Whole Genome Enabled Phylogenetic and Secretome Analyses of Two *Venturia nashicola* Isolates

Maxim Prokchorchik ^{1†}, Kyungho Won^{2†}, Yoonyoung Lee ¹, Cécile Segonzac ^{3,4}, and Kee Hoon Sohn ^{1,5*}

¹Department of Life Sciences, Pohang University of Science and Technology, Pohang 37673, Korea

²National Institute of Horticultural and Herbal Science (NIHHS), Rural Development Administration (RDA), Naju 58216, Korea

³Department of Plant Science, Plant Genomics and Breeding Institute and Research Institute of Agriculture and Life Sciences, College of Agriculture and Life Sciences, Seoul National University, Seoul 08826, Korea

⁴Plant Immunity Research Center, College of Agriculture and Life Sciences, Seoul National University, Seoul 08826, Korea

⁵School of Interdisciplinary Bioscience and Bioengineering, Pohang University of Science and Technology, Pohang 37673, Korea

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Venturia nashicola is a fungal pathogen causing scab disease in Asian pears. It is particularly important in the Northeast Asia region where Asian pears are intensively grown. *Venturia nashicola* causes disease in Asian pear but not in European pear. Due to the highly restricted host range of *Venturia nashicola*, it is hypothesized that the small secreted proteins deployed by the pathogen are responsible for the host determination. Here we report the whole genome based phylogenetic analysis and predicted secretomes for *V. nashicola* isolates. We believe that our data will provide a valuable information for further validation and functional char-

acterization of host determinants in *V. nashicola*.

Keywords : effector analysis, phylogenetic analysis, *Venturia nashicola*

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[†]These authors contributed equally to this work.

*Corresponding author.

Phone) +82-54-279-2357, FAX) +82-54-279-2199

E-mail) khsohn@postech.ac.kr

ORCID

Maxim Prokchorchik

<https://orcid.org/0000-0001-9118-6450>

Yoonyoung Lee

<https://orcid.org/0000-0002-5080-7633>

Cécile Segonzac

<https://orcid.org/0000-0002-5537-7556>

Kee Hoon Sohn

<https://orcid.org/0000-0002-9021-8649>

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Venturia nashicola is a member of *Venturiaceae* family that includes several important fungal pathogens of plant *Rosaceae* family, such as *Venturia inaequalis*, *V. pyrina*, and *V. carpophila* (Ishii and Yanase, 2000; Tanaka and Yamamoto, 1964). *Venturia nashicola* causes scab disease in Asian pears and is considered as a serious threat to the Asian pear industry. So far there is no scab disease resistant Asian pear cultivar available to growers (Ishii and Yanase, 2000; Park et al., 2000). Even though some agro-materials were shown to suppress the growth and sporulation of *V. nashicola*, in the field conditions, this disease is controlled by multiple fungicide applications in each growing season (Park et al., 2000; Song and Seo, 2018). Recently, emergence of several cases of fungicide resistance development was reported for *V. nashicola* (Ishii, 2012; Kwak et al., 2017). This emphasizes the need for developing scab resistant Asian pear cultivars and better understanding of the interactions between pear and *V. nashicola*.

There are several members of *Venturiaceae* family known as scab-causing pathogens including *Venturia inaequalis*, *V. nashicola*, *V. pyrina*, *V. carpophila*, and *V. effusa* (Gonzalez-Dominguez et al., 2017). Interestingly, these

species have distinct and narrow host range (Sivanesan, 1977). *Venturia inaequalis*, infecting apple but not pear, is the most studied species of *Venturiaceae* family (Bowen et al., 2011; MacHardy, 1996). Several genome analyses have been reported in recent years, providing a platform for development of scab resistant apple cultivars (Deng et al., 2017; Passey et al., 2018; Shiller et al., 2015). In contrast, significantly less effort was given on other *Venturiaceae* family members. Recently, draft genome sequence of *Venturia pyrina* which infects European pear but not Asian pear has been published (Deng et al., 2017). In addition, draft genomes of the peach scab causing fungus *V. carpophila* and pecan infecting *V. effusa* have been reported (Bock et al., 2016; Chen et al., 2017). More recently, genome sequences of several *V. nashicola* isolates have been presented (Johnson et al., 2019; Prokchorchik et al., 2019).

Plant pathogens deliver specific effector proteins into the host plant cells in order to promote virulence. On the other hand, plants have evolved specific immune receptors to recognize corresponding effector proteins directly or indirectly, and activate disease resistance (Jones and Dangl, 2006). To date, many effectors were discovered using genetic map-based cloning approach and proteome analysis in fungal and oomycete plant pathogens (Selin et al., 2016; Uhse and Djamei, 2018). In addition, recent progress on plant pathogen genomics enabled researchers to accelerate prediction of effectors. For example, comparative genom-

ics approach helped to identify the Ave1 effector from a fungal pathogen *Verticillium dahliae* that is recognized in tomato plants carrying *Ve1* disease resistance gene (de Jonge et al., 2012).

In *V. inaequalis*–*Malus* pathosystem, 17 genetically characterized disease resistance loci were reported (Bus et al., 2011). Of these, two resistance loci, *Rvi6* and *Rvi15*, were studied in detail but the avirulence determinants from *V. inaequalis* have not been identified to date (Joshi et al., 2011; Schouten et al., 2014; Vinatzer et al., 2001). The genetic basis of scab resistance in Asian and European pears has not been fully established, although some molecular markers linked to scab resistance have been developed (Bouvier et al., 2012; Won et al., 2014).

In this study, we used the whole genome sequences of several major *Venturiaceae* species to reconstitute the phylogenetic relationships between them. Furthermore, we predicted the small secreted proteins that may function as virulence or avirulence effectors during host plant infection. The data presented in this study would be valuable to better understand the host-specific virulence mechanism of *V. nashicola*.

Previously we have sequenced and annotated the genomes of two *Venturia nashicola* isolates, MAFF615029 and PRI2 collected from Japan and Korea, respectively (Prokchorchik et al., 2019). To define their phylogenetic relationships to the other members of *Venturia* genus, we

Fig. 1. Phylogenetic tree representing the relationships in the *Venturia* genus. Protein sequences of all the translated genes predicted in the strains were clustered and total 6,650 conserved cluster trees were used to build a majority rule consensus tree with 70% branch support threshold. Bootstrap branch support is provided as node labels.

aimed to construct a phylogenetic tree consisting of the strains for which whole genome sequence is available. As our study focuses on very closely related *Venturia* isolates we aimed to use the whole genome level data in order to be able to reliably resolve the closest relatives, which is in contrast to limited gene set alignments used previously for phylogenetic analysis (Yun et al., 2013; Zhao et al., 2012). We used whole genome predicted protein sets of 10 *Venturia* isolates for constructing phylogenetic tree. The protein sets were either retrieved from Mycocosm database (<https://genome.jgi.doe.gov/mycocosm/home>) for *V. pyrina* ICMP11032 and *V. inaequalis* ICMP13258, or generated in-house using Fungap pipeline applied to genome sequences of *V. carpophila* JP3-5 (NCBI BioProject: PRJNA321389), *V. effusa* 3Des10b (NCBI BioProject: PRJNA285422), *V. inaequalis* 1389 (NCBI BioProject: PRJNA261633), *V. inaequalis* 1639 (NCBI BioProject: PRJNA261633), *V. inaequalis* EU-B04 (NCBI BioProject: PRJNA407103), and *V. inaequalis* 05/172 (NCBI BioPro-

ject: PRJNA354841). Protein sequences of all the genes annotated in the *Venturia* isolates were clustered using OrthoMCL v2.0.9 software (Li et al., 2003) with BLAST e-value 10^{-7} . Only clusters conserved in all the isolates were considered for phylogenetic analysis (total 6,650 clusters). Hidden Markov Model (HMM) profiles of each gene cluster were built using HMMER3 v3.2.1 (Mistry et al., 2013). These were then compared between strains and neighbor-joining phylogenetic trees were built based on these comparisons using the pHMM-Tree (Huo et al., 2017). Each tree was bootstrapped 100 times and the best tree was used for further analysis. All cluster trees were used to build a majority rule consensus super tree with 70% branch support threshold using Geneious Prime 2019.04 (Fig. 1). Similar to recent studies (Yun et al., 2013; Zhao et al., 2012), the five *V. inaequalis* isolates were grouped together and *V. nashicola* isolates analyzed in this study showed close relationship to *V. pyrina* (Fig. 1). These data indicate that *V. pyrina* and *V. nashicola* form a separate clade from

Fig. 2. Whole genome comparison of *Venturia nashicola* PR12 and MAFF615029 confirms their close relation to each other. (A) Small genetic variants analysis shows that deletions are the most common of small genetic variants. Analysis was performed using NucMer and visualized by Assemblytics. (B) Larger genetic variants are abundant to similar extent. Analysis was performed as described in A. (C) Whole-genome comparison of assembled contigs of *V. nashicola* isolates reveals no large genomic re-arrangements. Genomes were analyzed with Sibelia and results were visualized with Circos.

V. inaequalis isolates (Fig. 1).

In order to further confirm the genetic relatedness of *V. nashicola* MAFF615029 and PRI2 isolates, we performed a whole genome comparison. We first aimed to discover single nucleotide polymorphisms (SNPs) using Snippy (v. 4.3.8) (Seemann, 2015). To begin with, comparison between *V. nashicola* MAFF615029 and PRI2 only was run, then Snippy was run in pairwise manner comparing different *Venturia* isolates to *V. nashicola* MAFF615029. Finally, snippy core command was used to call the core SNPs between all the strains compared. We could identify total 105,564 SNPs when comparing the MAFF615029 and PRI2 isolates only. To compare the amount of SNPs between more distant relatives of *V. nashicola* we generated the core SNPs shared in all the genomes compared to *V. nashicola* MAFF615029. We could identify 542 core SNPs when comparing MAFF615029 and PRI2, 14,304 SNPs when comparing *V. nashicola* MAFF615029 and *V. pyrina*, 35,549 SNPs when comparing *V. nashicola* MAFF615029 and *V. inaequalis* ICMP13258, 46,735 SNPs when comparing *V. nashicola* MAFF615029 and *V. effusa* and 48,263 SNPs when comparing *V. nashicola* MAFF615029 to *V. carpophila*. These results confirmed the close relation of *V. nashicola* strains between each other suggested by our phylogenetic analysis.

We further performed structural genome variations discovery in PRI2 genome in comparison to MAFF615029 isolate. We used NucMer with following parameters ‘-maxmatch -l 100 -c 500’ (Delcher et al., 2002) and then visualized using Assemblytics (Nattestad and Schatz, 2016). We could identify 777 significant variants. Majority of them were small, mostly 50-500 nucleotides (nt) deletions and, interestingly, insertions, deletions, repeat expansions and contractions were represented relatively equally among more significant (500-10,000 bp) variants (Fig. 2A and B). However, this could be the consequence of short-read assembly of PRI2 isolate genome.

Next, we analyzed *V. nashicola* MAFF615029 and PRI2 genomes for larger genomic re-arrangements. For that we used Sibelia with following parameters ‘-s fine -q -v --gff --correctboundaries’ (Minkin et al., 2013) and visualized the results using Circos (Krzywinski et al., 2009). There were no large genomic rearrangements detected inside the large scaffolds (Fig. 2C). Overall the genomes surveyed show very high similarity to each other and most of the differences are located in the unaligned short scaffolds. For example, there are 21 predicted genes in *V. nashicola* PRI2 and there are 586 predicted genes in *V. nashicola* MAFF615029 located in such scaffolds.

Bioinformatic prediction of effectors may help to iden-

Fig. 3. A schematic representation of the effector prediction pipeline used in this study. The descriptions and sources of these programs are indicated in main text.

tify the virulence and avirulence determinants in fungal species. We used a pipeline for effector annotation based on multiple criteria outlined in Fig. 3. Predicted genes were translated and checked for the presence of signal peptide using SignalP v4.1 (Nielsen, 2017). Proteins with predicted signal peptides were further surveyed for the absence of transmembrane helices using TMHMM (Krogh et al., 2001). All the proteins longer than 400 AA were removed and the short-listed proteins were further assessed for potential targeting in the plant cell using TargetP (Emanuelsson et al., 2007). Resulting set of proteins was subjected to several analyses to score and prioritize effector candidates. In short, the short-listed candidates were subjected to EffectorP2 analysis (Sperschneider et al., 2018) and each effector candidate was assigned a score between 0 and 10 based on the effector prediction confidence. Some fungal and oomycete effectors were shown to be localized to the plant cell nucleus (Ahmed et al., 2018; Vargas et al., 2016) and therefore the short-listed candidates were evaluated for the presence of nuclear localization signal (NLS) using NLStradamus (Nguyen Ba et al., 2009), based on the NLS prediction confidence a score from 0 to 5 was assigned. In

Fig. 4. Effector repertoires of *Venturia nashicola* isolates in comparison to *V. inaequalis* and *V. pyrina*. (A) Effector families presence overlap between *V. nashicola* PRI2 and MAFF615029 isolates able to infect Asian pear cultivars. (B) Effector families presence overlap between *V. nashicola* PRI2, MAFF615029 isolates which are able to infect Asian pear cultivars and *V. pyrina* ICMP11032 able to infect European pear cultivars. (C) Effector families overlap between *V. nashicola* PRI2 and MAFF615029 isolates which are able to infect Asian pear cultivars and *V. pyrina* ICMP11032 and *V. inaequalis* ICMP13258, infecting European pear and apple respectively. (D) Table representing the presence and absence of putative effector families in *Venturia nashicola*, *V. pyrina* and *V. inaequalis* isolates. Green or red color indicates presence or absence of effector gene, respectively. Purple color indicates the presence of multiple orthologous effector genes. The putative effector families that are commonly present in all 4 isolates are not shown.

addition, short-listed proteins were blasted against Fungal Stress Related Database (FSRD) (Karányi et al., 2013) and PHI-base (Pathogen-Host Interaction database) (Urban et al., 2017) databases. Depending on BLAST hit coverage and similarity level a score from 0 to 10 was assigned for comparison with each of two databases. Finally, short-listed proteins were assessed for the known fungal/oomycete effector motifs, such as RxLR, DEER, RxLX[EDQ], [KRHQA][DENQ]EL, [Y/W]xC, RSIVEQD (Sonah et al., 2016) using a custom python script and a score from 0 to 6 was assigned. A sum of individual tool scores was used as an integrated effector prediction confidence score and was used to rank the effector candidates in a final short-list. Effector candidates with overall score 0 were discarded from the final short-list. As the results we

identified the following numbers of potential effectors in *Venturia* strains analyzed: *V. nashicola* MAFF615029, 451; *V. nashicola* PRI2, 424; *V. pyrina* ICMP11032, 477; and *V. inaequalis* ICMP13258, 673 (see Supplementary Tables 1-4, Supplementary FASTA-files 1-4). We focused on these 4 representative isolates in order to identify the putative effectors that are specific to the *Venturia* species infecting European pear, Asian pear or apple. The putative effectors were clustered using OrthoMCL v2.0.9 with BLAST e-value of 10^{-7} and resulting effector families and single effector genes were assessed for presence or absence in the *Venturia* strains. We identified the core effector repertoire of two *V. nashicola* strains as 353 conserved effectors (Fig. 4A). When we expanded effector comparison to include two strains of *V. nashicola* and one *V. pyrina* strain,

we could identify 235 core effectors. In addition to core effectors we identified 118 potential effectors specific to *V. nashicola* strains and 183 potential effectors specific to *V. pyrina* (Fig. 4B). These putative effectors may act as host-range determinants between *V. nashicola* and *V. pyrina* enabling an avirulent recognition in non-host plant species. Finally, we expanded the effector comparison to include *V. inaequalis* ICMP13258 putative effectors. In this case we could identify 150 prospective core effectors for *Venturia* genus and 82 effector families specific for *V. nashicola* strains infecting Asian pear (see Supplementary Table 5), 134 effector families specific for *V. pyrina* infecting European pear (see Supplementary Table 6) and 200 effector families specific for *V. inaequalis* infecting apple (Fig. 4C, Supplementary Table 7). Overall presence and absence patterns of effector families in repertoires of *V. nashicola*, *V. pyrina* and *V. inaequalis* seem to correlate with the host range of the pathogen (Fig. 4D).

Based on our sequencing data we were able to analyze the phylogeny of representative isolates of *Venturia* genus using the whole genome supertree approach. We also employed the information from multiple gene clusters using the HMM profile comparison instead of protein alignment of single copy genes. Overall, our phylogenetic tree supports the phylogenetic analysis reported previously (Yun et al., 2013; Zhao et al., 2012). We also confirm a close relation between *V. nashicola* and *V. pyrina* species.

Fungal effector prediction is one of the most challenging tasks in contemporary plant pathology research. Availability of whole genome assemblies and utilizing a range of computational tools should increase the effector prediction sensitivity. Here we used integrated approach for identifying effectors where results of different analytic tools are evaluated to rank and shortlist the effector candidates for further analysis. We analyzed the presence of effector families in various *Venturia* species infecting different plant hosts. This provides a valuable resource for future research aiming to confirm effectors that play important roles in host specialization of *Venturia* species.

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Electronic Supplementary Material

Supplementary materials are available at Plant Pathology Journal website (<http://www.ppjonline.org/>).

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