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ORIGINAL ARTICLE

Genome-wide analysis of autophagy-related genes in banana highlights *MaATG8s* in cell death and autophagy in immune response to Fusarium wilt

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Abstract

Key message ***MaATG8s*** **play important roles in hypersensitive-like cell death and immune response, and autophagy is essential for disease resistance against** ***Foc*** **in banana.**

Abstract Autophagy is responsible for the degradation of damaged cytoplasmic constituents in the lysosomes or vacuoles. Although the effects of autophagy have been extensively revealed in model plants, the possible roles of autophagy-related gene in banana remain unknown. In this study, *32 MaATGs* were identified in the draft genome, and the profiles of several *MaATGs* in response to fungal pathogen *Fusarium oxysporum* f. sp. *cubense* (*Foc*) were also revealled. We found that seven *MaATG8s* were commonly regulated by *Foc*. Through transient expression in *Nicotiana benthamiana* leaves, we highlight the novel roles of *MaATG8s* in conferring hypersensitive-like cell death, and *MaATG8s*-mediated hypersensitive response-like cell death is dependent on autophagy. Notablly, autophagy inhibitor 3-methyladenine (3-MA) treatment resulted in decreased disease resistance in response to *Foc4*, and the effect of 3-MA treatment could be rescued by exogenous salicylic acid, jasmonic acid and ethylene, indicating the involvement of autophagy-mediated plant hormones in banana resistance to Fusarium wilt. Taken together, this study may extend our understanding the putative role of *MaATG8s* in hypersensitive-like cell death and the essential role of autophagy in immune response against *Foc* in banana.

Keywords Autophagy-related gene (ATG) · Banana (*Musa acuminata*) · Autophagy · Reactive oxygen species (ROS) · Cell death · Immune response · Fusarium wilt

Abbreviations

APX	Ascorbate peroxidase
ATG	Autophagy-related gene
CAT	Catalase
CDD	Conserved domain database
CDS	Coding sequence
dpi	Day post inoculation
DAB	Diaminobenzidine
DAPI	4′,6-Diamidino-2-phenylindole
EL	Electrolyte leakage
ELISA	Enzyme-linked immunosorbent assay
ETH	Ethylene
Fo	*Fusarium oxysporum*
Foc	*Fusarium oxysporum* f. sp. *cubense*
Focn	*Fusarium oxysporum* f. sp. *conglutinans*
Fol	*Fusarium oxysporum* f. sp. *lycopersici*

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Yunxie Wei, Wen Liu and Wei Hu contributed equally to this work.

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GSDS	Gene structure display server
GFP	Green fluorescent protein
hpi	Hour post inoculation
H_2O_2	Hydrogen peroxide
HR	Hypersentive response
JA	Jasmonic acid
MDA	Malondialdehyde
MDC	Monodansylcadaverine
MEME	Multiple Em for Motif elicitation
3-MA	3-Methyladenine
MW	Molecular weight
NCBI	National Center for Biotechnology Information
NBT	Nitro blue tetrazolium
$O_2^{\cdot-}$	Superoxide radical
PAMP	Pathogen-associated molecular pattern
PTI	PAMP-triggered immunity
PR	Pathogensis-related gene
RGAP	Rice genome annotation project
ROS	Reactive oxygen species
SA	Salicylic acid
SOD	Superoxide dismutase
TAIR	The Arabidopsis information resource
WT	Wild type

Introduction

Autophagy is responsible for the degradation of damaged cytoplasmic constituents in the lysosomes or vacuoles, so it is a conserved mechanism in yeast, animals and plants (Boya et al. 2013; Thompson and Vierstra 2005). Although three autophagy pathways (macroautophagy, microautophagy and chaperone-mediated autophagy) are existed, macroautophagy is usually taken as autophagy, and it has been extensively investigated in yeast, animals and plants (Boya et al. 2013). The basal autophagy is kept at a relative low level under control conditions, and it may be quickly and largely induced to achieve cellular homeostasis under stress conditions (Boya et al. 2013; Han et al. 2015; Zhou et al. 2014b).

To date, a series of autophagy-associated genes (*ATGs*) involved in autophagy have been identified (Wang et al. 2015; Zhou et al. 2014a, b, 2015). *ATGs* are first identified in yeast, and 32 *ATGs* are involved in the autophagy (Thompson and Vierstra 2005). In recent 15 years, many *ATGs* have been characterized in various plant species, including 25 *AtATGs* in *Arabidopsis thaliana* (Hanaoka et al. 2002), 33 *OsATGs* in *Oryza sativa* L. (Xia et al. 2011), 24 *SlATGs* in *Solanum lycopersicum* (Wang et al. 2015; Zhou et al. 2014b), 30 *NtATGs* in *Nicotiana tabacum* L. (Zhou et al. 2015), 29 *CaATGs* in *Capsicum annuum* (Zhai et al. 2016). Through identification and functional analysis of plant *ATGs*, the in vivo roles of autophagy have been largely revealed, including plant development and stress responses, including seed germination, seed and anther development, cell morphogenesis and differentiation, senescence, various abiotic stresses, hypersensitive response, plant-pathogen interaction (Chung et al. 2009; Kwon et al. 2013; Zhou et al. 2014b).

Almost all roles of autophagy in plants are obtained from the comparative phenotype analysis of wild type (WT) and ATG-knockout or overexpressing plants. *AtATG6* is essential for pollen germination, fertility and pathogen-related cell death (Fujiki et al. 2007; Harrison-Lowe and Olsen 2008; Patel and Dinesh-Kumar 2008; Qin et al. 2007), *OsATG6* and *TaATG6* play important roles in abiotic stress response (Rana et al. 2012) and powdery mildew immunity (Yue et al. 2015), respectively. *AtATG9* is involved in senescence and plant response to nitrogen and carbon-starvation (Hanaoka et al. 2002). *AtATG8* is essential for abiotic stress and starvation stresses (Slávikoká et al. 2005, 2008). *AtATG2*, *AtATG10* and *AtATG18a* are involved in mildew-induced cell death (Wang et al. 2011a, b), *AtATG5* and *AtATG7* are essential for plant response to various abiotic and biotic stresses (Lenz et al. 2011a, b; Zhou et al. 2013, 2014c). SlATG10 and SlATG18f are direct targets of HsfA1a and essential for drought stress response in tomato (Wang et al. 2015). Although the effects of *ATGs* and autophagy have been extensively revealed in investigated in model plants (*Arabidopsis*, rice and tomato), the possible roles of them in banana remain unknown. As one of the most popular fresh fruits worldwide, banana (*Musa acuminata*) is cultivated in the tropical and subtropical regions (Hu et al. 2015b, c; Liu et al. 2015a, b; Xu et al. 2014; Zhang et al. 2015). In recent years, banana is severely affected by the fungal pathogen *Fusarium oxysporum* f. sp. cubense (*Foc*) (Deng et al. 2015; Ploetz 2006, 2015; Ploetz et al. 2015; Silva et al. 2016; Tan et al. 2015). The disease has been rapidly and largely spreading to banana production areas worldwide, causing substantial yield losses (Bai et al. 2013; Guo et al. 2014; Li et al. 2012, 2013a, b; Wang et al. 2012; Wu et al. 2013). There are many evidences indicating the involvement of autophagy in plant immune responses (Chung et al. 2009; Kwon et al. 2013; Zhou et al. 2014b). However, no *ATG* has been identified in banana, and the possible roles of autophagy in banana remains unknown.

To extend our understanding of the in vivo roles of autophagy and possible utilization of *ATGs* in banana, a comprehensive identification and expression analysis of *MaATGs* were performed in this study. Moreover, molecular identification of *MaATG8s* reveals its novel roles in hypersensitive-like cell death, as well as immune response. Additionally, autophagy inhibitor 3-methyladenine (3-MA) treatment highlights the essential role of autophagy in banana resistance to Fusarium wilt.

Materials and methods

Plant materials and growth conditions

BX variety is widely cultivated in China, with long fingers, high yield and long-term storage. FJ variety is widely cultivated in Hainan province in China, because of its good flavor and tolerance to various abiotic stresses. After obtaining from Banana Tissue Culture Center (Danzhou city, Institute of Banana and Plantains, Chinese Academy of Tropical Agricultural Sciences), the five-leaf stage seedlings were grown in soil in the greenhouse. The greenhouse was controlled at 28 °C, under 12-h light/12-h dark cycles, with 120–150 μmol quanta m^{-2} s^{-1} irradiance.

Comprehensive identification and phylogenetic analyses of *MaATGs*

The candidate *MaATGs* were first searched using the keyword of autophagy in *M. acuminata v1* (Banana) database of Phytozome v10.3 (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Macuminata). Based on the sequences of *AtATGs* and *OsATGs* that were downloaded from The Arabidopsis Information Resource (TAIR) v10 (http://www.Arabidopsis.org) and Rice Genome Annotation Project (RGAP) v7 (http://rice.plantbiology.msu.edu), these candidate *MaATGs* were confirmed by National Center for Biotechnology Information (NCBI)'s conserved domain database (CDD) (http://www.ncbi.nlm.nih.gov/cdd) (Marchler-Bauer et al. 2015) and Pfam (http://pfam.xfam.org) (Finn et al. 2016).

After complete alignment by Clustalx 1.83 software, the DNA coding sequences of 32 *MaATGs* were used for the construction of phylogenetic tree construction by MEGA5.05 software according to the neighbor-joining method (Tamura et al. 2011). At last, the ProtParam software (http://web.expasy.org/protparam) was used to obtain the molecular weight (MW) and theoretical pI of all *MaATG* proteins.

Gene structure and conserved motif analyses of *MaATGs*

The coding sequences (CDS) and genomic sequences of 32 *MaATGs* were downloaded from *M. acuminata v1* (Banana) database of Phytozome v10.3, and were used to identify the exon, intron, upstream and downstream of all *MaATGs* by GSDS v2.0 (http://gsds.cbi.pku.edu.cn/index.php) (Hu et al. 2015a). Moreover, the MEME v4.11.0 (http://meme-suite.org/tools/meme) was used to identify the conserved motifs of 32 *MaATGs* based on their sequences.

Transcriptomic data analysis

The transcriptomic data of banana roots in response to pathogen infection of race 1 and tropical race 4 (*Foc1* and *Foc4*) were downloaded from published data (Li et al. 2013a). For the assay, sterile tissue cultivated banana roots were inoculated by control, *Foc1* or *Foc4*. Root samples were collected after 3, 27 and 51 h inoculation for banana roots.

Construction of heatmap based on hierarchical cluster analysis

After hierarchical cluster analysis by CLUSTER program (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) (Larkin et al. 2007), the heatmap of gene expression was constructed by Java Treeview (http://jtreeview.sourceforge.net) (Saldanha 2004).

Vector construction and transient expression in *Nicotiana benthamiana* leaves

For transient expression, the coding sequences of *MaATG8s* were amplified by PCR (Supplemental Table S1), and cloned into multiple clone sites of pEGAD vector (Cutler et al. 2000) in the C-terminal of green fluorescent protein (GFP) by double enzyme digestion and ligation, to form the constructs of *35S::GFP-MaATG8s*. After confirmation of sequences through DNA sequencing, the recombinant plasmids were syringe infiltrated into 4-week-old *N. benthamiana* leaves through *Agrobacterium tumefaciens* strain GV3101 as Sparkes et al. (2006) described. At 2 dpi, green fluorescence and 4′,6-diamidino-2-phenylindole (DAPI)-stained cell nuclei were then detected using a confocal laser-scanning microscope (TCS SP8, Leica, Heidelberg, Germany).

Determination of physiological parameters

The physiological parameters including hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\cdot-}$) as well as diaminobenzidine (DAB) and nitro blue tetrazolium (NBT) staining, relative electrolyte leakage (EL), chlorophyll, malondialdehyde (MDA) and callose deposition in plant leaves were quantified as Wei et al. (2016a, b) described. For H_2O_2 and $O_2^{\cdot-}$ quantification, plant leaves were extracted using 50 mM sodium phosphate buffer (pH 7.8). For H_2O_2 content assay, the extraction was mixed with 0.1% (w/v) titanium sulfate reagent [in 20% (v/v) H_2SO_4] at 1/1 (v/v) to form the peroxide-titanium complex.

Thereafter, the endogenous H_2O_2 level in the complex solution was examined at the absorbance of 410 nm. For $O_2^{\cdot-}$ content assay, the endogenous $O_2^{\cdot-}$ level in the plant extraction was quantified using plant $O_2^{\cdot-}$ enzyme-linked immunosorbent assay (ELISA) kit (Jianglai Biotechnology, Shanghai, China), according to the manufacturer's protocols.

RNA isolation and quantitative real-time PCR

Total RNA isolation, purification and first-strand cDNA synthesis were performed using RNAprep Pure Plant Kit (TIANGEN, DP441, Beijing, China), RNase-free DNase (NEB, M0303S, USA) and RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, K1622, Waltham, Massachusetts, USA), according to the manufacturer's instruction. Thereafter, the cDNA, primers (Supplemental Table S2) and TransStart Tip Green qPCR SuperMix (TransGen Biotech, AQ141, Beijing, China) were performed in LightCycler® 96 Real-Time PCR System (Roche, Basel, Switzerland) for quantitative real-time PCR.

Quantification of callose deposition

For callose deposition quantification, plant leaves were infiltrated in alcoholic lactophenol solution and 0.01% (w/v) aniline blue solution for 1 h as Hauck et al. (2003) described. After mounted in 50% (v/v) glycerol, callose depositions in plant leaves were visualized using fluorescence microscope (DM6000B, Leica, Heidelberg, Germany) and quantified using the ImageJ software.

Quantification of autophogasome by monodansylcadaverine (MDC) staining

The autophogasome was examined by MDC staining as Zhou et al. (2014a) described. Plant leaves were incubated in phosphate-buffered saline buffer containing 100 μM MDC (Sigma-Aldrich, 30432) phosphate buffer for 30 min. After washing three times by phosphate buffer, MDC-stained autophogasomes were visualized using a confocal laser-scanning microscope (TCS SP8, Leica, Heidelberg, Germany).

Determination of endogenous plant hormone levels

The endogenous salicylic acid (SA), jasmonic acid (JA) and ethylene (ETH) were extracted and quantified using ELISA Kits, including Plant SA ELISA Kit, Plant JA ELISA Kit and Plant ET ELISA Kit (Jianglai Biotechnology, Shanghai, China), as the manufacturer's protocols described.

Statistical analysis

At least three biological replicates were performed for every experiment. All the data in this study were analyzed using ANOVA and student *t* test, the significant differences were shown as asterisk symbols (*).

Results

Comprehensive identification and evolutionary analysis of *MaATGs*

After confirmation using CDD and Pfam databases, the locus name, chromosome location and predicted information of 32 *MaATGs* were listed in Supplemental Table S3. The neighbor-joining phylogenetic tree was constructed to investigate the evolution of 32 *MaATGs*. Seven *ATGs* (*ATG1*, *ATG2*, *ATG5*, *ATG6*, *ATG7*, *ATG9* and *ATG12*) contain only one member, whereas *MeATG8s* has the maximum ten members (Fig. 1; Table S3).

Gene structure and conserved motif analysis of *MaATGs*

To further reveal the structural features of *MaATGs*, gene structures and conserved motifs of 32 *MaATGs* were analyzed using Gene Structure Display Server (GSDS) v2.0 and Multiple Em for Motif Elicitation (MEME) v4.11.0 softwares. We found that the exon lengths, numbers and the conserved motifs of *MaATGs* showed direct relationship with their evolution, especially in the subfamily of *MaATG8s*, *MaATG18s*, *MaATG3s* and *MaATG4s* (Figures S1–S3). The closer gene structures and motifs as well as the link between them and the evolutionary history suggest the possible functional redundancy of these genes in the same subfamily.

The expression profile of *MaATGs* in response to *Foc* infection

Based on the published data (Li et al. 2013a), we analyzed the transcripts of *MaATGs* under biotic stress (*Foc1* and *Foc4*) from banana roots. In briefly, 12 of 32 *MaATGs* were clearly affected by *Foc* infection (Fig. 2). Notably, except *MaATG12*, all these gene transcripts were normally up-regulated by *Foc* at least in one time-point (Fig. 2). Among these genes, *MaATG8b*, *8j*, *18a* were generally up-regulated by *Foc1* and *Foc4*. *MaATG18b* was first up-regulated and later down-regulated by *Foc1* and *Foc4*, while *MaATG18d* was first down-regulated and later up-regulated by *Foc4*.

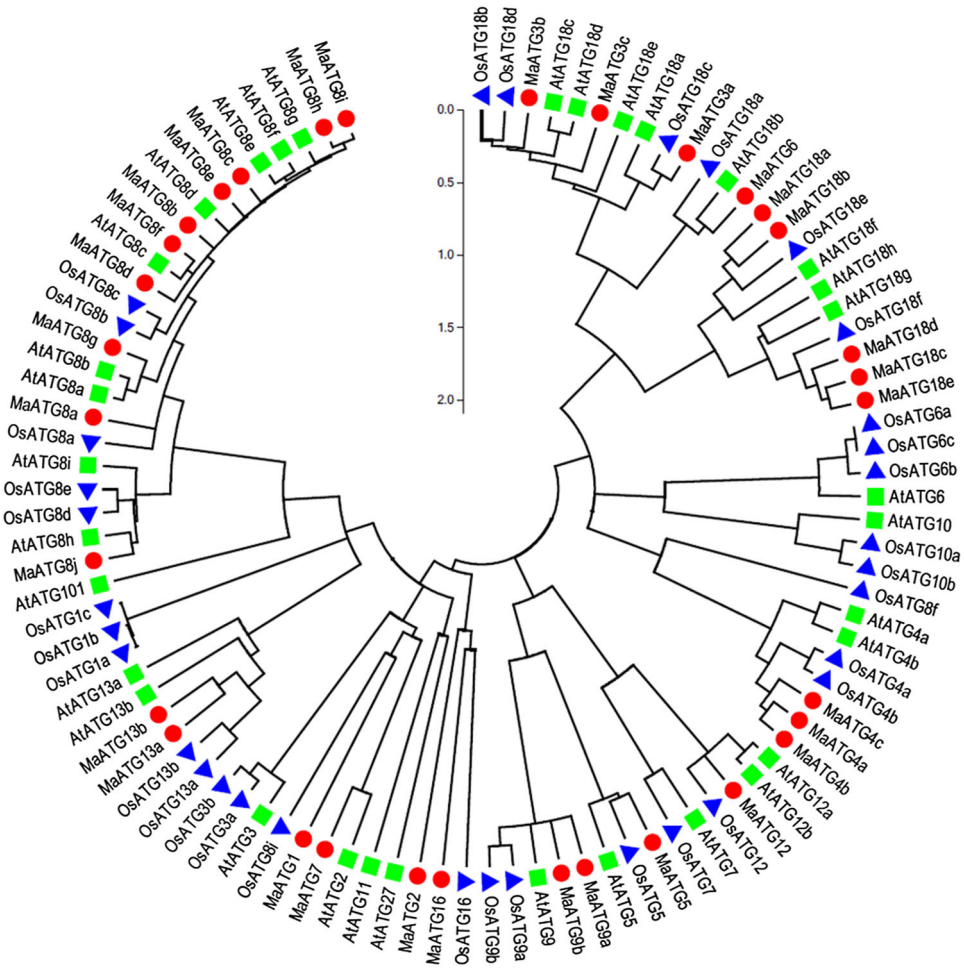


Fig. 1 The phylogenetic tree showing relationship of 32 *MeATGs* that was constructed using Clustalx 1.83 and MEGA5.05 softwares

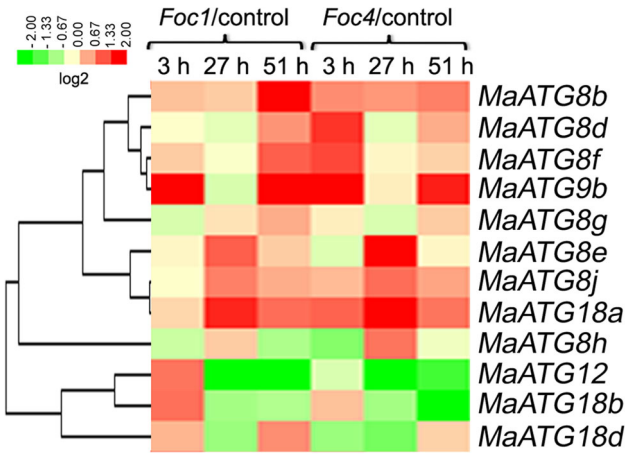


Fig. 2 The expression profile of *MaATGs* in response to pathogen infection of *Foc1* and *Foc4*. The transcriptomic data were downloaded from Li et al. (2013a). For the assay, banana roots were inoculated by control, *Foc1* or *Foc4* for 3, 27 and 51 h. The heatmap of gene expression was constructed using CLUSTER program and Java Treeview

Transient expression of *MaATG8s* confers hypersensitive response-like cell death and immune responses

In this study, seven *MaATG8s* (*MaATG8b*, *8d*, *8e*, *8f*, *8g*, *8h*, *8j*) were most up-regulation by *Foc1* and *Foc4* at all test time points. To further analyze the role of these genes in immune responses, we constructed the recombination vectors expressing MaATG8b/d/e/f/g/h/j fused with in the C-terminal of GFP under the control of 35S promoter. After transient expression in leaves of *N. benthamiana* for 2 days, seven 35S::GFP-MaATG8s (MaATG8b, d, e, f, g, h, j) proteins were detected predominantly in both nucleus and cytosol in the leaf cells (Fig. 3).

Notably, transient expression of seven *MaATG8s* in *N. benthamiana* leaves resulted in different degrees of cell death and hypersensitive response (HR) symptoms in comparison to transient expression of vector alone (control) (Fig. 4a). To further confirm the accumulation of ROS, we performed DAB and NBT staining assays (Fig. 4b, c) as well as quantification

of endogenous H_2O_2 and $O_2^{\cdot-}$ (Fig. 4d, e). The results showed that most of the *N. benthamiana* leaf disks expressing *MaATG8s* displayed higher levels of H_2O_2 and $O_2^{\cdot-}$ than vector transformation, especially *MaATG8h*. Additionally, the EL of leaf disks from *N. benthamiana* leaves expressing these genes was significantly higher than those from vector transformation (Fig. 4f), except *MaATG8f*. Meanwhile, the quantification of MDA was performed, and the MDA concentration was varied (Fig. 4g). These data indicate that *MaATG8s* are involved in plant immune response, as well as underlying HR and ROS modulation.

To further anatomize the potential mechanisms of *MaATG8s*-mediated ROS accumulation and immune response, we analyzed the effects of *MaATG8s* on regulating the expression of several key genes involving ROS and defense pathways. The results showed that *MaATG8s* transient expression in *N. benthamiana* leaves significantly increased the transcript levels of ROS-related genes [*superoxide dismutase* (*SOD*), *catalase* (*CAT*) and *ascorbate peroxidase* (*APX*)], defense-related genes [*RbohA*, *RbohB*, *pathogenesis-related gene 1* (*PR1*), *PR2* and *PR5*] and *NtATG8s* as revealed by quantitative real-time PCR (Fig. 5). Correspondingly, the *N. benthamiana* leaves expressing these genes (except *MaATG8j*) exhibited significantly more callose depositions than those expressing vector alone (Fig. 6). These results suggest that *MaATG8s* might be involved in ROS, defense signaling, autophagy signaling, and callose-associated cell wall to participate in plant immune response.

Additionally, the endogenous levels of immunity-associated hormones (SA, JA and ETH) in *MaATG8s* transiently expressed leaves were also analyzed. Our results showed that the *N. benthamiana* leaves expressing *MaATG8s* have varying levels of endogenous hormones compared with those expressing vector alone (Figure S4), indicating the possible involvement of these hormones in *MaATG8s*-mediated HR and immune response.

Fig. 4 Transient expression of *MaATG8s* confers hypersensitive response-like cell death. Symptoms of transient expressing *Nicotiana benthamiana* leaves that were visualized under UV light (**a**), DAB staining (**b**) and NBT staining (**c**). The endogenous levels of H_2O_2 (**d**), $O_2^{\cdot-}$ (**e**), EL (**f**) and MDA (**g**) in transient expressing *Nicotiana benthamiana* leaves. *Asterisk symbols* (*) were shown as significant difference in comparison to vector at $p < 0.05$

MaATG8s-mediated hypersensitive response-like cell death is dependent on autophagy

When *MaATG8s* were transcient expressed in *N. benthamiana* leaves, we found that the autophagic activity was significantly increased, as evidenced by MDC-stained autophogasomes (Fig. 7a). Then autophagy inhibitor (3-methyladenine, 3-MA) was used to investigate whether *MaATG8s* influence on hypersensitive-like cell death is dependent or independent on autophagy. In the present of 5 mM 3-MA, *MaATG8s*-increased autophgic activity and -mediated hypersensitive response-like cell death is alleviated, with no significant difference in comparison to vector (Fig. 7a, b). These results suggested that *MaATG8s*-mediated hypersensitive response-like cell death is dependent on autophagy.

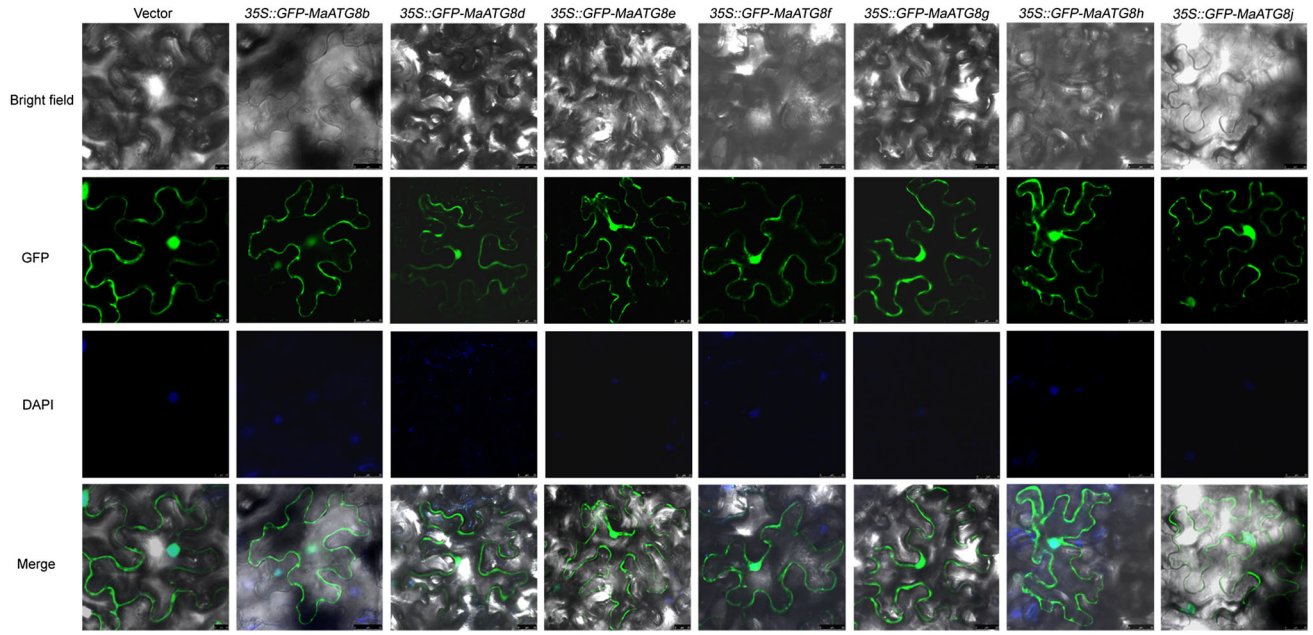
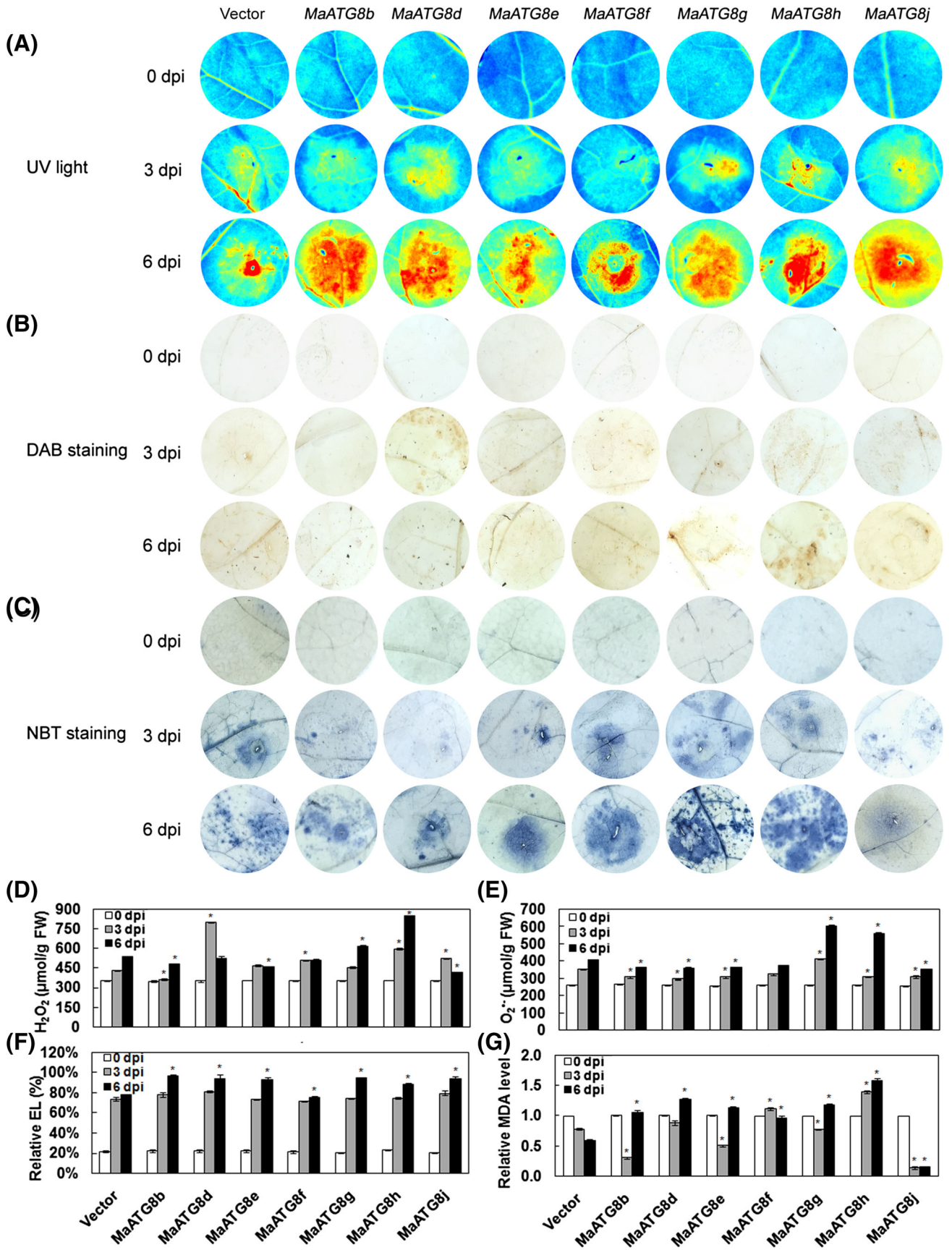


Fig. 3 Subcellular localization of MaATG8s. Transient expression of GV3101 cell culture with vector and different recombinant plasmids in 4-week-old *Nicotiana benthamiana* leaves, and DAPI-stained cell nuclei was shown in *blue color*. *Bars* 25 μm (color figure online)


Vector
MaATG8b
MaATG8d
MaATG8e
MaATG8f
MaATG8g
MaATG8h
MaATG8j
(A)
0 dpi
UV light
3 dpi
6 dpi
(B)
0 dpi
DAB staining
3 dpi
6 dpi
(C)
0 dpi
NBT staining
3 dpi
6 dpi
(D)
H2O2 (μmol/g FW)
0 dpi
3 dpi
6 dpi
(E)
O2·- (μmol/g FW)
(F)
Relative EL (%)
(G)
Relative MDA level
Vector
MaATG8b
MaATG8d
MaATG8e
MaATG8f
MaATG8g
MaATG8h
MaATG8j

3-MA treatment results in disease sensitive in response to *Foc4* in banana

To better understand the phenotypes during fungal infection, five-leaf stage banana roots were pre-treated with control, 2-mM autophagosome formation inhibitor 3-MA or 5 mM 3-MA for 2 days, followed by inoculated with *Foc4* strain tagged with GFP. As shown in Fig. 8a, 3-MA treatment resulted in significant reduction of MDC-stained autophogasomes in comparison to mock treatment. At 12- and 24-h post inoculation (hpi), roots pre-treated with 3-MA showed obviously more fluorescence than control roots (Fig. 8b). Accordingly, the symptoms were more severe and the relative EL was higher in 3-MA-treated plants than in control plants at 4-day post inoculation (dpi) of *Foc4* (Fig. 8c, d). Meanwhile, we quantified the endogenous levels of plant hormones (SA, JA and ETH) in banana and found that 3-MA treatment significantly affected the accumulation of disease-related plant hormones (SA, JA and ETH) (Fig. 8e–g). For instance, 3-MA-treated plants exhibited higher levels of SA at 0 hpi of *Foc4* in leaves and root, as well as at 12 hpi of *Foc4* in leaves, but lower levels of JA and ETH at 0 hpi in leaves than control (Fig. 8e–g). All the results suggested that *MaATGs*-mediated autophagy may be involved in disease resistance in banana.

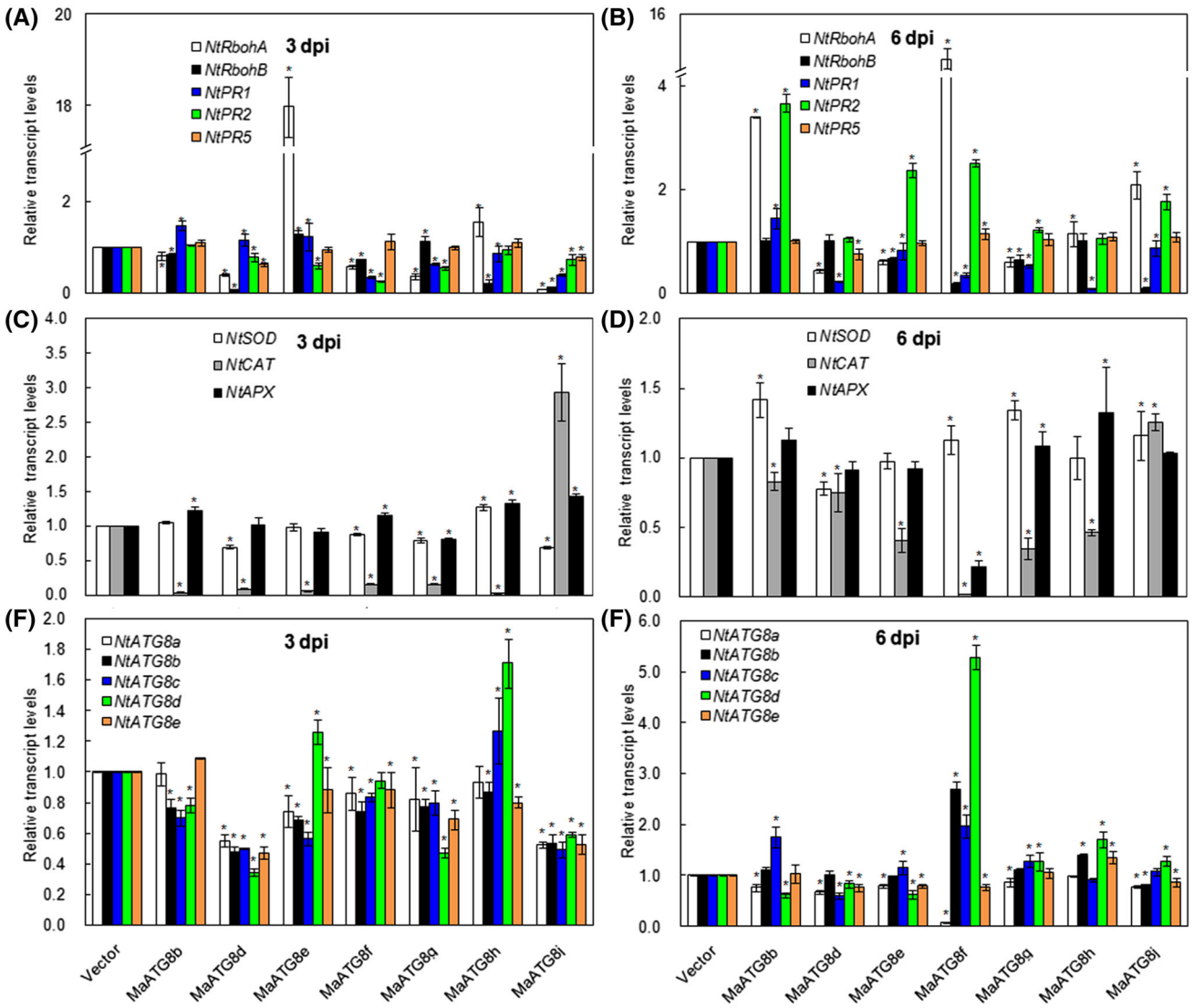


Fig. 5 *MaATG8s* regulate the transcripts of ROS and defense-related genes. The expression levels of ROS-related genes in transient expressing *Nicotiana benthamiana* leaves at 3 dpi (**a**) and 6 dpi (**b**) of GV3101 cell culture harboring the plasmids. The expression levels of defense-related genes in transient expressing *Nicotiana benthamiana* leaves at 3 dpi (**c**) and 6 dpi (**d**) of GV3101 cell culture harboring the plasmids. **e**, **f** The expression levels of *NtATG8s* in transient expressing *Nicotiana benthamiana* leaves at 3 dpi (**a**) and 6 dpi (**b**) of GV3101 cell culture harboring the plasmids. *Asterisk symbols* (*) were shown as significant difference in comparison to vector at $p < 0.05$

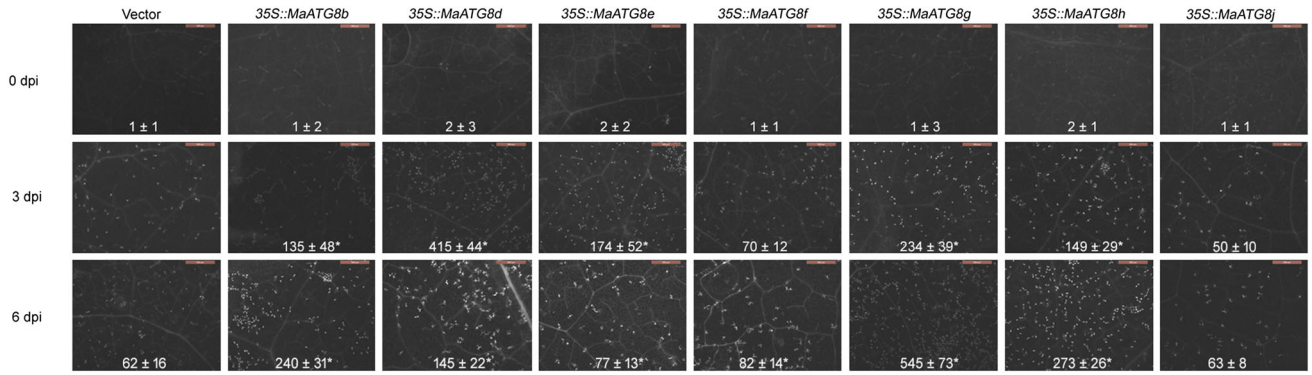


Fig. 6 The effects of *MaATG8s* transient expression on callose depositions. *White dots* indicate callose depositions that are stained with aniline *blue*. *Bars* 500 μm. *Asterisk symbols* (*) were shown as significant difference in comparison to vector at $p < 0.05$

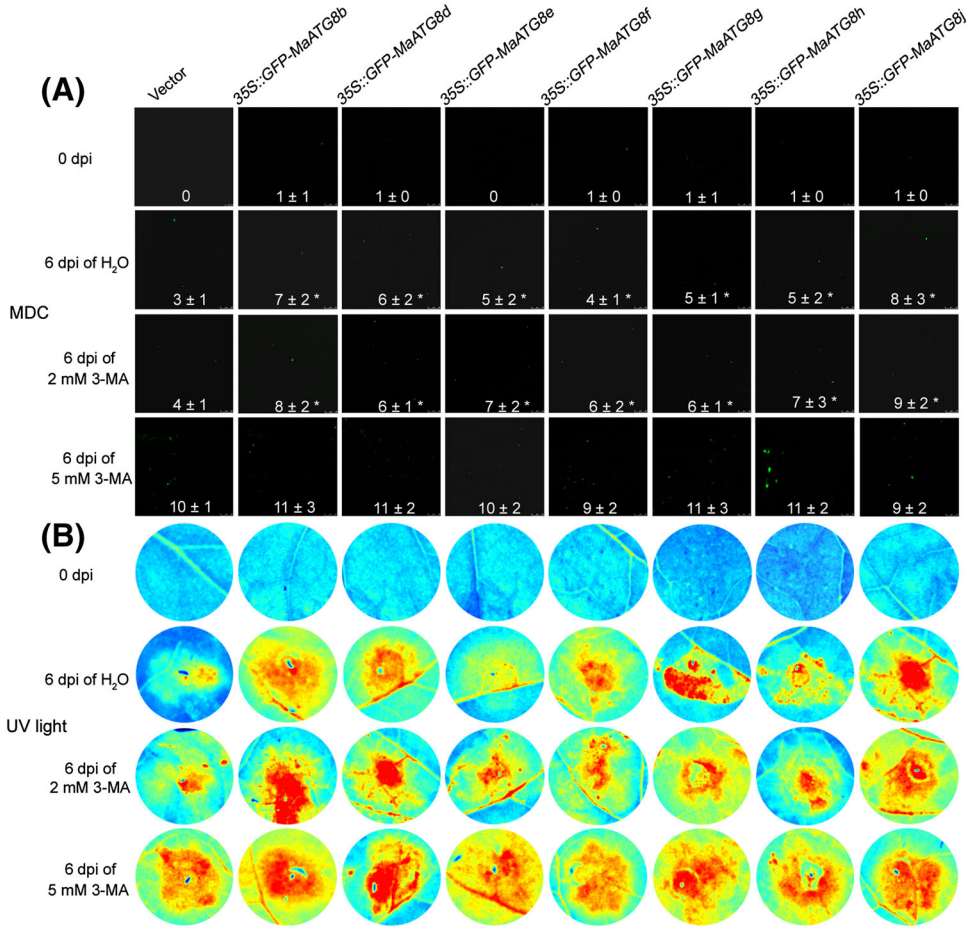


Fig. 7 The effect of autophagy inhibitor (3-MA) on *MaATG8s*-mediated hypersensitive response-like cell death. **a** The autophagic activity of MDC staining were shown and quantified as relative value, which of vector at 0 dpi was set as 1. *Bars* = 25 μm. **b** Symptoms of transient expressing *Nicotiana benthamiana* leaves that were visualized under UV light. For the assays, 4-week-old *Nicotiana benthamiana* leaves were syringe infiltrated with GV3101 cell culture harboring the plasmids. And 5 mM 3-MA were sprayed in the leaves 1 h after the infiltration. *Asterisk symbols* (*) were shown as significant difference in comparison to vector at $p < 0.05$

The effects of SA, JA and ETH treatment on autophagy-mediated banana resistance to Fusarium wilt

To further understand the possible mechanisms of hormones involved in *MaATG8s*-mediated plant immune, five-leaf stage banana roots were treated with different hormone combinations (control, 5 mM 3-MA, 5 mM 3-MA plus 10 μM SA, 5 mM 3-MA plus 10 μM JA, 5 mM 3-MA plus 10 μM ETH) for 2 days and then inoculated with *Foc4* strain tagged GFP. To screening suitable concentration of hormones, banana roots were firstly pre-treated with control, 2, 10 and 50 μM SA, 2, 10 and 50 μM JA, 2, 10 and 50 μM ETH before this test. As shown in Figure S5A, B, 10 μM was the suitable concentration for SA, JA and ETH treatment, since

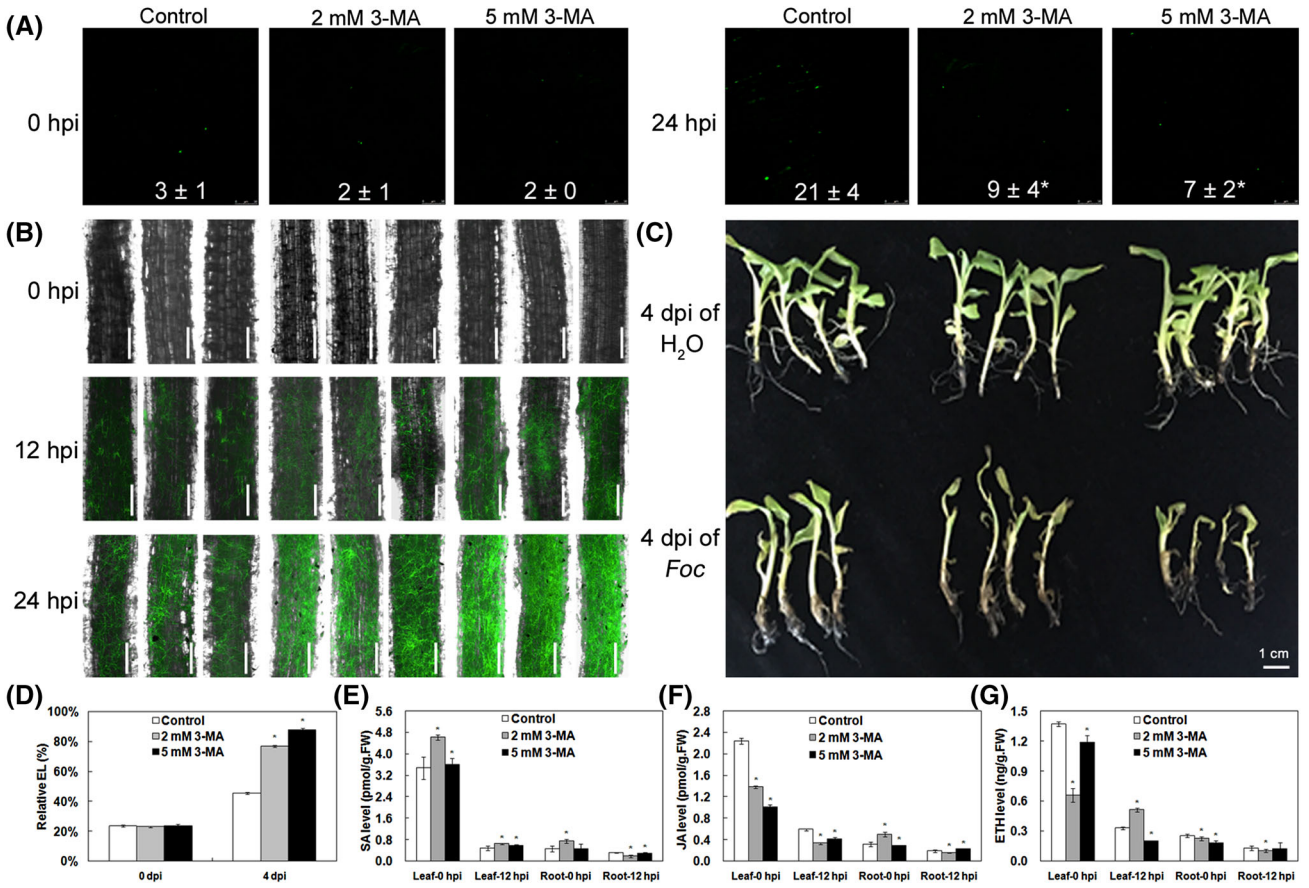


Fig. 8 Autophagy inhibitor (3-MA) treatment results in decreased disease resistance in response to *Foc4* in banana. **a** The autophagic activity of MDC staining were shown and quantified as relative value, which of mock treatment was set as 1. *Bars* 25 μm. **b** Micrographs of banana roots with different treatments (0, 2 and 5 mM 3-MA) at 0, 12, 24 hpi with GFP-tagged *Foc4* strain. *Bars* 200 μm. **c** The symptoms of banana seedlings with different treatments (0, 2 and 5 mM 3-MA) at 4 dpi with control and *Foc4* strain. *Bar* 1 cm. **d** The relative EL of banana leaves after 0 and 4 dpi of *Foc4* in banana leaves. The endogenous levels of SA (**e**), JA (**f**) and ETH (**g**) in banana leaves and roots after 0 and 12 hpi of *Foc4*. For the assay, five-leaf stage banana roots were pre-treated by control (mock), 2 mM 3-MA and 5 mM 3-MA for 2 days, thereafter were inoculated by *Foc4*. *Asterisk symbol* (*) indicate significant difference in comparison to control treatment

the phenotypes under this concentration were significantly different in comparison to control. Thus, 10 μM SA/JA/ETH were chosen for further analysis. At 3 and 6 day post inoculation (dpi) of *Foc4*, 3-MA and hormones co-treated plants exhibited similar phenotypes to those of control plants, while phenotypes of 3-MA-treated plants were severer (Fig. 9a). Consistently, the EL and chlorophyll of 3-MA and hormones co-treated plants showed no significant difference to control plants (Fig. 9b, c), while 3-MA-treated plants showed higher EL, but lower chlorophyll levels (Fig. 9b, c). In short, 3-MA and hormones co-treated plants had less significant effects on these phenotypes than 3-MA-treated plants (Fig. 9). The results indicated that these hormones may play roles in 3-MA and *MaATGs*-mediated plant immune.

Discussion

Fusarium oxysporum (*Fo*) is a widespread soil-borne fungus in global agriculture, causing vascular wilt disease through invading root surface and further colonization of plant root and xylem tissue (Berrocal-Lobo and Molina 2004; Di et al. 2016). With the isolation from various ranges of host species, every particular *Fo* strain typically produces disease in a specific plant host. To date, at least 120 *formae speciales* (ff. spp.) *Fo* strains have been isolated, including *Foc* from banana, *F. oxysporum* f. sp. *conglutinans* (*Focn*) from *Arabidopsis*, *F. oxysporum* f. sp. *lycopersici* (*Fol*) from tomato, etc. (Di et al. 2016, Katan and Di Primo 1999). In recent years, the wide and rapid spread of *Foc* largely affects the banana production in the tropical and subtropical climates (Deng et al. 2015; Ploetz

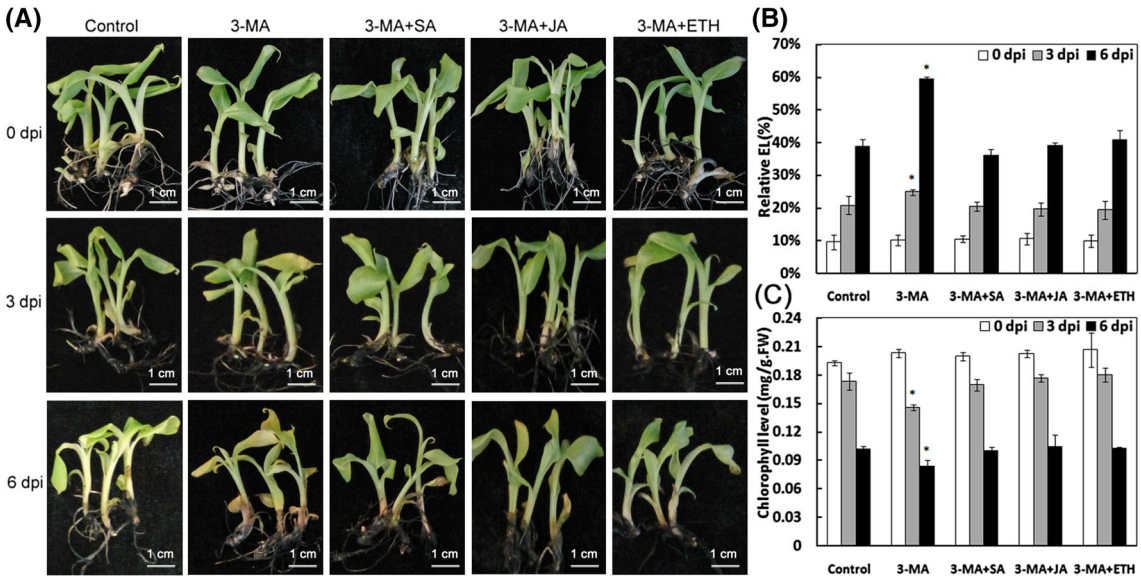


Fig. 9 The effects of SA, JA and ETH treatment on autophagy-mediated disease resistance in response to *Foc4* in banana. **a** The symptoms of banana seedlings with different treatments (control, 5 mM 3-MA, 10 μM SA, 10 μM JA, 10 μM ETH, 5 mM 3-MA plus 10 μM SA, 5 mM 3-MA plus 10 μM JA, 5 mM 3-MA plus 10 μM ETH) at 0, 3 and 6 dpi with *Foc4* strain. *Bars* 1 cm. The relative EL (**b**) and chlorophyll levels (**c**) of banana leaves at 0, 3 and 6 dpi with *Foc4* strain. For the assay, five-leaf stage banana roots were pre-treated by control (mock), 5 mM 3-MA, 5 mM 3-MA plus 10 μM SA, 5 mM 3-MA plus 10 μM JA, 5 mM 3-MA plus 10 μM ETH for 2 days, thereafter were inoculated by *Foc4*. *Asterisk symbol* (*) indicate significant difference in comparison to control treatment

2006, 2015; Ploetz et al. 2015; Silva et al. 2016; Tan et al. 2015). However, the enlargement cultivated area and the improved cultivated technique have very limited effect on the biological control of the Fusarium wilt, and no high resistant banana variety has been identified so far. Thus, the identification of candidate disease-related genes and revelation of underlying mechanism of defense response are very important for banana industry.

Unlike animals, plants must overcome or endure variable environmental stress (Yue et al. 2015). Pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), one of the plants main defense mechanisms, can combat most pathogen infections, and play significant roles in plant immune response (Apel and Hirt 2004; Jones and Dangl 2006; Kwon et al. 2013). In this study, *MaATGs* show coincident roles in some PTI responses, such as the transcript profile in response to *Foc*, ROS accumulation, hypersensitive-like cell death, defense-related gene expression and callose deposition. The induction of PTI response by *MaATGs* confers their effects on the basal immunity. Autophagy plays critical roles in degradation of damaged cytoplasmic constituents in the lysosomes or vacuoles to maintain cell, tissue and organism homeostasis (Boya et al. 2013; Thompson and Vierstra 2005). In recent years evidence accumulated that autophagy plays a key role in protecting plants against diverse pathologies infection through three ways, namely pathogen-induced hypersensitive cell death, SA- and JA-regulated defense and virus-induced RNAi (Zhou et al. 2014b). Using mutants and transgenic silenced plants, it has been revealed that a number of autophagy-related genes (*ATGs*) play roles in plant innate immunity, including *AtATG2*, *AtATG5*, *AtATG6*, *AtATG7*, *AtATG9*, *AtATG10*, *AtATG12*, *AtATG18a*, *TaATG6s* (Patel and Dinesh-Kumar, 2008; Hofius et al. 2009; Lenz et al. 2011a, b; Wang et al. 2011a, b; Yue et al. 2015). Among them, *AtATG6*, *AtATG7* and *AtATG9* showed positive role in plant resistance to bacterial pathogen (Patel and Dinesh-Kumar 2008; Hofius et al. 2009). In contrast, *AtATG5*, *AtATG10* and *AtATG18a* displayed the opposite results (Lenz et al. 2011a, b). For fungal pathogen (powdery mildew), *TaATG6s* play a weak, positive role (Yue et al. 2015). However, the mutants of *AtATG2*, *AtATG5*, *AtATG7*, *AtATG10* and *AtATG18a* enhanced resistance compared with wild types (Wang et al. 2011a, b). Consequently, the identification and functional analysis of *MaATGs* in banana could provide direct link between autophagy and immune response, as well as the potential mechanism of *MaATGs* in programmed-like cell death and ROS accumulation.

In this research, we successfully identified 32 *MaATG*s in banana and cloned the coding sequences of seven *MaATG8s* (Fig. 1; Table S1). The expression profiles of these genes response to biotic stresses (*Foc1* and *Foc4*) were analyzed by transcriptomic analysis, suggesting the possible role of them as well as autophagy in immune response in banana (Fig. 2). Overall, the transcripts of most *MaATG8s* were up-regulated by *Foc* infection (Fig. 2), the same as that of *TaATG6s* by powdery mildew infection

(Yue et al. 2015). And *TaATG6s* mutants were more sensitive to powdery mildew than wild type (Yue et al. 2015), so we concluded that *MaATG8s* could play positive role in fungal pathogens response. More importantly, we found that transient expression of seven novel *MaATG8s* (*MaATG8b*, *d*, *e*, *f*, *g*, *h*, *j*) led to hypersensitive-like cell death in leaves of *N. benthamiana*, which was dependent on ROS accumulation and endogenous hormone (Figs. 4, S4). SOD, CAT and APX are crucial antioxidant metabolic enzymes by catalyzing $O_2^{-\cdot}$ into H_2O_2 and O_2 (Lamb and Dixon 1997; Apel and Hirt 2004). *RbohA* and *RbohB* are important regulators of H_2O_2 accumulation and plant immunity. *PR1*, *PR2* and *PR5* are widely known marker genes of innate immune response (Kwon et al. 2013; Qian et al. 2015). ATG8 protein is well known crucial proteins of autophagosome formation and expansion (Kirisako et al. 2000). Therefore, further analysis of these gene expression in *N. benthamiana* leaves transiently expressing *MaATG8s* (Fig. 5) suggested that these genes might exert their function through ROS accumulation and immune response. Moreover, the *N. benthamiana* leaves expressing *MaATG8s* triggered the burst of callose depositions (Fig. 6), suggesting that these genes might be involved in the modulation of callose-associated cell wall and papillae-associated plant defense. In addition, *MaATG8s*-mediated hypersensitive response-like cell death is dependent on autophagy (Fig. 7).

So far, plant hormone signaling networks, especially SA, JA and ETH, are reported to be important regulators of plant–microbe interactions. Although it is clear about the involvement of SA, JA and ETH in plant–pathogenic fungi interaction, these hormones have complex effects in modulating disease and resistance, depending on the pathogen lifestyles as well as the genetic constitution of the host (An and Mou 2011; De Vleesschauwer et al. 2013; Denancé et al. 2013; Di et al. 2016; Ma and Ma 2016; Robert-Seilaniantz et al. 2011; Yan and Dong 2014; Yang et al. 2013). In general, SA signaling activates and confers disease resistance against biotrophic and hemibiotrophic pathogens, whereas JA and ET signaling triggers improved disease resistance against necrotrophic pathogens (Di et al. 2016; Glazebrook 2005). Consistently, exogenous application of proper (10 μM) concentration of SA, JA and ETH confers improved disease resistance against *Foc4* in banana, as evidenced by relative EL and chlorophyll level (Figure S5). Notably, 5 mM 3-MA treatment significantly reduced resistance against *Foc4* in banana, with accompany of reduced SA, JA and ETH levels in both roots and leaves (Fig. 8). Moreover, the rescue of autophagy-mediated disease resistance by co-treatment with SA, JA or ETH (Fig. 9) indicated the essential role of these hormones in autophagy-mediated banana resistance to *Foc4*.

Taken together, 32 *MaATGs* were comprehensively identified in banana genome, and the transcript profiles of these genes were also analyzed. Based on the common regulation of seven *MaATG8s*' transcripts by *Foc*, we highlight the novel roles of *MaATG8s* in conferring hypersensitive-like cell death through autophagy, as confirmed by ROS accumulation, callose depositions, the transcripts of multiple defense-related genes, and MDC staining. Notably, we found that autophagy inhibitor (3-MA) treatment resulted in decreased disease resistance in response to *Foc4* in banana, through modulation of endogenous levels of SA, JA and ETH, at least partially.

To our knowledge, this is the first study showing the putative roles of *MaATG8s* in hypersensitive-like cell death and autophagy in immune response against *Foc* in banana.

Author contribution statement H S and CH designed the experiments. YW and WH performed the experiments. WL and HS analyzed the data. WH, GL, CW, WL and HZ contributed reagents/materials/analysis tools. WL and HS wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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