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Developing Specific Markers and Improving Genetic Mapping for a Major Locus *Tyr1* of Citrus Nematode Resistance

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Abstract The NBS-LRR class resistance-gene candidate sequences (Pt8a and Pt9a) were used to develop new specific markers to the citrus nematode resistance gene locus *Tyr1*. By high-density colony screening, over 200 positive clones were pulled out from the BAC library. A few of the clones were found to be closely linked with the *Tyr1* region, because the primers from these clones insert sequence produced polymorphism which matched up with the phenotype after bulked segregant analysis. By primer walking approach, three integrate NBS-LRR class resistance-gene sequences were tagged and identified separately in three clones (7A4, 4L17 and 29F20). More specific markers were developed from these tagged sequences and relatively high-density genetic maps were constructed by incorporating the newly developed markers and previously developed markers in the '9145 family'. New markers were applied in '9401 family' trying to estimate roughly the genetic distance between the Ctv and Tyr1 region.
Keywords Citrus; Nematode; Resistance gene; Molecular marker; Genetic map

Background

Citrus nematode [CN, *Tylenchulus semipenetrans* (Cobb 1914)] has been one of the major parasitic pests that cause loss in production and a reduction in quality of fruit throughout the world (Duncan and Cohn, 1990). Citrus rootstock breeding for nematode resistance is also one of the objectives of modern citrus genetic research and molecular engineering. Although the inheritance of citrus nematode resistance is not yet clear, the QTLs of the citrus nematode resistance had been mapped with RAPD markers indicating a major gene confers resistance to citrus nematode (Ling et al., 2000).

The construction of high-density genetic map is very important for tagging the genes or QTLs, as well as for map-based cloning (MBC) and marker-assisted selection (MAS). The development of DNA markers has resulted in the genetic linkage map construction in most crop species, including perennial crop of citrus. Several loci of horticultural significance in citrus had been tagged with RAPDs, SCARs, CAPS and other markers, such as the citrus tristeza resistance gene (*Ctv*) and the major gene for citrus nematode resistance (*Tyr1*) (Gmitter et al., 1996; Deng et al., 1997; 2000; Fang et al., 1998; Ling et al., 2000). In recent years, the way of resistance-gene analogs (RGAs) or resistancegene candidates (RGCs) analysis by degenerate primers has been quickly and broadly extended. The NBS-LRR (nucleotide-binding site, Leucine-rich repeat) class of resistance genes have been proved to be most prevalently existed (Shen et al., 1998; Speulman et al., 1998; Meyers et al., 1999; Deng et al., 2000; Noir et al., 2001; Madsen et al., 2003; Lee et al., 2003). These genes share striking structural similarities, which seems to encode receptors that detect the presence of specific pathogens, and suggests that certain signaling reaction are common to all or most plant defense systems (Lee et al., 2003). Ten classes of NBS-LRR sequences were found in citrus with RGC-derived primers (Deng et al., 2000). Three of the RGC-derived markers

(designated as 18P33a, Pt9a and pt8a) were associated with *Ctv*, and one fragment (Pt8a) was linked closely with the *Tyr1*.

In this study, we selected the Pt8a and Pt9a positive BAC clones from USDA 17-47 BamH Ⅰ BAC library, sequencing and redesigning primers to screen the '9145 family' and plus analysis by BSA, newly developed markers to integrate into the genetic map of citrus nematode resistance loci of *Tyr1*, and three NBS-LRR class gene sequences were obtained by Pt9 fragment based BAC clone primer-walking, from which more specific markers closely linked with *Tyr1* were developed and its improved genetic map was constructed.

1 Results

1.1 Identification of BAC clones using Pt8a and Pt9a as probes

Over 200 positive BAC clones were screened out of the *Bam*H Ⅰ BAC library, using Pt8a and Pt9a as probes, under moderate stringency hybridization condition. From which, 23 clones were selected by PCR amplification with 'Pt9F+R' according to the length of the products and 28 clones were selected with 'Pt8F+R'. Over ten clones were finally chosen to sequence their insert's ends after amplification of the Pt8a and Pt9a fragments from these clones and polymorphisms were revealed between the nematode resistant and susceptible plants by restriction digestion of the amplified product with specific cutter.

1.2 Identification of SCARs and CAPS markers linked to CN resistant locus "*TyrI*"

The new primers designed from the insert end sequences were used in the bulked segregation analysis of the'9145 family'. Fourteen pairs of primers (Table 1) from seven clones (3p21, 45b9, 4L17, 34d12, 40b15, 7A4 and 63F7) were found the kind of polymorphisms existed in CN family by BSA at first. But, after further screening among the'9145 family', several pairs of primers from clone 34d12, 63F7 were taken out, which are not matching up with the phenotypes of known resistant or susceptible individuals. Some kind of departure from the '*Tyr1*' locus were observed in the bulked segregant analysis of '4L17L/*Hin*dⅢ' (Figure 1C), indicating that the marker was not tightly linked with the CN resistance gene.

After southern hybridization, Pt8a and Pt9a fragment were present as single or two copies in 4L17, 29F20 and 7A4 BAC clones and they were chosen to go sequencing by primer walking. From the 7A4 clone, a 6 319 bp length sequence (GenBank accession number: AY336943) was obtained from the both sides of the 'Pt9F+R' fragment. BLAST searches of the GenBank database revealed high sequence similarities between the ORF domains of the 6 319 bp length sequence and known plant disease resistance genes. A series of new, more specific primers were designed based on the new sequence, mainly from the ORF domains. Several pairs of primers have been selected as the more specific markers to the *TyrI* locus after doing BSA, screening the '9145 family' and other known- phenotype populations or individuals, among which the '7A4(1407)/*Bfa* Ⅰ' and '7A4(2168)/*Bfa* Ⅰ' are the better candidates. As shown in Figure 1B, the polymorphic band from '7A4(1407)/*Bfa* Ⅰ' was clearly revealed and the difference were easier to be judge from, and their reliability and repeatability were also confirmed under the standard protocol and ordinary lab condition.

1.3 Linkage-map construction

A genetic linkage map of citrus nematode resistance with 9 previously obtained markers and 10 newly developed markers (Table 1) were constructed by JoinMap 2.0 (Figure 2C). The nine previously developed markers were selected out by BSA of '9145 family' and their segregation data were obtained by screening all the individuals of the family. All the previously developed markers were mainly served as anchoring markers in the mapping population. Among these markers, OPX10, OPO04, OPO07, OPW14 were RAPD markers, SCO07, SCAD08, SCAm02, SCT08 were SCAR markers origionally from RAPD markers, Pt8a was locus co-segregating with *Tyr1* (Ling et al., 2000, Deng et al., 2001). Y65F+R marker was newly developed from *Ctv* candidate genomic sequence.

Table 1 Markers with polymorphisms detected among '9145 family': between two pairs of bulks made with the extreme resistant (R) and susceptible (S) individuals genomic DNA and their parents Swingle citrumelo and LB6−2

Marker	Primer	Sequence	Tm (℃)	Digestion enzyme	Polymorphism
7A4R	5'−upper (F)	GGCATCTCTATGAACTCTACAG	50	*Alu* Ⅰ	Codominant
	3'−lower (R)	AATAGAGGACAAGTCGATATG			
7A4L	5'−upper (F)	TTTGGAACTTATCAACTACA	56	−	Codominant, P/A
	3'−lower (R)	AGATGAGTTCTCCTTTTATAGTTA			
7A4(1407)	5'−upper (F)	GTTGGAAAGACGACTCTGTTA	53	*Bfa* Ⅰ	Codominant
	3'−lower (R)	CAAACAGAACATTACTTTTCAATC			
7A4(2168)	5'−upper (F)	CAATGTTTGATAAAGTATGGAGA	50	*Bfa* Ⅰ	Codominant
	3'−lower (R)	TCTTCTCCCTTGATAACGACTTTAC			
40b15L	5'−upper (F)	AAGTGTAGCTGTAGTATGATACC	48	−	Codominant, P/A
	3'−lower (R)	TTAGATAATGGATTTCATAACT			
45b9T	5'−upper (F)	GGATCCTTGTAGTAATTTGTTGT	55	*Hinf* Ⅰ	Codominant
	3'−lower (R)	GGAAGGCGGAAATTTACTGTT			
4L17R	5'−upper (F)	GATGTAGGGGTCTTTATGTAA	58	*Cfo* Ⅰ	Codominant
	3'−lower (R)	GTCGCTTTGCATGTTGTA			
4L17L	5'−upper (F)	TAGAAACCCCAATTCAAAAG	58	*Hin*dⅢ	Codominant
	3'−lower (R)	GACTTGAGAAGCAGAGAATGTC			
3p21S	5'−upper (F)	TCGAAGACTTGCAAGATTGTT	55	*Msp* Ⅰ	Codominant
	3'−lower (R)	CAATTTCCCAACTTGGTTGT			
Y65F+R	5'−upper (F)	GCATCCCTCTTCCTAATTCACA	50	*Rsa* Ⅰ	Codominant
	3'−lower (R)	TCTGCCAAGCCTGAAACATC			

Note: P/A: Presence or absence of DNA bands

The anchoring markers order of the first round linkage group (Figure 2C) in '9145 family' was not different from the one (Figure 2B) generated by MAPMAKER EXP3.0b (Deng et al., 2000) and the one (Figure 2A) in R family. The second round map (Figure 3A) construction with the more specific markers included showed almost the same markers order and genetic distance with the first round built map (Figure 2C) except that the locus of 7A4L was different. After incorporation of the specific marker 7A4(1407) and 7A4(2167), 7A4L locus had changed its position from the top of the Pt8 to the lower of Pt8 on the map, far away from the presumed CN resistance "*Tyr1*" locus (between Pt8 and SCO07). In this linkage map, nine GRC-derived markers' loci spanned 12.1 cM of between the "*Tyr1*" and the "*Ctv*" regions (SCAR marker SCAD08 were supposed to be the *Ctv* locus). 7A4R was the closest marker locus to "*Tyr1*" at distance of 1.5 cM, 7A4(1407), 7A4(2168), 4L17L and 40b15 flanked "*Tyr1*" at distance of 2.1 cM. 225 individuals were randomly chosen from '9401 family' to screen their genotype segregation with the eight markers that were located between *Ctv* and *Tyr1* regions in '9145 family' linkage map. The map constructed with the data of 225 individuals' marker segregation showed in Figure 3B. Eight markers were successfully mapped into one linkage group and the map covered 87.5 cM, with average map density of 11 cM per marker. Compared with CN resistance map (Figure 3A), the orders of three common markers 4L17R, 4L17L and SCO07 were completely altered in the linkage map of '9401 family'. But the order of another five markers basically stayed in the same.

The two markers 4L17R and 4L17L that were from the two borders of BAC clone insert (about the length of 80 kb) is just 3 cM apart in genetic map of '9145' population, far less than the genetic distance of the two ends of 7A4 (6.6~7.1 cM), but is 87.5 cM apart in genetic map of '9401' population. To the best of our knowledge, marker SCAD08 is very close to the *Ctv* locus and marker SCO07 is co-segregated with *Tyr1* locus. From the linkage

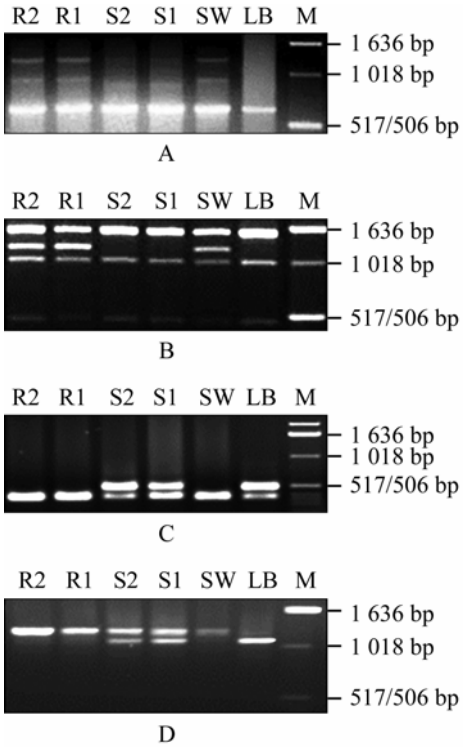

Figure 1 The polymorphism between two pairs of bulks in 12 individuals' genomic DNA

Note: A: RGC-derived 7A4L; B: RGC-derived 7A4(1407)/*Bfa* Ⅰ; C: RGC-derived 4L17L/*Hind* Ⅲ; D: RGC-derived 4L17R/Cfo Ⅰ; R1,R2: Nematode-resistant individuals in '9145 family'; S1,S2: Nematode-susceptible individuals in '9145 family'; SW: Swingle citrumelo; LB: LB6-2; M: 1 kb DNA ladder

groups in Figure 3, the genetic distance between the two resistance genes' loci is about 12.1 cM in '9145 family' and 17.2 cM in '9401 family'. The marker Y65F+R/*Rsa* Ⅰ developed from the *Ctv* resistance candidate sequence was the only one located between the *Ctv-Tyr1* regions in '9401' linkage map, but there are much markers located between the *Ctv-Tyr1* regions in '9145' linkage map.

2 Discussion

2.1 The bulked segregant analysis in citrus genetics

The bulked segregant analysis has been successfully and widely applied in much field of research, especially in genetic markers selection and other difference discriminating analysis (Mansur et al., 1993), and has accelerated the mapping of many genes in different plant species (Mohan et al., 1997).

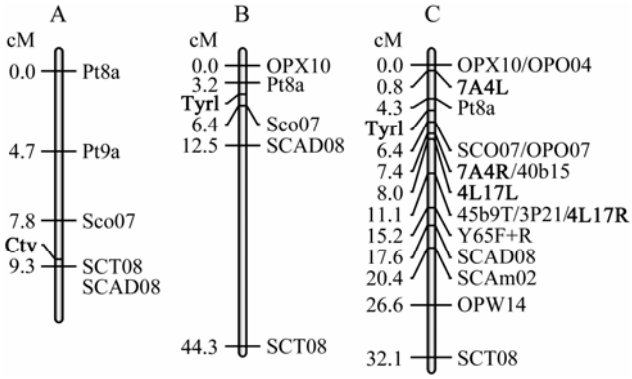

Figure 2 Genetics linkage map of the nematode resistance loci "*Tyr1*" (Deng et al., 2000)

Note: A and B were linkage maps constructed before and be used here for referendum and comparing; C: The first round building of linkage map of the nematode resistance loci "*Tyr1*" with the previously obtained multiple markers and the newly developed markers in the '9145 family', 'LB6−2'× 'Swingle'BC1 population

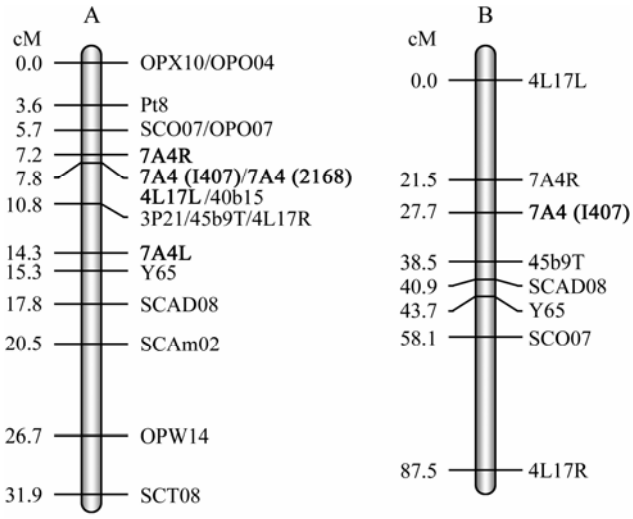

Figure 3 Genetic linkage map of molecular markers in '9145 family'(A) and '9401 family' (B)

Note: The specific markers 7A4(1407) and 7A4(2168) were incorporated in the second round map construction of '9145 family'

Several genes in citrus for controlling important horticultural traits, such as CTV resistance (Gmitter et al., 1996; Deng et al., 1997), CN resistance (Deng et al., 2000; Ling et al., 2000), dwarfing (Cheng and Roose, 1995), and fruit acidity (Fang

and Roose, 1997) had been tagged by molecular markers using BSA. Ling et al. (2000) used the second bulks of increased individuals to confirm the validity of markers that was selected by the first bulk screening.

In this work, bulks of different number of individuals between 6 and 12 were tried to select markers and the results were almost the same. A few of the markers selected by BSA, such as 34d12, 63F7R, not matching up with the known phenotypes of the individuals among '9145 family', although they were much well fitted to the phenotypes of the bulked segregating samples and even their parents, were taken out in the earlier stage. Some markers such as 3p21S, 45b9T and 4L17L, after screening the phenotype-known rootstocks, were thought to be not specific and unsuitable for application in MAS of citrus rootstock breeding. So, BSA is just the first and initial steps for marker development, but not the last. Much works of selection and evaluation needs to done before extended application might be expected in genetic identification of MAS and MBC in breeding.

2.2 The RGC gene clustering in citrus and the genetic distance between Ctv and *Tyr1*

By this study, it is obvious that the gene *Ctv* for resistance to CTV and the major gene *Tyr1* for resistance to citrus nematode are closely linked on the chromosome of CN family (9145). Both Pt8a and Pt9a markers are co-segregated with *Ctv* locus not only in R family (Deng et al., 2000), but also their derived markers co-segregated with *Tyr1* in 9145 family. This can also be confirmed by the previously mapped many *Ctv*-linked markers such as SCAD08, SCAm02, SCT08, SCO07 and a Ctv candidate sequence-derived marker Y65R+F that are also associated with resistance and mapped with *Tyr1* in the 9145 family. The *Ctv-Tyr1* region surely contains a major cluster of resistance genes. In addition to previously cloned RGC sequences, three NBS-LRR class gene sequences were obtained by BAC clone primer walking (GenBank accession number: AY336943). This phenomenon has also been found in other plants resistance genes research (Meyers et al., 1999; Shen et al., 1998).

The incorporation of markers from linkage with *Tyr1* to the *Ctv* locus region of'9401 family' was the first try to get an initial picture of major clustering of resistance genes in *Ctv-Tyr1* genomic region. The results showed that the two resistance genes loci were not so far away from each other. The structural rearrange ment in the *Ctv-Tyr1* chromosomal region was approved by the different markers' order in'9145' and '9401' family linkage map (Figure 3A; Figure 3B), although the eight markers' linkage in 9401 family was rather roughly constructed. A mutation or recombination event occurred in the *Ctv* region was also found in at least one group of *P. trifoliata* (Fang et al., 1998). The *R* gene cluster between *Ctv-Tyr1* regions might have arisen from the kinds of recombination and rearrangement events.

2.3 Other factors affecting the efficiency of the linkage map

Once the linkage mapping of the genes of interest with high resolution is complete, the genes may become targets for map-based cloning. As demonstrated in some model plants, molecular marker technologies have been providing powerful tools for tagging genes of interest rapidly for subsequent marker-assisted selection (MAS) and map-based cloning (MBC). The degree of correctness and accurateness of the genetic map is presumably based on its density of the existing markers. Well-saturated markers are proved to be decisive to localize the gene of interest to a short genomic region and facilitate the cloning the target sequence. The success will depend on the following two factors: (1) the size of the population; (2) the development of new markers and more specific markers.

One of the essential requirements for MAS in breeding program is the specificity and precision of markers with low error rate in scoring, that is as close to the gene as possible for its utility across all population, which is relied on the size of population used for mapping. If the linked marker used for selection is at a distance away from the gene of interest, leading to cross-overs between the marker and the gene, a high percentage of

false-positive/negatives will be produced in the screening process.

The fairly small size of CN population has been the bottle-neck which limit the progress and hinder the whole process for the not large enough population for mapping. It is of great difficulty to score for phenotype, not only for the time-consuming and labor-intensive process of the CN resistance evaluation, but also for the difficulty to distinguish among the individuals by present evaluation methods. If some new techniques were developed to quicken up the screening for CN resistance, it would be a great progress. In banana, a biochemical method 'butanol/HCl' assay of condensed tannin' was suggested for screening of resistance to R.similes (Collingborn et al., 2000). This would be a brand-new way for future efforts to solve the problem of CN resistance evaluation.

2.4 The potential for applications of these specific markers

Much works confirmed that the NBS-LRR class resistance genes inherited from *Poncirus trifoliate* were heterozygous alleles in the progenitor and the F_1 progeny from it were segregating. However, only a few F_1 hybrids from *P. trifoliata* have inherited the CN resistance. Among the citrumelo hybrids (*C. paradisi* Macf.×*P. trifoliata*) only Swingle citrumelo was reported to be highly resistant to *T. semipenetrans* (O'Bannon and Ford, 1977). The hybrid Carrizo citrange, having been utilized in Florida for its tolerance to the burrowing nematode (*Radopholus similes Cobb*), is susceptible to the citrus nematode. Therefore, the genes conferring resistance to citrus nematode by the *Tyr1* locus is completely different from the genes conferring resistance the burrowing nematode. P. trifoliata had been and has been the only genetic donator to the resistance to citrus nematode. Lately, several new selections identified to be highly resistant to *T. semipenetrans* were also from F_1 progeny of *Poncirus trifoliata* (Galeano et al., 2003). Although the present linkage map is not well saturated with molecular markers, a few of the RGC-derived specific markers actually provide enough information for us to go MAS. As presented in much of the species and rootstocks screening results, the reliability and repeatability of the RGC-derived markers had been confirmed, especially the more specific marker 7A4(1407) and 7A4(2168), derived from the NBS-LRR sequences of BAC clone insert, proved to be highly reproducible in the rootstock resistance screening evaluation, are the better candidates for making MAS feasible in citrus rootstock breeding.

3 Materials and Methods

3.1 Plant materials

Intergeneric hybrids including 62 individuals, designated'9145 family', from LB6-2 [Clementine mandarin (*C. reticulata*)×Hamlin orange (*C. sinensis*)]×Swingle citrumelo (*C. paradisi*×*P. trifoliata*) was used as the linkage group of the major gene *Tyr1* with molecular markers for their well defined characterization of nematode resistance (Ling et al., 2000). 225 individuals out of 687 offspring from a larger population backcross between DPI 4-5 ['Nakon'pummelo (*Citrus grandis* L.)] and USDA 17-47 ['Thong Dee'pummelo (*Citrus grandis* L.) × Pomeroy trifoliate orange (*P. trifoliata*)], designated "9401 family", was also utilized here to screen the segregation of the newly developed markers and to determine the genetic distance of *Ctv* and *Tyr1*.

3.2 DNA extraction and bulked segregant analysis

Genomic DNAs of each individual were extracted from fully expanded tender leaves, following a procedure described previously (Durham et al., 1992). Two pairs of DNA bulks were constructed based on the analysis results of Ling et al. (2000). Each pair of DNA bulks was generated by randomly pooling the extracted DNAs from the 6 to 12 individuals comprising each extreme of the phenotype distribution. For the CN resistant bulk (R1 and R2) and susceptible bulk (S1 and S2), DNA samples were pooled at equal ratios and diluted to 10 ng/μL.

3.3 High-density colony screening and characterization of Pt8 and Pt9 positive BAC clones

To set up the relationship of nematode resistance

locus *Tyr1* with the newly found NBS-LRR class genetic markers (Deng et al., 2000), BAC clones were separately screened with the probe of Pt8a and Pt9a fragment. The BAC library used was constructed from *Bam*H Ⅰ-partially digested high-molecular weight genomic DNA of USDA 17-47 and consisted of more than 24 000 clones with an average insert size of 115 kb. Sixteen high-density colony filters of citrus BAC clones were screened as described previously (Deng et al., 2001). BAC DNA of positive clones was prepared using a modified alkaline procedure described by Zhang et al. (1996). DNA preps were digested with *Hin*dⅢ and *Bam*H Ⅰ at 37℃ for 4 h, and run on 1% agarose gels in 1×TAE Buffer. The gels were stained with ethidium bromide and documented. DNA fragments in gels were transferred onto nylon membranes under alkaline conditions. Southern hybridization was conducted by using DIG (digoxigenin)-labeled probes. Manufacturer's recommendations were followed in probe labeling, hybridization, and detection (Roche Diagnostics Corporation, Indianapolis, IN, USA).

3.4 Primer design, PCR amplification, and restriction digestion

Both ends of the inserts from the selected clones were sequenced by the pair of primers (T7 and BAC4). New primers were designed from the end sequence using OLIGO 6. Amplifications were performed on a GeneAmp PCR System 9700 thermal cycler (PE Applied Biosystem) in a 15 μL reaction volume; each reaction contained 10 mmol/L Tris-HCl (pH 8.3), 2.0 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 10 pmol of each forward and reverse primers, 0.6 unit of *Taq* polymerase, 100~150 ng genomic DNA. The initial denaturation was at 93℃ for 2 min, followed by 42 cycles of 1 min at 92℃, 1 min at 50~60℃(depend on the primers combination), and 2 min at 72℃. When restriction digestions were required to reveal polymorphisms between the two parents, 12 μL of each PCR reaction was incubated at 37℃ 3~16 h with 3~5 units of restriction enzymes in a 18 μL volume. PCR products or their digests were separated on 1.6% agarose gels. The gels were mixed up with ethidium bromide at 500 ng/mL before electrophoresis and then visualized with ultraviolet image system. Markers defined by a pair of specific primers that based on the sequencing of the two ends of genomic clones were referred as sequence characterized amplified regions (SCARs) markers (Williams et al., 1991). Markers defined by a pair of specific primers and restriction digestion enzymes were referred as CAPS markers (Konieczny and Ausubel, 1993). Fragments associated with citrus nematode resistance were identified using the BSA approach as described above, firstly by BSA and secondly as in accordance with the individuals' nematode resistance phenotype.

3.5 Primer walking-based sequencing of BACs

BAC DNA was prepared from 1 000 mL of cell culture, using the Qiagen plasmid maxi kit (Qiagen, Valencia, CA, USA). Sequencing was performed by the UF-DSCL, as described previously. Initial sequence was obtained with primer Pt8(F+R) and Pt9(F+R). From this and subsequent new sequences, primers were designed with the assistance of computer program OLIGO 6 and used to walk on BACs for sequencing in both directions. After primer walking, numerous new primers were designed from the newly obtained sequence to screen the‘9145 family’ and plus BSA analysis to develop the better, more specific marker with length of over 1 000 bases.

3.6 Map construction with the Join-Map 2.0

A total of nineteen molecular markers closely linked to *Tyr1*, including four RAPD markers (Gmitter et al., 1996) and four SCARs markers(Deng et al., 1997) were used to screen the 9145 family, and eight of them to screen ‘9401 family’. Based on the segregation data of the above markers separately in‘9145 family’ and ‘9401 family’, marker orders and genetic distances were calculated with JoinMap version 2.0, using a LOD value threshold of 3.0, recombination threshold value of 0.45, jump threshold of 1.0 and triplet threshold of 7.0. The Kosambi function was used to convert recombination units into genetic distance.

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