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Characterization and expression of the wall-associated kinase/wall-associated kinase-like (*WAK/WAKL*) family in response to *Botrytis cinerea* infection in strawberry (*Fragaria×ananassa*)

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Abstract

Background Gray mold caused by *Botrytis cinerea* is a major threat to the production of strawberry. An increasing number of studies have reported that wall-associated kinase/wall-associated kinase-like (WAK/WAKL) played an important role in the recognition of oligogalacturonic acids (OGs) and the induction of plant defense, but there have been no systematic studies of *FaWAK/FaWAKL* in strawberry.

Results In this study, we identified 167 *FaWAK/FaWAKL* gene family members within the strawberry (*Fragaria×ananassa*) genome. The phylogenetic analysis showed the *FaWAK/FaWAKL* gene family has been divided into five groups, and they were unevenly distributed on 46 chromosomes. An analysis of the *cis*-regulatory elements suggested the *FaWAK/FaWAKL* gene family was more sensitive to abscisic acid and methyl jasmonate. A total of 36 *FaWAK/FaWAKL* genes were activated by *B. cinerea* according to an RNA-seq analysis, and 8 of them strongly responded to *B. cinerea* and exogenous treatment with OGs, particularly *FaWAK35*. Transient overexpression of *FaWAK35* increased the strawberry resistance to *B. cinerea*.

Conclusion This study conducted a comprehensive analysis of *FaWAK/FaWAKL* and provides foundational insights for further exploration of *FaWAK/FaWAKL* genes in strawberry resistance to *B. cinerea*.

Keywords *Fragaria×ananassa*, *Botrytis cinerea*, Plant-pathogen interaction, *WAK/WAKL*, Oligogalacturonic acids, Expression analysis

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Background

Strawberry (*Fragaria×ananassa*) is an economically important horticultural crop, which is widely popular around the world because it is nutritious and has a unique flavor. In 2020, a total of 14 billion USD of strawberry was produced around the world [1]. However, strawberry fruit are susceptible to many fungal pathogens because they are tender and juicy [2]. Gray mold caused by *Botrytis cinerea* is one of the most destructive fungal diseases in the field and after harvest. *B. cinerea* is a necrotrophic fungus that is not specific to particular hosts. Over 1,400 crop species have been reported to be susceptible to this pathogen. *B. cinerea* has been recognized as the second largest plant disease in the world, and it causes global economic losses of up to hundreds of billions of dollars a year [3].

Plants are confronted with various biotic stresses during their development and growth and have evolved a complex system to recognize infections and activate their own immune response against pathogens. When pathogens infect plants, the pathogen will release small molecules to attack the plant cells, while the plant cells also secrete substance responding to the pathogen attack. There are various pattern recognition receptors (PRRs) on the plant cell membrane, and they can recognize the small molecules released by the pathogens or plants. The plants then will produce pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) in response to the signal substances secreted by the pathogen or plant, respectively. This leads to the activation of the pattern-triggered immunity (PTI) [4]. As a typical necrotrophic fungus, *B. cinerea* primarily completes infection by releasing pectinases (PGs) to degrade the cell wall. During this process, the plants initiate pectinase inhibitor proteins (PGIPs) against infection. The interaction between PGs and PGIPs promotes the production of long-chain oligogalacturonic acids (OGs), which can be recognized by wall-associated kinase/wall-associated kinase-like (WAK/WAKL) on the cell membrane and induce resistance to disease [5, 6, 7].

The group of WAK/WAKL is a subfamily of receptor-like protein kinases (RLKs) with an intracellular Ser/Thr kinase domain and a distinct extracellular structure [8]. Their extracellular structure contains a galacturonan-binding domain (GUB_WAK_bind) and/or a calcium-binding epidermal growth factor-like domain (EGF_CA_bind) to perceive signals [8]. The *WAKs/WAKLs* play an important role in resistance to plant disease. *SlWAK1* has been reported to be involved in the regulation of the PRR-mediated immune response through the FLS2/FLS3 complex in tomato (*Solanum lycopersicum*) [9]. *GhWAK* in cotton (*Gossypium hirsutum*) affected the jasmonic acid (JA) and salicylic acid (SA) signaling pathways and regulated resistance to *Verticillium dahliae* [10]. The overexpression of *CsWAKL08* confered resistance to *Xanthomonas asaxonopodis* pv. *citri* via the control of reactive oxygen species and JA signaling in citrus (*Citrus sinensis*) [11]. Furthermore, *OsWAK1* and *OsWAK25* in rice (*Oryza sativa*) have also been shown to be associated with the resistance of plants to pathogens [12, 13]. In strawberry fruit, whether the *WAK/WAKL* family members are involved in disease resistance remains largely unknown. Our previous study found that many strawberry *WAK/WAKL* genes were significantly upregulated in response to infection with *B. cinerea*. In this study, we characterized the *FaWAKs/FaWAKLs* in the genome of octoploid cultivated strawberry (*Fragaria×ananassa*) and examined their pattern of expression in response to infection with *B. cinerea* and treatment with exogenous OGs. This study will enrich the information of the *FaWAK/FaWAKL* genes in the defense responses against *B. cinerea* and provide a theoretical basis to effectively control gray mold on strawberry fruit.

Results

Identification of the *FaWAK/FaWAKL* family members in *Fragaria×ananassa*

The *WAK/WAKL* family members in *Fragaria×ananassa* were identified using 68 *Rosa chinensis* WAK/WAKL members as a reference to perform BLASTP (E value < 0.001) in the octoploid cultivated strawberry genome database. A total of 6,366 homologous sequences were obtained. The HMM files of the calcium-binding EGF-like domain (EGF_CA_bind, PF07645.18), galacturonan-binding domain (GUB_WAK_bind, PF13947.9) and kinase domain (Pkinase, PF07714.20) from the Pfam database were then used for an hmmsearch. The EGF_CA, GUB_WAK_bind and Pkinase hmmsearch led to the identification of 86, 396 and 6,650 candidate sequences, respectively (Fig. 1). Finally, we verified a total of 167 non-redundant *FaWAK/FaWAKL* family genes, where 40 candidate genes contained EGF_CA, GUB_WAK_bind and Phkinase conserved domains, and considered as *FaWAK* genes. The others were *FaWAKL* genes, which 123 contained GUB_WAK_bind and Phkinase conserved domains and 4 contained EGF_CA and Phkinase conserved domains (Fig. 1). *FaWAK1-FaWAK40* and *FaWAKL1-FaWAKL127* were named based on the order of the genes on the chromosome (Supplementary Table 1).

Studies have shown that the *WAL/WAKL* family members play a vital role in the defense responses of plants to fungal pathogens [16]. Our previous study used RNA-seq to show that 36 *FaWAK/FaWAKL* genes were significantly upregulated around the infection site when strawberry fruit were inoculated with *B. cinerea*, including one *FaWAK* and 35 *FaWAKLs* that contained

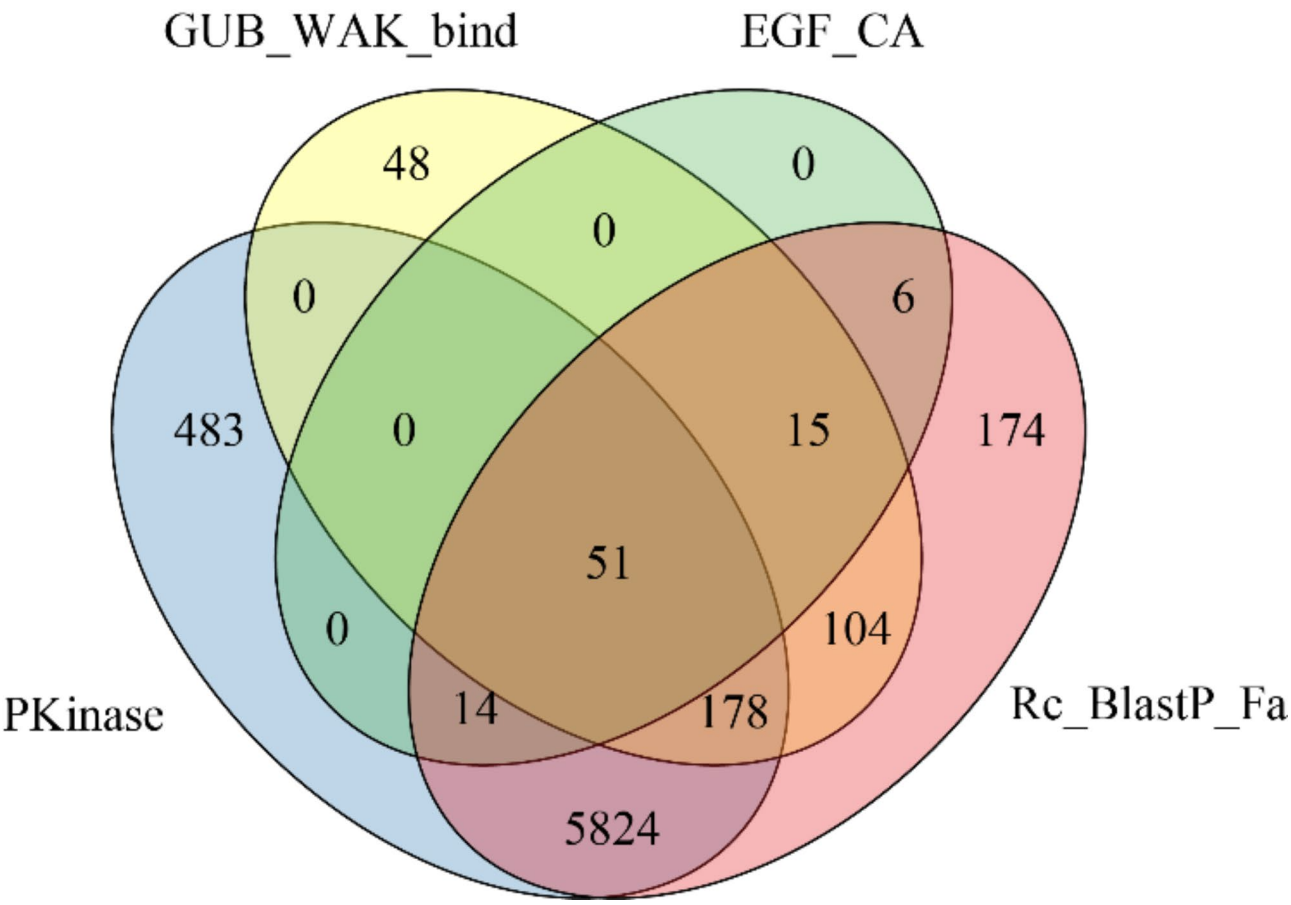

Fig. 1 Venn diagram of the number of predicted *FaWAKs/FaWAKLs* or their conserved motifs by using Blastp or hmm search. Rc_BlastP_Fa represented the number of predicted *FaWAKs/FaWAKLs* by using RcWAK/RcWAKL protein sequences to search for homologous proteins in *Fragaria×ananassa*, with E value < 0.001. GUB_WAK_bind, EGF_CA and Pkinase represented number of proteins carrying galacturonan-binding domain, calcium-binding EGF-like domain, or a kinase domain, respectively

GUB_WAK_bind and Phkinase conserved domains. The *FaWAKLs* that contained the EGF_CA and Phkinase conserved domains did not respond to infection with *B. cinerea* (Table 1).

Physiological and biochemical analysis of the FaWAK/FaWAKL family members in *Fragaria×ananassa*

We conducted a series of physiological and biochemical analyses to better characterize the FaWAK/FaWAKL proteins. The FaWAKs/FaWAKLs varied greatly in their length. The shortest was FaWAKL125, which encoded 399 amino acids. The longest was FaWAKL30, and it encoded 845 amino acids. Proteins with more than 600 amino acids accounted for 90.42% of all the proteins. The molecular weight of the FaWAKs/FaWAKLs ranged from 44.52 kDa to 94.11 kDa. The hydropathicity of all the FaWAKs/FaWAKLs was higher than −0.5, which indicated their hydrophilia. The isoelectric points ranged from 4.91 to 8.62. The detailed information about FaWAKs/FaWAKLs, including their accession number, chromosomal location, amino acid length, molecular weight, isoelectric point, instability index, and hydropathicity, are listed in Supplementary Table 1.

Upon the characterization of the 36 members of FaWAK/FaWAKL that responded to *B. cinerea*, we found that their length varied. The number of amino acids ranged from 339 to 800, and the molecular weight ranged from 44.52 kDa to 91.06 kDa (Supplementary Table 1). The isoelectric points ranged from 5.06 to 8.14. Therefore, there was no similar physiological and biochemical characterization for the 36 FaWAKs/FaWAKLs that responded to *B. cinerea*.

Chromosomal locations of the *FaWAK/FaWAKL* genes

The *FaWAKs/FaWAKLs* were mapped to 46 chromosomes of the *Fragaria×ananassa* genome using Map to Char software (Fig. 2; Supplementary Table 1). The *FaWAK/FaWAKL* family genes were unevenly distributed on the 46 chromosomes. A high density of *FaWAK/FaWAKL* was located in several specific regions, such as 3-4-1, 6-1-2, 4-4-1 and 6-1-1, that contained 13, 9, 8 and 8 genes, respectively. In contrast, 13 chromosomes only

Table 1 Expression of *FaWAK/FaWAKL* genes under *B.cinerea* infection

Gene	Accession number[a]	Log2 FC[b]	FDR[c]
FaWAK35	FxaYL_731g0847360	9.607	7.40E-102
FaWAKL2	FxaYL_142g0862890	3.566	2.10E-29
FaWAKL3	FxaYL_211g0427200	1.244	3.82E-06
FaWAKL4	FxaYL_212g0591940	3.197	5.37E-38
FaWAKL5	FxaYL_221g0476700	3.216	5.11E-31
FaWAKL6	FxaYL_222g0427240	3.633	8.27E-44
FaWAKL7	FxaYL_231g0402590	2.141	7.88E-20
FaWAKL8	FxaYL_232g0501230	4.496	1.11E-60
FaWAKL36	FxaYL_421g0555090	1.217	1.78E-03
FaWAKL37	FxaYL_421g0555110	9.283	2.39E-17
FaWAKL39	FxaYL_431g0648120	2.767	1.04E-32
FaWAKL42	FxaYL_432g0521770	5.683	3.59E-31
FaWAKL44	FxaYL_441g0251030	4.247	3.30E-25
FaWAKL46	FxaYL_441g0256960	1.572	2.03E-07
FaWAKL49	FxaYL_441g0257000	2.346	1.92E-07
FaWAKL53	FxaYL_442g0225670	1.596	7.14E-06
FaWAKL54	FxaYL_511g0685800	5.695	2.77E-43
FaWAKL55	FxaYL_511g0685770	4.396	5.00E-37
FaWAKL56	FxaYL_512g0654980	3.545	2.04E-28
FaWAKL57	FxaYL_512g0655010	2.854	1.49E-24
FaWAKL58	FxaYL_522g0621350	1.026	4.90E-05
FaWAKL60	FxaYL_532g0374770	5.076	3.71E-56
FaWAKL76	FxaYL_621g0059500	11.049	6.79E-43
FaWAKL79	FxaYL_622g0058250	3.981	2.26E-22
FaWAKL84	FxaYL_631g0012560	11.370	2.65E-49
FaWAKL85	FxaYL_631g0012450	10.642	1.78E-35
FaWAKL86	FxaYL_632g0045100	1.179	6.27E-06
FaWAKL87	FxaYL_632g0045070	9.692	9.64E-22
FaWAKL89	FxaYL_632g0012280	4.773	1.01E-23
FaWAKL96	FxaYL_711g0960330	1.665	6.34E-08
FaWAKL100	FxaYL_712g0945690	1.949	9.01E-09
FaWAKL115	FxaYL_731g0869290	10.293	5.92E-30
FaWAKL119	FxaYL_732g0854970	1.815	2.47E-09
FaWAKL120	FxaYL_732g0853100	10.386	1.65E-31
FaWAKL125	FxaYL_742g0973800	2.060	8.02E-06
FaWAKL126	FxaYL_742g0973790	1.775	9.19E-08

[a] Available at https://www.rosaceae.org/Analysis/14723107

[b] Expression of *FaWAK/FaWAKL* genes under *B. cinerea* infection from RNA-seq dataset

[c] FDR: false discovery rate

had one *FaWAK/FaWAKL* gene, and 11 chromosomes had none of these genes. The unbalanced distribution is an indication of how the *FaWAK/FaWAKL* genes varied genetically during the evolutionary process. The 36 *FaWAKs/FaWAKLs* that responded to *B. cinerea* were mapped to 26 chromosomes, and 13 chromosomes had only one *FaWAK/FaWAKL* gene.

Phylogenetic and structural analyses of the *FaWAK/FaWAKL* genes in *Fragaria×ananassa*

To evaluate the evolutionary relationship between the *FaWAKs/WAKLs* and the defense-related *WAKs/WAKLs* reported in different species and illustrate the potential role of *FaWAKs/FaWAKLs* in defense response, we compiled a total of 16 *WAK/WAKL* genes that were related to defense responses in plants and used the Neighbor-Joining method to establish a phylogenetic tree. The 16 *WAKs/WAKLs* were members of *A. thaliana*, cotton, rice, tomato, wheat (*Triticum aestivum*), maize, and rose (*Rosa chinensis*). The results showed that the *FaWAKs/FaWAKLs* were divided into five clusters, which were labeled with different colors. Group I contained the maximum number of *FaWAKs/FaWAKLs* (56) and clustered together without any defense-related *WAKs/WAKLs* from other species. A total of 15 *FaWAKs/FaWAKLs* were upregulated by infection with *B. cinerea*. Group II contained 8 *FaWAKs/FaWAKLs* that clustered with *ZmWAK-RLK1*, and five *FaWAKs/FaWAKLs* were upregulated by *B. cinerea*. Group III contained 18 *FaWAKs/FaWAKLs*, and six of them grouped with *OsWAK25* and formed a subgroup, while nine *FaWAKs/FaWAKLs* that responded to *B. cinerea* infection were clustered to another subgroup. *RcWAK4*, *AtWAKL10*, *AtWAKL22* and 38 *FaWAKs/FaWAKLs* were clustered into group IV. In this group, five *FaWAKs/FaWAKLs* were upregulated by *B. cinerea* infection, and *FaWAKL89* was the most closely related to *RcWAK4* evolutionarily. *AtWAK2*, *AtWAKL1*, *GhWAKL7A*, *SlWAK1* and 47 *FaWAKs/FaWAKLs* were clustered into Group V, and only *FaWAK35* was upregulated. No *FaWAK/FaWAKL* was grouped into group VI (Fig. 3).

Conserved analysis of the motifs of the FaWAKs/FaWAKLs

To study the composition of the conserved motifs of FaWAKs/FaWAKLs, we used the MEME online website to identify the conserved motifs of WAK proteins. A total of 10 conserved motifs were identified. The results showed that 149 FaWAKs/FaWAKLs had motifs 6, 8, 2, 1, 9, 7, 3, and 5, and the motif distribution was generally consistent (Supplementary Table 2).

We selected the 36 FaWAKs/FaWAKLs that had responses to *B. cinerea* to draw the diagram of gene structure. The results showed that most of the FaWAKs/FaWAKLs had motifs 6, 8, 2, 1, 9, 7, 3, and 5 except FaWAKL125, which only contained motifs 10, 6, 8, and 2. FaWAKL2/36/46/49/53/96/100 had motif 10, which was highly conserved and divided in Group I (Fig. 4).

Prediction of the putative *cis*-regulatory elements in the promoters of *FaWAKs/FaWAKLs*

The 2000 bp region upstream of the translation start site of *FaWAK/FaWAKL* genes was considered the

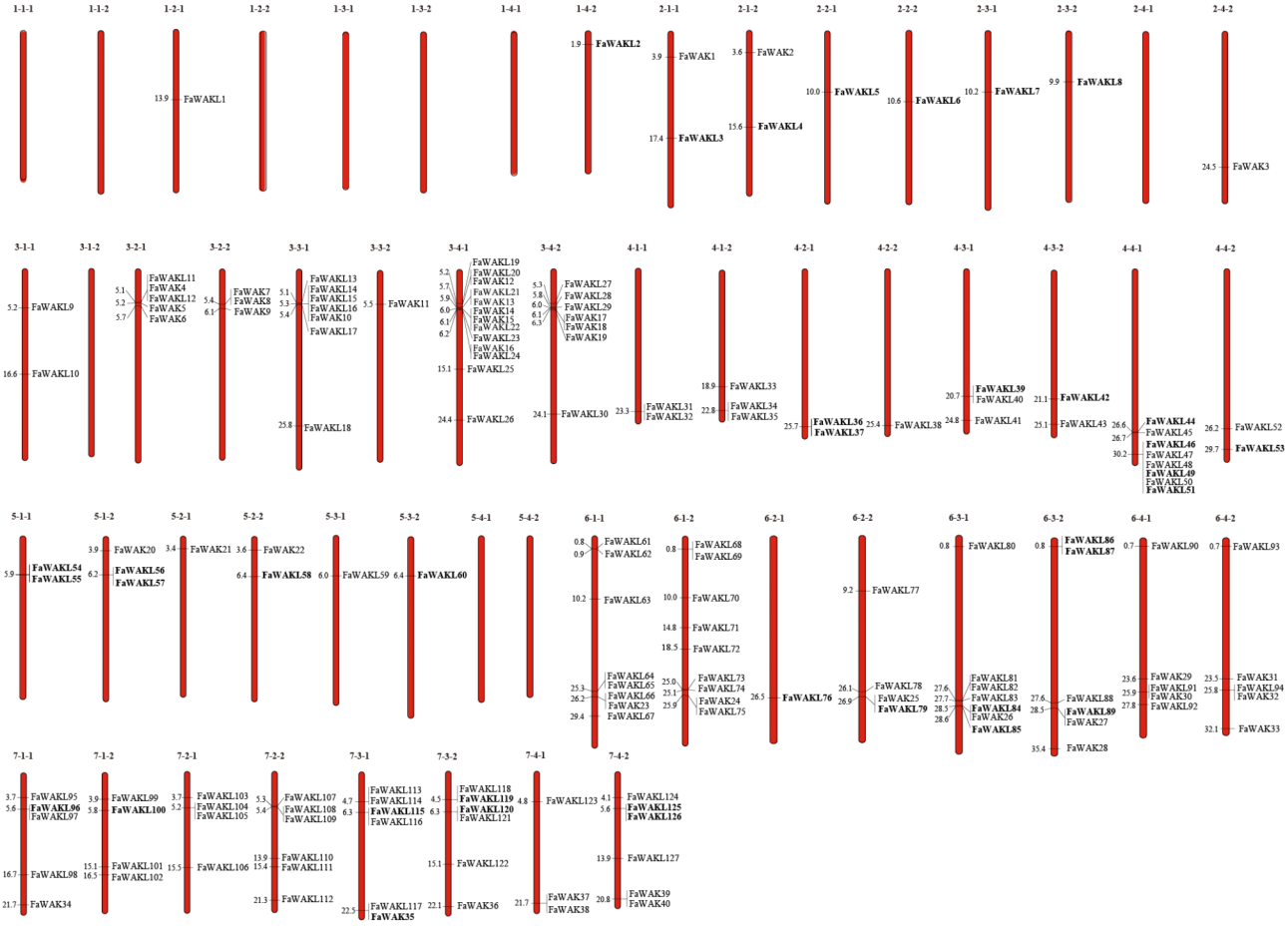

Fig. 2 The location of *FaWAK/FaWAKL* genes on '*Fragaria×ananassa*' chromosome. Boldness indicated the 36 members of *FaWAK/FaWAKL* that responded to *B. cinerea* infection

promoter and analyzed for the potential roles of *cis*-regulatory elements (Table 2). These *cis*-regulatory elements were classified into three main groups, including those that responded to hormones, abiotic stress, and light responses. There were numerous *cis*-elements related to the hormonal signals, which comprised up to 47.5% of the total *cis*-regulatory elements. Among them, most of the *cis*-regulatory elements were associated with abscisic acid (ABA) and methyl jasmonate (MeJA), which indicated that the *FaWAK/FaWAKL* gene family was more sensitive to ABA and MeJA. A total of 11 hormone-responsive regulatory elements were identified, including elements associated with ABA (ABRE), MeJA (TGACG-motif and CGTCA-motif), gibberellin (GA) (P-box, GARE-Motif, and TATC-box), auxin (AUX) (AuxRR-core, TGA-element, and TGA-box), ethylene (ET and ERE), and salicylic acid (SA) (TCA-element), respectively. ABRE, TGACG-motif, and CGTCA-motif were enriched in most of the *FaWAK/FaWAKL* promoters (Supplementary Table 3).

Further analysis of the 36 *FaWAKs/FaWAKLs* that responded to *B. cinerea* revealed that the *cis*-regulatory elements were primarily ABRE, TGACG motifs and CGTCA motifs. This indicated they were primarily regulated by ABA and MeJA. The primary *cis*-regulatory element in response to abiotic stress was ARE, which suggested that they might be involved in the regulation of oxidative compounds and antioxidants. In addition, the G-box was the primary photoresponsive *cis*-regulatory element (Fig. 5).

Analysis of the *FaWAK/FaWAKL* genes in response to infection with *B. cinerea* and stimulation with OGs

To further understand the roles of *FaWAKs/FaWAKLs* in strawberry, we selected 10 *FaWAKs/WAKLs* for additional validation and analysis based on their expression in a previous study with RNA-seq. When the strawberry fruits at the white stage was infected by *B. cinerea*, the levels of expression of *FaWAK35,FaWAKL60,FaWAKL8,FaWAKL7,FaWAKL39,FaWAKL4*, and *FaWAKL6* increased by 22.63-, 21.20-, 21.03-, 17.23-, 16.94-, 14.80-, and 14.61- fold, respectively (Fig. 6, $P \leq 0.001$).

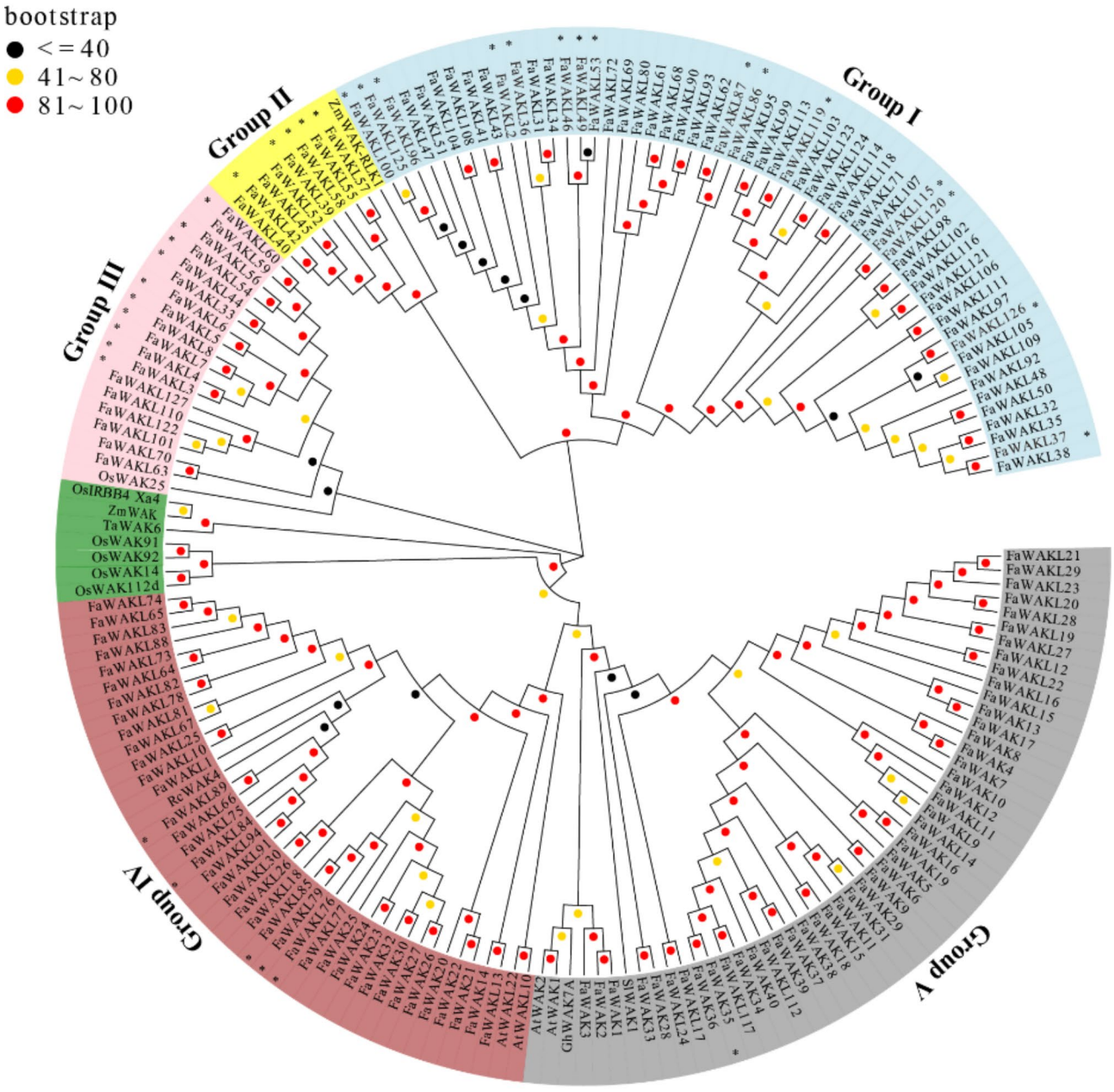

Fig. 3 Phylogenetic analysis of *FaWAK/FaWAKL* genes with defense-related *WAKs/WAKLs* from other plant species. Complete alignments of the strawberry and the defense-related *WAKs/WAKLs* from other plant species, including ***A.*** *thaliana*, cotton (*Gossypium hirsutum*), rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), wheat (*Triticum aestivum*), maize (*Zea mays*), and rose (*Rosa chinensis*), were used to construct a phylogenetic tree using the Neighbor-Joining method. The bootstrap values are indicated on the nodes of the branches. Group I-V were labled in blue, yellow, pink, red, and grey. * indicated the 36 members of *FaWAK/FaWAKL* that responded to ***B.*** *cinerea* infection

Analysis of the *FaWAK/FaWAKL* genes in response to stimulation with OGs

In this study, OGs with an oligomerization degree of 10–15 was used to evaluate the levels of expression of the *FaWAK/FaWAKL* genes in response to stimulation by the OGs. First, we tested the effects of the treatment with OGs on the development of disease on strawberry fruits with the artificial inoculation of *B. cinerea.* The results indicated that the treatments with OGs significantly inhibited the development of lesion on the strawberry fruits. After 24 h of inoculation, 40% of the fruit developed lesions on the control fruits, while no OGs-treated fruit developed lesions ($P \leq 0.05$; Fig. 7A). After 48 h and 72 h of inoculation, the lesions on the fruits treated with OGs decreased by 58.99% and 32.77%, respectively, compared to the control ($P \leq 0.05$; Fig. 7B and C).

An additional analysis showed that the treatment with OGs stimulated the expression of the *FaWAKs/*

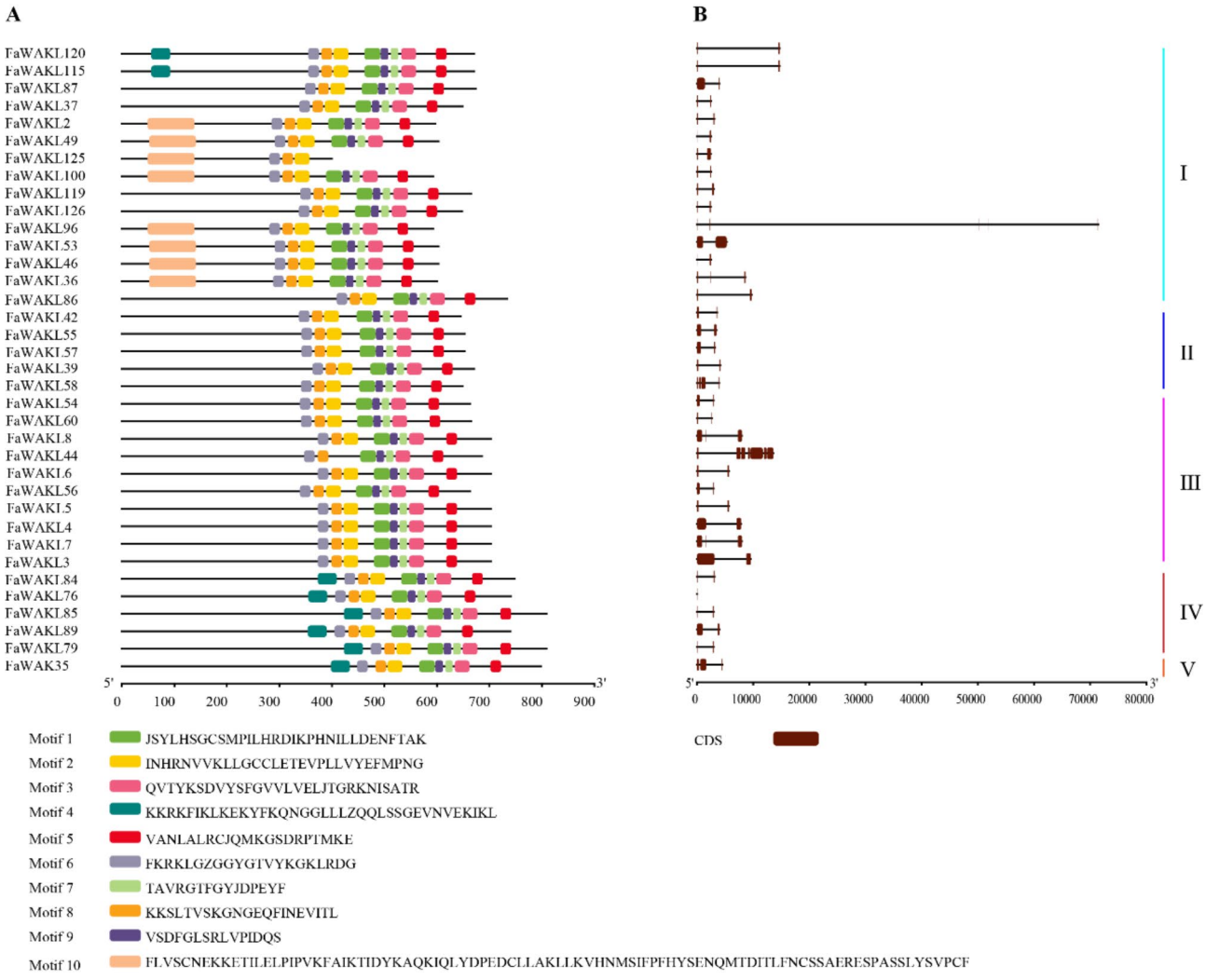

Fig. 4 The conserved motif (**A**) and coding sequence (**B**) analyses of the 36 members of FaWAK/FaWAKL that responded to *B. cinerea* infection. The motif compositions were predicted using MEME software, and the 10 conserved motifs are represented by different colors. Coding sequence (CDS) were represented by crimson box

Table 2 Promoter *cis*-regulatory elements enrichment analysis of *FaWAK/FaWAKL* genes in '*Fragaria×ananassa*'

Abiotic stress		Hormone response						Light response
		ABA	JA	GA	AUX	ET	SA	
I	323 (30.70%)	112 (10.56%)	216 (20.53%)	76 (7.22%)	56 (5.32%)	2 (2.00%)	25 (2.38%)	223 (21.20%)
II	46 (25.84%)	32 (17.98%)	16 (8.99%)	10 (5.62%)	7 (3.93%)	4 (2.25%)	0 (0.00%)	63 (35.39%)
III	104 (33.44%)	41 (13.18%)	42 (13.50%)	21 (6.75%)	12 (3.86%)	13 (4.18%)	10 (3.22%)	68 (21.86%)
IV	172 (23.99%)	110 (15.34%)	118 (16.46%)	48 (6.69%)	41 (5.72%)	16 (2.23%)	24 (3.35%)	188 (26.22%)
V	210 (24.48%)	142 (16.55%)	148 (17.25%)	52 (6.06%)	42 (4.90%)	11 (1.18%)	15 (1.75%)	238 (27.74%)

FaWAKLs. After 48 h of treatment, the levels of expression of all the genes increased and peaked, and the changes in *FaWAK35* and *FaWAKL86* were the most significant. After treatment with the OGs, the levels of expression of *FaWAK35* and *FaWAKL89* increased by 28.92- and 45.08-fold, respectively (Fig. 8).

FaWAK35 participated in strawberry resistance to *B. cinerea*

To further illustrate the potential role of *FaWAKs/FaWAKLs* in strawberry fruit against *B. cinerea* infection, we transiently expressed *FaWAK35* in strawberry fruits and infected the fruits with *B. cinerea*. *FaWAK35* was the most strongly up-regulated *FaWAKs/FaWAKLs* upon *B. cinerea* infection through RNA-seq analysis and our qRT-PCR confirmation. Meanwhile, *FaWAK35*

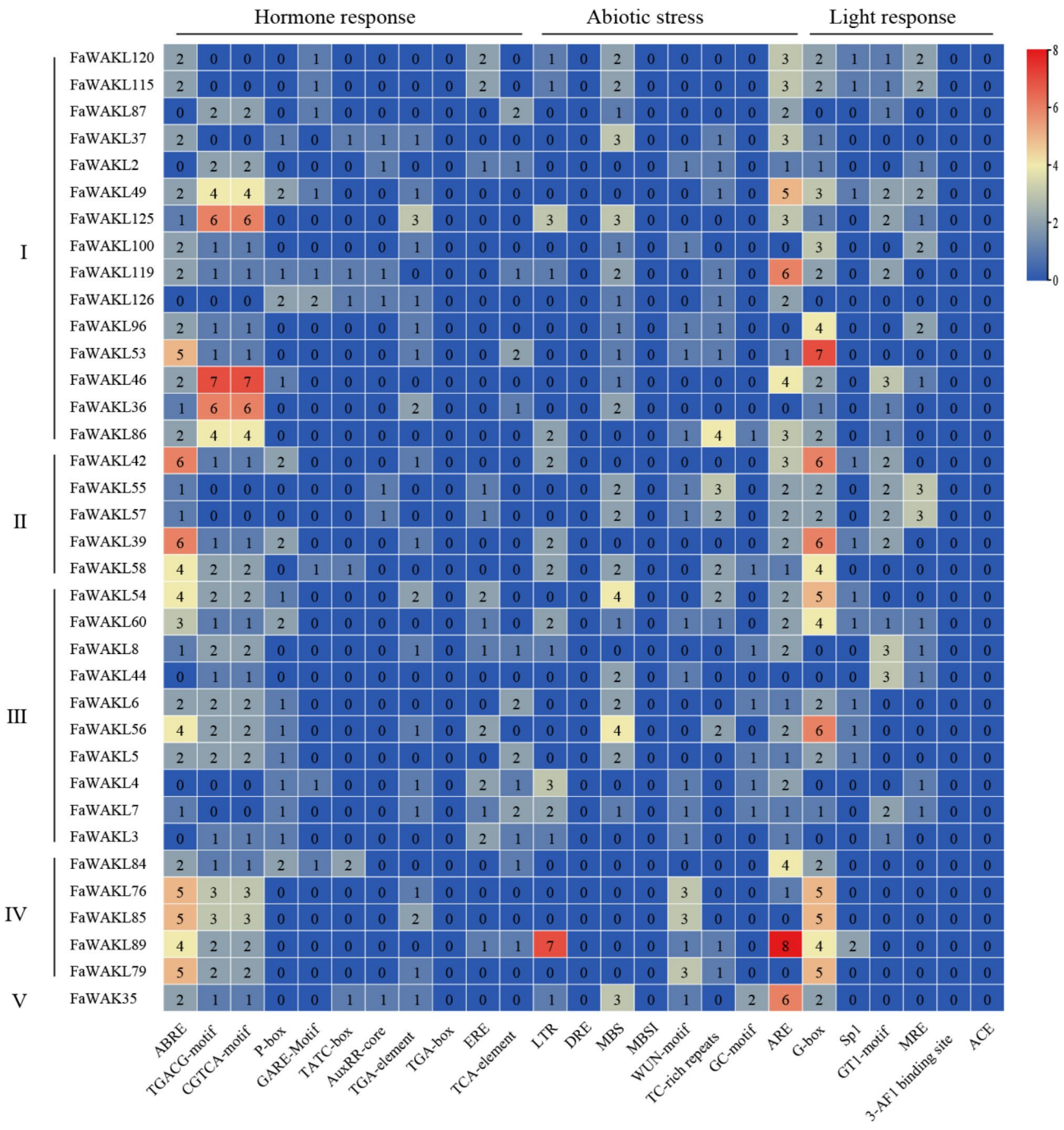

Fig. 5 Potential *cis*-regulatory elements of the 36 members of *FaWAK/FaWAKL* that responded to *B. cinerea* infection. The number of each *cis*-regulatory element was shown, and the color changed from blue to red as the number increased. All *cis*-regulatory elements were classified into three groups, including hormones, biotic, and light

was significantly upregulated by OGs treatment. Thus, *FaWAK35* was considered an important candidate gene for strawberry resistance to *B. cinerea*.

To clarify whether *FaWAK35* was involved in strawberry defense response, we overexpressed *FaWAK35* in strawberry fruits, and inoculated the fruits with *B. cinerea*. The overexpressed strawberry fruits exhibited a significant decrease in lesion development, compared to the control. After 96 of inoculation, the lesion on *FaWAK35-OE* fruits was 28.75% lower than that on the control fruits ($P \leq 0.05$; Fig. 9A and C). Finally, the over-expressed efficiency was confirmed by RT-qPCR (Fig. 9B). These results suggested that *FaWAK35* played

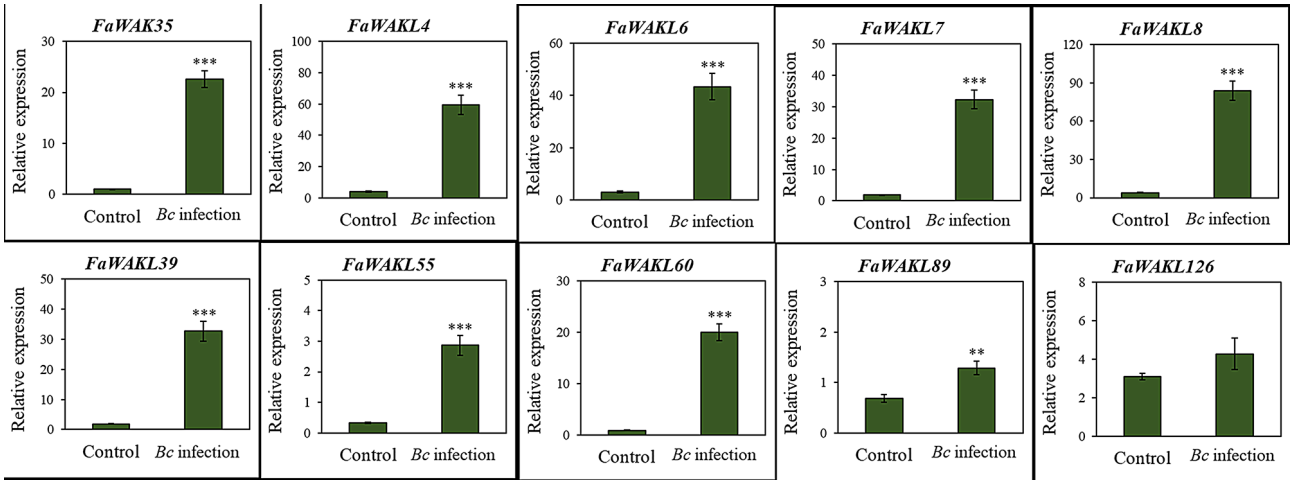

Fig. 6 The expression analysis of *FaWAKs/FaWAKLs* in strawberry fruits after *B. cinerea* infection. Each value is the mean for three replicates, and the vertical bar indicates the standard error.*** $P \leq 0.001$; ** $P \leq 0.01$

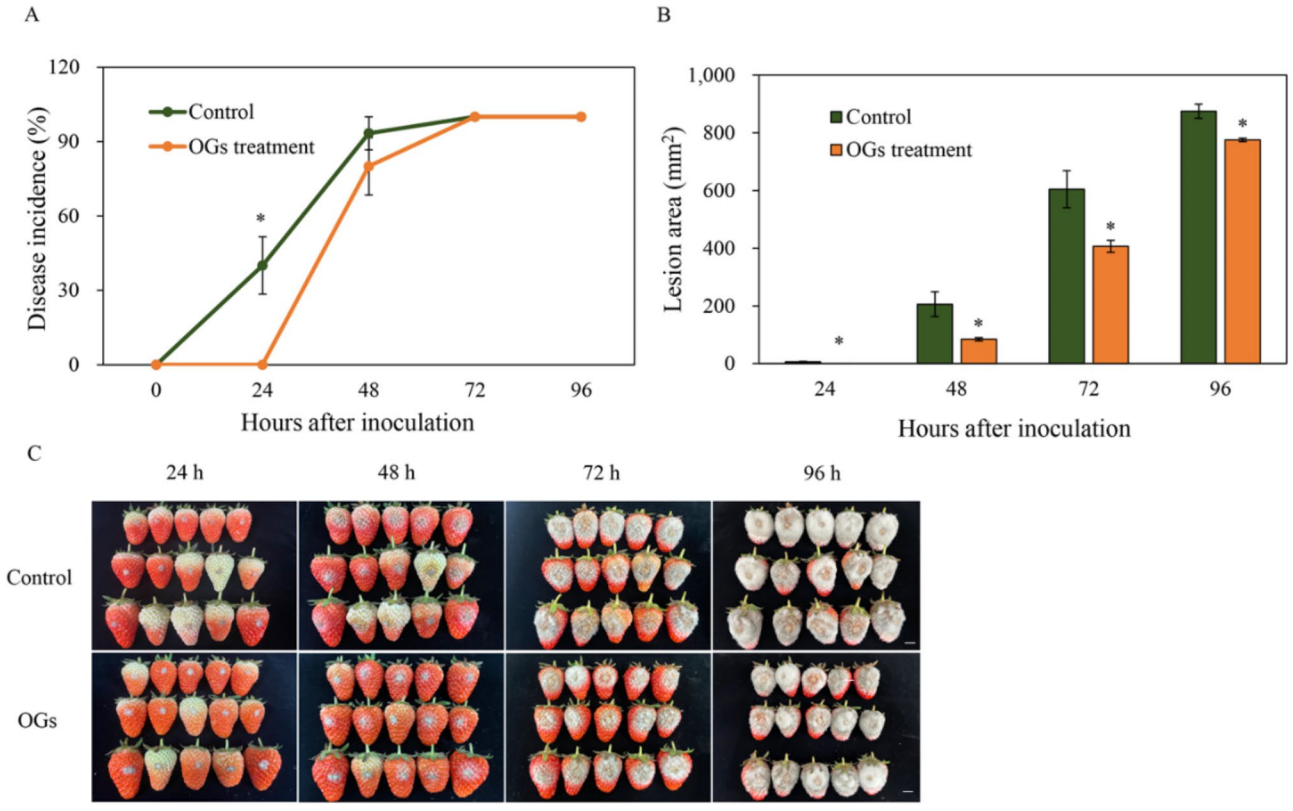

Fig. 7 Effects of oligogalacturonic acids treatment on disease development of strawberry fruits artificially inoculated with *B. cinerea*. (**A**) Disease incidence; (**B**) Lesion development; (**C**) Changes in symptoms, bars = 1.0 cm. Each value is the mean for three replicates, and the vertical bar indicates the standard error. Within the same day, * $P \leq 0.05$

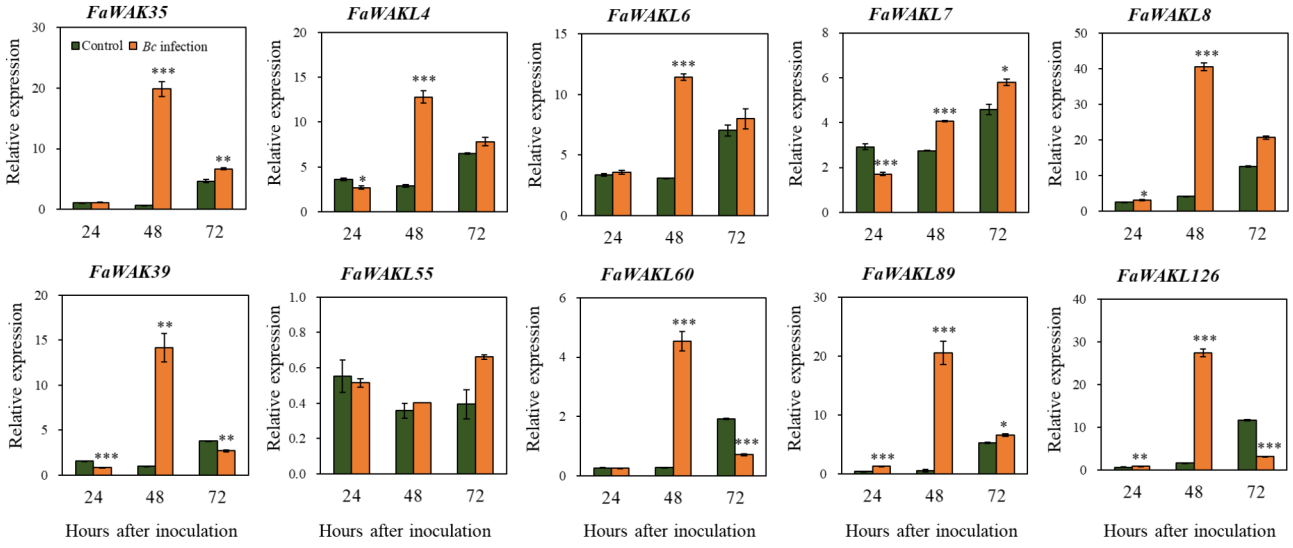

Fig. 8 The expression analysis of *FaWAKs/FaWAKLs* in strawberry fruits after oligogalacturonic acids treatment. Each value is the mean for three replicates, and the vertical bar indicates the standard error. Within the same day, *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$

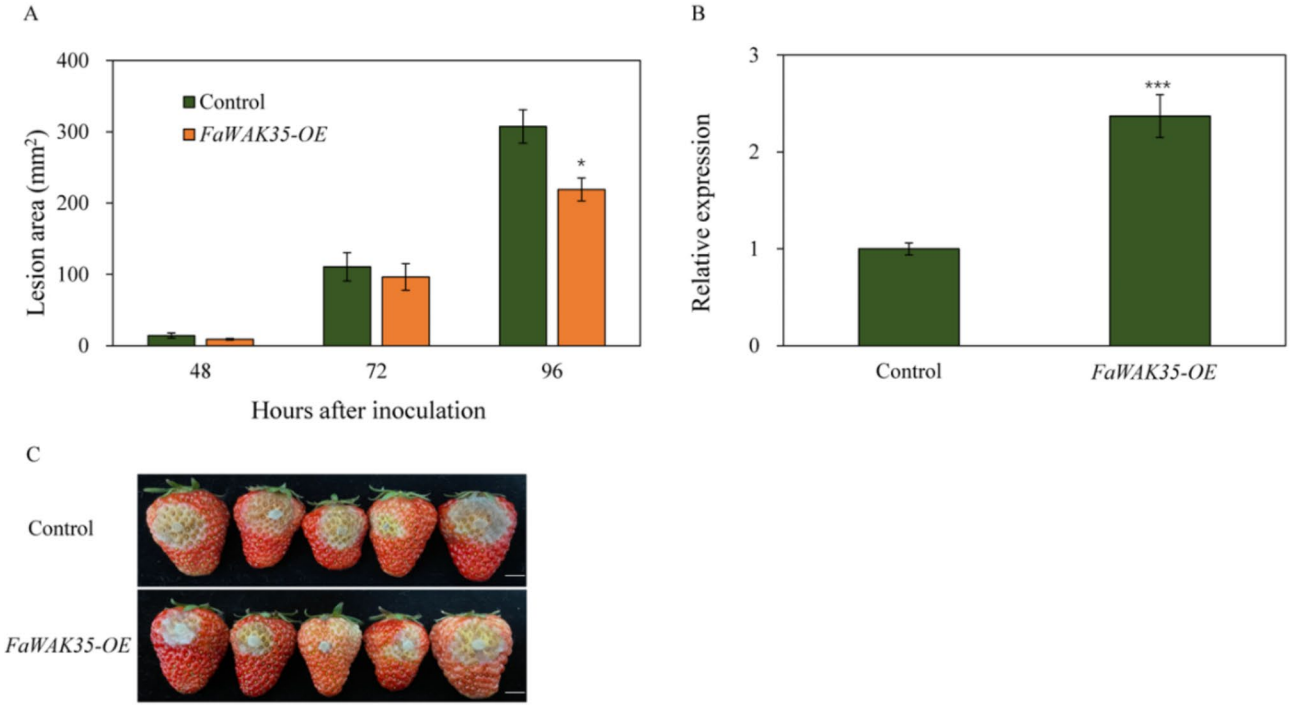

Fig. 9 Function analysis of strawberry wall-associated kinase *FaWAK35*. (**A**) Lesion development on strawberry fruits inoculated with *B. cinerea* on *FaWAK35-OE* and control fruit. (**B**) Quantification of *FaWAK35* expression in *FaWAK35-OE* and control fruit. (**C**) Changes in symptoms at 96 h post inoculation, bars = 1.0 cm. Each value is the mean for three replicates, and the vertical bar indicates the standard error. Within the same day, *** $P \leq 0.001$; * $P \leq 0.05$. OE: overexpression

and important role in strawberry fruit resistance to *B. cinerea*.

Discussion

The plant resistance that is initiated by the PRRs located on the cell membrane recognizes pathogenic signals, such as DAMPs or PAMPs. The RLKs are a class of PRRs that are widely located on the surface of plant cells. They contain extracellular signal-sensing and intracellular kinase structures and play a crucial role in the transduction of extracellular signals by perceiving the changes in polysaccharides, proteins, lipids and other ligands [14]. The WAK/WAKL comprise an important group of RLKs for necrotrophic pathogens like *B. cinerea* [15]. The

genome-wide characterization of *WAK/WAKL* has been successively reported in diploid wild strawberry (*Fragaria vesca*) and some other members of the Rosaceae, and studies have confirmed the important role of the *WAK/WAKL* genes in rose [15, 16]. However, to our knowledge, there has not been any systematic study of the *FaWAK/FaWAKL* genes in octoploid cultivated strawberry (*Fragaria×ananassa*), and the functions of *FaWAKs/FaWAKLs* remain largely unclear. In this study, we used the octoploid cultivated strawberry genome as a reference to perform a genome-wide analysis of the *FaWAK/FaWAKL* genes. We predicted the potential function of the *FaWAK/FaWAKL* genes in strawberry through physiological and biochemical characterization, chromosomal location, phylogenetic analysis, putative *cis*-regulatory elements, and gene expression in response to infection with *B. cinerea* and exogenous treatment with the OGs.

We identified all 167 *FaWAK/FaWAKL* genes in octoploid cultivated strawberry. There are 36 *FvWAKs*/*FvWAKLs* in diploid wild strawberry [15]. The increase in number may be related to gene duplication events. There are different numbers of *WAK/WAKL* genes of different species, and 125, 96, 29, 91, and 68 *WAK/WAKL* gene family members were identified from rice [17, 18], cabbage (*Brassica oleracea* var. *capitata*) [19], contton [20], barley [8], and rose [16], respectively, indicating that members of the *WAK/WAKL* gene family varied extensively and expanded among different species. In *A. thaliana*, there are five *WAK* and 21 *WAKL* genes [21, 22]. The *AtWAK* members have different extracellular domain sequences, with similarities that range from 40 to 64%. This suggested that their extracellular domains may bind different ligands and receive varied environmental signals, which indicated that the *WAK/WAKL* gene family is functionally redundant and differentiated [21]. In this study, the analysis of the *FaWAK/FaWAKL* gene family also confirmed this point, with 40 *FaWAK* and 127 *FaWAKL* genes verified. The unbalanced distribution of the *FaWAK/FaWAKL* genes on the chromosome also indicated that there was genetic variation during the evolutionary process, which was consistent with other studies on the *Rosaceae*.

Combined with the transcription analysis, we selected 36 *FaWAK/FaWAKL* genes that were upregulated by infection with *B. cinerea*. All 36 *FaWAK/FaWAKL* genes contained the GUB_WAK_bind extracellular domain, which suggested that the GUB_WAK_bind domain might contribute to the recognition of *B. cinerea* in strawberry fruit. The cysteine-rich GUB_WAK_bind is a unique domain of WAKs, and it has been reported to play an important role in transmitting signals, such as pectin fragments that activate the corresponding physiological processes [6]. However, the 36 *FaWAKs/FaWAKLs* that responded to *B. cinerea* did not show a similar characterization based on an analysis of its physiology/biochemistry and chromosomal locations.

In the phylogenetic analysis, the *FaWAK/FaWAKL* family members were divided into five groups. An evolutionary relationship analysis of barley (*Hordeum vulgare*) and rose also divided the *WAKs/WAKLs* into five groups [16]. A phylogenetic analysis of *FaWAKs/FaWAKLs* with 16 plant defense response-related *WAK/WAKL* family members enables us to hypothesize about the function of the *FaWAKs/FaWAKLs*. In this study, eight *FaWAK/FaWAKL* genes were divided with *ZmWAK-RLK1* into group II, and five of them were stimulated by infection with *B. cinerea*. *ZmWAK-RLK1* has been reported to play a critical role against maize leaf blight caused by *Exserohilum turcicum* [23]. A total of 18 *FaWAK/FaWAKL* genes and *OsWAK25* were divided into group III. *OsWAK25* has been reported to increase resistance to bacterial leaf blight (*Xanthomonas oryzae*) and rice blast (*Magnaporthe oryzae*) [24]. However, 10 *FaWAK/FaWAKL* genes that responded to *B. cinerea* were far from the *OsWAK25* in the evolutionary relationship. In group IV, *FaWAKL86* had a close evolutionary relationship with *RcWAK4*. *RcWAK4* has been studied to increase the resistance of rose petals to *B. cinerea* [16]. In group V, *AtWAK1*,*AtWAK2*,*GhWAK7A*, and *SlWAK1* have been identified as able to increase the resistance of plants to *B. cinerea*, *V. dahliae*, *Fusarium oxysporum*, and *Pseudomonas syringae* [9, 25]. *FaWAK35* clustered into the same branch with the four *WAKs*. Moreover, *FaWAK35* was upregulated 22.63-fold after infection with *B. cinerea*, and 28.92-fold after the treatment with OGs.

The *cis*-acting elements are short DNA sequences in gene regulatory regions, which are closely related to potential gene functions and regulatory mechanisms. In this study, a large number of *cis*-acting elements related to hormone response were detected. Plant hormones are important signaling components that mediate the resistance of plants to pathogens. JA primarily induces plant immune responses to necrotrophic pathogens, while SA primarily induces plant immune responses to biotrophic and semi-biotrophic pathogens [26]. The analysis of *cis*-acting elements in the promoters indicated that the elements that are affected by ABA and JA respond more intensively. ABRE can specifically recognize the gene promoter ABRE *cis*-acting elements and participate in the regulation of ABA response [27]. The TGACG motif and CGTCA motif *cis*-acting elements are used to respond to JA. They all play an important role in plant resistance. The three *cis*-acting elements were the most abundant elements in the *FaWAK/FaWAKL* family.

The patterns of expression of the *FaWAKs/FaWAKLs* induced by *B. cinerea* could provide candidate genes for their possible involvement in defense response to *B. cinerea*. In this study, the levels of expression of

FaWAK35,*FaWAKL60*,*FaWAKL8*,*FaWAKL6*,*FaWAKL4*,*FaWAKL39*, and *FaWAKL7* were strongly increased by *B. cinerea*. Transient over-expression of *FaWAK35* in strawberry fruit exhibited increased resistance to *B. cinerea*, indicating its potential function in defense response It has been shown that *RcWAK4* in rose petals was upregulated by *B. cinerea* infection, and the transient silencing of *RcWAK4* in rose petals increased their susceptibility to *B. cinerea* [16]. The extracellular domains of *WAK/WAKL* can bind signal molecules, such as oligogalacturonic acid and pectin fragments, and activate resistance to plant diseases through various mechanisms [28]. In this study, the treatment with OGs inhibited the development of lesions on strawberry fruit inoculated with *B. cinerea*, as well as stimulating the expression of the *FaWAKs/FaWAKLs*, particularly *FaWAK35* and *FaWAKL89*. Together, these results suggested that *FaWAK35* as an important *FaWAK/FaWAKL* member involved in the response of strawberry fruit against *B. cinerea*.

Conclusions

A genome-wide characterization of the *FaWAK/FaWAKL* family was performed in this study and primarily included physiological and biochemical properties, chromosome localization, gene structure analysis, phylogenetic relationships, and an analysis of the expression of genes induced by *B. cinerea*. We identified a total of 167 non-redundant *FaWAK/FaWAKL* family members in the whole genome of *Fragaria×ananassa*. Our qRT-PCR analysis indicated a few of *FaWAK/FaWAKL* genes upregulated with *B. cinerea* infection and involved in the resistance to *B. cinerea* was induced by the OGs in strawberry fruit. *FaWAK35* was confirmed to be involved in strawberry fruit resistance to *B. cinerea* by transient expression. This study lays the foundation for further exploration of the *FaWAK/FaWAKL* genes in the resistance of strawberry to *B. cinerea.*

Materials and methods

Fruit and fungal materials

Strawberry fruits (*Fragaria×ananassa*) were harvested from a greenhouse in Beijing, China, and all the strawberries were cultivated under commercial management. Fruits with uniform size and color and free of physical damage and disease lesions were selected for the experiment.

The *Botrytis cinerea* strain was purchased from the Agricultural Culture Collection of China (ACCC) and stored at 4 °C. For mycelial production, the strain was inoculated on potato dextrose agar (PDA) medium and incubated at 25 °C with 12 h of light for 14 days.

Identification and characterization of the *FaWAK/FaWAKL* family members in *Fragaria×ananassa*

The complete genome of the octoploid cultivated strawberry (*Fragaria×ananassa*) was downloaded from https://www.rosaceae.org/Analysis/14723107 and used as a reference. First, we performed a Blastall homology search in the *Fragaria×ananassa* library based on the 68 members of WAK/WAKL in *Rosa chinensis* (E value < 0.001), since both strawberry and rose belong to *Rosaceae*, and the characterization of *WAK/WAKL* family in *Rosa chinensis* was reported [16]. Secondly, based on the identification of the *FaWAKs/FaWAKLs*, the HMM files of EGF_CA (PF07645), GUB_WAK_bind (PF13947) and PKinase_Tyr (PF07714) were downloaded from the Pfam database (http://pfam.xfam.org) for hmmsearch with an E-value < 0.001 [16]. Finally, the prediction of the genes with conserved domain database (https://www.ncbi.nlm.nih.gov/cdd/?term=) and TMHMM Sever (https://services.healthtech.dtu.dk/services/TMHMM-2.0) were co mbined. The candidate *FaWAK/FaWAKL* genes that contained the PKinase structural domain, EGF_CA domain or GUB_WAK_bind domain, transmembrane structure, and signal peptide were selected. The gene chromosome location distribution was mapped by Map to chat.

Gene structure and phylogenetic analysis of the *FaWAKs/FaWAKLs*

The structures of the *FaWAKs/FaWAKLs* were performed using Batch CD-Search (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi). The motifs were analyzed using MEME (https://meme-suite.org/meme), and the promoters were predicted using PlantPAN 3.0 (http://plantpan.itps.ncku.edu.tw/plantpan3/index.html). These results were uploaded to TBtools for integration and visualization [29].

We used the ClustalW tool to align the multiple sequences of the *FaWAK/FaWAKL* genes with the *WAK/WAKL* genes in other species. Subsequently, MEGA7 software was used to build the phylogenetic tree using the Neighbor-Joining method with a bootstrap of 1,000 replicates. The *WAK/WAKL* family members from the other plants included *A. thaliana* (*AtWAK1*,*AtWAK2*,*AtWAKL10*, and *AtWAKL22*), cotton *(GhWAK7A)*, rice *(OsWAK14*,*OsWAK91*,*OsWAK92*,*OsWAK112d*,*OsWAK25*, and *OsIRBB4_Xa4)*, tomato *(SlWAK1)*, wheat *(TaWAK6)*, maize *(ZmWAK*,*ZmWAK-RLK1)*, and rose *(RcWAK4)*, which had been shown to be associated with plant defense responses in a previous study.

Patterns of expression of the *FaWAK/FaWAKL* genes in *Fragaria×ananassa following infection withB. cinerea*

Strawberry fruits were artificially inoculated with the mycelia of *B. cinerea* and incubated at 25 °C and 80%

relative humidity (RH) with 12 h of light. After 48 h, the fruit tissue around the lesion was sampled and stored at -80 °C for subsequent experiments. Uninfected fruit were sampled as the control. There were three replicates, and each replicate contained 20 fruits. The experiment was repeated three times.

The RNA-seq data of the strawberry fruit in response to *B. cinerea* were obtained from the octoploid cultivated strawberry genome database (https://www.rosaceae.org/Analysis/14723107). The clean data were mapped to the genome, and the Fragments Per Kilobase per Million reads (FPKM) were used to evaluate the levels of gene expression, and log2 (FPKM treatment/ FPKM control) was used to calculate the differential level of expression of the genes.

Real-time quantitative reverse transcription PCR (RT-qPCR) was used to confirm the results of RNA-seq. The total RNA was extracted using an E.Z.N.A. Total RNA Kit (Omega Bio-Tek, Norcross, GA, USA) with some modifications. The first-strand cDNA was synthesized from 1 g of total RNA using an R223 Kit (Vazyme Biotech Co., Ltd., Dalian, China). The primers were designed on the NCBI and listed in Supplementary Table 4. An ABI QuantStudio 1 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) was used in the standard mode with a Q712 Kit (Vazyme Biotech Co., Ltd.). The relative expression of the target gene was calculated using the $2^{-\Delta\Delta CT}$ analysis, and the *FaACTIN* gene was used as the internal control.

Patterns of expression of the *FaWAK/FaWAKL* genes in *Fragaria×ananassa* stimulated by the OGs

Strawberry fruits were treated with OGs with an oligomerization degree 10–15 at the concentration of 100 µL/mg and inoculated with *B. cinerea* mycelia as described above. The inoculated fruits were incubated at 25 °C and 80% RH with 12 h of light, and the lesion area was calculated to evaluate the development of the lesions. The fruits were treated with ddH_2O as the control. There were three replicates for each treatment, and one replicate contained 20 fruits. The experiment was repeated three times.

The strawberry fruits were treated with the OGs as described above and placed at 25 °C and 80% RH with 12 h of light. At 24 h, 48 h, and 72 h of treatment, the fruits were sampled and stored at -80 °C for the analysis of the expression of *FaWAKs/FaWAKLs* as described above. The fruits were treated with ddH_2O as the control. There were three replicates for each treatment, and one replicate contained 20 fruits. The experiment was repeated three times.

Overexpression of *FaWAK35* and *B. cinerea* inoculation assays

The coding sequence (CDS) of *FaWAK35* was isolated and ligated into clone vector pRI101, empty pRI101 was used as a control. The constructs were transformed into *Agrobacterium* strain GV3101, respectively, and cultured on LB medium (50 mg/mL kanamycin and rifampicin, respectively). For each construct, a single colony was picked and cultured in liquid LB medium containing the same antibiotics overnight, and then cultivated to OD 600 value of 0.8. The liquid was centrifuged and the collected cells were suspended with infiltration buffer containing 10 mM MES, 10 mM $MgCl_2$, and 100 µm acetosyringone (pH 5.6), respectively. After standing at room temperature for 2 h, the buffer was injected slowly into the strawberry fruits at white stage using a 1-mL syringe. The strawberry fruits were collected after four days, and inoculated with *B. cinerea* mycelia as described above. The inoculated fruits were incubated at 25 °C and 80% RH with 12 h of light, and the lesion area was calculated to evaluate the development of the lesions. There were three replicates for each, and one replicate contained 20 fruits. The experiment was repeated three times.

Abbreviations

ABA	Abscisic acid
DAMPs	Damage-associated molecular patterns
EGF_CA_bind	Calcium-binding epidermal growth factor -like domain
GUB_WAK_bind	Galacturonan-binding domain
JA	Jasmonic acid
MeJA	Methyl jasmonate
OGs	Oligogalacturonic acids
PAMPs	Pathogen-associated molecular patterns
PGs	Pectinases
PGIPs	Pectinase inhibitor proteins
PRRs	Pattern recognition receptors
PTI	Pattern-triggered immunity
RLKs	Receptor-like protein kinases
SA	Salicylic acid
WAK/WAKL	Wall-associated kinase/Wall-associated kinase-like

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06405-0.

Supplementary Material 1

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Author contributions

JY, ZH, and CX conceived and designed the experiments; CX, XK, and YZ performed the experiments; QP and MW assisted in the experiments; ZH and CX analyzed the data; JY and ZH wrote the paper. All the authors have read and approved the final version of the manuscript. All the authors reviewed the manuscript.

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Data availability
The *FaWAK/FaWAKL* sequences used or analyzed during the current study are available in '*Fragaria×ananassa*' (https://www.rosaceae.org/Analysis/14723107). The data generated or analyzed during the current study are included in this published article and its supplemental data file. The RNA-seq data used in the current study is available in NCBI database with the accession number PRJNA1203349.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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