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TITLE: Mapping Genes for Bacterial Wilt Resistance in Tomato with DNA Genetic Markers

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Abstract

To locate genes associated with resistance to bacterial wilt (BW; *Pseudomonas solanacearum*) in tomato, we have analyzed an F2 population segregating for resistance using DNA genetic markers. The population was derived from a cross between a resistant tomato genotype, L285, and a susceptible cultivar, CLN286. Because this cross is intraspecific in nature and therefore low in DNA sequence polymorphism, 100 clones from the established tomato molecular map were surveyed with 30 restriction enzymes to find a total of 46 restriction fragment length polymorphisms (RFLPs). Additionally, seven random amplified polymorphic DNA (RAPD) markers were added to the map. At 10 days after inoculation, two genomic regions were found to be associated with BW resistance. One of the putative resistance loci, located on a linkage group consisting of RFLPs known to reside on chromosome six of tomato, showed a very high level of correlation with BW resistance, being significant at p=0.0004. At 14 days after inoculation, three genomic regions were correlated with BW resistance. These included the region on chromosome six, plus two others on linkage groups 7 and 10. The region on linkage group 7 was unusual in that the allele from the susceptible parent (CLN286) was the one associated with higher levels of resistance in the F2. Together, the three putative resistance loci explained 49% of the total variation in BW resistance in the F2. Five cases of significant gene x gene interactions were also observed.

Introduction

Bacterial wilt (BW) is a major disease of many important crops, including several in the *Solanaceae*. The causal agent, *Pseudomonas solanacearum*, invades the vascular system of the host and grows *in planta*, restricting transpiration of water. Wilting occurs, often accompanied by poor plant growth and even death. Although biological control of *P. solanacearum* has been recommended (Trigalet and Trigalet-Demery, 1990), the best method of control known is use of BW-resistant lines (Opeña et al, 1987). In the past, resistance to BW in tomato has generally not been stably maintained (Opeña and Hartman, personal communication), however a landrace of *Lycopersicon esculentum* known as line L285 was recently shown to possess moderately durable resistance in breeding programs conducted at the Asian Vegetable Research and Development Center (AVRDC) in Taiwan (Opeña and Hartman, AVRDC, personal communication). The resistance is thought to be polygenic, as indicated by the pattern of the resistance among F2 progeny, although the number of genes involved and their contribution is unknown.

Restriction fragment length polymorphisms (RFLP's), and more recently random amplification of polymorphic DNA (RAPDs), have been used to link DNA genetic markers to disease resistance in tomato (Behare, et al, 1991; Messeguer et al, 1991; Martin et al, 1991; Sarfatti et al, 1989; Young et al, 1988). Much of this success is due to the availability of a well-saturated RFLP map for tomato (Tanksley et al, 1992), one of the best among higher plants. Using DNA markers, breeding for complex characters such as BW resistance should

be much more straightforward. In particular, retaining resistance loci while simultaneously selecting against unwanted characters from a donor parent should be feasible with the use of DNA markers.

To locate the genes that underlie BW resistance in L285, we have initiated a mapping effort based on RFLP and RAPD markers. In this preliminary report, we describe experiments in which 53 DNA markers spread throughout the tomato genome were used to locate regions associated with BW resistance. The results indicate that at least four genomic regions contain putative loci for BW resistance, including one region that plays a major role in the response.

Materials and Methods

Plant Material. Bacterial wilt resistant L285, a *cerasiforme* type of tomato, was crossed with a cultivated but susceptible line, CLN286BC1F2-25-14-7 (henceforth called CLN286), at AVRDC and increased to produce the F2 progeny. The parents and 71 F2 progeny were grown in a soil:vermiculite:peat (1:1:1) mix in the greenhouse at the University of Minnesota in St. Paul, Minnesota. Leaves were collected from each plant for DNA extraction as described below. Three to four cuttings of each plant were then made for inoculation with *P. solanacearum* and disease scoring. Cuttings of lateral branches were rooted and transplanted into the soil mix and grown for 7 days prior to inoculation.

Disease Scoring. *P. solanacearum strain UW364*, a Race 1, Biovar 4 isolate

from tomato in China (Cook et al, 1989), was used in inoculation tests. Cells were grown on casamino acid, peptone, and glucose (CPG) medium for 48 hours, washed into sterile distilled water, and diluted to give an optical density (A600) of 0.100 (10^8 cells per mL). Cuttings of the parents and progeny were inoculated by placing 15 uL of the inoculum in the third leaf axil from the top and passing a needle through the drop into the stem of the plant. Control plants were inoculated at the same time with 15 uL of sterile distilled water. Virulence of the pathogen was determined by hypersensitivity tests on *Nicotiana tabacum*. Plants were maintained in the greenhouse at 28 - 30°C and preliminary disease symptoms scored seven days after inoculation (dai), and then again on 10 and 14 dai. On day 14 all plants that were not wilted or dead were re-inoculated to prevent escapes and plants were scored once more seven days after the second inoculation. Symptoms were indexed on a scale of one (no symptoms) to four (severe wilting and death).

RFLP Analysis. DNA extracted from the parents were surveyed to identify polymorphic clones. Tomato DNA was extracted using the protocol of (Dellaporta et al, 1983) with minor modifications. The extracted DNA was digested with one of 30 restriction enzymes, electrophoresed on 1% agarose gels, and Southern blotted onto Hybond N+ (Amersham, Inc.) membranes (Southern, 1975). One hundred tomato genomic clones kindly provided by Dr. S. Tanksley (Department of Plant Breeding, Cornell University, Ithaca, NY) were amplified by polymerase chain reaction before random hexamer labelling (Feinberg and Vogelstein, 1983) with α-32P-dCTP. Labelled clones were first hybridized to filters containing restriction enzyme digested parental DNA in order to identify band leagth polymorphisms. Each polymorphic

clone was then hybridized to filters containing parental and F2 DNA digested with the appropriate restriction enzyme for that clone.

RAPD analysis. Randomly amplified DNA polymorphisms between L285 and CLN286 and among the F2 progeny were identified using DNA extracted as described above. For each reaction, 2 ng of DNA was amplified with 15 ng of a 10-mer primer (Operon) and 1.75U Taq polymerase (Promega) as described in (Williams et al, 1990). A Coy Tempcycler (model 50) was used for 45 cycles of 1 min at 94°C, 1 min at 35°C and 2 min at 72°C. Amplified fragments were electrophoresed in 1.4% agarose.

Linkage analysis. Mapmaker software (Lander et al, 1987) was used to construct a linkage map for the L285 x CLN286 cross based on segregation data in the F2 for each polymorphic marker. Linkage was inferred with a minimum LOD (log of the odds ratio) score of 2.5. DNA markers associated with BW resistance were then identified using the statistical software, Statview-II (Abacus Concepts). In the analysis, both simple regression and single factor analysis of variance (ANOVA) were utilized with a significance level of $p < 0.01$. Two-factor ANOVA was also performed between putative BW resistance loci and the remaining DNA markers on the genetic map to uncover possible gene x gene interactions. In this analysis, a significance level of 0.01 was also used.

Results and Discussion

Genetic Map of L285 x CLN286 Cross. Because of the intraspecific nature of the cross between the parents, DNA polymorphism was low. Initially, 10 enzymes

(*BamHI, BstNI, DraI, EcoRI, EcoRV, HaeIII, HindIII, RsaI, TaqI, XbaI*) were used to survey the clones, but only 30% of the clones were polymorphic. A second set of 10 enzymes (*AseI, BclI, BsaBI, DpnI, HinfI, HhaI, MspI, NdeI, NsiI, ScaI*) were then used to prepare filters, which were probed together with filters from the first 10 enzymes. The rate of polymorphism almost doubled to 53%. The remaining non-polymorphic clones were then hybridized to filters made with a third set of enzymes (*AflII, AluI, BanI, BglII, BstEII, BstXI, KpnI, VspI, SspI, XmnI*) and an additional 8 polymorphic clones were identified. In a report by (Miller and Tanksley, 1990), the percent polymorphism between cultivated tomato and a *cerasiforme* genotype was low (41% with 5 enzymes); our results indicated that doubling the number of restriction enzymes surveyed led to a two-fold increase in polymorphisms. Nevertheless, 10 additional restriction enzymes increased the level of polymorphism only slightly.

Linkage analysis of the polymorphic clones produced the map shown in Fig. 1. Additional markers were added using RAPDs. The linkage groups generally agreed with the chromosomes of the published tomato map (Tanksley et al, 1992). However, several clones mapped to positions different to those previously reported, presumably because we mapped different segregating bands for those clones than in the earlier report. This is particularly striking for the linkage group tentatively assigned as chromosome 7. Because of this and other discrepancies, the chromosome assignments in this report should be considered tentative. Nonetheless, any statistical associations between DNA genetic markers and BW resistance would be unaffected by discrepancies in the assignment of linkage groups.

Some linkage groups were also poorly populated, with groups three, four, five, and eight having only a single marker. Still other linkage groups (1, 2, 6, and 11) consisted of two linkage blocks unlinked to one another, but inferred to be on the same chromosome based on the map of Tanksley et al. (1992). For all of these reasons, the saturation of DNA markers throughout the tomato genome was far from complete in this study and it was possible that some resistance loci might have been missed.

Disease Scoring. Disease indices of the resistant and susceptible parents were 1.0 and 4.0 respectively (data not shown), while the majority of the F2 fell between these values (Figure 2). Many F2 progeny showed a score of two, with the distribution slightly skewed toward resistance. The continuous nature of the disease severity in the F2 strongly indicated that multiple genes were involved in the response. Disease index of the F2 individuals changed only slightly between 10 and 14 days, although sufficiently to uncover different putative resistance loci (see below).

Map Locations of Putative BW Resistance Loci. Statistical analysis of BW disease index and DNA marker scores indicated that two genomic regions were associated with resistance 10 dai, one residing on linkage group six and another on linkage group eight (Table 1, Figure 1). The region on linkage group six was particularly significant, with five contiguous markers found to be significant and one (TG180) significant at the 0.0004 level (based on regression). Approximately 17% of the variation in BW reaction could be explained by this marker. Individuals homozygous for the TG180 allele from

L285 were 0.63 disease index units lower than homozygotes for the CLN286 allele.

In contrast, the genomic region on linkage group eight (TG181) was only barely significant (p=.0089) and had a much smaller impact on BW disease response. The effect of this putative resistance locus was also unusual in that the allele from CLN286 was associated with resistance, even though CLN286 is itself, more susceptible to BW disease. In this way, the putative resistance locus near TG181 resembled the one on linkage group seven described below.

By 14 dai, three genomic regions were found to have significant associations with BW disease, one each on linkage groups 6, 7, and 10 (Table 1, Figure 1). Results at 21 dai, seven days after surviving plants had been re-inoculated with *P. solanacearum*, gave essentially the same results as observed at 14 dai and are not shown. At 14 dai, the putative locus previously observed on linkage group eight was no longer considered significant, having a p-value of 0.0132. By contrast, the association between BW response and the putative resistance locus near TG180 on linkage group six had grown much stronger, with several contiguous markers now showing correlation at the p=0.0001 level. The proportion of variation explained by marker TG180 increased to 32% and the difference in disease index between individuals homozygous for the L285 allele compared to those homozygous for CLN286 had also increased to 0.98 units.

On linkage group 10, a single locus (TG230) was found to be associated with BW response. This marker just missed being counted as significant at 10 dai,

having a significance level of 0.0197 at that time. By 14 dai, the significance level was p=0.0033 and approximately 12% of the variation in disease could be explained by this locus. Homozygotes for the L285 allele at TG230 were more than 0.6 disease units lower than homozygotes for CLN286.

The effect of the putative resistance locus on linkage group seven at 14 dai resembled the one near TG181 on linkage group eight at 10 dai and differed fundamentally from the putative resistance loci near TG180 and TG230. In the case of the putative resistance locus on linkage group seven, the allele from CLN286 was the one associated with resistance. Five contiguous DNA markers were found to be correlated to BW response and two of the markers (TG51 and rA04) were significant at p=.0002. Individuals that were homozygous for the CLN286 allele at TG51 were approximately 0.66 disease units lower than homozygotes for the L285 allele.

<u>Interactions among Putative BW Resistance Loci.</u> In a genetically complex type of disease resistance, such as observed in the cross between L285 and CLN286, primary gene effects may be affected by interactions among resistance loci. To uncover putative gene x gene interactions associated with BW resistance, two factor ANOVA was performed between primary loci (identified by regression and/or single factor ANOVA) and the remaining loci on the molecular map. This analysis identified five significant interactions at 14 dai, three involving the putative resistance locus on linkage group six and two involving the putative locus on linkage group seven (Table 2).

Several interesting trends could be observed from the analysis gene x gene interactions. First, the putative resistance locus on linkage group six

interacted with two of the other genomic regions that had previously been found to be individually significant by single factor ANOVA (TG183 on linkage group seven and TG230 on linkage group 10). Likewise, the putative resistance locus on linkage group seven interacted with a marker on linkage group six (TG35b) that was also found to be significant by itself. Moreover, the putative resistance loci on both linkage groups six and seven interacted with the same locus on linkage group nine (TG16b), a marker that was not individually correlated to BW resistance.

Applications of DNA Markers Linked to BW Resistance Loci. The primary goal of this study was to map putative resistance loci for BW resistance. However, besides mapping putative resistance loci, DNA marker analysis also enables the selection of F2 plants suitable for use in breeding and genetic analysis. F2 plants that have retained putative resistance loci from L285, and at the same time carry a minimum of L285 DNA elsewhere in the genome can now be identified with DNA markers. In the process, individuals with crossovers near the putative resistance loci can also be pinpointed using DNA markers. This should be especially significant in the case of breeding BW resistance from L285, because resistance has been associated with small fruit size previously (Opeña and Hartman, personal communication).

At the same time, the contribution of different resistance loci can be studied individually, as it is now possible to select F2 plants that can be used to develop lines that carry only one or a few of the putative resistance loci. Considering the very different types of gene action observed for the different resistance loci uncovered in this study (such as the resistance locus on linkage

group six versus the locus on linkage group seven), this type of genetic dissection should be especially informative.

Finally, using DNA markers near resistance loci to select lines with tightly linked crossover may be useful for purposes other than minimizing linkage drag. In particular, crossover located near genes of interest can be used a basis for fine-structure mapping near those genes. Considering the fact that the putative resistance locus on linkage group six controls a very large fraction of the variation in BW response (greater than 30%), fine structure mapping around this locus may provide a basis for physical mapping and eventual gene cloning based on chromosome walking (Young, 1990). Although this type of research is still far in the future, the fact that tomato has desirable features such as a comprehensive yeast artificial chromosome library (S. Tanksley, personal communication) and high efficiency plant transformation (McCormick et al., 1986) may make it possible to clone some of the resistance loci uncovered in this study. If so, the major BW resistance locus of L285 may then be one of the first quantitative resistance genes to be cloned at the molecular level.

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Figure Legends

Figure 1. Linkage map of tomato, consisting of 46 RFLP and 7 RAPD markers. Marker positions are based on segregation in the L285 x CLN286 F2 population, as estimated by the Mapmaker (Lander, et al., 1987) program with a minimum LOD of 2.5. Linkage groups were tentatively assigned to "chromosomes" based on RFLPs common to the map of Tanksley et al. (1992). Those makers that map to a locus different than reported by Tanksley et al. (1992) are noted by the letter "b" at the end of the marker name. Genomic regions showing significant association with BW resistance at 10 and 14 days after inoculation are highlighted with a bar beside each of the regions. DNA markers showing correlation are noted, and the locus with the highest association is underlined. DNA marker TG16b, which was found to be significant only in terms of gene x gene interaction, is also noted.

Figure 2. Frequency distribution of BW disease index scores 14 days after inoculation. Three or four cuttings from each of 71 F2 plants were inoculated as described in the Material and Methods section and scored for BW disease 14 days later. Each group of cuttings was scored on a scale from 1 (no disease) to 4 (severe wilting and/or death).

TABLE 1. Marker loci showing significant relationship to BW resistance

Marker	Linkage Group	DAI	R-Squared	Probability (Regression)	Probability (ANOVA)	Type of Effect (a)	Phenotypic Effect (b)
TG180b	6	10	0.169	0.0004	0.0002	Dom(285)	(-)0.63
		14	0.318	0.0001	0.0001	Dom(285)	(-)0.98
TG51b	7	14	0.184	0.0002	0.0002	— (c)	(+)0.66
TG181	8	10	0.114	0.0089	— (d)	Rec(286)	(+).55
TG285	10	14	0.124	0.0033	0.0019	Dom(285)	(-).60

(a) Type of gene action, either additive (Add), recessive (Rec), or dominant (Dom). Numbers in paretheses indicate the parent (L285, "285" and CLN286, "286") carrying the allele that confers higher levels of resistance to BW.

(b) Difference in BW disease index between L285 homozygotes compared to CLN286 homozygotes.

(c) Type of gene action could not be inferred.

(d) Not significant.

Table 2. Loci showing significant gene x gene interactions

Primary Locus	Linkage Group	Interacting Locus	Linkage Group	Probability (ANOVA)
TG51b	7	TG35	6	0.0068
		TG16b	9	0.0076
TG118	6	TG183	7	0.0052
		TG16b	9	0.0008
		TG230	10	0.0022

Figure 1

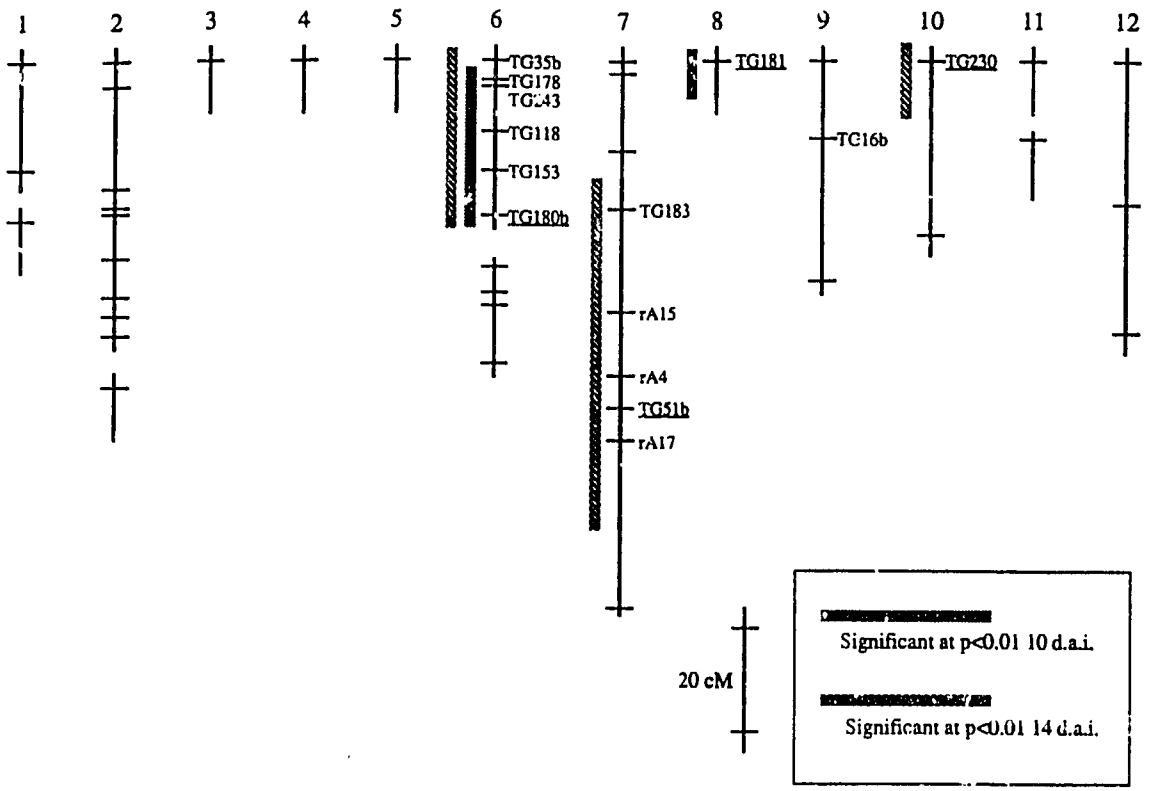
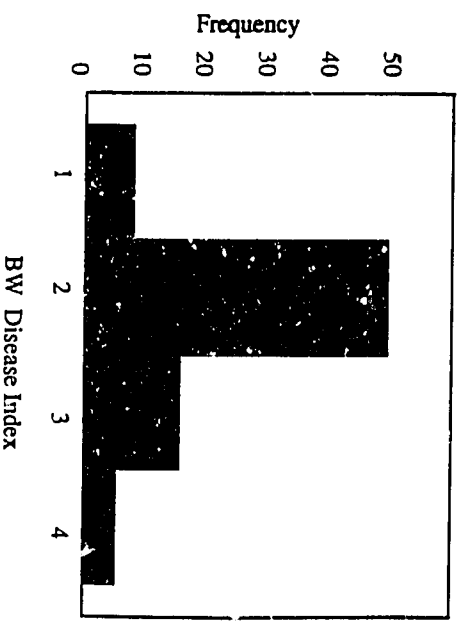


Figure 2



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