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Analysis of polymorphism and transcription of the effector gene *Avr1b* in *Phytophthora sojae* isolates from China virulent to *Rps1b*

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SUMMARY

The effector gene *Avr1b*-1 of *Phytophthora sojae* determines the efficacy of the resistance gene *Rps1b* in soybean. The sequences of the *Avr1b*-1 locus in 34 Chinese isolates of *P. sojae* were obtained and analysed by polymerase chain reaction (PCR) and inverse PCR. Four different alleles and a complete deletion mutation of the *Avr1b*-1 gene were identified. Molecular analysis of the deletion breakpoints in the *Avr1b*-1 locus revealed that an 8-kb DNA sequence containing *Avr1b*-1 was deleted and a 12.7-kb DNA sequence was inserted at the same locus. A truncated transposase gene was found and five transposable elements were predicted in the inserted sequence, suggesting that the deletion of *Avr1b*-1 might be attributed to transposon movement. The transcription of *Avr1b*-1 was analysed in virulent isolates containing four alleles of *Avr1b*-1 by real-time reverse transcription-PCR. In all virulent isolates, only those isolates containing the second allele transcripted *Avr1b*-1.

INTRODUCTION

Soybean [*Glycine max* L. (Merrill)] is an important oilseed and food crop. *Phytophthora sojae* (M. J. Kaufmann & J. W. Gerdemann) causes damping off of soybean seedlings and root rot of established plants, which is referred to as Phytophthora root and stem rot of soybean (Tyler, 2007). Worldwide, this disease causes approximately $1–2 billion in annual agricultural losses (Tyler, 2007). *Phytophthora sojae* is a hemibiotrophic oomycete that exhibits race-specific virulence to soybean. The compatibility of interaction between *P. sojae* and soybean follows the gene-for-gene model; thus, the *P. sojae*–soybean pathosystem serves as a model to study gene-for-gene-based resistance. So far, 15 disease resistance genes (*Rps* genes) at eight loci have been identified in soybean, including five at the *Rps*1 locus and three at the *Rps*3 locus (Burnham *et al*., 2003; Tyler, 2008). However, only one *Rps* gene, *Rps1k*, has been cloned from soybean to date (Gao *et al*., 2005). Four highly similar genes encoding coiled coil-nucleotide binding-leucine-rich repeat (CC-NB-LRR)-type proteins were isolated from the *Rps1k* locus. These genes conferred stable race-specific *Phytophthora* resistance in transgenic soybean plants and were grouped into two classes on the basis of their sequence identity (Gao *et al*., 2005).

Phytophthora sojae effector genes corresponding to 11 *Rps* genes have been genetically identified (May *et al*., 2002). Five *Avr* genes have been cloned from *P. sojae*: *Avr1b*-1 (Shan *et al*., 2004), *Avr1a* and *Avr3a* (Qutob *et al*., 2009), *Avr3c* (Dong *et al*., 2009) and *Avr4/6* (Dou *et al*., 2010). These *Avr* genes all encode secreted proteins with an RXLR-dEER protein translocation motif. This motif is necessary to deliver Avr proteins into plant cells (Dou *et al*., 2008a; Whisson *et al*., 2007). The C-terminus of these Avr proteins contains one or two conserved W motifs, excluding Avr4/6. It is thought that the C-terminus could suppress programmed cell death and also be recognized by the cognate Rps proteins in soybean (Dou *et al*., 2008b).

Effector genes are thought to facilitate pathogenic fitness in the absence of cognate *Rps* genes, but act as elicitors of host plant defence in the presence of cognate *Rps* genes. Under selective pressure of *Rps* genes, pathogens could evade recognition by host plants and invade them successfully by DNA modifications in cognate *Avr* gene loci (Stahl and Bishop, 2000). Thus, polymorphisms of *Avr* genes are generally abundant in isolates that are virulent to cognate *Rps* genes. From the analysis of polymorphisms in *Avr* genes, we can explain how Avr proteins evade Rps-mediated recognition at the molecular level and understand the evolution of *Avr* genes. *Avr1b*-1 was the first *Avr* gene to be cloned from *P. sojae* (Shan *et al*., 2004). Two types of polymorphism in virulent alleles of *Avr1b*-1 have been described: point mutations and the absence of transcripts (Shan *et al*., 2004). To further study polymorphisms in the *Avr1b*-1 locus, the *Avr1b*-1 sequence and transcriptional polymorphisms from 34 Chinese *P. sojae* isolates collected between 2001 and 2007 were examined. We found both of the previously known types of polymorphism: point mutations and the absence of transcripts (Shan *et al*., 2004). Further, a novel polymorphism, replacement of a fragment containing the *Avr1b*-1 gene with a transposase gene, was found. In all virulent isolates, only those isolates containing the second allele transcripted *Avr1b*-1.

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RESULTS

Polymorphism analysis of the *Avr1b*-1 gene in *P. sojae* isolates from China

The pathogenicity assay indicated that 11 of the 34 Chinese isolates were avirulent to the soybean cultivar with *Rps1b* (L77-1863) and the other 23 were virulent (Table 1 and Fig. S1, see Supporting Information). Two pairs of *Avr1b*-1 allele-specific primers, AVR1B1F/AVR1B1R and AVR1BS1F/AVR1BS1R (Table 2 and Fig. 1A), were developed to detect *Avr1b*-1. *Avr1b*-1could be amplified from any avirulent isolate and from 17 of the virulent isolates, whereas the same fragments were not amplified in the other six virulent isolates (Ps0715, Ps0716, Ps0717, Ps0718, Ps0719 and Ps0720) (Fig. 1B). These results suggest that there is variation in the DNA sequence of the *Avr1b*-1 locus in the six virulent isolates. The full length of *Avr1b*-1 was used as a probe in a Southern blot to verify the absence of *Avr1b*-1 in the six virulent isolates. One or two copies of the *Avr1b*-1 alleles were detected in avirulent isolates, whereas no band was detected in the six virulent isolates (Fig. 1C). These results show that the intact *Avr1b*-1 gene is deleted in the genomes of the six virulent isolates. This deletion is a new DNA polymorphism in the *Avr1b*-1 locus that has not been described previously (Shan *et al*., 2004).

Table 1 Disease reactions of soybean cultivars to isolates of *Phytophthora sojae* and the *Avr1b*-1 alleles.

Isolate	Origin		Disease reaction*		Type of *Avr1b*-1†
	Source	Year	L77-1863	Williams	
Ps0701	Soil	2007	R	S	I
Ps0702	Soil	2007	R	S	I
Ps0703	Soil	2007	R	S	I
Ps0704	Soil	2007	R	S	I
Ps0301	Soil	2003	R	S	IV
Ps0401	Soil	2004	R	S	IV
Ps0402	Soil	2004	R	S	IV
Ps0705	Soil	2007	R	S	IV
Ps0706	Soil	2007	R	S	IV
Ps0707	Soil	2007	R	S	IV
Ps0708	Soil	2007	R	S	IV
Ps0709	Soil	2007	S	S	I
Ps0403	Soil	2004	S	S	IV
Ps0301	Soil	2003	S	S	III
Ps0302	Soil	2003	S	S	III
Ps0404	Soil	2004	S	S	III
Ps0101	Soil	2001	S	S	II
Ps0102	Soil	2001	S	S	II
Ps0103	Soil	2001	S	S	II
Ps0104	Soil	2001	S	S	II
Ps0201	Soil	2002	S	S	II
Ps0405	Soil	2004	S	S	II
Ps0406	Soil	2004	S	S	II
Ps0710	Soil	2007	S	S	II
Ps0711	Soil	2007	S	S	II
Ps0712	Soil	2007	S	S	II
Ps0713	Soil	2007	S	S	II
Ps0714	Soil	2007	S	S	II
Ps0715	Soil	2007	S	S	V
Ps0716	Soil	2007	S	S	V
Ps0717	Soil	2007	S	S	V
Ps0718	Soil	2007	S	S	V
Ps0719	Soil	2007	S	S	V
Ps0720	Soil	2007	S	S	V

*The cultivar L77-1863 contains the *Rps1b* gene and the cultivar Williams is the universal susceptible cultivar. A soybean cultivar was considered to be susceptible (S) if 70% or more of the seedlings were killed and resistant (R) if 30% or less of the seedlings were killed.

†Four different alleles of the *Avr1b*-1 gene were identified and designated as *Avr1b*-1(I), *Avr1b*-1(II), *Avr1b*-1(III) and *Avr1b*-1(IV). A complete deletion mutation of the *Avr1b*-1 gene was also identified and designated as *Avr1b*-1(V).

The fragments amplified by AVR1B1F/AVR1B1R from 28 isolates were cloned into the vector pMD19 and sequenced. Four alleles of *Avr1b*-1 were identified in all sequenced isolates (Fig. 2). Twenty-three point mutations at the DNA level were identified in *Avr1b*-1 (Fig. S2, see Supporting Information), leading to variations in 17 amino acids (Fig. 2). We then defined the N (including RXLR and dEER) and C termini of the protein sequence on the basis of the cleaved site at the 65th amino acid. The Yn00 tool in the PAML v4.3 software package was used to count the ratio of the nonsynonymous substitution (d*N*) to synonymous substitution (d*S*) rate (Yang, 2007). The four sequences made up six pairwise comparisons for the test. The result showed that the d*N*/d*S* values of the full-length sequence and the C-terminus were higher than unity, suggesting evidence of selection pressure in the *Avr1b*-1 gene, especially at the C-terminus of the sequence (Table 3). Bayes empirical Bayes (BEB) analysis (Yang *et al*., 2005) suggested that Avr1b contains five residues (87A, 103G, 107D, 108K, 129N) that are under positive selection ($P > 0.95$). Consistent with this high selection pressure, the *Avr1b*-1 alleles show divergent active functions. The isolates containing alleles *Avr1b*-1(II) and *Avr1b*-1(III) were virulent to *Rps1b*. The isolates containing alleles *Avr1b*-1(I) and *Avr1b*-1(IV) were avirulent to *Rps1b*. However, there were two isolates (Ps0709 and Ps0403) that were virulent to *Rps1b* despite the presence of an avirulent *Avr1b*-1 allele [alleles *Avr1b*-1(I) and *Avr1b*-1(IV)].

In addition, sequencing results showed that there was a 10-bp deletion 320 bp upstream from *Avr1b*-1 in the isolates (isolates Ps0302, P0303 and Ps0404) containing *Avr1b*-1(III). Nevertheless, there were no differences among these isolates containing *Avr1b*-1(I), *Avr1b*-1(II) and *Avr1b*-1(IV) within a 0.5-kb region upstream of *Avr1b*-1.

Genomic structure of the deletion site of the *Avr1b*-1 gene

To understand how *Avr1b*-1 was deleted in the genomes of the six isolates, the genomic structure of the deletion site *Avr1b*-1 in a representative isolate, Ps0718, was compared with that of the sequenced isolate P6497 (Tyler *et al*., 2006). Four pairs of specific primers (AVR1B4F/AVR1B4R, AVR1B5F/AVR1B5R, AVR1B6F/

Table 2 List of primers used in this study.

Primer	Sequence 5′ to 3′	Remark
AVR1B1F	CTGTACCATATCACCATCAGAG	For *Avr1b*-1 allele-specific PCR
AVR1B1R	GTGCCAATACCACCAAGTTGAG	
AVR1BR1F	TCGAGCTCATGAAGAGGACG	For real-time RT-PCR
AVR1BR1R	GGTCCGCGATTGCCAACCAG	
ACTAF	ACTGCACCTTCCAGACCATC	
ACTAR	CCACCACCTTGATCTTCATG	
AVR1B4F	TGGCTAATCTCACATAGAACCG	For analysis of the deletion site of *Avr1b*-1
AVR1B4R	GTTGCCTCCAGTGACAGCATT	
AVR1B5F	AAGGCCTGTTAGACATGATGAC	
AVR1B5R	GGTGACAACTTGTCGGAATAGT	
AVR1B6F	TGGAAACTCGATGGTGAACGTC	
AVR1B6R	GGAGGTTACCGTTGTAGAATCC	
AVR1B8F	ACGGTTAAATCGACATACGCAG	
AVR1B8R	AGACACAGGTGATATGAGCGAC	
AVR1B9R	CTGGATAGTAACGAGTCAAGAC	
AVR1B10R	GAAGGATTTGTGTCGAGAGCAC	
AVR1B11R	AGAGACGGTAGAGGGAAGGTG	
AVR1B18F	GTAAAGCACATCCGTATCGTGTA	
AVR1B18R	GAAAGGAGGCTGCCTTGTGCAA	
Avh331_3R	CATATGATGTGTCGCTTAGCATC	
Avh331_3F	GTTTCTTCTTCAGCGTGCGTTC	For inverse PCR
AVR1B2R	CGAGATTTCCTCAGGATAATCTC	
Avh331_2F	GAGATTATCCTGAGGAAATCTCG	
AVR1B7R	GTTAGCTCACGAGATGATAGTAG	
AVR1B12R	TGCGTATGTCGATTTAACCGTG	
AVR1B12F	GTCGCTCATATCACCTGTGTCT	
AVR1B16F	AGGTAATCACACAGGTTCTCAC	
AVR1B16R	GTTACGTCTTAGGATAAGACCC	
AVR1BS1F	ATGCGTCTATCTTTTGTGCTTTC	For Southern blot
AVR1BS1R	TCAGCTCTGATACAGGTGAAAG	

RT-PCR, reverse transcription-polymerase chain reaction.

AVR1B6R and AVR1B8F/AVR1B8R) that were unique in the genome of P6497 were designed to find the left-hand deletion breakpoint (Table 2 and Fig. 3A). Polymerase chain reaction (PCR) results showed that the four predicted fragments could all be amplified from the genomic DNA of Ps0718 (Fig. 3B). Because the sequence between the primer AVR1B8R and the *Avr1b*-1 gene was a near repeat sequence, three reverse primers (AVR1B9R, AVR1B10R and AVR1B11R) were designed as counterparts of AVR1B8F (Table 2 and Fig. 3A). PCR results showed that none of the three predicted fragments could be amplified from the genomic DNA of Ps0718 (Fig. 3C). These results suggest that the left-hand deletion breakpoint lies between AVR1B8R and AVR1B9R. The gene *Avh331*, linked tightly with *Avr1b*-1, could also be amplified from the genomic DNA of Ps0718 with the gene-specific primers Avh331_2F/Avh331_3R (Table 2 and Fig. 3D). Sequencing results showed that there was a 0.7-kb deletion in the promoter region of *Avh331*, which was a repeat sequence in the genome of P6497 (Fig. 3A). This result suggests that the right-hand deletion breakpoint is on the left of the primer Avh331_2F. On the basis of these analyses, the length of the deletion fragment of *Avr1b*-1 should be shorter than 9 kb. To find the deletion breakpoints, a pair of primers (AVR1B8F/Avh331_3R) was used for amplification. However, no band was amplified from the genomic DNA of Ps0718 (data not shown).

To obtain the fragment of genomic DNA of Ps0718 between AVR1B8R and Avh331_2F, a DNA walk was conducted using an inverse PCR strategy. The DNA walk was first performed from the primer AVR1B8R. *Bsr*GI was used for the digestion of genomic DNA of Ps0718 and the primer pair AVR1B12F/AVR1B12R was used for inverse PCR. After PCR products had been cloned into a pMD19 vector and sequenced, a 1.9-kb DNA sequence was rightward extended from the primer AVR1B8R. Further DNA walks could not continue because of repeat sequences. Thus, a DNA walk was performed again from the primer Avh331_2F. Inverse PCR was successfully performed three times. *Eco*RI and Avh331_3F/AVR1B2R, *Cla*I and Avh331_2F/AVR1B7R, and *Mlu*I and AVR1B16F/AVR1B16R were used in turn for the digestion of genomic DNA and inverse PCR. A total 7.7-kb DNA sequence was leftward extended from the primer Avh331_2F. Then, a primer pair, AVR1B18F/AVR1B18R, was designed at the end of the newly obtained DNA sequences to link the two flanks of the deletion breakpoint. Finally, a 4-kb PCR product was obtained, cloned and sequenced. In this manner, the *Avr1b*-1 locus in Ps0718 was characterized.

Compared with the sequences of P6497, the sequences of Ps0718 diverged 174 bp downstream from primer AVR1B8R and 714 bp upstream from primer Avh331_2F, thus marking the positions of the left-hand and right-hand breakpoints. An 8-kb DNA

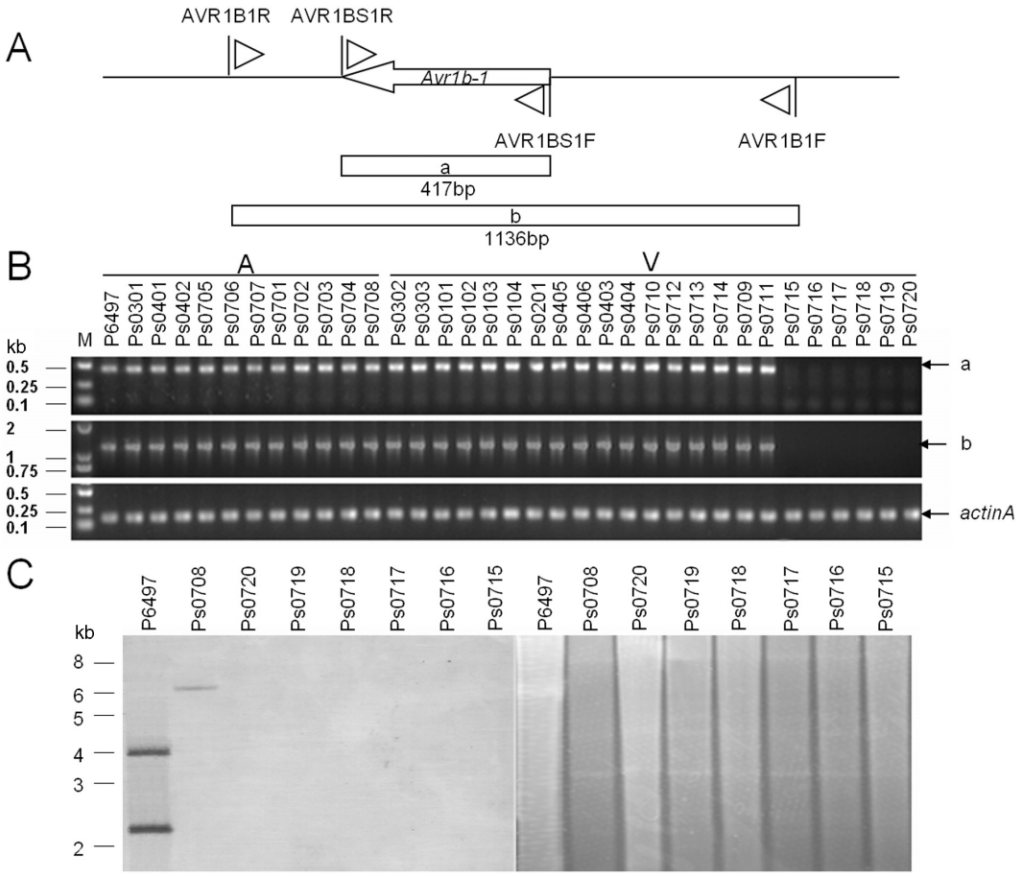

Fig. 1 Identification of the deletion mutation of the *Avr1b*-1 gene. (A) Schematic representation of primer locations in the *Avr1b*-1 locus. The primers AVR1B1F and AVR1B1S1F are forward primers and AVR1B1R and AVR1BS1R are reverse primers. (B) Polymerase chain reaction (PCR) amplification of the *Avr1b*-1 gene in different regions from Chinese isolates using two pairs of primers. A indicates an avirulent isolate and V indicates a virulent isolate. (C) Southern blot analysis of *Avr1b*-1. Genomic DNAs were digested with *Hin*dIII and probed with the coding region of *Avr1b*-1 (fragment a). A photograph of the ethidium bromide-stained gel is shown on the right and the Southern blot of the gel is shown on the left.

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Avr1b-1(I)     1   MRLSFVLSLVVAIGYVVTCNATEYSDETNIAMVESPDLVRRSLRNGDIAG  50
Avr1b-1(IV)    1   .................................................  50
Avr1b-1(III)   1   ..P..............................................  50
Avr1b-1(II)    1   .................................................  50

Avr1b-1(I)    51   GRFLRAHEEDDAGERTFSVTDLWNKVAAKKLAKAMLADPSKEQKAYEKWA 100
Avr1b-1(IV)   51   .................................................. 100
Avr1b-1(III)  51   ..................L.......G..T......K......E..KM.. 100
Avr1b-1(II)   51   ..................L.......G..T......K......E..KM.. 100

Avr1b-1(I)   101   KKGYSLDKIKNWLAIADPKQKGKYDRIYNGYTFHLYQS*  139
Avr1b-1(IV)  101   ...............................R...*  139
Avr1b-1(III) 101   ..K.T.QD..D.........K..............N*  139
Avr1b-1(II)  101   ..K.T.QD..D..................D.....*  139
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Fig. 2 Sequence comparison of *Avr1b*-1 alleles at the amino acid level. Identities are indicated by dots and substitutions by the substituted residue.

sequence containing *Avr1b*-1 was deleted in the genome of Ps0718 and a 12.7-kb DNA sequence was inserted at the same locus (Fig. 3A).The insertion sequence has been submitted to GenBank. The accession number is JF968506.

The 12.7-kb sequence was cut into nine 1.5-kb fragments for BLAST. Each sequence was used to BLASTN against the *P. sojae* trace file database with an *e*-value of 1*e*-5 to cut off. A total of 1926 BLAST hits was obtained with alignment lengths from 29 to 1088 bp. The number of BLAST hits for each base pair in the 12.7-kb sequence was also analysed (Fig. 4). The results showed that the insertion sequence was made up of many repeat sequences. Based on the Repbase web server (http://www.girinst.org/censor/index.php) (Kohany *et al*., 2006), five transposable elements were predicted in the 12.7-kb sequence (Fig. 4). There were four DNA transposons (EnSpm, one; MuDR, one; Sola, two) and one LTR retrotransposon (Gypsy). Moreover, a truncated transposase gene was found in the inserted sequence. These results suggest that the deletion of *Avr1b*-1 may be attributed to transposon movement.

Table 3 d*N*/d*S* analysis of pairwise comparisons of *Avr1b*-1 alleles.

Pairwise comparisons		Full sequences			N-terminus†			C-terminus		
Seq. 1	Seq. 2	d*N*/d*S**	d*N*	d*S*	d*N*/d*S*	d*N*	d*S*	d*N*/d*S*	d*N*	d*S*
Avr1b-1(I)	*Avr1b*-1(II)	2.8309	0.0618	0.0218	–	0	0	∞	0.0054	0
Avr1b-1(I)	*Avr1b*-1(III)	2.9739	0.0651	0.0219	0.4203	0.0073	0.0175	3.5858	0.1051	0.0293
Avr1b-1(II)	*Avr1b*-1(III)	∞	0.0031	0.0000	0.4203	0.0073	0.0175	3.7585	0.1112	0.0296
Avr1b-1(IV)	*Avr1b*-1(I)	1.1845	0.0126	0.0107	–	0	0	3.3488	0.0987	0.0295
Avr1b-1(IV)	*Avr1b*-1(II)	5.1620	0.0552	0.0107	–	0	0	3.5203	0.1047	0.0298
Avr1b-1(IV)	*Avr1b*-1(III)	5.4542	0.0585	0.0107	0.4203	0.0073	0.0175	∞	0.0167	0

*The '∞' in column d*N*/d*S* refers to pairwise comparisons in which d*N* > 0 and d*S* = 0, and '–' refers to pairwise comparisons in which d*N* = 0 and d*S* = 0.
†The N-terminus refers to the sequence from the 1st to 195th site, and the C-terminus refers to the sequence from the 196th site to the end, except the stop codon.

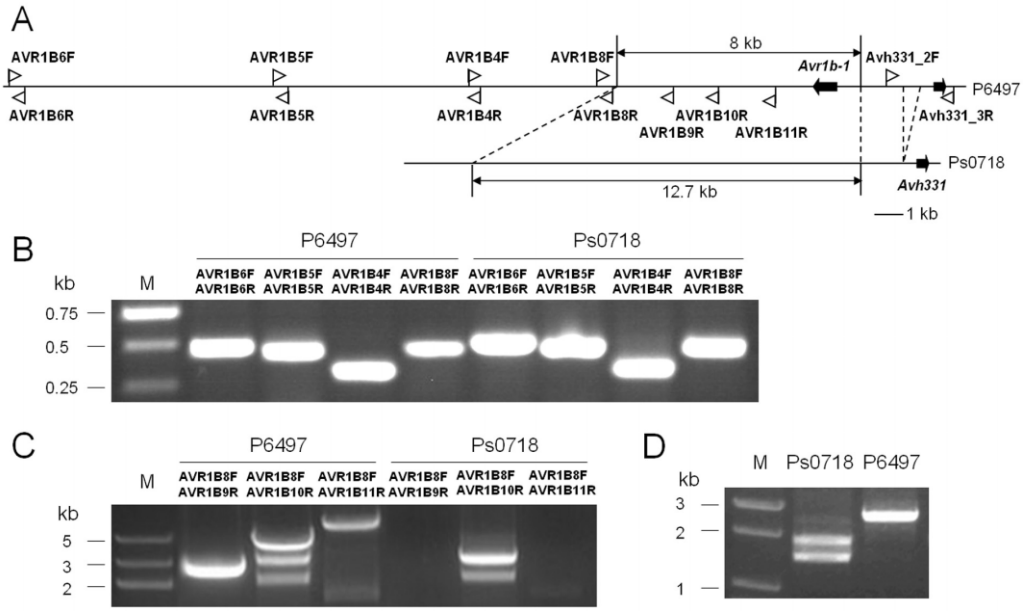

Fig. 3 Analysis of the genomic structure of the deletion site of the *Avr1b*-1 gene. (A) Schematic representation of primer locations in the full-length 30-kb region of the *Avr1b*-1 locus and genomic structure of the deletion site of the *Avr1b*-1 gene. (B, C) Polymerase chain reaction (PCR) amplification of the left regions of the *Avr1b*-1 locus from P6497 and Ps0718 using seven pairs of primers. (D) The primer pair Avh331_2F/Avh331_3R amplified the *Avh331* gene from P6497 and Ps0718.

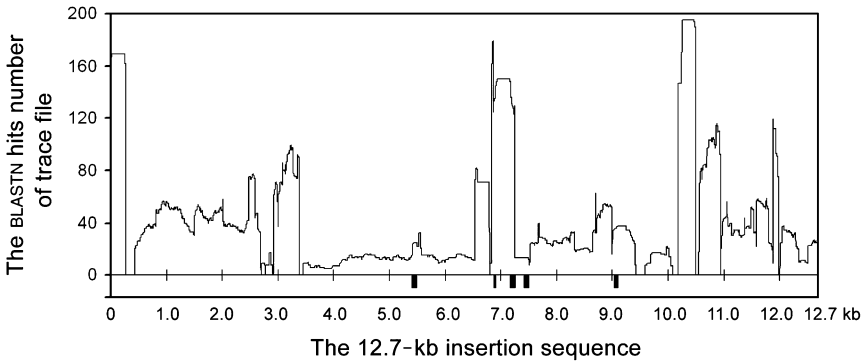

Fig. 4 Bioinformatics analysis of the 12.7-kb insertion sequence. The curve above the *y*-axis shows the distribution of the number of BLAST hits of the trace file for each base pair in the 12.7-kb sequence. The blocks under the *y*-axis show the location of the predicted transposable elements. From left to right, the predicted transposable elements are MuDR, Sola, Sola, EnSpm and Gypsy, respectively.

Transcriptional analysis of the *Avr1b*-1 gene in Chinese *P. sojae* isolates

A pair of *Avr1b*-1 gene-specific primers, AVR1BR1F/AVR1BR1R (Table 2), was designed in the conserved regions of *Avr1b*-1 to detect the transcription of *Avr1b*-1 by real-time reverse transcription (RT)-PCR. Nine isolates (Ps0702, Ps0708, Ps0709, Ps0403, Ps0404, Ps0302, Ps0201, Ps0405 and Ps0710), representative of the four alleles of *Avr1b*-1, were selected for transcriptional analysis of *Avr1b*-1. RNA was isolated from *P. sojae* isolates at 12 h

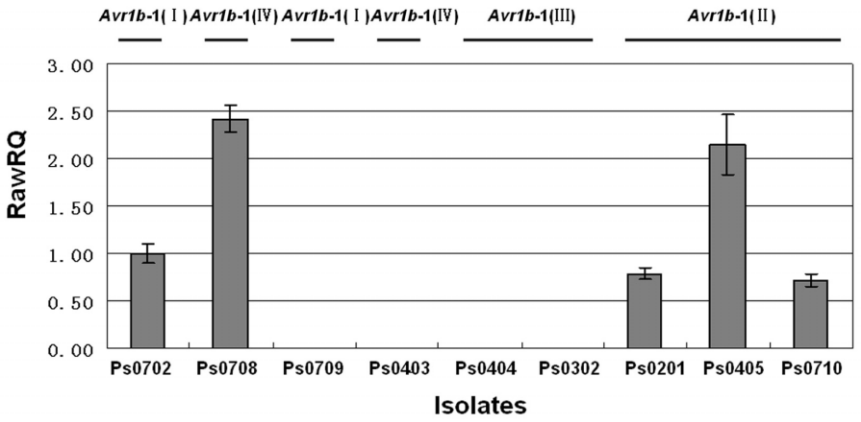

Fig. 5 Transcriptional analysis of the *Avr1b*-1 gene in the Chinese *Phytophthora sojae* isolates. Transcription of the *Avr1b*-1 gene was analysed in Chinese *P. sojae* isolates by real-time reverse transcription-polymerase chain reaction (RT-PCR). A pair of *Avr1b*-1 gene-specific primers (AVR1BR1F/AVR1BR1R) designed in the conserved regions of *Avr1b*-1 was used for real-time RT-PCR. RNA was isolated from *P. sojae* isolates at 12 h post-inoculation (hpi) on soybean leaves. The relative abundance of *Avr1b*-1 transcripts among isolates was normalized by comparison with the avirulent isolate Ps0702 (relative transcript level, 1.00).

post-inoculation (hpi) on soybean leaves. Transcriptional analysis showed that *Avr1b*-1 was transcribed in the isolates containing allele *Avr1b*-1(II), but not transcribed in the isolates containing allele *Avr1b*-1(III) (Fig. 5). *Avr1b*-1 was transcribed in isolate Ps0702 containing *Avr1b*-1(I) and isolate Ps0708 containing *Avr1b*-1(IV), but not transcribed in isolate Ps0709 containing *Avr1b*-1(I) or isolate Ps0403 containing *Avr1b*-1(IV). Thus, the isolates Ps0709 and Ps0403 were virulent to the soybean cultivar with *Rps1b*, unlike most other isolates containing *Avr1b*-1(I) or *Avr1b*-1(IV).

DISCUSSION

The polymorphism and transcription of the *Avr1b*-1 gene were studied in detail in *P. sojae* isolates from China. Five different alleles, including a complete deletion mutation of the *Avr1b*-1 gene, were identified. *Avr1b*-1 was transcribed in all isolates, except for those with deletions, those with *Avr1b*-1(III) and two isolates containing the alleles *Avr1b*-1(I) and *Avr1b*-1(IV). *Avr1b*-1 was transcribed in isolates containing *Avr1b*-1(II).

There is only one copy of *Avr1b*-1 in the genome of *P. sojae* (Tyler *et al*., 2006). However, Southern blot results of *Avr1b*-1 identified two hybridizing restriction fragments in P6497, probably because the paralogue *Avh*1, which is highly similar to *Avr1b*-1, is also present in the P6497 genome (Shan *et al*., 2004). The full-length probe of *Avr1b*-1 could hybridize to *Avh*1, as the sequence similarity between *Avh*1 and *Avr1b*-1 was 95% at the nucleotide acid level.

Shan *et al*. (2004) identified four alleles of *Avr1b*-1 in isolates P6954 [*Avr1b*-1(I)], P7076 [*Avr1b*-1(II)], P7074 [*Avr1b*-1(III)] and P7064 [*Avr1b*-1(IV)]. Corresponding GenBank accession numbers are AF449622, AF449624, AF449625 and AF449620, respectively. We also identified four alleles of *Avr1b*-1 among the Chinese isolates in the present study. The *Avr1b*-1(II) and *Avr1b*-1(III) alleles in the Chinese isolates differed at the 101st amino acid site from the sequences of the P7074 and P7076 alleles reported by Shan *et al*. (2004). However, the differences were not present in the sequences of the P7074 and P7076 alleles deposited by Shan *et al*. (2004) at the National Center for Biotechnology Information (NCBI), or in the genome sequences of P7074 and P7076 available at the Virginia Bioinformatics Institute (vmd.vbi.vt.edu). As a result, the four alleles of *Avr1b*-1 from the Chinese *P. sojae* isolates are the same as the four alleles identified by Shan *et al*. (2004). The ratios of d*N* and d*S* were counted, and the d*N*/d*S* values of the full-length sequence and the C-terminus were all higher than unity, suggesting that *Avr1b-1* suffered a selection pressure, especially in the C-terminus. BEB analysis suggested that Avr1b contained five residues that were under positive selection. These five residues were all in the C-terminus. These findings are consistent with the view that RXLR effectors are modular proteins, with the N-terminus involved in secretion and host translocation and the C-terminal domain dedicated to the modulation of host defences inside plant cells (Win and Kamoun, 2008; Win *et al*., 2007).

Significantly, a new DNA polymorphism, deletion of the *Avr1b*-1 gene, was identified in six Chinese *P. sojae* isolates. An 8-kb DNA sequence containing *Avr1b*-1 was deleted at the *Avr1b*-1 gene locus and a 12.7-kb DNA sequence was inserted at the same locus. Sequencing analysis showed that the inserted sequence was a complex fragment consisting of DNA fragments from different scaffolds in the P6497 genome. An incomplete gene homologous to a transposase gene of *Candida albicans* was found at the right hand of the inserted sequence, and five transposable elements were predicted in the inserted sequence. The results suggest that the deletion of *Avr1b*-1 may be attributed to the movement of transposons. There are many transposons in the genome of *Magnaporthe oryzae*, and genetic changes caused by these transposons are one of the mechanisms responsible for race variation (Kang *et al*., 2001; Zhou *et al*., 2007). The expansion of the *P. infestans* genome is associated with repeated elements (transposons) and effector genes localized to expanded, repeat-rich and gene-sparse regions of the genome, suggesting an association between transposons and virulence (Raffaele *et al*., 2010). Our results indicate that transposon movement may also be a mechanism for virulence variation in *P. sojae*.

Förster *et al*. (1994) identified four principal genetic lineages of *P. sojae*, each of which carries a distinctive allele of *Avr1b*-1 (Shan *et al*., 2004). The discovery of a new deletion allele of *Avr1b*-1 in Chinese isolates raises the question of whether these isolates may represent a previously unidentified lineage of *P. sojae* or a much more recent mutation that occurred in *Avr1b*-1 within the genetic background of one of the four known lineages. China is the centre of diversity of soybean and, if it is also the centre of diversity of *P. sojae*, new genetic lineages of *P. sojae* would be expected among Chinese isolates.

Most of the isolates containing *Avr1b*-1(I) and *Avr1b*-1(IV) were avirulent to the soybean cultivar with *Rps1b* (L77-1863). However, two isolates, Ps0709 containing *Avr1b-1*–1(I) and Ps0403 containing *Avr1b*-1(IV), were virulent to this cultivar. Transcriptional analysis showed that no *Avr1b*-1 mRNA could be detected in the Ps0709 and Ps0403 isolates. No *Avr1b*-1 transcription resulted in the pathogenicity of the two isolates on L77-1863. Sequencing results showed that there were no differences between Ps0709 and the avirulent isolate Ps0702 within a 0.5-kb region upstream of *Avr1b*-1 or within 0.2 kb downstream. Similarly, there were no differences between the sequences of Ps0403 and the avirulent isolate Ps0708 upstream or downstream of *Avr1b*-1. Thus, the lack of *Avr1b*-1 mRNA accumulation in Ps0709 and Ps0403 was caused by something other than DNA variation in the promoter and terminator regions. Shan *et al*. (2004) reported similarly that the virulent isolate P6497, which contains *Avr1b*-1(I), but no *Avr1b*-1, transcripts, did not differ in sequence from the avirulent isolate P6954 which contains *Avr1b*-1(I) transcripts. Shan *et al*. (2004) showed that a second gene, *Avr1b*-2, linked with *Avr1b*-1, is a *trans*-acting factor for *Avr1b*-1 and is required for the accumulation of *Avr1b*-1 mRNA (Shan *et al*., 2004). *Avr1b*-1 may not have been transcribed in isolates Ps0709 and Ps0403, because the two isolates lack the *Avr1b*-2 gene product. In our study, *Avr1b*-1 was also not transcribed in three isolates (Ps0302, P0303 and Ps0404) containing *Avr1b*-1(III). Sequencing results showed that there was a 10-bp deletion 320 bp upstream from *Avr1b*-1 in these three isolates, suggesting that the deletion in the promoter region may have been responsible for the loss of transcription, a different mechanism than in Ps0709 and Ps0403.

Under selective pressure from *R* genes, avirulent isolates can overcome the resistance by distinct mechanisms. DNA modifications in the corresponding *Avr* genes can include deletions, point mutations or insertions, resulting in either complete loss of the Avr protein or the production of altered forms that no longer trigger a defence response in plants carrying the *R* genes (Farman *et al*., 2002; Stergiopoulos *et al*., 2007; Stevens *et al*., 1998; Zhou *et al*., 2007). In this study, we identified at least three mechanisms, including point mutations in the coding region, deletion of the *Avr1b*-1 gene and loss of *Avr1b*-1 transcription, by which *P. sojae* could evade recognition by host plants containing *Rps*1b. These findings may help us to understand how *P. sojae* isolates overcome *Rps1b* at the molecular level.

EXPERIMENTAL PROCEDURES

Phytophthora sojae isolates, culture and pathogenicity assays

Fifty-five isolates of *P. sojae*, all purified by single zoospore isolation, were used in this study (Table 1). The isolate P6497 (from B. M. Tyler, Virginia Bioinformatics Institute, Blacksburg, VA, USA) was selected as a standard control for the analysis of DNA modifications in the *Avr1b*-1 locus, as the genome sequence of the isolate has been completed (Tyler *et al*., 2006). Other isolates were obtained from soybean field soils collected in China. Thirty-four isolates recovered from 2001 to 2007 were used for analysis of the polymorphisms in the *Avr1b*-1 locus (Cui *et al*., 2010; Wang *et al*., 2006). Twenty isolates recovered in 2009 were used to evaluate the accuracy of identification of isolates possessing virulence to *Rps1b* by real-time RT-PCR. All isolates were stored at 12 °C on 10% V8 juice agar, and grown on plates containing V8 juice agar (10% V8 juice filtered through four layers of cheesecloth, 1% $CaCO_3$ and 1.5% agar) for pathogenicity assays at 25 °C in the dark, or grown in 10% V8 juice at 25 °C for the production of mycelia to be used for DNA and RNA preparation.

The virulence of *P. sojae* isolates was evaluated on the *Rps*1b-containing cultivar L77-1863 and the non-*Rps*-containing cultivar Williams (from B. M. Tyler, Virginia Bioinformatics Institute) by the hypocotyl split inoculation method (Cui *et al*., 2010). Seven-day-old soybean seedlings were inoculated with 2 mm × 4 mm mycelium with 10 plants per plastic pot (diameter, 10 cm). Immediately after inoculation, inoculated plants were maintained in high humidity and darkness at 25 °C for 12 h. Thereafter, inoculated plants were kept in the glasshouse at 25 °C under a 14-h photoperiod and scored at 5 days post-inoculation as susceptible or resistant. Pathogenicity determination was repeated twice with each isolate. A differential was considered to be susceptible if 70% or more of the seedlings were killed, resistant if 30% or less of the seedlings were killed, and intermediate if 30%–70% of the seedlings were killed (Jackson *et al*., 2004; Ryley *et al*., 1998).

DNA extraction, PCR amplification, cloning and sequence analysis

Genomic DNA of *P. sojae* isolates was isolated from mycelia following the protocol described by Tyler *et al*. (1995). Two pairs of primers from different regions of the *Avr1b*-1 locus were designed and synthesized based on the genomic DNA sequence of *Avr1b*-1 (GenBank Accession No. AY426744) to detect the existence of *Avr1b*-1 and clone it. PCR amplification took place in a Peltier thermal cycler (PTC-200, MJ Research, Waltham, MA, USA) using the following PCR programme: one cycle at 95 °C for 3 min for initial denaturation, 29 cycles at 95 °C for 30 s, 58–60 °C (varied with the different primer pairs) for 30 s, 72 °C for 1 min, and a final extension of 72 °C for 5 min. Each PCR consisted of the following components: 5 μL of 10 × PCR buffer (Takara, Dalian, China), 3 μL of 25 mM $MgCl_2$ (Takara), 1 μL of 2.5 mM deoxynucleoside triphosphate (dNTP) (Takara), 1 μL of each 10 μM primer, 2 U of Taq polymerase

(Takara), 20 ng of genomic DNA and distilled water, in a final reaction volume of 50 μL. The PCR products were separated by electrophoresis on 1.0% (w/v) agarose gels in 1 × TAE (Tris-acetate-EDTA) buffer, stained with ethidium bromide, visualized and photographed using a Bio-Rad Gel Photographic System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A 2-kb DNA ladder (Takara) was included on each gel as a molecular size standard. All PCRs were repeated three times.

PCR products amplified from *P. sojae* using *Avr1b*-1 primers were purified using a Takara Agarose Gel DNA Purification Kit (Takara), cloned into a pMD19 vector (Takara) and sequenced by Invitrogen Inc., Shanghai, China. Three clones of the *Avr1b*-1 gene were sequenced in each isolate. Sequence analysis and alignments were performed using the software Bioedit 7.0.

Southern hybridization

To verify the deletion of *Avr1b*-1 in the genomes of Ps0715, Ps0701, Ps0717, Ps0718, Ps0719 and Ps0720, DNA blots were performed using a DIG High-Prime DNA Labelling and Detection Starter Kit I (Roche Applied Science, Penzberg, Germany). The *Avr1b*-1 probe was amplified from the genomic DNA of P6497 with the primer pair AVR1BS1F/AVR1BS1R (Table 2) and labelled with DIG-dUTP (alkali-labile). Samples of 5 μg genomic DNA of P6497, Ps0708, Ps0715, Ps0716, Ps0717, Ps0718, Ps0719 or Ps0720 were digested with the restriction enzyme *Hin*dIII prior to DNA blot analysis. The entire hybridization process was conducted according to the manufacturer's instructions.

Inverse PCR

An inverse PCR protocol (Ochman *et al*., 1988) was used for the analysis of the genomic structure of the deletion site of *Avr1b*-1. Approximately 2 μg of genomic DNA from Ps0718 were subjected to overnight digestion with 50 U of a desirable restriction enzyme (Takara) in a total reaction volume of 100 μL. The digested genomic DNA was purified by a Takara DNA Fragment Purification Kit (Takara). Approximately 0.2 μg of digested genomic DNA was then ligated with 20 U of T4 DNA ligase in a total reaction volume of 100 μL at 12 °C for 12 h to promote the formation of monomeric ligation products. Two microlitres of ligation product were added to 48 μL of PCR mix containing: 5 μL of 10 × LA PCR buffer (Invitrogen Inc.), 5 μL of 25 mM $MgCl_2$, 8 μL of 2.5 mM dNTP (Invitrogen Inc.), 2 μL of each 10 μM primer (Invitrogen Inc.), 0.5 μL of LA Taq polymerase (Invitrogen Inc.) and 25.5 μL of distilled water. The primers used are listed in Table 2. The conditions used for PCR were as follows: 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 5 min. The final extension step was 72 °C for 10 min. PCR products were purified, cloned into a pMD19 vector and sequenced as described above.

RNA extraction and SYBR Green real-time RT-PCR assay

To isolate RNA from *P. sojae* isolates on infection, soybean leaves were inoculated with small pieces of mycelia by the 'leaf sandwich' method (Chen *et al*., 2007) as follows. Mycelia grown in 10% V8 juice for 3 days were carefully placed on the leaf of a 10-day-old Williams soybean seedling and covered with another leaf. Then, the leaves with mycelia were placed in a Petri dish sealed with Parafilm and incubated at 25 °C in the dark. After 12 h, RNAs of the mycelia and leaves were isolated together using a NucleoSpin RNA II RNA Extraction Kit (Macherey-Nagel, Bethlehem, PA, USA) following the procedures described by the manufacturer. To remove contaminated genomic DNA in the RNA preparations, 10 μg of total RNA were treated with 4 U of RNase-free DNase I (Takara) at 37 °C for 30 min, and then transcribed to cDNA using a PrimeScript RT-PCR Kit (Takara) following the manufacturer's recommended procedures.

To analyse the transcription of *Avr1b*-1 in the *P. sojae* isolates possessing virulence to *Rps1b*, a real-time RT-PCR assay was performed in 20-μL reactions including 20 ng of cDNA, 0.2 μM of a gene-specific primer for *Avr1b*-1 or reference *Actin* gene (Table 2), 10 μL SYBR Premix ExTaq (Takara) and 6.8 μL dH_2O. PCRs were performed on an ABI PRISM 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 31 s to calculate cycle threshold (Ct) values, followed by a dissociation programme of 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s to obtain melt curves. The 7300 System Sequence Detection Software (Version 1.4) was used to obtain relative expression levels of each sample. The real-time RT-PCR assay was repeated three times.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Soybean seedlings inoculated on the hypocotyls with Ps0702 and Ps0709.

Fig. S2 Sequence comparison of *Avr1b-1* alleles at the nucleotide level.

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