Identification of Resistance Genes to *Phytophthora sojae* in Domestic Soybean Cultivars from China Using Particle Bombardment

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Abstract

Phytophthora root and stem rot caused by *Phytophthora sojae* is a destructive disease that afflicts soybean plants throughout the world. The use of resistant soybean cultivars is the primary means of managing this disease, as well as the most effective and economical approach. There are abundant soybean germplasm resources in China that could be deployed for breeding programs; however, the resistance genes (*Rps* genes) in most cultivars are unknown, leading to uncertainty concerning which are resistant cultivars for use. The resistance genes *Rps1a*, *Rps1c*, and *Rps1k* prevent root and stem rot caused by most *P. sojae* isolates within a Chinese field population. This study identified three *Rps* genes in Chinese domestic soybean cultivars using three related avirulence genes by particle bombardment. The complex genetic diversity of soybean cultivars and *P. sojae* strains has made it difficult to define single *Rps* genes without molecular involvement. Gene cobombardment is a method for

identifying *Rps* genes quickly and specifically. We showed that cultivars Dongnong 60 and Henong 72 contained *Rps1a*, while Hedou 19, Henong 76, 75-3, Wandou 21020, Zheng 196, Wandou 28, Heinong 71, and Wandou 29 all contained *Rps1c*. The cultivars Jidou 12, Henong 72, Heinong 71, and Wandou 29 contained *Rps1k*. The cultivar Henong 72 contained both *Rps1a* and *Rps1k*, while Wandou 29 and Heinong 71 contained both *Rps1c* and *Rps1k*. We then evaluated the phenotype of 11 domestic soybean cultivars reacting to *P. sojae* using the isolates P6497 and Ps1. The 11 domestic cultivars were all resistant to P6497 and Ps1. This research provides source materials and parent plant strains containing *Rps1a*, *Rps1c*, and *Rps1k* for soybean breeding programs.

Keywords: Avr genes, gene co-bombardment, *Phytophthora sojae*, *Rps* genes, soybean cultivars

Phytophthora root and stem rot (PRR) caused by *Phytophthora sojae* Kaufm. & Gerd. is a destructive soybean disease found throughout the world (Schmitthenner 1985). This disease causes soybean seedling damping off and root rot of established plants, costing approximately \$1 to 2 billion in annual agricultural losses worldwide (Tyler 2007).

The use of resistant soybean cultivars is the most effective and economical means of managing this disease (Sugimoto et al. 2012). Many plant–pathogen interactions are determined by resistance (*R*) genes that enable plants to recognize pathogens and induce a defense response (Grant et al. 1998). Two types of host resistance to *P. sojae* have been described: species-specific resistance determined by single dominant genes (*Rps* genes) (Gao et al. 2005; Sahoo et al. 2017) and broad-spectrum partial, nonspecific resistance conferred by several minor genes (field resistance, general resistance, rate-reducing resistance, and tolerance) (Abeysekara et al. 2016; Dorrance 2018). Molecular markers linked to *Rps* genes and quantitative trait loci (QTLs) of partial resistance to *P. sojae* in soybean plants have

been reported in different populations (Abeysekara et al. 2016; Sepiol et al. 2017).

The use of resistant cultivars controlled by a single resistance (*Rps*) gene to combat the disease has been shown to be an effective method to prevent disease (Schmitthenner 1985). The first *R* gene was cloned into maize 25 years ago and, since then, many *R* genes have been identified and cloned in different plants (Kourelis and van der Hoorn 2018), providing significant information for the engineering and use of resistant plants. To date, at least 27 *Rps* genes have been identified in soybean plants, including *Rps1a*, *Rps1b*, *Rps1c*, *Rps1d*, *Rps1k*, *Rps2*, *Rps3a*, *Rps3b*, *Rps3c*, *Rps4*, *Rps5*, *Rps6*, *Rps7* (Dorrance et al. 2004), *Rps8* (Sandhu et al. 2005), *Rps9* (Wu et al. 2011), *Rps10* (Zhang et al. 2013), *Rps11* (Ping et al. 2016), *Rps12* (Sahoo et al. 2017), *RpsYD25* (Fan et al. 2009), *RpsYu25* (Sun et al. 2011), *RpsYD29* (Zhang et al. 2014), *RpsUN1* (Li et al. 2016), *RpsJS* (Sun et al. 2014), *RpsSN10* (Yu et al. 2010), *RpsYB30* (Zhu et al. 2007), *RpsWaseshiroge* (Zhang et al. 2013), and *RpsZS18* (Zhong et al. 2018).

Rps1a was the first *Rps* gene widely deployed in soybean grown in the United States (Schmitthenner 1985). In the past several decades, *Rps1k* has become the most stable and widely used gene in major soybean-producing regions of the United States (Gao et al. 2005; Yan and Nelson 2019). *Rps1d* and *Rps1k* were the most effective resistance genes within Japan in 2010 (Sugimoto et al. 2012). *Rps1c* was also employed in the United States for many years (Beuerlein et al. 2000; Slaminko et al. 2010).

There are abundant soybean germplasm resources in China for use in breeding programs incorporating a variety of techniques. The hypocotyl inoculation method was commonly used in resistance evaluation assays (Huang et al. 2016; Slaminko et al. 2010; Zhu et al. 2007). Jiang et al. (2017) used a method in which, *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). Arsenault-Labrecque et al. (2018)

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developed a simple hydroponic assay to study resistance in soybean. However, *Rps* genes in most cultivars are uncharacterized, particularly in domestic cultivars, leading to uncertainty during resistant cultivar usage. Previous reports on cultivars containing *Rps1a*, *Rps1c*, and *Rps1k* suggest that these strains could be resistant to most of the *P. sojae* isolates in China (Cui 2010; Tang et al. 2011; Yang et al. 2019). Thus, use of cultivars containing these three *Rps* genes may be effective in the management of soybean disease in China.

To investigate *Rps* genes in domestic soybean cultivars within China, we collected 40 domestic soybean cultivars from the major soybean-producing regions and used a gene cobombardment method. This study provides source materials and identification of parent plants that contain *Rps1a*, *Rps1c*, and *Rps1k* for soybean breeding programs.

Materials and Methods

Soybean cultivars and *P. sojae* cultivation. Seed of cultivars Williams (*rps*), Harlon (*Rps1a*), Williams 79 (*Rps1c*), and Williams 82 (*Rps1k*) were provided by Prof. Han Xing at Nanjing Agricultural University. Cultivar Hedou 19 was from Shandong Province; Henong 72, Henong 76, Heinong 71, and Dongnong 60 were from Heilongjiang Province; 75-3 was from Fujian Province; Jidou 12 was from Hebei Province; Wandou 21020, Wandou 28, and Wandou 29 were from Anhui Province; and Zheng 196 was from Henan Province. The ancestral parents and origins are provided in Supplementary Table S1. Soybean cultivars were grown in a greenhouse at 25°C with a cycle of 14 h of light and 10 h of darkness.

P. sojae isolates P6497 and Ps1 used in this study were stored in 10% vegetable (V8) juice agar medium at 10 to 12°C in the dark. Each isolate was isolated from a single zoospore. The isolates were grown in 10% V8 juice agar medium at 25°C in the dark for transferring and inoculation.

Inoculation assay. Soybean seedlings were grown in vermiculite soaked with sterilized water at 25°C during the day (14 h) and at night (10 h) for 4 days prior to harvest for inoculation assays (Dong et al. 2009). The vermiculite was washed off for disease assays. A 5-mm-diameter mycelial plug, cut from the growing edge of 7-day-old *P. sojae* cultures, was placed on the hypocotyls of etiolated seedlings, 2 to 3 cm from the base of the cotyledon, mycelial-side down. This was done for 10 plants per cultivar and repeated two times. A 5-mm-diameter inoculum-free 10% V8 agar plug was used as a blank control. The seedling roots and inoculation sites were covered with papers soaked with sterilized water to maintain humidity, with plastic bag coverings used to maintain humidity for 4 days. The inoculated plants were then placed in the dark at 25°C.

Resistance phenotypes were determined at 4 days postinoculation. A cultivar was characterized as resistant when more than 7 of 10 plants were similar to the blank control or exhibited soft red-brown patches only at the inoculated sites with 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. If the 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 the number of healthy plants ranged from 4 to 6, then the cultivar was identified as having intermediate resistance. If there were differences between the two assays, the assay was repeated for a third time and the response of two of the three assays was recorded. Assays producing intermediate resistance reactions were

repeated until the result was consistent. The phenotypes of each cultivar were scored as resistant, susceptible, or intermediate.

Plasmid construction. Avirulence (*Avr*) genes *Avr1a*, *Avr1c*, and *Avr1k* alleles without a signal peptide were cloned from genomic DNA of *P. sojae* isolate P6497 (Supplementary Table S2). For gene cobombardment experiments, all the amplified fragments were purified by the Agarose Gel DNA Purification Kit (TaKaRa) following the manufacturer's instructions. The purified fragments were infused using a ClonExpressII One Step Cloning Kit (Vazyme, Nanjing, China) into pFF19, which was linearized by restriction enzyme *Sma*I (New England Biolabs, Ipswich, MA, U.S.A.) (Timmermans et al. 1990). Individual colonies of each construct were tested by PCR and verified by sequencing. The cloning primers are shown in Table 1.

Gene cobombardment of soybean leaves. *Avr1a*, *Avr1c*, and *Avr1k* alleles were amplified using a pair of primers without a signal peptide, infused into plasmid pFF19 (replacing β -glucuronidase [*GUS*]), and digested by *Sma*I (Kong et al. 2015). Double- or quadruple-barreled particle bombardment assays were performed on 12- or 13-day-old soybean leaves containing *GUS* DNA and empty vector (EV), or *GUS* and *Avr* genes, using a Bio-Rad He/1000 Particle Delivery System (Hercules, CA, U.S.A.) (Dou et al. 2008b) to enable target leaves to be bombarded with two DNA preparations simultaneously.

In total, 60 mg of 0.6- μ m gold microcarrier particles (Bio-Rad) was washed twice with 75% ethanol and three times with sterile deionized water, then suspended in 50% sterile glycerol to a concentration of 60 mg/ml, and 50 μ l was placed in each tube. For bombardment experiments, particles (50 μ l) were combined with 10 μ g of *GUS* plasmid DNA (PFF19-*GUS*) and test DNA or EV (pFF19) as the control in a 1.5-ml centrifuge tube. Then, 70 μ l of 2.5 M CaCl_2 was added to the reaction, followed by 30 μ l of freshly prepared 0.1 M spermidine. The preparation was vortexed vigorously for 5 min, then pulsed for another 5 min. The particle preparation was then concentrated to 10 μ l by brief centrifugation.

Soybean leaves were hydrated for 2 days in the dark at 25°C. The leaves were stained for 12 to 24 h at 37°C with 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid (cyclohexylammonium salt) at 0.5 mg/ml, 80 mM sodium phosphate, 0.4 mM potassium ferricyanide, 0.4 mM potassium ferrocyanide, 10 mM disodium EDTA (pH 8.0), and 0.1% (vol/vol) Triton X-100, then destained in 75% ethanol until the chlorophyll was destained enough to take clear photos.

For each pair of photos, the ratio of the number of blue spots with various *Avr* gene constructs to the EV control was calculated. Each assay consisted of five pairs of photos and was conducted at least twice. For quadruple-barreled particle bombardment assays, the numbers of blue spots of the three *Avr* genes were compared with the EV expressed in the same leaves. Each assay consisted of five pairs of images and at least three biological replicates were performed.

Results

The three *Avr* genes are recognized by the cognate *Rps* genes.

To investigate the resistance genes of domestic soybean cultivars in China, three similar *Avr* genes were transiently expressed in soybean leaves. The *Avr* genes *Avr1a*, *Avr1c*, and *Avr1k* were constructed into plasmid pFF19 without a signal peptide. The *Avr* genes, together with the reporter gene *GUS*, were expressed in leaves of the Williams (*rps*), Harlon (*Rps1a*), Williams 79 (*Rps1c*), and Williams 82 (*Rps1k*)

Table 1. Primers used in this study

Primers	Sequence 5'→3'
pFF19- <i>Avr1a</i> -F	GCTTTCGCGAGCTCGGTACCCGGGATGGCAGCAACTGATGCCGAC
pFF19- <i>Avr1a</i> -R	GGTCGACTCTAGAGGATCCCGGGGTACCAGTCTTTATAATTCCTG
pFF19- <i>Avr1c</i> -F2	GCTTTCGCGAGCTCGGTACCCGGGATGGCAGCAACTGATGCCGAC
pFF19- <i>Avr1c</i> -R2	GGTCGACTCTAGAGGATCCCGGGGCTAATGACCTCTCAAGTGGAAATAC
pFF19- <i>Avr1k</i> -F	GCTTTCGCGAGCTCGGTACCCGGGATGCTACTTGCCTCCACCTCCAG
pFF19- <i>Avr1k</i> -R	GGTCGACTCTAGAGGATCCCGGGGTACAGTAATCATGATGCTGTTC

cultivars using double-barreled particle bombardment. Blue spots indicating surviving cells were counted. Our results showed that there was no significant difference in the number of surviving cells in Williams (*rps*) leaves expressing the three *Avr* genes compared with that of the EV. The amount of pFF19-*Avr1a* gene expressed in Harlon (*Rps1a*) was adjusted to 100, 300, 500, 700, or 1,000 ng, and the results showed that plasmid amounts of 500 ng and higher significantly induced cell death in Harlon leaves. When the amount of pFF19-*Avr1c* gene expressed in Williams 79 (*Rps1c*) was adjusted to 100 ng, 500 ng, 1 μ g, 2 μ g, 4 μ g, or 8 μ g, we observed that 4 to 5 μ g and higher of pFF19-*Avr1c* significantly induced cell death in Williams 79 leaves. For the *Avr1k* gene, we altered the plasmid quantity to 100 ng, 500 ng, 1 μ g, 2 μ g, 4 μ g, 8 μ g, 10 μ g, 12 μ g, and 14 μ g, and demonstrated that 10 μ g and higher of plasmid significantly induced cell death in Williams 82 (*Rps1k*) (Fig. 1).

Identification of *Rps* genes in domestic soybean cultivars. Although there are abundant soybean germplasm resources in China, resistance to *P. sojae* and the presence of *Rps* genes in these resources remains unknown. We collected 40 domestic soybean cultivars in major soybean production regions from China to identify the *Rps* genes contained in those domestic cultivars. The leaves of 12- to 13-day-old soybean plants of 40 domestic cultivars were harvested to transiently express three *Avr* genes. The cultivars Williams, Dongnong 60, Jidou 12, Henong 72, Hedou 19, Henong 76, 75-3, Wandou 21020, Wandou 29, and Hefeng 50 all showed cell death when the three *Avr* genes with GUS were transiently expressed using double-barreled particle bombardment assays. There were significantly fewer spots in cultivars Dongnong 60 and Henong 72 expressing *Avr1a* compared with the EV, indicating that the cultivars Dongnong 60 and Henong 72 contain *Rps1a*. Similarly, Hedou 19, Henong 76, 75-3, Wandou 21020, and Wandou 29 all contain *Rps1c*. The cultivars Jidou 12, Henong 72, and Wandou 29 contain *Rps1k*.

Fig. 1. Three avirulence (*Avr*) genes are recognized by cognate single dominant resistance (*Rps*) genes. **A**, Response of *Avr1a*, *Avr1c*, and *Avr1k* transiently expressed in Williams (*rps*), Harlon (*Rps1a*), Williams 79 (*Rps1c*), and Williams 82 (*Rps1k*) cultivars. Blue spots represent surviving cells and red asterisks indicate cell death. **B**, Ratio of β -glucuronidase (*GUS*)-positive blue spots following bombardment with *Avr* genes compared with the empty vector (EV) control. Error bars represent standard error from three independent replicates. Asterisks (**) indicate $P < 0.01$ determined by *t* test.

The cultivar Henong 72 contains both *Rps1a* and *Rps1k*, while Wandou 29 contains both *Rps1c* and *Rps1k*. The cultivars Williams and Hefeng 50 were used as controls (Fig. 2; Supplementary Table S1).

The *Avr* genes were transiently expressed in the cultivars Williams, Zheng 196, Henong 71, Wandou 28, and Kenfeng 23 using quadruple-barreled particle bombardment assays (Fig. 3). The ratio of blue spots that expressed the three *Avr* genes compared with the EV was calculated for each cultivar. Our results indicated that Zheng 196 contains *Rps1c*, Henong 71 contains both *Rps1c* and *Rps1k*, and Wandou 28 contains *Rps1c*, whereas Kenfeng 23 contains none of the three *Rps* genes.

In all, 11 of the 40 domestic soybean cultivars from China contained *Rps1a*, *Rps1c*, *Rps1k* or a combination of the three *Rps* genes (Supplementary Table S1). Three cultivars contained *Rps1a*, nine cultivars contained *Rps1c*, and five cultivars contained *Rps1k*. Two cultivars contained *Rps1c* and *Rps1k* while one cultivar contained *Rps1a* and *Rps1k*. Twenty-nine cultivars contained none of the three *Rps* genes.

Phenotypic evaluation of resistance of domestic cultivars in China to *P. sojae*. To further investigate the resistance of cultivars containing the three *Rps* genes, we evaluated the reaction of 11 domestic soybean cultivars to *P. sojae* using the isolates P6497 (pathotype 1b, 7) and Ps1 (pathotype 7), which contain the three *Avr* genes: *Avr1a*, *Avr1c*, and *Avr1k*. The Williams cultivar was used as a susceptible control and the Hefeng 50 cultivar was used as a resistant control. The 11 cultivars containing *Rps* genes were all resistant to isolates Ps1 (pathotype 7) and P6497 (pathotype 1b, 7) (Fig. 4).

Discussion

Since the first *R* gene (*Hm1*) found in *Zea mays* was cloned in 1990, several different *R* genes in various plants have been identified and cloned (Kourelis and van der Hoorn 2018). Molecular identification and functional determination are crucial for molecular engineering and correct usage of *R* genes in plant breeding programs. The genome of the soybean plant is approximately 1.1 Gb and genome duplications occurred approximately 59 and 13 million years ago, resulting in a highly duplicated genome with nearly 75% of genes present in multiple copies (Schmutz et al. 2010). The complexity of the soybean genome makes it difficult to clone soybean genes. An accurate soybean genome sequence of Williams 82 was completed in 2010; this has facilitated identification of many soybean traits (Schmutz et al. 2010).

Generally, resistance to plant pathogens is controlled by a single *Rps* gene and would only be effective for 8 to 15 years due to rapid evolution of pathogens under high selection pressure in the field (Schmitthenner 1985). Stacking multiple *Rps* genes or combining QTLs into a single cultivar is considered an effective strategy to ensure durable, broad-spectrum resistance (Dorrance et al. 2009). Recently, *RpsX* in the soybean cultivar Xiu94-11 was reported to exhibit broad-spectrum PRR resistance (Zhong et al. 2019). Nevertheless, single dominant *Rps* genes have been highly effective in managing stem rot disease. To date, at least 28 *Rps* genes have been identified in the soybean genome; several nucleotide-binding site leucine-rich repeat receptor (NLR) genes have also been recorded (Gao et al. 2005; Ping et al. 2016). There are few cultivars currently identified with *Rps* genes, particularly domestic soybean cultivars.

Most *R* genes were previously cloned using a map-based cloning strategy in rice, maize, wheat, and soybean (Dai et al. 2007; Kourelis and van der Hoorn 2018; Zhao et al. 2013; Zhong et al. 2019). The traditional method of identifying *Rps* genes was to expose cultivars to *P. sojae* isolates and see if resistance was present. A hypocotyl-slit inoculation method was commonly used in soybean resistance identification assays (Huang et al. 2016; Slaminko et al. 2010; Zhu et al. 2007). Jiang et al. (2017) used a modified method in which *P. sojae* isolate inoculum was mixed into soil and resistance was evaluated by the survival rate (percentage) of soybean seedlings. Arsenault-Labrecque et al. (2018) developed a simple hydroponic assay to study resistance in soybean using different isolates. Then, the *Rps* genes were postulated in soybean cultivars according to the

resistance to various isolates (Kyle et al. 1998; Wu et al. 2010). However, the complex genetic diversity of soybean cultivars and *P. sojae* strains has made it difficult to define single *Rps* genes without molecular involvement. Gene cobombardment is an established transient expression technique for soybean genomics (Dou et al. 2008 a,b; Huang et al. 2019; Kong et al. 2015; Na et al. 2013; Song et al. 2013). This is the first report to identify *Rps* genes in a significant

number of cultivars using this technique. We first used a quadruple-barreled particle bombardment assay, which allows expression of four genes simultaneously in soybean leaves and was more effective for identification. We then tested the cultivar phenotypes against isolates P6497 and Ps1, each of which contained the three *Avr* genes. The 11 domestic cultivars that expressed *Rps* genes were resistant to both isolates, suggesting that the *Rps* genes we identified will be useful

Fig. 2. Response of cultivars expressing the three avirulence (*Avr*) genes. **A**, The 10 cultivars transiently expressing the three *Avr* genes. Blue spots represent surviving cells and red asterisks indicate cell death. **B**, Ratio of β -glucuronidase (*GUS*)-positive blue spots following bombardment with *Avr* genes compared with the empty vector (EV). Cultivar Hefeng 50 was used as a control. Error bars represent standard error from three independent replicates. Asterisks (**) indicate $P < 0.01$ determined by *t* test.

against PRR. Wandou 28, Wandou 29, and Hedou 19 were all resistant to Ps1 (pathotype 7), Ps3 (1a, 7), Ps4 (1a, 1c, 7), Ps5 (1a, 1c, 6, 7), PsUSAR2 (1b, 2, 3c, 5, 7), Ps41-1 (1a, 1d, 2, 3b, 3c, 5, 7, 8), PsMC1 (1a, 1c, 1k, 2, 3b, 3c, 4, 5, 6, 7, 8), and PsJS2 (1a, 1b, 1c, 1d, 1k, 2, 3a, 3b, 3c, 4, 5, 6, 7, and 8) (Yang et al. 2020).

Rps1c, *Rps1a*, and *Rps1k* are the most commonly used resistance genes in the United States (Schmitthenner 1985; Slaminko et al. 2010). These *Rps* genes can defend against the most *P. sojae* isolates in a Chinese field population (Tang et al. 2011; Yang et al. 2019). We collected 40 domestic soybean cultivars from major Chinese soybean-producing regions, including Heilongjiang, Henan, Jiangsu, Anhui, and Fujian Provinces, to conduct this survey. Within a group of 11 domestic soybean cultivars, 2 cultivars contained *Rps1a* genes, 8 cultivars contained *Rps1c*, and 4 cultivars contained *Rps1k*. Meanwhile, 29 cultivars did not contain any of the three known *Rps* genes. Our results indicated that the resistance gene *Rps1c* was the most

common in Chinese domestic soybean cultivars. The two cultivars containing *Rps1a* were both from Heilongjiang Province. The cultivars containing *Rps1k* were from Anhui and Heilongjiang Province, while the cultivars containing *Rps1c* were distributed in every region tested.

We first identified *Rps* genes in soybean cultivars using double- or quadruple-barreled particle bombardment assays. Our research has provided knowledge of parent plants that contain *Rps1a*, *Rps1c*, and *Rps1k* for breeders to stack several *Rps* genes together to develop more resistant and durable soybean cultivars.

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Fig. 3. Response of cultivars expressing all three avirulence (*Avr*) genes. **A**, Four cultivars transiently expressing all three *Avr* genes simultaneously. Blue spots represent surviving cells while the red asterisks indicate cell death. **B**, Ratio of β -glucuronidase (*GUS*)-positive blue spots following bombardment with *Avr* genes compared with the empty vector (*EV*). Cultivar Kenfeng 23 was used as a control. Error bars represent standard error from three independent replicates. Asterisks (**) indicate $P < 0.01$ determined by *t* test.

Fig. 4. Phenotypic evaluation of resistance to *Phytophthora sojae* P6497. Cultivar Williams was used as a susceptible control and cultivar Hefeng 50 was used as a resistant control. R = resistant and S = susceptible.

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