Comparative Secretome Prediction and Analysis of Two *Phytophthora* spp.

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Abstract
Phytophthora spp. are widely distributed pathogens causing some of the most devastating diseases in plants. They accomplish parasitic colonization in hosts by virtue of an array of signaling proteins. Secretome analysis is one of the methods to attain molecular insights into species pathogenicity. In this study, we have analyzed the proteome of two species viz., P. ramorum and P. sojae and predicted extracellular secretory proteins particularly involved in host-pathogen interactions. The potential Phytophthora spp. secretome comprising of both the classical and non-classical secretory proteins was predicted with the aid of a stringent computational pipeline.

Out of the 15,743 P. ramorum and 19,027 P. sojae proteins which were computationally analyzed in the current study, 1396 (8.86%) and 1666 (8.75%) proteins were categorized as classical secretory proteins (CSPs) while a total of 71 (0.45%) and 96 (0.50%) proteins were categorized as non-classical secretory proteins (NCSPs) in P. ramorum and P. sojae respectively. In addition, 235 and 399 effector proteins were also predicted from P. ramorum and P. sojae respectively. The functional annotation of the effector proteins revealed the occurrence of SSPs (small specific proteins), virulence and avirulence factors which could prove to be future target to control the pathogenicity and to decipher its role in host-parasite interactions.

Keywords: *Phytophthora* spp., CSPs, NCSPs, SSPs.

Introduction

The genus *Phytophthora* is known worldwide for its pathogenicity on a wide range of plants. This eukaryotic plant pathogenic oomycete causes diseases not only on economically important agricultural crops, but also on ecologically valuable tree species of natural forests[1]. Pathogenesis is a dynamic and intricate process modulated by a plethora of extracellular signals secreted by both host plants and pathogen. Pathogens have been known to invade plants by virtue of various classical and non-classical secretory proteins (CSPs and NCSPs)[2].

The oomycetes accomplish parasitic colonization of plants by reprogramming the defense circuitry of host cells through an array of disease effector proteins[3]. Studies on molecular mechanisms underlying *Phytophthora*-host interactions have always elicited an interest in researchers as they could aid in the development of new plant protection strategies. The whole genome sequencing of certain *Phytophthora* spp., along with the development of high-throughput bioinformatics tools, have revolutionized the field of molecular plant- *Phytophthora* interactions. The proteins involved in the host- *Phytophthora* interactions might also play a role in ascertaining the host range[4].

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P. ramorum and *P. sojae* are the two species whose genomes have been sequenced. One of the interesting, but contrasting features of the two species is that *P. sojae* is host-specific whereas *P. ramorum*, has a broad host range. *P. ramorum* incites disease predominantly in forest tree species and poses a major threat to the natural ecological balance. The sudden oak death disease caused by *P. ramorum* affects the keystone species and is causing devastating damage to the ecosystems of Western America[5,6]. The pathogen has a wide host range affecting plant species from Aceraceae, Anacardiaceae, Betulaceae, Caprifoliaceae, Cupressaceae, Ericaceae, Fagaceae, Hippocastanaceae, Lauraceae, Pinaceae, Rhamnaceae and Rosaceae[7,8].

Diseases caused by *P. ramorum* have been reported from USA, Canada, Germany, the Netherlands, Belgium, Denmark, France, Slovenia, Spain, Sweden, England and the Republic of Ireland[9]. *P. sojae* is the causal agent of root rot and stem rot of soybean. The pathogen has a limited host range and zoospores of *P. sojae* are reported to be chemotactically attracted to the isoflavones - daidzein and genistein exuded by soybean roots[10]. In this study, we have carried out comparative secretome prediction in these two *Phytophthora* spp. and detailed analyses to investigate effector proteins which could be putatively involved in host-pathogen interactions.

Material and Methods

Secretome prediction: We have used complete predicted proteomes of two *Phytophthora* spp. *viz. P. ramorum* and *P. sojae* which are available on the fungal genomics database developed by the Joint Genome Institute[11]. The proteomes of these two species were analyzed and compared with the aid of a stringent computational pipeline developed specifically for this study, the schema of which is shown in figure 1. The presence of secretion signals in the sequences was investigated by the virtue of SignalP v.2[12]. Further, transmembrane helices were predicted with TMHMM[13,14].

The non-classical secretory proteins which are known to lack signal peptides at their N-terminal regions and are called 'leaderless' sequences, were predicted using SecretomeP[15]. TargetP[16] was employed for investigating the subcellular localization of the proteins. Non-plant option was chosen for the organism group and the default parameters were set for the tool. The sequences predicted as 'S' (secretory signal peptides) were selected for the further analysis. The total sequences predicted as 'S' were further analyzed to look for the presence of GPI-anchored proteins by the virtues of GPIsom[17]. Predicted proteins shorter than 40 amino-acids were excluded from the analysis.

Effector proteins and effector proteins cluster prediction: The total predicted secretory proteins (including classical and non-classical secretory proteins) obtained through the study were further analyzed by the virtue of EffectorP[18] for the prediction of effector proteins. Further, intra-species BLAST search was performed using the standalone BLAST server[19]. The predicted effector proteins obtained were annotated using Blast2GO[20]. Orthologous effector proteins clusters between *P. ramorum* and *P. sojae* were identified using Inparanoid[21] (standalone version 4.1) while taking into account the certain parameters *viz*. score cutoff 40 bits; sequence overlap cutoff 0.5; group merging cutoff 0.5 and scoring matrix BLOSUM62.

Results and Discussion

In this study, initially, the presence of extracellular secretory signals was predicted using a combination of SignalP, TMHMM and TargetP[22]. Out of the 15,743 proteins analyzed in the *P. ramorum* and 19,027 analyzed in *P. sojae*, 1396 and 1666 proteins were categorized as CSPs (Classical Secretory Proteins). Normally, eukaryotic secretory proteins have been known to follow classical ER-Golgi pathway, involving a characteristic feature of having a short N-terminal signal peptides[23]. However, several secretory proteins that lack the signal peptides are found to be exported via a non-classical secretion pathway. Therefore, in order to look for the presence of non-classical secretory proteins, SecretomeP was employed. The results obtained were subjected to GPIsom analysis to investigate the presence of GPI anchored proteins. We have predicted 71 and 96 proteins respectively from *P. ramorum* and *P. sojae* as NCSPs (Non-Classical Secretory Proteins).

Further, total predicted secretory proteins (CSPs and NCSPs) were taken into account for the prediction of effector proteins. A total of 235 and 399 effector proteins were predicted as candidate effectors in *P. ramorum* and *P. sojae* respectively with effector probability >0.5 by virtue of EffectorP as represented in figure 2. Annotation of effector proteins reveals the occurrence of 19 effectors to act as avirulence factors as mentioned in table 1. In this study, a total of 268 and 353 GPI-anchored proteins (GPI-APs) have been reported and predicted to be found on the external leaflet of the membrane in the respective species. An earlier study undertaken in *F. graminearum* suggests that GPI-APs possess a crucial role in growth, development and virulence as well as have the potential to act as diagnostic markers[24].

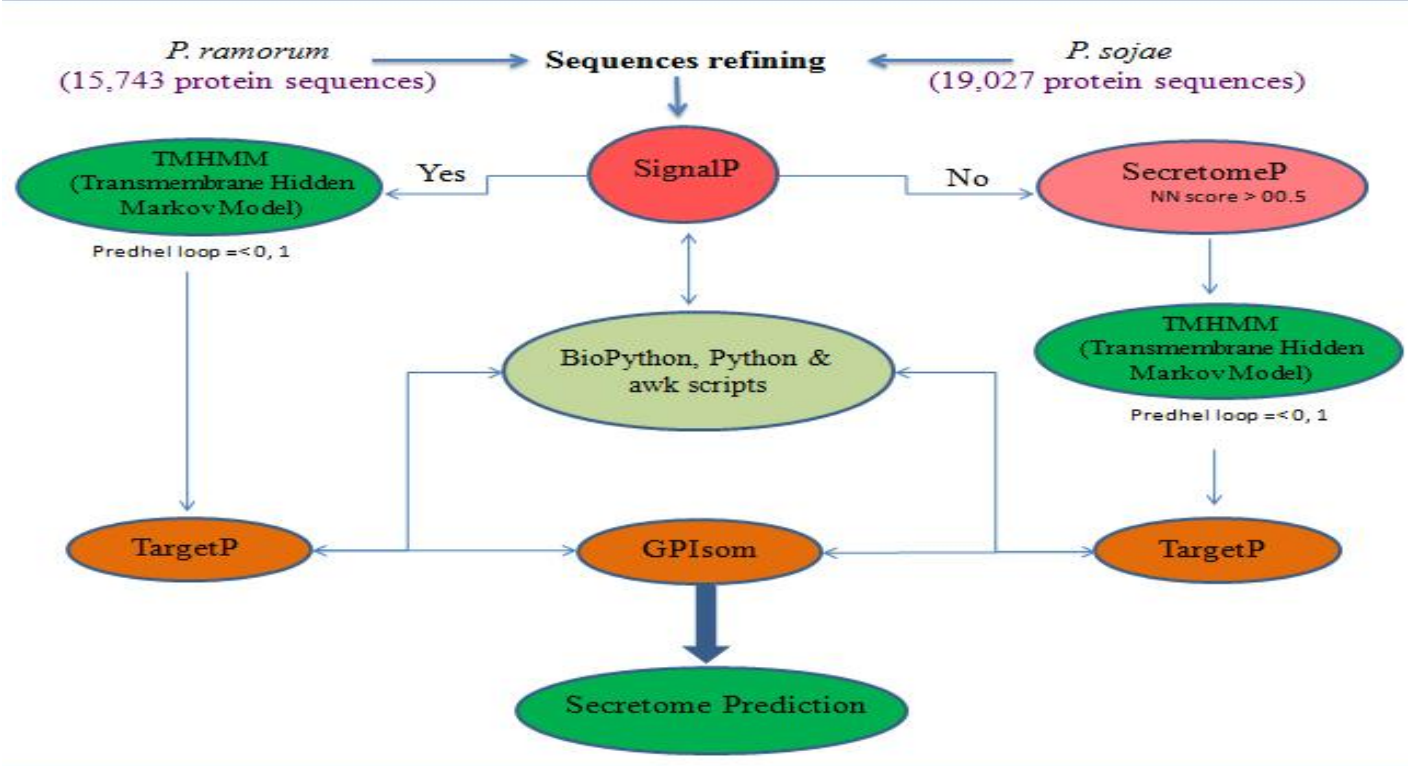

Figure 1: Computational pipeline used for the prediction of secretory proteins (i.e. classical and non-classical proteins).

We have compared the effector proteins predicted in both the species. In order to investigate sequence homology between *Phytophthora* spp., BlastP was performed with e-value set as equal to less than 1 using standalone NCBI-BLAST. The annotation results revealed that a majority of the effector proteins were found to function as virulence factors and toxins to facilitate infection and trigger defense responses based on recognition of avirulence factors and elicitors[25]. In this study, various elicitins as mentioned in table 2 which could be categorized as pathogen associated molecular patterns (PAMPS) for both the species were investigated.

Elicitins are species-specific avirulence factors which belong to a family of small proteins reported to be secreted by *Phytophthora* and *Pythium* sp. and are classified as oomycete PAMPs having a characteristic molecular feature of sterol-binding[26,27]. Previously, three elicitins specific to *P. infestans* have been reported, which are known to constitute a powerful strategy for crop protection by inducing defense response in plants[28]. Plants recognize the PAMPs by the virtue of plant receptors called pattern recognition receptors (PRRs) and get induced to distal systemic resistance[25,27].

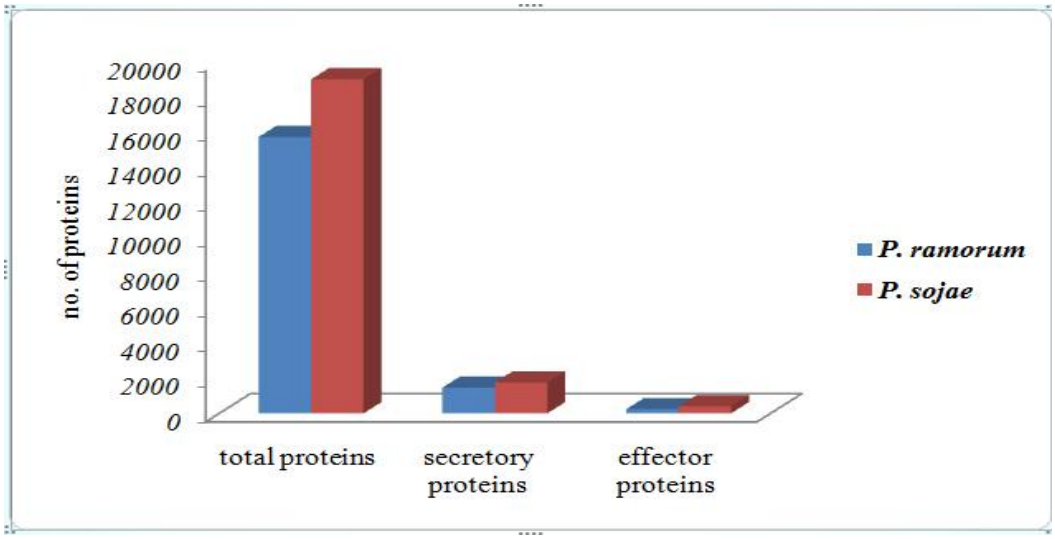

Figure 2: Graph showing comparative relation between proteomes, predicted secretory proteins (CSPs & NCSPs) and effector proteins in *P. ramorum* and *P. sojae*.

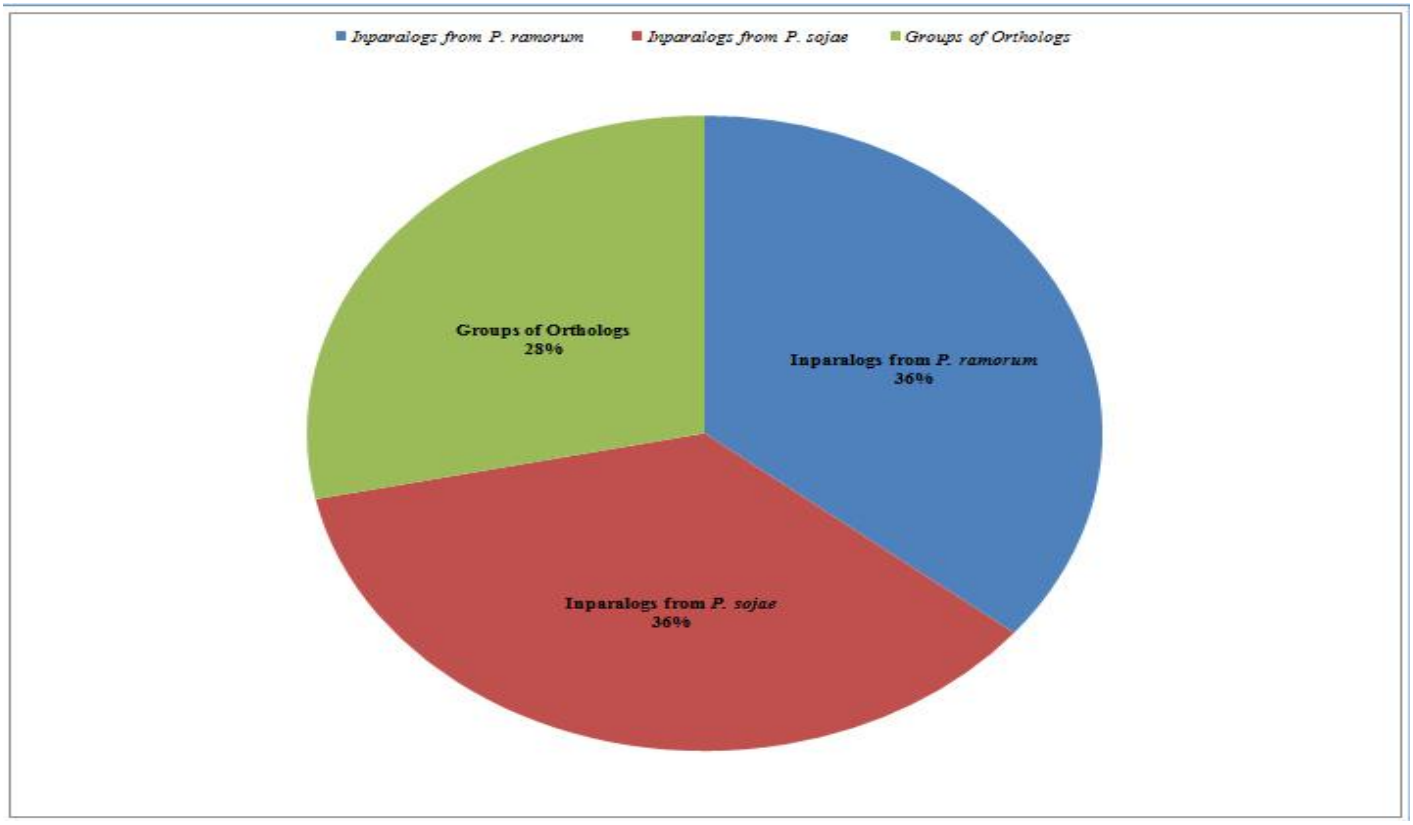

Figure 3: Representation of orthologous and paralogous protein clusters in the two species.

Table 1
Annotation results showing the occurrence of avirulence factors with Seq ID, functional description and the corresponding E-value in (1) *P. ramorum* and (2) *P. sojae*.

S.N.	*Phytophthora ramorum* ID	Description	E-value
1.	jgi|Phyra1_1|79329|	Avirulence 1b	4.5E-9
2.	jgi|Phyra1_1|81610|	Avirulence 1b	9.9E-36
3.	jgi|Phyra1_1|74075|	Avr1b-1 avirulence	2.1E-22
4.	jgi|Phyra1_1|80524|	Avr1b-1 avirulence	4.9E-43
5.	jgi|Phyra1_1|80530|	Avr1b-1 avirulence	2.5E-27
6.	jgi|Phyra1_1|80531|	Avr1b-1 avirulence	4.7E-25
7.	jgi|Phyra1_1|80533|	Avr1b-1 avirulence	4.5E-28

(1)

S.N.	*Phytophthora sojae* ID	Description	E-value
1.	jgi|Physo1_1|135585|	Avirulence 1b	1.9E-82
2.	jgi|Physo1_1|135621|	Avr1b-1 avirulence	2.3E-48
3.	jgi|Physo1_1|136046|	Avirulence 1b	4.6E-87
4.	jgi|Physo1_1|136207|	Avr1b-1 avirulence	1.2E-80
5.	jgi|Physo1_1|136214|	Avr1b-1 avirulence	2.0E-80
6.	jgi|Physo1_1|136280|	Avr1b-1 avirulence	1.8E-69
7.	jgi|Physo1_1|136284|	Avirulence 1b	4.4E-70
8.	jgi|Physo1_1|139437|	Avr1b-1 avirulence	1.4E-74
9.	jgi|Physo1_1|139466|	Avirulence 1b	1.9E-67
10.	jgi|Physo1_1|139921|	Avirulence 1b	1.1E-73
11.	jgi|Physo1_1|139923|	Avirulence 1b	1.1E-63
12.	jgi|Physo1_1|142101|	Avirulence 1b	4.9E-105
13.	jgi|Physo1_1|142795|	Avr1b-1 avirulence	1.7E-86
14.	jgi|Physo1_1|131199|	Avr1b-1 avirulence	1.4E-134
15.	jgi|Physo1_1|135099|	Avr1b-1 avirulence	1.8E-116
16.	jgi|Physo1_1|140616|	Avr1b-1 avirulence	2.1E-132

(2)

Table 2
Annotation results showing the occurrence of elicitins with Seq ID, functional description, and the corresponding E-value in (1) *P. ramorum* and (2) *P. sojae*.

S. N.	*Phytophthora ramorum* ID	Description	Length	E-value
1.	jgi|Phyra1_1|71532|	Elicitin-Like Mating M25	247	9.9E-179
2.	jgi|Phyra1_1|71746|	Elicitin RAL6	176	5.62E-90
3.	jgi|Phyra1_1|76630|	Elicitin RAL13C	137	2.37E-78
4.	jgi|Phyra1_1|77586|	Elicitin-Like Mating M25	251	0E0
5.	jgi|Phyra1_1|78652|	Elicitin Vex1	135	1.17E-79
6.	jgi|Phyra1_1|96566|	Elicitin RAL11D	138	4.9E-82

(1)

S.N.	*Phytophthora sojae* ID	Description	Length	E-value
1.	jgi|Physo1_1|108257|	Elicitin-Like Mating M25	242	2.74E-161
2.	jgi|Physo1_1|134055|	Elicitin SOL11C	136	2.56E-92
3.	jgi|Physo1_1|135435|	Elicitin Vex1	157	3.69E-98
4.	jgi|Physo1_1|144033|	Elicitin	135	1.07E-93
5.	jgi|Phyra1_1|108901|	Elicitin SOL6	172	4.77E-89

(2)

Moreover, SSPs (small secreted proteins) and small cysteine-rich protein have been reported and some of them were found to be common in the two species as depicted in the table 3. SSPs have been reported to be species-specific and could be potential targets to control the pathogenicity of the concerned species[29]. Small-cysteine rich proteins are known fungal effectors that trigger resistance or susceptibility in specific host plants[30]. RXLR effector is known to be one of the most diverse groups of cytoplasmic effector families associated with oomycetes plant pathogen which has the potential to get delivered into the host cell and suppresses its immunity[31]. In this study, we reported species-specific RXLR effector proteins as mentioned in the table 4.

Table 3
Comparative view of small cysteine-rich proteins with Seq ID, functional description and the corresponding E-value in (a) *P. ramorum* and (b) *P. sojae*. First two proteins were predicted to be common in both the species.

S.N.	*Phytophthora ramorum* ID	Description	Length	E-value
1.	jgi|Phyra1_1|43241|	Small cysteine rich SCR91	73	7.6E-11
2.	jgi|Phyra1_1|71697|	Small cysteine rich SCR108	112	1.0E-42
3.	jgi|Phyra1_1|78603|	Small cysteine rich	145	2.6E-82

(a)

S.N.	*Phytophthora sojae* ID	Description	Length	E-value
1.	jgi|Physo1_1|111409|	Small cysteine rich SCR91	72	1.5E-31
2.	jgi|Physo1_1|156264|	Small cysteine rich SCR108	206	9.7E-131

(b)

Table 4
Comparative view: Proteins belonging to RXLR family with Seq ID, functional description and the corresponding E-value in (a) *P. ramorum* and (b) *P. sojae*.

S.N.	*Phytophthora ramorum* ID	Description	Length	E-value
1.	jgi|Phyra1_1|78800|	RXLR-class effector Avh247	127	4.3E-88
2.	jgi|Phyra1_1|83417|	RXLR-class effector Avh108	138	1.6E-6
3.	jgi|Phyra1_1|79106|	RXLR effector	122	7.2E-23
4.	jgi|Phyra1_1|85154|	RXLR effector	182	1.8E-5

(a)

S.N.	*Phytophthora sojae* ID	Description	Length	E-value
1.	jgi|Physo1_1|139461|	RXLR effector family	190	4.4E-139
2.	jgi|Physo1_1|140615|	RXLR effector Avh205	119	7.4E-82
3.	jgi|Physo1_1|140618|	RXLR effector Avh205	127	3.2E-88

(b)

In this study, we have also investigated the occurrence of effector proteins clusters in the two species as represented in figure 3. The result depicts the presence of 129 in-paralogs from *P. ramorum* and *P. sojae* each. Also, 102 groups of orthologous have been predicted. The orthologous proteins clusters observed in the current study points towards the fact that *Phytophthora* spp. evolved through a remarkable degree of convergent evolution[32].

Conclusion

Computational analysis and manual evaluation of the secretome of *Phytophthora* spp. could provide researchers with a comprehensive set of secretory proteins. Nevertheless, detailed studies and experimental validation of the predicted proteins will definitely enable an understanding of the molecular basis of host-pathogen interaction. Furthermore, identification of critical avirulence factors and elucidation of their functions promises to provide insight into plant defense mechanisms and to create a basis for the development of associated and improved strategies for the control of plant disease.

Most importantly, in this study we have reported host - specific effector proteins which are species-specific and lack similarity to known proteins which will prove to be potential target for pathogen control. With the current study, we would like to anticipate that the predicted data will provide a basis for the development of improved disease control strategies and promote research in this indispensable and complex area.

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