncirus trifoliata* were less freeze tolerant than nonsalinized plants. The mixed results from these growth chamber studies with young seedlings, did not permit us to draw any general conclusions on whether salinization enhances freezing tolerance. This work was reported at the annual meeting of the American Society for Horticultural Sciences (Tozlu et al., 1993). This work relates to objective C.

2.3 Isolation of Genes Responsive to Salt and Low Temperature Stress

Three cDNA clones have been isolated that respond to salt stress. One clone *csa*, encoded the protein Cit-SAP, previously isolated by 2-D gel electrophoresis, and recently identified as phospholipid hydroperoxide glutathione peroxidase. The other cDNA clones were selected by differential screening. They have been sequenced and by homology searches, they were recognized as a late embryogenesis abundant (LEA) group 4 homolog and an oleosin which consists of a family of membrane proteins found associated with oil bodies of seeds and pollen. Both genes are preferentially expressed in response to salt stress, although the oleosin gene was not expressed in differentiated tissues even in response to salinization except for seeds. Interestingly, the *Lea5* protein was not responsive to cold stress. The role of these two genes in citrus salt stress responses continues to be investigated. This work is reported in three journal articles (Holland et al., 1993; Naot et al., 1995a; 1995b).

Two cDNA libraries were prepared from mRNA obtained from cold acclimated *P. trifoliata* and *C. grandis*. The *P. trifoliata* library was differentially screened with single-stranded cDNA prepared from nonacclimated and cold acclimated poly (A)⁺RNA and nine partial cDNA clones representing 5 hybridization classes were obtained. The cDNA library was rescreened with the largest clone of the cross-hybridizing group and two near full-length clones were isolated. Sequencing of the clones and homology searches revealed that they encoded proteins related to the group 2 late embryogenesis abundant proteins. The two clones were found to be from different genes and corresponded to two different size transcripts. These clones along with those from the other hybridization classes

were used to probe RNA blots. All revealed heightened steady-state levels in *P. trifoliata* exposed to low temperature. RNA blots for *C. grandis* also showed that several were differentially expressed in response to low temperature, but some were not detected at all. In one case, genomic DNA blots indicate that a homologous sequence for the gene is present, but apparently not expressed in response to low temperature. In another case, the gene was not detected in genomic blots indicating that gene is not present in *C. grandis* or that it has diverged to the point where it does not cross-hybridize at high stringency. The differential expression of these two genes between *P. trifoliata* and *C. grandis* does correlate with the well documented differences in their relative hardiness capacity. Whether there is a causal relationship with hardiness was not determined.

Our original hypothesis that cold and salt stresses may elicit common responses and share mechanistic bases for tolerance, seemed to be confirmed with the isolation of genes for LEA proteins which have been associated with drought, cold, salt and osmotic stresses. However, RNA blots showed that in droughted, and more importantly salt stressed citrus, the mRNAs for the Group 2 LEA genes were strongly repressed. If any group 2 LEAs in citrus are induced by drought or salt stress, then they are sufficiently divergent to not cross-hybridize at high stringency. Possibly adding credence to the divergence hypothesis is the fact that the group 2 LEA proteins encoded by the cDNAs isolated in this study are very unusual in several ways. While they have the lysine (K) rich motif characteristic of this class, the motif is one of the most divergent yet found differing at several residues. The encoded proteins have an atypical Q (glutamine) motif and the poly serine pattern normally found N-terminal to the K motif, is found at the carboxy-terminus of the protein. Only at the amino acid level is homology observed. At the DNA level no apparent homology was found in Blast searches. This work is to be reported in a an article that has been accepted by Plant Molecular Biology (Cai et al., 1995).

The *C. grandis* library has not been used to isolate any citrus specific clones, but it remains a valuable product of the project.

2.4 Hybridization Studies

The cDNA for the salt responsive protein Cit-SAP, which encodes a phospholipid hydroperoxide glutathione peroxidase, was used to probe several RNA blots of *Citrus grandis* and *Poncirus trifoliata* grown under non- and cold-acclimating conditions. The mRNA was expressed in both species and under both environmental conditions, but there was no apparent response to different temperature treatments.

The inserts of the cDNAs for cold regulated genes isolated from *Poncirus trifoliata* were hybridized with labeled RNAs from salinized and control citrus callus. The labeled RNAs failed to hybridize with the *Poncirus* cDNAs. RNA blots sent from Israel to Florida

containing samples representing salinized and control callus tissue were used for hybridization studies with the *Poncirus* cDNAs to determine whether salt stress altered the expression of these genes responsive to low temperature. The initial hybridization experiment did not work and we were unable to repeat the experiment.

2.5 Construction of a High Density Linkage Map for Citrus

We proposed to search for evidence that tolerance to two different stresses, cold and salinity, in citrus may involve common mechanisms. The US group would work on aspects of cold stress and the Israeli group would work on similar facets of salt stress, each group taking advantage of an established research program in their respective areas. At the outset, we concluded that the ultimate basis on whether common tolerance mechanisms for the two stresses would exist in citrus would be if loci for tolerance to one stress mapped to the same position as loci important for tolerance to the other stress. One of the Investigator's (GAM) had created a low density map for citrus based upon isozymes and RFLP markers. In order to map stress tolerance loci in citrus a linkage map with a much greater density of markers needed to be developed. Funds provided by BARD were used to employ a Post-doctoral Associate in genetics to add RAPD markers to the existing map using a BC₁ population from a [*Citrus grandis* x *Poncirus trifoliata*] x *Citrus grandis*. This was done and a new higher density linkage map for citrus was published in Theoretical and Applied Genetics in 1994 (Cai et al., 1994). Nine linkage groups were identified corresponding to the number of chromosomes found in citrus. The average distance between markers in the new linkage map is approximately 7.5 cM. It is estimated that between 70 and 80% of the genome is included in the linkage map. With this new map, it will be possible to more precisely map the location of important quantitative trait loci (QTLs) in citrus.

An unexpected finding in the mapping studies is the high proportion of RAPD markers (40%) showing a skewed segregation. While skewed segregation patterns were previously known for citrus, the magnitude observed here is greater than what has been recorded before. Often markers showing skewed segregation cluster together on the same linkage grouping. More than 40% of the skewed markers were found to map to linkage group I and the remaining markers were distributed on four other linkage groups. The analyses also showed that the recurrent parent *C. grandis* is much more heterozygous than previously thought. The BC₁ population was used to map the location of several genes isolated during the course of this project.

2.5 Mapping Stress Responsive Genes

The position of the *csa* gene was mapped in the citrus genome using the BC₁ population described in the mapping section. Genomic DNA blots for the BC₁ population were probed with labeled *csa* prepared from the cDNA isolated by Dr. Ben-Hayyim's group.

The results were obtained from genomic DNA digested with three different restriction enzymes. The analysis of the data indicated that *csa* mapped to the exact proximal end of linkage group 4. A second citrus gene that was cloned at Volcani, *ccr*, was also placed on the linkage map. A cDNA clone was sent to the US and it was used to probe the BC₁ population. It was found to hybridize with polymorphic bands that permitted loci to be placed on the linkage map. Two loci were mapped, one near the middle of linkage group I (C1701) and other (C1702) was 15.9 cM from the proximal end of linkage group II. A third clone isolated by D. Holland was also sent for mapping, but attempts to map it were prevented by poor hybridization results in two separate experiments.

Clone pBCORc115 which encodes a 19.8 kDa group 2 LEA-like protein described above was used to place cold responsive loci on the linkage map. The RFLP patterns using this clone suggested at least a four gene model in citrus. Three loci could be mapped. Two were found adjacent to one another on linkage group IV while the other was found on linkage group VIII (Cai et al., 1994).

The genes for other hybridization class cDNAs described above have not been mapped.

2.7 Mapping Freezing Tolerance QTLs

A major goal of this project was to characterize the freezing tolerance variation in the BC₁ population, and use that information to position QTLs for freezing tolerance on the linkage map. This part of the project has not been successful. We have attempted in three different experiments to accurately determine the freezing tolerance for each progeny in both the nonacclimated and acclimated state. Variation inherent in the experimental methodology and variation from experiment to experiment has prevented us from unambiguously assigning a definitive hardiness level to most progeny. Because of the limited range in hardiness between the F₁ and *C. grandis* (ca 6°C max), experimental variation with the number of plants available would have had to have been vanishingly small for us to have been successful. Since early efforts were limited by plant materials and so variable, the decision was made to utilize all the plants that we could produce for the salinization studies in the hope that we would be able to map loci linked with salt stress tolerance.

2.8 Mapping DNA Methylation Patterns in Citrus

As an offshoot of the mapping efforts a novel approach to identify methylation patterns in the intergeneric BC₁ was developed. DNA methylation was detected in the *Citrus* genome using a new method involving coupled restriction enzyme digestion and random amplification (CREDRA). Twenty seven methylation events were found using 7 random primers and 4 restriction enzymes. One locus controlling cytosine methylation at Sau3AI sites in a DNA band (1.2 Kb) amplified with OPS19 was identified and mapped to linkage

IV of this genome. DNA methylation was also detected using Southern-blot analysis using a cold acclimation responsive clone, pBORc115, as a probe and two restriction enzymes, PstI and BamHI. Two gene loci for cytosine methylation at PstI sites and BamHI sites were identified and also found to be linked to linkage IV. This new method offers a rapid and powerful way to examine methylation patterns in a segregating population without requiring genes of known sequence. This work has been submitted to the Plant Journal for review.

2.9 Mapping Salt Tolerance QTLs

Mapping efforts to identify salt tolerance QTLs required that we study the salt sensitivity of the progeny in the BC₁ and determine whether sensitivity was segregating in the population. Initially our goal was to vegetatively propagate each genotype to produce as many plants as possible with our goal being at least 10 plants for each. Some genotypes rooted readily from cuttings while others established roots very poorly. Some genotypes grew vigorously from cuttings and others very slowly. This created, for many genotypes, too few plants for the cold and salt stress screenings. To increase the numbers of plants available for the salt screening, the fourth cold stress screening planned was discontinued and all plants were used for salt stress studies.

The response of *P. trifoliata* to increasing salinity was examined to help us focus in on the correct salinity level to use in mapping studies. Na⁺ and Cl⁻ levels, along with other macro- and micro-nutrients, were determined to establish the accumulation patterns and observe their impact on normal mineral nutrition. Sodium levels were very high in fine roots relative to leaf tissue and this supports the concept that *P. trifoliata* excludes Na⁺ or sequesters it in the roots. Comparison of leaf and root tissue Cl⁻ content showed little differences and suggested that a chloride sequestering mechanism in the root is not operable in this plant. The results of this experiment is reported in an abstract to be presented in a poster at the 1995 Plant Physiology meeting.

In another experiment, the responses of *C. grandis*, *P. trifoliata* and their F₁ were compared following salinization at 0, 40 and 80 mM. At 40 mM, *P. trifoliata* showed less shoot growth inhibition than *C. grandis* or the F₁. *Poncirus* leaf Na⁺ was low, but Na⁺ was high in fine roots. In contrast, *C. grandis* had low leaf Cl⁻, but high stem and structural root Cl⁻ content. It appears that *C. grandis* preferentially stores Cl⁻ in stems and structural roots while *Poncirus* tries to sequester Na⁺ in the fine roots. These preliminary studies further demonstrated the potential for QTL mapping of salt stress responses in the BC₁. The results of this experiment is reported in an abstract to be presented in a poster at the 1995 Plant Physiology meeting.

In 1993, a small scale pilot salt stress screening was conducted with 35 genotypes. Two year old cuttings were divided into two equal groups for each genotype. One group was

fertilized daily with a nutrient solution to 4X soil water holding capacity. The other group was fertilized in exactly the same way except the solution contained 30 mM NaCl. Previous pilot studies conducted in growth chambers had indicated that citrus and *Poncirus* were relatively sensitive to salt stress. A salt concentration towards the lower end was chosen for this long-term experiment. The plants were salinized for a 9 month period in the greenhouse. Growth was analyzed by measuring shoot height and node number at three week intervals. The concentration of macro- and micro-elements along with Na⁺ and Cl⁻ were determined at the end of the experiment. Shoot vigor and inorganic element concentration data were subjected to QTL analysis and loci for vigor and leaf nutrient accumulation patterns were mapped.

Salinization with 30 mM reduced growth by an average of 30% versus the nonsalinized control plants. Some progeny showed little growth reduction while others were strongly inhibited. By the end of the 9 month treatment, only two genotypes were dead from the salt stress. Many exhibited foliar symptoms of salt stress; necrotic lesions, interveinal chlorosis, leaf curling, and leaf abscission while some genotypes showed no outward symptoms in response to salt treatment. Therefore, sensitivity to salt stress was indeed segregating in the progeny population. Shoot vigor was mapped for both the control and salinized plants. Since shoot growth appeared to segregate in the control plants, we were able map this trait. Five loci on linkage groups II, III, V were identified.

Interestingly five loci related to foliar Na⁺ accumulation were distinguished. Three loci accounted for significant variation in the Na⁺ leaf content of salinized plants and two loci were associated with leaf Na⁺ accumulation in control plants. Four of the five loci mapped to linkage group I. The other was found on linkage group II. That foliar Na⁺ accumulation might segregate in the population was expected. *Poncirus trifoliata* is considered to exclude Na⁺ while *Citrus* spp. are considered to be Cl⁻ excluders. Therefore since the parental patterns for sodium and chloride exclusion are considered to be opposite, the accumulation patterns in the progeny should vary and segregate. That Na⁺ and Cl⁻ accumulation patterns in foliar tissue could be mapped in this small experiment shows that important QTLs for salt uptake and accumulation can be identified. Like sodium, chloride accumulation varied substantially in both the control and salinized plants. One locus for Cl⁻ accumulation was found with salinized plants and two with control plants. These mapped to linkage groups IV and V.

Unexpectedly, some of the shoot vigor QTLs mapped to the same location as the QTLs for foliar sodium and chloride accumulation QTLs. Citrus leaves are long lived and it may be necessary for the optimal function of older leaves to keep the Na⁺ and/or Cl⁻ levels as low as possible. Even under low saline conditions, over time, Na⁺ and/or Cl⁻ may accumulate to inhibitory levels. Plants able to keep sodium and chloride levels low may exhibit the greatest shoot vigor. This work was reported in a poster at the 1994

American Society for Horticultural Science annual meeting and an abstract (Tozlu et al., 1994).

We have, from our progeny salinization experiment, been able to examine the relationship between foliar Na^+ and/or Cl^- accumulation and shoot growth reduction with surprising results. The results unmistakably show that under mild salt stress conditions (30 mM), that initially affect only growth, there is absolutely no relationship between shoot growth reduction and foliar Na^+ and/or Cl^- content. This is in agreement with NaCl pretreatment and salt tolerance induction studies with Sorghum (Amzallag et al., 1990). The growth reduction was not caused by an osmotic or water stress that might alter gas exchange parameters and in turn reduce photosynthetic assimilatory processes (Munns and Termaat, 1986, Munns 1993). The implication of these findings is that the function of the canopy, ie photosynthesis and metabolism, is not in any way related to the shoot growth reduction as has been suggested by Munns (1993). Therefore, the cause of the shoot growth reduction must be associated with the function of the roots in a saline environment (Munns and Termaat, 1986).

In 1994, a larger more comprehensive greenhouse salinization experiment was conducted. This experiment included 50 progeny genotypes with about 750 plants. The salt concentration was increased to 40 mM to slightly accelerate the onset of stress symptoms and to help us better gauge the segregation of tolerance in the population. As in 1993, shoot growth was monitored throughout the course of the experiment, and the nutrient levels in all tissues were determined. Fresh and dry weights of leaf, stem, structural, and fine roots were also determined. Macro and micro nutrient levels have been determined for all tissues along with Na^+ and Cl^- content. The experiment was terminated at the beginning of November 1994. The mineral analyses for the plants was only completed at the end of March 1995. More than 50,000 data points comprise the growth and mineral content database.

Some analysis of this enormous dataset has begun, but is not expected to be complete until the end of the year. The dataset has been superficially examined for completeness and quality. While variation continues to be large in some cases, the dataset appears to be quite good. We expect to accomplish the following mapping results with this data:

1. Map QTLs for shoot vigor under nonsalinized conditions.
2. Map QTLs for shoot vigor under salinized conditions.
3. Map QTLs for greatest shoot growth in salinized plants. QTLs different or unique from those found in the control plants will be by our operational definition linked to salt tolerance.
4. QTLs influencing shoot/root ratio in control plants should be mappable.

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5. We expect to definitively map loci associated with Na^+ and/or Cl^- accumulation in foliar and fine root tissue in control plants.
6. Loci associated with Na^+ and/or Cl^- accumulation in foliar and fine root tissue of salinized plants should be mapped.
7. Using sodium/chloride ratio data, we hope that loci for Na^+ and Cl^- exclusion and accumulation can be accurately mapped.
8. Using plant ion budgets, we may be able to map ion sequestration patterns in the BC_1 .
9. Although not a high priority, we should be able to map loci that influence the content of other macro- and micro-nutrients in the various tissues of control and salinized plants.

The growth and mineral content datasets will not only permit us to map loci linked with growth and salt stress tolerance in citrus, but nearly as important, they will provide us with the ability to test several hypotheses about how salt stress affects growth and causes injury. One assumption upon which our salt stress screening was based was that growth is the most sensitive indicator of stress. The sensitivity of growth is considered to ensue from either an osmotically induced closing of stomata that would restrict gas exchange and reduce photosynthesis, or an accumulation of Na^+ and/or Cl^- in the leaves which would inhibit photosynthetic enzymes. In either case growth would be reduced because photosynthesis would be diminished and there would be less fixed carbon available for growth. Results from our 1993 experiment and observations from the 1994 experiment already dispel this notion. Some progeny did not show severe reductions in growth, but did show an obvious progression of foliar symptoms of stress that well preceded reductions in growth. From the 1993 data, we conducted regression analyses of growth versus Na^+ or Cl^- content in the leaves. The results were that growth reduction was not correlated with higher levels of either ion. Therefore, the idea that growth would be reduced because high foliar levels of Na^+ and/or Cl^- inhibited photosynthetic metabolism is untenable and suggests that the *raison d'être* must reside in another part of the plant. We did not conduct gas exchange measurements or determine water relations parameters of control and salinized plants, so we cannot make irrefutable conclusions about the role of osmotic stress reduction in photosynthesis and its attendant influence on growth. However, the experimental design was formulated to minimize the osmotic component of salt stress. First, the salt concentration was lower (30 mM) than is usually used in salinization experiments and would not drastically contribute to the osmolarity of the soil solution. Second, the irrigation regime was such that salt accumulation in the soil due to evapotranspiration was prevented by extensive daily leaching of the soil medium. Thus while we cannot prove there was no osmotic effect, our continuous observation of the plants and the experimental design lead us to conclude that the growth reduction of some progeny was not mediated through an osmotic stress mechanism on gas exchange and photosynthesis.

A second determinant that our physiological dataset should provide insight into is the role ion exclusion versus capacity to tolerate ion accumulation in citrus salt stress responses. We know from the work of others that various species of Citrus differentially exclude and/or accumulate sodium and chloride. We will be able to determine a whole plant budget for each of these ions and from this determine which progeny may exclude one or both or accumulate one or both and correlate this with the sensitivity of growth to salinization and overall sensitivity. In this way we should be able to determine if exclusion is a tolerance factor, if sequestering in the stem or roots is a tolerance factor, if the capacity to endure accumulation in foliar tissue is a tolerance factor, or if all of these possibilities are tolerance factors.

We envision at least three important journal articles to emanate from the BC₁ salt stress experiments and the mapping of QTLs for salt responses and ion accumulation patterns. When these are combined with the articles in print or already in press, the high and unique productivity of this project will be evident.

2.10 Description of Cooperation

Antibodies prepared by GB-H and CLG have been exchanged and used in surveys to determine whether one stress elicits the accumulation of proteins responsive to another form of environmental stress. In an exchange visit, GB-H provided important input into the salt tolerance segregation screening of the BC₁ population. During that same exchange visit, the genetic locus for the salt responsive protein Cit-SAP was mapped using the BC₁ population. Two other clones isolated at the Volcani were provided and one *ccr* was successfully mapped. Hybridization studies were also conducted with exchanged clones and blots. While no joint authorship articles will stem from the completed work, resources developed in each location were utilized to obtain new and important information about the responses of citrus to the stresses of salinity and low temperature.

During this interval of funding several genes for citrus were successfully mapped. There is great interest by all the investigators of this project to attempt to place on the linkage map the remaining genes that were cloned during the course of this project. This includes a second *ccr* gene, the *Lea5* and the "oleosin" like gene in addition to three cold responsive genes.

3.0 CONTRIBUTIONS TO AGRICULTURE

While the scientific efforts of this project have yet to develop into applications that translate into new agricultural practices or materials, they have clearly added to our understanding of the responses of citrus to environmental stress. Furthermore, these

efforts have begun a process to establish the genetic information necessary to identify genes of importance for tolerance to salt and cold stress.

One of the major achievements of this project is the creation of a reasonably dense linkage map for citrus. This linkage map consists of three types of molecular markers; isozymes, RFLPs, and RAPDs (Fig. 1). A well populated linkage map is the first requisite for taking advantage of the state-of-the-art molecular genetics approach to identifying and characterizing genes of agricultural importance which belong to a large group of agronomic traits known to be controlled by a large number of genes. Such genes or regions of the genome are known as quantitative trait loci (QTL). With the availability of such a linkage map, it now becomes feasible to create special gene libraries known as YAC (yeast artificial chromosome), from which important genes can be identified and isolated by a process called map-based cloning. Of course having a linkage map makes possible the potential to genetically map any trait that segregates in the existing backcross population. This has already been proven true. We have taken growth data from the population of plants that were used to develop the linkage map, and identified a segment of the citrus genome which has a major influence on shoot vigor. Genes responsive to cold acclimation and salt adaptation have also been mapped to discrete locations within the citrus genome. The results of physiological experiments recently completed will determine whether any of the genes that have already been cloned, correspond to genes that make major contributions to salt and cold tolerance.

Genes have been isolated from salt adapted cells and plants during cold acclimation. One of the genes obtained from salt adapted cells has been identified as glutathione peroxidase (GP). It is known that salt stress, like many other types of environmental stress, increases various forms of oxidative stress. Previous to the linkage of glutathione peroxidase expression with salt adaptation in citrus cells, such an association was totally unknown. In a recent and exciting discovery, Drs. Eshdat, Ben-Hayyim and Holland have established that lipid hydroperoxides are the likely substrates for this enzyme. This has important implications, for it provides a mechanistic foundation in which to base further investigations of how high salt injures plants. Two other genes have been identified in salt adapted cells. One is for an oleosin-like gene which functions in lipid metabolism and the other is for a protein, *Lea5*, that belongs to a superfamily of genes linked with dehydration and desiccation in plants. Similarly, genes cloned from cold acclimating plants also belong to a similar superfamily of proteins linked with drought and dehydration.

Through physiological studies of salt stress in the population of plants that was used to construct the linkage map, we have obtained compelling experimental evidence that accumulation of sodium and/or chloride in the leaf tissue is not the cause of growth inhibition under moderately saline conditions. This is important because it indicates that the greatest sensitivity of citrus to salt stress involves processes that are unrelated to

the function of the leaf. More likely, the first impact of salt stress in citrus is in the root. Whether saline soil causes citrus to redistribute resources for growth to the root for increased biomass at the expense of the shoot is but one possibility. Answers to this and many other questions about salt stress may come from large scale experiments that were recently completed, but whose results have not yet been fully analyzed.

4.0 LIST OF PUBLICATIONS

4.1 Journal Articles

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Cai, Q, Guy, CL, and Moore, GA 1994. Extension of the linkage map in citrus using random amplified polymorphic DNA (RAPD) markers and RFLP mapping of cold acclimation responsive loci. *Theoret. Appl. Genet.* 89, 606-614.

Naot, D, Ben-Hayyim, G, Eshdat, Y, and Holland, D 1995. Salt stress induces the expression of a citrus homologue of an atypical late embryogenesis *Lea5* gene. *Plant Mol. Biol.* 27, 619-622.

Naot, D, Holland, D, Eshdat, Y, and Ben-Hayyim, G 1995. Induction of a gene encoding an oleosin-like homologue in cultured citrus cells exposed to salt stress. *Gene* (In press).

Cai, Q, Guy, CL, and Moore, GA 1995. An unusual group 2 lea gene family in citrus responsive to low temperature. *Plant Mol. Biol.* (Accepted).

Cai, Q, Moore, GA, and Guy, CL 1995. Inheritance and genetic mapping of a parental specific DNA methylation pattern locus in a backcross between *Citrus* X *Poncirus*. submitted.

4.2 Published Abstracts

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Tozlu, I, Moore, GA, and Guy, CL 1995. Response of *Citrus grandis*, *Poncirus trifoliata* and their F₁ to salinization. *Plant Physiol.* (In press).

US-1887-90 Common Responses to Salt and Low Temperature and Genetic Mapping

Faltin, Z, Ben-Hayyim, G, Beeor, T., Camoin, L., Strosberg, AD, and Eshdat, Y 1994. Isolation and structure-function study of a plant glutathione peroxidase-like protein expressed under salt stress. Fourth Internatl. Cong. Plant Mol. Biol.

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. HortScience 29, 511.

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Cai, Q, Guy C, and Moore, GA 1993. Genetic mapping of random amplified polymorphic DNA (RAPD) markers in citrus. Gordon Conference on Temperature Stress in Plants.

Cai, Q, Guy, C, and Moore, GA 1993. Genetic mapping of random amplified polymorphic DNA (RAPD) markers in Citrus. HortScience 28, 582.

Cai, Q, Guy, C, and Moore, GA 1992. Genetic mapping of random amplified polymorphic DNA (RAPD) markers in citrus. Plant Genome I, p. 20.

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