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Polymorphism in natural alleles of the avirulence gene *Avr1c* is associated with the host adaptation of *Phytophthora sojae*

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Abstract

Phytophthora sojae is a destructive pathogen of soybean that is widely distributed in the world. The interaction between *P. sojae* and soybean follows the gene-for-gene model. The use of resistant soybean cultivars is the primary and most effective method to combat the disease. However, variation in the *Avr* genes of the pathogen enables it to evade host defenses. We collected 81 isolates from four major soybean-production areas in China to analyze the polymorphism of *Avr* genes in *P. sojae* field population. The virulence of these isolates towards 14 differential soybean lines indicated complex pathotypes in *P. sojae* field population in China. In this study we found that *Rps1c*, which is cognate with *Avr1c*, could be deployed in Heilongjiang, Shandong, and Jiangsu Provinces but not in Anhui Province. To determine the mechanism by which *Avr1c* escapes recognition by *Rps1c*, we analyzed the polymorphism of *Avr1c* gene in 50 isolates of a field population of *P. sojae* and found multiple novel genotypes related to virulence and avirulence. By performing infection assays and gene co-bombardment, we showed that the K105 amino-acid residue was under strong positive selection and was a determinant of the avirulence of *Avr1c*. Structural analysis showed that K105 was exposed on the surface of the protein, suggesting it to be a critical site for interacting with *Rps* genes or their associated proteins.

Keywords: *Phytophthora sojae*, *Avr1c*, Polymorphism, Key site, Structure

Background

Phytophthora root and stem rot caused by *Phytophthora sojae* (M. J. Kaufmann & J. W. Gerdemann) is a destructive disease of soybean. Root rot of soybean caused by *P. sojae* was first observed in Indiana in 1948 and then in Ohio in 1951 (Schmitthenner 1985), which can result in soybean seedlings damping off and cause an annual loss of approximately $1–2 billion on soybean production worldwide (Tyler 2007).

The use of resistant soybean cultivars is the primary and most effective method to manipulate the disease (Sugimoto et al. 2012). *P. sojae* and soybean follows the gene for gene model and using resistant cultivars controlled by a single resistance (*Rps*) gene to combat the disease can result in complete resistance (Schmitthenner 1985). More than 28 *Rps* genes were identified so far, including *Rps1a* to *Rps12*, *RpsYD25*, *RpsYu25*, *RpsYD29*, *RpsUN1*, *RpsJS*, *RpsSN10*, *RpsYB30*, *RpsWaseshiroge* and *RpsZS18* (Sun et al. 2014; Sahoo et al. 2017), and only 4 genes encoding nucleotide binding-leucine rich repeat (NB-LRR) proteins were isolated from the *Rps1k* locus (Gao et al. 2005).

However, avirulence (*Avr*) genes are under strong selective pressure in *P. sojae* field population, which makes the resistance conferred by *Rps* genes to be overcome by *P. sojae* in 8–15 years (Dorrance et al. 2016). Nine *Avr* genes of *P. sojae* have been cloned to date: *Avr1a* (cognate *Rps* gene: *Rps1a*) (Qutob et al. 2009), *Avr1b* (*Rps1b*) (Shan et al. 2004), *Avr1c* (*Rps1c*) (Na et al. 2014), *Avr1d* (*Rps1d*) (Yin et al. 2013), *Avr1k* (*Rps1k*) (Song et al. 2013), *Avr3a/5* (*Rps3a*, *Rps5*) (Qutob et al. 2009), *Avr3b* (*Rps3b*) (Dong et al. 2011), *Avr3c* (*Rps3c*) (Dong et al. 2009), and *Avr4/6* (*Rps4*, *Rps6*) (Dou et al. 2010). *Avr1b-1* was the first *Avr*

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gene of *P. sojae* to be cloned. Point mutations and loss of transcript of *Avr1b-1* induced virulence (Shan et al. 2004). In addition, the transposable elements insertion resulted in deletion of *Avr1b-1* from Chinese isolates (Cui et al. 2012). *Avr1d* is present in *P. sojae* strains avirulent to *Rps1d*, but is absent from the genome of virulent strains (Na et al. 2013). The virulent alleles of *Avr4/6* compared with its avirulent ones have nucleotide substitutions and deletions in the 5'untranslated region but not in the protein-coding region (Dou et al. 2010). The transgenerational silencing of *Avr3a* by 25-nucleotide RNA molecules enhances the virulence of *Rps3a* (Qutob et al. 2013).

Rps1c is the most commonly used *Rps* gene in commercial soybean cultivars in the United States (Slaminko et al. 2010) and in domestic cultivars in China (unpublished data). However, the pathogens were under high selection pressure, causing complex pathotypes in yield population so that the resistant cultivars controlled by a single *Rps* gene were easy to be overcome (Dorrance et al. 2016). So monitoring of a pathogen population's adaptation to resistance (*R*) genes is essential for long-term management of plant diseases. In our work, we found that *Rps1c* could be deployed in most, but not all, soybean-production areas in China, indicating that *Avr1c* is under strong selective pressure. Deletions and silencing, but not point mutations, in the cognate avirulence gene *Avr1c* of *P. sojae* result in its escape from recognition by *Rps1c* (Na et al. 2014; Arsenault-Labrecque et al. 2018). To determine the mechanism by which *Avr1c* escapes recognition by *Rps1c*, we collected *P. sojae* isolates from major soybean-production areas in China and analyzed them together with several isolates stored in our laboratory.

Results

Virulence proportion of *P. sojae* population collected in China

To study the pathotypes of *P. sojae* in China, we collected 81 isolates from diseased plants and soil samples in 4 major soybean-production regions, including Heilongjiang at northeast China (36 isolates), Shandong (13 isolates), Anhui (19 isolates) and Jiangsu (13 isolates) Provinces at central-east China, during 2015–2016. All of these isolates were recovered by single zoospore and evaluated for pathotypes on 14 soybean differential lines with Williams as a blank control. Of the 81 isolates, more than 60% were virulent on soybean lines carrying *Rps5, Rps1d, Rps3c, Rps7* or *Rps8*, 21% were virulent on *Rps1k*, 22% were virulent on *Rps1c*, and 33% were virulent on *Rps1a* and *Rps3a* (Fig. 1a). These results indicated that resistance conferred by *Rps1k*, *Rps1c* and *Rps1a* are less likely to be overcome by these *P. sojae* isolates. In addition, 74 pathotypes were identified in these 81 isolates (Additional file 1: Table S1), with seven pathotypes isolated twice while the rest of pathotypes only once, showing complex pathotypes in Chinese *P. sojae* population. The isolate PsSD6 was virulent on only *Rps1a* gene, six isolates were virulent on two *Rps* genes, and approximately 75% of isolates were virulent on six or more *Rps* genes.

Of the 36 isolates obtained from Heilongjiang Province, less than 9% were virulent on *Rps1b, Rps1c* and *Rps6* while 19%, 22% and 27% were virulent on *Rps1k, Rps3a* and *Rps1a*, respectively. More than 50% isolates were virulent on *Rps1d, Rps2, Rps3b, Rps3c, Rps5* and *Rps8*, indicating that those *Rps* genes have already been overcome by *P. sojae* isolates in Heilongjiang Province. Of the 13 isolates obtained from Shandong Province, only one was virulent on *Rps1k* and *Rps3a* individually. Meanwhile, three isolates were virulent on *Rps1a* and *Rps1c.* Four or more isolates were virulent on 10 other *Rps* genes. In the case of the isolates collected from Anhui and Jiangsu Provinces, except that 23% isolates were virulent on *Rps1k* in Jiangsu, more than 30% isolates were virulent on each of the remaining 13 *Rps* genes (Fig. 1b). The survey showed that significant differences of pathotypes existed among different areas, and it's important to deploy cultivars carrying different *Rps* genes according to the virulence proportion of *P. sojae*.

Polymorphism of *Avr1c* in Chinese isolates

In this study, a *P. sojae* field population was collected from Heilongjiang, Shandong, Anhui and Jiangsu Provinces, the major soybean-production areas in China, where we found that 5.6%, 23.1%, 47.4% and 30.8% of the 81 isolates were virulent on *Rps1c*, respectively (Fig. 1). So cultivars containing *Rps1c* was nearly overcome in Anhui Province. To elucidate how the cognate avirulence gene *Avr1c* escaped recognition by *Rps1c*, we collected 50 (35 virulent and 15 avirulent) *P. sojae* isolates from China to analyze the polymorphism of *Avr1c* using P6497 as the control (Additional file 2: Table S2). Two pairs of primers were used to amplify the open reading frame (ORF), the upstream and downstream regions of *Avr1c* from genomic DNA (Additional file 3: Table S3).

Avr1c was absent in 14 virulent isolates (Fig. 2a), and 10 nucleotide sequences (Additional file 4: Figure S1) were detected in the remaining 21 virulent and 16 avirulent isolates. Alleles *Avr1c-3-1* and *Avr1c-3-2* encode the same amino acids (Additional file 5: Figure S2). None of the *Avr1c* sequences contained premature stop codons or frameshift mutations. Multiple variants of the nine predicted Avr1c amino acid sequences revealed a highly polymorphic family. There were 37 polymorphic sites among the nine amino acid genotypes (Fig. 2b, c), of which six were virulent and three

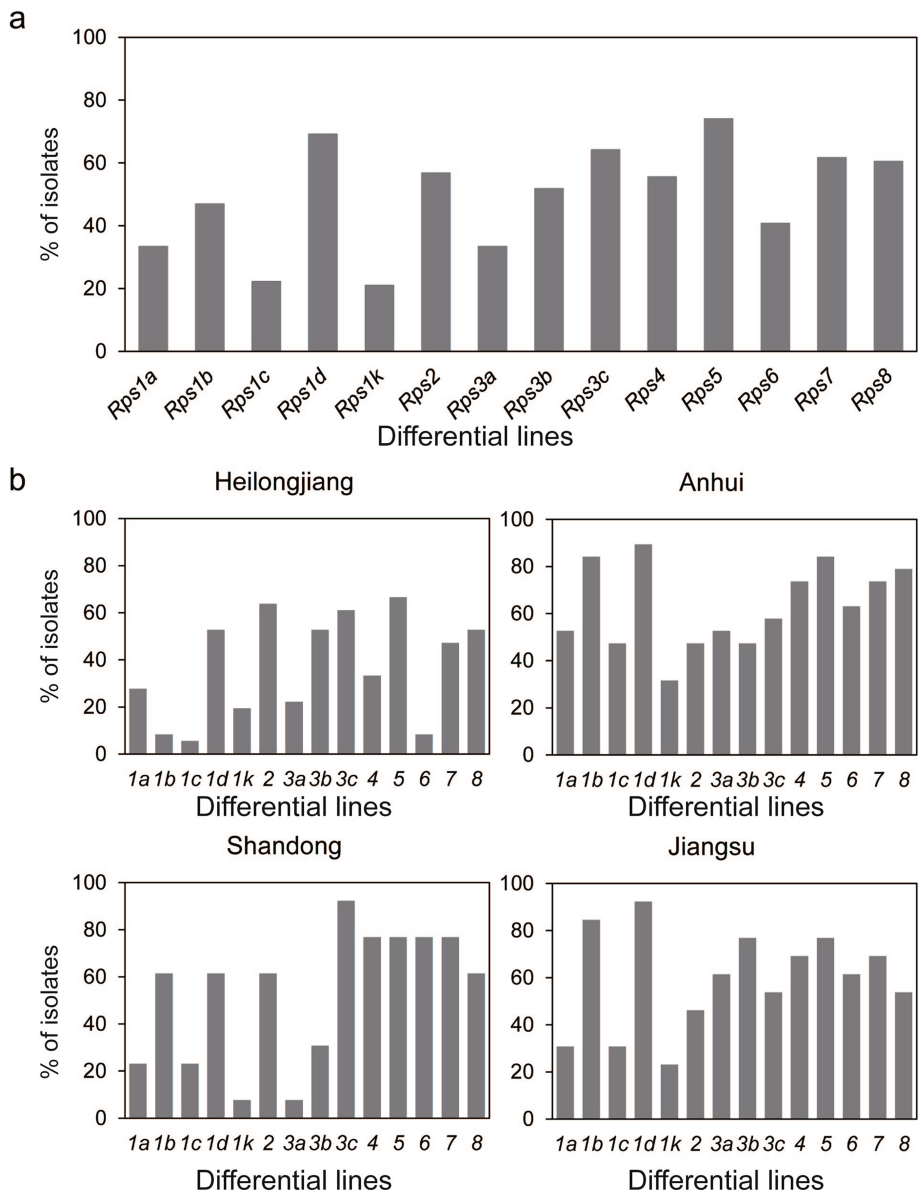

Fig. 1 The virulence proportion of *Phytophthora sojae* isolates on 14 differential lines collected in China. **a** The virulence proportion of all 81 isolates and **b** Proportion of isolates collected in Heilongjiang, Anhui, Shandong, and Jiangsu Province on 14 differential lines. Every assay was repeated independently twice. The x-axis indicated the differential lines containing the 14 *Rps* genes

were avirulent types. Among the avirulent isolates, thirteen contained the *Avr1c-1* allele, two contained the *Avr1c-2* allele, and one contained the *Avr1c-3* allele. Eight of the twenty-four virulent isolates contained the *Avr1c-1*, six harbored the *Avr1c-7*, three contained the *Avr1c-4*, and one each had the *Avr1c-5*, *Avr1c-6*, *Avr1c-8*, and *Avr1c-9* allele (Additional file 2: Table S2). We found that the absence of *Avr1c* alleles induced virulence in *Rps1c*, but the presence of *Avr1c-1* alleles did not always induce avirulence in *Rps1c*. Moreover, the presence of *Avr1c-5*, *Avr1c-6* and *Avr1c-7* alleles induced virulence in *Rps1c*.

Avr1c residues were under diversifying selection

The Yn00 tool in the PAML 4.9e package (Yang 2007) was used to count the ratio of the nonsynonymous substitution (d*N*) to synonymous substitution (d*S*) to determine the selection pressures underlying sequence diversification in the 10 *Avr1c* nucleotide sequences. The 10 nucleotide sequences made up 45 pairwise comparisons for the test. We found that d*N* was greater than d*S* ($\omega = dN/dS > 1$) in 42 of 45 pairwise comparisons, showing that the full-length sequences were under strong selection pressure in the *Avr1c* gene, particularly at the C-terminal domain (Additional file 6: Table S4). To detect Avr1c residues that

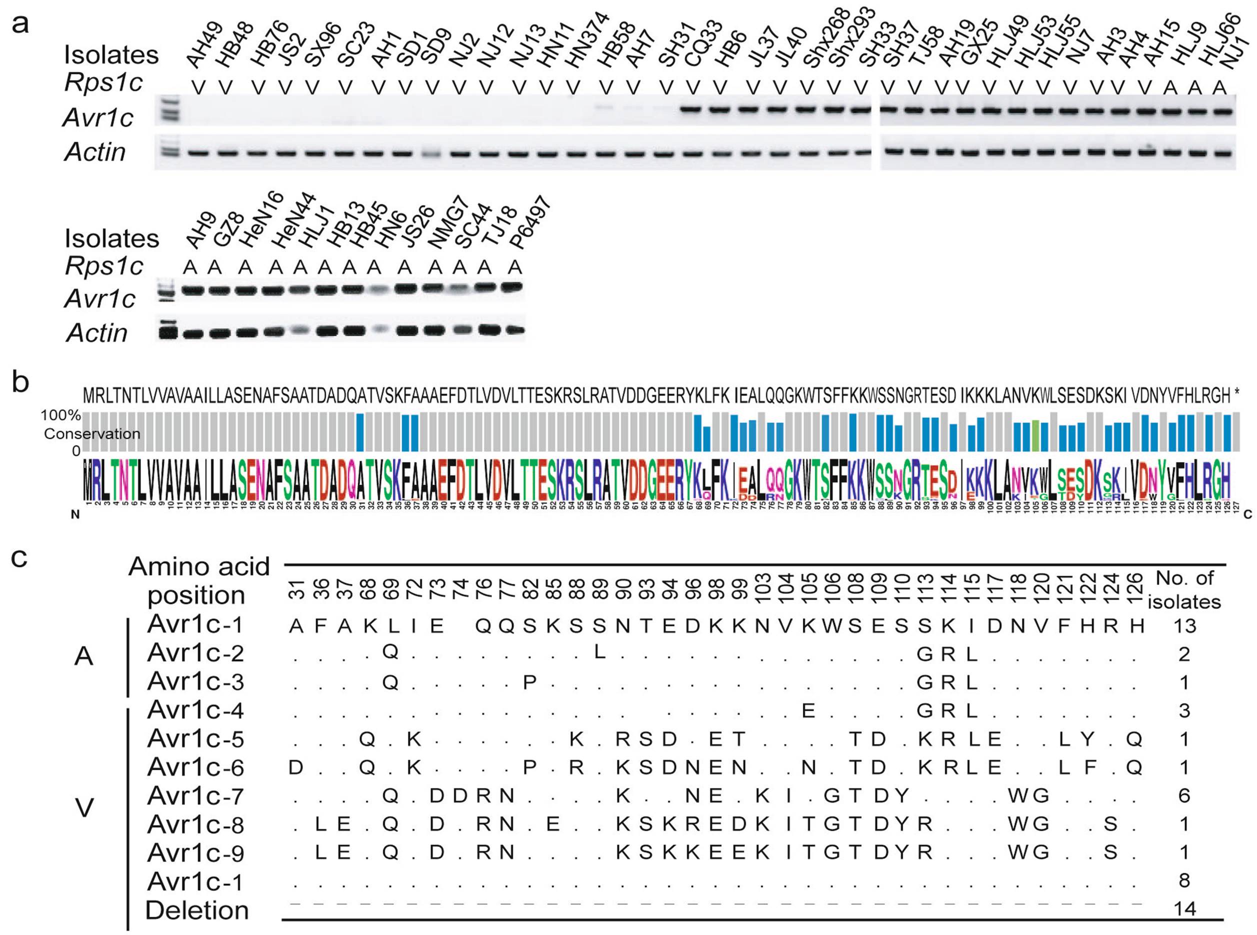

Fig. 2 Polymorphism analysis of *Avr1c*. **a** PCR amplification of *Avr1c*. A, the isolates were avirulent on *Rps1c*; V, virulent on *Rps1c*. P6497 was used as the control. **b** Amino-acid sequence of Avr1c-1. Conserved sites (grey), variation sites (blue), and key recognition site (green). The consensus sequence and sequence diversity are depicted as sequence logos. **c** Amino-acid sequences encoded by Avr1c variants. Identities are indicated by dots and substitutions by the substituted residue. *, stop codon

were positively selected, we used the maximum likelihood method and CodeML program in the PAML 4.9e package (Yang 2007). We collected all divergent *Avr1c* alleles and conducted the likelihood ratio test (LRT) between the null model M7 (β distribution) and the alternative model M8 (β + ω distribution). The data suggested that the *Avr1c* alleles are under positive selection. The Bayes empirical Bayes analysis revealed that the six amino acid sites (94 E, 96 D, 99 K, 105 K, 113 S, 118 N) were under positive selection with confidence > 99% (Table 1).

Table 1 Likelihood ratio test results for *Avr1c*

Model	Estimate parameters	lnL[a]	Sites under selection[b]	Model comparison	2ΔL[c]	χ2 Critical value	Degree of freedom
M7 β	p = 17.49869	− 935.39	Not allowed	M7 v M8	31.61	9.21	2
	q = 0.00500						
M8 β + ω	p0 = 0.69174	− 918.86	**94 E 96 D 99 K 105 K 113 S 118 N**				
	p = 60.85475						
	q = 90.46392						
	p1 = 0.30826						
	ω = 8.13961						

[a]lnL, log likelihood value
[b]Amino acid sites inferred to be under positive selection with a probability > 99% are in bold
[c]Likelihood ratio test: 2DL = 2 (lnLalternative hypothesis −lnLnull hypothesis)

Three variants in virulent isolates were recognized by *Rps1c*

To investigate whether those variants in virulent isolates evade recognition of *Rps1c* by mutation, we transiently expressed the divergent Avr1c protein fused GFP in *Nicotiana benthamiana* without signal peptide to ensure the expression (Additional file 7: Figure S3). Then the Avr1c variants were transiently expressed in *Rps1c* leaves by co-bombardment. The isolates containing Avr1c-2 and Avr1c-3 were all avirulent on *Rps1c*, so we speculated that Avr1c-2 and Avr1c-3 could be recognized by *Rps1c*. The only difference between Avr1c-8 and Avr1c-9 was a single change from K (lysine) to E (glutamic acid) at position 85 (Fig. 2c). We expressed Avr1c-1, Avr1c-4, Avr1c-5, Avr1c-6, Avr1c-7 and Avr1c-8, together with the reporter gene *GUS*, in leaves of Williams 79 (*Rps1c*). The results showed that there was no significant difference in the number of blue spots (surviving cells) in leaves expressing Avr1c-8 and Avr1c-4 compared with that of empty vector (EV), suggesting that Avr1c-8 and Avr1c-4 were not recognized by *Rps1c*, while there was significant difference in leaves expressing Avr1c-5, Avr1c-6, and Avr1c-7 compared with that of empty vector (EV) (Fig. 3), indicating that these three variants were recognized by *Rps1c*.

Avr1c was silenced in some virulent Chinese isolates

The Avr1c-1, Avr1c-5, Avr1c-6 and Avr1c-7 variants were all recognized by *Rps1c* when transiently expressed in *Rps1c* leaves. We hypothesized that the virulent isolates containing those four variants escaped the recognition of *Rps1c* by silencing. To test the transcript level of divergent *Avr1c* alleles in different isolates, we collected RNA samples at 0.5, 1.5, 3, 6, 9 and 12 hours post inoculation (hpi) to conduct RT-qPCR (Additional file 8: Figure S4). The results showed that the highest expression level of *Avr1c* occured at 3 hpi, and then reduced at 6 hpi. Therefore, we collected samples from different isolates containing the divergent *Avr1c* alleles at different infection stages and merged the samples to extract RNA and to conduct RT-qPCR. The results showed that *Avr1c* transcript was not detected in 16 virulent isolates containing the *Avr1c-1*, *Avr1c-5*, *Avr1c-6* and *Avr1c-7* alleles. These data suggested that *Avr1c* in these Chinese *P. sojae* isolates escaped recognition by *Rps1c* by gene silencing. Transcript levels of *Avr1c* in isolates containing the *Avr1c-4*, *Avr1c-8* and *Avr1c-9* alleles were quantified, although the expression level of the *Avr1c-4* was significantly lower than the *Avr1c-8* and *Avr1c-9* alleles (Fig. 4). We hypothesized that point mutations in the 10 virulent isolates with these three alleles were responsible for the escape from the recognition of *Avr1c* by *Rps1c*. This may explain why these alleles were recognized by *Rps1c*.

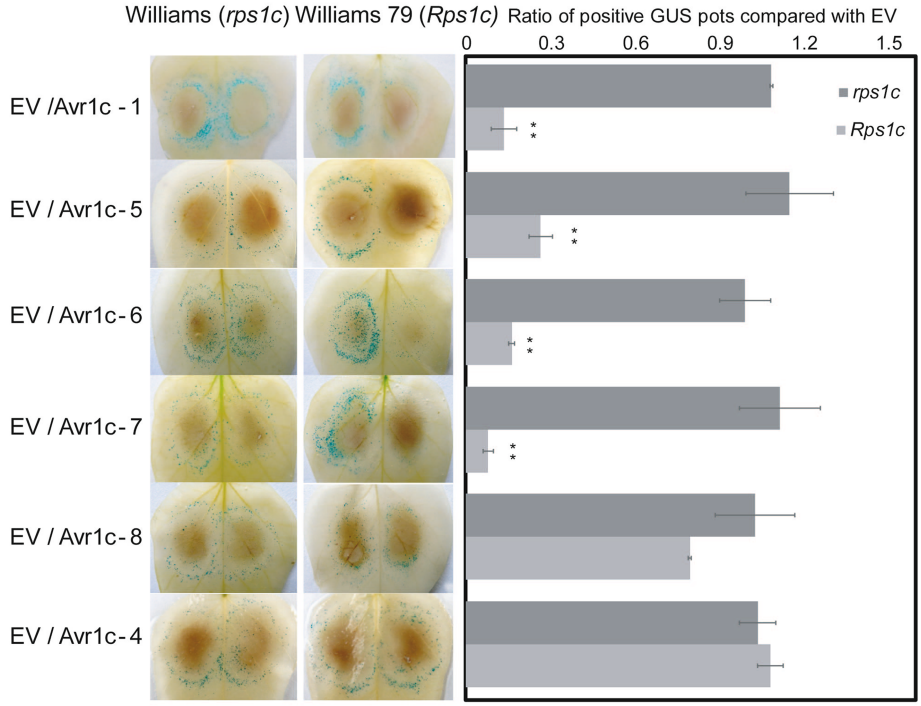

Fig. 3 The response of five Avr1c variants expressed in Williams (*rps1c*) and Williams 79 (*Rps1c*). Blue spots, surviving cells. Right, the ratio of GUS-positive blue spots following bombardment with Avr1c compared to EV. Bars represent standard errors from three independent replicates. **, $P < 0.01$ by *t*-test

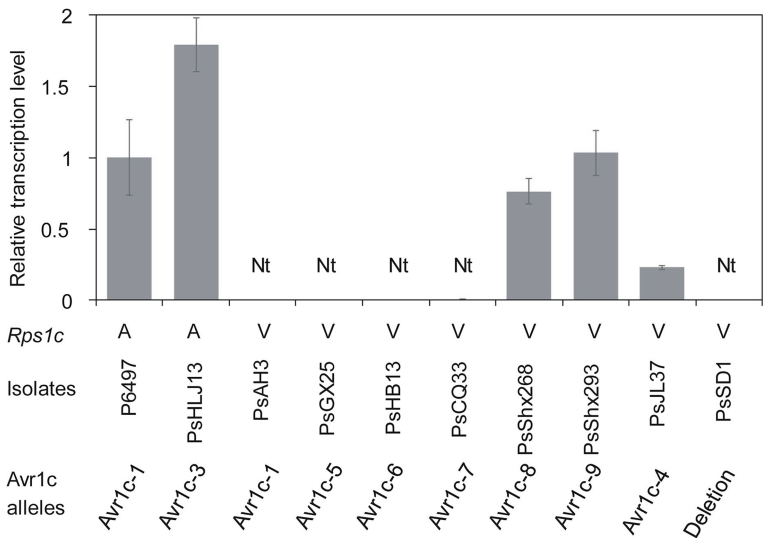

Fig. 4 Expression levels of *Avr1c* alleles determined by RT-qPCR. The samples at infection stages at 0.5, 1.5, 3, 6, 9, and 12 hpi were collected and mixed. The RNA of the merged samples were extracted to conduct RT-qPCR. The error bars were from three independent replicates. PsSD1 (*Avr1c* deleted) as the control. Nt, no transcription. A, the isolates were avirulent on *Rps1c*; V, virulent on *Rps1c*

C-terminal of Avr1c-1 is critical for recognition by *Rps1c*

The isolates containing Avr1c-4 and Avr1c-8 were virulent on *Rps1c*, and the two variants were not recognized by *Rps1c* when transiently expressed in *Rps1c* leaves. So we speculated that isolates containing Avr1c-4 and Avr1c-8 proteins escaped recognition by point mutation. Firstly, we infused Avr1c-$1^{1\text{-}104}$ with Avr1c-$8^{105\text{-}126}$ (Avr1c^{1N+8C}) and Avr1c-$8^{1\text{-}104}$ with Avr1c-$1^{105\text{-}126}$ (Avr1c^{8N+1C}) combining the sequence of Avr1c-4 and Avr1c-8. The Avr1c-1, Avr1c-8, Avr1c^{1N+8C} and Avr1c^{8N+1C} were transiently expressed in *Rps1c* leaves using gene co-bombardment. The number of blue spots of Avr1c^{8N+1C} was significantly smaller than that of the control empty vector (EV), while the blue pots of Avr1c^{1N+8C} were similar with empty vector (EV) (Fig. 5), indicating that the C-terminal of Avr1c-1 is important for recognition by *Rps1c*.

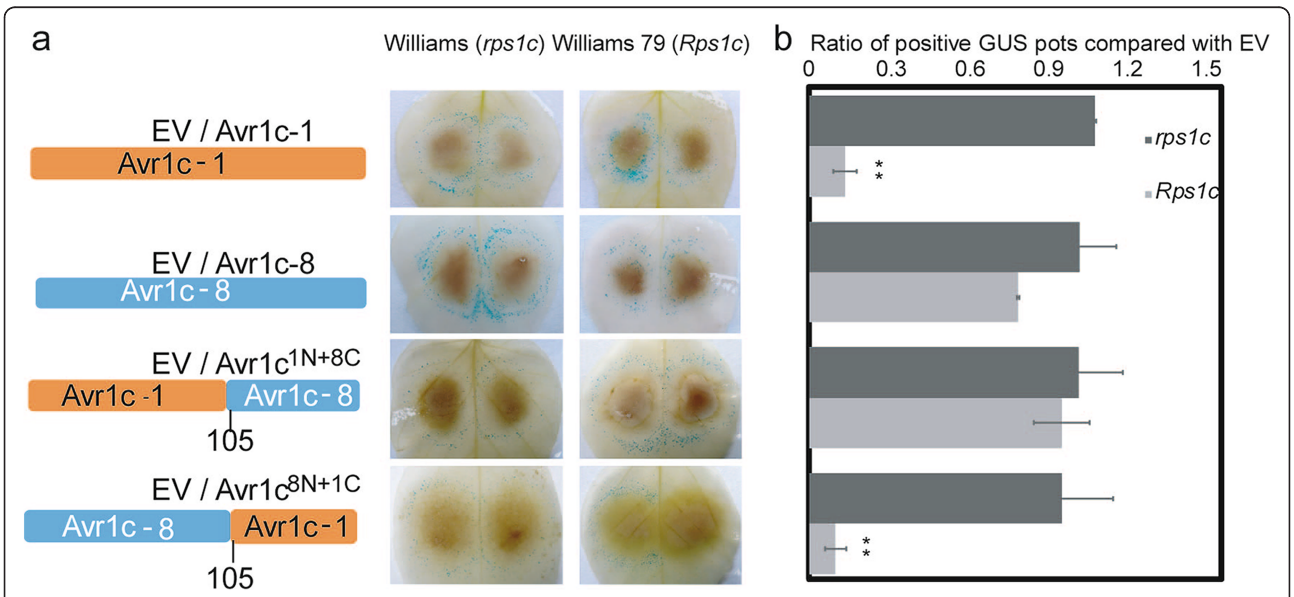

Fig. 5 C-terminal of Avr1c is critical for recognition by *Rps1c*. **a** The wild-type and mutants transiently expressed in *rps1c* and *Rps1c* leaves. The blue pots (surviving cells) of *rps1c* and *Rps1c* leaves transiently expressing Avr1c^{1N+8C}, Avr1c^{8N+1C}, and the wild-type. **b** Ratio of GUS-positive blue spots following bombardment with *Avr1c* variants and mutants compared to EV. Bars represent standard errors from three independent replicates. **, $P < 0.01$ by *t*-test

K105 is the key site of Avr1c recognized by *Rps1c*

The C-terminal 105–126 of Avr1c-1 was required for its recognition by *Rps1c*. Considering the Avr1c-5 and Avr1c-6 were all recognized by *Rps1c*, we speculated that the point mutations in Avr1c-5 and Avr1c-6 were all overcome. To further investigate the key amino acids that determined the recognition of Avr1c by *Rps1c*, we compared the Avr1c-1, Avr1c-2 and Avr1c-7, which were recognized by *Rps1c*, with Avr1c-4, Avr1c-8, which were not recognized by *Rps1c*. K (lysine) 105 and R (arginine) 124 were found to be different between the variants (Fig. 6a), indicating that the two amino acids may be important for recognition of Avr1c. We constructed the mutations Avr1c-1^{K105E}, Avr1c-1^{K105T}, Avr1c-8^{T105K} and Avr1c-4^{E105K}. All mutations were ligated into pFF19 and were transiently expressed in *Rps1c* leaves using gene co-bombardment. Following their bombardment into *Rps1c* leaves, the expression of Avr1c-8^{T105K} and Avr1c-4^{E105K} induced significantly more cell death as compared to empty vector, while Avr1c-1^{K105E} and Avr1c-1^{K105T} did not (Fig. 6b), which indicates that K105 is important for recognition of Avr1c by *Rps1c*. However, we expressed Avr1c-1^{R124S} and Avr1c-8^{S124R} in *Rps1c* leaves, and the blue pots in leaves expressing the two variants were similar to those in wild-type leaves with Avr1c-1 and Avr1c-8, respectively. There was no difference in cell death between Avr1c-1^{R124S} and Avr1c-8^{S124R} versus the wild type in *Rps1c* leaves, indicating that amino acid R124 was not a key point for Avr1c recognition by *Rps1c* (Fig. 6b). Interestingly, the key residue K105 we identified fell within the sites that were under positive selection (Table 1). Our results demonstrated that K105 is a key residue determining the recognition of Avr1c by *Rps1c* and K105 was under strong positive selection, suggesting that Avr1c evolved to escape from *Rps1c* recognition through substitution at this key residue.

Discussion

In this study, we tested the pathotypes of *P. sojae* isolates from four representative major soybean-production areas in China (Heilongjiang, Anhui, Shandong and Jiangsu Provinces), to monitor changes and shifts of *P. sojae* population. We found that the virulence proportion of *P sojae* isolates toward *Rps1a, Rps1b, Rps1c, Rps1k, Rps3a* and *Rps6* are less than 25% in Heilongjiang, indicating that cultivars containing these resistance genes could defend most isolates in Heilongjiang Province. In line with our data, Wen et al. (2018) collected *P. sojae* isolates from Heilongjiang Province in three continuous years and suggested that *Rps1c, Rps1k* and *Rps3a* were more effective than other *Rps* genes. In addition, our results demonstrated that *Rps1a, Rps1c, Rps1k* and *Rps3a* were effective in Shandong Province, *Rps1a, Rps1c* and *Rps1k* were effective in Jiangsu Province and only *Rps1k* was effective in Anhui Province. Since the virulence proportion of the isolates on *Rps1k* in Anhui was higher than 30%, the resistance gene *Rps1k* was nearly about to be overcome. It is reported that virulence towards *Rps1a, Rps1c* and *Rps1k*, which were widely deployed in the USA in the last many years, increased continually, but *Rps6* and *Rps8* were effective against the majority of isolates collected in northern regions of the sampled area (Dorrance et al. 2016). However, our data showed that *Rps6* and *Rps8* were not effective in China except Heilongjiang Province. Together, our results revealed increasing pathotype complexity of *P. sojae* population in China, and suggested the importance of stacked *Rps* genes in combination with high partial resistance to limit losses caused by *P. sojae* in breeding programs.

It is reported that *Rps1c* is the most commonly used *Rps* gene in commercial soybean cultivars in the United States (Slaminko et al. 2010). We identified *Rps* genes in 41 domestic cultivars in China, 9 of which contained *Rps1c*, indicating that *Rps1c* is the most commonly used *Rps* gene in domestic cultivars in China (unpublished data). However, *Avr* genes are under strong selective pressure in field populations of *P. sojae*, which drives *Rps* genes to be overcome. We found that the virulence proportion of *P. sojae* population on *Rps1c* was relatively lower in Heilongjiang, Shandong, and Jiangsu Provinces than in Anhui Province, indicating that cultivars containing *Rps1c* could defend against most isolates in Heilongjiang, Shandong, and Jiangsu Provinces, but not in Anhui Province. According to the *Rps* genes identification, 3 of 6 domestic cultivars from Anhui Province contained *Rps1c*, indicating that *Rps1c* is the most commonly used *Rps* gene in Anhui (unpublished data). It is thus important to investigate the variations of *Avr1c* gene in Chinese populations of *P. sojae*. This is the first report of the variation of *Avr1c* in Chinese *P. sojae*, and also the first to show that point mutations in *Avr1c* are responsible for its evasion of host recognition.

The results of YN00 indicated *Avr1c* was under positive selection, particularly the C-terminal (Additional file 6: Table S4). BEB analysis suggested that the six residues contained in Avr1c were under strong positive selection (Yang 2007). Our further analyses demonstrated that K105 fell within the sites that were under positive selection. Pro132 is essential for both avirulence and virulence of Avr3b, and substitution of serine for glycine at position 174 in PsAvr3c resulted in evasion of *Rps3c*-mediated immunity (Kong et al. 2015; Huang et al. 2019). Point mutations in *Avr* genes enable them to evade recognition by *Rps* genes. The *Hpa* isolate Hind2 evades recognition by *RPP4* due to a mutation in the functional NLS region in HaRxL103, resulting in altered subcellular localization (Asai et al. 2018). It is reported that the conserved motifs at the C-terminus of

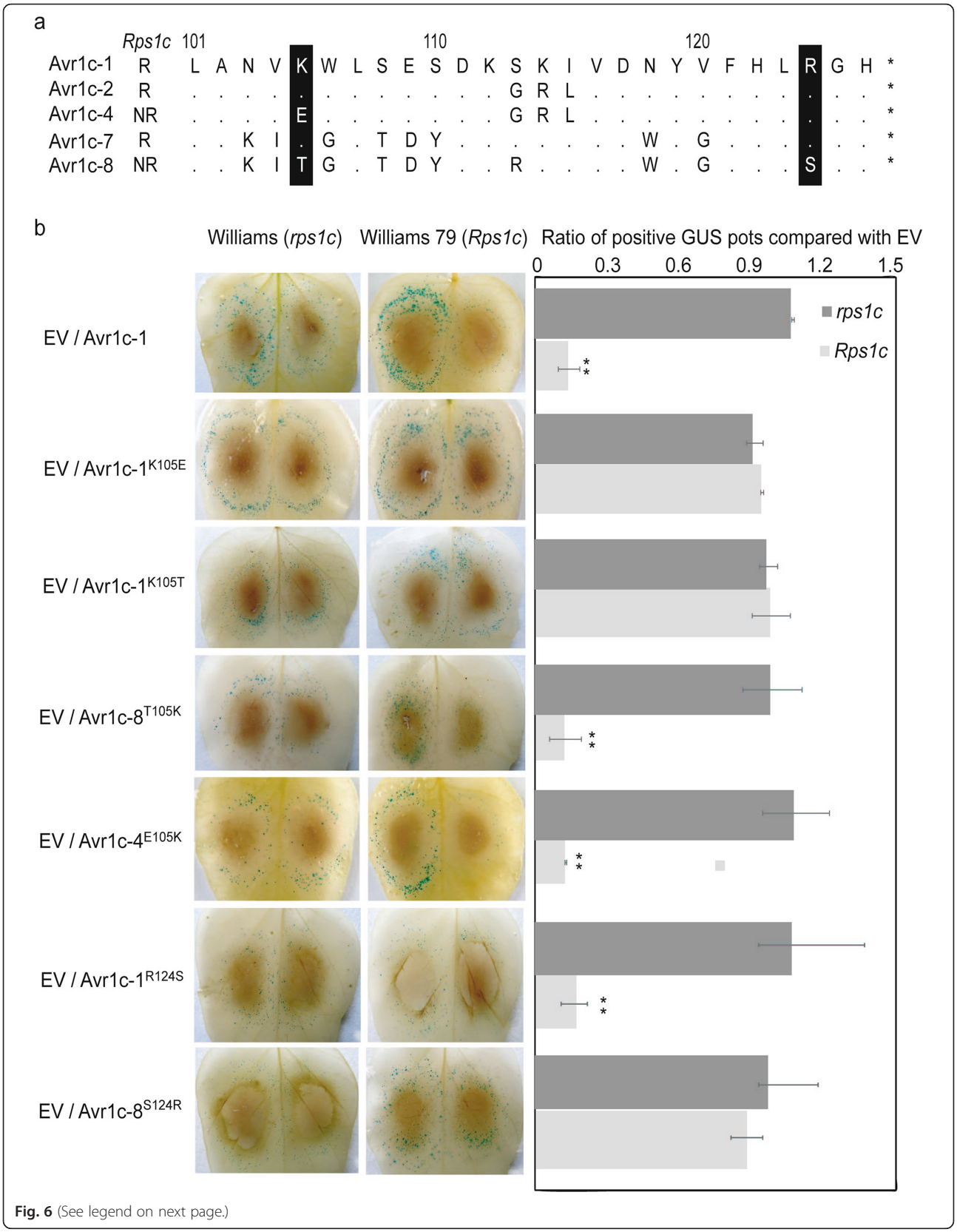

Fig. 6 (See legend on next page.)

(See figure on previous page.)
Fig. 6 The key site of *Avr1c* recognized by *Rps1c*. **a** Avr1c^{1N+8C}, Avr1c^{8N+1C}, and the wild-type allele expressed in *Rps1c* leaves. **b** Sequence analysis of the key site. R, recognized by *Rps1c*; NR, not recognized by *Rps1c*. **c** Mutants of *Avr1c-1*, *Avr1c-8*, and *Avr1c-4* expressed in *Rps1c* leaves. Right bar chart, frequency of GUS-positive blue spots following bombardment with *Avr1c* compared to EV. Bars represent standard errors from three independent replicates. **, $P < 0.01$ by *t*-test

Avr are critical for their interactions with *Rps* genes (Dou et al. 2008a; Du et al. 2018). In this study, we also found that C-terminal of Avr1c is critical for its recognition by *Rps1c*. Our results indicated K105 of Avr1c was important for the *Rps1c*-mediated response but is not a predicted NLS using the cNLS Mapper (Kosugi et al. 2009). However, R124, which was mutated in Avr1c-8 and Avr1c-9, was not a key position. Structural simulation using SWISS-MODEL indicated that the RXLR domain comprised a bundle of three α-helices (α1, Trp80-Asn90; α2, Glu94-Ala100; and α3, Ser113-Leu123). Amino acids K105, T105 and E105 in variants were exposed on the surface of the flexible loop between the W and Y motifs. The K105 residue was mapped on the exposed surface of Avr1c (Additional file 9: Figure S5a) between the W and Y motifs (Additional file 9: Figure S5b). Also, K105 was mapped on the ribbon diagram (Additional file 9: Figure S5b–d), suggesting its importance in interactions of Avr1c with *Rps* genes or target proteins, although the key site was mapped on the flexible loop. Thus, K105 may be a determinant of the interaction of Avr1c with *Rps* or target proteins. The next step is to identify the target proteins of Avr1c.

Many Avr effectors escape recognition by cognate *Rps* genes in *P. sojae* by deletion, such as *Avr1a, Avr1b, Avr1c* and *Avr1d* (Qutob et al. 2009; Cui et al. 2012; Na et al. 2013; Na et al. 2014; Arsenault-Labrecque et al. 2018). In three isolates, *Avr1c* was deleted in an 8.8 kb or 10.8 kb region containing two copies of *Avr1a*, one copy of *Avr1c*, and one copy of *Avh72* (Na et al. 2014; Arsenault-Labrecque et al. 2018). In our study, *Avr1c* was deleted in 14 of the 35 virulent isolates. We detected *Avr1a* and *Avh72* in some, but not all, of the isolates that lacking *Avr1c*. Whether the entire locus or only *Avr1c* was deleted needs to be investigated further.

Avr1c transcript was not detected in 16 virulent isolates harboring the *Avr1c-1*, *Avr1c-5*, *Avr1c-6* and *Avr1c-7* alleles using the specific primers. Nucleotide substitutions in the 5′-untranslated region can silence *Avr4/6* (Dou et al. 2010). In the region upstream of the *Avr1c* ORF (Additional file 10: Figure S6), we did not detect a silencing mutation. Arsenault-Labrecque et al. (2018) reported that the virulent isolates 5B, 5C and 45B had significantly lower expression of *Avr1c* compared with the avirulent isolate 28A. Furthermore, no causative mutation in *Avr1c* was detected, but unique mutations were present in 5B and 5C. In another study, *Avr3a* of *P. sojae* was detected in avirulent but not in virulent strains, and 25 nt RNA molecules were abundant in gene-silenced strains (Qutob et al. 2013). We hypothesized that small RNAs are responsible for silencing Avr1c, but the type of small RNA and whether the silencing was transgenerational needs further investigation.

The selective pressure exerted by *R* genes has driven avirulent isolates to evolve mechanisms for overcoming host resistance; e.g., deletions, point mutations or insertions, or silencing of *Avr* genes, which can result in complete loss of the Avr protein or the production of altered forms that do not trigger an *R*-gene-mediated defense response. We evaluated the variation of *Avr* genes and the mechanisms through which they evade recognition by cognate *R* genes. The ultimate aim is to engineer soybean cultivars with enhanced resistance to disease. We found that deletion, silencing and point mutations of *Avr1c* allow it to evade from *Rps1c* recognition, driving *P. sojae* to overcome *Rps1c*-mediated host defenses and providing molecular insight into the coevolution of *Avr* and *Rps* genes.

Conclusions

Phytophthora root and stem rot caused by *Phytophthora sojae* (M. J. Kaufmann & J. W. Gerdemann) is a destructive disease of soybean which causes approximately $1–2 billion in annual agricultural losses worldwide. *Rps1c* is the most commonly used resistance gene in commercial soybean cultivars in the United States and in domestic cultivars in China. However, the resistant cultivars controlled by a single *Rps* gene to combat the disease is easily overcome in a few years. We tested the pathotypes of *P. sojae* isolates from four representative major soybean-production areas in China to monitor changes and shifts of *P. sojae* population. Our results showed that the K105 amino-acid residue under strong positive selection is a determinant of the avirulence of *Avr1c*. Structural analysis showed that K105 was exposed on the surface of the protein, indicating that it to be a critical site for interacting with *Rps* genes or their associated proteins. We found that deletion, silencing, and point mutations of *Avr1c* allow it to evade from *Rps1c* recognition, driving *P. sojae* to overcome *Rps1c*-mediated host defenses and providing molecular insight into the coevolution of *Avr* and *Rps* genes.

Methods

Samples and *P. sojae* isolates collection

Soil samples and diseased plants were harvested in soybean fields from Heilongjiang, Anhui, Shandong

and Jiangsu Provinces, where 100 soil samples, 43 soil samples, 45 diseased plants samples, and 68 diseased plant samples were gathered, respectively. Soil baiting for *P. sojae* was conducted according to the methods of Schmitthenner et al. (1994). About 10 g of each finely ground soil sample was placed uniformly in a 9 cm plastic pot bathed with 4–7 mL sterilized and deionized water, then sealed with parafilm, and inversely cultured in a greenhouse at 25 °C, with a photoperiod of 14 h light/10 h dark for 5 days. Leaf discs were put into the pot quickly after the appropriate amount of sterilized and deionized water was added to incubate for 24–48 h in the greenhouse. The baited leaf discs were washed two times with sterilized and deionized water and fostered in the greenhouse for 48 h. The zoospore suspension containing 200–300 zoospores was coated on *Phytophthora* selective water agar containing 50 ppm ampicillin, 50 ppm rifampicin, 50 ppm quintozene (PCNB) and 5 ppm phenamacril. The single generated zoospore was selected and transferred to a plate with 10% V8 juice agar and stored at 12 °C in the dark. Isolation of *P. sojae* from diseased plants was conducted following Dorrance et al. (2008). Plant tissue was washed to remove soil particles and then rinsed with sterile distilled water, and blotted dry on sterile filter paper. The sections from the edge of the lesion were placed on *Phytophthora* selective agar media. After 3 days of incubation, hyphal tips of *Phytophthora* were removed aseptically and transferred to fresh V8 selective plates.

Pathotypes characterization of *P. sojae*

The virulence formula of *P. sojae* isolates was performed by hypocotyl inoculation technique (Dorrance et al. 2008). A set of 14 soybean differential lines including 'Harlon' (*Rps1a*), 'Harosoy 13XX' (*Rps1b*), 'Williams 79' (*Rps1c*), 'PI103091' (*Rps1d*), 'Williams 82' (*Rps1k*), 'L76–1988' (*Rps2*), 'Chapman' (*Rps3a*), 'PRX146–36' (*Rps3b*), 'PRX 145–48' (*Rps3c*), 'L85–2352' (*Rps4*), 'L85–3059' (*Rps5*), 'Harosoy 62XX' (*Rps6*), 'Harosoy' (*Rps7*), and 'PI 399073' (*Rps8*) (McBlain et al. 1991; Dorrance et al. 2004) were inoculated, and Williams (*rps*) was used as a susceptible control. Ten seeds of each differential line were grown in vermiculite in the greenhouse at 25 °C, with a photoperiod of 14 h light/ 10 h dark for 7 days. Make a slit about 3 mm long with the scalpel in the hypocotyls of the seedling 1 cm below the cotyledonary node. The mycelium culture was cut in 3 mm square strips and placed the strips into the slit. Moisturized the plants in a box for about 12 h to prevent the agar from drying and for the infection to take place in the greenhouse. Susceptible plants would die or develop distinct lesions 3 to 5 days after inoculation. Seven or more plants killed was susceptible, three or less killed was resistant, and four to six plants killed was intermediate resistant. Every assay was repeated twice.

DNA extraction, amplification, and sequence analysis

Genomic DNA was extracted and purified using a DNA-secure Plant Kit (Tiangen Biotech, Beijing, Co., Ltd., China) following the manufacturer's instructions, and was then stored at − 20 °C. *Avr1c* alleles were amplified in a 25 μL PCR reaction mix containing 2.5 μL of 10× PCR buffer (Mg^+; TaKaRa, Japan), 2 μL of 2.5 mM deoxynucleoside triphosphates (dNTP) (TaKaRa), 0.5 μL of each specific primer (10 μM), 1 μL (5 U/μL) of r*Taq* polymerase (TaKaRa), 30 ng of genomic DNA, and 18.75 μL of sterilized and deionized water. The reactions were performed at 95 °C for 5 min, followed by 32 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min and a final extension at 72 °C for 10 min. The PCR products were resolved by ethidium bromide electrophoresis in 1.0% (w/v) agarose gels in 1× Tris-acetate-EDTA buffer, and the bands were sequenced. *PsActin* was used as the control.

Sample preparation, RNA extraction, and real-time quantitative reverse transcriptase-polymerase chain reaction

The roots were immersed in a zoospore suspension, and mixed root samples were collected at 0.5, 1.5, 3, 6, 9, and 12 hpi and frozen in liquid nitrogen. The samples were mixed and total RNA was extracted using a Total RNA Kit I (Omega, Norcross, GA) following the manufacturer's instructions, and was stored at − 70 °C. The concentration of RNA was determined using a spectrophotometer (ND-1000; NanoDrop, Wilmington, DE) and diluted to 100 ng/μL using diethyl polycarbonate water (RNase- and DNase-free; Solarbio, Beijing, China). cDNA was synthesized using a PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa). Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) was conducted in a 20 μL reaction mix containing 20 ng of cDNA, 0.4 μL of primers (10 μM) specific for the target or reference gene (PsActin), 10 μL of SYBR Green Premix Ex *Taq* (Tli RNaseH Plus), 0.4 μL of ROX Reference Dye II (50×), and 6.8 μL of sterilized and deionized water. The primers used in this experiment were all confirmed by PCR from genomic DNA to ensure the specificity. RT-qPCR was conducted using a 7500/7500 Fast Real-Time PCR System (Applied Biosystems Inc., Foster City, CA) under the following conditions: 95 °C for 30 s followed by 40 cycles at 95 °C for 5 s, and 60 °C for 34 s; and dissociation at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The RT-qPCR analyses were performed in triplicate.

Plasmid construction

Avr1c alleles and mutants without a signal peptide were cloned from the genomic DNA of different *P. sojae* isolates. For the expression in *N. benthamiana*, the amplified fragments of variants were ligated into pBinGFP2 (N-terminal tag green fluorescent protein (GFP) fusion). For the bombardment experiment, all the amplified fragments were ligated into pFF19 (Timmermans et al. 1990). All site-directed mutants were cloned from genomic DNA by overlapping PCR using mutated primers. Individual colonies of each construct were tested by PCR and verified by sequencing. The cloning primers are shown in Additional file 3: Table S3.

Agrobacterium tumefaciens infiltration, protein extraction and western blotting

N. benthamiana plants were grown in a greenhouse house under a 16 h 25 °C:8 h 22 °C, day: night regime. *N. benthamiana* plants used for infiltration were 5–6 weeks old. All divergent Avr1c variants and mutants fused GFP constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation. *A. tumefaciens* strains were cultured in Luria Bertani liquid medium for 12–16 h at 30 °C in a shaking incubator. The cultures were collected by centrifugation and resuspended in infiltration buffer (10 mM magnesium chloride, 10 mM MES, pH 5.6, and 150 mM acetosyringone) to an OD600=0.6, and then infiltrated into *N. benthamiana* leaves. Two days after agroinfiltration, agroinfiltrated leaves were frozen in liquid nitrogen and ground to powder using a mortar and pestle. Approximately 0.5 g powder was added to 1.0 mL ice-cold extraction buffer (NP-40 (P0013F, Beyotime, Shanghai); 1 mM Phenylmethanesulfonyl fluoride (PMSF)) in a 2.0 mL centrifuge tube. The mixture was centrifuged at 14000 × g for 10 min at 4 °C, and 100 μL of supernatant was transferred to a 1.5 mL tube and then boiled in SDS-PAGE loading buffer for 5 min. The samples were loaded onto a 15% SDS-PAGE gel for protein electrophoresis.

The total protein in leaves of *N. benthamiana* expressing Avr1c variants and mutants were examined by western blotting with anti-GFP antibody. The separated proteins were transferred from the gel to polyvinylidene difluoride (PVDF) membrane and then blocked with PBSTM buffer (phosphate-buffered saline with 0.1% Tween-20 and 5% non-fat dry milk) for 30–40 min at room temperature with 60–70 rpm shaking; anti-GFP (1: 5000; #M20004, Abmart, Shanghai, China) was then added to PBSTM buffer and incubated at room temperature for 2 h, followed by five washes with PBST buffer. The membrane was then incubated with a goat anti- mouse antibody (Odyssey, no. 926–32,210; Li-Cor, Lincoln, NE, USA) at a ratio of 1: 10000 in PBSTM at room temperature in the dark for 30–40 min with 60–70 rpm shaking. The membrane was washed five times with PBST, and then visualized by excitation at 800 nm. The green fluorescent protein (GFP) was used as a control.

Gene co-bombardment on soybean leaves

Avr1c alleles and mutations were amplified using a pair of specific primers (Additional file 3: Table S3) that do not encode a signal peptide, and were ligated into the plasmid pFF19 (replacing *GUS*), which was digested by *Sma*I (Kong et al. 2015). Double-barreled particle bombardment assays were performed on 12–13 days old soybean leaves containing *GUS* DNA and empty vector (EV), or *GUS* and *Avr*, using a Bio-Rad He/1000 Particle Delivery System (Hercules, CA) (Dou et al. 2008b). Next, the soybean leaves were moisturized for 2 days in darkness at 25 °C. The leaves were stained for ~ 12 h at 37 °C with 0.5 mg/mL X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, cyclohexylammonium salt), 80 mM sodium phosphate, 0.4 mM potassium ferricyanide, 0.4 mM potassium ferrocyanide, 10 mM disodium EDTA (pH 8.0), and 0.1% (v/v) Triton X-100, and then destained in 75% ethanol. Each experiment was repeated at least three times.

Blue spots were counted and the ratio of blue spots in leaves expressing target gene compared with EV were calculated. The bars represent standard errors from at least three independent replicates and analyzed by *t*-test.

Structure of Avr1c variants

The structure of Avr1c variants were simulated using SWISS-MODEL (Waterhouse et al. 2018) and the RXLR effector PcAVR3a4 was used as a template, which has 23.08% sequence identity to Avr1c (Yaeno et al. 2011). Because mature AVR3a4 without the signal peptide (Asn22-Tyr122) was highly soluble and properly folded, and the N-terminal region (including the RXLR domain [Asn22-Arg58]) was disordered, so we used Avr1c$^{K79\text{-}H126}$ (deleted signal peptide Met1-Ala17 and Ser18-Gly78) in the simulation. The peptide Avr1c-1$^{K79\text{-}H126}$, Avr1c-4$^{K79\text{-}H126}$ and Avr1c-8$^{K79\text{-}H126}$ as target sequences were used to search for templates and build model.

Additional files

Additional file 1: Table S1. The origin and pathotypes of *Phytophthora sojae* isolates. (XLS 34 kb)

Additional file 2: Table S2. Reactions of soybean cultivars to *Phytophthora sojae* isolates and *Avr1c* alleles. (XLS 34 kb)

Additional file 3: Table S3. Primers used in this study. (XLS 30 kb)

Additional file 4: Figure S1. Nine nucleotide sequences of *Avr1c*. (JPG 1763 kb)

Additional file 5: Figure S2. Nine amino acid variants of Avr1c. A, the isolates were avirulent on *Rps1c*; V, virulent on *Rps1c*. (JPG 312 kb)

Additional file 6: Table S4. d*N*/d*S* analysis of pairwise comparisons of *Avr1c* alleles. (XLS 39 kb)

Additional file 7: Figure S3. The expression of the divergent Avr1c variants fused GFP in *Nicotiana benthamiana*. **a** and **b** The Avr1c variants and mutants were transiently expressed in *N. benthamiana* without signal peptide. The total protein of leaves expressed Avr1c variants and mutants were extracted and were examined by western blotting with anti-GFP antibody. Protein bands corresponding to Avr1c variants and mutants were indicated by red asterisks. The green fluorescent protein (GFP) was used as a control. (JPG 142 kb)

Additional file 8: Figure S4. Expression levels of *Avr1c* in different isolates determined by RT-qPCR. The RNA samples at infection stages at 0.5, 1.5, 3, 6, 9, and 12 hpi were collected to conduct RT-qPCR. The error bars were from three independent replicates. The *P. sojae* isolates P6497, AH14 and HLJ5 are avirulent on *Rps1c*. The isolate JL37 is virulent on *Rps1c*. (JPG 142 kb)

Additional file 9: Figure S5. Structural simulation of Avr1c forms. **a** Multiple sequence alignment of the effector domain of Avr1c-1, Avr1c-4, Avr1c-8 and homolog PcAVr3a4. PcAVR3a4 is from *P. capsici*. Avr1c-1, Avr1c-4 and Avr1c-8 are from *P. sojae*. The helical regions and corresponding amino acid positions for PcAVR3a4 are shown above the alignment and for Avr1c are shown below the alignment. **b** The surface structure of Avr1c-1 and the K105 residue. Ribbon diagram of (**c**) Avr1c-1; purple, side chains of K105; (**d**) Avr1c-4; and (**e**) Avr1c-8. W and Y are motifs in effector domain. (JPG 527 kb)

Additional file 10: Figure S6. The untranslated regions upstream the *Avr1c*. The isolates P6497 and PsNJ1 are avirulent on *Rps1c*. The isolates PsShx268, Shx293, CQ33, GX25, HB6, SH31, SH33, SH37 and TJ58 are virulent on *Rps1c*. The transcript of *Avr1c* was detected in isolates P6497, PsNJ1, PsShx268 and Shx293, but was not detected in isolates CQ33, GX25, HB6, SH31, SH33, SH37 and TJ58. No transcript of *Avr1c*, the transcript level of *Avr1c* was not detected in the isolates. (JPG 351 kb)

Abbreviations

d*N*: Nonsynonymous substitution; d*S*: Synonymous substitution; ORF: Open reading frame; RT-PCR: Reverse transcription polymerase chain reaction; RXLR-dEER: Arg-Xaa-Leu-Arg-Asp-Glu-Glu-Arg, X could be any amino acid

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Authors' contributions

YCW, SD, WY, JY, XZ and YW conceived the study; JY and XW performed the experiments; JY and BG analyzed the Avr1c model; and JH provided suggestions. JY analyzed the data. JY, WY, and YCW wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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