Banana fruit NAC transcription factor MaNAC5 cooperates with MaWRKYs to enhance the expression of pathogenesis-related genes against *Colletotrichum musae*

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SUMMARY

Plants respond to pathogen attack by the modulation of a large set of genes, which are regulated by different types of transcription factor (TF). NAC (NAM/ATAF/CUC) and WRKY are plant-specific families of TFs, and have received much attention as transcriptional regulators in plant pathogen defence. However, the cooperation between NAC and WRKY TFs in the disease response remains largely unknown. Our previous study has revealed that two banana fruit WRKY TFs, MaWRKY1 and MaWRKY2, are involved in salicylic acid (SA)- and methyl jasmonate (MeJA)-induced resistance against *Colletotrichum musae* via binding to promoters of pathogenesis-related (PR) genes. Here, we found that MaNAC1, MaNAC2 and MaNAC5 were up-regulated after *C. musae* infection, and were also significantly enhanced by SA and MeJA treatment. Protein–protein interaction analysis showed that MaNAC5 physically interacted with MaWRKY1 and MaWRKY2. More importantly, dual-luciferase reporter (DLR) assay revealed that MaNAC5, MaWRKY1 and MaWRKY2 were transcriptional activators, and individually or cooperatively activated the transcriptional activities of *MaPR1-1*, *MaPR2*, *MaPR10c* and *MaCHIL1* genes. Collectively, our results indicate that MaNAC5 cooperates with MaWRKY1 and MaWRKY2 to regulate the expression of a specific set of PR genes in the disease response, and to contribute at least partially to SA- and MeJA-induced pathogen resistance.

Keywords: banana fruit, *Colletotrichum musae*, NAC transcription factor, PRs, WRKY transcription factor.

INTRODUCTION

As sessile organisms, plants are constantly threatened by adverse environmental conditions, including pathogen infection. Fortunately, plants have evolved a variety of physiological and biochemical defence strategies to cope with pathogen attacks. Cascading mechanisms leading to plant defence often operate at the transcriptional level through intricate regulation, ultimately resulting in increased expression of defence-related genes (Le

Hénanff *et al.*, 2013; Tsuda and Somssich, 2015). Extensive studies have revealed that stress-responsive genes are mainly regulated by multiple transcriptionally and/or post-translationally activated transcription factors (TFs) (Llorca *et al.*, 2014; Thatcher *et al.*, 2012; Tsuda and Somssich, 2015). TFs are grouped into different families based on conserved structural domains. A wide range of TF families, such as WRKY, NAC, bZIP, AP2/ERF and bHLH, have been implicated in the control of plant defence responses (Alves *et al.*, 2013; Eulgem and Somssich, 2007; Licausi *et al.*, 2013; Nuruzzaman *et al.*, 2013; Puranik *et al.*, 2012; Singh *et al.*, 2002; Tsuda and Somssich, 2015).

NAC (NAM, ATAF1/2 and CUC2) is one of the largest plant-specific TF families, and is characterized by a highly conserved DNA-binding NAC domain at the N-terminus and a highly diversified transcriptional activation domain at the C-terminus (Olsen *et al.*, 2005; Puranik *et al.*, 2012; Shan *et al.*, 2012). Genetic and molecular studies of NAC TFs in plants have suggested that they regulate diverse biological processes, such as developmental programs, senescence and ripening, secondary cell wall formation and defence responses to biotic and abiotic stresses (Nakashima *et al.*, 2012; Nuruzzaman *et al.*, 2013; Puranik *et al.*, 2012). The involvement of NAC TFs in the plant defence response to pathogens is well established (Nuruzzaman *et al.*, 2013; Puranik *et al.*, 2012). The expression of many NAC genes from *Arabidopsis*, barley, rice, wheat and grape is enhanced during diverse biotic stresses and in response to defence-related phytohormones, such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) (Chen *et al.*, 2013; Feng *et al.*, 2014; Le Hénanff *et al.*, 2013; McGrann *et al.*, 2015; Nuruzzaman *et al.*, 2013; Wang *et al.*, 2015; Wu *et al.*, 2009; Yokotani *et al.*, 2014). Some NAC TFs may positively regulate the plant defence response by activating pathogenesis-related (PR) genes, inducing a hypersensitive response (HR) and cell death at the infection site (Nuruzzaman *et al.*, 2013; Puranik *et al.*, 2012; Yokotani *et al.*, 2014); contrastingly, other NAC TFs have been regarded as negative regulators of pathogen resistance by suppressing defence-related gene expression, implying their alliance with distinct regulatory complexes (Delessert *et al.*, 2005; Nuruzzaman *et al.*, 2013; Puranik *et al.*, 2012; Wang *et al.*, 2015). In addition, WRKYs are well-characterized plant-specific TFs that also play an important role in the regulation of defence-related gene expression via the W-box (CCGACT/C), a *cis* element found in

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a wide range of disease-responsive genes in various plant species (Rushton *et al.*, 2010, 2012). However, whether extensive cooperation between NAC and WRKY TFs exists during the defence response remains unclear.

Although extensive efforts have been made to identify different NAC TFs induced by biotic stress responses, their exact role in the defence response of economically important fruits is largely unknown. Bananas (*Musa acuminata*) are one of the world's important food crops, with a global production of about 138 million tons in 2010. These crops are part of a balanced human diet and a staple food for more than 400 million people in the tropics (Hölscher *et al.*, 2014). However, bananas are highly susceptible to disease infections, and anthracnose caused by *Colletotrichum musae* is the major post-harvest disease leading to severe post-harvest losses if not controlled (Chen *et al.*, 2011; Ma *et al.*, 2009). Thus, an understanding of the defence response mechanisms is helpful for maintaining banana quality and extending shelf-life. Our previous study has shown that pretreatment with SA or methyl jasmonate (MeJA) can induce resistance against *C. musae* infection in banana fruit, and two WRKY TFs, MaWRKY1 and MaWRKY2, can activate the expression of *PR* genes and possibly function in SA- and MeJA-induced pathogen resistance (Tang *et al.*, 2013). About 167 potentially functional NAC genes are found in the banana genome (Cenci *et al.*, 2014) and, in our previous work, six members of the NAC family involved in banana fruit ripening were comprehensively characterized (Shan *et al.*, 2012). In the present work, we found that banana fruit *MaNAC1*, *MaNAC2* and *MaNAC5* were up-regulated after *C. musae* infection, and were also significantly enhanced by SA and MeJA treatment. More importantly, *MaNAC5* physically interacted with MaWRKY1 and MaWRKY2 to cooperatively activate the transcriptional activities of *PR* genes, including *MaPR1-1*, *MaPR2*, *MaPR10c* and *chitinase*-like (*MaCHIL1*). Our findings expand the knowledge of the transcriptional regulation network in economically important fruit crops in relation to induced pathogen resistance.

RESULTS

Expression of *MaNAC* genes during SA- and MeJA-induced resistance against *C. musae*

It has been shown in our recent report that banana fruit begin to show disease symptoms at 9 days after inoculation with *C. musae*, and treatment with SA or MeJA significantly reduces the disease index and lesion diameter (Fig. S1, see Supporting Information), indicating that SA and MeJA treatment induce the resistance of banana fruit against *C. musae* (Tang *et al.*, 2013). In our previous work, six members of the NAC family involved in banana fruit ripening were comprehensively characterized (Shan *et al.*, 2012). To understand the possible role of banana fruit MaNACs in relation to the defence response, their expression in banana fruit inoculated

with *C. musae* after SA or MeJA treatment was investigated by quantitative real-time polymerase chain reaction (qRT-PCR). As shown in Fig. 1, among the six *MaNAC* genes, *MaNAC1*, *MaNAC2* and *MaNAC5* were pathogen inducible; moreover, SA or MeJA treatment induced higher levels of *MaNAC1*, *MaNAC2* and *MaNAC5* transcripts than those in the control fruit, especially at days 13, 5 and 9, respectively (Fig. 1). In addition, *MaNAC3* and *MaNAC6* were slightly affected by SA or MeJA treatment, whereas *MaNAC4* was repressed. These results suggest that *MaNACs* are differentially expressed during the defence response, and that *MaNAC1*, *MaNAC2* and *MaNAC5* may be more strongly associated with SA- and MeJA-induced resistance of banana fruit against *C. musae*. Thus, these three *MaNAC* genes were investigated in the following analysis.

MaNAC5 physically interacts with MaWRKY1 and MaWRKY2

Recent studies have raised the intriguing possibility of crosstalk between different types of TF (Lee *et al.*, 2010; Xu *et al.*, 2013). Two WRKY TFs, MaWRKY1 and MaWRKY2, have also been shown to be activated during *C. musae* inoculation, and either SA or MeJA treatment induced stronger MaWRKY1 and MaWRKY2 expression (Fig. S2, see Supporting Information), suggesting that MaWRKY1 and MaWRKY2 might be associated with SA- and MeJA-induced pathogen resistance of banana fruit (Tang *et al.*, 2013). Based on the results that *MaNAC1*, *MaNAC2* and *MaNAC5* might be involved in SA- and MeJA-induced resistance of banana fruit against *C. musae*, it could be speculated that possible interactions between MaNAC and MaWRKY proteins might exist.

To confirm this hypothesis, the Matchmaker Gold Yeast Two-Hybrid (Y2H) System (Clontech Laboratories, Inc., Mountain View, CA, USA) was used to investigate the interactions between MaNACs and MaWRKYs. *MaNAC1/2/5* and *MaWRKY1/2* coding sequences were subcloned into pGBKT7 and pGADT7 vectors, respectively, for Y2H assay as full-length *MaNAC1/2/5* exhibited no transactivation activity (autoactivation) in yeast when fused with the binding domain (BD) (Shan *et al.*, 2012). As shown in Fig. 2, yeast cells co-transformed with the positive control (pGBKT7-53 + pGADT7-T) and DBD (DNA-binding domain)-*MaNAC5* with AD (activation domain)-*MaWRKY1* or AD-*MaWRKY2* not only grew on selective medium (synthetic medium lacking tryptophan, leucine, histidine and adenine), but also turned blue in the presence of the chromogenic substrate X- α -Gal. However, yeast cells harbouring DBD-*MaNAC1* or DBD-*MaNAC2* with AD-*MaWRKY1* or AD-*MaWRKY2*, and the negative controls, did not grow on the selective medium or turn blue under the same conditions. These results indicate that *MaNAC5* may physically interact with *MaWRKY1* and *MaWRKY2*.

We next used the bimolecular fluorescence complementation (BiFC) assay to confirm the interactions between *MaNAC5* and

Fig. 1 Expression patterns of *MaNAC1–MaNAC6* (A–F) in banana fruit inoculated with *Colletotrichum musae*. Banana fruit was treated with 2 mM salicylic acid (SA) or 0.1 mM methyl jasmonate (MeJA) or water (control), inoculated with *C. musae*, and then stored at 25 °C with 90% relative humidity (RH) for 13 days. The expression level of each gene at different time points is given as a ratio relative to day 0 of the control, which was set to unity. Each value represents the mean $\pm$ standard error (SE) of three biological replicates. Different letters above the bars indicate a statistically significant difference at the 5% level. The physiological data related to induced resistance, including disease index and lesion diameter, are presented in Fig. S1, which has been published in the work of Tang *et al.* (2013).

MaWRKY1/2 in BY-2 protoplasts. A strong yellow fluorescent protein (YFP) fluorescence signal was detected in the nucleus of BY-2 cells expressing *MaNAC5*-pSPYNE (split YFP N-terminal fragment expression) and *MaWRKY1*-pSPYCE (split YFP C-terminal fragment expression) or *MaWRKY2*-pSPYCE, whereas no YFP fluorescence signal was observed in either cells expressing *MaNAC5*-pSPYNE with only pSPYCE or those expressing *MaWRKY1/2*-pSPYCE with only pSPYNE (Fig. 3A), indicating that *MaNAC5* interacted with *MaWRKY1* and *MaWRKY2* *in vivo*. Similar results were also observed when *MaNAC5*-pSPYCE was co-transfected with *MaWRKY1/2*-pSPYNE (Fig. 3B). Collectively, these results demonstrate that *MaNAC5* physically interacts with *MaWRKY1* and *MaWRKY2* in the nucleus of plant cells.

MaNAC5, MaWRKY1 and MaWRKY2 exhibit transactivation activities *in vivo*

Transcriptional activity is important for TFs to regulate downstream genes. To investigate the abilities of *MaNAC5*, *MaWRKY1* and *MaWRKY2* to activate transcription, the transactivation activities of *MaNAC5*, *MaWRKY1* and *MaWRKY2* were investigated using transient expression assay in tobacco leaves. We employed a dual-luciferase reporter plasmid harbouring five copies of the GAL4-DNA-binding element and minimal TATA region (5'-TATAAA-3') of the 35S promoter fused to the LUC (firefly luciferase) reporter; a REN (renilla luciferase) reporter under the

control of the 35S promoter at the same vector was used as an internal control for successful transfection, and an effector plasmid encoding full-length *MaNAC5*, *MaWRKY1* and *MaWRKY2* was fused to the GAL4 DBD (GAL4BD) (Fig. 4A). Compared with the GAL4BD (empty, pBD) negative control, *MaNAC5*, *MaWRKY1* and *MaWRKY2*, together with the transcriptional activator control GAL4BD-VP16, strongly activated the LUC reporter gene, as shown in Fig. 4B. The LUC/REN ratios of *MaNAC5*, *MaWRKY1*, *MaWRKY2* and GAL4BD-VP16 were 3.8-, 3.6-, 6.1- and 14.1-fold higher, respectively, than that of the negative control (Fig. 4B). Therefore, these results suggest that *MaNAC5*, *MaWRKY1* and *MaWRKY2* are transcriptional activators.

MaNAC5 acts cooperatively with MaWRKY1 and MaWRKY2 to activate *MaPRs*

WRKY TFs can transcriptionally regulate many *PR* genes via binding to a W-box [(T)(T)TGAC(C/T)] in their promoters (Rushton *et al.*, 2010). It has been shown in our previous study that *MaWRKY1* and *MaWRKY2* can bind directly to *MaPR1-1*, *MaPR2*, *MaPR10c* and *MaCHIL1* promoters in yeast one-hybrid assay (Tang *et al.*, 2013). It is well known that NAC TFs preferably bind to the so-called NAC recognition sequence (NACRS) or NAC binding sequence (NACBS) in promoters of their target genes (Puranik *et al.*, 2012). Sequence analysis has identified NACRS or NACBS in the *MaPR1-1*, *MaPR2*, *MaPR10c* and *MaCHIL1* promoters (Fig. S3, see Supporting

Fig. 2 Interactions between MaNACs and MaWRKYs detected in yeast two-hybrid (Y2H) assay. The coding regions of MaNACs and MaWRKYs were fused with DNA-binding domain (DBD) and activation domain (AD) vectors, respectively, as indicated, and co-transformed into the yeast strain Gold Y2H. The ability of yeast cells to grow on synthetic medium lacking tryptophan, leucine, histidine and adenine (Leu[–]Trp[–]His[–]Ade[–]), but containing 125 μ M Aureobasidin A, and to turn blue in the presence of the chromogenic substrate α -Gal, was scored as a positive interaction. Positive or negative controls as indicated were also included.

Information), suggesting that *MaPR1-1*, *MaPR2*, *MaPR10c* and *MaCHIL1* might also be direct targets of MaNAC5. Together with the fact that MaNAC5 interacted with MaWRKY1 and MaWRKY2, this raises the possibility that MaNAC5 cooperates with MaWRKY1 and MaWRKY2 for the activation of *MaPR1-1*, *MaPR2*, *MaPR10c* and *MaCHIL1* expression. To test this idea, we again investigated the transient expression system in tobacco leaves using *MaPRs pro::LUC* as the reporter construct and the plasmid expressing MaNAC5, MaWRKY1 or MaWRKY2 as the effector (Fig. 5A). As shown in Fig. 5B, the LUC/REN ratio was increased significantly when the *MaPR1-1*, *MaPR2*, *MaPR10c* or *MaCHIL1 pro::LUC* reporter construct was co-transfected with MaNAC5, MaWRKY1 or MaWRKY2, compared with the control that was co-transfected with the empty construct. More interestingly, when both MaNAC5 and MaWRKY1 or MaWRKY2 were co-transformed, the LUC/REN ratio was more strongly induced, and was much higher than the combined values observed with MaNAC5, MaWRKY1 or MaWRKY2 alone, respectively (Fig. 5B). These data suggest that MaNAC5, MaWRKY1 and MaWRKY2 regulate *MaPR1-1*, *MaPR2*, *MaPR10c* and *MaCHIL1* individually, or MaNAC5 forms a protein complex with MaWRKY1 or MaWRKY2 to act cooperatively in the activation of *MaPR1-1*, *MaPR2*, *MaPR10c* and *MaCHIL1* expression.

DISCUSSION

Over the past two decades, molecular and genetic studies have uncovered numerous TF family members that are critical in the regulation of appropriate transcriptional responses when plants are confronted by phytopathogens (Tsuda and Somssich, 2015). The NAC proteins comprise a large family of TFs with more than 100 members in plant species whose genomes have been completely sequenced so far (Nakashima *et al.*, 2012). Recently, genome-wide bioinformatics analysis has identified 167 potentially functional *MaNAC* genes in banana (Cenci *et al.*, 2014), although their involvement in physiological processes is largely unknown. Banana *MusaVND1*, an orthologue of *Arabidopsis VND1*, regulates secondary wall deposition and differentiation of xylem vessel elements (Negi *et al.*, 2015). In our previous studies, six banana *NAC* genes were characterized, and two, termed *MaNAC1* and *MaNAC2*, were ripening and ET induced, and were involved in banana fruit ripening via interaction with the ET signalling pathway (Shan *et al.*, 2012). In addition, *MaNAC1* is cold responsive, and may be associated with the cold tolerance of banana fruit through its interaction with the ICE1 (inducer of CBF expression 1)-CBF (C-repeat binding factor) cold signalling pathway (Shan *et al.*, 2014). NAC TFs have been demonstrated to be involved in responses to pathogen attack: for example, barley *HvNAC6* is transcriptionally induced in epidermal cells shortly after inoculation with *Blumeria graminis* f.sp. *hordei* (Bgh) (Jensen *et al.*, 2008); maize *ZmNAC41* and *ZmNAC100* are induced after *C. graminicola* infection (Voitsik *et al.*, 2013); and wheat *TaNAC4* and *TaNAC8* are also enhanced by pathogen infection (Xia *et al.*, 2010a, b). Similarly, the present work has shown that *MaNACs* are differentially expressed during the defence response, as *MaNAC1*, *MaNAC2* and *MaNAC5* were induced after *C. musae* infection, whereas *MaNAC4* was repressed (Fig. 1).

Induced resistance against pathogen attack has been demonstrated to be controlled by phytohormones, such as SA, MeJA and ET (Kazan and Lyons, 2014; Pieterse *et al.*, 2012; Robert-Seilantantz *et al.*, 2011). We have revealed that SA and MeJA treatments induce the resistance of banana fruit to *C. musae* (Tang *et al.*, 2013). *NAC* genes from different plant species have been shown to be regulated by these small molecules, for instance, the transcription level of pepper *CaNAC1* can be elevated by exogenous SA, ET and MeJA treatment (Oh *et al.*, 2005). Lin *et al.* (2007) showed that the *OsNAC19* transcript was induced by the exogenous application of ET, MeJA and ABA. Wheat *TaNAC4* and *TaNAC8* transcripts were induced to different extents following exogenous ET, MeJA and ABA treatment, but only slightly affected by SA (Xia *et al.*, 2010a, b). Interestingly, all of these molecules transiently up-regulated grape *VvNAC1* expression (Le Hénaff *et al.*, 2013), as well as rice *ONAC122* and *ONAC131* (Sun *et al.*, 2013); moreover, silencing of *ONAC122* or *ONAC131* can down-regulate the expression of *OsNH1* and *OsWRKY45*, both acting downstream of the SA signalling pathway, and *OsLOX*, which has

Fig. 3 Bimolecular fluorescence complementation (BiFC) assay in tobacco BY-2 protoplasts showing the interactions between MaNAC5 and MaWRKYs in living cells. (A) MaNAC5 and MaWRKYs proteins were fused with the N- or C-terminus of yellow fluorescent protein (YFP) (YNE and YCE), respectively. (B) MaNAC5 and MaWRKYs proteins were fused with the C- or N-terminus of YFP (YCE and YNE), respectively. Expression of MaNAC5 or MaWRKYs alone was used as negative control. VirD2NLS-mCherry, driven by the 35S promoter, was included in each transfection to serve as a control for successful transfection as well as for nuclear localization (NLS-mCherry). YFP indicates the fluorescence of YFP; 'Merge' is a digital merge of bright field and fluorescence images. The length of the bar indicated in the photographs is 25 μ m.

been shown to be involved in JA biosynthesis, implying that ONAC122 and ONAC131 may act as a point of convergence in the SA and JA signalling pathways (Sun *et al.*, 2013). Collectively, these previous reports have proposed reasonable links between NAC TFs and their putative function signalling pathways mediated by SA, ET, MeJA or ABA in relation to defence responses. Our results revealed that both SA and MeJA treatment induced *MaNAC1*, *MaNAC2* and *MaNAC5* expression, at days 13, 5 and 9, respectively (Fig. 1), suggesting their possible involvement in the defence responses mediated by SA and MeJA. However, the roles of MaNAC1, MaNAC2 and MaNAC5 in the SA and JA signalling pathways in relation to defence responses require further investigation.

The accumulation of PR proteins is potentially important for plants and fruits in response to pathogen attack (Robert-Seilanianz *et al.*, 2011). Transcriptional regulation of defence-responsive gene expression is a major feature of plant immunity and is governed by TFs and co-regulatory proteins associated within discrete transcriptional complexes (Moore *et al.*, 2011). As major downstream defence-responsive genes in plant pathogen resistance networks, PR genes are positively or negatively regulated by various TFs, including ERF (Licausi *et al.*, 2013), WRKY (Rushton *et al.*, 2010; Tang *et al.*, 2013), bZIP (Zander *et al.*, 2010), MYB (Shim *et al.*, 2013) and NAC (Puranik *et al.*, 2012; Yokotani *et al.*, 2014). NAC TFs can function as either transcriptional activators or transcriptional repressors (Nuruzzaman *et al.*, 2013; Xia *et al.*,

2010a, b; Yokotani *et al.*, 2014). For example, *Arabidopsis* ATAF2, ANAC019 and ANAC055/AtNAC3 act as repressors of PR gene expression and negatively control defence against *Botrytis cinerea* and the root-infective pathogen *Fusarium oxysporum* (Bu *et al.*, 2008; Delessert *et al.*, 2005), whereas, recently, it has been reported that rice OsNAC111 transactivates PR2 and PR8/OsChib3a directly and contributes positively to disease resistance (Yokotani *et al.*, 2014). In our study, a transient reporter assay demonstrated that MaNAC5 acts as a transcriptional activator that can directly promote the expression of the reporter gene driven by the *MaPR1-1*, *MaPR2*, *MaPR10c* or *MaCHIL1* promoter in tobacco leaves (Figs 4 and 5), indicating that MaNAC5 transactivates these genes directly. Interestingly, MaNAC5 can interact with MaWRKY1 and MaWRKY2 (Figs 2 and 3), which are also transcriptional activators, and activate *MaPR1-1*, *MaPR2*, *MaPR10c* or *MaCHIL1* directly (Figs 4 and 5). Previous studies have suggested that NAC TFs form heterodimers with other TFs to regulate stress response genes (Lee *et al.*, 2010; Xu *et al.*, 2013). In *Arabidopsis*, ANAC096 interacts with bZIP-type TFs, ABF2 and ABF4, to synergistically activate RD29A transcription in response to dehydration and osmotic stress (Xu *et al.*, 2013). In soybean, GmNAC81 cooperates with another NAC TF, GmNAC30, to activate the stress-induced programmed cell death (PCD) response through the induction of the cell death executioner VPE (Mendes *et al.*, 2013). Similarly, in the present investigation, we have demonstrated that MaNAC5

Fig. 4 MaNAC5, MaWRKY1 and MaWRKY2 have transcriptional activation activities in tobacco BY-2 protoplasts. (A) Diagram of the various constructs used in this assay. CaMV, *Cauliflower mosaic virus*; CPMV UTR, *Cowpea mosaic virus* untranslated region. (B) *Agrobacterium* strain EH105 carrying the LUC (firefly luciferase) reporter plasmid, together with different combinations of effector plasmids, was infiltrated into *Nicotiana benthamiana* leaves, and the luciferase activity at the sites of infiltration was measured 3 days after infiltration. Relative activity was expressed as the ratio of LUC to REN (renilla luciferase) activity. Data represent the mean $\pm$ SE of six biological replicates. Different letters above the bars indicate a statistically significant difference at the 5% level compared with pBD.

acts cooperatively with MaWRKY1 and MaWRKY2 in the activation of *MaPR1-1*, *MaPR2*, *MaPR10c* and *MaCHIL1* expression (Fig. 5). However, it should be pointed out that whether the MaNAC5–MaWRKYs protein complex binds to the promoter of *MaPR1-1*, *MaPR2*, *MaPR10c* or *MaCHIL1* should be further confirmed. To the best of our knowledge, this is the first report indicating that NACTFs cooperate with WRKY TFs to regulate *PR* genes in relation to the induced defence response. The interaction of MaNAC5 with MaWRKY1 or MaWRKY2 raises an interesting question of why plants use such interactions in the defence response. Under biotic stress, plants often need to rapidly optimize their physiological process for survival through the adjustment of defence-related gene expression (Denance *et al.*, 2013; Tsuda and Somssich, 2015). For a robust response to biotic stress, it is clearly advantageous to employ multiple TFs, as transcriptional reprogramming is a major

feature of the plant defence response (Tsuda and Somssich, 2015). Multiple cooperative interactions between different TFs could be critically important for the elicitation of the defence response. Thus, cooperative interactions between different types of TF, as observed in the case of MaNAC5 and MaWRKYs, may be an essential part of the mechanism for banana fruit adaptation to biotic stress, such as *C. musae* infection. In addition, it should be pointed out that, currently, it is not clearly understood why two other defence-responsive NAC TFs, MaNAC1 and MaNAC2, cannot interact with MaWRKY1 and MaWRKY2. Whether MaNAC1, MaNAC2 and MaNAC5 interact with each other to form a homodimer to activate the defence response also needs to be elucidated further.

In summary, *MaNAC1*, *MaNAC2* and *MaNAC5* are pathogen inducible and may be associated with induced resistance of banana fruit against *C. musae*. Moreover, MaNAC5, MaWRKY1 and MaWRKY2 are transcriptional activators, and individually or cooperatively activate the transcriptional activities of *MaPR1-1*, *MaPR2*, *MaPR10c* and *MaCHIL1*. These findings provide new insight into the regulatory pathway involved in NAC and WRKY TFs in relation to the induced pathogen resistance of economic fruit crops.

EXPERIMENTAL PROCEDURES

Plant materials and treatments

Pre-climacteric banana (*Musa acuminata*, AAA group, cv. Cavendish) fruit at 75%–80% maturation (about 12 weeks after anthesis), obtained from a local commercial plantation near Guangzhou, China, were used for this study. The selected banana fruits were randomly divided into three groups of 150 fingers each for the following treatments: 30 min in 10 L of distilled water containing 0 (control) or 2 mM SA, or 0.1 mM MeJA. The treated fruits were then inoculated with 20 μ L (1.0×10^5 spores/mL) of *C. musae*. SA or MeJA treatment, as well as *C. musae* isolation, culture and inoculation, were performed as described previously (Tang *et al.*, 2013). Inoculated fruits of each treatment were subsequently placed into 10 individual unsealed polyethylene plastic bags (thickness, 0.01 mm) and stored at 25 °C and 90% relative humidity (RH) for 13 days. The disease index and lesion diameter were recorded, as described by Tang *et al.* (2013). Samples were taken at 0, 1, 5, 9, 11 and 13 days, as banana fruit began to show disease symptoms at day 9 of *C. musae* inoculation. Banana peel 30 mm around the inoculation point from five bananas was collected for each sample. All of the samples were frozen in liquid nitrogen and stored at –80 °C for further use.

Gene expression analysis

Frozen tissues were ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted using the hot borate method (Wan and Wilkins, 1994). The extracted RNA was treated with DNase I (Promega Corporation, Madison, WI, USA), and first-strand cDNA was synthesized using M-MLV reverse transcriptase (Promega). qRT-PCR was carried out on a Bio-Rad CFX96 Real-Time PCR System using the SYBR®Green PCR Supermix Kit (Bio-Rad Laboratories Co., Ltd. Hercules, CA, USA) following the manufacturer's instructions. The expression levels were normalized to the expres-

Fig. 5 Co-operative activation of *MaPR1-1*, *MaPR2*, *MaPR10c* and *MaCHIL1* expression by MaNAC5 and MaWRKYs in a dual luciferase assay. (A) Diagram of the various constructs used in this assay. CaMV, *Cauliflower mosaic virus*; CPMV UTR, *Cowpea mosaic virus* untranslated region. (B) *Agrobacterium* strain EH105 carrying the LUC (firefly luciferase) reporter plasmid together with different combinations of effector plasmids was infiltrated into *Nicotiana benthamiana* leaves, and the luciferase activity at the sites of infiltration was measured 3 days after infiltration. The activities of LUC and REN (renilla luciferase) were measured sequentially, and the LUC/REN ratio was calculated as the final transcriptional activation activity. Data represent the mean $\pm$ standard error (SE) of six biological replicates. Different letters above the bars indicate a statistically significant difference at the 5% level compared with the empty effector.

sion of the *MaCAC* gene, according to our previous study on the selection of reliable reference genes under different experimental conditions (Chen *et al.*, 2011). Primers are listed in Table S1 (see Supporting Information).

Y2H assay

The Y2H assay was performed using the Matchmaker™ Gold Yeast Two-Hybrid System (Clontech), as described previously (Shan *et al.*, 2012). The coding sequences of MaNAC1/2/5 and MaWRKY1/2 were subcloned into pGBKT7 or pGADT7 vector to fuse with the DBD and AD, respectively, to create the bait and prey (primers are listed in Table S1). Then, the bait and prey constructs were co-transformed into yeast strain Gold Y2H by the lithium acetate method, and yeast cells were grown on DDO medium

(minimal medium double dropouts, synthetic dropout (SD) medium lacking leucine and tryptophan) for 3 days according to the manufacturer's protocol. Transformed colonies were plated onto QDO medium (minimal medium quadruple dropouts, SD medium lacking leucine, tryptophan, adenine and histidine), and QDO medium containing 4 mg/mL X- α -Gal (α -Gal) for blue colour development, to test the possible interaction between MaNAC1/2/5 and MaWRKY1/2 according to their growth status and the activity of α -galactosidase.

BiFC assay

For BiFC assay, full-length coding sequences of MaNAC5, MaWRKY1 and MaWRKY2 (without their stop codons) were subcloned into pUC-pSPYNE

or pUC-pSPYCE vector obtained from the laboratory of J. Harter and J. Kudla (Institut für Biologie und Biotechnologie der Pflanzen, Universität Münster) (Walter *et al.*, 2004). The specific primers are shown in Table S1. Expression of MaNAC5, MaWRKY1 or MaWRKY2 alone was used as negative control. The resulting constructs were co-transfected into BY-2 protoplasts by the polyethyleneglycol (PEG) method, as described previously (Shan *et al.*, 2012). The transfected protoplasts were then incubated at 22 °C for 24–48 h. YFP fluorescence was observed using a fluorescence microscope (Zeiss Axioskop 2 plus, Carl Zeiss Microscopy GmbH, Munich, Germany).

Dual-luciferase transient expression assay

The high-level simultaneous expression binary vector pEAQ (kindly gifted by Dr George P. Lomonosoff, Department of Biological Chemistry, John Innes Centre, Norwich Research Park, Norwich, Norfolk, UK) (Sainsbury *et al.*, 2009) was used in this assay. To construct the pEAQ-BD vector, first the coding region of the GAL4 DBD was amplified from the GAL4DBD vector (kindly provided by Dr Junping Gao, Department of Ornamental Horticulture, China Agricultural University, Beijing, China), and was subsequently ligated into the pEAQ vector. For the assay of the transcriptional activities of MaNAC5, MaWRKY1 and MaWRKY2, full-length MaNAC5, MaWRKY1 and MaWRKY2, respectively, without their stop codons were cloned into the reconstructed pEAQ-BD as effectors. The double reporter vector includes a native GAL4-LUC, and an internal control REN driven by the 35S promoter, which was modified based on the pGreenII 0800-LUC reporter vector. GAL4-LUC contains five copies of the GAL4 binding element and minimal TATA region of the 35S promoter of *Cauliflower mosaic virus* (CaMV), and these sequences are located upstream of LUC.

For the assay of the binding activities of MaNAC5, MaWRKY1 and MaWRKY2 to the *MaPR1-1*, *MaPR2*, *MaPR10c* and *MaCHIL1* promoters, the promoters were cloned into the pGreenII 0800-LUC double-reporter vector (Hellens *et al.*, 2005), whereas MaNAC5, MaWRKY1 and MaWRKY2 were cloned into the pEAQ vector as effectors. The primers used for the construction of the effectors and reporter vectors are listed in Table S1.

The constructed effectors and reporter plasmids were electroporated into *Agrobacterium tumefaciens* strain EHA105 and infiltrated into tobacco (*Nicotiana benthamiana*) leaves by needleless syringe. After 3 days, tobacco leaves were harvested to determine the LUC and REN activities using the dual-luciferase assay reagents (Promega Corporation, Madison, WI, USA). The analysis was carried out using a Luminoskan Ascent Microplate Luminometer (Thermo Fisher Scientific Inc. Waltham, MA, USA) according to the manufacturer's instructions, with a delay of 5 s and integrated measurements for 15 s. The transactivation ability of MaNAC5, MaWRKY1 and MaWRKY2, and the binding activity of MaNAC5, MaWRKY1 and MaWRKY2 to the *MaPRs* promoter are indicated by the ratio of LUC to REN. At least six assay measurements were included for each combination.

Statistical analysis

A completely randomized set-up was performed in all experiments. Data are plotted in the figures as means $\pm$ standard error (SE). Statistical comparison of the mean values was performed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test at the 0.05 confidence level using DPS7.05 software (Zhejiang University, Hangzhou, China).

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The authors declare that no conflict of interest exists.

REFERENCES

- Alves, M.S., Dadalto, S.P., Goncalves, A.B., De Souza, G.B., Barros, V.A. and Fietto, L.G. (2013) Plant bZIP transcription factors responsive to pathogens: a review. *Int. J. Mol. Sci.* **14**, 7815–7828.
- Bu, Q., Jiang, H., Li, C.B., Zhai, Q., Zhang, J., Wu, X., Sun, J., Xie, Q. and Li, C. (2008) Role of the *Arabidopsis thaliana* NAC transcription factors ANAC019 and ANAC055 in regulating jasmonic acid signaled defense responses. *Cell Res.* **18**, 756–767.
- Cenci, A., Guignon, V., Roux, N. and Rouard, M. (2014) Genomic analysis of NAC transcription factors in banana (*Musa acuminata*) and definition of NAC orthologous groups for monocots and dicots. *Plant Mol. Biol.* **85**, 63–80.
- Chen, L., Zhong, H.Y., Kuang, J.F., Li, J.G., Lu, W.J. and Chen, J.Y. (2011) Validation of reference genes for RT-qPCR studies of gene expression in banana fruit under different experimental conditions. *Planta*, **234**, 377–390.
- Chen, Y.J., Perera, V., Christiansen, M.W., Holme, I.B., Gregersen, P.L., Grant, M.R., Collinge, D.B. and Lyngkjær, M.F. (2013) The barley HvNAC6 transcription factor affects ABA accumulation and promotes basal resistance against powdery mildew. *Plant Mol. Biol.* **83**, 577–590.
- Delessert, C., Kazan, K., Wilsom, L.W., Straetend, V.D., Manners, J., Dennis, E.S. and Dolferus, R. (2005) The transcription factor ATAF2 represses the expression of pathogenesis-related genes in *Arabidopsis*. *Plant J.* **43**, 745–757.
- Denance, N., Sanchez-Vallet, A., Goffner, D. and Molina, A. (2013) Disease resistance or growth: the role of plant hormones in balancing immune responses and fitness costs. *Front. Plant Sci.* **4**, 155.
- Eulgem, T. and Somssich, I.E. (2007) Networks of WRKY transcription factors in defense signaling. *Curr. Opin. Plant Biol.* **10**, 366–371.
- Feng, H., Duan, X., Zhang, Q., Li, X., Wang, B., Huang, L., Wang, X. and Kang, Z. (2014) The target gene of *tae-miR164*, a novel NAC transcription factor from the NAM subfamily, negatively regulates resistance of wheat to stripe rust. *Mol. Plant Pathol.* **15**, 284–296.
- Hellens, R., Allan, A., Friel, E., Bolitho, K., Grafton, K., Templeton, M. and Laing, W. (2005) Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. *Plant Methods*, **1**, 13.
- Hölscher, D., Dhakshinamoorthy, S., Alexandrov, T., Becker, M., Bretschneider, T., Buerkert, A., Crecelius, A.C., De Waele, D., Elsen, A., Heckel, D.G., Heklau, H., Hertweck, C., Kai, M., Knop, K., Krafft, C., Maddula, R.K., Matthäus, C., Popp, J., Schneider, B., Schubert, U.S., Sikora, R.A., Svatoš, A. and Swennen, R. (2014) Phenalenone-type phytoalexins mediate resistance of banana plants (*Musa* spp.) to the burrowing nematode *Radopholus similis*. *Proc. Natl. Acad. Sci. USA*, **111**, 105–110.
- Jensen, M.K., Hagedorn, P.H., de Torres-Zabala, M., Grant, M.R., Rung, J.H., Collinge, D.B. and Lyngkjær, M.F. (2008) Transcriptional regulation by an NAC (NAM-ATAF1,2-CUC2) transcription factor attenuates ABA signalling for efficient basal defence towards *Blumeria graminis* f. sp. *hordei* in *Arabidopsis*. *Plant J.* **56**, 867–880.
- Kazan, K. and Lyons, R. (2014) Intervention of phytohormone pathways by pathogen effectors. *Plant Cell*, **26**, 2285–2309.
- Le Hénanff, G., Profizi, C., Courteaux, B., Rabenoelina, F., Gérard, C., Clément, C., Baillieux, F., Cordelier, S. and Dhondt-Cordelier, S. (2013) Grapevine NAC1

- transcription factor as a convergent node in developmental processes, abiotic stresses, and necrotrophic/biotrophic pathogen tolerance. *J. Exp. Bot.* **64**, 4877–4893.
- Lee, S.J., Kang, J.Y., Park, H.J., Kim, M.D., Bae, M.S., Choi, H.I. and Kim, S.Y. (2010) DREB2C interacts with ABF2, a bZIP protein regulating abscisic acid-responsive gene expression, and its overexpression affects abscisic acid sensitivity. *Plant Physiol.* **153**, 716–727.
- Licausi, F., Ohme-Takagi, M. and Perata, P. (2013) APETALA2/Ethylene responsive factor (AP2/ERF) transcription factors: mediators of stress responses and developmental programs. *New Phytol.* **199**, 639–649.
- Lin, R.M., Zhao, W.S., Meng, X.B., Wang, M. and Peng, Y.L. (2007) Rice gene OsNAC19 encodes a novel NAC-domain transcription factor and responds to infection by *Magnaporthe grisea*. *Plant Sci.* **172**, 120–130.
- Llorca, C.M., Potschin, M. and Zentgraf, U. (2014) bZIPs and WRKYs: two large transcription factor families executing two different functional strategies. *Front. Plant Sci.* **5**, 169.
- Ma, B.C., Tang, W.L., Ma, L.Y., Li, L.L., Zhang, L.B., Zhu, S.J., Zhuang, C.X. and Irving, D. (2009) The role of chitinase gene expression in the defense of harvested banana against anthracnose disease. *J. Am. Soc. Hort. Sci.* **134**, 379–386.
- McGrann, G.R., Steed, A., Burt, C., Goddard, R., Lachaux, C., Bansal, A., Corbitt, M., Gorniak, K., Nicholson, P. and Brown, J.K. (2015) Contribution of the drought tolerance-related stress-responsive NAC1 transcription factor to resistance of barley to Ramularia leaf spot. *Mol. Plant Pathol.* **16**, 201–209.
- Mendes, G.C., Reis, P.A., Calil, I.P., Carvalho, H.H., Aragão, F.J. and Fontes, E.P. (2013) GmNAC30 and GmNAC81 integrate the endoplasmic reticulum stress- and osmotic stress-induced cell death responses through a vacuolar processing enzyme. *Proc. Natl. Acad. Sci. USA*, **110**, 19 627–19 632.
- Moore, J.W., Loake, G.J. and Spoel, S.H. (2011) Transcription dynamics in plant immunity. *Plant Cell*, **23**, 2809–2820.
- Nakashima, K., Takasaki, H., Mizoi, J., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2012) NAC transcription factors in plant abiotic stress responses. *Biochim. Biophys. Acta*, **1819**, 97–103.
- Negi, S., Tak, H. and Ganapathi, T.R. (2015) Cloning and functional characterization of MusaVND1 using transgenic banana plants. *Transgenic Res.* **24**, 571–585.
- Nuruzzaman, M., Sharoni, A.M. and Kikuchi, S. (2013) Roles of NAC transcription factors in the regulation of biotic and abiotic stress responses in plants. *Front. Microbiol.* **4**, 248.
- Oh, S.K., Lee, S., Yu, S.H. and Choi, D. (2005) Expression of a novel NAC domain-containing transcription factor (CaNAC1) is preferentially associated with incompatible interactions between chili pepper and pathogens. *Planta*, **222**, 876–887.
- Olsen, A.N., Ernst, H.A., Leggio, L.L. and Skriver, K. (2005) NAC transcription factors: structurally distinct, functionally diverse. *Trends Plant Sci.* **10**, 79–87.
- Pieterse, C.M., Van der Does, D., Zamioudis, C., Leon-Reyes, A. and Van Wees, S.C. (2012) Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* **28**, 489–521.
- Puranik, S., Sahu, P.P., Srivastava, P.S. and Prasad, M. (2012) NAC proteins: regulation and role in stress tolerance. *Trends Plant Sci.* **17**, 369–381.
- Robert-Seilaniantz, A., Grant, M. and Jones, J.D. (2011) Hormone crosstalk in plant disease and defense: more than just jasmonate–salicylate antagonism. *Annu. Rev. Phytopathol.* **49**, 317–343.
- Rushton, D.L., Tripathi, P., Rabara, R.C., Lin, J., Ringler, P., Boken, A.K., Langum, T.J., Smidt, L., Boomsma, D.D., Emme, N.J., Chen, X., Finer, J.J., Shen, Q.J. and Rushton, P.J. (2012) WRKY transcription factors: key components in abscisic acid signalling. *Plant Biotechnol. J.* **10**, 2–11.
- Rushton, P.J., Somssich, I.E., Ringler, P. and Shen, Q.J. (2010) WRKY transcription factors. *Trends Plant Sci.* **15**, 247–258.
- Sainsbury, F., Thuenemann, E.C. and Lomonosoff, G.P. (2009) pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. *Plant Biotechnol. J.* **7**, 682–693.
- Shan, W., Kuang, J.F., Chen, L., Xie, H., Peng, H.H., Xiao, Y.Y., Li, X.P., Chen, W.X., He, Q.G., Chen, J.Y. and Lu, W.J. (2012) Molecular characterization of banana NAC transcription factors and their interactions with ethylene signalling component EIL during fruit ripening. *J. Exp. Bot.* **63**, 5171–5187.
- Shan, W., Kuang, J.F., Lu, W.J. and Chen, J.Y. (2014) Banana fruit NAC transcription factor MaNAC1 is a direct target of MaICE1 and involved in cold stress through interacting with MaCBF1. *Plant Cell Environ.* **37**, 2116–2127.
- Shim, J.S., Jung, C., Lee, S., Min, K., Lee, Y.W., Choi, Y., Lee, J.S., Song, J.T., Kim, J.K. and Choi, Y.D. (2013) AtMYB44 regulates WRKY70 expression and modulates antagonistic interaction between salicylic acid and jasmonic acid signaling. *Plant J.* **73**, 483–495.
- Singh, K., Foley, R.C. and Onate-Sanchez, L. (2002) Transcription factors in plant defense and stress responses. *Curr. Opin. Plant Biol.* **5**, 430–436.
- Sun, L., Zhang, H., Li, D., Huang, L., Hong, Y., Ding, X.S., Nelson, R.S., Zhou, X. and Song, F. (2013) Functions of rice NAC transcriptional factors, ONAC122 and ONAC131, in defense responses against *Magnaporthe grisea*. *Plant Mol. Biol.* **81**, 41–56.
- Tang, Y., Kuang, J.F., Wang, F.Y., Chen, L., Hong, K.Q., Xiao, Y.Y., Xie, H., Lu, W.J. and Chen, J.Y. (2013) Molecular characterization of PR and WRKY genes during SA- and MeJA-induced resistance against *Colletotrichum musae* in banana fruit. *Postharvest Biol. Technol.* **79**, 62–68.
- Thatcher, L.F., Kazan, K. and Manners, J.M. (2012) Lateral organ boundaries domain transcription factors: new roles in plant defense. *Plant Signal. Behav.* **7**, 1702–1704.
- Tsuda, K. and Somssich, I.E. (2015) Transcriptional networks in plant immunity. *New Phytol.* **206**, 932–947.
- Voitsik, A.M., Muench, S., Deising, H.B. and Voll, L.M. (2013) Two recently duplicated maize NAC transcription factor paralogs are induced in response to *Colletotrichum graminicola* infection. *BMC Plant Biol.* **13**, 85.
- Walter, M., Chaban, C., Schütze, K., Batistic, O., Weckermann, K., Näke, C. and Kudla, J. (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant J.* **40**, 428–438.
- Wan, C.Y. and Wilkins, T.A. (1994) A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.). *Anal. Biochem.* **223**, 7–12.
- Wang, F., Lin, R., Feng, J., Chen, W., Qiu, D. and Xu, S. (2015) TaNAC1 acts as a negative regulator of stripe rust resistance in wheat, enhances susceptibility to *Pseudomonas syringae*, and promotes lateral root development in transgenic *Arabidopsis thaliana*. *Front. Plant Sci.* **6**, 108.
- Wu, Y., Deng, Z., Lai, J., Zhang, Y., Yang, C., Yin, B., Zhao, Q., Zhang, L., Li, Y., Yang, C. and Xie, Q. (2009) Dual function of *Arabidopsis* ATAF1 in abiotic and biotic stress responses. *Cell Res.* **19**, 1279–1290.
- Xia, N., Zhang, G., Liu, X.Y., Deng, L., Cai, G.L., Zhang, Y., Wang, X.J., Zhao, J., Huang, L.L. and Kang, Z.S. (2010a) Characterization of a novel wheat NAC transcription factor gene involved in defense response against stripe rust pathogen infection and abiotic stresses. *Mol. Biol. Rep.* **37**, 3703–3712.
- Xia, N., Zhang, G., Sun, Y.F., Zhu, L., Xu, L.S., Chen, X.M., Liu, B., Yu, Y.T., Wang, X.J., Huang, L.L. and Kang, Z.S. (2010b) TaNAC8, a novel NAC transcription factor gene in wheat, responds to stripe rust pathogen infection and abiotic stresses. *Physiol. Mol. Plant Pathol.* **74**, 394–402.
- Xu, Z.Y., Kim, S.Y., Hyeon do, Y., Kim, D.H., Dong, T., Park, Y., Jin, J.B., Joo, S.H., Kim, S.K., Hong, J.C., Hwang, D. and Hwang, I. (2013) The *Arabidopsis* NAC transcription factor ANAC096 cooperates with bZIP-type transcription factors in dehydration and osmotic stress responses. *Plant Cell*, **25**, 4708–4724.
- Yokotani, N., Tsuchida-Mayama, T., Ichikawa, H., Mitsuda, N., Ohme-Takagi, M., Kaku, H., Minami, E. and Nishizawa, Y. (2014) OsNAC111, a blast disease-responsive transcription factor in rice, positively regulates the expression of defense-related genes. *Mol. Plant–Microbe Interact.* **27**, 1027–1034.
- Zander, M., La Camera, S., Lamotte, O., Métraux, J.P. and Gatz, C. (2010) *Arabidopsis thaliana* class-II TGA transcription factors are essential activators of jasmonic acid/ethylene-induced defense responses. *Plant J.* **61**, 200–210.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Effects of salicylic acid (SA) and methyl jasmonate (MeJA) treatment on disease index (A) and lesion diameter (B) caused by *Colletotrichum musae* in banana fruit.

Fig. S2 Effects of salicylic acid (SA) and methyl jasmonate (MeJA) treatment on the expression of MaWRKY1 (A) and MaWRKY2 (B) in banana fruit inoculated with *Colletotrichum musae*.

Fig. S3 Nucleotide sequences of MaPR1-1 (A), MaPR2 (B), MaPR10c (C) and MaCHIL1 (D) promoters.

Table S1 Summary of the primers used in this study.