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Identification of DNA markers of tobacco linked to bacterial wilt resistance

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Abstract Bacterial wilt caused by *Ralstonia solanacearum* is one of the most destructive soil-borne diseases in the world. Breeding resistant commercial varieties of tobacco is difficult because most donor candidates' resistance is controlled by polygenes. In this paper, we demonstrate the identification of useful DNA markers for bacterial wilt-resistant tobacco breeding. One hundred and seventeen markers were identified by the amplified fragment length polymorphism (AFLP) method between *W6*, a burley variety with resistance originating from a Japanese domestic variety, *Hatano*, and *Michinoku 1*, a commercial burley wilt-susceptible variety, using 3,072 primer combinations. These markers were analyzed in 125 doubled haploid lines, derived from F_1 hybrids between *W6* and *Michinoku 1*, and a linkage map consisting of ten linkage groups was drawn. The resistance phenotype of each of these lines was investigated on the basis of the average of disease severity obtained from field trials over two growing cycles. Quantitative trait loci (QTL) analysis was performed on the marker phenotypes and the resistance phenotype of each line. One QTL for the bacterial wilt resistance of *W6* and DNA markers associated with this QTL were identified on a linkage group consisting of 15 markers, 32 cM in length. This QTL explained more than 30% of the variance in resistance among these lines.

Keywords AFLP · *Nicotiana tabacum* · *Ralstonia solanacearum* · Disease resistance · QTL analysis

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Introduction

Bacterial wilt caused by *Ralstonia solanacearum E. F. Smith* is one of the most destructive soil-borne diseases in the world, affecting the cultivation of many economically important crops such as potato, *Solanum tuberosum* L., tomato, *Lycopersicon esculentum* L., eggplant, *S. melongena* L., and tobacco, *Nicotiana tabacum* L., (Kelman 1953). Several methods such as soil fumigation and crop rotation have been employed for the control of this disease. However, as the bacterium resides in the soil and enters the plant via the roots, chemical methods for control are ultimately ineffective. Therefore, one of the most useful control methods has been the introduction of resistant varieties.

The resistance of several species to bacterial wilt has been assumed to be under the control of polygenes and resistance phenotypes to be affected by environmental conditions (Kelman 1953; Hayward 1991). Recently, however, the bacterial wilt resistance of several varieties of tomato has been genetically analyzed using DNA markers. Several quantitative trait loci (QTL) affecting resistance to bacterial wilt have been found on chromosome 6, chromosome 7, and chromosome 12 (Thoquet et al. 1996a,b; Margin et al. 1999; Danesh et al. 1994; Wang et al. 2000).

To breed for durable bacterial wilt-resistant tobacco, sources of bacterial wilt resistance were genetically analyzed and classified into two groups based on their genetics. The resistance in one group is controlled by polygenes, such as those originating from T. I. 448A and DSPA; resistance in the other is controlled by a major gene, such as that originating from the Japanese domestic varieties, *Sumatra C* and *Xanthi* (Matsuda 1977). Among Japanese domestic varieties, Matsuda suggested that resistant varieties, such as *Hatano*, *Kokubu*, *Odaruma*, and *Awa*, had the major gene *Rps*, while highly resistant varieties such as *Enshu* and *Hatanodaruma* had not only *Rps* but also polygenes; those controlled only by polygenes became moderately resistant varieties, for example *Kirigasaku* and *Suifu*. However, no major resis-

tance gene such as *Rps*, nor QTL involved in polygene-controlled bacterial wilt resistance has been mapped. Furthermore, the presence of a major resistance gene has not been reconfirmed. In practice, greenhouse evaluations of resistance are very difficult in tobacco breeding; it has been necessary to evaluate the resistance of many breeding lines in the field, where variable conditions affect the resistant phenotype of each breeding line differently. Genetic analysis with DNA markers of bacterial wilt resistance in tobacco and the identification of DNA markers linked to this resistance will be important steps in breeding resistant tobacco varieties.

In this paper, we demonstrate a method of identifying DNA markers that could be used for the marker-assisted selection of bacterial wilt-resistant tobacco varieties; we also locate QTLs carried by tobacco variety W6 that affect the plant's resistance to bacterial wilt.

Materials and methods

Pathogen culture

The *Ralstonia solanacearum* strain used in this study, Ps35, had been previously obtained from naturally infested tobacco plants in Japan's Fukushima prefecture. The strain was grown in a CPG medium (0.01% casamino acids, 1% peptone, 1% glucose) without agar at 30 °C for 18 h.

Plant material

Michinoku 1, which was developed from a spontaneous mutant of *Burley 21* (Yoshida et al. 1985) and is one of the most commonly cultivated burley varieties in Japan, was used as the bacterial wilt susceptible parent. *W6*, which is a burley variety developed by Yoshida et al. (1992) from a Japanese domestic variety, *Hatano*, and similar in appearance to *Michinoku 1*, was used for the bacterial wilt resistant parent (Fig. 1). One hundred and twenty-five *Michinoku 1-W6* F_1 doubled haploid (F_1DH) lines were developed according to the method of anther culture by colchicine-treatment for haploid plantlets (Nakamura 1974).

Infested field preparation

We prepared the infested field at Shizuoka, Japan. In 1997, the year previous to our resistance tests, a susceptible variety of *Burley 49* was planted in the field and conventionally cultivated. To ensure a uniformly contaminated field, we spray-inoculated all plants with an isolate of *R. solanacearum*, Ps35 (4.0×10^{14} cfu/100 m^2) just after topping and subsequently plowed the plants into the ground after the tobacco season. The population density of *R. solanacearum* in the soil at the following spring was investigated according to Hara et al. (1995).

Resistance tests

Resistance tests were conducted in 1998 and 1999 in the infested field. One hundred and twenty-five F_1DH lines and the two parent cultivars were transplanted into the field on 10 April in 1998 and 8 April in 1999 to evaluate them for resistance to bacterial wilt. The population density of *R. solanacearum* in the field prior to transplanting was 5×10^2 and 9.3×10^2 cfu/g dry soil in 1998 and 1999, respectively. Fifteen plants per plot were planted 32 cm apart within rows. Entries were replicated four times in a randomized block design, with each replication containing four plots of parent cultivars. In 1998, the first disease reading was taken after the development of first symptoms and subsequent readings were taken every 2 weeks for a total of four readings, at 8, 10, 12, and 14 weeks after transplanting to the field. In 1999, the disease reading at 12 weeks was used to evaluate resistance. Disease symptoms were scored on a scale of 0 to 3, as follows: 0, no symptoms observed; 1, one or two leaves on lower stalk wilting; 2, half of all leaves on stalk wilting; 3, all leaves on stalk yellowish or wilting. Average disease index $\mathrm{ADI} = \sum_{i=1}^{n} [\text{disease index}/(3\times n)] \times 100$ (n = total number per plot) was used as a measure of resistance for each line.

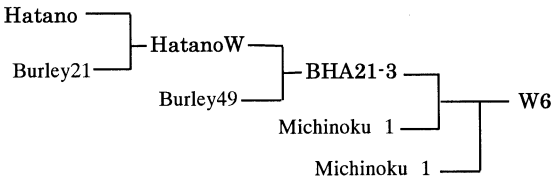

Fig. 1 The pedigree of bacterial wilt resistant variety *W6* originated from *Hatano*. *Letters* in indicate the resistant varieties or lines

Amplified Fragment length polymorphism (AFLP) analysis

AFLP analysis for the detection of DNA polymorphisms between *Michinoku 1* and *W6* was carried out according to the method of Vos et al. (1995). The combinations of 'frequent/rare cutter' used for digesting genomic DNA were *Mse*I/*Pst*I. The amplification primers consisted of a core sequence of an adaptor, an enzyme-specific sequence, and a selective extension. To reduce the background in fingerprinting patterns and increase the reliability of amplification of AFLP bands in this study, we selected the number of nucleotides of the selective extensions of *Mse*I-primers as four and that of *Pst*I-primers as three. The sequences of the AFLP adapters and amplification primers are shown in Table 1. A total of 3,072 combinations of *Pst*I/*Mse*I primer pairs were used. An ABI PRISM 377 DNA sequencer and GeneScan software (Applied Biosystems, Foster City, Calif) were used for the analysis of amplified fragments and for the identification of DNA polymorphisms between *Michinoku 1* and *W6*.

The DNA polymorphisms between *Michinoku 1* and *W6*, which were detected through segregation in the 14 plants of the F_1DH lines including the two parent cultivars, were used as DNA markers for the analysis of the entire F_1DH progeny.

Map construction

A linkage map of identified DNA markers was constructed using the marker data from the F_1DH lines and MAPMAKER software (Lander et al. 1987) with the Kosambi mapping function. Markers were assigned to linkage groups with a minimum LOD score of 3.0 and a map distance below 37.5 cM.

QTL analysis for resistance to bacterial wilt

The QTL controlling resistance to bacterial wilt was detected using the marker data and the resistance phenotype of each F_1DH line using MAPMAKER/QTL (Whitehead Institute, Cambridge, Mass.). The threshold for declaring an association with a marker and the QTL controlling resistance was LOD > 3.0.

Table 1 Adaptor and amplification primer sequences

	Sequences
Adaptor	
*Pst*I	5′-CTCGTAGACTGCGTACATGCA-3′ 3′-CATCTGACGCATGT-5′
*Mse*I	5′-GACGATGAGTCCTGAG-3′ 3′-TACTCAGGACTCAT-5′
Amplification primer	
PP	5′-GACTGCGTACATGCAGNNN
MP	5′-GATGAGTCCTGAGTAANNNN

Results

Resistance tests of F_1DH lines in the field

The ADI of the resistant parent, *W6*, ranged from 4 (8 weeks after transplanting, w.a.t.) to 31 (14 w.a.t.) and that of the susceptible parent, *Michinoku 1*, from 20 (8 w.a.t.) to 88 (14 w.a.t.) in 1998 (Table 2). The resistance of F_1DH lines showed a continuous distribution between the resistance of *Michinoku 1* and *W6* (Fig. 2). The experimental blocks did not affect the trait, and the broad-sense heritability on resistance was 0.54 (Table 3).

In 1998, ADI at 14 w.a.t., when variance among the F_1DH lines reached its maximum, was used for analysis. In 1999, the symptoms of disease developed earlier than in 1998; ADI of *Michinoku 1* was more than 70 at 12 w.a.t. Therefore, in 1999 ADI at 12 w.a.t. was used for analysis. In each line, the ADI in 1999 was highly correlated to the ADI figure from 1998 ($r^2 = 0.68$) (Fig. 3).

Identification of DNA markers and construction of linkage map

One hundred and seventeen genomic DNA markers, 66 of which originated from *W6* and 51 from *Michinoku 1*, were identified and used for the analysis of the F_1DH lines and for the construction of the linkage map. The sequences of selective extension products and the sizes of these markers are shown in Table 4. Of these markers, 106 were assigned to ten linkage groups and 11 remained unlinked (Fig. 4). Sixteen markers (13%) deviated from the expected 1:1 ratio, but most of the DNA markers showed Mendelian segregation (Table 4). The number of AFLP markers per linkage group varied from six to twenty. The total length of the linkage map was 383 cM, and the average interval length between each marker was 3.3 cM.

QTL analysis for resistance to bacterial wilt

One QTL strongly associated with resistance to bacterial wilt was observed on linkage group 5; it was 32 cM in length and appeared near marker M84 in both years'

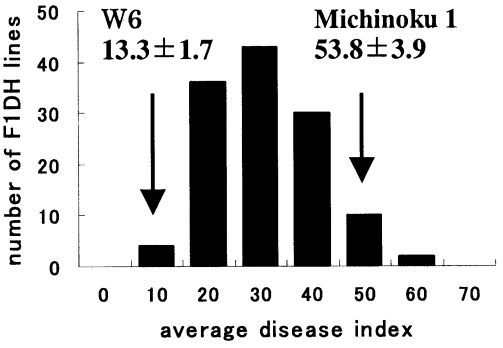

Fig. 2 Frequency distributions of the average disease index (ADI) of F_1DH lines at 12 w.a.t. in 1998

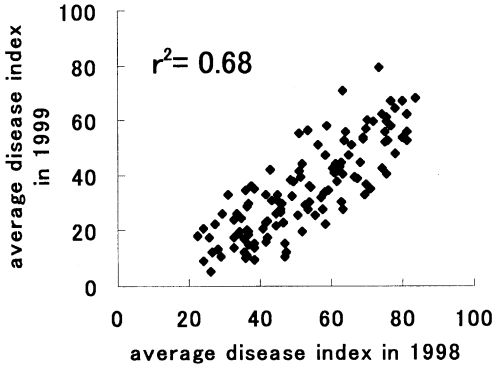

Fig. 3 Correlation diagram of average disease index (ADI) of F_1DH lines in 1998 and 1999

Table 2 Average disease index (ADI) of tobacco varieties observed during resistance tests

	Weeks after transplanting				
	1998				1999
	8	10	12	14	12
W6	4.1	7.1	13.3	30.8	16.5
Michinoku 1	20.1	35.3	53.9	87.7	73.3

Table 3 Analysis of variance for average disease index (ADI) in 1998[a]. Heritability on resistance was 0.54

Factor	SS	*df*	MS	*F*-ratio	*P*
Block	478	3	159	0.84	0.47
Plant	131,345	124	1,059	5.60	<0.01
Error	70,289	372	189		
Total	202,113	499			

[a] The average disease index at 14 w.a.t. in 1998 was used in this analysis

Table 4 Genomic DNA markers between *Michinoku 1* and *W6* by AFLP analysis using the combination of *Pst*I/*Mse*I

Linkage group	Marker	Sequence of selective extension of the amplification primers		Size of markers (bp)	Chi-square[a]	Linkage group	Marker	Sequence of selective extension of the amplification primers		Size of markers (bp)	Chi-square[a]
		*Pst*I	*Mse*I					*Pst*I	*Mse*I		
1	M36	ACC	CCGG	225	0.96	5	M17	AAA	CAGG	239	4.23*
1	M65	GGT	CATT	166	0.96	5	M84	CAG	CCTA	127	3.22
1	M62	GGT	CACT	166	0.51	5	M82	CAG	CCGA	128	2.61
1	M1	ACT	CTAC	280	0.29	5	M104	CCG	CCGA	128	2.31
1	M73	CAA	CTGA	203	0.00	5	M83	CAG	CCGG	128	2.06
1	M89	CAT	CAGA	98	2.61	5	M93	CAT	CTGT	450	0.52
1	M101	CAT	CCGA	98	3.27	5	M94	CAT	CTGT	447	1.18
2	M3	AAA	CTCA	360	0.39	5	M74	CCA	CTCA	336	0.03
2	M5	AAT	CATG	141	0.00	5	M108	CGT	CTTA	189	0.13
2	M61	GGT	CACT	56	0.00	5	M92	CCT	CTTA	185	0.12
2	M2	ACT	CGTG	129	0.03	5	M91	CCT	CTTA	200	0.12
2	M7	AAA	CTAT	300	0.03	5	M107	CGT	CTTA	200	0.52
2	M12	AAA	CACA	310	0.00	6	M26	ACT	CCTC	460	1.16
2	M14	AAA	CGCC	230	0.00	6	M44	GCA	CGAG	59	0.64
2	M19	AAG	CGGT	352	0.00	6	M22	ACA	CCTA	107	0.51
2	M27	ACT	CCTG	130	0.07	6	M45	GCA	CGGT	130	0.64
2	M40	GCT	CGAA	62	0.00	6	M46	GGA	CGAG	110	0.64
2	M53	GGT	CGCT	55	0.00	6	M55	GCA	CAGT	130	0.64
2	M56	GCT	CCAA	63	0.00	6	M63	GGA	CAAG	109	0.51
2	M6	AAA	CTAT	188	0.03	6	M76	CAG	CTGC	261	0.96
2	M85	CCA	CCGC	220	0.00	6	M42	GCC	CGAT	127	1.37
2	M87	CAT	CTCA	299	0.00	7	M50	GAT	CAAG	183	0.64
2	M43	GAA	CGTC	91	14.22**	7	M49	GAT	CAAG	184	0.39
2	M37	GAA	CGAC	89	6.95**	7	M64	GGT	CAAG	62	1.58
2	M102	CAT	CCGA	374	0.07	7	M48	GGT	CGCG	67	1.16
2	M88	CAT	CTGA	372	0.07	7	M47	GGT	CGAG	63	3.52
2	M113	CTA	CTGA	372	0.07	7	M67	GAG	CCTA	40	11.64**
3	M25	AGG	CCTC	74	1.16	7	M60	GAG	CTTA	40	4.64*
3	M15	AAA	CGCC	206	0.00	8	M79	CCA	CAAA	139	2.06
3	M9	AAA	CGTC	206	0.12	8	M81	CCA	CCAA	70	2.65
3	M10	AAA	CGTC	216	0.03	8	M117	CTC	CCAT	242	2.88
3	M116	CTC	CATT	196	0.19	8	M68	CAA	CGGC	94	3.22
3	M11	AGG	CCCC	294	0.19	8	M77	CAA	CCGC	113	2.88
3	M28	AGG	CCGC	295	0.19	8	M69	CAA	CGGC	110	2.88
3	M13	AGG	CGCC	294	0.29	8	M112	CTG	CAGT	56	2.93
3	M106	CGT	CTCA	182	5.54*	9	M96	CCT	CAGG	120	4.30*
3	M80	CAT	CGAA	270	0.40	9	M97	CCT	CATG	120	3.22
3	M21	AAT	CCAT	133	0.03	9	M86	CCT	CAAG	120	3.52
3	M34	AAT	CCGT	132	0.03	9	M72	CAG	CGAT	523	2.65
3	M20	AAT	CCAT	134	0.03	9	M105	CGA	CCTA	202	2.38
3	M33	AAT	CCGT	133	0.12	9	M90	CCT	CTAG	120	2.65
3	M4	ACT	CTCC	407	0.29	10	M71	CAG	CGAT	213	3.96*
4	M41	GCT	CGAA	113	0.39	10	M100	CCG	CTAT	210	5.08*
4	M39	GCT	CGCA	127	0.64	10	M70	CCA	CGTC	67	4.64*
4	M23	ACA	CCTC	50	0.80	10	M16	AAA	CGCC	197	3.90*
4	M38	GAA	CGCG	105	0.39	10	M32	AAT	CCGT	249	3.22
4	M103	CAT	CCTA	135	0.20	10	M78	CCA	CAAA	350	3.22
4	M8	ATG	CCCA	230	0.20	–	M24	ACA	CCTC	257	2.61
4	M58	GAC	CCAT	311	0.00	–	M29	AAA	CCGG	270	9.32**
4	M59	GAC	CCAT	310	0.07	–	M31	AAT	CCGT	260	0.00
4	M114	CTC	CAGT	458	0.20	–	M51	GCA	CTTG	251	1.18
4	M110	CGT	CTGT	458	0.03	–	M52	GCA	CTTG	249	1.18
4	M111	CGT	CTGT	454	0.03	–	M54	GGC	CAAT	274	54.22**
4	M115	CTC	CAGT	455	0.29	–	M57	GCC	CTAG	177	16.19**
4	M98	CCC	CTGT	458	0.07	–	M66	GGT	CCCT	51	0.29
4	M99	CCC	CTGT	456	0.07	–	M75	CAG	CTCG	198	0.19
5	M18	AAG	CGGT	68	2.31	–	M95	CAT	CACA	507	12.16**
5	M35	AGG	CCGT	72	0.80	–	M109	CGG	CTCG	213	0.29
5	M30	AAA	CCGG	238	3.90*						

[a] Loci marked *and ** deviated significantly from a 1:1 ratio at $P < 0.05$ and 0.01, respectively

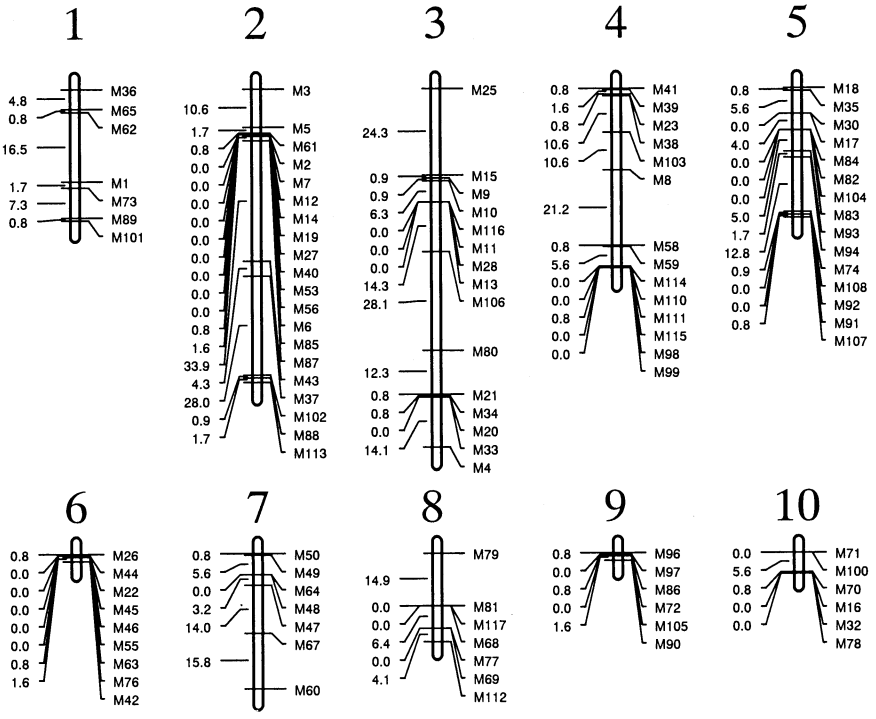

Fig. 4 Linkage map of F_1DH lines derived from a cross of *W6* and *Michinoku 1*. *Numbers* to the *left* of the linkage groups are map distances in centiMorgans calculated using the Kosambi function

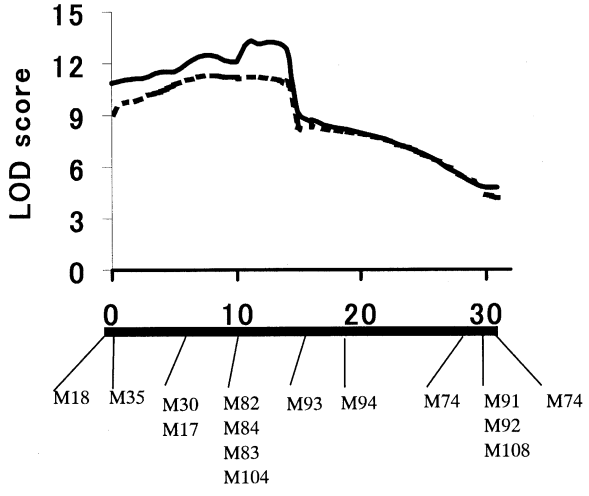

Fig. 5 LOD score scans of genetic linkage group 5. Different curves show results of analysis on two years of tests: in 1998, *continuous line*; in 1999, *dotted line*. Map distances (centiMorgans) are shown *below* the genetic linkage group (*bold straight lines* with marker names indicated) with LOD score values

tests. Using 1998 ADIs, the QTL had a maximum LOD score of 13.3, was located between markers M84 and M93, and explained 43.8% of the resistant variation. Using 1999 ADIs, the QTL had a maximum LOD score 11.3, was detected between marker M17 and M84, and accounted for 34.3% of the resistant variation (Fig. 5).

Discussion

The resistant parent *W6* used in this study was developed from a double backcross of *Michinoku 1* (Fig. 1). Apart from bacterial wilt resistance, it is similar in appearance to *Michinoku 1*. Therefore, it is likely that *W6* and *Michinoku 1* have similar genetic backgrounds excepting genomic regions relating to the resistance. In fact, we could map a QTL relating to the resistance using only 117 DNA markers, a number that is considered to be very low given the chromosome number of 48, and total genome size, 4.9×10^9 bp (Galbraith et al. 1983), of tobacco.

QTL analysis indicated that one QTL is present on linkage group 5. The QTL had the large effect on bacterial wilt resistance and explained 43.8% of the variation. This result supported a previous study (Matsuda 1977) using artificial inoculation in the greenhouse that the bacterial wilt resistance of *Hatano*, which was the origin of resistance in *W6*, was assumed to be controlled by only the partial dominant gene, *Rps*. In our field tests, however, resistance in F_1DH lines crossed between *W6* and *Michinoku 1* showed continuous distribution between the resistance of *W6* and *Michinoku 1*. At least under the condition of a uniformly contaminated field, the resistance of *W6* could be assumed to be controlled by polygenes. This contradiction may be explained by the fact that the AFLP method used in this study relied only on the restriction enzymes *Mse*I/*Pst*I. In barley, the distribution of AFLP markers detected by *Mse*I/*Eco*RI and *Mse*I/*Pst*I 'frequent/rare cutter' combinations between linkage groups was different (Powell 1997). As is the case in barley, the sites of *Pst*I in the tobacco genome

might not be evenly distributed. *Pst*I is a methylation-sensitive enzyme, and some of the polymorphisms might be influenced by methylation changes. For the detection of all QTLs on the genome of *W6* relating to bacterial wilt resistance, it would be necessary to create DNA markers using other 'frequent/rare cutter' combinations, such as *Mse*I/*Eco*RI, and/or a detection method for DNA polymorphisms other than AFLP.

The objective of the investigation reported here was to detect the QTL affecting field resistance to bacterial wilt and to identify DNA markers that can be used in tobacco breeding programs. This study was limited to one population of doubled haploid (DH) lines. Confirmation of the reliability of the identified QTL using another population of the progeny derived from *W6* is undergoing. Assuming confirmation, the identified QTL and the markers linking to this QTL will be useful in actual breeding programs for bacterial wilt-resistant *Burley* varieties; the identified QTL was strongly associated with resistance, explaining 44% and 34% of the resistance variance in our 1998 and 1999 trials, respectively. The preliminary selection of tentative resistance lines at the nursery using these linkage markers could reduce the number of evaluated lines in the field and the labor required for field resistance tests. In addition to being a first step toward the development of marker-assisted selection of tobacco breeding, the markers will provide a starting point for the cloning of genes affecting resistance to bacterial wilt.

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