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Extension of the linkage map in *Citrus* using random amplified polymorphic DNA (RAPD) markers and RFLP mapping of cold-acclimation-responsive loci

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Abstract Genetic mapping with RAPD markers has been initiated in *Citrus*. Reproducible polymorphism of amplified DNA fragments was obtained with approximately half of the 140 random primers tested, revealing 266 segregating loci. These were tested for linkage using 60 BC_1 progeny from an intergeneric cross of *Citrus grandis* (L.) Osb. × [*Citrus grandis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf.]. A core linkage map was constructed that consists of nine linkage groups containing 109 RAPD markers and 51 previously-mapped RFLP and isozyme markers. A further 79 markers that could not be ordered unambiguously because of their allelic constitution were associated with individual linkage groups and are shown in relation to the core map. The core map has a total length of 1192 cM with an average distance of 7.5 cM between loci and is estimated to cover 70–80% of the genome. Loci with distorted segregation patterns clustered on several linkage groups. Individual clusters of loci were skewed in allelic composition toward one or the other parent, usually *C. grandis*. This relatively-saturated linkage map will eventually be used to identify quantitative trait loci for cold and salt-tolerance in *Citrus*. As a beginning we have mapped three loci detected by a cold-acclimation-responsive cDNA.

Key words Genetic map · RAPD · RFLP · Molecular markers · Fruit breeding

Introduction

With the advent of molecular markers, genetic mapping has become important in basic genetic studies of plants as well as for crop improvement. The generation of linkage maps of plant genomes, traditionally based on morphological, biochemical and behavioral markers, has been accelerated by the use of DNA markers. Restriction fragment length polymorphism (RFLP) markers have been used to generate linkage maps in a number of species. These include plants where few prior linkage relationships had been previously determined, such as *Citrus* (Durham et al. 1992; Jarrell et al. 1992). However, RFLP assays are time-consuming, labor-intensive, and frequently require the use of radioisotopes. Recently, novel DNA markers, termed random amplified polymorphic DNA (RAPD) markers, have been developed (Welsh and McClelland 1990; Williams et al. 1990). RAPDs are based on random amplification of genomic DNA with single primers of arbitrary nucleotide sequence; they allow convenient and quick detection of polymorphism without requiring the use of radioactive isotopes. RAPD markers have been found useful for creating or expanding linkage maps in plant species (Williams et al. 1990; Klein-Lankhorst et al. 1991; Michelmore et al. 1991; Reiter et al. 1992, Tulsieram et al. 1992, Torres et al. 1993).

Genetic linkage maps perhaps provide the greatest potential for increasing the efficiency of cultivar improvement programs in tree fruit and other perennial crops with long generation times. A relatively-saturated linkage map would allow early screening procedures and the manipulation of quantitative traits based on marker-assisted selection (Tanksley et al. 1989). This would greatly reduce the time consumed in breeding compared to any traditional breeding scheme. Based on computer simulations in tomato, for instance, it was estimated that by using DNA marker-based whole-genome selection, the genotype of the recurrent parent, along with the transfer of desired genes, can be reconstructed in only three generations of 30 individuals in contrast to more than six generations of many more individuals with traditional backcross breeding (Tanksley et al. 1989). In addition, map-based cloning has become a reality in species with well-defined genetic maps (Leyser et al. 1993; Martin et al. 1993).

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Citrus is a tree fruit crop of world-wide economic importance. Conventional breeding methods are difficult in this genus because of biological constraints to crossing and long juvenile periods (Soost and Cameron 1975). However, it is a most attractive fruit tree crop for conducting linkage analysis. It is a diploid with a relatively low haploid chromosome number, n = 9, and small genome, 1C = 0.62 pg (Guerra 1984). The genus is highly polymorphic, and interspecific, as well as some intergeneric, hybrids can be produced. We have been constructing a linkage map in *Citrus* in order to eventually map quantitative trait loci (QTLs) governing cold- and salt-tolerance. We previously identified 11 linkage groups using an intergeneric BC_1 population, *Citrus grandis* (L.) Osb. cv Thong Dee × ['Thong Dee' × 'Pomeroy' trifoliate orange (*Poncirus trifoliata* L. Raf.)], and isozyme and RFLP markers (Durham et al. 1992). In this paper, we report further development of the linkage map in *Citrus* using RAPD markers and the mapping of some loci responding to cold acclimation.

Materials and methods

Plant materials

The intergeneric BC_1 population described in a previous paper (Durham et al. 1992) was used for this study. In this report, the two original parents used to create the F_1 hybrid are referred to as 'Thong Dee' (the *C. grandis*, or pummelo, parent) and 'Pomeroy' (the *Poncirus trifoliata*, or trifoliate orange, parent). The BC_1 population was derived from a backcross between *C. grandis* and an F_1 plant that was selected for its viability under freezing conditions.

DNA amplification and primer screening

Citrus genomic DNA extraction was done as previously described (Durham et al. 1992). A total of 140 random decamer oligonucleotide primers (kits A, E, F, G, O, Q and T from Operon Technologies Inc., Alameda Calif.) were used to amplify *Citrus* genomic DNA. Each 25-µl amplification reaction consisted of buffer [50 mM KCl, 10 mM TRIS-HCl (pH9.0), 1% Triton X-100] (Promega), 2 mM of $MgCl_2$, 0.8 mM dNTPs (200 µM of each dNTP), 0.4 µM primer, 1 U of *Taq* polymerase (Promega), and 15 ng of uncut genomic DNA. Each reaction mixture was overlaid with mineral oil. Amplification was conducted in a MJ PTC-100™ thermal controller with 96 wells (MJ Research, Inc.) under the following conditions: 1 min at 93 °C, 1 min at 35 °C, and 2 min at 72 °C for 42 cycles. The ramp time required in shifting from one temperature to another was automatically controlled. A final extension step at 72 °C for 8 min concluded amplification. Another model MJ thermal controller with 60 wells and a Coy TempCycler Model 50/60 were also used in this study to monitor variation in DNA amplification due to the use of different thermocyclers. The amplication products were separated in 2% agarose gels with TAE buffer by electrophoresis in a wide mini sub™ cell (Bio-Rad) and detected by ethidium bromide staining. In order to determine whether the detected polymorphisms were reproducible, amplification reactions were repeated with all of the primers that produced informative polymorphism. In some reactions, concentrations of various reaction components were varied to examine their effect on amplification.

Scoring of segregating markers and linkage analysis

To identify primers likely to detect polymorphism among the 'Thong Dee' × F_1 backcross population, all primers were initially screened using six plants: the two original parents 'Thong Dee' and 'Pomeroy', the F_1, and three BC_1 progeny plants. Those primers showing polymorphic banding patterns were used to screen the entire BC_1 progeny population of 60 plants to generate RAPD markers for linkage mapping. Since the evaluated population was a backcross, it was anticipated that in most cases the allelic configuration of the 'Thong Dee' and F_1 parents would be aa × Aa (i.e., band absent × band present) and that the expected segregation ratio in the BC_1 progeny would be 1:1. However, banding patterns suggesting Aa × aa and Aa × Aa parental allelic configurations were also observed. Goodness of fit to the hypothesized segregation ratios was determined by chi-square analysis using the LINKAGE-1 computer program (Suiter et al. 1983). MAPMAKER/EXP 3.0 (Lander et al. 1987; Lincoln et al. 1992) was used to order those loci with an aa × Aa configuration. Linkage groups were decided on the basis of a recombination fraction of 35 cM and a log likelihood of the odds (LOD) > 3. The framework order of markers was assigned to form a core map using the ORDER command at a ▲LOD ≥ 3.0, and then other markers were inserted in the core map using the BUILD command at a ▲LOD ≥ 2.0. The markers that could not be placed at this threshold level were added using the TRY command based on the best ▲LOD score or else shown in approximate relation to the core map if a more precise location could not be determined. The final map distances were estimated using the MAP command with the error detection function engaged. The error probability was estimated by comparing two sets of data scored separately by two people. The Kosambi (1944) centimorgan function was selected to define the map distance.

Markers with the Aa × aa configuration could not be directly placed on the core map, which consisted only of markers of the aa × Aa type. Joining the Aa × aa markers to the core map depended upon the analysis of the Aa × Aa type of markers, which could then be used to provide at least partial genetic information for linkage relationships between Aa × aa and aa × Aa types of markers. JoinMap (Stam 1993) was used for linkage analysis involving the putative loci in Aa × aa and Aa × Aa configurations. Significant linkage was determined using a minimal linkage LOD of 3.0. Finally, the linked markers were joined to the core map calculated with MAPMAKER by estimating pairwise recombination frequencies from all three types of raw data (Aa × aa, Aa × Aa and aa × Aa). The relative locations of Aa × aa and Aa × Aa types of markers on the core map were determined based on the closest linkage to the aa × Aa markers in the core map.

The amplified products evaluated as markers were named OP followed by a letter and a 2-digit number (for their Operon kit origin) and an additional 3-digit number indicating the size of the amplified product with a factor of ten base pairs. For example, the marker OPG14049 represents a DNA fragment with a size of 490 bp amplified with primer G14 from the Operon G kit.

RFLP analysis and mapping of cold-acclimation-responsive (COR) clones

Three COR cDNA clones, pBCORc011, pBCORc410, and pBCORc115, were isolated from a cDNA library constructed from mRNA obtained from cold-acclimated *Poncirus trifoliata* cv Pomeroy. These clones were maintained as cDNA inserts at the *Eco*RI/*Xho*I site of Bluescript *ks* + (Stratagene). The pBCORc115 plasmid contained the largest insert (850 bp), while pBCORc011 and pBCORc410 had inserts with sizes of 230 bp and 450 bp. The cDNA inserts used as probes were excised from the vector and labeled with ^{32}P-dCTP using a random primer labeling kit (BMB) as previously described (Durham et al. 1992). Approximately 4 µg of genomic DNA from each plant was digested with appropriate restriction enzymes. Following separation by agarose-gel (0.9% agarose in 1 × TAE) electrophoresis, the DNA was Southern-blotted to Hybond-N membrane (Amersham) as described by Ausubel et al. (1989). DNA hybridization was conducted at 42 °C for 12 h in a solution containing 0.25 M Na_3PO_4 (pH 7.2), 0.25 M NaCl, 1 mM EDTA, 7% SDS, 10% PEG and 50% formamide, to which 2–5 × 10^5 cpm/ml of labeled probe was added. The membrane was washed three times respectively in the following solutions: 2 × SSC + 0.2% SDS, 0.5 × SSC + 0.2%

SDS and 0.2 × SSC + 0.2% SDS, each for 15 min. The first two washes were done at room temperature and the last at 55 °C. Subsequently the membranes were exposed to X-ray film (Kodak) for 2 days. Initially the probes were hybridized to DNA from 'Thong Dee', 'Pomeroy', and the F_1 hybrid digested with four restriction enzymes: *Eco*RI, *Pst*I, *Hin*dIII, and *Bam*HI. The enzymes generating digested DNA in which the cDNA probes showed polymorphism were selected to digest the DNA from the BC_1 progeny plants. The RFLP data were scored as previously described (Durham et al. 1992).

Results and discussion

Random amplification of genomic DNA in *Citrus*

When 140 random primers were initially screened using six plants, including the two original parents, the F_1, and three BC_1 progeny, 69 (49%) consistently yielded amplified products and showed obvious polymorphic DNA banding patterns after agarose-gel electrophoresis and ethidium bromide staining. These 69 primers were subsequently used to amplify DNA from all 60 progeny of the BC_1 population for segregation and linkage analysis. The remaining 71 primers either failed to amplify DNA or showed no polymorphism in the initially-screened plants and were therefore eliminated from further analysis.

The numbers of amplified DNA bands, polymorphic bands, and scorable loci generated by individual primers are presented in Table 1. Some of the agarose-gel profiles of amplified products are shown in Fig. 1. The data summarized from the whole population showed that 4–19 DNA bands were amplified by a single primer and band sizes ranged from 200 bp to 3 kb. A high degree of polymorphism was revealed by these primers among the BC_1 population. In 43 of the 69 primers, over 50% of the amplified bands were polymorphic, and up to 11 polymorphic bands were produced with a single primer. All such polymorphic bands were considered to be a potential source of markers for mapping analysis. However, only those that proved to be easily scored and reproducible were finally used for mapping.

A concern in the use of RAPD markers is reliability; advantages in the use of RAPDs will be lost, and their applications will be greatly limited, if reliability is not

Table 1 The number of amplified bands, polymorphic bands, and scored loci detected with genomic DNA from the BC_1 population of *Citrus* and the selected primers

Primer	Sequence	Number of bands			Primer	Sequence	Number of bands		
		Amplified	Polymorphic	Scorable			Amplified	Polymorphic	Scorable
OPA01	GAGGCCCTTC	14	8	7	OPF10	GGAAGCTTGG	7	5	4
OPA02	TGCCGAGCTG	12	3	2	OPF14	TGCTGCAGGT	7	5	2
OPA04	AATCGGGCTG	7	3	3	OPF15	CCAGTACTCC	6	4	3
OPA05	AGGGGTCTTG	11	6	5	OPG02	GGCACTGAGG	8	3	3
OPA09	GGGTAACGCC	10	8	7	OPG03	GAGCCCTCCA	14	5	2
OPA10	GTGATCGCAG	9	7	6	OPG05	CTGAGACGGA	10	6	4
OPA11	CAATCGCCGT	5	3	3	OPG06	GTGCCTAACC	12	8	6
OPA12	TCGGCGATAG	4	1	1	OPG10	AGGGCCGTCT	12	6	3
OPA14	TCTGTGCTGG	6	3	3	OPG11	TGCCCGTCGT	7	4	3
OPA15	TTCCGAACCC	11	7	6	OPG12	CAGCTCACGA	13	6	4
OPA18	AGGTGACCGT	12	5	2	OPG13	CTCTCCGCCA	4	1	1
OPA19	CAAACGTCGG	15	9	4	OPG14	GGATGAGACC	9	5	3
OPE01	CCCAAGGTCC	18	10	7	OPG16	AGCGTCCTCC	9	2	1
OPE03	CCAGATGCAC	13	9	8	OPG17	ACGACCGACA	15	7	5
OPE04	GTGACATGCC	14	6	4	OPG18	GGCTCATGTG	7	5	3
OPE05	TCAGGGAGGT	12	8	5	OPG19	GTCAGGGCAA	10	5	1
OPE07	AGATGCAGCC	19	9	4	OPO04	AAGTCCGCTC	14	5	4
OPE08	TCACCAAGGT	10	7	7	OPO05	CCCAGTCACT	6	3	1
OPE12	TTATCGCCCC	12	8	5	OPO06	CCACGGGAAG	13	9	6
OPE14	TGCGGCTGAG	14	7	4	OPO07	CAGCACTGAC	8	4	2
OPE15	ACGCACAACC	18	8	6	OPO11	GACAGGAGGT	7	3	2
OPE16	GGTGACTGTG	15	9	4	OPO13	GTCAGAGTCC	8	4	3
OPE18	GGACTGCAGA	10	4	2	OPO14	AGCATGGCTC	10	5	5
OPE19	ACGGCGTATG	12	8	5	OPO15	TGGCGTCCTT	7	3	2
OPE20	AACGGTGACC	19	9	5	OPQ01	GGGACGATGG	15	7	3
OPF04	GGTGATCAGG	13	7	5	OPQ06	GAGCGCCTTG	12	5	4
OPF05	CCGAATTCCC	12	8	7	OPQ09	GGCTAACCGA	4	2	2
OPQ12	AGTAGGGCAC	7	4	3	OPT05	GGGTTTGGCA	8	6	5
OPQ14	GGACGCTTCA	10	7	6	OPT06	CAAGGGCAGA	11	7	6
OPQ15	GGGTAACGTG	8	6	6	OPT07	GGCAGGCTGT	15	9	4
OPQ16	AGTGCAGCCA	12	5	4	OPT08	AACGGCGACA	18	11	9
OPQ17	GAAGCCCTTG	3	1	1	OPT12	GGGTGTGTAG	12	9	6
OPQ18	AGGCTGGGTG	19	8	5	OPT14	AATGCCGCAG	7	5	4
OPT01	GGGCCACTCA	17	9	6	OPT17	CCAACGTCGT	9	3	2
OPT04	CACAGAGGGA	9	5	3					

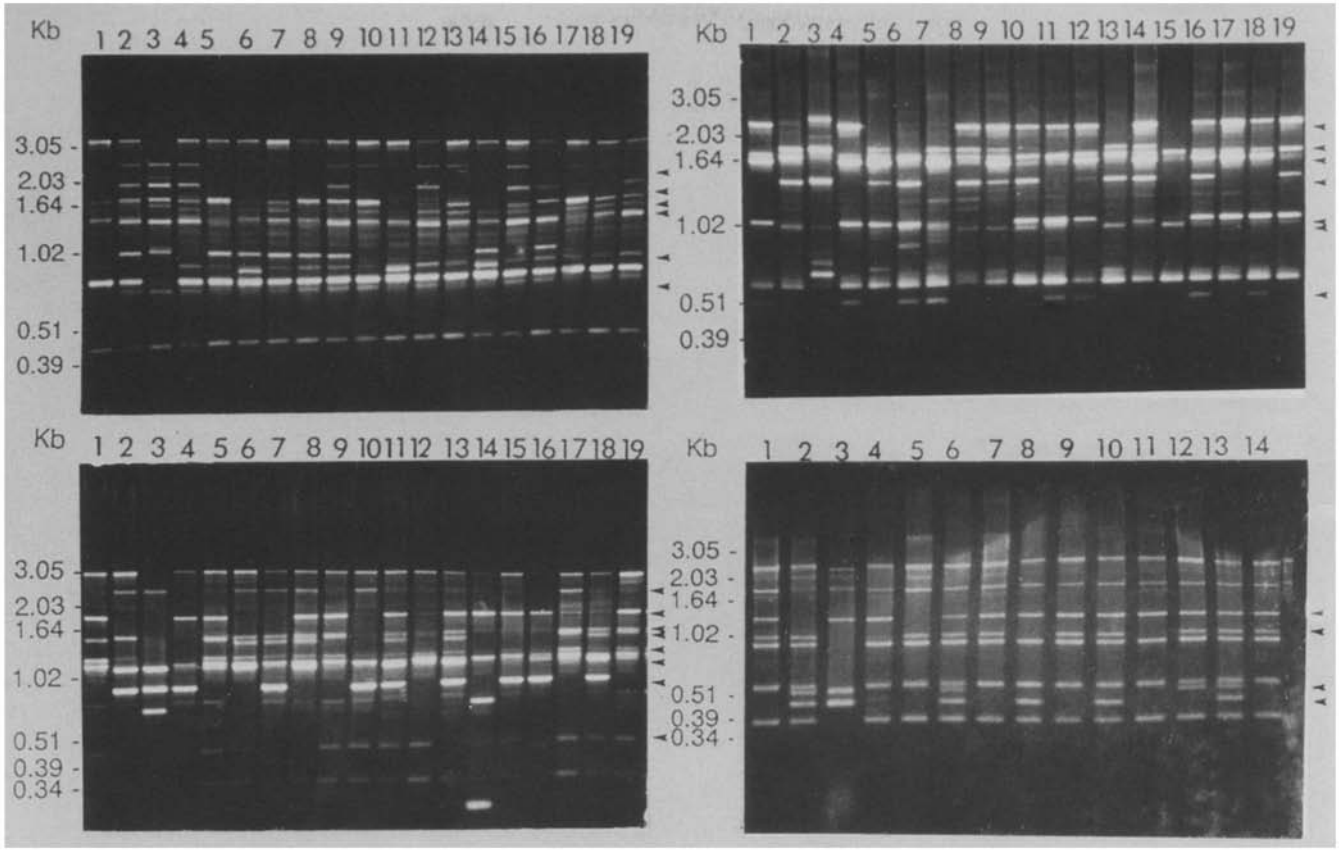


Fig. 1a–d Agarose-profiles of random-amplified DNA products from original parents, *C. grandis* (*lane 1*) and *P. trifoliata* (*lane 3*), their F_1 hybrid (*lane 2*), and some of the BC_1 (*C. grandis* × F_1) progeny plants (*all other lanes*). DNA was amplified with primer OPF04 (**a**), OPF05 (**b**), OPQ14 (**c**) and OPG14 (**d**). Amplified DNA was separated in a 2% agarose gel with 1 × TAE buffer at 60 V for 3 h. The *arrows* point to polymorphic bands

adequate. Others have done experiments to clarify the reproducibility of RAPD products by repeating random primer amplifications under consistent or varied working conditions. For example, Weeden et al. (1992) reported that randomly-amplified products from genomic DNA of 13 genera could be reproducibly obtained under permissively-varied working conditions; ten-fold variations of DNA template concentration and 2–5-fold variations of primer and magnesium concentrations had little affect on the stability of the amplified products. In this study, the reproducibility of the bands was tested for all of the primers selected as displaying polymorphism. Amplifications with primers from the A and G kits were repeated with all progeny plants, while reactions with the remaining primers were repeated with the six plants used for the initial screen. Some DNA bands, while showing polymorphism, were too faint to be scored reliably and were omitted from the mapping analysis. However, all of the DNA products that amplified well the first time that the reactions were done also amplified well when the reactions were repeated. Some of the DNA samples that amplified poorly, or failed to amplify the first time, amplified well in the repeat experiment. Very few DNA samples failed to amplify either time; this may be attributed to the poor DNA quality of these samples. Thus, our experiment confirmed the reproducibility of RAPD markers.

Minor variations in RAPD products were detected between amplifications carried out in different PCR machines. When the same reactions were done using two models of the MJ thermal controller, no variation in banding patterns was noted, but differences in banding intensities were observed; many of the bands amplified in the MJ 96-well thermal controller appeared to be more intense than those amplified in the MJ 60-well thermal controller. This may be because the microplate used in the MJ 96-well thermal controller was more suitable for DNA amplification than the microtubes used in the MJ 60-well thermal controller. Differences in band intensities were also observed when the Coy tempcycler and the 2 MJ thermal controllers were compared. Interestingly, the differences in intensity obtained seemed to be associated with the sizes of the amplified bands; the bands that were more intense when the Coy tempcycler was used were usually small in size, while the more intense bands obtained with the MJ instruments were mostly larger in size. This may be attributed to the ramp times between temperatures, which are longer in the Coy tempcycler than in the 2 MJ thermal controllers. In addition, two bands that were respectively the products of OPG12 and OPF15 and were well amplified in the Coy tempcycler were not amplified in the 2 MJ

thermal controllers. However, these two bands were not scored as markers and other minor variations of RAPDs between different PCR machines had little affect on the evaluation of RAPD markers in *Citrus* since no alteration in banding pattern was involved in these variations.

Nearly all RAPD markers are considered to be dominant, as DNA segments of the same length are amplified from some individuals but not others; only in rare cases do RAPD markers show a codominant segregation pattern (Williams et al. 1990). In the present study, no evidence was found to suggest any codominant segregation. Therefore all of the acceptable polymorphic bands were scored as dominant markers. However, an interesting phenomenon was observed with the following amplified bands: OPE07070, OPE20225, OPO13082, and OPO14064. These bands amplified from DNA from the F_1 but not from that of either parent. The bands segregated in the BC_1 progeny and three of them, OPE07070, OPE20225 and OPO14064, behaved in a Mendelian manner. This unexpected segregation pattern was not attributed to the production of artifacts or DNA contamination because repeated experiments confirmed that these bands were reproducible. This finding is very interesting because these bands may reflect the existence of interallelic interactions in RAPD markers. We propose the following possible mechanism. Two parents may contain two different types of homozygous alleles, both of which have a missing random primer-binding site at a different place in the amplified region. Thus neither of the parents display an amplified product. When the genomic DNA from the F_1 is used in an amplification reaction, however, an allelic complementation may occur through segmental homologous annealing between newly-amplified single strands and subsequent extension in two orientations until the two random primer-binding sites are reached. As a result, a hybrid DNA fragment extending between two random primer-binding sites can eventually be amplified. Additional experiments are required to test this hypothesis. Nevertheless, the hybrid-unique bands can be used as markers since they segregate in the BC_1 population in a fashion similar to the other RAPD markers. The data scored from these bands were included in the linkage analysis.

Segregation assays and linkage mapping

From a total of 373 polymorphic bands (Table 1), it was possible to score 266 as reproducible segregating loci for segregation and linkage analysis. The scored loci were of three different crosstypes: 146 of the loci were identified as aa × Aa (with no band in *C. grandis* but a band in the F_1), 48 were of type Aa × Aa (the band was present in both *C. grandis* and the F_1), and the remaining 72 were of type Aa × aa (a band was present in *C. grandis* but not in the F_1) (Table 2). Alleles at the 48 loci of the Aa × Aa type were expected to segregate in a 3:1 ratio in the BC_1 progeny, while those at all of the other loci were tested for goodness of fit to a 1:1 ratio. Chi-square analysis indicated that 31 of the 48 loci expected to segregate in a 3:1 ratio, and 128 of the 186 loci where expected segregation was 1:1, conformed to Mendelian expectations. Thus, a large proportion of the markers (40%) exhibited distorted segregation ($P < 0.05$); the values computed for these loci indicated that 74 of them were highly skewed ($P < 0.01$) while other loci showed a lower level of distortion ($P < 0.05$). This result was similar to that observed with RFLP markers in our previous study where 37% of the markers exhibited skewed segregation (Durham et al. 1992). Similar deviations from expected ratios have also been reported for other *Citrus* crosses. Nine of forty-six isozyme and RFLP loci (20%) identified in a similar intergeneric cross displayed segregation distortion (Jarrell et al. 1992), while 29% of the markers in an intraspecific *Citrus* cross had distorted ratios (Durham et al. 1992). Skewed ratios were detected in as many as seven of eight isozyme loci in certain families of a cross between *C. grandis* and *P. trifoliata* (Torres et al. 1985). Distorted segregation between alleles has been widely discussed; it has been commonly observed in both intra- and interspecific crosses and levels in *Citrus* are not particularly high when compared to those observed in other annual or perennial species (Helentjaris et al. 1986; Landry et al.

Table 2 The number, linkage status, crosstype, and distorted segregation of RAPD markers detected in the BC_1 population of *Citrus*

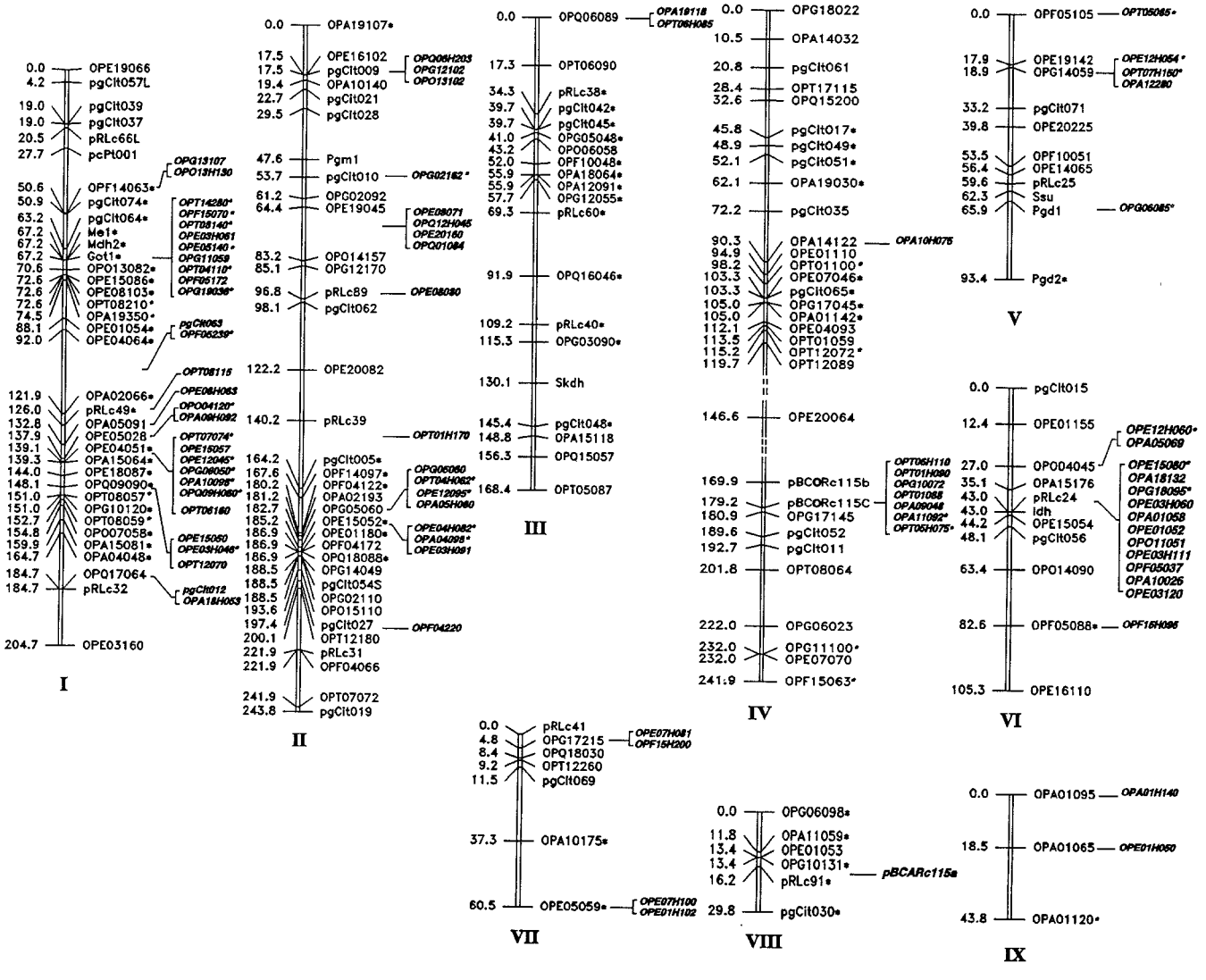
Linkage group	Number of markers	Contribution of crosstypes			Number of skewed loci[a]	
		aa × Aa	Aa × Aa	Aa × aa	C↑	P↑
I	53	32	7	14	31	2
II	40	31	7	2	6	5
III	15	14	1	0	7	0
IV	30	23	4	3	10	1
V	12	9	2	1	2	2
VI	21	7	4	10	2	2
VII	9	5	4	0	2	0
VIII	4	4	0	0	0	3
IX	5	3	2	0	0	2
Unlinked	77	18	17	42	19	11
Total	266	146	48	72	79	28

[a] C↑ = allelic segregation skewed toward *C. grandis* alleles and P↑ = segregation skewed toward *P. trifoliata* alleles

1987; Gebhardt et al. 1989; Pillen et al. 1992). The fact that many RFLP and RAPD markers exhibit distorted segregation indicates that DNA marker loci are not all neutral and both coding and noncoding sequences can be under positive or negative selection pressure (Landry et al. 1992). However, this does not preclude the use of such loci in mapping studies.

It has also been fairly common to find that markers that display segregation distortion are clustered on particular linkage groups (Landry et al. 1992; Prince et al. 1993). Jarrell et al. (1992) found that RFLP loci with distorted segregation clustered in three places in the *Citrus* genome. In the present study, 77 of the 107 distored RAPD loci were assigned to the current map. More than 40% of them clustered on linkage group I (Table 2; Fig. 2) and most of the remaining markers clustered on linkage groups II, III, IV and VIII. Areas of clustering in general corresponded to skewed areas identified with RFLP and isozyme markers in our previous study, although the areas were found to be much more extensive when the more numerous RAPD loci were available for analysis. Interestingly, however, RFLP markers that displayed segregation distortion in the cross constructed by Jarrell et al. (1992) did not usually show skewed allelic distribution in our population and vice versa. This distribution pattern suggests that most of the distorted loci in the *Citrus* genome may be linked to genes exposed to direct selection. In a

Fig. 2 Linkage map of *Citrus* consisting of RAPD markers consolidated with the previously-mapped RFLP and isozyme markers. This map was created based on segregation analysis data collected from an intergeneric BC_1 population of *Citrus*. All of the markers with designations beginning with 'OP' are RAPDs. RFLP markers are designated by 'pRLc' or 'pcPt' if they were obtained from a cDNA library and 'pgCit' if they were obtained from a *Pst*I genomic library (Durham et al. 1992). All other loci are identified by protein markers. The *prominent lines* designate the linkage groups of the core map with recombination values calculated with MAPMAKER (Lander et al. 1987) listed on the left. Markers in the core groups are all of the aa × Aa crosstype. Markers listed to the right of each core linkage group are those Aa × Aa and Aa × aa markers that could be located to the linkage groups using JoinMap (Stam 1993). Loci displaying distorted segregation are marked with *asterisks*. The *broken lines* in linkage group IV represent gaps where the significance level of linkage of the two adjacent markers is lower than the threshold LOD value (LOD < 3.0). Seventy-two RAPD markers remain unlinked to any group in this map



number of other studies it has been found that the clustered skewed loci were skewed toward one particular parent. This was also the case in our study. As shown in Table 2, almost all of the distorted loci skewed toward the recurrent parent *C. grandis* in three linkage groups, I, III, and IV, which contained most of the skewed loci, while all of the distorted loci skewed toward the donor parent *P. trifoliata* in groups VIII and IX. The presence of these large blocks of loci skewed toward one parent or the other may have implications for the use of this population for QTL analysis.

Unexpectedly, the segregation data in this study indicated that the recurrent parent, *C. grandis*, was highly heterozygous – 120 of the 266 scored RAPD loci were heterozygous in this species. This was in contrast to our previous study where 'Thong Dee' was homozygous at 41 of 48 RFLP loci (Durham et al. 1992). This situation, where *C. grandis* was heterozygous at many of the marker loci, resulted in allelic combinations other than those usually found in a backcross population and decreased the number of markers that could be unambiguously assigned to a map position. This unexpectedly-high level of heterozygosity might be due to RAPD amplification outside coding regions; RFLP markers might be more likely to map within coding regions since the probes used to generate them came from cDNA or *Pst*I genomic libraries.

All of the segregation data from the aa × Aa crosstype loci, along with the data from the previously-identified isozyme and RFLP markers, was imported to MAPMAKER for linkage analysis. Two-point and multiple-point analysis showed that 122 of the 146 RAPD loci were linked with a LOD level of > 3 and fell into nine groups. One group was easily distinguished from the others by its small size, as it contained only three markers. Within the remaining 24 markers that showed no linkage to these groups, there were four linked pairs: OPA10098/OPT06160, OPE18065/OPQ14138, OPF10130/OPT17045 and OPO14064/OPT08168. When the procedure described in Materials and methods was used to order loci within the map, 160 markers, including 51 previously-mapped isozyme and RFLP markers, could be assigned to nine linkage groups to form a core map (Fig. 2). The 11 linkage groups determined in the previous map were merged without difficulty into eight groups in the current map.

The total length of the current map is 1192 cM with a mean distance of 7.5 cM between markers. The length for individual linkage groups ranges from 29.8 cM to 243.8 cM. The largest interval between markers is less than 30 cM and most marker intervals have a distance of ≤ 5.5 cM. The three largest linkage groups, I, II and IV, cover more than 50% of the total map length and contain an even higher percentage of the total number of ordered markers. However, the linkage was only tentative in group IV because it contained a gap from OPT12089 to pBCORc115b, where confidence of linkage between the adjacent markers OPT12089/OPE20064 and OPE20064/pBCORc115b was lower than the threshold LOD value. There is also a rather large gap in the center of linkage group I. The genome of *Citrus* has been estimated to span from 1500 to 1700 cM (Liou 1990; Jarrell et al. 1992); thus, the current map may cover from 70 to 80% of the genome.

Since MAPMAKER does not accept mixed segregation data, linkage analysis involving the other 120 markers, that behaved as Aa × Aa and Aa × aa crosstypes, was performed using the JoinMap program (Stam 1993). As expected, when only Aa × aa and aa × Aa type raw data were loaded, no linkage was detected between these two types of markers. Since gamete formation in the recurrent parent is always independent from that of the F_1 parent, little information to show linkage between these two types of markers can be obtained from the BC_1 population. JoinMap analysis, which included the Aa × Aa markers, indicated that this type of marker could be linked to both Aa × aa and aa × Aa marker types. Therefore, when all three types of raw data were loaded to JoinMap, many of the remaining RAPD markers could be assigned to a linkage group; 61 of them (30 markers with the Aa × aa crosstype and 31 markers with the Aa × Aa crosstype) showed linkage to the nine core linkage groups at a level of LOD > 3. A further 14 markers could be grouped to form four additional linkage groups. One group consisted of five markers and each of the other groups had three markers. One marker, OPE01H050, showed linkage to both group III and group IX. This implies that these two groups may be eventually merged to the same linkage group. Although it was possible to assign these markers to linkage groups with Joinmap, the markers could not be ordered unambiguously within the groups because Aa × Aa type dominant markers could provide only partial linkage information. Therefore the markers are shown in their approximate relationship to the core map (Fig. 2).

When the core aa × Aa data was analyzed with Joinmap, no obvious discrepancies in map order were found. However, with the JoinMap program, map centimorgans were greatly reduced in the four large linkage groups (I–IV) when compared to the values obtained with MAPMAKER. Linkage group I covered 204.7 cM when calculated with MAPMAKER while it was only 150.5 cM in length when produced with JoinMap. The centimorgans of groups III and IV estimated with JoinMap accounted for only approximately 60% of those estimated with MAPMAKER and the total length of group II was reduced further, to less than 50%.

RFLP analysis and mapping of cold-acclimation-responsive clones

Three cDNA clones were used in initial screening, but only the largest clone, pBCORc115, showed polymorphism with *Eco*RI- and *Hin*dIII- digested DNA. This clone was subsequently used as a hybridization probe of Southern blots containing *Eco*RI-digested DNA from the 60 BC_1 progeny plants. A total of seven bands (of

sizes 3.50, 2.10, 1.95, 1.35, 1.25, 1.00, and 0.60 kb) were revealed by this probe (Fig. 3). Only one of the bands (0.60 kb) proved to be monomorphic; the other bands were polymorphic among the population. These polymorphic bands were scored as three loci that were deduced from the genetic behavior of the banding patterns in the BC_1 population; the loci were designated pBCORc115a, pBCORc115b, and pBCORc115c. All three loci appeared to be heterozygous in the recurrent parent *C. grandis* since none of the bands involved in the three loci were found to be persistent among the progeny. However, the alleles from the two parents were always distinguishable so that a *C. grandis*-donated codominant allele in the progeny could be simply scored as a recurrent parental type homozygote. Alleles that give rise to overlapping bands may exist between pBCORc115b and pBCORc115c. In addition, pBCORc115a and pBCORc115c were deduced to have a "null" allele (no hybridized signal) since in a few individuals of the progeny only one band from pBCORc115b was detected and no bands from the other two loci were found. Linkage analysis demonstrated that two of these three loci, pBCORc115b and pBCORc115c, were significantly linked with a recombination frequency of 13.6%, and they were significantly linked to the markers on group IV (Table 3). These two loci were mapped on linkage group IV between OPE20064 and OPG17145 (Fig. 2). As shown in Table 3, pBCORc115a was closely linked to pRLc91, and OPG10131 and was assigned to linkage group IX next to pRLc91.

pBCORc115 contains a 850-bp insert which is very close to the mRNA size (950 bp) revealed in Northern-blot assays (data not shown). Sequencing data have shown that this clone has a sequence similar to that of RAS15 (an cold responsive gene) in alfalfa (Monroy et al. 1993). At least four cDNA clones representing different members of this *Citrus* gene family have been obtained and all of them have different sequence elements from one another (unpublished data). These different members are expected to be encoded by different alleles from three polymorphic loci and a monomorphic locus. Work is in progress to further characterize this gene family.

Fig. 3 Southern-blot analysis showing segregation of the cold-acclimation-responsive loci in the BC_1 progeny. The Southern blot containing *Eco*RI-digested DNA from the two parents, F_1 and BC_1 progeny, was probed with a cold-acclimation-responsive cDNA clone pBCORc115. *Lanes 1, 2* and *3* are respectively *C. grandis*, the F_1, and *P. trifoliata*. The *other lanes* contain DNA from some of the BC_1 progeny. Three polymorphic loci, pBCORc115a (**A**), pBCORc115b (**B**) and pBCORc115c (**C**) were deduced from the RFLP phenotypes

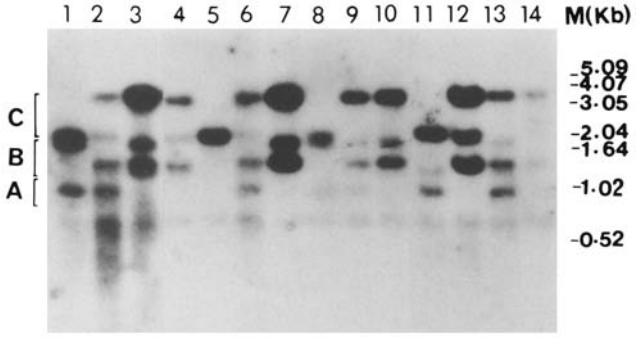


Table 3 Recombination percentage, map distance, and LOD value between the loci revealed by the cold-acclimation-responsive (COR) clone pBCORc115 and some mapped loci

Linked loci	Recombination percentage (%)	Map distance (cM)	LOD
pBCORc115c/OPG17145	5.9	2.4	10.4
pBCORc115c/pgCit052	11.9	11.3	8.4
pBCORc115c/OPE20064	24.1	37.7	3.3
pBCORc115b/pBCORc115C	13.6	11.2	7.6
pBCORc115b/OPG17145	17.6	13.6	5.0
pBCORc115b/OPE20064	25.9	26.7	2.8
pBCORc115a/OPG10131	10.3	–	3.9
pBCORc115a/pRLc91	6.3	–	4.3

Conclusions

The stability and general reproducibility of RAPDs have been further confirmed in this study with *Citrus*. This suggests that RAPDs are reliable as molecular markers for linkage mapping. However, this does not hold true for all RAPD markers; many of the originally-identified polymorphic bands in this study were subsequently excluded from segregation and linkage analysis because of poor resolution for scoring or less stability. Therefore, RAPDs must be carefully evaluated prior to their selection for mapping or for other purposes.

Nearly half of the RAPD markers detected in this study were heterozygous in the recurrent parent, *C. grandis*, suggesting higher than previously-expected heterozygosity in this species. We have collected a great deal of data of various types from the progeny population used in this study. In order to make best use of this data, we wish to have as saturated a linkage map as possible. Thus, it was somewhat disappointing that so many of the identified RAPD loci could not be unambiguously placed on the map. It will be necessary in the future to screen more markers than anticipated to more fully saturate this map. The construction of an integrated map to include those markers heterozygous in the recurrent parent is possible; the presence of the Aa × Aa type of RAPD markers allows the combining of linkage data from the Aa × aa type to that of the aa × Aa type of markers. However, codominant Aa × Aa markers would be more useful for integrating the other two types of markers in the map.

This study has provided the first population of RAPD markers for map construction in *Citrus*. It has demonstrated that RAPD markers are feasible for genetic mapping in this fruit tree crop. It is our goal to

construct a high-density linkage map to identify QTL for cold- and salt-tolerance in *Citrus*. To this end, each BC_1 progeny plant from the cross between very cold-sensitive *C. grandis* and extremely cold-hardy *P. trifoliata* is being propagated and will be tested for response to cold acclimation and salinization.

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