**Phytophthora sojae** Avirulence Effector Avr3b is a Secreted NADH and ADP-ribose Pyrophosphorylase that Modulates Plant Immunity

Suomeng Dong¹,², Weixiao Yin¹, Guanghui Kong¹, Xinyu Yang¹, Dinah Qutob³, Qinghe Chen⁴, Shiv D. Kale⁴, Yangyang Sui¹, Zhengguang Zhang¹,², Daolong Dou¹,², Xiaobo Zheng¹,², Mark Gijzen³, Brett M. Tyler⁴, Yuanchao Wang¹,²

¹ College of Plant Protection, Nanjing Agricultural University, Nanjing, China, ² Key Laboratory of Integrated Management of Crop Diseases and Pests (Nanjing Agricultural University), Ministry of Education, Nanjing, China, ³ Agriculture and Agri-Food Canada, London, Ontario, Canada, ⁴ Virginia Bioinformatics Institute, Blacksburg, Virginia, United States of America

### Abstract

Plants have evolved pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) to protect themselves from infection by diverse pathogens. Avirulence (Avr) effectors that trigger plant ETI as a result of recognition by plant resistance (R) gene products have been identified in many plant pathogenic oomycetes and fungi. However, the virulence functions of oomycete and fungal Avr effectors remain largely unknown. Here, we combined bioinformatics and genetics to identify Avr3b, a new Avr gene from *Phytophthora sojae*, an oomycete pathogen that causes soybean root rot. Avr3b encodes a secreted protein with the RXLR host-targeting motif and C-terminal W and Nudix hydrolase motifs. Some isolates of *P. sojae* evade perception by the soybean R gene Rps3b through sequence mutation in Avr3b and lowered transcript accumulation. Transient expression of Avr3b in *Nicotiana benthamiana* increased susceptibility to *P. capsici* and *P. parasitica*, with significantly reduced accumulation of reactive oxygen species (ROS) around invasion sites. Biochemical assays confirmed that Avr3b is an ADP-ribose/NADH pyrophosphorylase, as predicted from the Nudix motif. Deletion of the Nudix motif of Avr3b abolished enzyme activity. Mutation of key residues in Nudix motif significantly impaired Avr3b virulence function but not the avirulence activity. Some Nudix hydrolases act as negative regulators of plant immunity, and thus Avr3b might be delivered into host cells as a Nudix hydrolase to impair host immunity. Avr3b homologues are present in several sequenced *Phytophthora* genomes, suggesting that *Phytophthora* pathogens might share similar strategies to suppress plant immunity.

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* E-mail: wangyc@njau.edu.cn

### Introduction

Plant innate immunity, which has been continuously refined by challenges from a diversity of pathogens during evolution, employs at least two defense systems in response to pathogen attacks [1]. One is basal defense through host receptor recognition of conserved pathogen-associated molecular patterns (PAMPs), termed PAMP-triggered immunity (PTI). In most cases, the basal defense response can successfully prevent infections from becoming established. However, successful pathogens deliver secreted proteins (effectors) to suppress basal defense. Therefore, plants have developed a second immunity system that relies on plant resistance (R) protein perception of specific pathogen effectors and is called effector-triggered immunity (ETI). Pathogen effectors recognized by plant R proteins have historically been termed avirulence (Avr) effectors [1,2]. ETI usually results in faster and stronger plant resistance responses, including programmed cell death (PCD), reactive oxygen species (ROS) accumulation, and induction of plant hormone-mediated signal pathways [1]. Thus, the interaction between host R proteins and pathogen Avr effectors can determine the outcome of an infection.

Many plant *R* genes encode polymorphic proteins characterized by nucleotide binding (NB) and leucine-rich repeat (LRR) domains. These R proteins are located inside the cell, indicating that Avr effectors are usually delivered into plant cells by pathogens [2,3]. A motif, RXLR (Arg-any-Leu-Arg), was identified in the N-terminus of *Phytophthora* effectors such as *P. sojae* Avr1b and *P. infestans* Avr3a [3,4,5,6] and was experimentally shown to be a host-targeting motif that could translocate effectors into host cells [7,8,9]. Recent evidence has shown that *Phytophthora* RXLR effectors could enter host cells without pathogen machinery by binding to the host external lipid phosphatidylinositol-3-phosphate (PI3P), suggesting that RXLR-PI3P-mediated cell-entering mechanism is used by pathogens to deliver...
**Author Summary**

*Phytophthora*, a group of notorious oomycete pathogens, damages a very wide range of crop, vegetable, pasture and horticultural plants, generating great losses to agricultural production annually. Disease outcomes between plants and *Phytophthora* pathogens often depend on whether plants carry resistance (R) gene-encoded receptors than recognize the presence of pathogen avirulence (Avr) effectors. Previous studies identified a conserved host-targeting motif, RXLR (arginine, any, leucine, arginine), common to several *Phytophthora* Avr effectors. The genome sequencing of several *Phytophthora* species including *P. infestans* (potato late blight pathogen) and *P. sojae* (soybean root rot disease pathogen), resulted in the identification of a large reservoir of RXLR-carrying effector candidates. In this paper we identified an RXLR-carrying protein from *P. sojae* as Avr effector Avr3b based on genetic mapping, sequence polymorphisms, and transient expression. Avr3b carries a Nudix hydrolase motif at its C-terminus and enhances *Phytophthora* virulence. Biochemical assays revealed that Avr3b is a pyrophosphorylase with ADP-ribose and NADH as its preferred substrates. Furthermore, the enzymatic activity is required for Avr3b to promote virulence but is not required for recognition by Rps3b.

**Results**

**RXLR effector candidates for Avr3b**

Briefly summarized, previous studies have described the following features of Avr effectors [12,13,14]. (1) The transcripts of Avr effectors are detectable in avirulent strains; (2) all of the *Phytophthora* Avr effectors cloned so far possess the RXLR motif; and (3) many Avr proteins are polymorphic. Previous genetic studies also indicated that Avr3b segregates as a single dominant gene [34]. Thus, we hypothesized that Avr3b was likely encoded by an infection-expressed RXLR effector that is polymorphic among *P. sojae* strains. To identify expressed RXLR effector candidates in avirulent strains, we examined transcription of potential RXLR effector genes in *P. sojae* using digital gene expression (DGE), Affymetrix arrays, and expressed sequence tags (ESTs). The DGE method resulted in the identification of 81 expressed RXLR effector genes [35]. A BLAST search in the *P. sojae* EST database identified 16 expressed RXLR effector genes. After the addition of 59 previously identified expressed RXLR effector genes from microarray analysis [12,31], a total of 119 RXLR effectors were detected as expressed in the *P. sojae* Avr3b avirulent strain P6497 (Table S1).

Genomic and mitochondrial DNA RFLP results delineated *P. sojae* isolates P6497, P7064, P7074, and P7076 are representative isolates from each lineage. In addition to the published genome sequence of P6497, the re-sequencing of three *P. sojae* lines (P7064, P7074, and P7076) has recently been completed [31]. Investigation of the 131 expressed effector sequences among these lines revealed that six effectors (Avh20, Avh113, Avh238, Avh258, Avh288, and Avh307) showed a sequence polymorphism pattern consistent with the Avr3b phenotype; namely, identical in P6497 and P7074 (Avr3b avirulent strains) but variant in P7074 and P7076 (virulent strains) (Table 1). Furthermore, Avh6 showed a presence/absence polymorphism between avirulent and virulent strains (Table 1). Thus, seven effectors were selected as Avr3b candidates for further investigation.

**Genetic co-segregation of the Avh307 genotype with the Avr3b phenotype**

To determine whether candidate effectors might match Avr3b, we conducted genetic mapping to identify effectors that co-segregated with the Avr3b avirulence phenotype. First, we crossed P6497 and P7076, and identified 3 F$_1$ individuals. All the 3 F$_1$ individuals showed avirulence on Rps3b soybeans. Then, one F$_1$ individual was selected for self-crossing, resulting in a total of 71 F$_2$ individuals (*P. sojae* is diploid and homothallic, and wild-type strains show low levels of heterozygosity, hence segregation for most loci occurs in the F$_2$ generation). Avrulence assays of these progeny on soybean plants carrying resistance gene Rps3b U-box E3 ligase CMPG1, preventing it from being degraded by the proteasome system and subsequently blocking triggered cell death triggered by INF1 [33]. However, the virulence mechanism of most oomycete and fungal Avr effectors remains to be further explored.

Here, we identify a new oomycete Avr effector, Avr3b, from *P. sojae* that is recognized by soybean plants containing the *R* gene Rps3b. Biochemical assays validate the bioinformatic prediction that Avr3b has ADP-ribose/NADH pyrophosphorylase activity. Avr3b contributes to virulence through suppression of plant immunity and that suppression is dependent on enzyme activity. However, the enzyme activity of Avr3b is not required for recognition by Rps3b-containing plants.
Table 1. Seven expressed RXLR effectors showed Avr3b polymorphism.

| Name  | DGEa | Affya | ESTa | SP HMM valueb | SP Lengthc | RXLRd | dEERR | P7064d | P7074d | P7076d |
|-------|------|-------|------|--------------|------------|--------|-------|--------|--------|--------|
| Avh6  | +    |       |      | 0.998        | 25         | RFLR   | DNEER | Polymorphic | None   | None   |
| Avh20 | +    |       |      | 1.000        | 21         | RLLR   | DNEER | Identical | Polymorphic | Polymorphic |
| Avh113| +    | +     |      | 1.000        | 20         | RFLR   | EER   | Identical | Polymorphic | Polymorphic |
| Avh323| +    | +     | +    | 0.999        | 19         | RFLR   | DGKTEER | Identical | Polymorphic | Polymorphic |
| Avh258| +    |       |      | 1.000        | 24         | RRLR   | DLTAEEER | Identical | Polymorphic | Polymorphic |
| Avh288| +    | +     | +    | 0.999        | 20         | RRLR   | DLTAEEER | Identical | Polymorphic | Polymorphic |
| Avh307| +    |       |      | 1.000        | 17         | RSLR   | EER   | Identical | Polymorphic | Polymorphic |

a: Identification of expressed RXLR effectors by examination of three kinds of gene expression data: DGE, digital gene expression data generated by Solexa sequencing [33]; Affy, soybean Affymetrix array data [12,31]; and ESTs [61], the expression sequence tags were obtained by BLAST in VBI P. sojae unigenes (VBI microbial database).

b: An "+", as indicated in Table 1, is to indicate that transcripts of the gene were detectable using the method. All these data were derived from P. sojae strain P6497 (Rps3b avirulent). c: SP HMM (signal peptide prediction HMM probability) and SP length (signal peptide) were predicted using SignalP v3.0 (http://www.cbs.dtu.dk/services/SignalP/).

d: RXLR and dEER motifs were previously bioinformatically identified [42].

e: The sequences of RXLR effectors were obtained from three P. sojae lineages (P7064, P7074, and P7076) using 454 genome sequencing assembly (VBI microbial database)[31]; P7064 is an Avr3b avirulent lineage, and P7074 and P7076 are Avr3b virulent lineages. The translated amino acid sequences were compared with RXLR effectors in P6497. Polymorphism results defined as "identical" means that the amino acid sequences were perfectly identical; "none" means that the gene was missing in the genome assembly; and "polymorphic" represents mutations including SNPs, insertion, or deletion.

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Phytophthora Effector Avr3b is an Nudix Hydrolase

Table 1. Seven expressed RXLR effectors showed Avr3b polymorphism.

The proteins encoded by P. sojae Av genes trigger cell death when they are expressed in or enter into soybean cells containing their matching soybean Rps proteins. To test whether Avh307 alleles could trigger cell death in the presence of Rps3b, we conducted a soybean transient expression experiment by co-bombardment of soybean hypocotyls to measure cell death [37]. Plasmid constructs carrying Avh307 alleles encoding mature proteins without native signal peptides were delivered by particle bombardment into soybean hypocotyls, along with a beta-glucuronidase (GUS) reporter gene to measure cell survival. The Avh307P7076 allele triggered cell death in the presence of Rps3b, but not in the absence of Rps3b, as evidenced by a strong reduction in GUS staining (Figure 1C). In contrast, expression of Avh307P7076 did not reduce GUS staining in the presence of the Rps3b gene, nor in its absence (Figure 1D). Further confirmation of our results, we performed a double-barreled bombardment which could compare cell death in a more direct fashion [30]. These data confirmed that expression of the Avh307P7076 allele strongly triggered cell death on Rps3b soybeans, but no reaction on near-isogenic soybean lines that do not contain Rps3b (Figure S1). Avh307P7076, the virulence allele, did not induce significant cell death on Rps3b plants. Thus, both kinds of co-bombardment assays provided functional evidence that Avh307 is Avr3b, and that Avh307P7074 and Avh307P7076 are avirulence and virulence alleles, respectively, of Avh3b.
Phytophthora Effector Avr3b is an Nudix Hydrolase

A

B

C

D

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The Avr3b gene is expressed during infection and shows transcriptional polymorphisms between virulent and avirulent strains

To explore how Avr3b may be involved in P. sojae-soybean interactions, we examined the Avr3b transcriptional profile at different stages of infection and among different isolates. To define the transcriptional pattern of Avr3b, we isolated total RNA from mycelia, zoospores, cysts, germinating cysts, and from infected susceptible soybean leaves at 6 hours post-infection (hpi), 12 hpi, and 24 hpi. Real-time RT-PCR data indicated that Avr3b transcript levels are significantly elevated during infection (Figure 2A). Compared with in vitro grown mycelia, the Avr3b transcript level was elevated 20-fold, 150-fold, and 50-fold in the cyst, germinating cyst, and 24-hpi stages, respectively. These data indicate that Avr3b is strongly induced in pre-infection structures and during infection.

P. sojae Avr effector genes Avr1b, Avr1a, and Avr3a all showed transcriptional polymorphisms among P. sojae strains [12,13,14]. To test for transcriptional polymorphisms of Avr3b, we compared Avr3b transcript levels in the germinating cyst and 24-hpi stages between P. sojae isolates P6497 (avirulent) and P7076 (virulent) using real-time RT-PCR. In P6497 compared to P7076, Avr3b transcript levels were five times higher at the germination cyst stage and three times higher at the 24-hpi stage (Figure 2B), indicating that Avr3b exhibits significant transcriptional polymorphism. However, despite the relatively lower expression level, transcripts of the Avr3b virulence allele remain detectable, indicating that it might be required during infection.

Avr3b enhances Phytophthora virulence on N. benthamiana and decreases ROS accumulation

Several oomycete avirulence effectors can suppress aspects of plant immunity [8,32,38]. To determine whether Avr3b interferes with plant immunity, we transiently expressed recombinant Avr3bP6497 (without a signal peptide) fused with a FLAG tag in N. benthamiana using agroinfiltration with a potato virus X (PVX) vector. Total protein samples were isolated from agroinfiltrated plant leaves and subjected to western blot analysis, employing an anti-FLAG monoclonal antibody as a probe. The results show expression of a protein of the expected size (34-kDa) in the tissue infiltrated with the Agrobacterium cells carrying the FLAG:Avr3bP6497 construct (Figure 3A), consistent with expression of FLAG:Avr3bP6497 in N. benthamiana. Two Phytophthora pathogens of N. benthamiana, P. capsici and P. parasitica, were used to challenge the infiltrated tissue. After 48 hours following Agrobacterium infiltration, we inoculated the infiltrated regions with an agar plug (5×5 mm) containing freshly grown P. capsici and P. parasitica mycelia, then evaluated disease development. On leaves infiltrated with strains carrying a control gene (GFP), the diameter of the disease lesions was approximately 0.7 to 0.8 cm at 36 hpi; however, on leaves infiltrated with strains carrying Avr3bP6497 the lesion diameter expanded to 1.3 to 1.4 cm, as shown in Figure S2. To more precisely measure P. capsici infection, we measured the ratio of P. capsici DNA to N. benthamiana DNA using real-time PCR to determine the Phytophthora biomass in the infected plant tissues (Figure 3B). The data show that the P. capsici/N. benthamiana biomass ratio was significantly higher in Avr3bP6497-infiltrated leaves (38%) than in GFP leaves (24%) (P value < 0.01). Both the lesion size data and the biomass data suggest that Avr3b expression in planta enhanced the susceptibility of N. benthamiana to Phytophthora.

To explore possible mechanisms behind the increased Phytophthora susceptibility, trypan blue and diaminobenzidine (DAB) staining were performed to examine infected plant tissues for the distribution of hyphae and for ROS production, respectively. At 16 hpi, a higher density of infected mycelium was observed in Avr3bP6497-transformed tissues (Figure 3C), consistent with the real-time PCR data showing greater P. capsici biomass in Avr3bP6497-expressing tissues. Furthermore sporangia had formed within the infected regions of Avr3bP6497-expressing leaves, whereas none were found within the infected regions of GFP-expressing leaves (Figure 3C). In contrast, there was more extensive trypan blue staining of plant cells in infected tissues expressing the GFP control than in those expressing Avr3bP6497, suggesting that more defense-related cell death may have occurred in those tissues. Substantially less DAB staining of 16-hpi plant tissues was observed in infected regions of Avr3bP6497-expressing leaves compared to the GFP control, suggesting that ROS accumulation in response to infection was significantly reduced in the Avr3bP6497-expressing tissue (Figure 3D). Overall, transient expression of Avr3bP6497 in N. benthamiana leaf tissue resulted in a greater degree of Phytophthora infection with less ROS accumulation and less cell death, compared to the GFP control.

Avr3b-like RXLR-Nudix proteins are conserved in Phytophthora species, but not in other oomycetes

To examine the distribution of Avr3b homologs in other oomycete species, we performed a BLAST similarity search in several oomycete genome databases. A search of the P. sojae genome identified another 12 predicted genes that had the Nudix motif. A total of 15 Nudix hydrolases were found in the P. infestans genome; four of these hydrolases (PTTG00584, PTTG06308, PTTG15679, and PTTG15732) had a signal peptide and RXLR motif. The P. ramorum genome encoded a total of 14 Nudix hydrolases; three (PraAvh165, PraAvh268, and PraAvh281) were RXLR effectors. Only one RXLR Nudix hydrolase (ID: 102433) was defined in the JGI P. capsici genome. We also searched other plant and animal oomycete databases, including H. arabidopsidis, Pythium ultimum, and Saprolegnia parasitica without finding any predicted RXLR Nudix hydrolase effectors, suggesting that this gene family might be present only in the Phytophthora genus. Thus,
an Avr3b family composed of nine members from four Phytophthora species was identified (Table S4).

Sequence alignment of Avr3b family members plus two characterized Nudix hydrolases (AtNUDT7 from Arabidopsis and ScYSA1 from Saccharomyces cerevisiae) revealed that all of these sequences contain the conserved residues of the Nudix hydrolase motif (GX5EX7REUXEEXGU) (Figure 4A). The Nudix motif is located at the C-terminus of each of the oomycete effectors. The motif is located at the extreme C-terminus in many family members including P. capsici Pc102433, P. infestans PITG05846, PITG06308, PITG15679, and the three P. ramorum Nudix hydrolase effectors, a pattern similar to that of Avr3bP7076 (Figure S3). However, in Avr3bP6497 and PITG15732 the motif is closer to the middle of the protein, which is more similar to AtNUDT7 and ScYSA1.

Avr3b has ADP-ribose/NADH pyrophosphorylase activity

To examine whether Avr3b has Nudix hydrolase activity, and to identify possible substrates for it, we expressed FLAG:Avr3bP6497 (with the FLAG tag fused to the Avr3b N-terminus) in N. benthamiana (attempts to produce active protein in E. coli were unsuccessful). Western blot data showed a clear 34–35 kDa band which suggested the recombinant protein was expressed. As a positive control, we fused AtNUDT7 from Arabidopsis with a FLAG tag. We then conducted immuno-precipitation using anti-FLAG M2 affinity gel. After immuno-precipitation, western blotting of the elution samples showed clear signals, indicating that, Avr3b protein had been recovered (Figure 4B). To test the hydrolase activity of the precipitated FLAG:Avr3bP6497 protein and its possible substrates, relative hydrolase activity was measured as a ratio of FLAG:Avr3bP6497 samples over immuno-precipitated GFP, using a series of nucleotide derivatives as substrates. The results showed that FLAG:AtNUDT7 showed higher activity than Avr3bP6497. To confirm Avr3bP6497 that is a nudix hydrolase, and to validate that the hydrolase activity in the immunoprecipitates was due to Avr3b, we mutated Avr3bP6497 at four conserved residues (R220Q, E221Q, E225Q, E226Q simultaneously, Figure 4A) to generate a non-functional Nudix mutant [39], called Avr3bQQQQ. The hydrolase activity of

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**Figure 2. Avr3b is induced during infection and shows transcriptional polymorphisms.** (A) Avr3b transcript levels in different developmental and infection stages measured by real-time RT-PCR. Total RNA was extracted from mycelia (MY), zoospores (ZO), cysts (CY), germinating cysts (GC), and infected soybean leaves at 6 hours post-infection (hpi), 12 hpi, and 24 hpi. All inoculations for RNA isolation were performed on susceptible soybean cultivar Williams. Real-time RT-PCR analysis employed primers specific for Avr3b and the actin gene. Transcript level represent the P. sojae Avr3b mRNA levels compared with actin mRNA levels. Bars represent standard errors from 2 independent replicates each. (B) Avr3b transcriptional polymorphism between virulent (P7076) and avirulent (P6497) strains. Real-time RT-PCR analysis was conducted to compare Avr3b transcript levels at two infection-related stages, germinating cysts (GC) and infected leaves (24 hpi). Primers used in this assay amplified identical regions from Avr3bP6497 and Avr3bP7076. Bars represent standard errors from 2 independent replicates each.

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Phytophthora Effector Avr3b is an Nudix Hydrolase

A

α-FLAG

α-GFP

Ponceau S

34 kD

B

P. capsici/N. benthamiana biomass index

FLAG:Avr3b^P6497

GFP

C

GFP

FLAG:Avr3b^P6497

IS

SP

D

12 hpi

36 hpi

FLAG:Avr3b^P6497

GFP
immunoprecipitated Avr3bP6497 was significantly weaker than Avr3bP7076, on either ADPR or NADH, and not significantly greater than the GFP control (Figure 4C). Besides ADPR and NADH, other nucleotide derivatives including NADPH, NAD, Ap4A, FAD and Coenzyme A were also examined as possible substrates for Avr3bP6497. The Avr3bP6497 immunoprecipitate caused weak but significant hydrolysis of all these nucleotides except NAD, whereas there was no significant hydrolysis by the Avr3bQQQQ immunoprecipitate, except possibly of NADPH (Table S5). In our assay, the hydrolase data from AtNUDT7 (Figure 4C), a positive control, is consistent with previous reports [40]. Together, these data are consistent with the hypothesis that Avr3bP6497 contains ADP-ribose and NADH pyrophosphorylase activity.

To measure the elevation of total nuclear hydrolase activity in the plant tissue resulting from Avr3bP6497 expression, we examined the hydrolase activity of total plant extracts. Avr3bP6497, Avr3bQQQQ, GFP and AtNUDT7 were transiently expressed in N. benthamiana by using Agrobacterium infiltration. Western blotting of total plant extracts confirmed expression of the proteins (Figure 4D). Consistent with the results from the immunoprecipitated protein, the extracts from Avr3bP6497-expressing plant tissue showed significantly elevated hydrolase activity with NADH and ADPR than the control whereas expression of Avr3bQQQQ did not significantly elevate the hydrolase activity (Figure 4E). No significant elevation of hydrolase activity against NADPH, NAD, Ap4A, FAD or Coenzyme A could be detected in total extracts from Avr3bP6497- and Avr3bQQQQ-expressing tissue (Table S5). Thus, in planta expression of Avr3bP6497 elevated the ADP-ribose/NADH pyrophosphorylase activity in the plant tissue by approximately two-fold.

Avr3b virulence functions depend on its nudix motif, but its avirulence activity does not

To examine the relationship between Avr3b’s Nudix motif and its virulence and avirulence activities, we fused a FLAG tag to the N-terminus of Avr3bP6497, Avr3bQQQQ, Avr3bP7076 (virulence allele), Avr3b16-174 (N-terminus of Avr3bP6497), and Avr3b170-315 (C-terminal of Avr3bP6497) (Figure 5A). These proteins were then transiently expressed in N. benthamiana leaves using agroinfiltration. Western blots confirmed that all of the proteins were expressed in planta with the expected sizes (Figure 3B).

Next, N. benthamiana leaves expressing each protein were challenged with P. capsici and then stained with DAB to test for suppression of ROS production. Expression of Avr3bP6497, Avr3bP7076, and Avr3b170-315 all noticeably reduced ROS generation, whereas, expression of Avr3bQQQQ and Avr3b16-174 failed to do so, similar to expression of the GFP control (Figure 5C).

To measure the elevation in hydrolase activity caused by expression of the Avr3b proteins, total proteins from leaves expressing each construct were assayed with ADP-ribose and NADH as substrates. Protein extracts from leaf tissue expressing Avr3bP6497, Avr3bP7076, and Avr3b170-315 exhibited significantly elevated ADP-ribose and NADH pyrophosphorylase activity whereas leaves expressing Avr3b16-174 did not (Figure 5D, 5E). Avr3bQQQQ expression resulted in no significant elevation in ADP-ribose pyrophosphorylase activity, and a weak but not statistically significant elevation in NADH pyrophosphorylase activity (Figure 5D, 5E).

To determine the effect of each mutant on susceptibility to P. capsici infection, real-time PCR was used to quantify the pathogen biomass in infected plant tissues expressing each construct. The pathogen biomass was significantly higher in tissues expressing Avr3bP6497, Avr3bP7076, and Avr3b170-315 (p < 0.01) than in tissues expressing the GFP control, but was not significantly higher in tissues expressing Avr3b16-174 or Avr3bQQQQ (p > 0.05) (Figure 5F).

To quantitate the avirulence activity of each Avr3b allele and mutant, the double-barreled version of the co-bombardment assay was used. This assay allows precise quantification of cell death triggered by a construct by comparing the number of blue spots on one side of a leaf with the number produced by a parallel empty vector bombardment on the other side of the leaf [29]. Consistent with the qualitative assay results shown in Figures 1D and S1, Avr3bP6497, but not Avr3bP7076, significantly reduced the cell survival ratio to around 40% on leaves containing Rps3b, whereas no significant effector-triggered cell death was observed in control leaves lacking Rps3b (Figure 5G). Transient expression of Avr3bP6497 and Avr3b170-315 also reduced the cell survival ratio to around 40% in leaves with Rps3b but not in those without Rps3b. On the other hand, Avr3b16-174 did not trigger significant Rps3b-dependent cell death (Figure 5G). Therefore the avirulence activity of Avr3b (i.e. triggering of Rps3b-dependent cell death) required the C-terminal half of the protein, containing the Nudix motif, but did not require the Nudix motif to be intact.

Transient silencing of Avr3bP6497 impaired P. sojae virulence on susceptible soybean

To more directly test the contribution of Avr3b, to P. sojae virulence, transient silencing of Avr3b expression in P. sojae was carried out. In vitro synthesized Avr3bP6497 dsRNA was introduced into P. sojae P6497, and 16 lines were recovered. Avr3b transcript levels were determined in germinating cysts from two biological replicates of each line using Real-Time PCR. In five lines (T5, T7, T9, T10, T13) levels of the Avr3bP6497 transcript were significantly reduced (14%~29%) compared to P6497 (Figure 6A). In five non-silenced lines (T1, T2, T3, T12, T16) levels of the Avr3bP6497 transcript were consistently similar to P6497. In six lines, Avr3b transcript levels were not consistent between the two biological replicates; these lines were not examined further.

Zooploic inoculation of ciliated soybean hypocotyls was performed on susceptible soybean cultivar Williams. The disease
Figure 4. Avr3b is an ADP-ribose/NADH pyrophosphorylase. (A) Sequence alignment of the Nudix motif region from Avr3b homologs and characterized nudix hydrolases. Pc102433 is a predicted P. capsici RXLR effector, PITG05846, PITG06308, PITG15679, and PITG15732 are predicted P. infestans RXLR effectors. PrAvh165, PrAvh268, and PrAvh281 are P. ramorum RXLR effectors. AtNUDT7 is an ADP-ribose/NADH pyrophosphorylase from Arabidopsis. ScYSA1 is an ADP-ribose pyrophosphorylase from Saccharomyces cerevisiae. The black line refers to the Nudix hydrolase motif "GSXE7XREUXEEXGU." The threshold for amino acid shading in this alignment is 70%. Avr3bQQQQ is an Avr3b non-functional mutant; the glutamine (Q) substitutions in the nudix hydrolase motif are marked. (B) Western blot of purified Avr3b and control proteins. Immuno-precipitated recombinant proteins FLAG:AtNUDT7, FLAG:GFP, FLAG:Avr3bP6497 and predicted non-functional Avr3b mutant FLAG:Avr3bQQQQ were detected with anti-FLAG antibody. (C) Enzyme assays of purified Avr3b and control proteins. The hydrolase activities of FLAG:AtNUDT7, FLAG:Avr3bP6497 and FLAG:Avr3bQQQQ were measured separately with FLAG:GFP as a control for each assay. The relative hydrolase activity was calculated as a ratio of Avr3b/AtNUDT7 over GFP. Bars represent standard errors from 6 independent replicates each. Student’s t-test was used to compare whether testing sample activities are significantly different from the GFP control. The double asterisk and single asterisk indicates statistical significance p < 0.01 and p < 0.05, respectively. The letters represent statistical significance (P < 0.01) as measured by Duncan’s multiple range test. (D) Western blot of the total plant extract from the Agrobacterium infiltrated region. The supernatants from FLAG:AtNUDT7, FLAG:GFP, FLAG:Avr3bP6497 and FLAG:Avr3bQQQQ transgenic N. benthamiana leaves were electrophoresed and detected with anti-FLAG antibody. (E) Enzyme assays of the total protein extracts from the Agrobacterium infiltrated region. The relative hydrolase activities were measured as a ratio to extracts from a GFP-expression control. Bars represent standard errors from 4 independent replicates each. Student’s t-test was used to compare whether testing sample activities are significantly different from the GFP control. The double asterisk and single asterisk indicates statistical significance p < 0.01 and p < 0.05, respectively. The letters represent statistical significance (P < 0.01) as measured by Duncan’s multiple range test. doi:10.1371/journal.ppat.1002353.g004
lesion length was measured to quantify the virulence of Avr3b-silenced lines at 36 hpi, as shown in Figure 6B. The lesion lengths generated by the Avr3b-silenced lines were significantly smaller than those of the non-silenced lines and of P6497 (P < 0.01). Thus, the silencing of Avr3b expression in P6497 significantly impaired virulence.

The nudix motif is required for Avr3b\textsuperscript{P6497} to suppress ETI

To further elucidate the virulence mechanism of Avr3b, we tested if Avr3b could suppress ETI in soybean using a double-barreled bombardment protocol [31]. This experiment involves measuring ETI-associated cell death triggered by P. sojae effector Avr1b in the presence of soybean R gene Rps1b, in the presence or absence of Avr3b. The side-by-side comparison of cell death triggered by co-bombardment of Avr1b alone compared to Avr1b+Avr3b\textsuperscript{P6497} on Rps1b soybean indicated that Avr1b-triggered cell death was strongly suppressed in the presence of Avr3b\textsuperscript{P6497} as shown in Figure 7A.

To quantify suppression, and to determine whether the Nudix motif is required for suppression, an indirect and a direct assay were carried out (Figure 7B). For the indirect assay, the number of GUS-expressing spots surviving in the presence of Avr1b on Rps1b soybean was measured relative to a parallel GUS-only control in the presence or absence of Avr3b\textsuperscript{P6497} or Avr3b\textsuperscript{QDEQQ}. For the direct assay one barrel delivered DNA encoding Avr1b + Avr3b\textsuperscript{P6497} or Avr3b\textsuperscript{QDEQQ}, and the other barrel delivered DNA encoding Avr1b alone. The results of both the direct and the indirect assays showed that the survival of GUS-spots from Avr1b + Avr3b\textsuperscript{P6497} was significantly higher than from Avr1b alone, confirming that Avr3b could suppress Avr1b-triggered cell death. The survival of GUS-spots from Avr1b + Avr3b\textsuperscript{QDEQQ} was significantly less than from Avr1b alone and not significantly higher than from Avr1b alone indicating that Avr3b\textsuperscript{P6497} suppression of Avr1b mediated cell death is dependent on the nudix motif activity.

Discussion

Oomycete and fungal avirulence effectors form a highly diverse class of proteins, with few if any common sequence signatures. Therefore, the cloning of most oomycete and fungal Av effectors has relied heavily on genetic mapping and/or genomic subtraction techniques. The discovery of the RXLR host-targeting signal motif in oomycetes has provided a rapid way to identify new Av effectors in conjunction with mapping or screening strategies. For instance, RXLR effector prediction and high-throughput functional screening aided in the discovery of the P. infestans genes Avr3a, Avr-blb1, and Avr-blb2 [11,16]. By combining RXLR effector prediction with transcriptional patterns, two RXLR effectors encoded by the P. sojae avirulence genes Avr3a and Avr3c were identified [12,14]. Here, in addition to RXLR effector prediction and transcriptional data, sequence polymorphism analysis [41] was also used for the discovery of the P. sojae Av effector Avr3b. This powerful combination of methods could significantly accelerate the identification of additional oomycete and fungal avirulence effectors.

The avirulence allele of Avr3b, Avr3b\textsuperscript{P6497}, encodes a protein with an intact RXLR motif, a W-motif [5,30], a Nudix hydrolase motif and two cysteine residues. In the virulence allele, Avr3b\textsuperscript{P7076}, a premature stop codon creates a 90-amino-acid C-terminal deletion that removes one cysteine and places the Nudix motif at the extreme C-terminus of the protein. In the non-deleted region, sequence variation results in 46 amino acid substitutions and two deletions, making Avr3b the most polymorphic Av effector among all reported P. sojae Av genes [12,13,14,15]. Transcripts from Avr3b\textsuperscript{P7076} accumulate at a significantly lower level than from Avr3b\textsuperscript{P6497}. However, it is unlikely that Avr3b\textsuperscript{P7076} is a pseudogene because transcripts for this gene are detectable and in planta transient expression of Avr3b\textsuperscript{P7076} resulted in pyrophosphorylase enzyme activity and suppression of plant immunity.

Most RXLR effectors in Phytophthora have diverged in sequence to such a degree that the identification of orthologous effectors across species is often difficult or impossible. This is likely a result of the rapid diversification of RXLR effectors caused by the evolutionary host-pathogen arms race [3,6,18,23,42]. However, P. sojae Avr3b has one ortholog in P. capsici, three in P. ramorum, and four in P. infestans, forming an Avr3b-like Nudix RXLR effector family. Sequence alignment of the Avr3b family members shows that most of the sequence similarity occurs in the C-terminal region of the Nudix hydrolase motif (Figure S3). The key residues for Nudix hydrolase activity are conserved, consistent with enzyme activity being required for normal function. Avr3b from P. sojae has two cysteine residues, a feature that is uncommon in intracellular RXLR effectors but is not unusual for apoplastic effectors from oomycetes or fungi. However the positions of the cysteine residues are not conserved in other Avr3b-like effectors. Whether these cysteine residues are required for avirulence or virulence activities needs further investigation. With regard to expression of the Avr3b orthologs, microarray data shows that two P. infestans Avr3b-like effectors PITG06308, PITG15679 are up-regulated by 2.5 and 2.2 fold at 2 dpi, compared to mycelium stages, respectively [23], suggesting Avr3b-like effectors might also function during P. infestans infection. The orthologs’ conservation among Phytophthora species suggests that they that provide a common virulence mechanism. However, this remains to be tested.

Recently evidence has shown that P. sojae, P. infestans, P. capsici, M. oryzae, M. lini and other pathogen effectors can enter inside plant cells, presumably to promote virulence [7,8,9,10,43,44].
Figure 6. Transient silencing of Avr3b impaired P. sojae virulence on soybean. In vitro synthesized Avr3b dsRNA was introduced into P. sojae P6497, generating 16 lines. Avr3b transient transformants and recipient strain P6497 were examined for Avr3b transcript levels and virulence. RNA samples were extracted at the germinating cyst stage. (A) Avr3b transcript levels were determined by Real-time PCR using P. sojae actin transcripts as a reference and then normalized to the wildtype (P6497). Bars represent standard errors from 2 independent RNA isolation and Real-time PCR replicates. Letters indicated above each column represent statistical significance based on Duncan's multiple range test ($p < 0.01$). (B) The disease lesion lengths in etiolated soybean hypocotyls (susceptible cultivar Williams) infected with zoospores of Avr3b transient transformants and P6497 at 36 hpi. Means and standard errors from at least 10 measurements are shown. Two statistical tests was used. The letters indicated above each column represent statistical significance based on Duncan's multiple range test ($p < 0.01$). The asterisk indicates statistical significance based on student's t-test ($p < 0.01$).

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Figure 7. Avr3b suppresses ETI triggered by P. sojae effector Avr1b. Suppression of Avr1b-triggered PCD in soybean cultivar L77–1863 (Rps1b) by Avr3bP6497 and Avr3bQQQQ measured by indirect and direct double barrel particle bombardment assays. (A) Side-by-side comparison of cell death triggered by co-bombardment of Avr1b alone compared to Avr1b+Avr3bP6497 on Rps1b soybean. Numbers in parenthesis indicate total GUS positive spots counted for each treatment on the leaf shown. Co-bombardments occur horizontally and are separated by dashed lines. Replicate in the presence (light grey bars) or absence (white bars) of Avr3bP6497 or Avr3bQQQQ. For the direct assay the number of GUS-expressing spots surviving in the presence of Avr1b on Rps1b soybean, was measured relative to a parallel GUS-only control +EV (423) and GUS+Avr3bP6497 (178). For the indirect assay the number of GUS-expressing spots surviving in the presence of Avr1b+Avr3bP6497 on Rps1b soybean, was measured relative to a parallel GUS-only control +EV (196) and GUS+Avr3bP6497 (438). The direct ratio was then multiplied by the cell survival in the presence of Avr1b alone on Rps1b Soybean (white bars) to enable comparison to the results of the indirect assays. Based on the Wilcoxon signed ranks test (direct assays) or the Wilcoxon rank sum test (indirect assays and comparisons of two direct assays), outcomes that were significantly different p < 0.001 are marked with different letters.

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Figure 7. Avr3b suppresses ETI triggered by P. sojae effector Avr1b. Suppression of Avr1b-triggered PCD in soybean cultivar L77–1863 (Rps1b) by Avr3bP6497 and Avr3bQQQQ measured by indirect and direct double barrel particle bombardment assays. (A) Side-by-side comparison of cell death triggered by co-bombardment of Avr1b alone compared to Avr1b+Avr3bP6497 on Rps1b soybean. Numbers in parenthesis indicate total GUS positive spots counted for each treatment on the leaf shown. Co-bombardments occur horizontally and are separated by dashed lines. Replicate bombardments are separated by the solid black line. (B) Quantitation of suppression by indirect and direct co-bombardment assays. For the indirect assay the number of GUS-expressing spots surviving in the presence of Avr1b on Rps1b soybean, was measured relative to a parallel GUS-only control +EV (423) and GUS+Avr3bP6497 (178). For the direct assay the number of GUS-expressing spots surviving in the presence of Avr1b+Avr3bP6497 on Rps1b soybean, was measured relative to a parallel GUS-only control +EV (196) and GUS+Avr3bP6497 (438). The direct ratio was then multiplied by the cell survival in the presence of Avr1b alone on Rps1b Soybean (white bars) to enable comparison to the results of the indirect assays. Based on the Wilcoxon signed ranks test (direct assays) or the Wilcoxon rank sum test (indirect assays and comparisons of two direct assays), outcomes that were significantly different p < 0.001 are marked with different letters.

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Discrete targeting motifs have been identified in these effectors that are required for their translocation into host cells [10,43,45,46]. Like previously reported oomycete avirulence effectors [3,11,12,14,15,16,17,18,20], Avr3b has a signal peptide leader and an RXLR motif at its N-terminus. In our assays, expression of Avr3b in soybean or N. benthamiana cells without its signal peptide resulted in avirulence and virulence activities, suggesting that Avr3b normally acts inside plant cells; it should be able to enter into plant cells during infection. The RXLR motif was also conserved in the Avr3b-like Nudix RXLR effector family. Except for PrAvh268 from P. infestans (RFLR) and PrAvh268 from P. ramorum (RSLH), all the other Avr3b-like Nudix RXLR effectors have the sequence “RSLR” (Figure S3). The dEER motif that is associated with the RXLR motif was not as highly conserved as the RXLR motif in the Avr3b family. We failed to identify any Avr3b-like Nudix RXLR effectors in the genome of H. arabidopsidis, which has a reduced set of RXLR effectors [22] or in the genome of P. ultimum, which has no other RXLR effector genes [47]. Sequencing of additional oomycete pathogen genomes will be required to confirm whether this family is only present in Phytophthora species.

Nudix hydrolases are a family of pyrophosphatases containing the highly conserved Nudix motif GX5EX7REUXEEXGU. The family is widely distributed in many organisms, including viruses, bacteria, archaea, and eukaryotes [39]. Nudix hydrolases catalyze the hydrolysis of a variety of nucleoside diphosphate derivatives linked to a second moiety with varying degrees of specificity [48]. The substrates of Nudix hydrolases identified so far include di- and triphosphates and their oxidized forms, dinucleoside polyphosphates, nucleotide sugars, NADH, coenzyme A, and the mRNA cap [39,48]. In the genome of the model plant Arabidopsis, 29 putative Nudix hydrolases have been identified, and the substrates of a few Arabidopsis Nudix hydrolases have been characterized [49]. Like Avr3b, the Arabidopsis Nudix hydrolases AtNUDT2, AtNUDT7, and AtNUDT6 use both ADP-ribose and NADH as substrates [40,50]. Interestingly, among these hydrolases, AtNUDT7 was reported to be a pathogen-responsive gene whose induction depends on plant defense regulatory genes EDS1 and PAD4 [51]. Furthermore, AtNUDT7 knockout mutants showed enhanced resistance against Pseudomonas syringae and H. arabidopsidis, suggesting that ADP-ribose/NADH pyrophosphatases may act as negative regulators of plant immunity to pathogenic bacteria and oomycetes [51]. Therefore, it is reasonable to suppose that pathogen effectors like Avr3b might mimic negative regulators of plant immunity such as AtNUDT 7 to repress plant defense.

Recently, a few putative type-three secretion system (TTSS) effectors with the Nudix motif were identified from the plant pathogenic bacteriumRalstonia solanacearum [52], suggesting that this pathogen might also translocate Nudix proteins into plant cells as virulence factors. However, some TTSS effectors were not tested for enzyme activity nor was their contribution to virulence measured. Recombinant Avr3b expressed in planta elevated NADH and ADP-ribose pyrophosphatase activities in plant extracts, converting NADH to a reduced form of nicotinamide mononucleotide (NMNH) plus AMP, and ADP-ribose to AMP plus ribose 5-P. The NADH and ADP-ribose pyrophosphatase activity co-purified with Avr3b protein in immunoprecipitation experiments, and the activity was not present when the nudix motif mutant Avr3bP8882 was expressed. Therefore, it is very likely that Avr3b protein has NADH and ADP-ribose pyrophosphatase activity. However, because Avr3b was not purified to homogeneity, we cannot absolutely rule out an indirect stimulation and co-purification of a plant Nudix hydrolase with wildtype Avr3b in these experiments. NADH/NAD+ turnover plays an important role in maintaining the ROS balance. Recent reports have shown that AtNUDT7 and AtNUDT2 modulate redox homeostasis in response to biotic or abiotic stress by nucleotide recycling from free ADP-ribose molecules [40,53,54,55]. Thus, we
hypothesize that inside plant cells Avr3b can suppress pathogen-triggered ROS accumulation by reducing NADH content and recycling nucleotides from ADP-ribose. However, these mechanisms remain to be further investigated.

Rps3b recognizes the presence of Avr3b through the Avr3b C-terminal region (170 to 315 aa) but not the N-terminal region (16 to 174 aa) (Figure 3G). This is consistent with previous reports indicating that the C-terminal sequences of most P. sojae, P. infestans and H. arabidopsidis avirulence effectors show signs of positive selection [11,30,31,36]. In contrast to other avirulence effectors, the Avr3b^P6497 C-terminal region contains an identifiable enzyme domain, a Nudix hydrolase domain. In this study, we generated a mutant version of the protein, Avr3b^QQQQ, that apparently lacks Nudix enzyme activity but nonetheless could still trigger a defense response in the presence of Rps3b. This result suggests that Rps3b recognition might not depend on Nudix hydrolase activity. However, Avr3b^QQQQ retains residual hydrolase activity. Thus we can not rule out the possibility that Avr3b avirulence activity also required Nudix activity because the threshold for Avr3b-mediated avirulence activity is likely to be much lower than for its virulence activity. Due to the numerous polymorphic sites present within the Avr3b^170-515 domain, the particular residues responsible for Avr3b-Rps3b recognition cannot readily be discerned. Only Avr3b mutants retaining Avr3b APD-ribose and NADH pyrophosphorylase activity could suppress ETI and increase Phytophthora biomass in infected plant tissue, suggesting that the enzymatic activity is required to promote Phytophthora virulence.

Phytophthora genomes encode hundreds of RXLR effector genes, suggesting many of these effectors might be redundant in function [57]. For examine, either overexpression or silencing of P. sojae Avr gene Avr3a/5 does not significantly effect P. sojae virulence on susceptible soybean [19]. Overexpression of Avr1b in P. sojae makes transformants more aggressive on soybean [30], but the effector is naturally silenced in some isolates [13]. On the other hand silencing of Avr3a impaired P. infestans pathogenicity on N. benthamiana [33], and silencing of Avr172 and Avr238 in P. sojae also impaired virulence [31], suggesting that these effectors are essential virulence factors. In this paper, transient silencing of Avr3b^P6497 compromised the virulence of transformant recipient strains on susceptible soybean cultivar, identifying Avr3b also as an essential virulence factor. This is consistent with our finding that both Avr3b virulence and avirulence alleles are transcribed and both proteins retain Nudix enzyme activity. In conclusion, Avr3b plays dual roles (avirulence and virulence) in the P. sojae - soybean interaction. The virulence allele Avr3b^P7076 may be considered to be a compromise between host recognition pressures and pathogen fitness.

Materials and Methods

Plant and microbial culture, and virulence scoring

Detailed information about the P. sojae strains used in this study is listed in Table S6. The P. capsici (Pc263) and P. parasitica (P24-3) strains were obtained from the Phytophthora species collection at Nanjing Agricultural University. All of these isolates and the P6497 × P7076 F1 progeny and F2 progeny used in this paper were routinely maintained on 2.5% vegetable (V8) juice medium at 25°C in the dark [38]. Phytophthora sojae mycelia, zoospores, and cysts were prepared as previously described [59]. Cysts germinating at 25°C for 6 hours were used for this study. For RNA samples, infection assays with P. sojae were performed by sandwiching P. sojae mycelium between pairs of soybean leaves [60]. The mycelium was removed at 6 hpi, and the infected soybean leaves were collected at 12 hpi and 24 hpi. All of the collected samples were immediately frozen in liquid nitrogen and stored at −80°C until used for RNA isolation.

Soybean (Glycine max) cultivar Williams (Rps3b), Williams isoline L38-1479 (Rps3b), and PX146–36 (Rps3b) from the collections at Nanjing Agricultural University and Northeast Agricultural University (Harbin, China) were used to score the virulence of P. sojae cultures. Williams isoline L77–1963 (Rps3b) was from the collection at the Virginia Bioinformatics Institute. Exiliated soybean seedlings were grown in vermiculite soaked with water at 25°C without light for 4 days before harvest for co-bombardment. For light-grown soybeans, ten soybean seeds were sown in 10 cm pots (a minimum of three pots per isolate) containing a soil (70%) and vermiculite (30%) mix soaked with water. Soybeans were grown in a greenhouse with a 16-hour photoperiod at 25°C. Soybeans were grown for 7 days for virulence assays, or for 13 to 14 days for use in co-bombardment. Phytophthora sojae cultures were grown on 0.9% (w/v) V8 agar plates 5 to 7 days prior to light-grown plant inoculations. The virulence of P. sojae cultures was scored in exactly the same manner as that previously described [14]. A minimum of three independent replicates of the disease assay were performed for each P. sojae culture tested. N. benthamiana plants were grown at 25°C with a 16 hr photoperiod in a styrofoam cups containing disinfected soil. Plants of 5 to 6 weeks old were used for agroinfiltration.

Bioinformatics

In total, 395 predicted RXLR effectors in P. sojae P6497 were previously predicted [42]. For DGE expression data, RNA samples were collected at Nanjing Agricultural University, and sequencing and analysis were performed at Beijing Genomics Institute. Affymetrix microarray data were previously reported [12,31]. All RXLR sequences were used as queries for BLAST searches of the P. sojae EST unigene database [http://vmdvibi.vt.edu] [61], with an E-value cutoff at e^−20. We identified a total of 131 RXLR effectors that are expressed in P. sojae P6497. P. sojae 454 genome sequencing data (P0764, P0774, and P0776) [31] were accessed from Virginia Microbial Database [http://vmdvibi.vt.edu]. Nudix homologs were identified in two ways: genome annotation searching and BLAST searching. The P. sojae, P. capsici and P. ramorum homolog searches were performed at the Joint Genome Institute database [http://genome.jgi-psf.org], the H. arabidopsidis search was performed at Virginia Microbial Database, and the P. infestans search was conducted at the Broad Institute P. infestans database [http://www.broadinstitute.org]. For Pythium ultimum genome searching, the genome sequence was downloaded from the Pythium genome database available at http://pythium. plantbiology.msu.edu and a local BLAST search was conducted. SignalPv3.0 [http://www.cbs.dtu.dk/services/SignalP] was used for secretion signal peptide prediction. Protein domain and motif analyses were conducted using the NCBI conserved domain database [http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml] and Motif Scan [http://mrytics.isb-sib.ch/cgi-bin/motif_scan]. Sequence alignment was performed using BioEdit2005 [http://www. mbio.ncsu.edu/bioedit/bioedit.html]. The Avr3b-like family member sequences have been deposited into NCBI GenBank with accession numbers (Table S7).

Generation of F2 mapping population and scoring of genotypes

F1 hybrids were derived from P6497 (Avr3b) × P7064 (avr3b) crosses. Oospores from F1 hybrids were produced as described [14]. Single germinating oospores were harvested for DNA
soybean transient expression assays

The Avr3b\(^{P6497}\) and Avr3b\(^{P7076}\) genes, encoding regions corresponding to the predicted signal peptide sequences, were amplified using specific primers and cloned into a 35S promoter-derived vector. Constructs for bombardment of \(P. sojae\) hypocotyls were transformed into the Agrobacterium \(\) tumefaciens strain \(\) EHA105. \(\) Double-barreled particle bombardment assays on soybean leaves were performed as previously described [30]. Both hypocotyl co-bombardment and double-barreled particle bombardment were conducted using a Bio-Rad (USA) He/1000 particle delivery system. For each paired shot, the log ratios of the ratio of the spot counts obtained from the \(Rps\) 3b and non-\(Rps\) 3b leaves were then compared using the Wilcoxon rank-sum test.

Virulence assay of Phytophthora on \(N. benthamiana\)

\(\) Agrobacterium-infiltrated \(N. benthamiana\) plants were grown in a greenhouse for 48 hours, and transformed leaves were then detached and maintained on half-strength MS medium in a petri dish. Next, 2.5% V8 juice agar plugs (0.5 x 0.5 cm) infested with fresh \(P. capsici\) or \(P. parasitica\) mycelia were inoculated onto the infiltrated regions. The diameter of the disease lesion was photographed and measured at 36 hpi. Total DNA isolated from \(P. capsici\)-infected regions (2 x 2 cm) was isolated at 36 hpi. Real-time PCR was used to quantify the ratio of host to pathogen ratio DNA sequences, employing primers specific for the \(N. benthamiana\) and \(P. capsici\) housekeeping actin genes (Table S8). Three independent biological replicates were conducted. Diseased plant tissues at 16 hpi were stained by trypan blue and DAB according as described [63]. Quantification DAB was performed by analyzing DAB staining image by a combination of Photoshop and Quantity One. GFP was considered to be 100% standard.

Western blot and immuno-precipitation assays

Total plant protein was isolated as described [63] for Western blot and immuno-precipitation assays. The protein samples were quantified using a BioPhotometer (Eppendorf, Germany). A standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) protocol was used for protein separation. The amount of total protein loaded per gel lane varied from 80 \(\mu\)g to 120 \(\mu\)g depending on each experiment. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes using a semi-
wet apparatus (Bio-Rad) according to the product instructions. Western blotting was performed as a standard protocol. Anti-FLAG monoclonal antibody (Sigma-Aldrich) and anti-mouse IgG peroxidase conjugate (Beyotime Biotech, China) were used as the primary and secondary antibodies. The membrane was treated with Chemiluminescent Peroxidase Substrate-1 (Thermo Scientific Pierce, No. 34080, USA) for 5 min. The membrane was briefly drained and exposed to BioMax (Kodak, USA) light film several times (depending on results) for exposure signal development.

For immuno-precipitation, anti-FLAG M2 affinity gel (Sigma-Aldrich) was used. The gel suspension (40 µL) was transferred into a fresh 1.5 mL test tube and centrifuged in a pre-cooled rotor at 6000 g for 30 s. The resin was washed twice with 1 mL TBS (50 mM Tris HCL, 150 mM NaCl, pH = 7.4), and all washing buffer was removed. Total protein lysate was clarified by centrifugation (10000 g, 2 min) then 1000 µL of the supernatant was added to the washed resin, and the mixture was incubated using a roller shaker for 4 hours at 4°C. The suspension was centrifuged at 6000 g and the supernatant was removed. The remaining resin was washed three times with 1 mL of TBS. A total of 100 µL elution buffer (0.5 M Tris-HCl buffer, 150 ng/µL 3xFLAG, pH = 7.2) was then added to the resin, and the samples were incubated using a roller shaker for 10 min at room temperature. The resin was centrifuged for 30 s at 6000 g, and the supernatants were transferred to fresh tubes previously loaded with 10 µL of 0.5 M Tris HCl and 1.5 M NaCl, pH 7.4. The purified protein samples were used for gel electrophoresis and enzyme activity assay.

**Nudix hydrolase activity assays**

A general method for the Nudix hydrolase activity assay was performed to test for Avr3b enzymatic activity [40,53]; a 50-µL reaction mixture contained 50 mM Tris-HCl, pH 8.5, 5 mM MgCl2, 1 mM dithiothreitol, 2 units of calf alkaline phosphatase, and certain amount of protein samples. Equal amount of protein (5 µg for purified protein, 2 mg for total plant protein extract) was loaded for hydrolase activity assay in each reaction. After incubation for 1 hr at 37°C, the reaction was stopped by adding 150 µL of 1 N H2SO4. For color development, 100 µL of water and 700 µL of a freshly made mixture containing 600 µL of 3.4 mM ammonium molybdate and 100 µL of 570 mM ascorbic acid was used. The reaction tubes were incubated in a 45°C dry bath for 10 min for color development and then cooled on ice. The solutions were measured using the Du-640 spectrophotometer (Beckman, USA) at A502. The reaction without protein was used as a blank control for each substrate. For direct assay of purified proteins, immuno-precipitation eluate was directly added into enzymatic assay reaction mixture.

For hydrolase activity assay of total plant protein extract, two regions per leaf transiently expressing FLAG:GFP and FLAG:Avr3bP6497 were created in N. benthamiana leaves using Agrobacterium infiltration. A total of 0.1 g of GFP- or Avr3b-infiltrated leaf tissue was collected from each leaf. For both kinds of protein preparations, the relative hydrolase enzyme activity was calculated as a ratio of Avr3b extract (A502 reading) over GFP extract (A502 reading). For NADH and ADPR, 17 and 21 biological replicates were performed, respectively. For FAD, Ap4A, NAD and NADPH, 4 biological replicates were conducted.

**In vitro dsRNA synthesis and P. sojae transformation**

Avr3b in vitro dsRNA synthesis was carried out as described [64]. Primers AVH307-T7F/R with T7 promoter sequence added to the 5’ end of both primers (see Table S8) were used to amplify a C-terminal specific Avr3b DNA fragment from the Avr3bP6497 allele. The PCR product was cloned into pMD19 vector (TaKaRa) and sequenced. AVH307-T7F/R primers were also used to generate Avr3b in vitro dsRNA by using the MegaScript RNAi kit (Ambion AM1626). A total of around 200 µg dsRNA was obtained. The transfection was performed as described [30,59] with a few modifications [32]. About 100 µg of Avr3b dsRNA was added into 1 mL MMg solution (0.4 M mannitol, 15 mM MgCl2, 4 mM MES, pH 5.7) containing 5000–10000 P. sojae P6497 protoplasts. After transformation, the regenerated protoplasts were suspended in liquid pea agar (40°C) containing 0.5 M mannitol. The visible colonies could be observed after 36 h incubation at 25°C. A total of 16 single colonies were randomly selected and propagated on V8 agar plates for RNA extraction and virulence assays.

**Supporting Information**

Figure S1 Photographs of soybean leaves after double-barreled co-bombardment, GUS staining, and leaf destaining. Numbers in parenthesis indicate total GUS positive spots counted for each treatment. Numbering indicates the sequential order of co-bombardments on a given leaf. Co-bombardments occur horizontally and are separated by dashed lines. Different co-bombardments are separated by solid black lines. (A) Avr3bP6497 (B) Avr3bP7076 co-bombardeed with empty vector control on Rps3b soybean. (C) Avr3b co-bombardeed with empty vector control on rps3b soybean. (TIF)

Figure S2 P. parasitica and P. capsici lesion sizes on N. benthamiana plants transiently expressing Avr3b. (A) P. parasitica and P. capsici were inoculated onto Avr3b- or GFP-expressing leaves; a photograph was taken at 36 hpi. The white dotted line indicates the disease lesion region. (B) The diameter of the disease lesion was measured and the mean and standard error of at least five independent replicates are shown. (TIF)

Figure S3 Amino acid sequence alignment of the Avr3b family members. The threshold for shading is 70%. Blue box indicates RXLR host targeting motif, green box indicates Nudix hydrolase motif. (TIF)

Table S1 119 expressed P. sojae RXLR effector genes. (DOC)

Table S2 Cleaved Amplified Polymorphic (CAP) markers for Avr3b genotyping. (DOC)

Table S3 RXLR effectors that are located in the Avh307 region. (DOC)

Table S4 Predicted Phytophthora Nudix hydrolase RXLR effectors. (DOC)

Table S5 The hydrolytic activities of both pure Avr3b protein and plant protein extract against nucleotide derivatives. (DOC)

Table S6 List of P. sojae strains used in this study. (DOC)

Table S7 Sequences referred in this paper. (DOC)
Table S8. Primers used in this study. (XLS)

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Author Contributions

Conceived and designed the experiments: SD ZZ DD MG BMT YW. Performed the experiments: SD WY GK XY DQ QC YS. Analyzed the data: SD WY GK XZ BMT YW. Contributed reagents/materials/analysis tools: SD WY GK DQ SK QC ZZ. Wrote the paper: SD MG BMT YW.

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