An Improved Method for the Identification of Soybean Resistance to *Phytophthora sojae* Applied to Germplasm Resources from the Huanghuaihai and Dongbei Regions of China

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Abstract

Phytophthora root and stem rot (PRR) caused by *Phytophthora sojae* is a destructive disease afflicting soybean. The use of resistant cultivars is the most effective method to combat PRR. PRR resistance was assessed in 223 soybean cultivars from Huanghuaihai and Dongbei, major soybean-producing regions in east central and northeastern China. To evaluate levels of soybean resistance to *P. sojae*, we used eight representative *P. sojae* isolates and a modified etiolated hypocotyl-slit inoculation method. The cultivars Wandou21020, Xu9302-A, Kedou10, and Lidi055 showed resistance to all eight isolates; 14 cultivars showed intermediate resistance to all eight *P. sojae* isolates, and 53 cultivars were resistant to seven isolates. Thirty-three cultivars (15%) were susceptible only to the

highly virulent PsJS2 isolate, which is consistent with the reactions of the Chapman differential line that carries *Rps3a*. The diverse reaction patterns seen in germplasm from different regions (provinces/cities) in this study reflect the variety of PRR-resistant soybean sources in China. Our research indicates that sources of *P. sojae* resistance are present in the major soybean production areas of China. This study provides useful information for soybean breeding programs.

Keywords: improved method, *Phytophthora sojae*, resistance genes, soybean germplasm resources

Phytophthora root and stem rot (PRR), caused by *Phytophthora sojae* (M. J. Kaufmann & J. W. Gerdemann), is a destructive disease in soybean, causing approximately \$1 to 2 billion in losses worldwide annually (Tyler 2007). *P. sojae* infects soybean via chemotaxis of zoospores that are attracted to the isoflavones daidzein and genistein, which are secreted by soybean roots throughout the growing season (Morris and Ward 1992). Infection results in seedling damping off, root and stem rot, leaf yellowing and wilting, and sometimes death of adult soybean plants (Schmitthenner 1985). This disease imposes serious limitations on soybean production, causing yield losses of 4 to 100% (Sugimoto et al. 2012). In some years, PRR was the second most important yield-limiting disease of soybean in the northern central United States (Doupnik 1993). The disease was first reported in 1955 (Kaufmann and Gerdemann 1958); since that time, at least 55 races have been reported (Grau et al. 2004; Jackson et al. 2004; Leitz et al. 2000; Morgan and Hartwig 1965). The reported 223 pathotypes and complex pathogenic variation of *P. sojae* (Dorrance et al. 2016) make it difficult to control. *P. sojae* is widespread in China and has been reported in Heilongjiang, Jiangsu, Anhui, Fujian, Henan, Shandong, Xinjiang, and Sichuan provinces (Chen et al. 2004, 2016; Zhang et al. 2010).

Methods to control the disease include resistant and tolerant cultivars, fungicide application, and biological and cultural methods (Schmitthenner 1985). Fungicide seed treatment of cultivars with

partial resistance has been beneficial in controlling PRR (Dorrance and McClure 2001; Dorrance and Schmitthenner 2000; Dorrance et al. 2009). The use of resistant soybean cultivars is the primary and most effective method to combat the disease (Sugimoto et al. 2012), and numerous sources of resistance have been identified (Jiang et al. 2017; Kyle et al. 1998; Lohnes et al. 1996; Slaminko et al. 2010).

To date, at least 27 single genes conferring resistance to *P. sojae* (*Rps* genes) have been identified, including *Rps1a*, *Rps1b*, *Rps1c*, *Rps1d*, *Rps1k*, *Rps2*, *Rps3a*, *Rps3b*, *Rps3c*, *Rps4*, *Rps5*, *Rps6*, *Rps7*, *Rps8*, *Rps9*, *Rps10*, *Rps11*, *Rps12*, *RpsYD25*, *RpsYu25*, *RpsYD29*, *RpsUN1*, *RpsJS*, *RpsSN10*, *RpsYB30*, *RpsWaseshiroge*, and *RpsZS18* (Fan et al. 2009; Lin et al. 2013; Ping et al. 2016; Sahoo et al. 2017; Sandhu et al. 2005; Sun et al. 2014; Wu et al. 2011; Yao et al. 2010; Yu et al. 2010; Zhang et al. 2013; Zhu et al. 2007). *Rps1a* was the first *Rps* gene widely deployed in the United States (Schmitthenner 1985). During the past several decades, *Rps1k* has become the most stable and widely used gene in the major soybean-producing regions of the United States (Gao et al. 2005). *Rps1d* and *Rps1k* were the most effective resistance genes in Japan in 2010 (Sugimoto et al. 2012). Nevertheless, the eventual evolution of new strains of *P. sojae* will likely confer the ability to overcome these single *Rps* genes. Moreover, persistent use of stable soybean cultivars with partial resistance is appropriate for yield production (Dorrance and McClure 2001; Sugimoto et al. 2012). On average, single *Rps* genes have been found to be effective for 8 to 15 years (Schmitthenner 1985). It is important to evaluate soybean cultivar resistance to *P. sojae* to control this disease.

Lohnes et al. (1996) examined the distribution of *Phytophthora*-resistant soybean accessions from central China and classified their reactions to races 1, 3, 7, and 25 of *P. sojae*. They observed resistance to be more common in accessions from the eastern provinces of central China, especially Anhui and Jiangsu, where the use of resistant soybean lines was most frequent. Kyle et al. (1998) investigated the distribution and diversity of resistant soybean in southern China, evaluating the resistance of 628 accessions to races 1, 3, 4, 5, 7, 10, 12, 17, 20, and 25 of *P. sojae* using a hypocotyl inoculation technique. Most of the accessions were resistant to eight or more of the 10 races from the provinces of Hubei, Jiangsu, and Sichuan in

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southern China. Zhang et al. (2010) evaluated 62 soybean cultivars from Heilongjiang province in northeastern China for resistance to eight races, and they found that 44 cultivars were resistant to at least one race. A large number of studies have shown extensive amounts of resistant soybean germplasm to be present in southern, central, and northeastern China (Chen et al. 2016; Kyle et al. 1998; Zhang et al. 2010).

Huanghuaihai and Dongbei are regions in east central and north-eastern China, which are major areas of soybean production (Yin et al. 2017). To evaluate the levels of soybean resistance to *P. sojae*, we tested 223 soybean cultivars for their responses to eight representative *P. sojae* isolates following inoculation using a modified etiolated hypocotyl-slit inoculation method.

Materials and Methods

Soybean cultivars. The seeds of soybean cultivars from at least 12 provinces or cities of China (Fig. 1) were provided by Professor Shi Sun at the Chinese Academy of Agricultural Sciences (CAAS). Detailed information about these seeds is provided in Supplementary Table S1. The soybean germplasm resources used in this study are new cultivars developed by breeding programs at academies of agricultural sciences in different provinces. It was unknown whether these cultivars were *Phytophthora* resistant. Seeds from a set of 14 differential soybean lines were provided by Professor Han Xing at Nanjing Agricultural University. The development of the differentials is shown in Table 1. We used 14 differential soybean lines, each

carrying a single *Rps* gene, to confirm the pathotypes of *P. sojae*, produce standard reaction types, and validate the modified inoculation method (Supplementary Table S2). The differentials were Harlon (*Rps1a*), Harosoy 13XX (*Rps1b*), Williams 79 (*Rps1c*), PI 103091 (*Rps1d*), Williams 82 (*Rps1k*), L76-1988 (*Rps2*), Chapman (*Rps3a*), PRX146-36 (*Rps3b*), PRX145-48 (*Rps3c*), L85-2352 (*Rps4*), L85-3059 (*Rps5*), Harosoy 62XX (*Rps6*), Harosoy (*Rps7*), and PI 399073 (*Rps8*). Williams, which has none of the 14 *Rps* genes, was used as a susceptible control (Dorrance et al. 2004; McBlain et al. 1991), and Hefeng 47 was used as a second control from China (resistant to Ps1, Ps3, and Ps5; susceptible to Ps4, Ps41-1, PsMC1, PsJS2, and PsUSAR2 (Table 1).

***P. sojae* isolates and cultivation.** The eight representative *P. sojae* isolates were collected and provided by Professor Zhendong Zhu (CAAS), including isolates representing Ps1, Ps3, Ps4, and Ps5 originating from Heilongjiang province (Zhang et al. 2010); PsUSAR2 from the United States (provided by A. F. Schmitthenner); PsMC1 from Anhui province; PsJS2 from Jiangsu province; and Ps41-1 from Heilongjiang province (Zhang et al. 2014). The pathotypes of the eight isolates were confirmed using the hypocotyl-slit inoculation method (Dorrance et al. 2008) on the 14 differential soybean lines carrying a single *Rps* gene. The *P. sojae* isolates were stored in 10% V8 juice agar medium at 12°C in the dark.

Characterization of soybean resistance. Etiolated seedlings were inoculated using a modified etiolated hypocotyl inoculation

Fig. 1. The origin of soybean cultivar seeds in China. The numbers of soybean cultivars for each province (city) are also shown.

method. Etiolated soybean seedlings were grown in vermiculite at 25°C during the day (14 h) and night (10 h) for 4 days, after which the vermiculite was washed off for disease assays. A 15-mm cork borer was then used to cut material from the growing edge of 5- to 7-day-old *P. sojae* cultures. A 15-mm-diameter agar plug was placed mycelial side down to the hypocotyl where a slit had been slightly punctured with a knife tip, 1 to 2 cm below the cotyledon. A 15-mm-diameter inoculum-free 10% V8 agar plug was used as a noninoculated control (Supplementary Fig. S1). We then wrapped the inoculation site in absorbent paper (~10 cm²) saturated with sterilized water. Five plants of each soybean cultivar were inoculated with each *P. sojae* isolate and then wrapped with tin foil. The inoculated plants were placed in the greenhouse at 25°C, with 14 h of light and 10 h of darkness. Every assay was repeated twice for a total of 10 plants per cultivar per isolate.

The resistance phenotype was determined at 4 days postinoculation on the basis of the reaction of five plants. A cultivar was characterized as resistant (R) when more than three of five plants were similar to the noninoculated control or exhibited soft red-brown patches only at the inoculated sites and had normal root growth. If the water soaked or red-brown patch had expanded from the inoculated sites on more than three plants and the roots were barely developed, the cultivar was classified as susceptible (S). If the disease lesion appeared only at the inoculation site (whether or not it encompassed the stem) and the roots were normal compared with the control in more than three plants, or if there were two healthy and three diseased plants or two diseased and three healthy plants, then the cultivar was identified as having intermediate resistance (IR). If there were differences between the two assays, we repeated the assay a third time and recorded the response shown in two of the three assays. Assays producing intermediate resistance reactions were repeated until the result was stable (Supplementary Table S3, detailed results for partial cultivars). Intermediate resistance was classified as “resistance” in the reaction pattern analysis (Kyle et al. 1998).

Results

Pathotypes of the representative *P. sojae* isolates. The identified pathotypes of the *P. sojae* isolates are shown in Figure 2A. Among these isolates, PsJS2 overcame all 14 *Rps* genes and contained no known avirulence (*Avr*) genes (pathotype: 1a, 1b, 1c, 1d, 1k, 2, 3a, 3b, 3c, 4, 5, 6, 7, and 8), whereas Ps1 (pathotype: 7), Ps3 (1a, 7), and Ps4 (1a, 1c, 7) were comparatively weaker, overcoming one, two, and three *Rps* genes, respectively.

The reaction types of the 14 differential soybean lines inoculated with eight representative *P. sojae* isolates are shown in Table 1. According to the reaction types, lines containing the *Rps1a*, *Rps1b*, *Rps1c*, *Rps1d*, *Rps3a*, and *Rps6* genes were distinguishable, whereas

those with the *Rps1k*, *Rps2*, *Rps3b*, *Rps3c*, *Rps4*, *Rps5*, *Rps7*, and *Rps8* genes were not. Thus, the eight representative *P. sojae* isolates can be used to identify hosts with the *Rps1a*, *Rps1b*, *Rps1c*, *Rps1d*, *Rps3a*, and *Rps6* genes and to identify cultivars that might have novel *Rps* genes.

Resistance of the soybean germplasm resources. We found that >80% of the tested soybean cultivars were simultaneously resistant to the isolates Ps1, Ps3, and Ps5 (82, 81, and 88%, respectively). Among the isolates, such as PsUSAR2 (pathotype: 1b, 2, 3c, 5, 7), Ps41-1 (1a, 1d, 2, 3b, 3c, 5, 7, 8), and PsMC1 (1a, 1c, 1k, 2, 3b, 3c, 4, 5, 6, 7, 8), the proportions of resistant cultivars were reduced to 16, 40, and 36%, respectively. Only 5% of soybean cultivars exhibited resistance to isolate PsJS2. In addition, 16% of the 223 cultivars showed intermediate resistance to Ps1, and 14, 35, 10, 24, 16, 33, and 18% showed intermediate resistance to Ps3, Ps4, Ps5, PsUSAR2, Ps41-1, PsMC1, and PsJS2, respectively (Fig. 2A).

All 223 cultivars showed intermediate resistance to at least two isolates, and >56% were resistant to six or more isolates. Wandou21020, Xu9302-A, Kedou10, and Lidi055 were resistant to all eight isolates. Fourteen cultivars showed intermediate resistance to eight isolates, and 54 were resistant to seven isolates (Fig. 2B); thus, many of the resistant cultivars showed only intermediate resistance. Average numbers of soybean-resistant *P. sojae* isolates were similar among individual provinces or cities, ranging between five and six isolates (Fig. 2C), indicating that resistant cultivars are abundant in all of these regions. Most resistant soybean cultivars were resistant to four isolates (Fig. 2B and C). Thus, overall, the examined soybean germplasm resources exhibited a broad spectrum of resistance to *P. sojae*.

Reaction patterns and resistance gene postulation. A comparison of the reaction patterns of each tested cultivar with those of the differential lines was used to postulate the *Rps* gene complement. The 223 cultivars developed 35 different reaction patterns (Fig. 3). The most common of these, occurring in 34 cultivars (15%), was RRRRRRRS, indicating resistance to *P. sojae* isolates Ps1, Ps3, Ps4, Ps5, PsUSAR2, Ps41-1, and PsMC1 but susceptibility to PsJS2. This reaction pattern is consistent with that of the Chapman differential line, which carries *Rps3a*. The second most common reaction pattern (RRRSRRS) was observed in 22 soybean cultivars (10%), which were postulated to have *Rps1b*. An RRRRRSRS pattern was consistent with *Rps1d*, which was observed in 10 soybean cultivars. The RRRRSSSS reaction pattern, which was consistent with that of differentials carrying *Rps2*, *Rps3c*, *Rps5*, or a combination of these, was observed in 11 cultivars. Five cultivars with an RRRRRRSS reaction pattern and three with RRRRRSSS were postulated to have *Rps1k* and/or *Rps4*, and *Rps3b* and/or *Rps8*, respectively. Resistance gene complements could not be deduced for 30 other reaction types,

Table 1. Reactions of 14 differential soybean lines inoculated with eight representative *Phytophthora sojae* isolates

Soybean line ^a	<i>Rps</i> gene	<i>P. sojae</i> isolates ^b								Reaction pattern	Reference for differential line origin
		Ps1	Ps3	Ps4	Ps5	PsUSAR2	Ps41-1	PsMC1	PsJS2		
Harlon	<i>Rps1a</i>	R	S	S	S	R	S	S	S	RSSSRSSS	Schmitthenner et al. (1994)
Harosoy 13XX	<i>Rps1b</i>	R	R	R	R	S	R	R	S	RRRSRRS	Buzzell et al. (1987)
Williams 79	<i>Rps1c</i>	R	R	S	S	R	R	S	S	RRSSRRSS	Moots et al. (1983)
PI 103091	<i>Rps1d</i>	R	R	R	R	R	S	R	S	RRRRRSRS	Buzzell and Anderson (1992)
Williams 82	<i>Rps1k</i>	R	R	R	R	R	R	S	S	RRRRRRSS	Moots et al. (1983)
L76-1988	<i>Rps2</i>	R	R	R	R	S	S	S	S	RRRRSSSS	Kilen et al. (1974)
Chapman	<i>Rps3a</i>	R	R	R	R	R	R	R	S	RRRRRRRS	McBlain et al. (1991)
PRX-146-36	<i>Rps3b</i>	R	R	R	R	R	S	S	S	RRRRSSSS	Buzzell et al. (1987)
PRX-145-48	<i>Rps3c</i>	R	R	R	R	S	S	S	S	RRRRSSSS	Dorrance et al. (2004)
L85-2352	<i>Rps4</i>	R	R	R	R	R	R	S	S	RRRRRRSS	Athow et al. (1980)
L85-3059	<i>Rps5</i>	R	R	R	R	S	S	S	S	RRRRSSSS	Buzzell and Anderson (1981)
Harosoy 62XX	<i>Rps6</i>	R	R	R	S	R	S	S	S	RRRSRRSS	Buzzell et al. (1987)
Harosoy	<i>Rps7</i>	S	S	S	S	S	S	S	S	SSSSSSSS	Anderson and Buzzell (1992)
PI 399073	<i>Rps8</i>	R	R	R	R	R	S	S	S	RRRRSSSS	Burnham et al. (2003)
Williams	<i>rps</i>	S	S	S	S	S	S	S	S	SSSSSSSS	Wagner and Bernard (1991)
Hefeng 47	<i>rps</i>	R	R	S	R	S	S	S	S	RRSRSSSS	

^a The genetic background and the source of each *Rps* gene were obtained from Dorrance et al. (2004) and McBlain et al. (1991).

^b R = resistant and S = susceptible.

which may indicate new *Rps* genes, a combination of novel *Rps* genes, or a combination of novel and known *Rps* genes.

Some reaction patterns were more common in soybean cultivars from particular provinces or cities. For example, 21% of cultivars from Henan province had a RRRRSRRS pattern (postulated to have *Rps1b*), which was significantly >10% observed among all tested cultivars. In addition, 22, 22, and 17% of cultivars from the Dongbei region (Heilongjiang, Jilin, and Inner Mongolia) had the patterns RRRSSRS, RRRSSSS (postulated to have *Rps2*, *Rps3c*, and/or *Rps5*), and RRRSSRR, respectively; 10% of those from Shandong province had RRSRSRS, 8% of those from Hebei province had RSSSSSS, and 17% of those from Shanxi and Shaanxi provinces had RRSRRRS (Fig. 3). These results indicate that the examined soybean germplasm resources had diverse reaction patterns of *P. sojae* resistance.

Discussion

Many surveys have been conducted over the past few decades to identify soybean resistance to *P. sojae*; however, the *P. sojae* isolates used in these studies were inconsistent. Until recently, eight *P. sojae* isolates were used to represent the major pathotypes and are currently used to identify soybean resistance in China (Li et al. 2017). However, it appears that these isolates are not representative of all pathotypes occurring in China.

Jiang et al. (2017) modified a common *P. sojae* resistance identification assay to make it simpler and more cost-effective (Dorrance et al. 2008; Schmitthenner et al. 1994; Stewart and Robertson 2012). In that type of assay, *P. sojae* inoculum was mixed into soil and resistance was evaluated by the survival rate (percentage) of soybean seedlings (mixing agar homogenate containing *P. sojae* into the soil [MAPS]). This method was then applied to evaluate *P. sojae* resistance in soybean mini core collections, revealing wide variation in resistance among individual varieties (Jiang et al. 2017). However, the MAPS method requires longer than 3 weeks from the growth of *P. sojae* to the scoring of seedling survival rates. Arsenaault-Labrecque et al. (2018) developed a simple hydroponic assay to study resistance in soybean. However, differences in zoospore production among individual isolates have caused difficulties in mass resistance identification using this method. We assessed *P. sojae* resistance in soybean by modifying the etiolated hypocotyl-slit inoculation method of Dorrance et al. (2004) and Dong et al. (2009), using absorbent paper and tin foil wrap to maintain humidity and exclude light. A previous method described by Dorrance et al. (2004) used absorbent paper to wrap all etiolated seedlings of each cultivar, whereas our method used absorbent paper to wrap every plant from each cultivar to ensure that the mycelium touched hypocotyls and the tin foil to segregate each cultivar from others and maintain the darkness condition of *P. sojae*. Dong et al. (2009) used

Fig. 2. Characterization of *Phytophthora sojae* resistance for the 223 soybean cultivars. **A**, The number of resistance soybean cultivars on different *P. sojae* isolates. **B**, The number of resistance soybean cultivars on different numbers of *P. sojae* isolates in R and IR+R levels, respectively. **C**, The average numbers of soybean-resistant *P. sojae* isolates among the provinces (cities). S = susceptible, IR = intermediate resistance, and R = resistance.

The eight differential isolates allowed us to postulate the involvement of single genes *Rps1a*, *Rps1b*, *Rps1c*, *Rps1d*, *Rps3a*, and *Rps6*, but not other specific *Rps* genes, according to the reaction types. Reactions in the differential lines L76-1988 (*Rps2*), PRX145-48 (*Rps3c*), and L85-3059 (*Rps5*) were so similar that it was impossible to identify the relevant *Rps* genes accurately. Similarly, we were not able to predict the involvement of specific *Rps* genes in cultivars with the RRRRRRSS (*Rps1k* or *Rps4*) or RRRRRSSS (*Rps3b* or *Rps8*) reaction patterns. There were no reaction types consistent with the

To place minimal selection pressure on *P. sojae* populations, it is recommended to deploy cultivars with partial resistance, which is predicted to be more durable, and many soybean cultivars exhibit partial resistance (Abeysekara et al. 2016; Schneider et al. 2016). Although the deployment of *Rps* genes induced high selection pressures on *P. sojae* populations, causing the evolution of populations that overcome the *Rps* gene resistance, resistance mediated by major *Rps* genes is a highly effective and complete resistance in field production (Dorrance et al. 2003).

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Fig. 3. Reaction patterns of the 233 soybean cultivars. Reaction patterns followed the isolate order Ps1, Ps3, Ps4, Ps5, PsUSAR2, Ps41-1, PsMC1, and PsJS2. The numbers of cultivars are shown, and the percentage of the indicated reaction pattern in the indicated provinces (cities) is displayed as the background in the heatmap. Asterisks indicate a significantly higher percentage of the reaction pattern in the indicated provinces (cities), compared with that in all 233 soybean cultivars, using an hypergeometric test ($P < 0.05$).

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