Genome-wide characterization and expression of the TLP gene family associated with *Colletotrichum gloeosporioides* inoculation in *Fragaria* × *ananassa*

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Abstract

Background. *Colletotrichum gloeosporioides*, a soil-borne fungal pathogen, causes significant yield losses in many plants, including cultivated strawberry (*Fragaria* × *ananassa*). Thaumatin-like proteins (TLPs) are a large and complex family of proteins that play vital roles in host defense and- other physiological processes in plants.

Methods. To enhance our understanding of the antifungal activity of TLPs in *F.* × *ananassa*, genome-wide identification of the *FaTLP* gene families and their expression patterns were investigated in *F.* × *ananassa* plants upon pathogen infection. Moreover, we used RNA sequencing (RNA-seq) to detect the differences in the expression patterns of TLP genes between different resistant strawberry cultivars in response to *C. gloeosporioides* infection.

Results. In total~~Totally,~~ 76 TLP genes were identified from the octoploid cultivated strawberry genome, with a mean length of 1439 bp, and they were distributed on 24 chromosomes of *F.* × *ananassa*. The *FaTLP* family was divided into ten groups (Group I–X) according to the comparative phylogenetic results, among which, Group VIII contained the highest number of TLP family genes. qPCR analysis indicated a clear upregulation of *FaTLP40*, *FaTLP41*, *FaTLP43*, *FaTLP62*, *FaTLP68*, and *FaTLP75* after *C. gloeosporioides* infection in the octoploid strawberry under evaluation.

Conclusions. Altogether, the data indicated that there were some differences in TLP gene expression patterns among different resistant strawberry cultivars~~,~~ and that faster defense responses of TLPs to pathogenic fungi were observed in resistant cultivars. These results ~~provide a description of~~describe the TLP gene family members found in octoploid strawberry and their potential biological functions in plant defense against pathogenic fungi.

Introduction

Thaumatin-like proteins (TLPs) are important members of a highly complex gene family named pathogenesis-related protein group 5 (PR5), which is highly homologous with the sweet-tasting thaumatin protein produced by ~~the~~ the fruit of *Thaumatococcus daniellii* (*Wel & Loeve, 1972*; *Velazhahan et al., 1999*; *Christensen et al., 2002*; *Loon et al., 2006*). TLPs are widely distributed in plants, including angiosperms (*Loon et al., 2006*) and gymnosperms (*O'Leary et al., 2007*; *Liu et al., 2010*). In recent years, they have been identified in a wide range of plants, including moss, barley, maize, rice, Arabidopsis, and Populus (*Zhao & Su, 2010*; *Cao et al., 2016*; *Singh et al., 2017*). In plants, these proteins exhibit- a series of responses against biotic and abiotic stress factors, such as pathogen invasion, drought, wounding, freezing, and salinity (*Petre et al., 2011*). Furthermore, they also play roles in a variety of physiological and developmental processes, including organ formation, fruit ripening, and seed germination (*Salzman et al., 1998*; *Seo et al., 2008*).

The octoploid cultivated strawberry (*Fragaria* × *ananassa*) is an economically important perennial horticultural crop (*FAO, 2017*) widely cultivated in China, and the main cultivar is 'Benihoppe' from Japan. 'Benihoppe' is susceptible to a range of diseases, particularly anthracnose, which is one of the most destructive fungal diseases of strawberries results in considerable losses in production (*Hammerschlag et al., 2006*; *Dean et al., 2012*), especially at the seedling stage and early stages after transplanting. On the east coast of China, anthracnose in strawberry is mainly caused by the fungus *Colletotrichum gloeosporioides* (*Zhang et al., 2017*), which can infect all aerial plant parts, and the most severe symptoms are dwarf-stem and foliar lesions.

PR proteins, including TLPs, exhibit significant antifungal activity in plants (*Velazhahan et al., 1999*). As a consequence of fungal infection, TLPs are expressed in many plant species during the induction of resistance (*Liu et al., 2010*). They have multiple functions in inhibiting hyphal growth and spore germination of various pathogenic fungi (*Woloshuk et al., 1991*; *De Freitas et al., 2011*) or binding β-1,3-glucans to degrade fungal cell walls (*Grenier et al., 2000*; *Osmond et al., 2001*; *Zareie et al., 2002*), thereby stimulating plant defensive responses against pathogenic fungi. Because of their effective antifungal activity, TLP genes have been promising candidates for plant transformation in plant disease resistance. Overexpression of defense-related TLP genes in transgenic Arabidopsis (*Rout et al., 2016*), rice (*Datta et al., 1999*), tobacco (*Singh*

et al., 2013), grape (*He et al., 2017*), wheat (*Mackintosh et al., 2007*), and potato (*Acharya et al., 2013*) significantly enhanced plant resistance to fungal diseases.

Considering the importance of TLP genes in plant defensive responses against diseases, we deemed it would be worthwhile to investigate the TLP gene family in strawberry. To date, the genome of octoploid strawberry has been sequenced and published (*Edger et al., 2019*), which provides opportunities to further investigate the genetics and genomics of strawberry. To our knowledge, the composition of the members of the *FaTLP* gene family and their mechanisms underlying defense against *C. gloeosporioides* remain to be clarified. In the present study, we performed genome-wide identification and characterization of the TLP gene family in octoploid cultivated strawberry (*F.* × *ananassa*). Transcriptome sequencing and expression analyses were also conducted. Our results provide novel insights into the distribution, expression, and function of TLP genes in strawberry.

Materials & Methods

Identification and characterization of thaumatin-like proteins in *F.* × *ananassa*

The hidden Markov model (*Eddy, 1998*) was constructed using the HMMER software (version 3.0) based on the thaumatin-like protein sequences of Arabidopsis (https://www.arabidopsis.org) and rice (https://rapdb.dna.affrc.go.jp); the model was then used as a standard sequence to isolate all possible homologs in *F.* × *ananassa.* BLASTp was performed to retrieve TLP protein sequences for *F.* × *ananassa* with a cutoff e-value of e^{-10}. Only those aligned sequences were considered as candidate sequences for further analysis. The SMART (http://smart.embl-heidelberg.de/) and Pfam (http://pfam.sanger.ac.uk) databases were used to ensure the accuracy of the identification results, and only the sequence containing the TLP domain (PF00314) was determined as the final TLP sequence (*Ivica et al., 2012*; *El-Gebali et al., 2018*). The molecular weight (kDa) and theoretical isoelectric point (pI) of each gene were predicted using ExPASy ProtParam (http://web.expasy.org/protparam/) (*Gasteiger et al., 2003*).

Phylogenetic analysis of TLP genes

Amino acid sequences of TLP proteins identified in *Fragaria* × *ananassa*, *Fragaria vesca*, Arabidopsis, and rice were used in our phylogenetic analysis. ClustalW (http://www.clustal.org/) (*Larkin et al., 2007*) was used for multiple sequence alignment, after which a phylogenetic tree

was generated with the neighbor-joining method using the MEGA6.0 program (http://www.megasoftware.net/) (*Tamura et al., 2013*) with 1000 bootstrap replicates.

Protein motif analysis

MEME software (http://meme.nbcr.net/meme/, v4.11.0) was used to predict the motifs of TLP proteins in *F.* × *ananassa*. Motif window length was set from 10 to 100 bp, and the maximum number of motifs was set at 20.

Chromosome distribution analysis

To determine the physical locations of *FaTLP* genes, the starting positions of all *FaTLP* genes identified from the *F.* × *ananassa* genome database were determined. A diagram of chromosome locations of *FaTLP* genes was generated using MG2C (http://mg2c.iask.in/mg2c_v2.0/).

Plant materials

Seedlings of 'Benihoppe' (susceptible) and 'Kaorino' strawberry (resistant) (*Mangandi et al., 2015*; *Han et al., 2019*) were cultivated at the Zhejiang Academy of Agricultural Sciences. Experimental plants of the two strawberry cultivars were propagated from runners and rooted in 10-cm diameter pots. No fungicides were applied, and fertilizer was applied proportionally as needed.

Infection with *Colletotrichum gloeosporioides*

The pathogenic fungus *C. gloeosporioides* was cultured and kindly provided by the Institute of Plant Protection and Microbiology, of the Zhejiang Academy of Agricultural Sciences. The *C. gloeosporioides* fungal suspension was cultured in 100-mL liquid potato dextrose medium by shock for seven days (25 °C, 150 rpm). After filtration to remove hyphae, the conidia concentration was adjusted to 10^6 spores/mL using a hemocytometer. Three hundred plants were divided into three subgroups containing 100 plants each: control, susceptible, and resistant groups. Sixty-day-old strawberry seedlings were used for fungal inoculation. The leaves of strawberries in the susceptible and resistant groups were sprayed until dripping with the spore suspension (10^6 spores/mL) using an atomizer. Control plants were inoculated with sterile water. Twenty randomly selected leaves from each group were sampled at 2, 6, 12, 24, and 48 h, and at

3, 4, 5, 6, and 7 d after inoculation. The samples were immediately frozen in liquid nitrogen and stored at -80 °C for further processing. Three replicates were sampled at each time point.

Transcriptome analysis

To determine the transcriptome profile of different resistant strawberry cultivars in response to *C. gloeosporioides*, 12 samples were used for RNA-seq analysis: 'Kaorino'-infected (24 h ~~post~~ post-inoculation, hpi), 'Kaorino'-uninfected, 'Benihoppe'-infected (24 hpi), and 'Benihoppe'-uninfected, with three replicates per treatment. The RNA-seq transcriptome library was prepared using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). HisAT2 (v2.1.0) (*Kim et al., 2019*) was used for sequence alignment, and an annotated genome (Fragaria × ananassa Camarosa Genome Assembly v1.0.a1) available from the Genome Database for Rosaceae (https://www.rosaceae.org/species/fragaria_×_ananassa), was used as a reference. The fragments per kilobase million (FPKM) value (*Malone & Oliver, 2011*) was used to identify differentially expressed genes (DEGs) between two different samples. DESeq2 software (*Anders & Huber, 2010*) was used for differential expression analysis.

RNA isolation and quantitative RT-PCR (qRT-PCR) analysis

Total RNA was isolated using the modified CTAB method (*Chang et al., 2007*). ~~Integrity~~ The integrity of the RNA samples was examined using a U-0080D Protein nucleic acid spectrophotometer (HITACHI, Japan). cDNA was synthesized from 2 μg of total RNA using TranScript® II One-Step gDNA Removal and cDNA Synthesis SuperMix (TransScript®, China). Then, qPCR was carried out in a LightCycler® 96 real-time PCR system (Roche, Switzerland) with DNA Green Master (Roche, Switzerland). The primers used for the validation of DEGs are shown in Table S5. The *Actin* gene ~~were~~ was used for the reference gene. Each sample was repeated in triplicate.

Statistical analysis

Statistical analysis was carried out using SPSS 16.0 software (SPSS Inc., USA). ~~Significance~~ The significance level was $p<0.05$.

Results

Genome-wide identification of TLP genes in *Fragaria* × *ananassa*

To understand the potential roles of TLPs in strawberry, cultivated strawberry (*F.* × *ananassa*) was used for genome-wide identification and characterization of TLP genes. A total of 76 TLP gene members (designated as '*FaTLP*') were identified from *F.* × *ananassa*, which was more than the number of TLP genes in many plant species (*de Jesus-Pires et al., 2020*). Among the 76 *FaTLPs* identified, *FaTLP68* was the longest (over 4370 bp), *FaTLP5* was the shortest (384 bp), and the mean length was approximately 1439 bp. The molecular weights of these TLP genes ranged from 13.36 to 138.40 kD, with PI values between 4.27 and 8.77. Most of these genes were 200–400 aa in length with 1–2 introns and 2–3 exons, although several genes had over 10 introns/exons. Detailed information on the TLP genes, including names, Coding sequence (CDS) lengths, molecular weights, and pI values, is shown in Table 1.

Phylogenetic relationships of TLPs in major plant species

To investigate the evolutionary relationship of TLP gene families, we performed a phylogenetic analysis using three representative plant species other than cultivated strawberry, including *Arabidopsis thaliana*, *Oryza sativa*, and *Fragaria vesca.* According to the information of the constructed phylogenetic tree, 76 *FaTLPs* were classified into 10 groups, named Groups I to X (Figure 1A). Group VIII contained the most TLP family genes, with 16 genes in *F.* × *ananassa*, followed by Group V (15), Group VI (14), and Group II (9). Groups III and IX contained the least TLP family genes, with two genes each.

Chromosomal distribution of TLP family genes in *Fragaria* × *ananassa*

To elucidate the distribution of TLP family genes on the chromosomes of *F.* × *ananassa*, we performed a chromosomal localization analysis. The results showed that the members of *FaTLP* were distributed among 24 of all the 56 chromosomes of *F.* × *ananassa* (Figure 1B). Among them, Chr6-1 had the most genes (seven genes), followed by Chr2-1, Chr2-3, and Chr7-2 (six genes), and Chr7-1 and Chr7-4 (five genes). Additionally, in most cases, for the same paraphyletic group of genes, they were not distributed on a certain chromosome, that is, all seven genes in Group VII were distributed on seven chromosomes (Chr1-1, Chr1-2, Chr1-3, Chr1-4, Chr2-1, Chr2-2, and Chr2-3), and all nine genes in Group II were distributed on seven chromosomes (Chr6-1, Chr6-3, Chr6-4, Chr7-1, Chr7-2, Chr7-3, and Chr7-4). However, there

are a few exceptions; thus, for example, five genes of Group VIII were distributed on the same chromosome, Chr6-1. Moreover, the genes were not evenly distributed on a certain chromosome but instead, in most cases, genes were distributed at both ends of the chromosome (such as Chr1-1, Chr2-2, Chr2-3, Chr3-2, Chr4-2, Chr4-3, Chr5-4, Chr6-3, Chr7-1, and Chr7-2), and there were fewer TLP genes near the centromere (such as Chr5-2 and Chr6-4) than near the ends. These results were similar to those of previous reports (*Wang et al., 2020*).

Conserved motifs of TLP genes

The diversity of motif compositions of TLP genes in *F.* × *ananassa* was assessed using the MEME software, and a total of 15 conserved motifs were obtained. The distribution of these 15 motifs in the TLPs under study is shown in Figure 2 and Figure 3. Only one gene, *FaTLP10*, contained all 15 conserved motifs. In addition, four genes (*FaTLP55*, *FaTLP56*, *FaTLP59*, and *FaTLP62*) contained 14 motifs but did not include motif11. Ten motifs were present in most of the TLP genes in *F.* × *ananassa*, including motif1, 2, 3, 4, 5, 6, 7, 10, 11, and 15. The position of motif5 was in the front, followed by motif11, 3, 10, 6, 2, 7, and 4, and the positions of motif1 and motif15 were in the back. Among them, motif6 was the most conserved TLP domain, and it was identified in all TLP proteins. In addition, motif2 and motif7 were lost in only one gene (*FaTLP5*), and motif3 was lost in two genes (*FaTLP9* and *FaTLP33*). These ten conserved motifs were also common in all groups (Group I to X). Moreover, some members of Group V (*FaTLP10*, *FaTLP11*, *FaTLP55*, *FaTLP56*, *FaTLP58*, *FaTLP59*, *FaTLP62*, *FaTLP64*, and *FaTLP68*) shared several unique motifs, namely, motif8, 9, 12, 13, and 14. These results indicated that the TLP genes in each group shared several unique motifs and may have certain functional similarities. Moreover, these motifs were relatively conserved, which is why they may be used as markers for the identification of TLP genes and might be important functional components of the TLP gene family.

Transcriptome changes in different resistant strawberry cultivars in response to *C. gloeosporioides* infection

To better understand the transcriptome profile of different resistant strawberry cultivars in response to *C. gloeosporioides*, RNA-seq analysis was assessed on 12 samples (‘Kaorino’-infected, ‘Kaorino’-uninfected, ‘Benihoppe’-infected, and ‘Benihoppe’-uninfected) with three

replicates per treatment. Approximately 576 million raw reads were obtained, and the clean reads were mapped to the *F.* × *ananassa* genome (Table S1). Based on p-adjust<0.05, and $log_2FC \geq 1$, a total of 10462 and 13682 DEGs were detected in the 'Kaorino'-infected/uninfected (resistant (R) group) and 'Benihoppe'-infected/uninfected (susceptible (S) group) leaves, respectively. In addition, 5490 genes were downregulated in the S-group compared to 4765 in the R-group ($log_2FC \leq -1$) (Figure S1, Table S2). Furthermore, more genes were upregulated in the S-group (8192) than in the R-group (5697) ($log_2FC \geq 1$) (Figure S1, Table S3). Based on sequence homology, DEGs were classified into 48 functional groups belonging to three main GO ontologies: cellular components, molecular functions, and biological processes. Among these DEGs, 2993 (R-group) and 3564 genes (S-group) were involved in the GO categories "response to stimulus" (Figure S2, Figure S3) which contained three subgroups associated with fungal resistance: GO:0050832, GO:0009817, and GO:0009620 (Figure S4). Six DEGs annotated as TLPs were categorized into these functional subgroups, indicating that these DEGs varied greatly in response to *C. gloeosporioides* (Table S4). According to our identification of the TLP gene family in octoploid strawberry, these six differentially expressed TLP genes were identified as *FaTLP40*, *FaTLP41*, *FaTLP43*, *FaTLP62*, *FaTLP68*, and *FaTLP75*. The RNA-seq results showed that at 24 hpi, *FaTLP40* and *FaTLP41* were upregulated and *FaTLP75* was downregulated in the resistant group, while *FaTLP62* was upregulated in the susceptible group and *FaTLP43* was downregulated and *FaTLP68* was upregulated in both groups.

Quantitative real-time PCR analysis of TLP genes in response to *C. gloeosporioides* infection

To further understand the roles of TLP genes in strawberry, we investigated the expression profiles of *FaTLP* genes in *F.* × *ananassa*. The qRT-PCR results of these TLP genes in different resistant strawberry cultivars after infection with the fungal pathogen showed a wide range of expression responses (Figure 4B). The induction expression of *FaTLP68* was significant in the resistant cultivar ('Kaorino') at 12 hpi, although it decreased over time, whereas the upregulation of *FaTLP68* was detected at 24 hpi in the susceptible cultivar ('Benihoppe'), and its expression peak appeared later than that of the resistant cultivar. *FaTLP40* and *FaTLP41* were also highly induced in both strawberry cultivars but there were two expression peaks. This induction increased over time and reached the first peak at 24 hpi and the second peak at 4 d ~~post~~ post-

inoculation (dpi) in the resistant cultivar, whereas in the susceptible cultivar, the first expression peaks of *FaTLP40* and *FaTLP41* occurred at 48 hpi. The expression of *FaTLP62* was considerably upregulated at 24 hpi only in the susceptible cultivar. These results showed a trend similar to that observed using RNA-seq. Nevertheless, a gradual downregulation was observed for *FaTLP43* and *FaTLP75* after pathogen infection in 'Kaorino', which significantly increased at 12 hpi and 48 hpi, respectively, followed by a decrease until 7 dpi, which was not entirely consistent with the results of the transcriptome analysis showing that *FaTLP43* and *FaTLP75* were downregulated at 24 hpi. However, in the susceptible cultivar, the expression patterns of *FaTLP43* and *FaTLP75* were similar during the initial stage after inoculation but the maximum peak occurred at 4 dpi (one day after disease symptoms were visible to the naked eye, Figure 4A). Overall, the qPCR analysis indicated a clear upregulation of the six abovementioned TLP genes upon *C. gloeosporioides* infection in the octoploid strawberry under study.

Discussion

Thaumatin-like proteins (TLPs) are a PR family that plays key roles in plant defense. In the present study, 76 TLP gene members were identified in *F.* × *ananassa*, and their characteristics, phylogenetic relationships, motif organization, and chromosomal location were investigated. Previous studies have indicated that plant TLPs usually consist of approximately 200 amino acids, with a molecular weight of 21–26 kD (*Velazhahan et al., 1999*). Most TLPs contain 10 to 16 conserved motifs (*Hulo et al., 2008*). Furthermore, the chromosomal distribution of TLP genes was found to not be uniform (*Wang et al., 2020*), and phylogenetic studies using Arabidopsis, Oryza (*Shatters et al., 2006*), and Populus (*Zhao & Su, 2010*) species found that their TLPs clustered into ten paraphyletic groups, consistently with the findings reported herein. Further, these results indicated that the TLP genes belonging to the same phylogenetic group or sharing several unique motifs may have certain functional similarities.

TLPs are universal in plants, and their expression can be induced by various environmental stress factors, including wounding, heat, chilling, and pathogen stress (*Velazhahan et al., 1999*; *Loon et al., 2006*). The expression of TLP genes is responsive to infection by a variety of fungal pathogens, such as Colletotrichum, Podosphaera, Phytophthora, Fusarium, and Neurospora (*Woloshuk et al., 1991*; *Narasimhan et al., 2003*; *Rather et al., 2015*). TLP proteins disrupt the stability of fungal membranes as well as hyphae and spore formation to prevent fungal damage

or reduce disease symptoms (*Roberts et al., 1990; Woloshuk et al., 1991*; *De Freitas et al., 2011*). In this study, six genes encoding TLP proteins were identified by transcriptome data, namely, *FaTLP40*, *FaTLP41*, *FaTLP43*, *FaTLP62*, *FaTLP68*, and *FaTLP75*, were also found to be associated with plant responses to infection with *C. gloeosporioides*. Further qPCR analysis verified a clear upregulation of these six TLP genes among different resistant strawberry cultivars upon infection with *C. gloeosporioides*. Upregulation or overexpression of TLP genes often results in enhanced antifungal activity against a diversity of pathogenic fungi (*Datta et al., 1999*; *Fagoaga et al., 2001*; *Kalpana et al., 2006*). Thus, for example, overexpression of barley TLP-1 in transgenic wheat lines showed improved pathogen resistance to *Fusarium graminearum* (*Mackintosh et al., 2007*). Similarly, increased TLP gene expression in transgenic tobacco plants enhanced resistance to *Pythium aphanidermatum* and *Rhizoctonia solani* (*Rajam et al., 2007*), while overexpression of the TLP gene *VaTLP* improved downy mildew resistance in *Vitis vinifera* (*He et al., 2017*). In turn, significant upregulation of *FaPR5-1* and *FaPR5-2* was observed in the salicylic acid-primed defense response of octoploid strawberry to *Podosphaera aphanis* (*Feng et al., 2020*). Altogether, these results suggest that *FaTLP40*, *FaTLP41*, *FaTLP43*, *FaTLP62*, *FaTLP68*, and *FaTLP75* may have potential functions in plant resistance responses to *C. gloeosporioides.*

Additionally, our results demonstrated that differentially expressed TLP genes were delayed in the susceptible cultivar compared with the resistant cultivar; indeed, some genes were even expressed after the appearance of visible disease symptoms (Figure 4). In ‘Kaorino’, *C. gloeosporioides* infection caused milder symptoms with earlier upregulation of *FaTLP40*, *FaTLP41*, *FaTLP43*, *FaTLP68*, and *FaTLP75*, indicating that this resistant cultivar ~~is able to~~can activate defense responses faster. Similar results were reported for the response of strawberries to infection with *Verticillium dahliae* (*Besbes et al., 2019*) and *Podosphaera aphanis* (*Feng et al., 2020*). These results suggested that, compared to susceptible cultivars, resistant cultivars or cultivars with induced resistance showed better defense responses against pathogenic fungi. Therefore, we concluded that the difference in resistance between different strawberry cultivars to *C. gloeosporioides* might relate to differences in the response efficiency of resistance-related proteins such as TLP upon infection by pathogenic fungi, especially at the initial stage of infection.

Conclusions

In this study, we performed genome-wide identification and characterization of thaumatin-like proteins in octoploid strawberry. A total of 76 TLP genes (*FaTLP1–76*) were identified by genome-wide screening. Comparative phylogenetic analysis classified the TLPs into ten groups. Furthermore, the functions of TLPs in *F.* × *ananassa* were analyzed. Our qRT-PCR analysis indicated a clear upregulation of six TLP genes in strawberry leaves infected with *C. gloeosporioides*. Furthermore, our results showed differences in TLP gene expression patterns among different resistant strawberry cultivars. We concluded that faster defense responses of TLPs to pathogenic fungi might be a major reason why the resistant strawberry cultivar 'Kaorino' showed greater anthracnose resistance than the susceptible cultivar 'Benihoppe'. Our results provide a useful basis for future studies on the antifungal function of TLP genes in *F.* × *ananassa*.

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