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MeSAP10 plays an important role in cassava disease resistance via integrated genomic screening and functional analysis

Authors

Yixuan Wang[#], Mingzhi Lin[#], Ke Li[#], Yuhua Chen, Linling Zheng, ..., Liyun Yang[*]

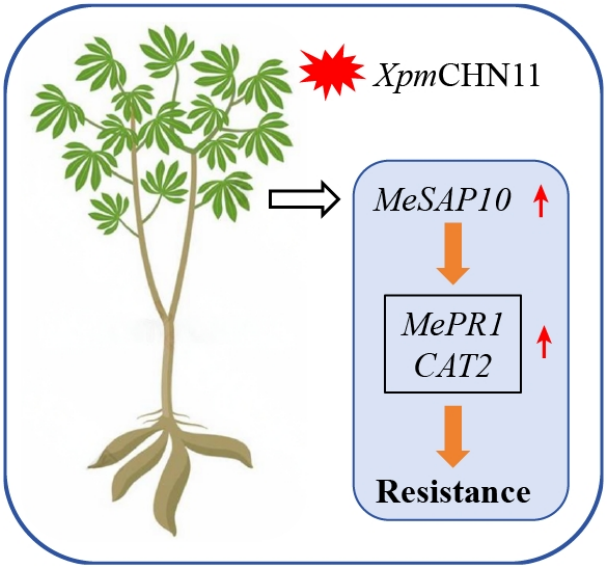
Correspondence

996674@hainanu.edu.cn (Yang L)

In Brief

This study identified 16 *SAP* gene family members in cassava. Among them, *MeSAP10* is induced by *Xpm*CHN11 infection and subsequently upregulates the expression of defense-related genes *MePR1* and *MeCAT2*, thereby enhancing cassava resistance to *Xpm*CHN11.

Graphical abstract



Highlights

- This study firstly identified 16 *SAP* gene family members in cassava, and systematically characterized their expression patterns in response to plant hormones (SA, JA, and ABA), as well as cold, drought, salt, and H_2O_2 treatments.
- Overexpression of *MeSAP10* significantly enhanced cassava resistance to *Xpm*CHN11, while silencing of *MeSAP10* gene increased cassava susceptibility to *Xpm*CHN11.
- *MeSAP10*-mediated cassava resistance to *Xpm*CHN11 is associated with the upregulation of defense-related genes *MePR1* and *MeCAT2*.

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MeSAP10 plays an important role in cassava disease resistance via integrated genomic screening and functional analysis

Yixuan Wang[1#], Mingzhi Lin[2#], Ke Li[2,3#], Yuhua Chen[1], Linling Zheng[1], Honggang Wang[1], Xiaohui Yu[1], Xiaolei Niu[1], Liyun Yang[1*] and Yinhua Chen[1]

[1] *State Key Laboratory of Tropical Crop Breeding, Sanya Institute of Breeding and Multiplication, Hainan University, Sanya, Hainan 572025, China*
[2] *Sanya Research Institute, Chinese Academy of Tropical Agricultural Sciences, Sanya, Hainan 572025, China*
[3] *Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou, Hainan 571101, China*
[#] Authors contributed equally: Yixuan Wang, Mingzhi Lin, Ke Li
[*] Correspondence: 996674@hainanu.edu.cn (Yang L)

Abstract

Stress-associated proteins (SAPs) are A20/AN1 zinc-finger proteins that participate in plant growth and development, and in responses to diverse stresses. Although *SAP* genes have been studied in many plants, their functions in cassava remain largely unexplored, particularly those involved in pathogen resistance. In this study, 16 *MeSAPs* with conserved zinc-binding motifs were identified in cassava. The phylogenetic analysis of *MeSAP* proteins and their orthologs from *Arabidopsis* and rice indicated that they can be classified into five groups. Analyses of gene structure, conserved motifs, and chromosomal distribution indicated relatively high conservation within the *MeSAP* gene family. Numerous elements related to stress and plant hormones were identified through prediction of the *cis*-acting elements in the *MeSAP* gene promoters. Moreover, transcriptome sequencing and quantitative real-time PCR demonstrated that *MeSAP10* was significantly upregulated under *Xpm*CHN11 infection. Functional characterization demonstrated that silencing *MeSAP10* increased cassava susceptibility to *Xpm*CHN11, accompanied by downregulation of pathogenesis-related genes *MePR1* and *MeCAT2*. In contrast, *MeSAP10* overexpression conferred enhanced disease resistance with simultaneous upregulation of the defense-related genes, *MePR1* and *MeCAT2*. These findings provide new insights into the *SAP* gene family in cassava, and identify *MeSAP10* as a valuable target for breeding disease-resistant varieties.

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Introduction

Cassava (*Manihot esculenta* Crantz) originates from the Amazon region and is primarily cultivated in tropical areas. It is one of the world's starch-rich crops, serving as a staple food in tropical regions like Africa[1]. Additionally, it is utilized as feed and as a raw material in various industries[2]. Cassava bacterial blight (CBB), caused by *Xanthomonas phaseoli* pv. *manihotis* (*Xpm*) stands out as the most destructive disease affecting cassava production[3]. CBB played a significant role in the famine in Zaire between 1970 and 1975, leading to a staggering loss of up to 80% of cassava production in Central Africa[3]. Identification and characterization of resistant genes responsive to *Xpm* infection in cassava will provide valuable genetic resources for developing stress-resistant cassava cultivars.

Stress-associated proteins (SAPs) are typical A20/AN1 zinc-finger proteins with physiological functions in regulating abiotic stress signaling and defense responses to disease in plants[4–6]. There are 14 genes in *Arabidopsis*, 18 in rice, 17 in *Pinus massoniana*, 17 in potato, and 27 in sunflower[7–11]. Based on the number and structure of zinc-finger motifs, *Arabidopsis* SAP proteins can be classified into three groups (I-III). Group I comprises of a A20 zinc finger domain characterized by multiple Cys2-Cys2 finger motifs, while Group II includes an AN1 zinc finger domain containing multiple Cys2-His2 finger motifs, or both. Group III consists of proteins with additional Cys2-His2 RING motifs at the C-terminus[10]. Previous studies have shown that SAPs enhance the ability of plants to respond to abiotic stress. Overexpressing *ZmSAP8* enhanced the tolerance to drought stress in *Arabidopsis*, with higher seed germination and longer root length[12]. Overexpression of *ZmSAP1* or *ZmSAP7* significantly enhances salt stress tolerance in *Arabidopsis* and rice[13]. Overexpression of *NtSAP9* significantly enhances freezing tolerance in tobacco, whereas knockout of *NtSAP9* resulted in reduced freezing tolerance[14]. Meanwhile, the *SAP* gene family participates in regulating plant growth and development. For example, in *Artemisia annua, AaSAP1* positively regulated the development of glandular trichomes, thereby increasing the production of artemisinin[15]. *Oryza sativa OsZFP185* regulates plant growth and stress responses by affecting GA and ABA biosynthesis in rice[16]. Moreover, studies have shown that *SAPs* play important roles in plant immunity. For instance, overexpression of tomato *SlSAP3* led to increased accumulation of reactive oxygen species (ROS) upon flg22 treatment, accompanied by elevated expression of immune marker genes triggered by PAMP, such as *SlPTI5* and *SlLRR22*[17]. Further research revealed that *SlSAP3* positively regulated tomato resistance to the *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) pathogen through the SA signal[17]. Overexpression of rice *OsSAP1* enhanced plant resistance against virulent bacterial pathogens, and upregulated the expression of defense genes in tobacco[18]. Silencing or knocking out of *SAP12* in tobacco and *Arabidopsis* increased plant susceptibility to *Meloidogyne incognita*[5]. Therefore, investigating the *SAP* gene family is crucial for understanding its functional diversity across multiple plant species.

However, the molecular mechanisms underlying SAP-mediated stress tolerance and pathogen resistance remain incompletely understood. Multiple regulatory pathways appear to be involved in SAP function. First, SAP proteins participate in signal transduction through protein-protein interactions with RLCKs under abiotic stress[19]. For instance, OsSAP11 interacts with OsRLCK253 via its A20

zinc-finger domain, thereby enhancing water-deficit and salt stress tolerance in transgenic *Arabidopsis,* and modulating the expression of endogenous stress-response genes[20]. Additionally, post-translational modifications of SAP proteins, particularly phosphorylation and ubiquitination play critical roles in regulating their function. In *Arabidopsis*, AtSAP9 interacts with RADIATION SENSITIVE23d (Rad23d) to target ubiquitinated proteins for proteasome degradation, thereby fine-tuning abiotic stress responses and homeostasis of stress-related proteins[21]. Furthermore, SAP proteins act as transcriptional regulators by binding to *cis*-elements in the promoter regions of stress-related genes[19]. GaZnF, a potential SAP-family transcription factor, binds to MYB-box elements in the *GUSP1* promoter, suggesting that SAP proteins can directly or indirectly regulate stress-related gene expression in response to drought, salt, and heavy metal stresses[22]. MiPDI1 interacts with SlSAP12, an ortholog of the redox-regulated AtSAP12, and played a significant role in plant responses to abiotic and biotic stresses[5]. Despite extensive characterization of *SAP* functions in various plant-pathogen interactions, the molecular mechanisms by which SAP proteins contribute to resistance in cassava against *Xpm* have not yet been elucidated.

In the present study, we identified and characterized the *SAP* gene family containing A20/AN1 zinc-finger domains from the cassava genome and assessed their transcriptional responses to various abiotic and biotic stresses. Identification, characterization, and elucidation of evolutionary patterns of the cassava *SAP* gene family, along with analyzing their involvement in diverse stress responses could substantially advance the development of strategies to enhance stress tolerance and pathogen resistance in cassava. Additionally, our research specifically focuses on the characterization of *MeSAP10* and its role in response to the Chinese strain of *Xpm* (*Xpm*CHN11). This investigation offers valuable insights for the functional characterization of *SAP* gene family members in cassava.

Materials and methods

Cultivation of plant materials

Cassava cultivar South China 8 (SC8) obtained from the National Cassava Germplasm Nursery and the Chinese Academy of Tropical Agricultural Sciences, was used in this study. SC8 was cultivated as previously described[23]. Briefly, SC8 stems were planted in a substrate composed of a 1:1 mixture of nutrient soil and vermiculite in a greenhouse. The plants were grown under controlled conditions with a 16 h light and 8 h dark cycle at 28 °C with a relative humidity maintained between 60% and 80%.

Hormone treatment and *Xpm*CHN11 inoculation

For phytohormone analysis, the callus was cultured in MS medium, supplemented with 100 μM ABA and JA, respectively. For H_2O_2 treatments, the callus was cultured in MS medium supplemented with 100 mM H_2O_2. Samples were collected at 0, 2, 6, 12, and 24 h after treatments. For salinity and drought treatments, the callus tissues grown in pots was subjected to 150 mM NaCl and 20% PEG6000, respectively. Samples were collected at 0, 2, 6, 12, 24, and 48 h after treatments. The SC8 seedlings with consistent growth conditions were inoculated with *Xpm*CHN11 following a previously reported method[23]. The 3rd and 4th leaves were sampled at 0, 3, 6 h, and 1, 3, 6 d after inoculation. All samples were quickly frozen in liquid nitrogen for subsequent RNA isolation.

RNA extraction and quantitative real-time PCR (qRT-PCR) assay

Total RNA was extracted from leaves using the RNAplant plus kit (TIANGEN, China), and the RNA was used for cDNA synthesis with SPARKscript II RT Plus Kit (with gDNA eraser) (Sparkjade, China). qRT-PCR was performed by using ChamQ Universal SYBR qPCR Master Mix (Vazyme, China) following protocols on analytikjena qTOWER^3 84G (Jena, Germany). The cycling program was 95 °C for 5 s, 55 °C for 15 s, and 68 °C for 20 s, for 40 cycles. *MeTubulin* (Manes. 08G061700) and *MeUBQ10* (Manes. 07G019300) were selected as the internal reference gene. Relative expression levels of genes were calculated using the $2^{-\Delta\Delta C_T}$ method[24]. Primer sequences are listed in Supplementary Table S5.

Identification and phylogenetic analysis of *SAP* genes in cassava

Cassava SAP genomic, coding, and amino acid sequences were retrieved from Phytozome v12.1 (https://phytozome.jgi.doe.gov/pz/portal.html). Conserved domains were identified using HMMER against the Pfam database[25,26]. Putative cassava *SAP* proteins were identified by BLASTP using the amino acid sequences of *Arabidopsis* SAP (TAIR 10, E-value ≤ 1e −10) as queries, and A20/AN1 domains were further confirmed using NCBI CDD (www.ncbi.nlm.nih.gov/cdd). Physicochemical properties, amino acid length/composition, isoelectric points (pIs), and molecular weights (MWs), were calculated using the ExPASy website (https://web.expasy.org), and subcellular localization was predicted using PSORT (www.genscript.com/psort.html). Phylogenetic analysis was performed in MEGA 7.0 using the Neighbor-Joining method with 1,000 bootstrap replicates. SAP protein sequences from *Arabidopsis*, rice, and cassava were downloaded from the Phytozome database (https://phytozome.jgi.doe.gov).

Chromosomal distribution, gene structure, and evolutionary relationship of cassava *SAP* genes

The chromosomal locations of cassava *SAP* genes were plotted using MapInspect software following the procedure described (http://mapinspect.software.informer.com). The gene structures of *SAPs* were based on gene models downloaded from the Phytozome 12.1 website as described above, and the conserved motifs of SAP proteins were predicted using MEME (http://meme-suite.org/tools/meme). These characteristics were visualized on the phylogenetic tree using ITOL 4 (https://itol.embl.de)[27]. Gene duplication analysis of cassava *SAP* gene family and collinearity analysis of orthologs between *M. esculenta and A. thaliana* as well as between *M. esculenta and O. sativa,* were performed using MCScanX with default parameters, and the results were visualized using Circos[28,29].

Cis-elements analysis of cassava *SAP* genes

To investigate the promoter regions of the *SAP* genes, the 2 kb genomic DNA upstream sequences of 16 *SAP* family genes were extracted and subjected to TSSP (www.softberry.com)[30].

Expression patterns of *MeSAP* genes

To analyze *MeSAP* gene expression across cassava tissues, and after *Xpm* infection, RNA-seq datasets (Arg7, KU50, and wild W14) were downloaded from the NCBI (Supplementary Tables S2–S4). Reads were mapped using HISAT2[31], and transcripts were assembled and quantified as FPKM (Fragments per kilobase of transcript per million mapped reads) with StringTie using default settings[32]. Differentially expressed genes in mock- and *Xpm*-infected samples

were identified using the DESeq2 R package with a false discovery rate < 0.001, and at least a two-fold expression difference[33]. Heatmaps were generated in TBtools using log_2-transformed FPKM values[34].

Construction and disease resistance analysis of CsCMV-mediated *MeSAP10* gene-edited plants

For construction of the pCsCMV-*MeSAP10* vector, the *MeSAP10* target fragment was predicted using the VIGS tool (https://vigs.solgenomics.net) and cloned into the pCsCMV-NC vector. For pCsCMV2-*MeSAP10*, the coding sequence (CDS) of *MeSAP10* was amplified from cDNA prepared from SC8 cassava leaves and inserted into the pCsCMV2-NC vector[35,36]. Primers are listed in Supplementary Table S5. For inoculation, pCsCMV-NC, pCsCMV-*MeSAP10*, pCsCMV2-NC, and pCsCMV2-*MeSAP10* were introduced into *Agrobacterium tumefaciens* GV3101 together with the p19 plasmid. Subsequently, *agrobacterium* was collected and resuspended in infiltration buffer (1 M $MgCl_2$, 0.5 M 2-morpholinoethanesulfonic acid hydrate, and 150 mM acetosyringone, pH 5.6) to an OD_{600} = 0.6, and then infiltrated into leaves of 4-week-old cassava plants as previously described.

Statistical analysis

All statistical analyses were conducted using IBM SPSS Statistics version 20 software. One-way ANOVA and Student's *t*-test were used to determine significant differences, with $p < 0.05$ considered statistically significant. GraphPad Prism version 8.0.2 was used for generating graphs.

Results

Identification of *SAP* gene family members in cassava

Using amino acid sequences of *Arabidopsis* SAP family proteins as query sequences (E-value ≤ 1e−10) (Supplementary Fig. S1), 16 SAP family proteins were identified from the cassava genome via BLASTP, and their corresponding coding genes were named *MeSAP1*–*MeSAP16* (Fig. 1). Detailed information regarding the *MeSAP* family genes are shown in Supplementary Table S1. Overall, the length of the amino acid sequences for these SAPs ranged from 125 (MeSAP2) to 293 (MeSAP10). Predictions of isoelectric point (pI) and protein molecular weight (MW) for MeSAPs revealed that pI values ranged from 7.99 (MeSAP4) to 9.37 (MeSAP15), while protein MWs varied between 13.66 kDa (MeSAP2) and 32.42 kDa (MeSAP10), respectively. Predicted subcellular localization indicated that all SAP members were localized in the nucleus (Supplementary Table S1).

MeSAP1 to *MeSAP16* were unevenly distributed across the 10 cassava chromosomes (Chr.). The number of *MeSAPs* located on a single chromosome varied from one to four, with Chr. 2, 6, 8, 10, 11, and 15 containing one gene each, Chr. 7, 9, and 14 containing two genes, and only Chr. 1 containing four genes. There was no evident positive correlation between chromosome length and the number of *SAP* family genes.

Gene structure and conserved motifs of *MeSAPs*

To elucidate the phylogenetic relationship and classification of *MeSAPs*, a neighbor-joining (NJ) phylogenetic tree was constructed. The tree revealed that the 16 MeSAP proteins clustered into five groups (Supplementary Fig. S1). Among these proteins, 5 SAP proteins were classified into Group I, 6 into Group II, whereas Group III contained only SAP13. Group IV comprised SAP10 and SAP16, and Group V included SAP1 and SAP15. To explore the structural diversity of the cassava *SAP* family genes, gene structure analyses of the 16 *MeSAP* genes were conducted based on their evolutionary relationships. Remarkably, all genes were found to lack introns. 14 SAP proteins shared a common structural feature: a highly conserved AN1 and A20 domain, whereas MeSAP10 and MeSAP16 possessed two AN1 motifs (Fig. 2, Supplementary Fig. S2). Interestingly, SAPs containing only a single A20 domain were not identified in cassava, consistent with observations in *Arabidopsis*, maize, and tomato, but not rice[11,37]. In agreement with alignment and phylogenetic analysis, members of each group exhibited similar patterns of conserved motifs.

Evolutionary analysis of *MeSAPs* between cassava and other species

To further investigate the evolutionary relation of the cassava *MeSAP* gene family through duplication events, a homologous-block

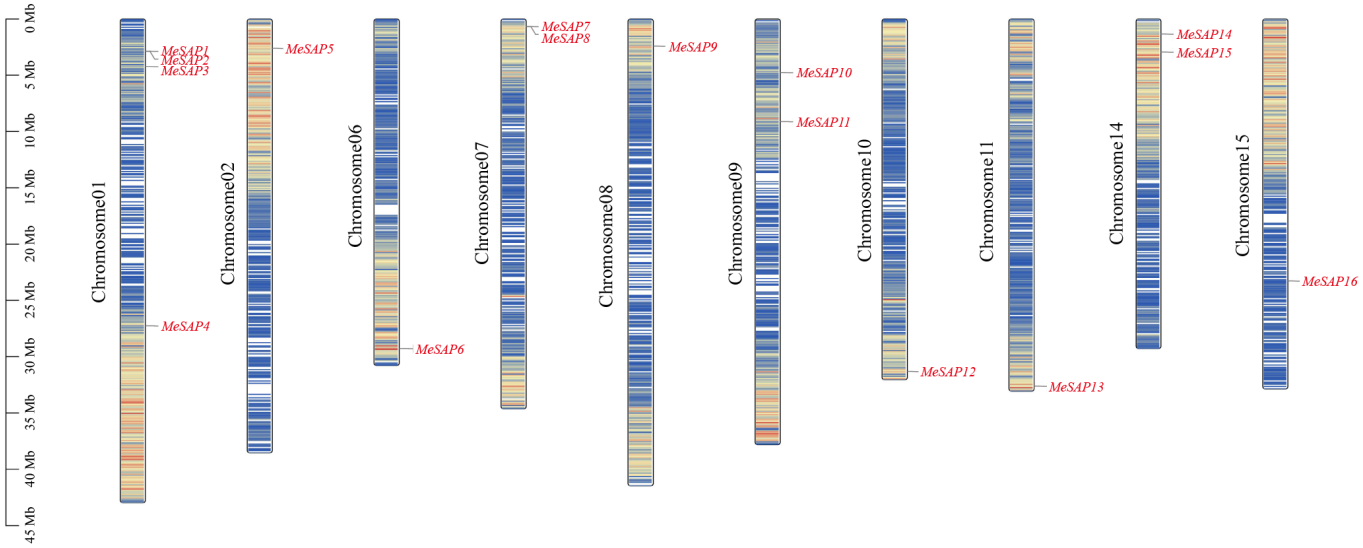

Fig. 1 Distribution of the *SAP* genes among 10 cassava chromosomes. The scale of the left side represents the length of chromosome displayed in megabase (Mb).

Fig. 2 The structure, clustering, and conserved motif analysis of cassava MeSAP proteins. The left side illustrates the gene structure of the cassava *MeSAP* gene family. The right side displays the conserved motifs of cassava MeSAP proteins.

map was constructed (Fig. 3a). The results revealed that 10 *MeSAP* genes are located in orthologous blocks with corresponding homologous *MeSAP* genes. These duplicated *MeSAP* genes were distributed across cassava Chr. 1, 2, 6, 7, 8, 9, 10, 11, and 14. The 12 pairs of *MeSAP* genes included *MeSAP4* and *MeSAP5*, *MeSAP4* and *MeSAP15*, *MeSAP5* and *MeSAP14*, *MeSAP6* and *MeSAP7*, *MeSAP6* and *MeSAP12*, *MeSAP6* and *MeSAP14*, *MeSAP7* and *MeSAP12*, *MeSAP7* and *MeSAP14*, *MeSAP9* and *MeSAP10*, *MeSAP9* and *MeSAP13*, *MeSAP10* and *MeSAP13*, and *MeSAP12* and *MeSAP14*. *MeSAP6*, *MeSAP7* and *MeSAP14* were associated with three syntenic gene pairs, suggesting their potential importance in the evolution of the *MeSAP* gene family. To further elucidate the phylogenetic mechanisms of the cassava *SAP* gene family, syntenic maps were constructed using cassava, *Arabidopsis*, and rice (Fig. 3b, c). The results revealed eight gene pairs with collinearity between cassava and rice, and sixteen gene pairs with collinearity between cassava and *Arabidopsis*. These results suggested a closer relationship between cassava *SAP* genes and *Arabidopsis* compared to rice. Among them, *MeSAP6*, *MeSAP9*, *MeSAP13* and *MeSAP14* had orthologous genes in both rice and *Arabidopsis*, indicating the existence of these orthologous pairs prior to ancestral divergence. However, orthologous genes of *MeSAP4*, *MeSAP5* and *MeSAP10* were only found in *Arabidopsis*, while orthologous genes of *MeSAP11* and *MeSAP12* were only found in rice. This result suggests that these orthologous pairs may form after the divergence of dicotyledonous and monocotyledonous plants.

Analysis of *cis*-elements in the promoter regions of cassava *MeSAP* genes

To investigate the regulatory mechanisms governing the expression of cassava *SAP* genes, putative *cis*-elements within their promoter regions were predicted using the Softberry website (www.softberry.com) (Fig. 4). Eight *cis*-elements associated with stress tolerance, namely MYB element, anaerobic induction element (ARE), wound response elements (WUN-motif), G-box, F-box, MYB binding sites (MBS), WRKY binding sites (W-box), and stress-responsive elements (TC-rich repeats), were identified in the promoter regions of *MeSAP* genes. Notably, the MYB element was prominently present in most promoters, except for *MeSAP13*. The ARE, WUN-motif, and G-box were observed in the promoters of the majority of *SAP* genes, indicating their potential involvement in stress responses, particularly osmotic stresses. However, F-box, MBS, W-box, and TC-rich repeats were detected in only a few cassava *SAP* gene promoters. Moreover, eight phytohormone-responsive elements were detected in the promoters, including ABRE elements, ERE elements, and CGTCA motifs, which mediate plant responses to abscisic acid (ABA), ethylene (Eth), and methyl jasmonate (MeJA), respectively. Additionally, TCA elements, TGA elements, and GARE-motifs were found in a subset of *SAP* genes, potentially mediating plant responses to salicylic acid (SA), and gibberellin (GA). These findings suggest that cassava *SAP* genes may play diverse roles in hormone responses, highlighting their multifunctional nature in regulating stress and hormonal signaling pathways.

Expression profiles of *MeSAPs* in different tissues

To understand the potential functions of *MeSAP* genes during cassava growth and development, the expression patterns of cassava *SAP* genes in leaves and different stages of roots were extracted for analysis based on transcriptome data from cassava varieties W14, KU50, and Arg7 (Supplementary Table S2). The analysis revealed that most *MeSAP* genes, except *MeSAP3*, exhibited low expression levels in the leaves of all varieties and in the roots of Arg7 and KU50, at 120 and 150 d. Notably, *MeSAP* genes, excluding *MeSAP2* and *MeSAP3* showed high expression during the early stages of root development in KU50. Additionally, *MeSAP3*, *MeSAP8*, *MeSAP14* and *MeSAP16* displayed lower expression levels in the roots of W14 compared with other tissues at different stages of root development (Supplementary Fig. S3a). However, 12 *MeSAP* genes exhibited higher expression levels in the roots of W14. These findings align with previous studies conducted on other plants, which have reported lower transcription levels of most *SAP* genes under normal conditions[25,37].

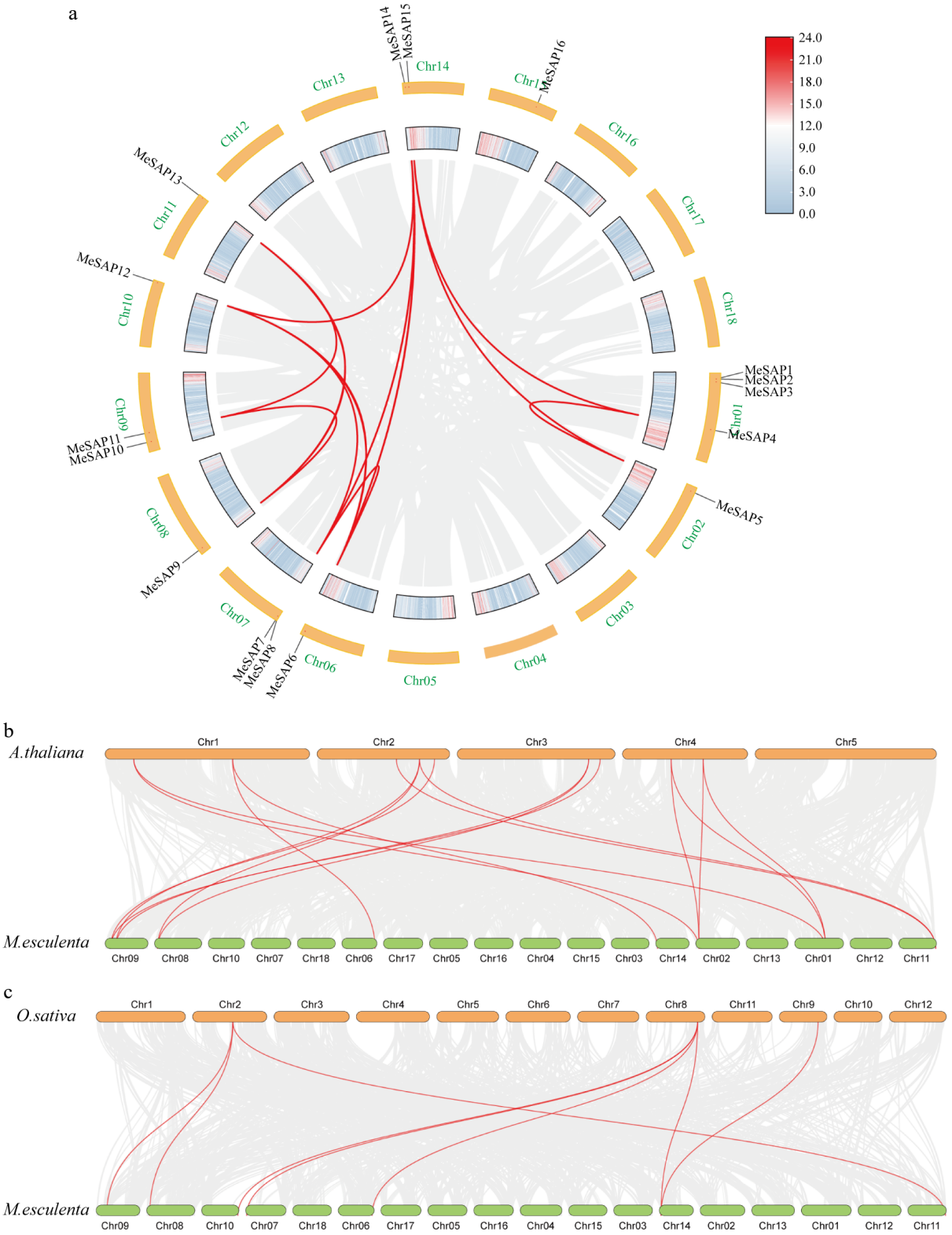

Fig. 3 Duplication and collinearity analysis of *SAP* genes between cassava, *Arabidopsis*, and rice. (a) Duplicated *MeSAP* genes on the cassava chromosomes. (b) Duplicated *SAP* genes on the cassava and rice chromosomes. (c) Duplicated *SAP* genes on the cassava and *Arabidopsis* chromosomes. Gray lines indicate all synteny blocks in the cassava genome, and the red lines indicate duplicated *SAP* gene pairs.

Expression profiles of *MeSAP*s under different pathogen strains

To explore the functional divergence of *SAP* genes, transcriptome data from cassava treated with different pathogen strains were obtained from the National Center for Biotechnology Information (NCBI) (Supplementary Tables S3, S4). The Fragments Per Kilobase of transcript per million mapped reads (FPKM) values of *MeSAP* genes infected with *Xam*668 were utilized to generate a heat map depicting the transcription levels of *MeSAP* genes[38]. The heat map

	Stress								Hormone							
	MYB	F-box	MBS	W-box	ARE	WUN-motif	TC-rich repeats	G-box	ABRE	ERE	CGTCA-motif	TCA element	TGA element	P-box	GARE-motif
MeSAP1	3	1			3		1	2	1		1				1
MeSAP2	5	1	1		2		1	3	3					1	1
MeSAP3	4				2	2				5		1	1		2
MeSAP4	3			1	2			4	2	1	4		1	1	
MeSAP5	3		1		3	3		3	2	3	1				
MeSAP6	3		2	1	2	1	1	2	3	1	2	1			
MeSAP7	1				2	1		7	7		4		1		
MeSAP8	6				1		1	1	1	1	1		1	1	
MeSAP9	7		1			4		3	3	3			1		
MeSAP10	4	1				5				9	2	1			1
MeSAP11	2			1	3			1	1	2					
MeSAP12	2			1	1	2		2	2	5	2	1			
MeSAP13				1	2	1				9	2	1			
MeSAP14	5	1			5	1	1			1		1			1
MeSAP15	2				1					6					
MeSAP16	4		1		1	1		6	6	6	3		1		

Fig. 4 The *cis*-elements type and numbers distribution in the promoter regions of cassava *MeSAP* genes.

results revealed that *MeSAP14*, *MeSAP15*, and *MeSAP16* exhibited higher expression at 50 h post *Xam*668 inoculation, while *MeSAP11* and *MeSAP13* displayed high levels of expression at 24 h post-inoculation, which decreased in later stages. Furthermore, *MeSAP7* and *MeSAP9* were induced by *Xam*668 in the early stages but exhibited lower expression in later stages (Supplementary Fig. S3b). The expression levels of these genes under *Xanthomonas phaseoli* pv. *manihotis* (*Xpm*) infection was also assessed. *MeSAP1*, *MeSAP4*, and *MeSAP8* displayed peak expression at 6 h after *Xpm* inoculation, while *MeSAP3* and *MeSAP6* showed elevated expression at 1 and 3 d after inoculation. *MeSAP15* and *MeSAP16* exhibited high expression at 6 d after inoculation. In contrast, *MeSAP5*, *MeSAP7*, *MeSAP10*, *MeSAP12*, and *MeSAP13* remained at low expression levels following *Xpm* inoculation (Supplementary Fig. S3c).

qPCR validation of expression profiles of *MeSAPs* under abiotic stresses

To examine the effects of abiotic stresses and hormonal treatments on *MeSAP* gene expression, the expression patterns of 13 *MeSAP* genes were analyzed using qRT-PCR under various conditions, including ABA, H_2O_2, NaCl, PEG6000, and cold treatments (Fig. 6). Among them, *MeSAP10* was notably induced by ABA, H_2O_2, NaCl, PEG6000 and low temperature, but downregulated by JA treatment. In response to *Xpm*CHN11 infection, *MeSAP10* was significantly upregulated, suggesting its potential key role in cassava disease resistance (Fig. 5). These results indicate that *MeSAP10* is a key gene involved in cassava responses to both abiotic and biotic stresses.

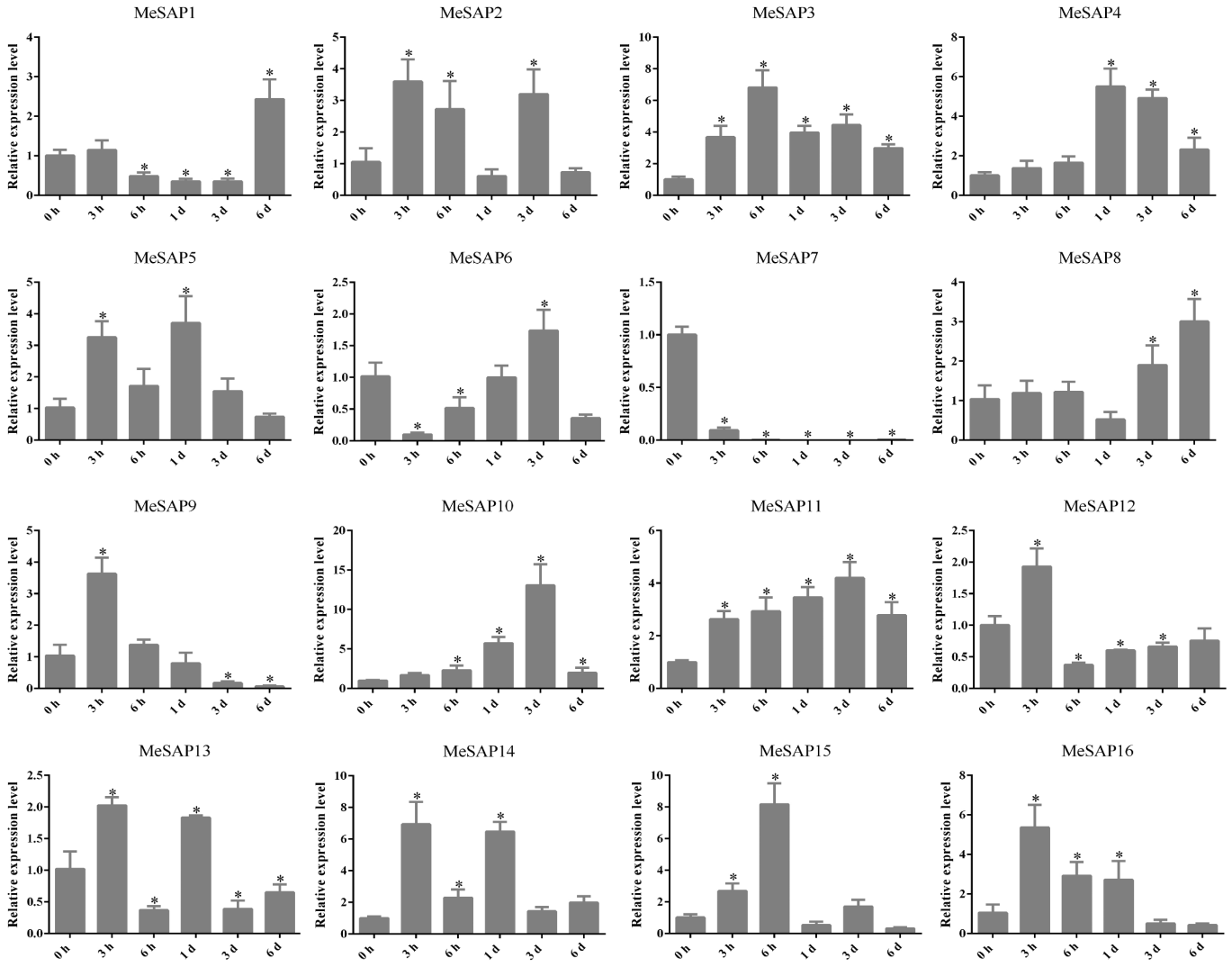

Fig. 5 Expression of cassava *SAP* genes induced with *Xpm*CHN11. Data is mean ± SE pooling from three biological replicates. * Indicates a significant difference ($p < 0.05$) between the control and treatments.

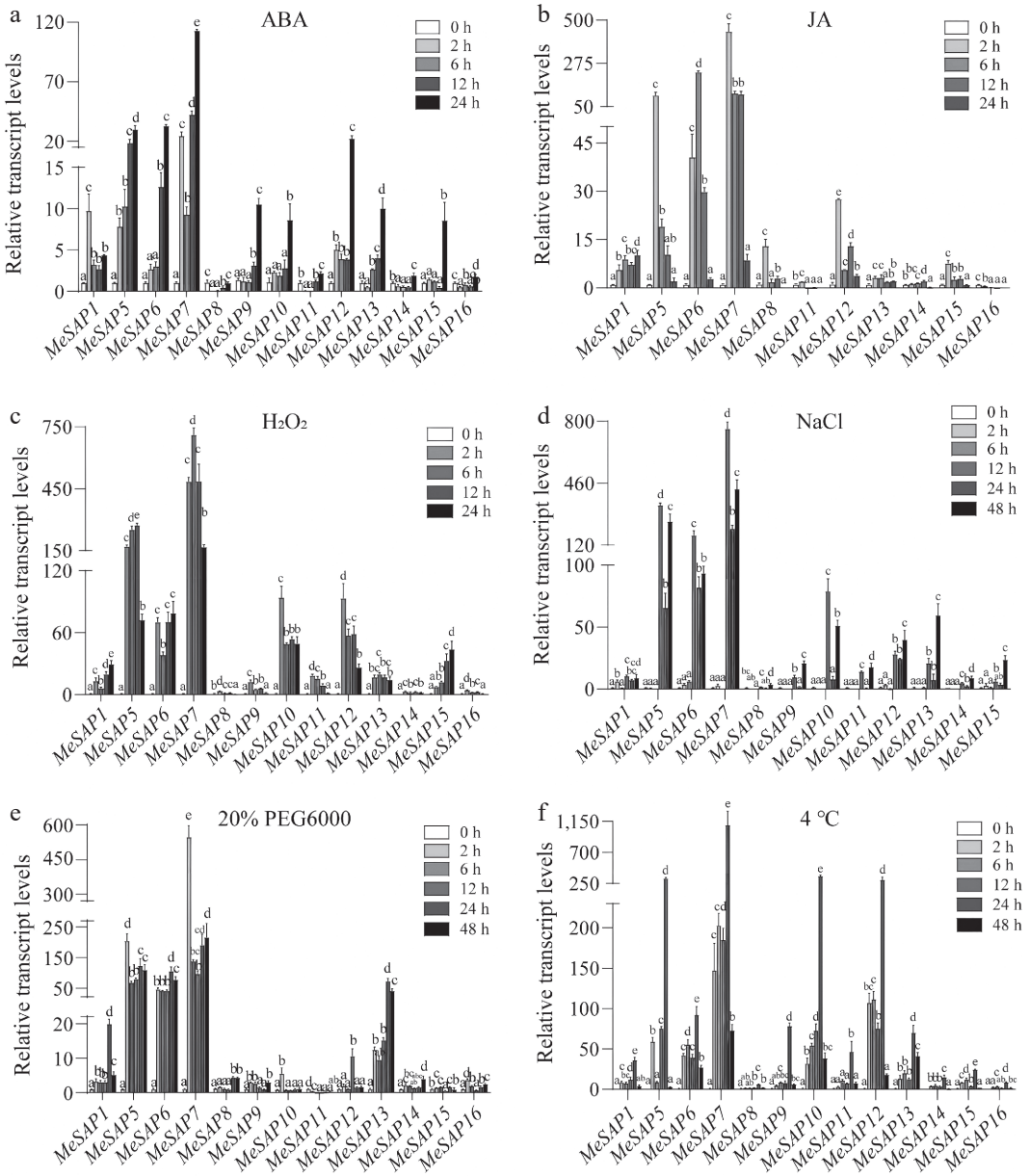

Fig. 6 Expression profiles of the *MeSAP* gene family under abiotic stresses. Expression patterns of the *MeSAP* gene family under (a) ABA treatment, (b) JA treatment, (c) H_2O_2 treatment, (d) NaCl treatment, (e) 20% PEG6000 treatment, (f) 4 °C treatment. Data is mean ± SE pooling from three biological replicates. Statistical significance was assessed by One-way ANOVA. Letters indicate significant differences between groups ($p < 0.05$).

MeSAP10 improves cassava resistance to *Xpm*CHN11

To explore the role of *MeSAP10* in response to *Xpm*CHN11 in cassava, we generated *MeSAP10*-silenced plants using CsCMV-mediated gene silencing technology, with plants infected with the pCsCMV-NC empty vector as a negative control. After 4 weeks, qRT-PCR analysis revealed that the expression level of *MeSAP10* in pCsCMV-*SAP10* plants was reduced to 20% of that in pCsCMV-NC plants (Fig. 7b). Subsequently, leaves of pCsCMV-NC and pCsCMV-*MeSAP10* plants were inoculated with *Xpm*CHN11. The results revealed that silencing of *MeSAP10* in cassava plants increased susceptibility to *Xpm*CHN11 infection compared to pCsCMV-NC plants leaves, as evidenced by larger lesions in *MeSAP10*-silenced plants at 6 d post-inoculation (dpi) (Fig. 7a, c). Moreover, *Xpm*CHN11 growth in *MeSAP10*-silenced leaves was significantly higher than in pCsCMV-NC plants (Fig. 7d). Additionally, the expression of *MeSAP10*, *MePR1*, and *MeCAT2* was significantly downregulated in *MeSAP10*-silenced plants compared to pCsCMV-NC plants (Fig. 7e).

Furthermore, we generated *MeSAP10*-overexpressing plants (designated as pCsCMV2-*MeSAP10*) via CsCMV-mediated gene overexpression system, with pCsCMV2-NC plants used as the control. qRT-PCR analysis revealed that the expression level of *MeSAP10* in pCsCMV2-*MeSAP10* plants was 29.68-fold higher than that in pCsCMV2-NC plants (Fig. 7g). Subsequently, leaves of pCsCMV2-NC and pCsCMV2-*MeSAP10* plants were inoculated with *Xpm*CHN11. The results demonstrated that, compared with pCsCMV2-NC leaves, *SAP10*-overexpressing plants exhibited enhanced resistance to *Xpm*CHN11 at 6 dpi, which was manifested by a smaller lesion area on the leaves (Fig. 7f, h). In addition, the content of *Xpm*CHN11 in *SAP10*-overexpressing plants leaves was significantly lower than that in pCsCMV2-NC leaves at 6 dpi (Fig. 7i). Consistently, the expression levels of *MeSAP10*, *MePR1*, and *MeCAT2* were significantly

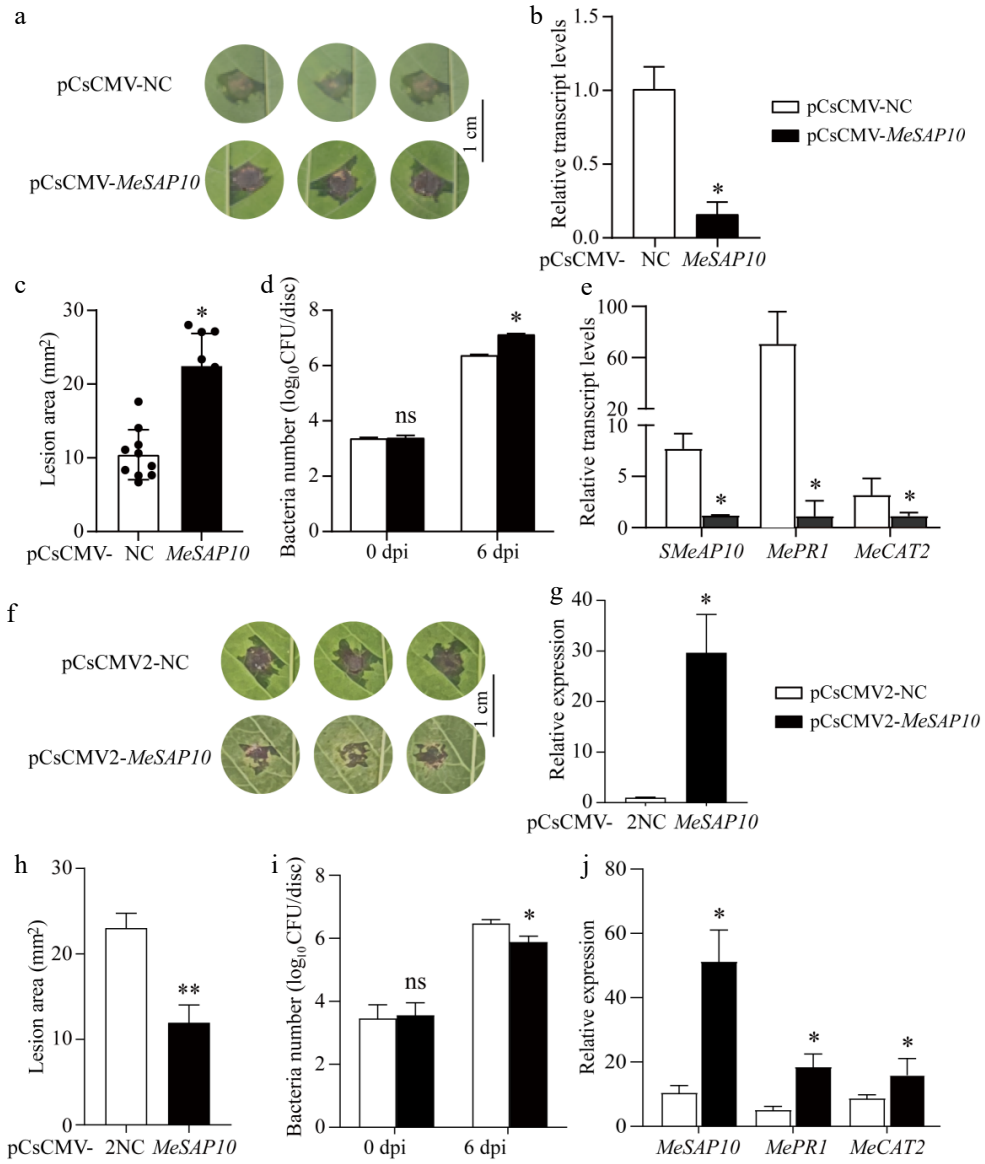

Fig. 7 Functional analysis of *MeSAP10* in response to *Xpm*CHN11. (a) Lesion phenotypes of pCsCMV-NC and pCsCMV-*MeSAP10* plants after inoculation with *Xpm*CHN11 at 6 d post-inoculation (dpi), scale bar = 1 cm. (b) Silencing efficiency of *SAP10* in pCsCMV-NC and pCsCMV-*MeSAP10* plants. (c) Lesion area of pCsCMV-NC and pCsCMV-*MeSAP10* plants after inoculation with *Xpm*CHN11 at 6 dpi. (d) Bacteria number in leaves of pCsCMV-NC and pCsCMV-*MeSAP10* plants after inoculation with *Xpm*CHN11 at 6 dpi. (e) Expression levels of *MeSAP10*, *MePR1*, and *MeCAT2* in leaves of pCsCMV-NC and pCsCMV-*MeSAP10* plants at 3 dpi. (f) Lesion phenotypes of pCsCMV2-NC and pCsCMV2-*MeSAP10* plants after inoculation with *Xpm*CHN11 at 6 d post-inoculation (dpi), scale bar = 1 cm. (g) Expression levels of *MeSAP10* in pCsCMV2-NC and pCsCMV2-*MeSAP10* plants. (h) Lesion area of pCsCMV2-NC and pCsCMV2-*SAP10* plants after inoculation with *Xpm*CHN11 at 6 dpi. (i) Bacteria number in leaves of pCsCMV2-NC and pCsCMV2-*MeSAP10* plants after inoculation with *Xpm*CHN11 at 6 dpi. (j) Expression levels of *MeSAP10*, *MePR1*, and *MeCAT2* in leaves of pCsCMV2-NC and pCsCMV2-*MeSAP10* plants at 3 dpi. Statistical significance was assessed by Student's *t*-tests. * Represents significant differences between pCsCMV-NC and pCsCMV-*MeSAP10* plants, and between pCsCMV2-NC and pCsCMV2-*MeSAP10* plants (* $p < 0.05$, ** $0.001 \leq p < 0.01$).

upregulated in *MeSAP10*-overexpressing leaves compared to those in pCsCMV2-NC leaves (Fig. 7j). These results collectively demonstrate that *MeSAP10* plays an important role in plant immune responses by positively regulating cassava resistance to *Xpm*CHN11.

Discussion

Proteins containing the A20/AN1 zinc finger domain play crucial roles in plant responses to biotic and abiotic stresses[6,39]. While *SAPs* are known to function in stress tolerance as ubiquitin ligases, redox sensors, or gene expression regulators, the precise mechanism remains elusive. Recent studies have shown that constitutive expression of *SAP* genes in various plant species, including *Arabidopsis*, rice, maize, and soybean enhances tolerance to multiple abiotic stresses[10,16,37,40]. However, *SAP* genes in cassava have not been fully identified, and their functions in cassava remain poorly understood.

We identified 16 *MeSAP* genes in cassava, which were categorized into three subgroups through whole-genome screening. Among them, MeSAP10 and MeSAP15 contained two AN1 domains, while the remaining 12 genes had one A20 domain and one AN1 domain. This finding suggests that the A20/AN1 type is the predominant arrangement among zinc-finger family members in

cassava, consistent with observations in tomato and *Dendrobium officinale*[41,42]. All the SAP proteins contained the A20/AN1 type domain, except for MeSAP14, which only had one AN1 domain in cassava. Previous research has indicated that lower organisms, such as *Saccharomyces cerevisiae* and *Aspergillus fumigatus*, possess only AN1 zinc finger domains, suggesting the ancient origin of the AN1 zinc finger domain compared to the A20 domain[41,43]. During the evolution of living organisms, gene duplication occurs and is considered a major cause of genome complexity and functional expansion[44]. In cassava, gene duplication mainly occurs on Chr. 1, 2, 6, 7, 10, and 14.

In *Arabidopsis*, *SAP* genes have been found to confer defense against pathogens in plants. For instance, *AtSAP9* was induced by pathogens, PAMP molecules, and phytohormones, leading to increased susceptibility to non-host pathogen *Pseudomonas syringae* pv. *phaseolicola*, indicating its crucial role in basal resistance[21]. Furthermore, an orchid SAP protein, Pha13, and its *Arabidopsis* homologue, AtSAP5, was reported to serve as an important regulatory hub in plant antiviral immunity[45]. Transcriptome data revealed that eight *MeSAP* family members were regulated by *Xpm*, with their expression profiles differing under high-virulence *Xpm* compared to low-virulence *Xpm*. Some *MeSAP* genes, such as *MeSAP3*, *MeSAP15*, and *MeSAP16*, responded to *Xam*668 and *Xpm*CHN11 inoculation. Notably, *MeSAP4* was only induced by *Xpm*CHN11 and not *Xam*668 (Supplementary Fig. S3c). Conversely, *MeSAP9*, *MeSAP11*, *MeSAP13*, *MeSAP14*, *MeSAP15*, and *MeSAP16* were markedly induced by *Xam*668. *MeSAP4*, *MeSAP8*, *MeSAP15*, and *MeSAP16* responded to *Xpm*CHN11. In contrast, *MeSAP7* was downregulated under *Xam*668 and *Xpm*CHN11 treatment. We further validated these results using qPCR in cassava leaves treated with *Xpm*CHN11, which showed consistency with the qRT-PCR results, indicating that *SAP* family members responded to *Xpm*CHN11 infection and may play crucial roles in pathogen defense. Furthermore, some *MeSAP* family members may respond differently to pathogens. For example, *MeSAP10* did not respond to *Xam*668 but was significantly induced by *Xpm*CHN11 (Fig. 5). Integration of results from different phytohormone and biotic stress assays suggested that *MeSAP10* may play an important role in plant resistance, possibly through the H_2O_2 pathway in response to *Xpm*CHN11.

Previous studies have emphasized the pivotal roles of phytohormones in regulating plant tolerance and resistance. It was observed that the promoters of all cassava *SAP* genes contain *cis*-acting elements related to stress and hormone responses, such as drought, low temperature, defense, and ABA responses (Fig. 4). Several *SAP* genes, including *MeSAP4*, *MeSAP6*, *MeSAP7*, *MeSAP10*, *MeSAP12*, *MeSAP13*, and *MeSAP16* were found to contain more CGTCA elements, indicating the presence of *cis*-elements related to phytohormones. Interestingly, promoters of the *SAP* genes also contained ERE motifs responsive to ethylene, such as *MeSAP3*, *MeSAP10*, *MeSAP12*, *MeSAP13*, *MeSAP15*, and *MeSAP16*, containing more ERE elements. However, the relationship between *SAP* family gene expression and ethylene could not be confirmed through qRT-PCR analysis. Furthermore, *MeSAP5*, *MeSAP9*, and *MeSAP10* contained more WUN motifs, which respond to wounding stress. The potential defense mechanisms of *SAP* genes against phytohormone stresses and their involvement in pathogen defense through hormone signal transduction warrant further investigation. Subsequent studies are needed to validate these hypotheses and elucidate the regulatory mechanisms underlying the expression of *SAP* genes under hormone stresses.

In conclusion, we conducted a genome-wide analysis of *SAP* family members in cassava, identifying a total of 16 *SAP* genes. A comprehensive characterization of *SAP* genes was performed, including analyses of gene structures, characteristic domains, and phylogenetic relationships. Moreover, we found that 16 cassava *SAP* genes identified from cassava were induced by abiotic stresses and pathogen treatments through RNA-seq and qRT-PCR analysis. Additionally, we found that *MeSAP10* strongly responded to *Xpm*CHN11 infection, and silencing of *MeSAP10* in cassava enhanced its susceptibility to *Xpm*CHN11, while overexpression of *MeSAP10* significantly enhances its resistance (Fig. 7). All these results may help us further understand the mechanisms of cassava defense to stress conditions and provide candidate genes for breeding stress-resistant cultivars. Future research should be conducted to study the function of plant *SAP* family genes under stress conditions.

Author contributions

The authors confirm their contributions to the paper as follows: writing–original draft: Wang Y, Li K; visualization: Wang Y, Li K, Niu X, Yang L, Wang H; validation: Wang Y; data curation: Chen Y, Yu X; writing–review and editing: Zheng L, Yang L, Lin M; supervision, conceptualization, project administration: Chen Y. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest.

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