Inhibition of PbeXTH1 and PbeSEOR1 is required for the Valsa canker resistance contributed by Wall-associated kinase gene MbWAK1

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Abstract

Wall-associated kinases (WAKs) were determined the role for perceiving pathogenic signals and initiation of plant immune responses. However, the roles of the family members in host resistance against *Valsa* canker, a serious fungal disease of apple and pear, are largely unknown. Here, we identified *MbWAK1*in *Malus baccata*, a resistant germplasm, was differentially expressed during infection by *Valsa mali* (*Vm*) and multiple stress-related signals. Over-expression *MbWAK1* enhanced the *Valsa* canker resistance of apple and pear fruits and 'Duli-G03' (*Pyrus betulifolia*) suspension cells. A large number of phloem, cell wall and lipid metabolic process-related genes were differentially expressed during overexpressed suspension cells lines respond to *Valsa pyri* (*Vp*) signals. Among these, the expression of xyloglucan endotransglucosylase/hydrolase gene *PbeXTH1* and sieve element occlusion-related gene *PbeSEOR1* were significantly inhibited. Transient expression of *PbeXTH1* or *PbeSEOR1* obviously compromised the expressional induction of *MbWAK1* and the resistance contributed by *MbWAK1*. In addition, *MbWAK1* interacted with *PbeXTH1* and *PbeSEOR1*, as well as several patterns recognize receptors, such as *MdBAK1* and *MdCERK1*. Our results enriched the molecular mechanisms for *MbWAK1* against *Valsa* canker and resistant breeding.

Key Message

The regulatory role of *MbWAK1* on *Valsa* canker is affected by *PbeXTH1* and *PbeSEOR1*.

Introduction

During long-term of co-evolution between plants and pathogenic microorganisms, plants have formed the sophisticated immune system to deal with the invasion (Jones et al., 2006). Upon infection, plant membrane localized pattern-recognition receptors (PRRs) can recognize the signals from pathogen/damage-associated molecular patterns (PAMPs/DAMPs) and activate horizontal disease resistance, known as PAMPs-triggered immunity (PTI) (Zhou et al., 2016). After that, the pathogens can employ effectors to inhibit PTI, but the signals can be sensed by the R protein and initiate vertical disease resistance, defined as effector-triggered immunity (ETI) (Boller et al., 2009). In normal, PTI contributes to plant basal resistance to various microbes, whereas ETI plays a central role in host-specific resistance. Compared to ETI, PTI is more important for host resistance against necrotrophs (Cui et al., 2015). And PTI responses include reactive oxygen species (ROS) bursts, activation of mitogen-activated protein kinase (MAPK) cascades, callose deposition, etc. (Nicaise et al., 2009)

Receptor-like kinases (RLKs) are the key PRRs and regulate various biological processes in plants, including development and immunity responses (Luo et al., 2017). Typically, RLKs are composed of a signal peptide, an N-terminal domain, a transmembrane region, and a C-terminal kinase domain (Gandhi et al., 2023). Based on the diversity of extracellular domains, RLKs can be classified into 14 types, such as leucine-rich repeat (LRR), *Catharanthus roseus*-like (CrRLK), lysin motif (LysM), thaumatin, leaf rust kinase-like (LRK), wall-associated kinase (WAK), etc. (Jose et al., 2020). Several RLKs, such as the

brassinosteroid insensitive1-associated kinase1 (BAK1), the flagellin sensing 2 (FLS2), wall-associated kinase 1 (WAK1), botrytis-induced kinase 1 (BIK1) and chitin elicitor receptor kinase 1 (CERK1), were determined as typical PRRs and responsible for PAMPs perception and immune response induction (Saur et al., 2016; Roux et al., 2011; Petutschnig et al., 2010; Gimenez et al., 2009). Among these, CERK1 and WAK1 were shown to regulate immune responses against to infection of necrotrophic microbes (Yang et al., 2022).

WAKs possess extracellular region epidermal growth factor (EGF)-like domain. There are five members, WAK1-5, were determined in *Arabidopsis thaliana* (He et al., 1999). Some of these have been suggested as sensor for the environmental and intracellular signals (Verica et al., 2002). Five tandemly arranged also regard as the key regulators for plant growth and responses to attacking of various pathogens. WAKs act as typical receptors for cross-linked pectin to regulate cell expansion, and they can also bind to oligogalacturonic (OGs) to activate stress responses (Gramegna et al., 2016; Kohorn et al., 2016). To date, the vital role of some WAKs in perceiving pathogenic signals and initiation of immune responses was determined in *A. thaliana*, tomato, and cotton, etc. (Delteil et al., 2016; Feng et al., 2021; Kohorn et al., 2009; Zhang et al., 2020). Multiple immunity-related signals, such as methyl jasmonate (MeJA), salicylic acid (SA), and ethylene (ET), callose deposition, system acquired resistance (SAR), are also involved in the immune response regulated by WAKs (Brutus et al., 2010; Li et al.,2020; Lin et al., 2021).

Valsa canker, caused by *Valsa* species, is one of the most serious fungal diseases in apple and pear-producing areas in China and East Asia (Cui et al., 2022). For a long term, resistant breeding has been recognized as an efficient, durable, economically and environmentally acceptable practice for controlling the widespread spread of diseases and reducing fungicide applications (Zhou et al., 2019; Deng et al., 2020). To speed up the progress of resistant breeding, it is more and more important to investigate disease-resistance mechanism and identify disease-resistance genes. With multiple characteristics such as strong environmental adaptability and high resistance to *Valsa* canker, *Malus baccata* was widely used as the rootstocks in the apple-growing regions of northern China (Abe et al., 2010). In the previous study, we screened a WAK-RLK gene, *MbWAK1* (MABA014609), which was differentially expressed in ‘Dongbeishanjingzi’ (*M. baccata*) suspension cells in response to signals from *Valsa* canker (Mao et al., 2021).

In this investigation, we identified that *MbWAK1* was up-regulated in resistant germplasm *M. baccata* when it respond to *Valsa* canker signals. Functional investigations displays that *MbWAK1* positively regulate *Valsa* canker resistance and initiate immune responses. Overexpression of *MbWAK1* strongly inhibited the expression of the *PbeXTH1* and *PbeSEOR1*. Further functional analysis reveals that these two downstream genes could significantly weaken the *Valsa* canker resistance and related immune responses contributed by *MbWAK1*. Our results supplied a potential basis for the resistant mechanism regulated by WAK genes on *Valsa* canker resistance of apple and pear.

Material and method

Plant material and growth conditions

Suspension cells of the 'Duli-G03' (*P. betulifolia*) and 'Dongbeishanjingzi' (*M. baccata*) were induced from leaves of tissue-cultured plantlets and cultured at 25 ºC in darkness with shaking at 100 rpm. The pathogenic *Vp* isolate Vp-001 and *Vm* isolate Vm-A-003 were isolated from diseased phloem tissues of pear (*P. bretschneideri*) 'Yuluxiang' and apple (*M. domestica*), respectively (Sun et al. 2023). For inoculation tests, the strains were darkly cultured on potato dextrose agar (PDA) for 5 days at 25 ºC.

Bio-informatics analysis

Structural domain composition was analyzed by online software SMART (http://smart.emblheidelberg.de/). Using Arabidopsis Information Resource (TAIR, https://www.arabidopsis.org/), the Genome Database for Rosaceae (GDR, https://www.rosaceae.org/), and the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) to retrieve sequences, then the phylogenetic tree were constructed by using the neighbor-joining method in MEGA 5.0 (http://www.megasoftware.net). Prediction of *cis*-acting elements was performed using Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plant-care/html/).

Preparation of *Vp* and *Vm* metabolites

Ten mycelia agar discs of *Vp* or *Vm* with 5 mm diameter were transferred into 100 mL of potato deoxy glucose broth (PDB) and darkly cultured 3 d at 25 ºC. The supernatant was harvested as the *Vp* metabolites (*Vp*M) and *Vm* metabolites (*Vm*M) via centrifugation (5500 rpm, 10 min) and filtration (13-mm PES syringe with a 0.22 μm filter).

The treatments

For hormonal treatment, methyl jasmonate (MeJA) or abscisic acid (ABA) with final concentration of 50 μM were added into pre-cultured 'Dongbeishanjingzi' cell suspensions and darkly cultured for 0, 1, 3 and 6 h at 25 ºC, respectively. The samples were collected for RNA extraction and qRT-PCR assay. For infection test, *Vm* was prepared for incubation of apple fruits, whereas *Vp* was used for pear fruits and 'Duli-G03' cells (WT and transgenic cell lines). *Vm*M and *Vp*M were collected for metabolism treatment of suspension cells of 'Dongbeishanjingzi' and 'Duli-G03' (WT and transgenic cell lines), respectively. In brief, *Vm*M or *Vp*M were added into the cell suspensions with the final concentration as 10% or 20% (v/v). The cells were harvested at 0, 1, 3 and 6 h of *Vm*M or *Vp*M exposure for further qRT-PCR assay and cell viability detection.

Subcellular localization of *MbWAK1*

The coding sequence (CDS) of *MbWAK1* was cloned independently into pBI121-GFP vector (Tiandz, Inc., China). The primer sequences are listed in Table S1. The plasmids of *Pro35S::MbWAK1-GFP* and *Pro35S::GFP* were transformed into *Agrobacterium tumefaciens* strain GV3101, and then the *Nicotiana*

benthamiana GFP fluorescence signals were detected at 488 nm using confocal laser-scanning microscope (Olympus FV1200).

qRT-PCR analysis

Total RNA of the samples was isolated by using a Plant RNAout kit (Tiandz Inc., Beijing, China). The purity, concentration and integrity of RNA were checked by using agarose gel electrophoresis and an ultrami-cro spectrophotometer (Nanodrop and Agilent 2100). The specific steps of quantitative PCR and determination of *Actin* gene were referred as the method described previously in Sun et al. (2023). The primer sequences were listed in Table S2. Each qRT-PCR analysis was biologically replicated at least three times. The fluorescence value change curve and fusion curves were analyzed. The relative transcript levels were calculated via the modified $2^{-\Delta\Delta CT}$ method (Livak et al., 2001).

Transformation of suspension cells

Full-length *MbWAK1* was inserted into the pFGC-5941 vector. The recombinant plasmid was transformed into *A. tumefaciens* strain GV3101 and was further delivered into 'Duli-G03' suspension cells via Agrobacturium-mediated transformation method (Mao et al., 2021). Finally, over-expression cell lines were screened by using glufosinate-ammonium (15 g/mL), PCR detection and qRT-PCR assay.

Bimolecular fluorescence complementation (BiFC) assays

For the BiFC assays, the following construct combinations were used: *MbWAK1-nY/PbeBAK1-cY*, *MbWAK1-nY/PbeBIK1-cY*, *MbWAK1-nY/PbeCERK-cY*, *MbWAK1-nY/PbeFLS2-cY*, *MbWAK1-nY/PbeXTH1-cY*, *MbWAK1-nY/PbeSEOR1-cY*, *MbWAK1-nY/cY*, *nY/PbeBAK1-cY*, *nY/PbeBIK1-cY*, *nY/PbeCERK-cY*, *nY/PbeFLS2-cY*, *nY/PbeXTH1-cY* and *nY/PbeSEOR1-cY*. The constructs were transformed into *A. tumefaciens* GV3101 for subsequent infiltration of *N. benthamiana* leaves. At 24 h post-infiltration, the YFP fluorescence signals were detected at 514 nm using confocal laser-scanning microscope (Olympus FV1200).

Transient expression in fruits and suspension cells

For transient expression of target genes in fruits, CDS of each gene was cloned into the pFGC-5941 vector (Tiandz, Inc., China). The empty vector was used as a control. The recombinant plasmid and empty vector were transformed into *A. tumefaciens* strain GV3101, respectively, and then penetrated into the fruit of 'Yanfu 3' apple (*M. domesticus*) or 'Huangguan' pear (*P. bretschneideri*) (Zhao et al., 2020). Similarly, for transient expression of suspension cells, the GV3101 strain carrying recombinant plasmid or empty vector were activated and then re-suspended in MS liquid medium containing 100 μM acetosyringone (AS). The suspensions were resuscitated for 3 h on a shaker at 28 °C, 180 rpm, and then added 5 mL of resuspension solution to the pre-treated 'Duli-G03' suspension cells or *MbWAK1* over-expression suspension cells. The cells were harvested for further analysis at 3 d post co-culturing at 25 °C.

Cell activity assay

Cell activity was determined by MTT assay (Yu et al., 2023). Briefly, 200 μL cells with various treatments were wished three times by PBS (pH=7.4) and re-suspended into 180 μL PBS. After that, the cells were added with 20 μL MTT solutions (0.4 mg/mL) and stained for 3 h at 37 ºC. The excess MTT aqueous solution was removed via PBS washing. The washed cells were re-suspended in 200 μL Dimethyl Sulfoxide (DMSO). The absorbance value was detected at 480 nm using multi-function enzyme labeler (Tecan Spark). The assays were biologically replicated at least 5 times.

Statistical analysis

Differences between means were determined in Microsoft Excel 2010 using Student's *t*-test.

Result

Bioinformatics analysis and subcellular localization of *MbWAK1*

MABA014609 encoded a protein with 664, which is composed of a signal peptide at the N-terminus, extracellular domain GUB_WAK_bind (PF13947) and EGF (PF00008), a transmembrane region, and an intracellular kinase domain S_TKc (SM000220) (Figure 1A). The construction of the phylogenetic tree displayed that MABA14609 was most closely related to *A. thaliana* WAKs (WAK1, AT1G21250; WAK3, AT1G21240; and WAK5, AT1G21230) and was named *MbWAK1* (Figure 1B). Further subcellular localization assay revealed that MbWAK1 was localized on the plasma membrane (Figure 1C). These results indicated that *MbWAK1* was a typical WAK member.

MbWAK1 responded to signals of MeJA, ABA and *Vm*

On the promoter region of *MbWAK1*, multiple predicted *cis*-elements were involved in response to stress or related signals, such as ABA, MeJA, and defense and stress signals (Table S3). Further expressional assays confirmed that *MbWAK1* was responsive to signals of ABA and MeJA (Figure 2A, B). The expressional data from previous transcriptomic analysis exhibited that the expression *MbWAK1* was up-regulated to 1.79-, 2.41-, and 2.64-fold of control when the 'Dongbeishanjingzi' suspension cells treated with *Vm* metabolism (*Vm*M) for 1, 3 and 6 h. Further qRT-PCR also revealed the continual up-regulation during the suspension cells exposured to *Vm*M (Figure 2C). In addition, we analyzed the expression pattern of *MbWAK1* in different tissues of *M. baccata*. Results showed that the expression level of *MbWAK1* was the highest in radical, followed by perennial roots, which was divided into 4.3- and 2.9-fold of the average expression level of each tissue, but the expression level was lower in annual branches, perennial branches, and leaves (Figure 2D). Above all, these results indicated that *MbWAK1* responded to *Vm* and multiple stresses-related signals.

Over-expression of *MbWAK1* enhanced *Valsa* canker resistance of 'Duli-G03' suspension cells

To further study the function of *MbWAK1* on *Vm*, we transformed *MbWAK1* into 'Duli-G03' suspension cells and selected three lines with the highest expression of *MbWAK1* for further study (Figure 3A). Then we inoculated *Vm* into the suspension cells of wild-type 'Duli-G03' (DL) and over-expression cell lines (*MbWAK1-1*, *MbWAK1-3*, *MbWAK1-4*) to detect the development of lesions. At 3 d post-incubation, the lesions on all over-expression lines showed a smaller diameter than that on wild-type suspension cells at the same time point (Figure 3A, C). The results were further confirmed by using MTT staining (Figure 3B). Furthermore, we compared the tolerance of 'Duli-G03' and *MbWAK1-3* suspension cells during exposured to *Vp*M. Obviously, the cell viability of the *MbWAK1-3* was consistently higher than that of 'Duli-G03' under the same treatment (Figure 3D). The above results indicated that over-expression of *MbWAK1* enhanced the *Valsa* canker resistance of 'Duli-G03' suspension cells.

MbWAK1 interacts with multiple PRRs.

BAK1, *FLS2*, *CERK1* and *BIK1* are well-characterized PRRs in *A. thaliana*, which play an important role in the perception of PAMPs and the induction of immune response (Boller et al., 2009). We identified four genes (*PbeBAK1*, Chr15.g00306; *PbeFLS2*, Chr5.g06311; *PbeCERK1*, Chr8.g55620 *and PbeBIK1*, Chr3.g20096) in the *P. betulifolia* genome, which are homologous to above PRRs in *A. thaliana* PRRs. The interactions between *MbWAK1* and the RLKs were determined using bimolecular fluorescence complementation (BiFC) assays (Figure 4). We observed that *MbWAK1* had a strong interaction with *PbeCERK1* and a weak interaction with the other three RLKs. Therefore, *MbWAK1* may play a key role in plant immunity.

MbWAK1 caused expressional changes of cell wall and phloem related genes

To further understand the regulatory mechanism of *MbWAK1*, we performed the RNA sequencing when the 'Duli-G03' and *MbWAK1-3* suspension cells were treated with *Vp*M (10%). Subsequently, 1364, 1191, 1280 and 1188 differentially expressed genes (DEGs) were detected at time points of 0 h (CK), 1 h (T1), 3 h (T2), and 6 h (T3), respectively (Figure 5A). Functional enrichment of DEGs exhibited that the GO terms "lipid metabolic process" and "Lignin catabolitic process" obviously enriched in up-regulated DEGs, whereas "cellular glucan metabolic process", "xyloglucan metabolic process", and "phloem development" in down-regulated DEGs (Figure 5B, C). Then the reliability of RNA-seq data were confirmed by detecting expression of candidate 9 genes using qRT-PCR assay (Figure S1). Within enrich GO terms, all 5 sieve element occlusion genes and 13 xyloglucan endotransglucosylase/hydrolase genes were down regulated, whereas all 9 laccase genes and lipid metabolite process-related genes were up-regulated (Figure 5D). These above results suggested that over-expression of *MbWAK1* resulted in the induction of lipid and laccase metabolic process during suspension cells response to *Vp*M, but in reduction of the xyloglucan endotransglucosylase/hydrolase and sieve element occlusion accumulation.

Over-expression of *MbWAK1* inhibited the expression of *PbeXTH1* and *PbeSEOR1*

Among enriched GO terms, Chr4.g38267 and Chr13.g23519 were significantly down-regulated in *MbWAK1-3* compared to the wild-type 'Duli-G03'. Blast analysis on the TAIR database showed that these

two genes were homologous of *ATXTH16* (AT3G23730) and *ATSEOR1* (AT3G01680) in *A. thaliana*, therefore named as *PbeXTH1* and *PbeSEOR1*, respectively. Analysis of the protein domain displayed that *PbeXTH1* has a xyloglucan endotransglucosylase (XET) domain, whereas *PbeSEOR1* possessed a SEO N-terminal domain (SEO-N), and a SEO C-terminal domain (SEO-C) (Figure 6A). We further examined the expression patterns of *PbeXTH1* and *PbeSEOR1* in 'Duli-G03' and *MbWAK1-3* suspension cells treated with 10% *Vp*M and 20% *Vp*M. Consistently with RNA-seq data, over-expression of *MbWAK1* significantly inhibited the expression of *PbeXTH1 and PbeSEOR1* during the cells treated with or without *Vp*M (Figure 6B-G). Above all, the expression of *PbeXTH1* and *PbeSEOR1* were significantly inhibited by over-expression of *MbWAK1*.

PbeXTH1 and *PbeSEOR1* compromised the *Valsa* canker resistance derived from *MbWAK1*

We further performed a transient expression to explore the role of *PbeXTH1* and *PbeSEOR1* in resistance to *Valsa* canker, and whether these two genes can influence the function of *MbWAK1*. The results further confirmed that both pear and apple fruits with over-expression of *MbWAK1* obviously reduced the lesion diameter compared with empty vector. In addition, transient expression of *PbeXTH1* enlarged the lesion area, but *PbeSEOR1* only displayed a weak enlargement. Importantly, compared to OE-*MbWAK1*, the lesion sizes were significantly enlarged on apple and pear fruits of OE-*MbWAK1*+OE-*PbeXTH1* and OE-*MbWAK1*+OE-*PbeSEOR1* (Figure 7). qRT-PCR demonstrated that all target genes were well overexpressed at the injection site (Figure 7C, G). Above all, these results suggested that the positive regulation of *MbWAK1* on *Valsa* canker resistance of fruits was significantly compromised by over-expression of both *PbeXTH1* and *PbeSEOR1*.

PbeXTH1 and *PbeSEOR1* suppressed the tolerance of *MbWAK1*-3 against *Vp*M

To further explore the influence of the two genes on *MbWAK1* function, we transiently overexpressed *PbeXTH1* and *PbeSEOR1* in *MbWAK1*-3. Compared to the control (empty vector), the cell viability of *MbWAK1*-3 was significantly decreased with 10% *Vp*M or 20% *Vp*M exposure for 6 h (Figure 8A, B). Subsequent qRT-PCR demonstrated that *PbeXTH1* and *PbeSEOR1* were well overexpressed in *MbWAK1*-3 (Figure 8D, E). Interestingly, the expression of *MbWAK1* in *MbWAK1-3* was largely suppressed at all time points by transient expression of *PbeSEOR1*, and at 1 h of *PbeXTH1* (Figure 8C). These data therefore suggested that the *Vp*M tolerance of 'Duli-G03' suspension cells caused by *MbWAK1* could be significantly weakened by the over-expression of *PbeXTH1* and *PbeSEOR1*.

MbWAK1 interacted with *PbeXTH1* and *PbeSEOR1*

Given the clear influence of *PbeXTH1* and *PbeSEOR1* on the function of *MbWAK1*, we detected whether MbWAK1 interacted with PbeXTH1 and PbeSEOR1 by using BIFC assay. As expected, the results confirmed that MbWAK1 directly interacted with PbeXTH1 and PbeSEOR1 (Figure 9A). Furthermore, we also analyzed the influence of *PbeXTH1* and *PbeSEOR1* on the expression defense-related genes treated or untreated with 20% *Vp*M. Compared with the control (*MbWAK1-3*), over-expression of *PbeXTH1* or *PbeSEOR1* both resulted in down-regulation of the SA-related gene *PbePR1*, the R-gene *PbeEDS1*, the ROS-

related gene *PbeTaNOX*, the PTI-related gene *PbeFRK1* and JA-realted gene *PbeLOX1* at later time points (Figure 9B). Collectively, these results showed that the immune responses induced by *MbWAK1* were at least partially inhibited by *PbeXTH1* and *PbeSEOR1*.

Discussion

Valsa canker, caused by *Valsa* species, is one of the most serious fungal diseases in apple and pear-producing areas in China and East Asia (Cui et al., 2022). Investigations on the resistant mechanism and related key genes are thus critical for the genetic improvement on the *Valsa* canker resistance of fruit crops in *Malus* and *Pyrus* species. Currently, we identified a WAK member, *MbWAK1*, is a positive regulator for *Valsa* canker resistance. Furthermore, we determined that inhibition of the *PbeXTH1* and *PbeSEOR1* are required for the resistance contributed by *MbWAK1*.

Plants employ PRRs for rapid and accurate detection of the potential danger caused by microbes and pests (Hu et al., 2017). Plants PRRs are either RLKs or RLPs, in which RLKs are part of the property-specific membrane protein complex and they have a key mediator of plants immunity (Cayrol et al., 2016). RLKs exhibit a wide range of extra-cellular motifs well suited for the recognition of PAMPs/DAMPs, including leucine-rich repeat sequences, lectin and lysine motifs and EGF-like extracellular structural domains (Dardick et al., 2012; Macho et al., 2014). Among theose, WAKs possess the EGF-like extracellular structural domain and have an important role in perceiving of pathogenic signals and initiating the immune responses (Zuo et al 2015; Delteil et al 2016). *WAK1*, the most studied gene in WAK gene family, was first confirmed that take crucial roles on protection of plant tissues via induction of salicylic acid during plant-pathogen interactions (He et al., 1998). Subsequently, a number of WAKs have been identified that are involved in the regulation of plant immunity. *Htn1*, encodes a WAK protein in maize, which is used to control leaf blight in northern maize (Hurni et al., 2015). In rice, *OsWAK91* is required for H_2O_2 production and sufficient to enhance defense gene expression during infection (Delteil et al., 2016). In one case, the *Snn1*, one WAKs gene in wheat, is a susceptibility factor to promote infection of the SnTox1 toxin produced by the *Parastagonospora nodorum* (Shi et al., 2016). Here, we identified that *MbWAK1* acts as a positive regulator in *Valsa* canker resistance and initiates immune responses (Fig. 1, 2, 3). To our knowledge, the roles of the members in this RLK subfamily on disease resistance were not yet systemically investigated in *Malus* or *Pyrus* species.

Many studies have showed that a number of PRRs co-regulate the downstream of the immune responses when the plant resist to pathogenic microorganisms (Zipfel et al., 2014). For example, FLS2 and FLS3 bind the flagellin-derived MAMPs flg22 and flgII-28, and in concert with the co-receptor BAK1 to activate intracellular immune signaling (Sun et al., 2013). In the downstream of the immune response, the receptor-like cytoplasmic kinase BIK1 interacts directly with FLS2, EFR, and CERK1. After activation of these PRRs, BIK1 will be rapidly phosphorylated and then detached from the receptor to activate the downstream signaling (Lu et al., 2010; Zhang et al., 2010). In addition, the WAK1 protein in tomato associates in a complex with FLS2 and FLS3 and plays an important role in the later stages of flagellin-induced PTI (Zhang et al., 2020). In our study, we also found that *MbWAK1* is associated with BAK1,

FLS2, BIK1 and CERK by BIFC (Fig. 4). Indeed, *GhWAK7A* as an important component in the regulation of cotton's response to fungal wilt pathogens, which promotes chitin-induced GhLYK5-GhCERK1 dimerization and directly interacts with both *GhLYK5* and *GhCERK1* (Wang et al., 2020). Therefore, we suggested that the *MbWAK1* was collaborating with other PRRs to regulate the *Valsa* canker resistance.

Enhancing of the cell wall integrity is crucial for resistance contributed by WAKs. In rice, *Xa4* encodes a WAK protein which confers race-specific durable resistance against *Xanthomonas oryzae pv. Oryzae* via increases the mechanical strength of the cell wall and reduces plant height (Hu et al., 2017). In our study, the expression of two genes, *PbeXTH1* and *PbeSEOR1*, were significantly suppressed when the *MbWAK1* over-expressing cell lines were treated with *Vp*M (Fig. 6b, c, d). *PbeXTH1* involves in a cell wall-modifying protein, which also have the capacity to loosen cell walls (Miedes et al., 2013). Over-expression of *CaXTH3* in *A. thaliana* and tomato enhances drought and salt tolerance in transgenic plants (Cho et al., 2006; Choi et al., 2011). However, the regulate mechanism of xyloglucan endotransglucosylase/hydrolases (XTHs) on plant immunity is largely elusive. In our study, we confirmed that *PbeXTH1* negatively regulates *Valsa* canker resistance. Further over-expression of *PbeXTH1* compromises the resistance and related immune responses contributed by *MbWAK1*. Thus, we suggest that suppression on expression of the *PbeXTH1* is one of the major factors in the resistance caused by over-expression of the *MbWAK1*.

PbeSEOR1, a SEOR gene, encode the common phloem proteins (P-proteins) and associate with plug sieve plates after wounding (Ernst et al., 2012). *ATSEOR1* (AT3G01680), the homologue of *PbeSEOR1* in *A. thaliana*, occurs as conjugates in the form of filaments in sieve elements with *AtSEOR2* (At3G01670) (Anstead et al., 2012). The *ATSEOR1ko* mutant line hosts a considerably lower number of phytoplasmas, even though the phloem flow is not affected. This observation leads to the hypothesis that an unknown *SEOR2*-associated mechanism assists the plant to combat the pathogens (Pagliari et al., 2017). Further investigation display that *SEOR2* interferes with phytohormonal pathways in *A. thaliana* midrib tissues in order to establish the early defensive responses (Bernardini C et al., 2020). Our data showed that expression of *PbeSEOR1* was significantly suppressed in *MbWAK1* over-expressed cell lines. Further over-expression of *PbeSEOR1* damaged resistance contributed by *MbWAK1* and the associated immune responses (Fig. 7, 8, 9). Thus, we suggest that the expressional inhibition of the *PbeSEOR1* is another major factor in the resistance derived from *MbWAK1*.

In conclusion, we demonstrated that *MbWAK1* is collaborating with other PRRs to positively regulate *Valsa* canker and induce immune responses. Overexpression of *MbWAK1* in 'Duli-G03' suspension cells significantly suppressed the expression of *PbeXTH1* and *PbeSEOR1*. Further analysis revealed that the inhibition of the expression of both *PbeXTH1* and *PbeSEOR1* were essential for the resistance and immune response caused by *MbWAK1*. Our results offered new insights into the molecular mechanisms of plant immunity of *Valsa* canker and resistant breeding in future.

Declarations

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Author's contributions ZC conceived, designed, and coordinated the study. LY, MX, and WC performed the experiments. LY, ZY and DH performed collected analyzed, and deposited the data. ZC, and LY proofread the final draft, SE, YH and CZ revised the manuscript. All authors have read and approved the manuscript.

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Conflict of interest The author declares no competing interests

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Figures

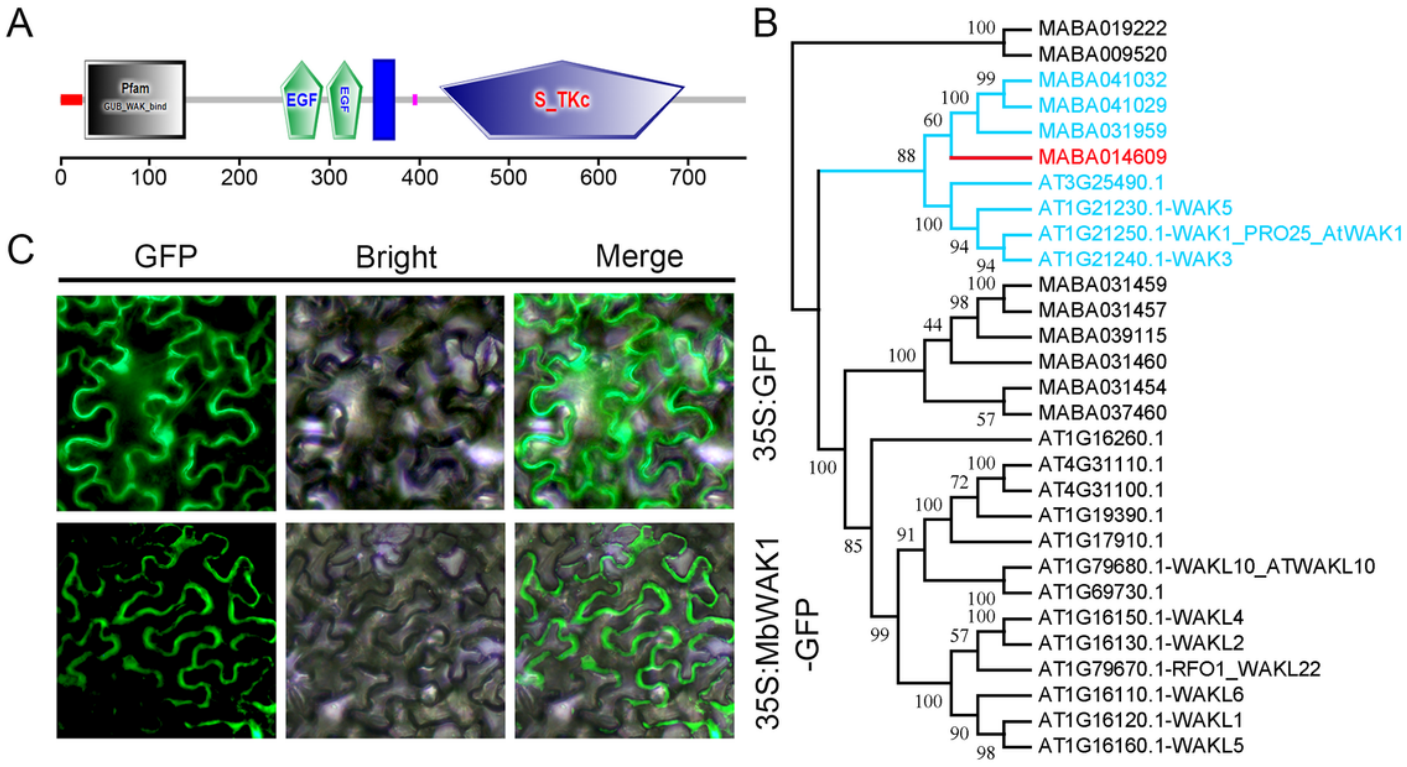

Figure 1

Bioinformatics analysis and subcellular localization of *MbWAK1*. (A) The structural domain of *MbWAK1*consists of an N-terminal signal peptide, a extracellular GUB_WAK_bind domain (PF13947), two EGF domains (PF00008), a transmembrane region, and an intracellular kinase domain S_TKc (SM000220). (B) The evolutionary analysis of WAKs in apple 'Dongbeishanjingzi' (*M. baccata*) and *A. thaliana*. (C) *MbWAK1* is localized at the plasma membrane.

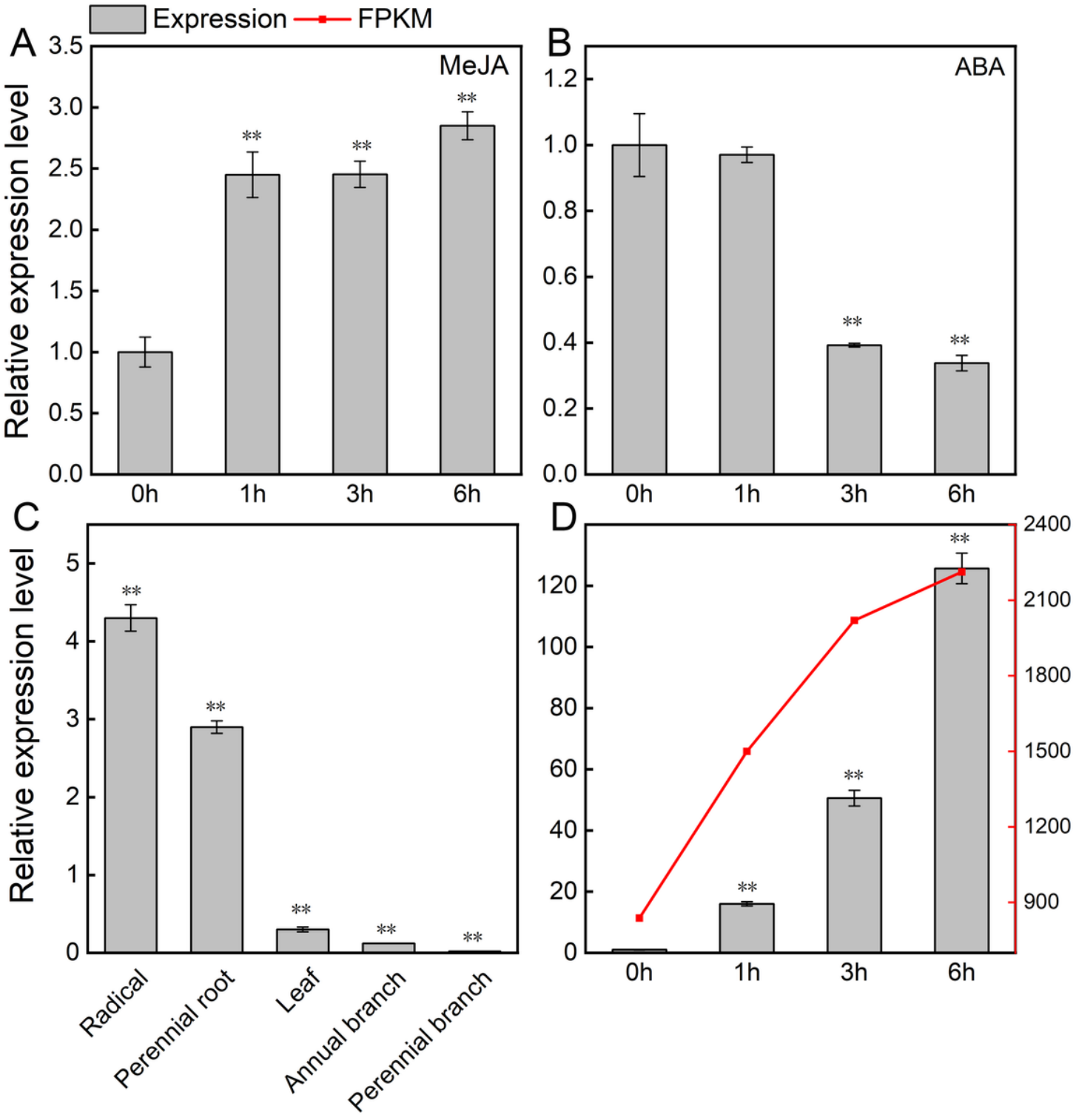

Figure 2

Expression pattern of *MbWAK1* in 'Dongbeishanjingzi' suspension cells under MeJA, ABA, and *Vm*M treatments and tissue-specific analysis of *MbWAK1*.(A) Expression pattern of *MbWAK1*under MeJA treatment. (B) Expression pattern of *MbWAK1* under ABA treatment. (C) Expression pattern of *MbWAK1* under *Vm*M treatment, the expression data (PRJNA792943) and qRT-PCR assays. (D) Expression patterns of *MbWAK1* in different tissues including radical, perennial root, leaf, annual and perennial branch. Data

were means (±SD), n=3. Significant differences compared with the control were determined using Student's *t*-test: *p<0.05, **p<0.01.

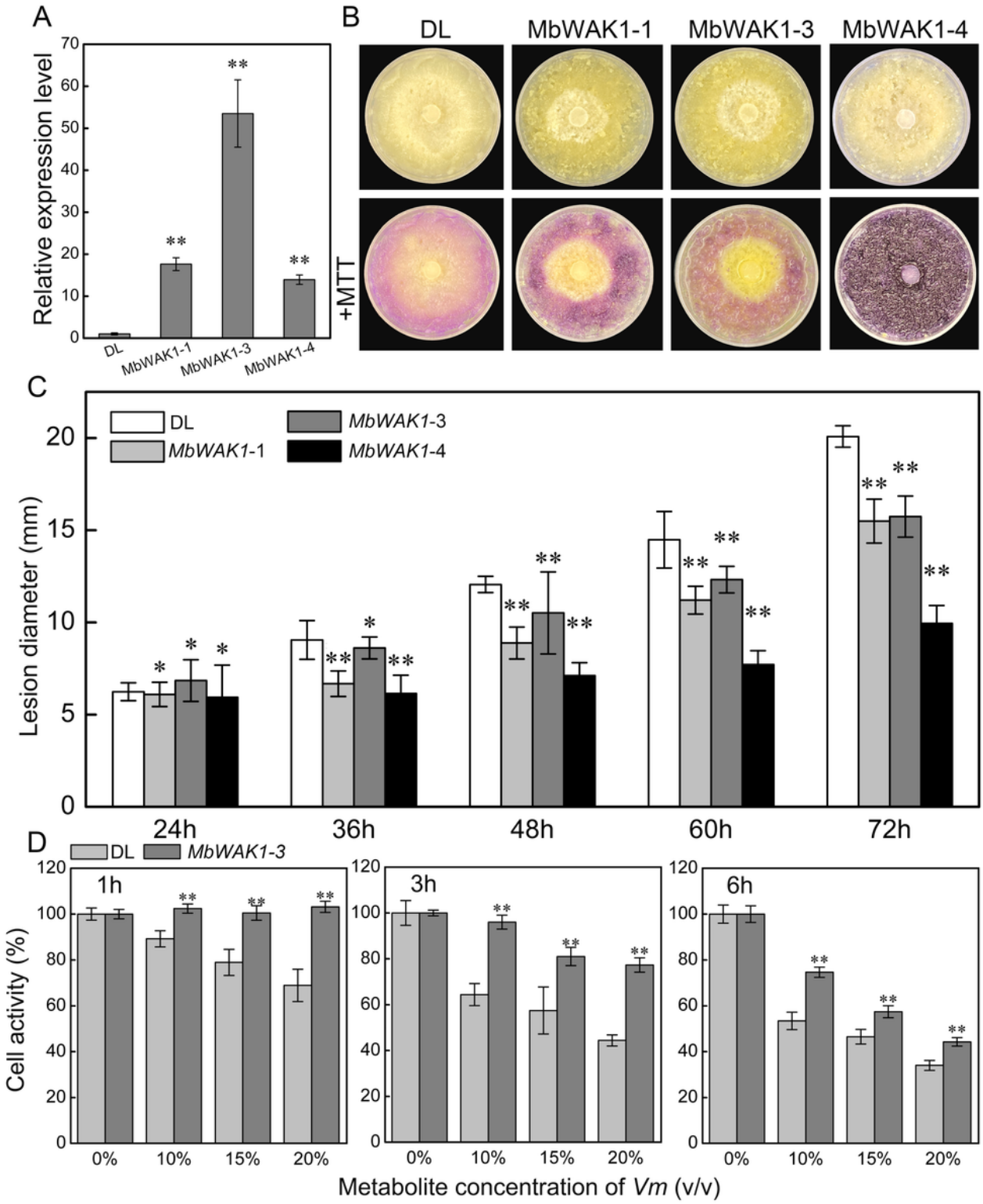

Figure 3

*MbWAK1*enhances the resistance of suspension cells of the 'Duli-G03' to *Vp*. (A) and Relative expression levels of *MbWAK1* in the control and over-expression cell lines. (B) Representative pictures of the lesions

in control and *MbWAK1* over-expression cell lines after 3 d post inoculation with *Vp*, distinguishing dead cells and live cells (purple) after the addition of MTT. (C) The lesion diameter changes of control and *MbWAK1* over-expression cell lines after inoculation with *Vp*. (D) Cell viability of the control and *MbWAK1-3* over-expression cell line with different concentrations treatment of *Vp*M.

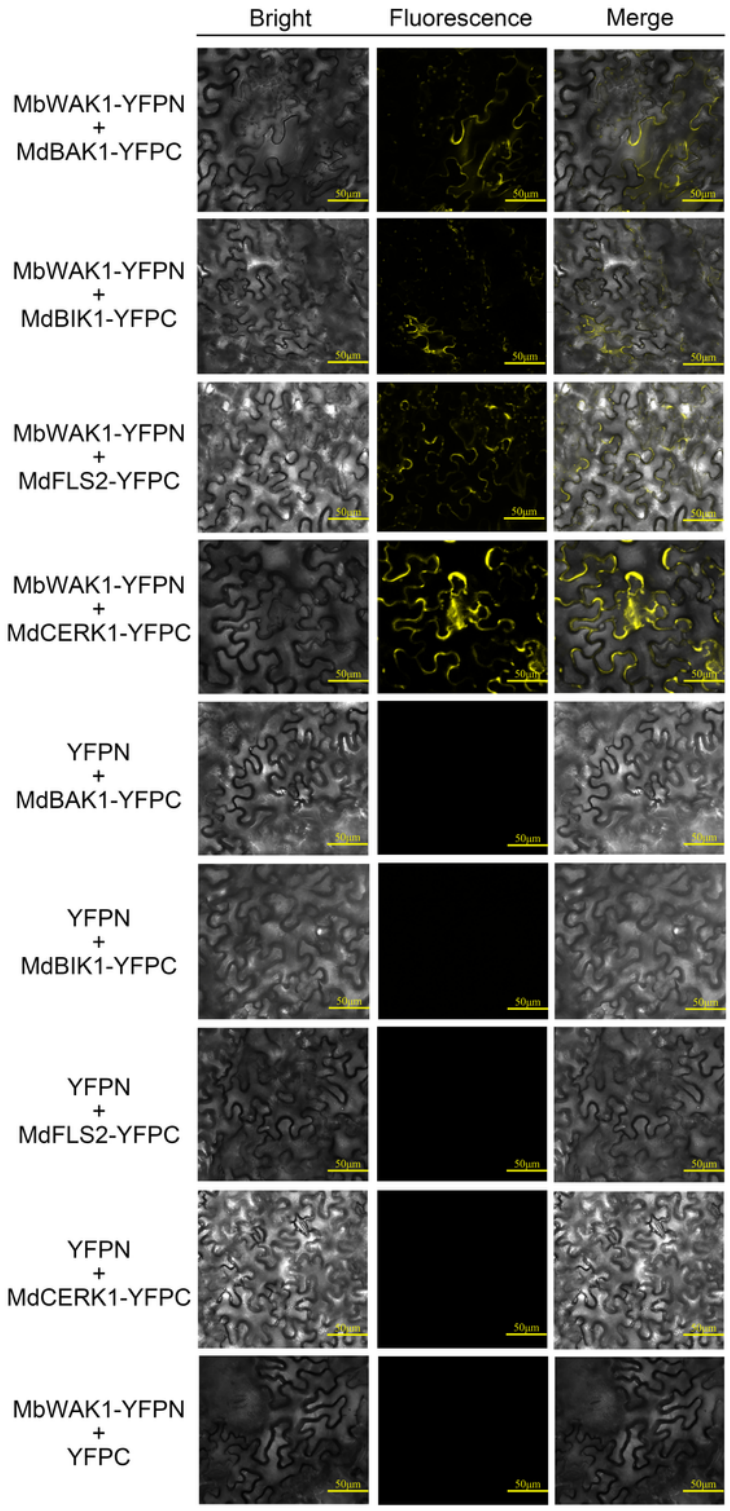

Figure 4

BiFC assays show that *MbWAK1* interacts with *MdBAK1*, *MdBIK1*, *MdFLS2*, and *MdCERK* in *N. benthamiana* leaves. Bar: 50 μm.

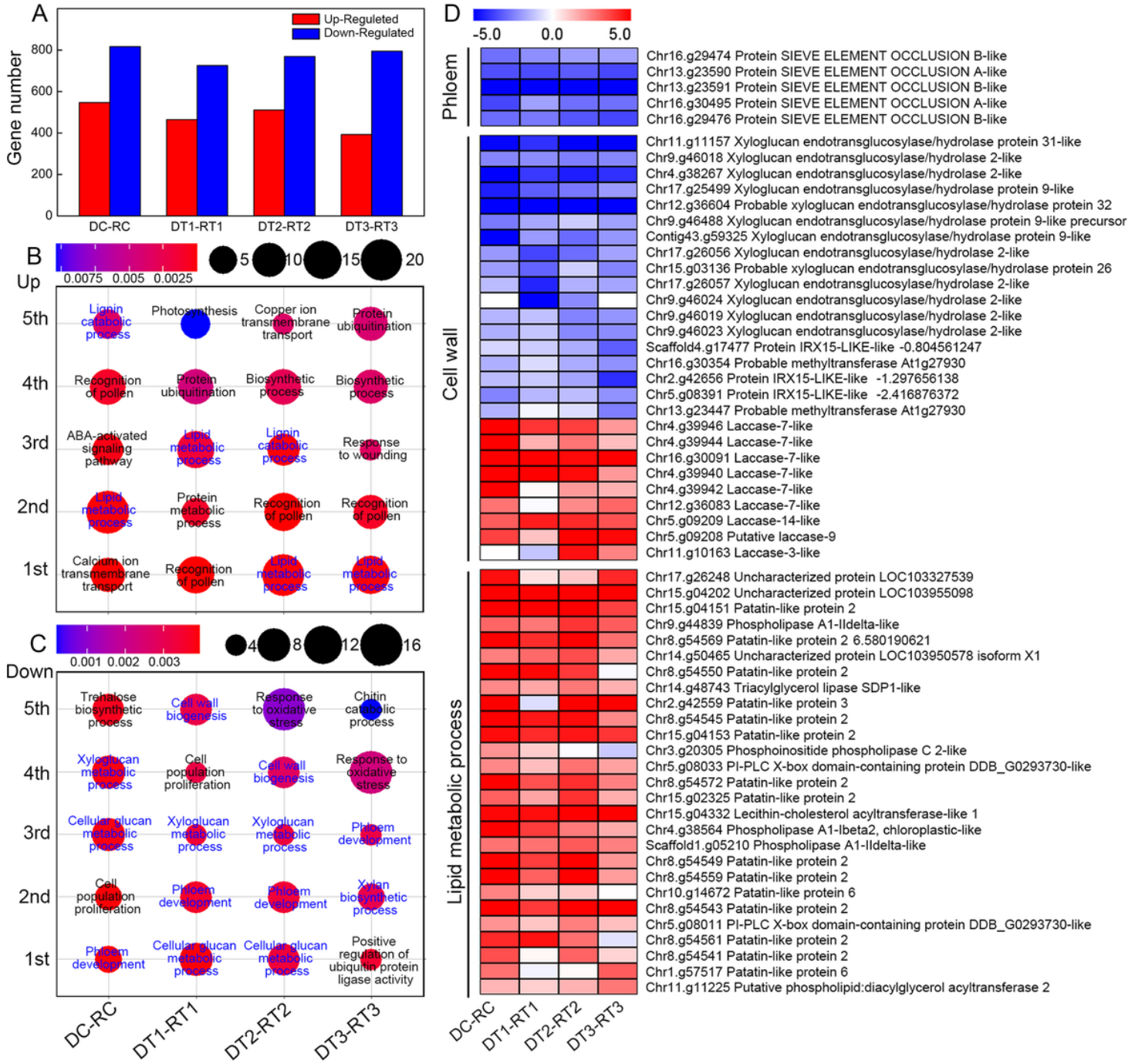

Figure 5

Over-expression of *MbWAK1* in 'Duli-G03' suspension cells activates the defense response after *Vp*M treatment. RNA-seq analyses were performed with *Vp*M treatment after 0 (C), 1 (T1), 3 (T2), and 6 h (T3), respectively. Samples from 'Duli-03'were referred to as DC, DT1, DT2 and DT3, but *MbWAK1* over-expression line (*MbWAK1-3*) as RC, RT1, RT2 and RT3. (A) The number of differentially expressed genes (DEG) in *MbWAK1-3* compared to 'Duli-G03' at various time points (B, C). GO enrichment analyses of up-regulated DEGs and down-regulated DEGs, the five most enriched GO terms are listed. (D) Expression

patterns of all DEGs associated with phloem, cell wall, and lipid metabolic process pathway genes in (B) and (C).

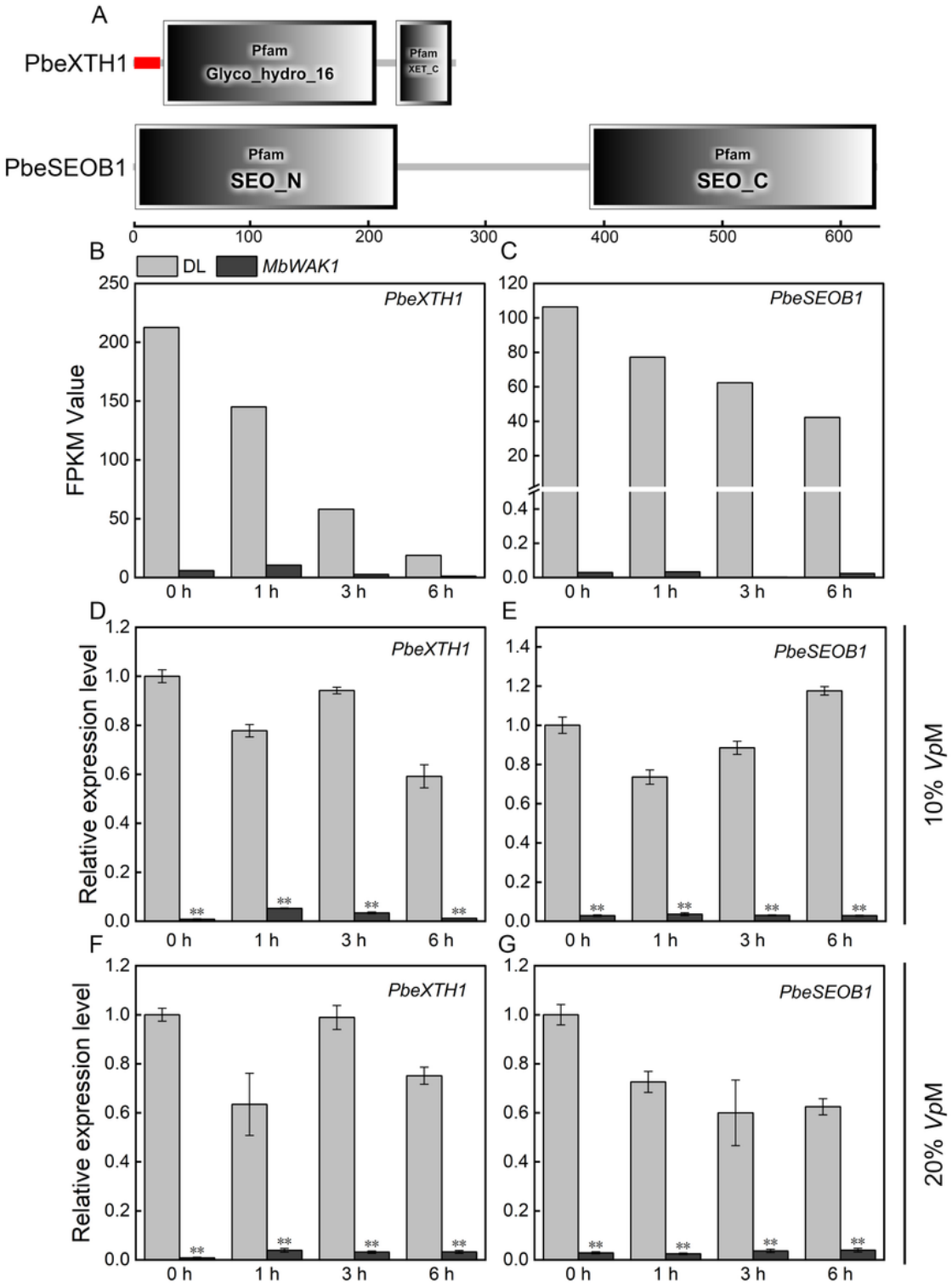

Figure 6

Over-expression of *MbWAK1* in 'Duli-G03' suspension cells is antagonistic to the expression of *PbeXTH1*and *PbeSEOR1*. (A) The structural domain of *PbeXTH1* and *PbeSEOR1*, *PbeXTH1* are consisting

of a XET domain and *PbeSEOR1* consisting of a SEO N-terminal domain (SEO-N), and a SEO C-terminal domain (SEO-C). (B, C) Expression pattern of *PbeXTH1* and *PbeSEOR1* with *Vp*M treatment in *MbWAK1* over-expression cell lines. The expression data were obtained from RNA-sequencing assays. (D, E) Expression of *PbeXTH1* and *PbeSEOR1* in *MbWAK1* over-expressing cell lines with 10% *Vp*M treatment. (F, G) Expression of *PbeXTH1* and *PbeSEOR1* in *MbWAK1*over-expressing cell lines with 20% *Vp*M treatment. Data are means (±SD), n=3. Significant differences compared with the control were determined using Student's *t*-test: **p*<0.05, ***p*<0.01.

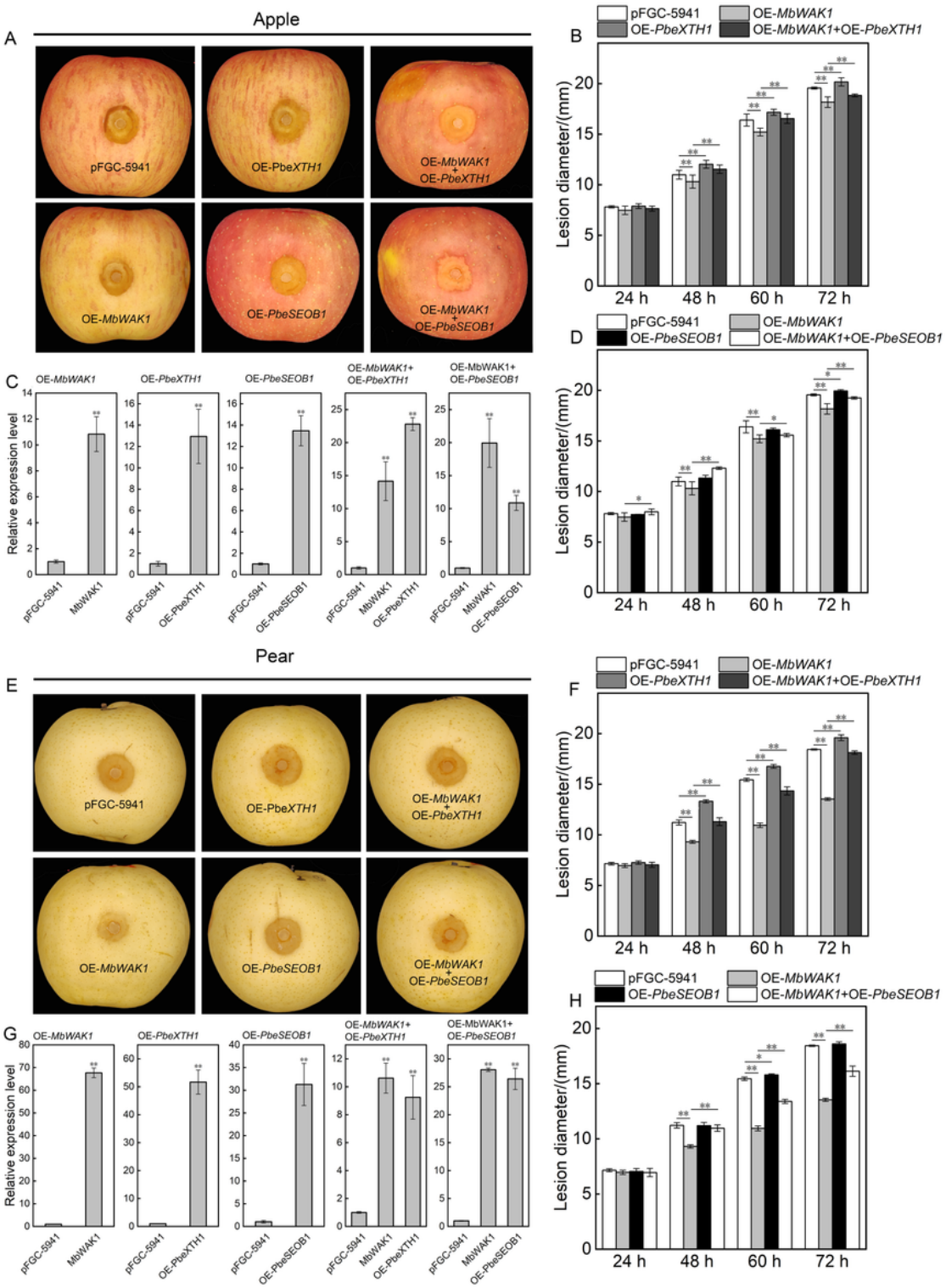

Figure 7

Co-overexpression of *MbWAK1* and *PbeXTH1* or *PbeSEOR1* compromised the *Valsa* canker resistance of *MbWAK1-3*. (A, E) Image of *Valsa* canker incidence at different gene expression sites of 'Yanfu 3' apple and 'Huangguan' pear at 72 h of inoculation with *Vp*. (B, D, F, H) The lesion diameters on the 'Yanfu 3' apple and 'Huangguan' pear at different time points after inoculation with *Vp*. In terms of significant differences, the OE-*MbWAK1*, OE-*PbeXTH1*, and OE-*PbeSEOR1* lines are compared with the pFGC-5941 (empty vector), while OE-*MbWAK1*+OE-*PbeXTH1* and OE-*MbWAK1*+OE-*PbeSEOR1* are compared with OE-*MbWAK1*. (C, G) The expression of the target gene at different site is examined using the pFGC-5941 as a control. All data in the graphs are means (±SD), n=3, and significant differences compared are determined using Student's *t*-test: **p*<0.05, ***p*<0.01.

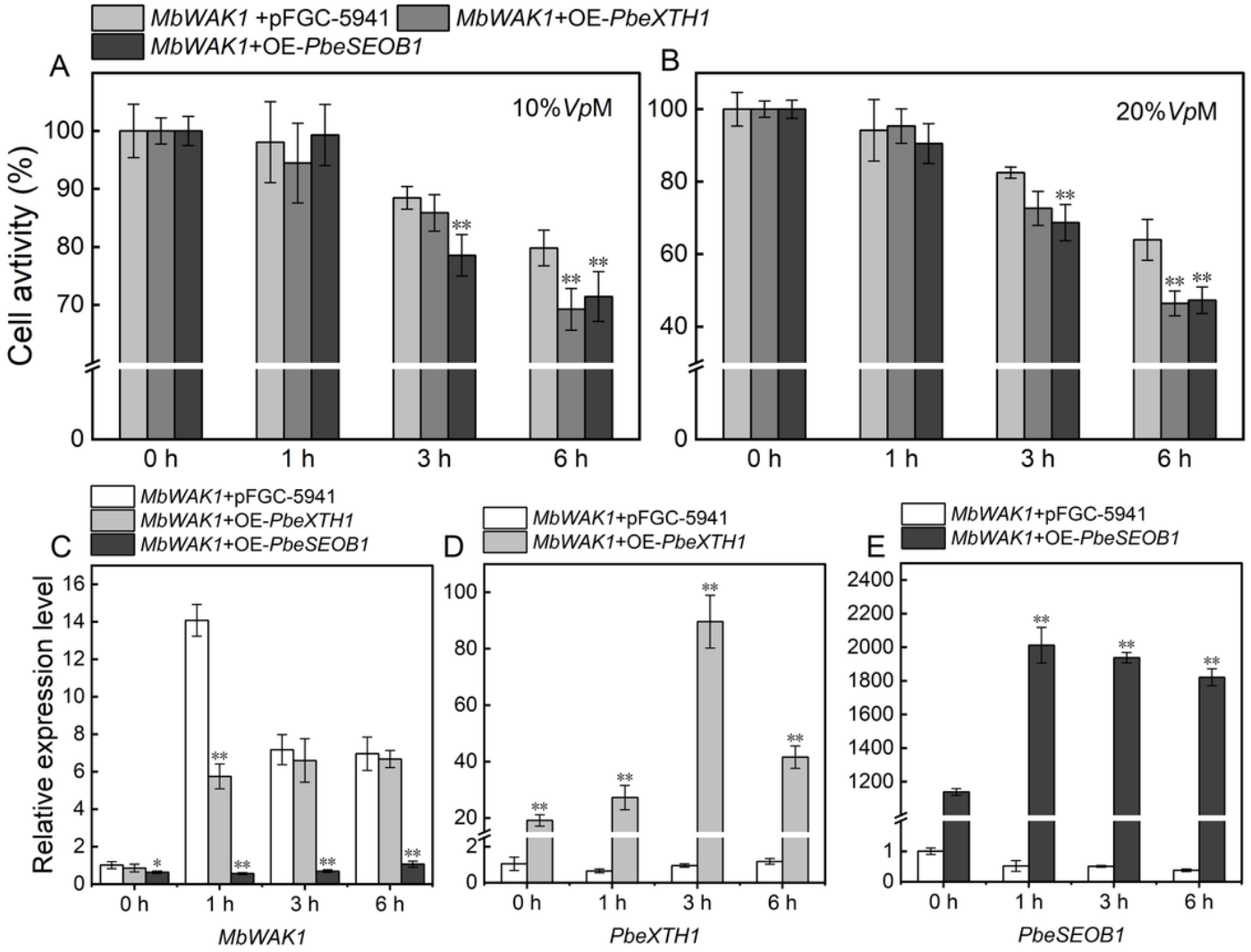

Figure 8

Over-expression of *PbeXTH1* and *PbeSEOR1* reduced the resistance of OE-*MbWAK1* to *Valsa* canker. (A, B) Cell activity analysis at different time points after 10% and 20% *Vp*M treatments, using *MbWAK1* over-expression cells treated with the pFGC-5941 as a control. (C, D, E) Transient expression of *PbeXTH1* and

PbeSEOR1 in *MbWAK1*over-expression cells and the cell activity of *MbWAK1*, *PbeXTH1*, and *PbeSEOR1* when exposed to 10% and 20% *Vp*M. Using *MbWAK1* over-expression cells treated with the pFGC-5941 as a control. All data in the graphs are means (±SD), n=3, and significant differences compared were determined using Student's *t*-test: *p<0.05, **p<0.01.

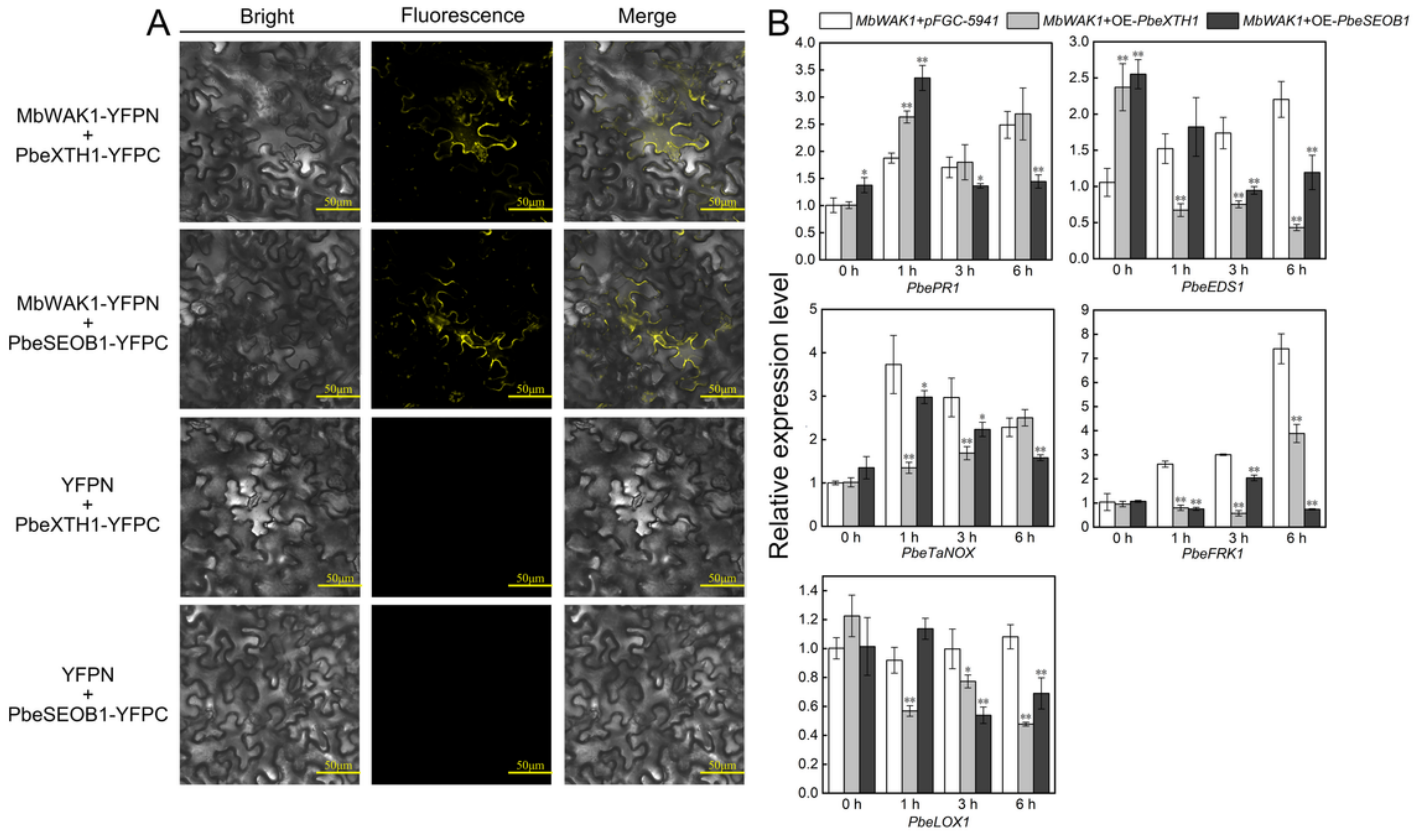

Figure 9

PbeXTH1 and PbeSEOR1 interact with *MbWAK1* and transient expression of *PbeXTH1 and PbeSEOR1* changes the expression levels of defense marker genes in *MbWAK1* suspension cells. (A) BiFC assays show that *MbWAK1*interacts with *PbeXTH1 and PbeSEOR1* in *N. benthamiana* leaves. (B) Relative expression of *PbePR1, PbeEDS1, PbeTaNOX*, *PbeFRK1* and *PbeLOX1*, as determined by qRT-PCR. *Actin* was used as the reference and the expression is relative to that in the control (0 h), the value of which was set as 1. All data in the graphs are means (±SD), n=3, and significant differences compared were determined using Student's *t*-test: *p<0.05, **p<0.01.

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