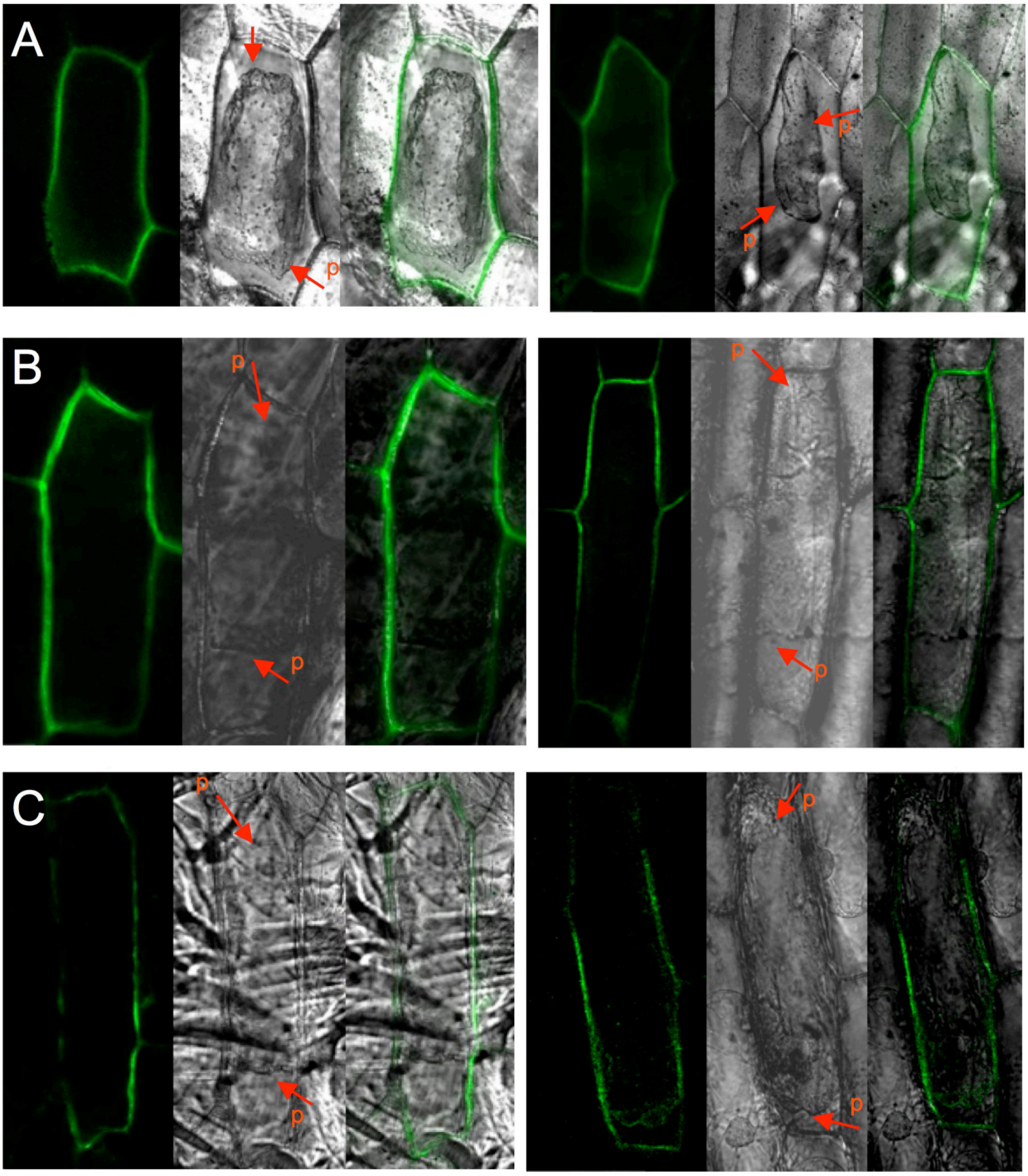
Supplemental Data. Dou et al. (2008) RXLR-mediated entry of *Phytophthora sojae* effector Avr1b into soybean cells does not require pathogen encoded machinery



Supplemental Figure 1. Plasmolyzed onion bulb epidermal cells expressing secreted Avr1b-GFP fusion proteins.

DNA encoding various fusions of Avr1b with *Aequorea coerulescens* green fluorescent protein (GFP) was bombarded into onion epidermal cells. 24 hours later, plasmolysis was performed for 15 min in 0.8 M mannitol.

Each cell is shown three times in adjacent panels: fluorescent image (left), bright-field image (middle) and merged image (right). “p” indicates the plasma membrane in plasmolyzed cells.

(A) Fusion of GFP to the secretory leader of Avr1b alone (sGFP);
(B) GFP fused to Avr1b RXLR2^{AAAA} mutant including its secretory leader;
(C) GFP fused to Avr1b dEER^{A6} mutant including its secretory leader.

No	Name	Applications	Sequence (from 5' to 3')*
1	UF	*Hind* III and *Xma* I sites added to rpL41 promoter to drive G418 resistance gene	ataagcttgaatTCTGGCGTTCATCTCCGACG
2	UR		ttcccgggTGGATGCTCAGATGctagcGTC
3	HamF	*Bremia* Ham34 promoter internal primer	TTCTCCTTTTCACTCTCACG
4	HamR	*Bremia* Ham34 Terminator internal primer	AGACACAAAATCTGCAACTTC
5	Avr1bReF	*Avr1b*-1 internal primers for PCR	ACCTTCAGCGTGACTGACCT
6	Avr1bReR		GCGATTGCCAACCAGTTCT
7	ActinF	*P. sojae Actin* gene internal primers for the reference in RT-PCR	CGACATCCGTAAGGACCTGT
8	ActinR		TTCGAGATCCACATCTGCTG
9	PrimerC	*Kpn* I sites added flanking *Avr1b*-1 for insertion downstream of HAM34 promoter	ggggtaccgacaacaATGCGTCTATCTTTTGTGCT
10	PrimerD		ggggtaccTCAGCTCTGATACCGGTGAA
11	Avr1bF	*Xho* I site and initiation codon added to 5' end of mature *Avr1b*-1 for insertion downstream of CaMV 35S promoter	atcgcactcgagctttcgcagatcccgggggggcaatgagatatgACTGAGTACTCCGACGAA
12	Avr1bR	*Xho* I site to 3' end of *Avr1b*-1 for insertion downstream of CaMV 35S promoter	atcgcactcgagcttgtcgatcgacagatccggtcggcatctactTCAGCTCTGATACCGGTG
13	Avr1bfull_F	Same as Avr1bF but for secretory *Avr1b*-1	atcgcactcgagctttcgcagatcccgggggggcaatgagatATGCGTCTATCTTTTGTG
14	Motif1F	Introduction of RXLR1^{AAAA} mutation, creating a *Pst* I site	TCGTCCGTgctgcagctgctAACGGCGACATTGCCGGTGG
15	Motif1R		TCGCCGTTagcagctgcagcACGGACGAGATCTGGAGATT
16	Motif2F	Introduction of RXLR2^{AAAA} mutation, creating a *Pst* I site	CCGGTGGAgctgcagctgctGCTCATGAAGAGGACGATGC
17	Motif2R		TCATGAGCagcagctgcagcTCCACCGGCAATGTCGCCGT
18	Motif1+2F	Introduction of RXLR1^{AAAA} & RXLR2^{AAAA} mutations, creating 2 *Pst* I sites	gctgcagctgctAACGGCGACATTGCCGGTGGAgctgcagctgctGCTCATGAAGAGGACGATGC
19	Motif1+2R		agcagctgcagcTCCACCGGCAATGTCGCCGTTagcagctgcagcACGGACGAGATCTGGAGATT
20	Motif3F	Introduction of dEER^{A6} mutation, creating a *Pst* I site	gctgcagcagctGCGGGGgctgctACCTTCAGCGTGACTGACCT
21	Motif3R		agcagcCCCCGCagctgctgcagcATGAGCTCGAAGAAATCTTC
22	HpAvh341F	5' flanking *Xma* I site added to Hp Avh341 for insertion downstream of HAM34 promoter	attcccggggacaacaATGCGACTCCACTACGTG
23	HpAvh341R	For fusion with *Avr1b*-1	GTCAGTCACGCTGAAGGTIATCGAGAACGCCATGCCCAT
24	PsAvh171F	5' flanking *Xma* I site added to PsAvh171 for insertion downstream of HAM34 promoter	attcccggggacaacaATGGGCCTCCACAAGGGCT
26	PsAvh171R	For fusion with *Avr1b*-1	GTCAGTCACGCTGAAGGTITAGGTGGTGTAGTCCGAC
26	1bRXLR(-)F	For deletion of N-terminus of *Avr1b*-1	aacccgggacaacaatgACCTTCAGCGTGACTGAC
27	Avr1b_EcoRI	Primers for making moving Avr1b expression cassette into pUC19 vector with *Xma* I and *Kpn* I sites flanking *Avr1b*-1 gene	GGAGgaaTTcGCTGGCTGGTGGCAGGAT
28	Avr1b_HindIII		GTATTGGCTAGAGaAGCTTGCCA
29	Avr1b_genegun_KpnI		AGAAACTCGAGCTTGTCGATCGACAGATCCGGTCGGCAggTACcTCAGCTCTGATAC
30	M2_F1	Replacement of RFLR with mutation encoding RFRL	GAAGATTTCgaCttGCTCATGAAG
31	M2_R1		CTTCATGAGCaaGtcGAAATCTTC
32	M2_F2	Replacement of RFLR with the mutation encoding FRLR	TGCCGGTGGAtttagaCTTCGAGCTC
33	M2_R2		GAGCTCGAAGtctaaaTCCACCGGCA
34	M2_F3	Replacement of RFLR with the	GAAGATTTgcaCGAGCTCAT

35	M2_R3	mutation encoding RFAR	ATGAGCTCGtgcAAATCTTC
36	M2_F4	Replacement of RFLR with the mutation encoding QFLQ	GGTGGAcagTTTCTTCaaGCTCATGAAG
37	M2_R4		AGCttGAAGAAActgTCCACCGGCAATG
38	GFPF	For fusion with *Avr1b*-1 to make *Avr1b*-1-*AcGFP*	CTTTCACCGGTATCAGAGCl<u>ggtaccg</u>cca<u>cc</u>l<u>atg</u>GTGAGCAAG GGCGCCGAG
39	GFPR	Addition of *Kpn* I after stop codon of GFP	AA<u>GgtaCC</u>tcaCTTGTACAGCTCATCCAT
40	Avr1bSac	Addition of NgoMIV site upstream of RFLR	TCAT<u>GAGCTC</u>GAAGAAATCTTCC<u>gCCGGC</u>AATG
41	AvrRFLQ	Replacement of RFLR with the mutation encoding RFLQ	CATT<u>GCCGGc</u>GGAAGATTTCTTCaAGC
42	AvrQFLR	Replacement of RFLR with the mutation encoding QFLR	CATT<u>GCCGGc</u>GGAcaATTTC
43	AvrRFVR	Replacement of RFLR with the mutation encoding RFVR	CATT<u>GCCGGc</u>GGAAGATTTgTTC
44	AvrKFLR	Replacement of RFLR with the mutation encoding KFLR	CATT<u>GCCGGc</u>GGA<u>Aag</u>TTTCTTC
45	Avr9R1	Replacement of RFLR with the sequence encoding the 9 arginine motif	cgtcgacgtcggcgacgcGCTCATGAAGAGGACGATG
46	Avr9R2		CATT<u>GCCGGc</u>GGAcgacggcgacgtcgacgtcggcgacg
47	AvrTAT1	Replacement of RFLR with the sequence encoding the TAT motif	taagaaacgccgtcagcgacgtcgaGCTCATGAAGAGGACGATG
48	AvfrTAT2		CATT<u>GCCGGc</u>GGAtatggacgtaagaaacgccgtcagc
49	GBPF1	Replacement of RFLR-dEER with the sequence from Pf GBP130	gaaggagaagactacactccggaaaagcaagcaaaag|ACCTTCAGCGTGACTGACC
50	GBPF2		ctcgtatactggcagagggcgaagatacctgcgcaaggaaggagaagactacact
51	GBPF3		gtatgagaaagcggtagattacggctttcgagagtctcgtatactggcagag
52	GBPR		ctaccgctttctcatacttatc|TGCGTTGCAGGTCACGAC
53	HRPF1	Replacement of RFLR-dEER with the sequence from Pf HRPII	tgtcgacgatgcgcaccatgcacaccatgttgcagatl|ACCTTCAGCGTGACTGACC
54	HRPF2		aacctcaacaagagactgttgcacgagacacaagcacatgtcgacgatgcgcacc
55	HRPF3		acaataacctgtgtagtaagaatgctaaaggcttgaacctcaacaagagactg
56	HRPR1		actacacaggttattgttaaal|TGCGTTGCAGGTCACGAC
57	1615F1	Replacement of RFLR-dEER with the sequence from Pf 1615c	cagtcaatgcattacaagaaatgttagatgatagtgtc|ACCTTCAGCGTGACTGACC
58	1615F2		ctcaagcagttggagttcatcacattggaagagaagacagtcaatgcattacaag
59	1615F3		aagatcaactcgtcatctacttatacacacagtagaatactcaagcagttggagttc
60	1615R1		agatgacgagttgatcttgttgtaactl|TGCGTTGCAGGTCACGAC
61	AvrGFPF	Replacement of codons encoding nine arginine motif in pR9GFP with Avr1b residues 33 to 71 from wild type Avr1b or from Avr1b RXLR or dEER mutants	gatct<u>agatct</u>GTGGAATCTCCAGATCTC
62	AvrGFPR1		GTCATATGGATAGCCGGACATlGTCAGTCACGCTGAAGGT
63	AvrGFPR2		GATC<u>CCATGG</u>AGCCAGCATAGTCTGGGACGTCATATGGATAGCCGGA

Supplemental Table 1. Oligonucleotides used for PCR and plasmid construction.

Uppercase letters indicate bases that match the initial template. Lower case letters indicate mutations or 5' extensions that do not match the initial template. Restriction sites introduced into the amplicon are underlined. A pipe (|) indicates the boundary between *Avr1b*-1 sequences and fused sequences (Avh, GFP or *Plasmodium* RXLX motif) in the fusion oligonucleotides.

No	Plasmid Name	Sources	Construct	Construction strategy
1	pUN	pHAMT35N (*5*)	*Npt* II gene for G418 resistance fused to *P. sojae* rpL41 promoter in pUC19	Ham34 promoter of pHAMT35N replaced by *P. sojae* rpL41 promoter using primers UF and UR
2	pHamAvr1b	pHAMT35N	*Avr1b*-1 gene fused to Ham34 promoter and terminator for *P. sojae* transformation in pUC19	NptII gene of pHAMT35N replaced with *P. sojae Avr1b*-1 using PrimerC and PrimerD
3	M1	pHAMT35N	*Avr1b*-1(RXLR1^{AAAA}) mutant fused to Ham34 promoter and terminator	Same as pHamAvr1b with mutation introduced by primers motif1F and motif1R
4	M2	pHAMT35N	*Avr1b*-1(RXLR2^{AAAA}) mutant fused to Ham34 promoter and terminator	Same as pHamAvr1b with mutation introduced by primers motif2F and motif2R
5	M1+2	pHAMT35N	*Avr1b*-1(RXLR1^{AAA}A,2^{AAAA}) mutant fused to Ham34 promoter and terminator	Same as pHamAvr1b with mutation introduced by primers motif1+2F and motif1+2R
6	M3	pHAMT35N	*Avr1b*-1(dEER^{A6}) mutant fused to Ham34 promoter and terminator	Same as pHamAvr1b with mutation introduced by primers motif3F and motif3R
7	pHamAvh341	pHAMT35N	*HpAvh341* fused with C-terminal domain of *Avr1b*-1, driven by Ham34 promoter and terminator	NptII gene replaced with HpAvh341-Avr1b fusion using primers HpAvh341F, HpAvh341R and PrimerD
8	pHamAvh171	pHAMT35N	Ps *Avh171* (=*Avr*4/6) fused with C-terminal of *Avr1b*-1 driven by Ham34 promoter and terminator	NptII gene replaced with Ps Avh171-Avr1b fusion using primers PsAvh171F, PsAvh171R and PrimerD
9	pHamAvr1bCt	pHAMT35N	C-terminus of *Avr1b*-1 gene fused to Ham34 promoter and terminator for *P. sojae* transformation in pUC19	NptII gene of pHAMT35N replaced with *P. sojae Avr1b*-1 C terminus using primers 1bRXLR(-)F and PrimerD
10	UNM1	pUN and M1	Ham34::Avr1b (RXLR1^{AAAA}) inserted together with rpL41::NptII for *P. sojae* transformation	Selection and Gus expression cassettes in pCambia1305.2 replaced by inserts from pUN and M1, respectively
11	UNM2	pUN and M2	Ham34::Avr1b (RXLR2^{AAAA}) inserted together with rpL41::NptII for *P. sojae* transformation	Selection and Gus expression cassettes in pCambia1305.2 replaced by inserts from pUN and M2, respectively
12	UNM1+2	pUN and M1+2	Ham34::Avr1b (RXLR1^{AAAA}A,2^{AAAA}) inserted together with rpL41::NptII for *P. sojae* transformation	Selection and Gus expression cassettes in pCambia1305.2 replaced by inserts from pUN and M1+2, respectively
13	UNM3	pUN and M3	Ham34::Avr1b(dEER^{A6}) inserted together with rpL41::NptII for *P. sojae* transformation	Selection and Gus expression cassettes in pCambia1305.2 replaced by inserts from pUN and M3, respectively
14	pCambiaAvr1b	pCambia1305.2	CaMV 35S promoter fused to full length *Avr1b*-1 in GUS-containing vector	HMT gene in pCambia1305.2 replaced by *Avr1b*-1 using primers Avr1bfull_F and Avr1bR
15	pCambiaM2	pCambia1305.2	CaMV 35S promoter fused to *Avr1b*-1 (RXLR2^{AAAA}) in GUS-containing vector	HMT gene in pCambia1305.2 replaced by Avr1b(RXLR2) using primers Avr1bfull_F and Avr1bR
16	pCambia-mAvr1b	pCambia1305.2	CaMV 35S promoter fused to leaderless *Avr1b*-1 in GUS-containing vector	HMT gene in pCambia1305.2 replaced by mature *Avr1b*-1 using primers Avr1bF and Avr1bR
17	pCambia-mM2	pCambia1305.2	CaMV 35S promoter fused to leaderless *Avr1b*-1(RXLR2^{AAAA}) in GUS-containing vector	HMT gene in pCambia1305.2 replaced by mAvr1b(RXLR2) using primers Avr1bF and Avr1bR
18	pCa-GUS(-)	pCambia1305.2	Empty vector as the control	GUS expression cassette was removed by *Sph* I restriction and re-ligation for co-transformation experiments
19	pCaAvr1b	pCambiaAvr1b	CaMV 35S promoter fused to *Avr1b*-1 in GUS-free vector	
20	pCaM2	pCambiaM2	CaMV 35S promoter fused to *Avr1b*-1 (RXLR2^{AAAA}) in GUS-free vector	
21	pCa-mAvr1b	pCambia-mAvr1b	CaMV 35S promoter fused to leaderless *Avr1b*-1 in GUS-free vector	
22	pCa-mM2	pCambia-mM2	CaMV 35S promoter fused to leaderless *Avr1b*-1(RXLR2^{AAAA}) in GUS-free vector	
23	pUCAvr1b	pCambiaAvr1b	CaMV 35S promoter fused to *Avr1b*-1 with *Xma* I site	Avr1b expression cassette was inserted into pUC19 by PCR with primers Avr1b_EcoRI and Avr1b_HindIII

24	pUCAvr1bXK	pUCAvr1b	*Kpn* I site added to 3' end pUCAvr1b to facilitate later constructions	Amplification with primers Avr1b_EcoRI, Avr1b_HindIII and Avr1b_genegun_KpnI
25	pUCmAvr1b(dEER)	pUCAvr1bXK and M3	CaMV 35S promoter fused to mature *Avr1b*-1(dEER^{A6}) using *Xma* I and *Kpn* I sites	Ligation of *Xma* I-*Kpn* I fragment from M3 into pUCAvr1bXK
26	pUCmAvr1bGFP	pAcGFP1-N	Fusion of leaderless *Avr1b*-1 with *AcGFP* from pAcGFP1-N (Clontech, # 632485) placed under control of CaMV 35S promoter	AcGFP fused to relevant Avr1b sequence by PCR with primers Avr1bF or Avr1bfull_F plus GFPF and GFPR. PCR product inserted into pUCAvr1bXK using *Xma* I and *Kpn* I
27	pUCsAvr1bGFP	pUCAvr1bXK	Fusion of normal *Avr1b*-1 with *AcGFP* placed under control of CaMV 35S promoter	
28	pUCGFP	pUCsAvr1bGFP	CaMV 35S promoter fused to *AcGFP*	Deletion of Avr1b sequences by self-ligation of *Xma* I/*Age* I restricted pUCsAvr1bGFP
29	pUCsGFP		CaMV 35S promoter fusion of Avr1b secretory leader to *AcGFP* placed under control of CaMV 35S promoter	Deletion of Avr1b sequences encoding mature protein by self-ligation of *Nco* I restricted pUCsAvr1bGFP
30	pUCM2GFP	pUCAvr1bXK	*Avr1b*-1(RXLR2^{AAAA})-*AcGFP* fusion placed under control of CaMV 35S promoter	AcGFP fused to relevant Avr1b sequence by PCR with primers Avr1bfull_F, GFPF and GFPR. PCR product inserted into pUCAvr1bXK using *Xma* I and *Kpn* I
31	pUCM3GFP		*Avr1b*-1(dEER^{A6})*::AcGFP* fusion placed under control of CaMV 35S promoter	
32	pUCAvr1b(R9)GFP		*Avr1b*-1(RXLR2^{Arg9})::*AcGFP* fusion placed under control of CaMV 35S promoter	
33	pUCAvr1b(TAT)GFP		*Avr1b*-1(RXLR2^{TAT})::*AcGFP* fusion placed under control of CaMV 35S promoter	
34	pUCNgo	pUCAvr1bXK	Addition of NgoMIV site upstream of RFLR in N-terminus of Avr1b (T150C) in pUCAvr1bXK to facilitate later manipulations	GCCGGT in was mutated to GCCGGc using primer Avr1bSac
35	pUCRFRL	pUCAvr1bXK	CaMV 35S promoter fused to *Avr1b*-1 genes carrying indicated mutations	Wild type Avr1b in pUCAv1bXK was replaced with the corresponding mutants using *Xma* I and *Kpn* I
36	pUCFRLR			
37	pUCRFAR			
38	pUCQFLQ			
39	pUCQFLR	pUCNgo		Avr1b in pUCNgo was replaced with the corresponding mutants by *NgoMIV*I and *Kpn* I
40	pUCKFLR			
41	pUCRFLQ			
42	pUCRFVR			
43	pUCAvr9R			
44	pUCAvrTAT			
45	pUCAvrPfGBP	pUCAvr1bXK		Wild type Avr1b in pUCAvr1bXK was replaced with the corresponding mutants using *Xma* I and *Kpn* I
46	pUCAvrPfHRP			
47	pUCAvrPf1615			
48	pAvr1bGFP	pR9GFP	Replacement of codons encoding nine arginine motif in pR9GFP with Avr1b residues 33 to 71 from wild type Avr1b or from Avr1b RXLR or dEER mutants	Amplification with primers AvrGFPF, AvrGFPR1 and AvrGFPR2
49	pAvr1b(M1+2)GFP			
50	pAvr1b(M3)GFP			

Supplemental Table 2. Description of Plasmids Used

Transgene	Transformation Strategy[a]	Numbers of Positive Clones				Clones used in this study[f]
		G418 Growth[b]	PCR[c]	RT-PCR[d]	Efficiency[e]	
GUS	Single plasmid	22	NT	4[g]	18.2%	1
sAvr1b(WT)	Co-transformation	44	6	2	4.5%	2
sAvr1b(RXLR1^{AAAA})	Single plasmid	9	7	3	33.3%	3
sAvr1b(RXLR2^{AAAA})	Single plasmid	20	13	5	25.0%	2
sAvr1b(RXLR1^{AAAA}, 2^{AAAA})	Single plasmid	9	6	4	44.4%	2
sAvr1b(dEER^{A6})	Single plasmid	3	1	1	33.3%	1
sAvr1b(dEER^{A6})	Co-transformation	12	2	1	8.3%	1
Ps *Avh171-Avr1bCt*	Co-transformation	28	6	2	7.1%	2
Hp *Avh341-Avr1bCt*	Co-transformation	22	5	2	9.1%	2

Supplemental Table 3. Efficiency of PEG-mediated *P. sojae* protoplast transformation.

a For each transformation, 0.5~2x10^7 protoplasts were used and 1-3 transformation experiments were carried out. In some cases the transgene was inserted into the same plasmid as the selectable marker and in some cases the transgene was introduced by co-transformation.

b Number of G418 resistant colonies surviving after three rounds of selection on 50 ug/ml G418.

‡ Number of colonies in which the transgene could be detected by PCR from genomic DNA. NT = not tested.

d Number of colonies in which Avr1b mRNA could be detected by RT-PCR. In colonies with no detectable Avr1b transgene mRNA, the endogenous Avr1b gene was often silenced.

e percentage of G418 resistant colonies confirmed to express the transgene by RT-PCR.

f All the RT-PCR positive clones had similar expression levels for a give transgene.

g The 22 putative GUS gene transformants were assessed for transgene expression by staining for beta-glucuronidase activity with 5-bromo-4-chloro-3-indolyl- beta-D-glucuronic acid (X-gluc), rather than RT-PCR.