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Comparative secretome analysis of Indian wheat leaf rust pathogen *Puccinia triticina*

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ABSTRACT

The secretome of two races, 77-5 and 106 of wheat leaf rust pathogen *Puccinia triticina* with known virulence and avirulent trait, respectively were analyzed in this study. The secretome analysis revealed 546 putative secretory proteins (PSPs) present in the race 77-5, and 481 PSPs in race 106. Race-specific PSPs analysis also showed that race 77-5 had higher number of PSPs (19.72%) in comparison to race 106. Various other gene families like pathogenicity and virulence factor were also expanded in higher number in the race 77-5 and few of them having multiple domains which are known for pathogenicity, were exclusively present in this virulent race. The candidate secretory effector proteins (CSEP) analysis also showed that the virulent race contained 8.98% higher CSEP proteins compared to the avirulent race 106. The results suggest that these genes are playing important roles in their respective race-specific manner. Surprisingly the carbohydrate metabolism-related enzymes were found 5.26% higher in the avirulent race in comparison to the virulent one, and few of them have shown race specificity. The *in-silico* expression analysis of the selected candidate's genes also revealed their role in the pathogenesis process. The three dimensional (3D) structure predictions were performed for few of the candidate genes that were highly expressed. In this comparative secretome analysis, our findings provide a baseline for the characterization of effectors and avirulence factors in different races of *P. triticina*.

Key words: Effector proteins, Puccinia, Rust pathogen, Secretome, Wheat

Wheat (*Triticum aestivum*) is one of the top most produced and consumed crops in India as well as across the globe. Despite being one of the important agricultural crops, a large fraction of it get lost because of various biotic and abiotic stresses. Among biotic stresses, damages caused by pathogens are one of the leading causes of yield loss. Among the pathogens, the most notorious organism causing infection to wheat are obligate biotrophs such as the powdery mildew *Blumeria graminis* and the rust pathogens, *Puccinia* species. However, the yield loss caused by rust pathogens is much higher and covers a larger area of the world than others (German *et al.* 2007, Herrera-Foessel *et al.* 2011). Among wheat rust pathogens, leaf rust, also known as *Puccinia triticina* is one of the most widespread and destructive pathogens as compared to the other wheat pathogens, viz. *Puccinia striiformis* and *Puccinia graminis* (Thind 1998, Draz *et al.* 2015). In India, primarily two *P. triticina* races, i.e. race 77 and 106 are reported to be prevalent in wheat-growing regions (Bhardwaj 2013). The majority of infection in Indian wheat varieties is caused by race 77 and its different pathotypes. Until now, 13 different pathotypes have been reported for race 77 and therefore regarded as virulent race of *P. triticina* (Nayar *et al.* 1996, Bhardwaj 2011). In contrast to this, the race 106, which has no known pathotypes till date, is considered as an avirulent race (Bhardwaj 2013).

In view of the above, the present study was planned to find the differences in the race 77-5 and race 106 of *P. triticina* by exploiting genome-wide secretory proteins. The presence/absence, expansion as well as expression analysis of the selected candidates can shed light on the virulence spectrum of both races, viz. race 77 and race 106.

MATERIALS AND METHODS

Data Set: The predicted proteomes of the *P. triticina* race 77-5 and race 106 were utilised from the data generated by Kiran *et al.* (2016). The analysis was performed in the

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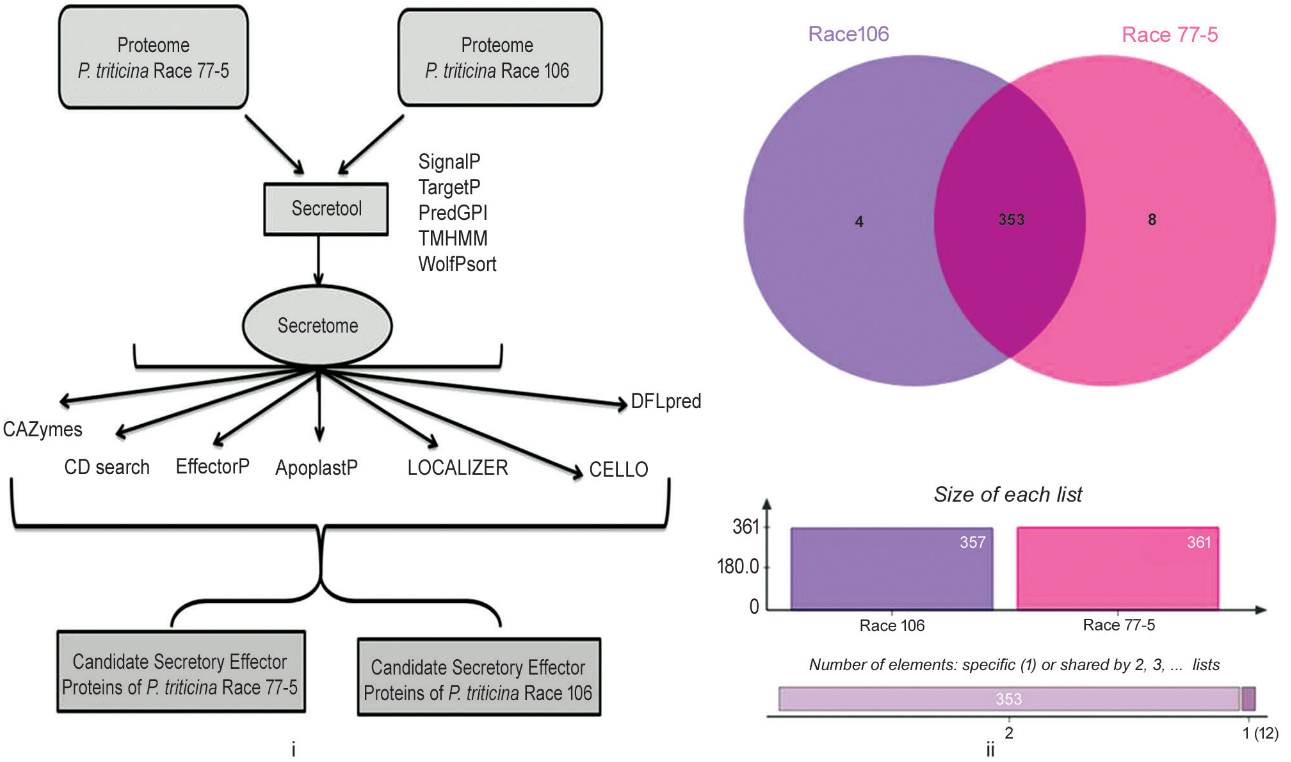

Fig 1 Secretome and orthologs analysis. (i) A pipeline used in genome-wide identification of secretory proteins; (ii) The orthologous gene identification in virulent 77-5 and avirulent 106 races.

year 2019 at National Agri-Food Biotechnology Institute (NABI), Mohali, Punjab.

Secretome and orthology analysis: The secretome analysis was done by the software Secretool using default parameters (Cortazar *et al.* 2014). The CD-search was used to find the conserved domains in the putative secretory proteins (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The nonspecific hits obtained from the CD search were removed manually. The orthologous genes identification was done using Orthovenn software (http://www.bioinfogenome.net/OrthoVenn).

Effector, subcellular localization, intrinsic domain and Cell Wall Degrading Enzymes Prediction (CWED): The putative effector, apoplastic localisation and intrinsic domain-containing proteins were identified by using the Effector P, ApolplaticP and DFLpred tools, respectively (Meng *et al.* 2016, Sperschneider *et al.* 2016, 2018). The subcellular localisation, nuclear localization sequences (NLs) and transit peptides were predicted using CELLO and Localizer tools (Yu *et al.* 2006, Sperschneider *et al.* 2017). The carbohydrate active enzymes were identified using dbCAN server (http://cys.bios.niu.edu/dbCAN2).

Expression analysis and 3D structure prediction: The CLC Genomics v. 12 was used for expression analysis of pathogenicity genes as well as species-specific genes. In this analysis, the data was taken from the publically available NCBI SRA database (PRJNA393407). The heatmap using the expression values was plotted using the Multiple Experiment Viewer (MEV) (http://en.bio-soft.net/chip/MeV.html). The 3D structure prediction was done using the I-Tasser server using ab initio method (Yang *et al.* 2015).

RESULTS AND DISCUSSION

Genome-wide identification of secretory proteins and their orthologs analysis: The secretool software uses a combined pipeline that contains essential tool like SignalP, TMHMM, PredGPI and WolfPsort were employed for the genome-wide identification of the secretory proteins in both the races 77-5 and 106 (Fig 1). The analysis revealed that race 77-5 has 546 putative secretory proteins (PSPs), while race 106 has 481 proteins. The orthologs analysis of these PSPs showed that both the races form 353 common clusters, whereas 8 and 4 clusters were unique to race 77-5 and 106, respectively. Further analysis of unique clusters disclosed that 77-5 contained 170 proteins as single copy presence in the genome however, race 106 had only 114 single copy proteins existence in the genome. Majority of the species-specific proteins did not show any Pfam hit hence were called as novel ones. The presence of less number of PSPs as well as race-specific proteins in the race 106 correlate with avirulent nature of the pathogen when they were compared with virulent race 77-5 (Table 1). The characteristic features of most of the potential candidate effector proteins were reported to be the species-specific nature or uniqueness to a specific race. Such race-specific proteins have already been known to play a role in the disease development (Stergiopoulos I and de Wit P J 2009). Similarly, we also found some race-specific proteins in both the races, with varying numbers, however higher numbers of these proteins present in virulent race 77-5 suggests its

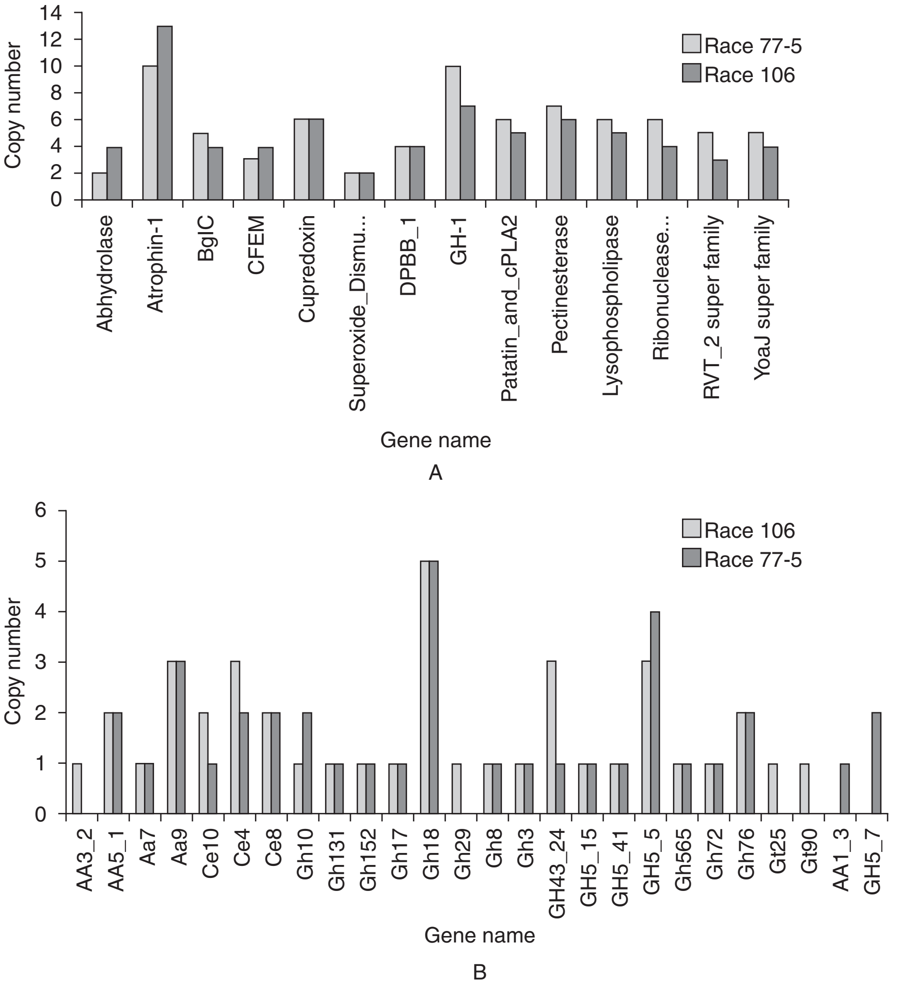

Fig 2 (A) Cell wall degrading enzymes Prediction (CWED); (B) major domains present in virulent and avirulent races of *P. triticina.*

pathogenicity advantages over the avirulent race 106.

In silico characterization of candidate effector molecules: The effector P analysis showed that race 77-5 has 196 effector proteins with a high score (>0.7) compared to race 106 having only 164 effectors. Moreover, the results obtained for the apoplastic and intrinsic protein analysis was consistent with Effector P results and showing higher proteins for race 77-5 than race 106 (Table 1). In contrast to the higher number of secretory proteins and race-specific proteins present in race 77-5, we found a higher number of the cell wall degrading enzymes in race 106 (Fig 2). Although race 106 is reported to be less virulent than race 77-5, it still carries pathogenicity genes, which are essential for its survival and mild disease response.

Identification of novel secretory proteins: To gain more insight into secretome, we looked into the common as well as a unique set of proteins reported to be involved in pathogenicity and virulence from other pathogens. The predominant domains are shown in Fig 2A. Among the NCBI CD searched proteins, the atrophin protein was found to have maximum hits in race 106 (13 copies) compared to the race 77-5 (10 copies). The atrophins are serine repeat-containing proteins having alternate basic and acidic residues. The exact functions of atrophins have not been yet understood. However, many studies showed that these proteins might be behaving like transcriptional co-repressor (Wood *et al.* 2005). The ribonuclease proteins are reported to be present in effector proteins in *Blumeria graminis* that interferes with mRNA degradation pathways (Pilego *et al.* 2013, Schafer *et al.* 2017). In race 77-5, 6 RNaseH like proteins and 5 reverse transcriptases were presents. In contrast to this, race 106 had only three reverse transcriptases and 4 RNaseH like proteins. The presence of two additional secretory reverse transcriptase genes in race 77-5 might be one of the unique strategies utilized by the race 77-5 to tackle host defence. In addition to these genes, few others like Pectinesterases, Patatins and Lysophospholipase were also found in higher number in race 77-5 than that of the present in race 106. The presence of the high number of these cell wall degrading enzymes could be possibly associated with the detection mechanism of the host plant, as various studies have reported that the Glycosyl Hydrosyl (GH) and other cell wall degrading enzymes may be a potential pathogen-associated molecular pattern (PAMP) (Fig 2 B). For example, the Glycosyl Hydrosyl 12 (GH12) of oomycetes pathogen, *Phytophthora sojae* is known to activate innate immunity when expressed in soybean as well as in tobacco cells (Ma *et al.* 2015).

Besides the common domains containing proteins, obtained in the CD search, in both the races, the only unique proteins present in race 77-5 as well as in race 106 are given in Table 2. The reprolysin (M12B) family

Table 1 Characteristics of putative effector proteins in virulent and avirulent races of *P. triticina*

Race	77-5	106
Classically secretory proteins	546	481
Non-classically secreted proteins	12342	7781
Race-specific proteins	170	114
Effector proteins	196	164
Apoplastic proteins	237	197
Extracellular	388	326
NLS and transit peptide	167	164
Intrinsic domain	72	66
CWED	36	40

Table 2 Selected race-specific secretory proteins present in *P. triticina* Race 77-5 when compared to Race 106

Gene Race 77-5	Protein Family	Function	Copies	Size in kDa	Isoelectric Point
Pt_77_5_contig05782_5_4	Reprolysin (M12B) family zinc metalloprotease	Endopeptidases	1	97.09	5.09
Pt_77_5_contig01183_2_5	Rax2 superfamily	Cell polarity	2	155.5	5.32
Pt_77_5_contig14077_1_1	Cyclases	Biosynthesis of lantibiotics	1	10.7	4.5
Pt_77_5_contig00596_3_10	Cytokinin-bind superfamily	Cytokinin degradation	1	51.25	9.29
Pt_77_5_contig03224_2_4	Protein kinase	Signaling	1	19.34	6.20
Gene Race106					
pt_106_contig09324_1_9	SugarP_isomerase	Sugar phosphate isomerization	1	95.52	7.91
pt_106_contig04720__16	Pectinesterase super family, ATP synthase delta (OSCP) subunit	Energy production and conversion. Cell wall degradation	1	79.25	9.36
pt_106_contig04014_7_7	FKBP superfamily	Peptidyl-prolyl cis-trans isomerization	1	15.96	6.77

zinc metalloprotease are zinc-dependent peptide cleaving proteins that are generally secreted as a proenzyme. The members of this family are also known as admalysins and reported to present in snake venom and few proteins of mammals like fertillin (Rawlings *et al.* 1995, Seals and Courtneidge 2003). In tick, these play an important role in host-pathogen interaction, by degrading the fibrinogen and gelatin as well as altering the homeostasis. One more candidate cyclases were present exclusively in race 77-5. The cyclases are involved in the production of lantibiotics that have antimicrobial activities; however, these lantibiotics are reported to be produced by bacterial species only (LI *et al.* 2006, Nair *et al.* 2017). The expression analysis using transcriptomic data also supported the presence of cyclases gene in the infection cycle. A cytokinin degradation gene family member was also present in unique genes of race 77-5. In plants, cytokinin plays multiple crucial roles ranging from cell growth to responding to various stresses including biotic stresses. Manipulation of cytokinin level has been reported as one of the mechanisms to tackle host defense in various pathosystem (Kazan and Lyons 2014). Some pathogens, for example, *Pseudomonas syringae* affect the cytokinin signalling pathways, while some others manipulate the level of cytokinin by de novo synthesizing or by degradation.

In fungal pathogens like *Magnaporthe oryzae* (Jiang *et al.* 2013, Chanclud *et al.* 2016) *Cladosporium fulvum* (Murphy *et al.* 1997) and *Ustilago maydis* (Bruce *et al.* 2011), cytokinin has been reported to be synthesized *de novo*. Moreover, few studies have reported that the cytokinin inducing proteins of fungi are known as effector proteins (Chanclud *et al.* 2016, Hinsch *et al.* 2016). One of the cytokinin producing genes of *M. oryzae* is designated effector as it plays an important role in manipulating the host defense system (Chanclud *et al.* 2016). Some of the mycorrhizal fungi, such as *Rhizopogon ochraceorubens* too express cytokinin-producing candidates (Miller 1967, Crafts and Miller 1974) to hijack the defence signalling pathways in the host plant (Miransari *et al.* 2011)

The 3D structure prediction and expression analysis: The expression analysis of various pathogenicity and effectors of race 77-5 showed numerous genes expressed at the time of infection. However, the maximum genes expression was observed for Pt_77_5_contig19561_6_5 (Atrophin), pt_77_5_contig19295_2_5 (Lytic transglycolase), pt_77_5_contig25542_5_10 (GH1) andpt_77_5_contig19576_4_10 (Pectinesterase) (supplementary Fig 1). In addition to this, 11 identified effectors in race 77-5 showed expression in both resistance and susceptible transcriptomic data, suggesting the role of these genes in plant-pathogen interaction. For 3D structure prediction, all four candidates with high expression mentioned above (Atrophin gene, Lytic transglycolase, GH1 and Pectinesterase) were used (Supplementary Fig 2).

The identification of various secretory proteins and virulence factor in pathogens can shed light on various strategies adopted by pathogens to disarm plant defence system. In case of *P. triticina*, races 77-5 and 106 that harbour contrasting virulence features and could be used for a better molecular understanding about the race-specific virulence spectrum in the biotrophic pathogens. Presence of unique genes in virulent race 77-5 and their absence in avirulent race 106 could be potential pathogenesis associated candidates in race 77-5 however, vice versa of this could be the source of avirulence candidate genes in race 106.

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