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Secreted In Xylem (SIX) genes across isolates of flax pathogenic ascomycete *Fusarium oxysporum* f. sp. *lini*

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Motivation and Aim: Secreted In Xylem (SIX) genes include 14 unrelated families of small cysteine-rich secreted proteins (also called effectors), originally found in xylem sap of plants severing from fusariosis, and occurred exclusively in pathogenic strains of *Fusarium oxysporum* [1]. SIX genes are used by fungus to interact with different molecules in different pathways of plant immunity to prevent immune response [2]. These 14 SIX genes families are grouped in different ways in various special forms and even within one form [3]. It was shown that deletion of some SIX genes leaded to reduced virulence on host plant, unlike our knowledge about structure and function of the most SIX genes are still remained elusive [4]. First, we aimed to investigate repertoire, structure and phylogeny of SIX genes in 13 isolates of *F. oxysporum* f. sp. *lini* (*Folini*) with different virulence to flax, which genomes published earlier [5–7]. Then, we tried to determine whether SIX genes taking part in progression of fusariosis infection on flax or not, and how they are influenced on fungal virulence and pathogenicity.

Methods and Algorithms: SIX genes in *Folini* genomes were detected by homology search with ncbi blast+ toolkit. introns and exact gene borders in the genomes were analyzed with MIT GENSCAN web server and FindORF tool [8]. ML phylogeny was created with IQ-TREE 1.6.12 wherefore the best fitted model was predicted for our dataset by implemented ModelFinder [9]. According to its prediction we chose Tamura–Nei model and then constructed ML tree with non-parametric UltraFast Bootstrap algorithm [10]. Genomic DNA of *Folini* isolates for PCR was extracted with DNeasy Plant Kit (QIAGEN), while total RNA was obtained with RNeasy Kit (QIAGEN). Fungal protoplast preparation and transformation with pCT74 vector performed according to [11] with some modifications.

Results: Firstly, we established a repertoire of SIX gene families in thirteen genomes of *Folini* by homology search with known sequences of SIX genes from other special forms. We found that eleven genomes had similar repertoire and consisted of *SIX1*, *SIX7*, *SIX10*, *SIX12* and *SIX13* families. We could not find any SIX genes in other two genomes (F365 and F482), that was a surprise. After that, we validated SIX genes repertoire by PCR analysis on presence/absence of their sequences in genomic DNA. The PCRs confirmed our genomic search.

As SIX genes cannot be predicted by Augustus, we aimed to obtain exact gene borders, gene sequence and coding sequence of each SIX gene copy found in genomes by

homology search. *SIX1* of *Folini* did not have any paralogous sequences and always presented as a single-copy gene in each studied genome. Six1 proteins had 277 a. a. length and was identical in all genomes except F418 and F456. Based on the differences in length and polymorphic loci within sequences between the genomes we assigned copies from chromosome 12 as *SIX7a*, and as *SIX7b* copies found on chromosome 15. *SIX10* gene in *Folini* genomes always situated on chromosome 12 and usually presented in one intact copy of 619 bp length. Structural analysis of *SIX10* reveals a single large conserved intron of 166 bp length. *Folini SIX12* orthologs can be grouped in two sequence variants of which *SIX12a* are present on both chromosomes 12 and 13, while *SIX12b* was found only on chromosome 13. *SIX13* gene sequence was the largest among other *Folini* SIX genes with estimated length 1275 bp, did not have paralogs in individual genomes, always occurred on chromosome 12, and had two large introns (168 bp and 160 bp). Another interesting finding was the presence of approximately one-half of *SIX8* gene sequence (493 bp of 1010 bp complete cds) on chromosome 13 in *Folini* genomes. Unfortunately, we could not find any correlation between SIX genes features typical for a given isolate and its virulence.

Interestingly, that genes *SIX7a*, *SIX12a* and *SIX13* formed a mini-cluster on chromosome 12 in MI39 genome, and the same cluster we found on chromosome 12 in F418 genome. Such mini-cluster were previously detected in Fol4287 isolate of *F. oxysporum* f. sp. *lycopersici* [12].

On the *SIX1* gene phylogenetic tree we observed clustering of *Folini* strains, surprisingly together with *F. oxysporum* f. sp. *cubense* (*Focub*) strain. Moreover, the clade which contains *Folini* also is settled by two separate subclades containing the rest of *Focub* and *F. oxysporum* f. sp. *canariensis* members that infect banana (*Musa* spp.) and canary palm (*Phoenix canariensis*), respectively. Phylogenetic inference on *SIX7* genes in *Folini* demonstrated differences between *SIX7a* and *SIX7b* sequence variants, since they occupy very distinct clades on the phylogenetic tree. On the *SIX10* gene phylogenetic tree we observed that all *Folini* strains were grouped into a separate clade distinct from other formae speciales. *SIX12* phylogeny clearly showed the divergence of *SIX12a* and *SIX12b* sequence variants which were placed into the separate clades in the phylogenetic tree. Interestingly, *SIX12b* clade also includes at least two members of *F. oxysporum* f. sp. *pisi*, that reaffirms the hypothesis of the horizontal gene transfer event between these formae speciales, which possibly took place in a recent past. Phylogenetic inference on *SIX13* gene demonstrated that *Folini* orthologs formed a distinct clade close to the clades consisted of *F. oxysporum* f. sp. *fragariae* and *F. oxysporum* f. sp. *niveum*, members of that causing severe on strawberry (Rosaceae) and watermelon (Cucurbitaceae), respectively.

To investigate whether SIX genes are involved in the virulence and could play a role during flax infestation process, we performed differential expression gene analysis from RNA-seq data in MI39 strain and validated it by RealTime-PCR. No expression of any studied SIX genes were found in mycelial culture. The most differentially expressed genes were *SIX1* and *SIX13*, and they also have the highest level of expression compare to other SIX genes. Expression in susceptible flax cultivar LM-98 began earlier than in resistant cultivar Atalanta: on third- and fifth-day post inoculation, respectively.

To further investigate role of SIX genes during plant tissue colonization we aimed to create mutant MI39 strain with deletion of *SIX13* gene, as one of the strongest differentially expressed SIX gene *in planta*. To obtain this mutant we firstly characterized stages of fusarium infectious in flax by creating GFP producing MI39

mutant to get easy microscopic observation of fungal structures and damaged plant cell. We transformed *Folini* MI39 wild type strain by pCT74 vector with PEG/$CaCl_2$ method and obtained transformant MI39_GFP2, which constantly expressed GFP under strong eukaryotic promoter.

Conclusion: The repertoire of SIX genes in *Folini* genomes is quite conserved and consists of five families: *SIX1*, *SIX7*, *SIX10*, *SIX12* and *SIX13*, with notable exception for two low-virulent strains F365 and F482. Each of *SIX1*, *SIX10* and *SIX13* genes usually present in one copy per genome, while *SIX7* and *SIX12* genes have paralogs and extra-copies in the individual genomes. In contrast to bulk of effectors that mostly placed in conservative genome part, SIX genes were exclusively found on variable chromosomes 12, 13 or 15. Differential expression analysis of SIX genes revealed that none of them was expressed in LCM. During infestation on flax all of detected SIX genes in MI39 genome have expressed *in planta*, but with different level of expression. We created MI39_GFP2 mutant which constantly expressed GFP and described stages of fusarium infection during flax colonization.

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