

## Addendum

# Adaptive evolution has targeted the C-terminal domain of the RXLR effectors of plant pathogenic oomycetes

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**Key words:** plant-microbe interactions, effectors, gene families, positive selection

Plant pathogenic microbes deliver effector proteins inside host cells to modulate plant defense circuitry and enable parasitic colonization. As genome sequences from plant pathogens become available, genome-wide evolutionary analyses will shed light on how pathogen effector genes evolved and adapted to the cellular environment of their host plants. In the August 2007 issue of *Plant Cell*, we described adaptive evolution (positive selection) in the cytoplasmic RXLR effectors of three recently sequenced oomycete plant pathogens. Here, we summarize our findings and describe additional data that further validate our approach.

A diverse number of plant pathogens, including bacteria, oomycetes, fungi and nematodes, deliver effector proteins inside host cells to modulate plant defense circuitry and enable parasitic colonization.<sup>1-8</sup> Because these so-called cytoplasmic effectors function inside plant cells and produce phenotypes that extend to plant cells and tissues, their genes are expected to be the direct target of the evolutionary forces that drive the antagonistic interplay between pathogen and host.<sup>9,10</sup> In a study published in the August 2007 issue of *Plant Cell*, we and our collaborators examined the extent to which positive selection (adaptive evolution) has shaped the evolution of the cytoplasmic effectors of three recently sequenced oomycete plant pathogens *Phytophthora sojae*, *Phytophthora ramorum*, and *Hyaloperonospora parasitica* (Genome Sequencing Center at Washington University).<sup>11</sup>

## Oomycete RXLR Effectors are Modular Proteins

Four oomycete *Avr* proteins have been described in the past three years and were found to contain a secretory signal peptide followed by a conserved domain featuring the motif RXLR, flanked by a high frequency of acidic (D/E) residues.<sup>1,3,12</sup> The RXLR motif defines a domain that functions in delivery of effector proteins into host cells.<sup>13</sup> It

is similar in sequence and position and is functionally interchangeable with the plasmodial host translocation (HT)/Pexel motif that functions in delivery of parasite proteins into the cytoplasm of red blood cells of mammalian hosts.<sup>14</sup> Also, the RXLR motif is not required for the effector activities of *P. infestans* AVR3a when this protein is directly expressed inside plant cells consistent with a role in targeting rather than effector activity.<sup>15</sup> Altogether these findings led to the view that oomycete RXLR effectors are modular proteins with two major functional domains.<sup>3</sup> While the N-terminal domain encompassing the signal peptide and RXLR leader functions in secretion and targeting, the remaining C-terminal region carries the effector activity and operates inside plant cells.

## Ab Initio Identification of RXLR Effectors: Rationale

In the initial part of our study, we aimed to develop a method for ab initio identification of RXLR effector genes in the sequenced genomes. Our approach was to first determine the defining features of validated oomycete RXLR effectors in order to develop a robust set of data mining criteria. We therefore, developed an unbiased list of 43 oomycete RXLR proteins consisting of validated effectors and their closest homologs. Also, to objectively address the extent to which the tetrapeptide RXLR sequence is overrepresented and positionally constrained in *Phytophthora*, we examined the distribution of the RXLR sequence in the proteomes of these species compared to 46 other eukaryotes. These analyses indicated that the RXLR sequence is significantly overrepresented and positionally constrained in the secretomes of *Phytophthora* relative to other eukaryotes and formed the basis of the ab initio algorithm.

## Ab Initio Identification of RXLR Effectors: Further Validation

Since the publication of our study, two new avirulence genes, PsAvr1a and PsAvr3a, were reported from *Phytophthora sojae* by Mark Gijzen laboratory, London, Ontario, Canada (GenBank accessions ABQ81647 and ABO47652). Interestingly, PsAvr1a and PsAvr3a fulfill our criteria for RXLR effectors and were identified by our ab initio algorithm (Supplemental Table S2 of the Win et al. paper). In Table 1, we list the features of PsAvr1a and PsAvr3a, and their 34 homologous genes. The mean values for protein size, position of RXLR, and position of EER sequence obtained with this new set of validated RXLR effectors are remarkably similar to those we reported earlier.

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**Table 1 New validated RXLR effectors. The new validated effectors are based on two *Phytophthora sojae* avirulence proteins, PsAvr1a and PsAvr3a, reported by the laboratory of Mark Gijzen, London, Ontario, Canada and their homologs (E value <10<sup>-4</sup>)**

Description	Accession <sup>a</sup>	Species	Evidence	Length <sup>b</sup>	Signal Peptide Length <sup>b</sup>	SignalP v2.0 HMM score	SignalP v2.0 NN score	RXLR Position <sup>c</sup>	EER position <sup>c</sup>
Avirulence effector protein PsAvr3a	ABQ81647, Ps_scaffold_80_R245	<i>P. sojae</i>	Avr effector	111	20	0.998	0.910	43	
Avirulence effector protein PsAvr1a	ABO47652, Ps_scaffold_1058_F4	<i>P. sojae</i>	Avr effector	121	25	0.999	0.813	54	64
Unknown protein similar to PsAvr1a	Pr_scaffold_103_F268	<i>P. ramorum</i>	homolog	121	21	0.994	0.850	54	
Unknown protein similar to PsAvr1a	Pr_scaffold_13_F1570	<i>P. ramorum</i>	homolog	129	25	0.998	0.880	48	68
Unknown protein similar to PsAvr1a	Pr_scaffold_17_F1241	<i>P. ramorum</i>	homolog	112	21	0.997	0.815	51	59
Unknown protein similar to PsAvr1a	Pr_scaffold_207_F26	<i>P. ramorum</i>	homolog	138	23	0.997	0.898	56	69
Unknown protein similar to PsAvr1a	Pr_scaffold_251_R3	<i>P. ramorum</i>	homolog	139	21	1	0.894	54	73
Unknown protein similar to PsAvr1a	Pr_scaffold_26_R566	<i>P. ramorum</i>	homolog	141	21	1	0.898	54	75
Unknown protein similar to PsAvr1a	Pr_scaffold_26_R615	<i>P. ramorum</i>	homolog	152	21	1	0.884	58	86
Unknown protein similar to PsAvr1a	Pr_scaffold_34_F586	<i>P. ramorum</i>	homolog	293	21	1	0.938	52	67
Unknown protein similar to PsAvr1a	Pr_scaffold_50_R933	<i>P. ramorum</i>	homolog	140	23	1	0.822	52	
Unknown protein similar to PsAvr1a	Pr_scaffold_52_F517	<i>P. ramorum</i>	homolog	151	22	0.994	0.811	52	70
Unknown protein similar to PsAvr1a	Pr_scaffold_64_F233	<i>P. ramorum</i>	homolog	293	21	1	0.947	52	67
Unknown protein similar to PsAvr1a	Pr_scaffold_64_F343	<i>P. ramorum</i>	homolog	294	21	1	0.932	52	67
Unknown protein similar to PsAvr1a	Pr_scaffold_65_R231	<i>P. ramorum</i>	homolog	162	21	1	0.953	58	83
Unknown protein similar to PsAvr1a	Pr_scaffold_75_F477	<i>P. ramorum</i>	homolog	136	23	0.999	0.870	53	69
Unknown protein similar to PsAvr1a	Pr_scaffold_91_R166	<i>P. ramorum</i>	homolog	154	21	0.999	0.892	57	81
Unknown protein similar to PsAvr1a	Ps_scaffold_118_R508	<i>P. sojae</i>	homolog	98	21	0.998	0.869	54	71
Unknown protein similar to PsAvr1a	Ps_scaffold_122_R489	<i>P. sojae</i>	homolog	125	25	0.999	0.858	48	68
Unknown protein similar to PsAvr1a	Ps_scaffold_27_R1297	<i>P. sojae</i>	homolog	305	21	0.996	0.951	51	
Unknown protein similar to PsAvr1a	Ps_scaffold_3_R4103	<i>P. sojae</i>	homolog	130	21	0.994	0.863	54	70
Unknown protein similar to PsAvr1a	Ps_scaffold_36_F644	<i>P. sojae</i>	homolog	137	23	1	0.856	53	74
Unknown protein similar to PsAvr1a	Ps_scaffold_68_F347	<i>P. sojae</i>	homolog	162	21	1	0.898	50	61
Unknown protein similar to PsAvr3a	Pr_scaffold_1497_R5	<i>P. ramorum</i>	homolog	126	19	0.997	0.934	41	56
Unknown protein similar to PsAvr3a	Pr_scaffold_33_F760	<i>P. ramorum</i>	homolog	126	19	0.998	0.942	41	56
Unknown protein similar to PsAvr3a	Pr_scaffold_33_F786	<i>P. ramorum</i>	homolog	125	19	1	0.932	41	56
Unknown protein similar to PsAvr3a	Pr_scaffold_33_R44	<i>P. ramorum</i>	homolog	128	19	0.998	0.942	41	56
Unknown protein similar to PsAvr3a	Pr_scaffold_34_R60	<i>P. ramorum</i>	homolog	127	19	0.997	0.943	41	56
Unknown protein similar to PsAvr3a	Pr_scaffold_6_R2337	<i>P. ramorum</i>	homolog	203	20	1	0.941	43	61
Unknown protein similar to PsAvr3a	Pr_scaffold_6_R2603	<i>P. ramorum</i>	homolog	204	20	1	0.935	43	61
Unknown protein similar to PsAvr3a	Ps_scaffold_106_F265	<i>P. sojae</i>	homolog	131	20	0.999	0.930	45	
Unknown protein similar to PsAvr3a	Ps_scaffold_106_R557	<i>P. sojae</i>	homolog	131	20	0.999	0.930	45	
Unknown protein similar to PsAvr3a	Ps_scaffold_24_F382	<i>P. sojae</i>	homolog	137	20	1	0.954	44	
Unknown protein similar to PsAvr3a	Ps_scaffold_31_F1779	<i>P. sojae</i>	homolog	167	20	1	0.934	43	56
Unknown protein similar to PsAvr3a	Ps_scaffold_31_R1171	<i>P. sojae</i>	homolog	120	20	1	0.950	40	58
Unknown protein similar to PsAvr3a	Ps_scaffold_87_F189	<i>P. sojae</i>	homolog	145	22	1	0.879	43	75
Means	155.94	21.1	0.99	0.90	48.9	66.6			
Means reported by Win et al (2007)	158.3	20.7	0.99	0.86	45.0	62.1			

The two *P. sojae* avirulence proteins were reported after we applied the gene mining pipeline described in Win et al (2007) and therefore validate the approach. This list of 36 genes complements the 43 validated effectors described in Table 1 of Win et al. (2007). <sup>a</sup>GenBank accession number is provided where available. Otherwise, accession numbers correspond to sequences listed in Table S2 of Win et al (2007). <sup>b</sup>Length in amino acids. <sup>c</sup>Position counting from N-terminus.

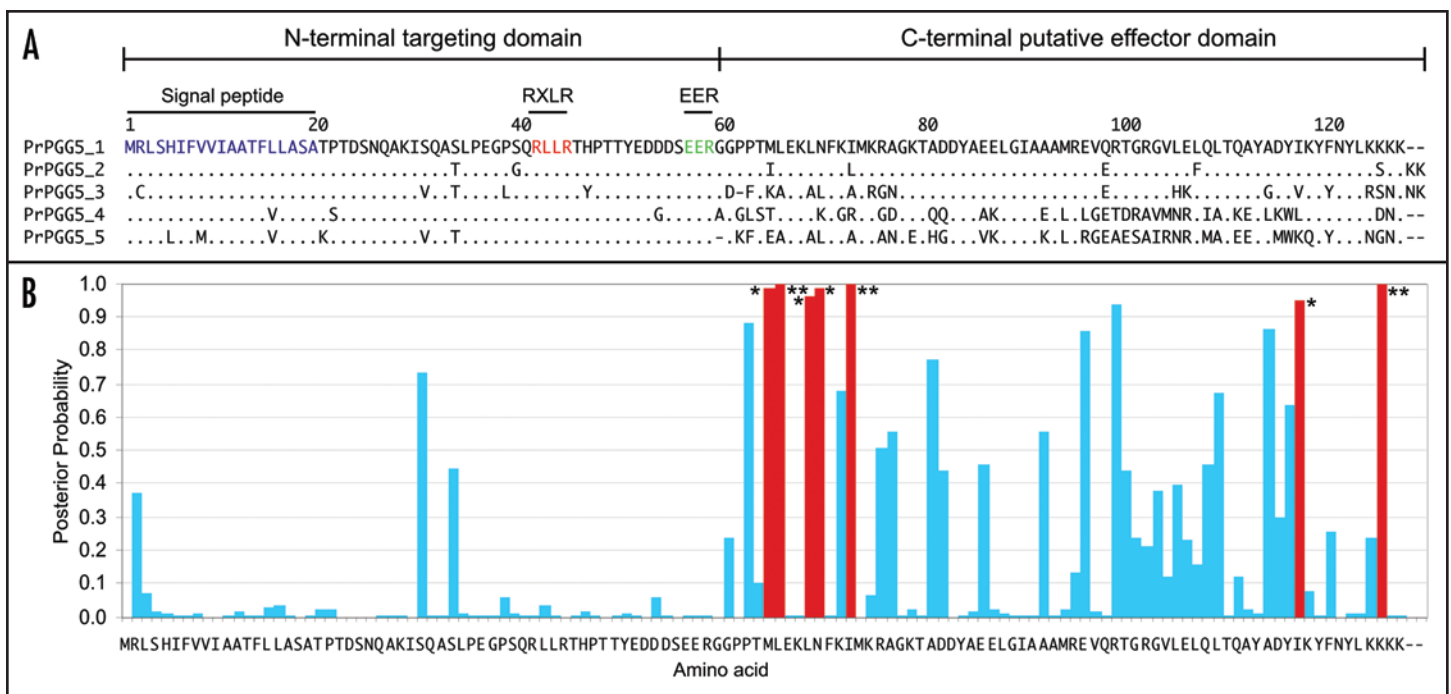


Figure 1. An example of a paralogous gene group (PGG) with evidence of positive selection focused mainly on the C-terminal effector domain. (A) Multiple sequence alignment of the five *Phytophthora ramorum* proteins that form PrPGG5. Identical amino acids are indicated by dots. (B) Posterior probabilities estimated by Bayes Empirical Bayes analysis for the model M8 in PAML software package were plotted for each amino acid site in PrPGG5. Positively selected sites are shown in red. \* $p > 95\%$  and \*\* $p > 99\%$ .

## Patterns of Positive Selection are Consistent with the Modular Structure of RXLR Effectors

The genome-wide catalogs of RXLR effector genes from the three oomycete species revealed complex and diverse sets of RXLR effector genes that have undergone relatively rapid birth and death evolution. We obtained robust evidence of positive selection in more than two thirds of the examined paralog families of RXLR effectors. Positive selection has acted on paralogous RXLR gene families targeting for the most part the C-terminal region. These findings are consistent with the view that RXLR effectors are modular proteins with the N-terminus involved in secretion and host translocation and the C-terminal domain dedicated to modulating host defenses inside plant cells. In Figure 1, we illustrate the remarkably biased distribution of the positively selected sites towards the C-terminal region for PrPGG5, one of the paralogous gene groups of *P. ramorum*.

## Conclusion

In summary, we reported and validated a method for ab initio mining of RXLR effectors in oomycete genome sequences. We applied this method to develop genome-wide catalogs of RXLR effectors and demonstrate that adaptive evolution has shaped the structure of these genes. Future studies will determine the extent to which the positively selected genes and residues identified in our study are functionally important.

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