The Role of Chitinase Gene Expression in the Defense of Harvested Banana Against Anthracnose Disease

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ABSTRACT. The pathogenic fungus *Colletotrichum musae* infects developing green bananas (*Musa* spp. AAA group), but remains latent until the fruit ripens. The aim of this research was to determine whether the appearance of disease symptoms is regulated by chitinase gene expression following treatment of fruit with benzothiadiazole (BTH) and methyl jasmonate (MeJA), and with physical (heat) and chemical (H₂O₂ and Ca²⁺-related) treatments. In bananas inoculated with *C. musae*, BTH and MeJA lowered disease severity and stimulated higher gene expression compared with the untreated controls during ripening. However, in naturally infected bananas, BTH and MeJA treatments slightly reduced transcription of the chitinase gene in green bananas, but they prolonged gene expression in ripe bananas and significantly reduced disease severity. The combination of H₂O₂ and the NADPH oxidase inhibitor, diphenylene iodonium, down-regulated chitinase gene expression and compromised disease resistance compared with H₂O₂ alone. Heat treatment (HT) or the combination of HT followed by CaCl₂ reduced disease, but only the latter significantly upregulated chitinase gene expression. The combination of HT and a calcium ionophore (A23187) resulted in different disease indices and different levels of gene expression depending upon the order of application: HT followed by A23187 induced higher gene expression and lower disease. The results suggest that disease resistance of green bananas could be related to high and prolonged levels of chitinase gene expression, and chitinase could be involved in harvested banana's anthracnose resistance activated by different defense pathway signals, such as BTH, MeJA, H₂O₂, and Ca²⁺.

Banana is an important crop produced in tropical or sub-tropical climates, including parts of China. There, as elsewhere, bananas are subject to disease infections that can lead to severe postharvest losses if not controlled, typically using chemicals such as copper, thiabendazole, and prochloraz.

Bananas possess some natural defenses to diseases, especially against *Colletotrichum musae*, the causal organism of anthracnose and the main problem disease of banana in China (Jiang et al., 1997; Yang et al., 2000). Natural defense is apparent from the absence of disease in immature fruit, even though they may be latently infected early in their development (Brown and Swinburne, 1980). In most instances, the initial symptoms of anthracnose appear as small brown lesions scattered over the surface of the fruit.

Systemically acquired resistance (SAR) is a defense mechanism induced by most pathogens that cause tissue necrosis (Anfoka and Buchenauer, 1997; Ryals et al., 1994). SAR provides protection in uninfected parts of the plant against a spectrum of pathogens and it is correlated with the expression of pathogenesis-related (PR) proteins, some, such as chitinase, with antimicrobial activity (Lawrence et al., 1996; Mauch et al., 1984). Expression of PR genes serves as a convenient marker for monitoring SAR (Dong, 1996). Because chitinase in plant tissues degrades chitin in fungal cell walls and can inhibit fungal growth (Arlorio et al., 1992; Leah et al., 1991), expression of chitinase-encoding genes is thought to be an important biochemical mechanism of plant defense against fungal pathogens (Evans and Greenland, 1998; Geddes et al., 2008; Lawrence et al., 1996; Mauch et al., 1984; Yamamoto et al., 2000). However, there are studies showing that the accumulation of chitinase is not necessarily correlated with induction of disease resistance in plants (Anand et al., 2003; Jakobek and Lindgren, 1993; Narusaka et al., 1999; Neuhaus et al., 1991; Punja and Zhang, 1993; Schickler and Chet, 1997). These studies have revealed a complex picture in which the role of chitinases in disease resistance depends upon the specific interaction of pathogen, plant or plant organ, and chitinase (Punja and Zhang, 1993; Schickler and Chet, 1997).

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Plants use multiple pathways to transduce pathogenic signals and activate the hypersensitive response (HR), SAR, and other defense responses. The onset of SAR is associated with increased endogenous levels of salicylic acid (SA) (Malamy et al., 1990), and exogenous SA application also induces SAR and PR gene expression (Ward et al., 1991). Treatment of plants with functional analogs of SA, benzothiadiazole (BTH), also induces SAR (Gorlach et al., 1996), as does treatment with jasmonic acid (JA) and methyl jasmonate (MeJA). The two latter compounds mediate a different pathway from the SA-dependent one (Glazebrook et al., 2003). These pathways do not function independently, but rather, influence each other through a complex network of regulatory interactions (Kunkel and Brooks, 2002).

Networks are apparent in defense responses to chemical and physical stimuli. For example, heat treatment controlled decay of fruit by inducing disease resistance (Chen et al., 2006; Pavoncello et al., 2001), but Ca^{2+} made the heat treatment even more efficacious (Klein et al., 1997; Sams et al., 1993). Signaling pathways involving Ca^{2+} include the production of reactive oxygen species, such as H_2O_2 , that activate defense gene expression (Nürnberg and Scheel, 2001). Ca^{2+} plays a key role as a second messenger in defense-signaling pathways (Lecourieux et al., 2006), and Ca^{2+} -dependent activation of NADPH oxidase is a source of plasma-membrane- H_2O_2 . As diphenylene iodonium (DPI) is an inhibitor of NADPH oxidase (Qin et al., 2004) and the calcium ionophore A23187 increases fluxes of Ca^{2+} across the plasma membrane (Gong et al., 1998; Jiang and Zhang 2003; Pei et al., 2000), it should be possible to manipulate Ca^{2+} fluxes to determine whether this can regulate chitinase induction and hence, plant defense.

Here, we extend our earlier study (Zhu and Ma, 2007) on chitinase gene expression and disease resistance following treatment of fruit with BTH and MeJA to determine the possible relationship between chitinase gene expression and resistance to anthracnose disease in harvested bananas during development from the green to the ripe stage. We also tested physical (heat) and chemical (H_2O_2 - and Ca^{2+} -related) treatments to determine the factors likely to control chitinase gene expression and defense against anthracnose disease to provide some molecular evidences that natural defense can be used in the control of anthracnose in harvested bananas.

Materials and Methods

PLANT MATERIAL. Banana (cv. Brazilian) fruit were harvested from groves in the Wanqingsha Township, Panyu District, Guangzhou City, China. Fruit were over several seasons when at commercial maturity, and only those normal and healthy in appearance without physical injury or disease symptoms were selected. As there are differences in maturity between proximal and distal hands on a bunch, the hands at either end were discarded and the fingers were separated. The fingers from different hands or bunches were mixed and randomly assigned to the treatments. Within 12 h of harvest, fruit were rinsed twice in tap water, and then air-dried before treatments were applied. The experiments were repeated two to three times.

FUNGAL INOCULUM. *C. musae* was isolated from infected bananas and cultured on potato dextrose agar [PDA (consisting of 200 g of diced potato, 20.0 g of sucrose, 15.0 g of agar, and 1.0 L of distilled water)] for 7 d at 25 °C. The culture

plates were flooded with distilled water and the spore suspensions obtained were adjusted to 10^5 spores/mL using a hemocytometer.

TREATMENTS. BTH (5 mM), MeJA (0.1 mM), CaCl_2 (20 mM), A23187 (5 μM), H_2O_2 (1 mM), or DPI (5 μM) were applied in a fine mist until runoff to 60 fruit per treatment. The control fruit were sprayed with distilled water in the same way. Where combination treatments were applied, the bananas were permitted to dry before the second was applied. Heat treatments imposed were based on our previous experiments (Chen et al., 2006). Bananas were dipped in water at 52 °C for 3 min, cooled at room temperature for 3 h, and then chemically treated, if required.

The treated and untreated (control) fruit were placed in polyethylene bags with 0.5 mm-diameter holes and then stored at 22 °C and 95% relative humidity (RH). They were assessed daily for the development of disease until ripe.

In the first experiment, after BTH or MeJA treatment, the fruit were divided into two sets and one set was inoculated with a spore suspension of *C. musae*, and the other set remained noninoculated. For the inoculated set, each banana was inoculated with 20 μL of *C. musae* spores in suspension following wounding at each end using a sterile needle. Each wound was 1.5 mm in depth. Inoculated fruit were wrapped in perforated polyethylene and kept at 22 °C and 95% RH and assessed daily for the development of disease until ripe. In subsequent experiments, only noninoculated (naturally infected) fruit were used.

EVALUATION OF RIPENING STAGES AND INDUCED DISEASE RESISTANCE. The ripening stages were determined using the 1 to 7 scale of Kader (2005), where stage 1 = hard and green; stage 2 = green with a trace of yellow; stage 3 = more green than yellow; stage 4 = yellow with a green hint; stage 5 = all yellow with a green tip on the crown; stage 6 = all yellow; stage 7 = yellow with brown "sugar spots." The ripening index (RI) was calculated from these scores: $RI = \sum(N_X \times X) / \sum N_X$, where X represents ripening stage (1–7), and N_X represents the number of fruit at the corresponding stage.

Disease severity was estimated using the 0 to 4 scale of Zhu and Ma (2007), where 0 = no occurrence of disease; 1 = the area of decay occupied <25% of the fruit surface; 2 = the area of decay occupied 25% to 50% of the fruit surface; 3 = the area of decay occupied 50% to 75% of the fruit surface; and 4 = the area of decay occupied >75% of the fruit surface. The disease index (DI) was calculated from these scores: $DI = [\sum(N_Y \times Y) \times 100] / 4 \sum N_Y$, where Y represents disease severity (0–4), and N_Y represents the number of fruit with the corresponding severity score. The DI ranged from 0 to 100. Disease percentage (DP) refers to the percentage of bananas with disease symptoms, and was calculated $(100 \times I_d / I_t)$, where I_d was the number of inoculated sites that developed disease, and I_t the total number of inoculation sites.

Disease incidence was calculated as percentage based on the number of inoculated sites that developed disease over the total number of inoculations.

EXTRACTION AND HYBRIDIZATION. Total RNA was extracted from peel of six bananas from all the treatments following the method of Wan and Wilkins (1994). RNA (10 μg per lane) was separated on 1.0% (w/v) agarose gel containing 1.1% (v/v) formaldehyde, blotted to Hybond-N membranes (Amersham Biosciences, Piscataway, NJ), and cross-linked by ultraviolet. The chitinase cDNA clone Banchi, isolated from banana, was radio-labeled with ^{32}P -dCTP by random priming and was used as a probe for hybridization.

RT-PCR was performed with an ImProm-II™ Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's protocol. The forward and reverse primers used for amplification of chitinase AF 416677 (*Musa acuminata* endochitinase) were 5'-TTACTGCTTCGTCCAGGAACA GAA-3' and 5'-AGCAAGTCGAGTACCTCTTGTAGA-3', giving a 429-bp fragment. PCR amplification was performed for a total of 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. A 0.4-kb amplified PCR product was purified using a DNA gel extraction kit from Qiagen (Qiagen, Hilden, Germany), and cloned into *E. coli* using a TOPO TA cloning kit (Invitrogen Life Technologies, Carlsbad, CA). The methods used followed the manufacturers' instructions. Positive clones were identified by PCR and were confirmed by sequencing (99% identity with AF 416677).

DESIGN AND ANALYSIS. The experiments were completely randomized designs. Treatments were applied to three replications of 20 fruit for disease and maturity measurements, and three replications of two fruit for gene expression. SAS (SAS Institute, Cary, NC) was used to analyze the results, and Duncan's multiple range test was used to determine significant differences between treatments for disease and ripening data ($P < 0.05$).

Results

DISEASE RESISTANCE AND CHITINASE GENE EXPRESSION IN BANANAS INOCULATED WITH *C. MUSAE*. Compared with the controls, BTH and MeJA significantly reduced lesion diameter and disease incidence in banana fruit inoculated with *C. musae* (Figs. 1 and 2). Northern blots showed that BTH and MeJA induced stronger chitinase expression than in the inoculated-only control banana fruit, and MeJA induced higher expression than BTH (Fig. 3). Compared with the control, BTH slowed the ripening of bananas ($P < 0.05$) when assessed 7 and 12 d after treatment (Fig. 4, A and B), whereas MeJA did not.

DISEASE RESISTANCE AND CHITINASE GENE EXPRESSION IN BANANAS TREATED WITH BTH AND MeJA. Bananas were green when harvested and they stayed green for 4 d before turning yellow, indicating a normal course of ripening (Fig. 5, A and B). The control bananas showed no disease symptoms at the green stage, but they developed the characteristic spot symptoms of

Fig. 1. Effects of benzothiadiazole (BTH) and methyl jasmonate (MeJA) on lesion diameter of bananas inoculated with *Colletotrichum musae*. Bananas were sprayed with BTH (5 mmol·L⁻¹) or MeJA (0.1 mmol·L⁻¹). The controls received distilled water. The inoculation was conducted within 12 h of treatment. After inoculation, bananas were put into plastic bags and stored at 22 °C. Data were from evaluations made 12 d following inoculation, the same day as the images in A were taken. Significance of differences is indicated by letters above the bars ($P < 0.05$). Standard errors are shown. The experiments were repeated three times with similar results.

Fig. 2. Effects of benzothiadiazole (BTH) and methyl jasmonate (MeJA) on disease incidence of bananas inoculated with *Colletotrichum musae*. Bananas were sprayed with BTH (5 mmol·L⁻¹) or MeJA (0.1 mmol·L⁻¹). The controls received distilled water. The inoculation was conducted within 12 h of treatment. After inoculation, bananas were put into plastic bags and stored at 22 °C. Data were from evaluations made 12 d following inoculation, the same day as the images in Fig. 1A were taken. Significance of differences is indicated by letters above the bars ($P < 0.05$). Standard errors are shown. The experiments were repeated three times with similar results.

Fig. 3. Northern blots showing the effects of benzothiadiazole (BTH) and methyl jasmonate (MeJA) on chitinase (*MaChit*) gene expression in bananas inoculated with *Colletotrichum musae*. Bananas were sprayed with BTH (5 mmol·L⁻¹) or MeJA (0.1 mmol·L⁻¹). The controls received distilled water. The inoculation was conducted within 12 h of treatment. After inoculation, bananas were put into plastic bags and stored at 22 °C. RNA gel blot analysis was performed with a ³²P-labeled probe for *MaChit*. The experiments were repeated three times with similar results. DAI = days after inoculation.

anthracnose disease 2 d after becoming yellow (i.e., on day 8). BTH- and MeJA-treated bananas were more mature than the controls 6 d after treatment (Fig. 5, A and B), but then, although all bananas ripened rapidly, BTH-treated fruit remained slightly less mature ($P < 0.05$) than the controls up to 10 d post-treatment (Fig. 5B). On day 12, BTH- and MeJA-treated bananas showed significantly lower ($P < 0.05$) DI and DP than the controls (Figs. 6 and 7).

Northern blots showed that there were high levels of chitinase expression in all green bananas, especially within 1 day of harvest (Fig. 8). Maximum expression occurred at 24 h, and levels remained constant for the duration of the green stage.

Chitinase gene expression in ripe (yellow) bananas was strongest on day 6 (Fig. 8), although expression levels varied greatly between the treatments. BTH induced stronger expression, and MeJA weaker expression, than in the controls. The time courses of gene expression also differed between the treatments. The controls displayed rapid decline in gene expression as the fruit proceeded to senescence, and no expression was detected on day 12. The BTH and MeJA treatments, however, produced fluctuating levels of expression during the same period, but importantly, there was still strong expression on day 12.

MANIPULATION OF CHITINASE GENE EXPRESSION IN PEELS OF BANANA FRUIT IN RELATION TO DISEASE DEFENSE AND RIPENING. H₂O₂ treatment of peels resulted in lower DI and DP than H₂O₂ plus DPI (Figs. 9 and 10). Together with the control,

Fig. 4. Effects of benzothiadiazole (BTH) and methyl jasmonate (MeJA) on disease symptoms (A), ripening index during storage time (B) of bananas inoculated with *Colletotrichum musae*. Bananas were sprayed with BTH (5 mmol·L⁻¹) or MeJA (0.1 mmol·L⁻¹). The controls received distilled water. The inoculation was conducted within 12 h of treatment. After inoculation, bananas were put into plastic bags and stored at 22 °C. The ripening stages were determined using the 1 to 7 scale of Kader (2005). In B, significance of differences is indicated by letters above the bars ($P < 0.05$). Standard errors are shown. The experiments were repeated three times with similar results.

the combination treatment produced weaker chitinase expression than H₂O₂ alone (Fig. 11). Compared with the control, H₂O₂ plus DPI slightly, but significantly ($P < 0.05$), increased the ripening rate between 4 and 8 d after treatment (Fig. 12).

Bananas that were heat-treated had significantly ($P < 0.05$) less disease than the controls (Figs. 13 and 14), but the combination of heat and CaCl₂ treatment resulted in even less disease. The heat-treated bananas showed similar chitinase gene expression to the control fruit (Fig. 15), but the combination of heat and CaCl₂ markedly intensified expression. The treatments did not influence ripening ($P > 0.05$) (Fig. 16).

When banana fruit were exposed to the calcium ionophore A23187 before heat treatment (A23187 plus HT), the disease (Fig. 17) and ripening (Fig. 18) indices were higher, and chitinase gene expression was lower (Fig. 19) than when the heat treatment was applied first. Mean DP for the combination treatments was significantly ($P < 0.05$) lower than HT alone and the control (Fig. 20). Ripening was only different on days 8 and 10 for the A23187 plus HT treatments (Fig. 18). Compared with the control, all treatments produced lower disease indices (Fig. 17 and 20), but only the HT-first treatment was higher in chitinase expression. The A23187-first treatment did not have much influence upon chitinase expression compared with the control (Fig. 19), but disease was suppressed (Figs. 17 and 20).

Fig. 5. Effects of benzothiadiazole (BTH) and methyl jasmonate (MeJA) on progression of ripening (A and B). Banana fruit were sprayed with distilled water (control), 5 mmol·L⁻¹ BTH or 0.1 mmol·L⁻¹ MeJA, put into plastic bags, and stored at 22 °C. The ripening stages were determined using the 1 to 7 scale of Kader (2005), where stage 1 = hard and green; stage 2 = green with a trace of yellow; stage 3 = more green than yellow; stage 4 = yellow with a green hint; stage 5 = all yellow with a green tip on the crown; stage 6 = all yellow; stage 7 = yellow with brown "sugar spots." In B, significance of differences is indicated by letters above the bars ($P < 0.05$). Standard errors are shown. The experiments were repeated three times with similar results.

Fig. 6. Effects of benzothiadiazole (BTH) and methyl jasmonate (MeJA) on disease index. Banana fruit were sprayed with distilled water (control), 5 mmol·L⁻¹ BTH or 0.1 mmol·L⁻¹ MeJA, put into plastic bags, and stored at 22 °C. Data for disease index were from evaluation 12 d after treatment. Significance of differences is indicated by letters above the bars ($P < 0.05$). Standard errors are shown. The experiments were repeated three times with similar results.

Fig. 7. Effects of benzothiadiazole (BTH) and methyl jasmonate (MeJA) on disease percentage. Banana fruit were sprayed with distilled water (control), 5 mmol·L⁻¹ BTH or 0.1 mmol·L⁻¹ MeJA, put into plastic bags, and stored at 22 °C. Data for were from evaluation 12 d after treatment. Significance of differences is indicated by letters above the bars ($P < 0.05$). Standard errors are shown. The experiments were repeated three times with similar results.

Fig. 9. Effects of H₂O₂ and the combination of H₂O₂ and diphenylene iodonium (DPI) on disease index of harvested bananas. Bananas were sprayed with H₂O₂ (1 mmol·L⁻¹), H₂O₂ plus DPI (DPI at 5 μmol·L⁻¹ 3 h after H₂O₂ treatment), or distilled water (control). After treatments, bananas were placed into plastic bags and stored at 22 °C. Data for disease index were from evaluation 10 d following treatment. Significance of differences is indicated by letters above the bars ($P < 0.05$). Standard errors are shown. The experiments were repeated two times with similar results.

Fig. 8. Northern blots show the effects of benzothiadiazole (BTH) and methyl jasmonate (MeJA) on chitinase (*MaChit*) gene expression in banana peel. Banana fruit were sprayed with distilled water (control), 5 mmol·L⁻¹ BTH, or 0.1 mmol·L⁻¹ MeJA, put into plastic bags, and stored at 22 °C. RNA gel blot analysis was performed with a ³²P-labeled probe for *MaChit*. The experiments were repeated three times with similar results.

Fig. 10. Effects of H₂O₂ and the combination of H₂O₂ and diphenylene iodonium (DPI) on percentage disease of harvested bananas. Bananas were sprayed with H₂O₂ (1 mmol·L⁻¹), H₂O₂ plus DPI (DPI at 5 μmol·L⁻¹ 3 h after H₂O₂ treatment), or distilled water (control). After treatments, bananas were placed into plastic bags and stored at 22 °C. Data were from evaluation 10 d following treatment. Significance of differences is indicated by letters above the bars ($P < 0.05$). Standard errors are shown. The experiments were repeated two times with similar results.

Discussion

All bananas, including the controls, showed strong chitinase expression at the green stage (Fig. 8), and these fruit did not develop anthracnose even though they may be latently infected with *C. musae*. This suggests that chitinase could play a role in disease resistance of green bananas. As the fruit ripened and senesced [from 6 to 12 d (Fig. 5B)], the intensity of chitinase expression decreased rapidly in the controls (Fig. 8), and this may have contributed to the loss of disease resistance. This conclusion is supported by observations on the BTH- and MeJA-treated banana fruit that showed, whether inoculated with *C. musae* or not, relatively high levels of chitinase gene expression throughout the experiment (Fig. 3) or on day 12 (Fig. 8), and correspondingly, there was lower disease incidence and DP (Figs. 1, 2, 6, and 7).

Two additional lines of evidence support the conclusion that chitinase gene expression lead to active chitinase enzyme and disease resistance.

First, H₂O₂, an activator of defense gene expression (Nürnberg and Sheel, 2001), stimulated chitinase gene expression and gave some measure of disease protection, whereas the addition of DPI [an inhibitor of NADPH oxidase (Qin et al., 2004) and a potential source of H₂O₂] down-regulated chitinase expression and compromised disease resistance in the fruit (Figs. 9–11). This result may have been affected by DPI reducing endogenous H₂O₂ production and hence inhibiting

Fig. 11. Northern blots showing effects of H₂O₂ and the combination of H₂O₂ and diphenylene iodonium (DPI) on chitinase (*MaChit*) gene expression in banana peel. Bananas were sprayed with H₂O₂ (1 mmol·L⁻¹), H₂O₂ plus DPI (DPI at 5 μmol·L⁻¹ 3 h after H₂O₂ treatment), or distilled water (control). After treatments, bananas were placed into plastic bags and stored at 22 °C. RNA gel blot analysis was performed with a ³²P-labeled probe for *MaChit*. The experiments were repeated two times with similar results.

Fig. 12. Effects of H₂O₂ and the combination of H₂O₂ and diphenylene iodonium (DPI) on the progression of ripening. Bananas were sprayed with H₂O₂ (1 mmol·L⁻¹), H₂O₂ plus DPI (DPI at 5 μmol·L⁻¹ 3 h after H₂O₂ treatment), or distilled water (control). After treatments, bananas were placed into plastic bags and stored at 22 °C. The ripening stages were determined using the 1 to 7 scale of Kader (2005), where stage 1 = hard and green; stage 2 = green with a trace of yellow; stage 3 = more green than yellow; stage 4 = yellow with a green hint; stage 5 = all yellow with a green tip on the crown; stage 6 = all yellow; stage 7 = yellow with brown “sugar spots.” Significance of differences is indicated by letters above the bars ($P < 0.05$). Standard errors are also shown. The experiments were repeated two times with similar results.

Fig. 13. Effects of heat treatment (HT) and the combination of HT plus CaCl₂ on disease index of harvested bananas. For the heat treatment (HT), banana fruit were dipped in water for 3 min at 52 °C, and for HT plus CaCl₂, 20 mmol·L⁻¹ CaCl₂ was sprayed onto bananas 3 h after HT. The controls were dipped in water at 22 °C for 3 min. After treatments, bananas were placed into plastic bags and stored at 22 °C. Data were from evaluation 6 d after treatment. Significance of differences is indicated by letters above the bars ($P < 0.05$). Standard errors are also shown. The experiments were repeated two times with similar results.

Fig. 14. Effects of heat treatment (HT) and the combination of HT plus CaCl₂ on disease percentage of harvested bananas. For the heat treatment (HT), banana fruit were dipped in water for 3 min at 52 °C, and for HT plus CaCl₂, 20 mmol·L⁻¹ CaCl₂ was sprayed onto bananas 3 h after HT. The controls were dipped in water at 22 °C for 3 min. After treatments, bananas were placed into plastic bags and stored at 22 °C. Data were from evaluation 6 d after treatment. Significance of differences is indicated by letters above the bars ($P < 0.05$). Standard errors are also shown. The experiments were repeated two times with similar results.

Fig. 15. Northern blots showing effects of heat treatment (HT) and the combination of HT plus CaCl₂ chitinase (*MaChit*) gene expression in harvested banana fruit. For the heat treatment (HT), banana fruit were dipped in water for 3 min at 52 °C, and for HT plus CaCl₂, 20 mmol·L⁻¹ CaCl₂ was sprayed onto bananas 3 h after HT. The controls were dipped in water at 22 °C for 3 min. After treatments, bananas were placed into plastic bags and stored at 22 °C. RNA gel-blot analysis was performed with a ³²P-labeled probe for *MaChit*. The experiments were repeated two times with similar results.

Fig. 16. Effects of heat treatment (HT) and the combination of HT plus CaCl₂ on ripening index during storage. For the heat treatment (HT), banana fruit were dipped in water for 3 min at 52 °C, and for HT plus CaCl₂, 20 mmol·L⁻¹ CaCl₂ was sprayed onto bananas 3 h after HT. The controls were dipped in water at 22 °C for 3 min. After treatments, bananas were placed into plastic bags and stored at 22 °C. The ripening stages were determined using the 1 to 7 scale of Kader (2005), where stage 1 = hard and green; stage 2 = green with a trace of yellow; stage 3 = more green than yellow; stage 4 = yellow with a green hint; stage 5 = all yellow with a green tip on the crown; stage 6 = all yellow; stage 7 = yellow with brown “sugar spots.” Significance of differences is indicated by letters above the bars ($P < 0.05$). Standard errors are also shown. The experiments were repeated two times with similar results.

Fig. 17. Effects of heat treatment (HT) and the combination of heat treatment (HT) and A23187 on disease index of bananas during storage (D). For the HT plus 23187 treatment, banana fruit were dipped in water for 3 min at 52 °C, cooled at room temperature for 3 h, and then sprayed with A23187 (5 μmol·L⁻¹). For the A23187 plus HT treatment, bananas were first sprayed with A23187 (5 μmol·L⁻¹), held for 3 h, and then exposed to HT. After treatment, bananas were placed into plastic bags and stored at 22 °C. Data were from evaluation 10 d following treatment. Significance of differences is indicated by letters above the bars ($P < 0.05$). Standard errors are also shown. The experiments were repeated two times with similar results.

activation of defense gene expression. A direct effect of H₂O₂ on *C. musae* can probably be ruled out because of the chitinase expression observed (Fig. 11). Second, and in contrast, the HT plus CaCl₂ treatment markedly upregulated chitinase gene expression and elevated disease resistance in banana fruit (Figs. 13–15). These results suggest that in ripe bananas, enhanced expression of the chitinase gene enables induction of defense against anthracnose disease.

There is variation in chitinase expression in bananas, even at harvest. In Figs. 8, 11, and 19, some expression was found at harvest, whereas in Fig. 15, there was very little expression at that time. Because the samples were analyzed within 12 h of harvest, and not immediately at harvest, it is not yet clear whether chitinase expression is constitutive or induced by harvest. Nevertheless, chitinase expression does not appear to be ripening-related, as expression levels vary in several treatments (Figs. 11 and 15), but ripening was similar to the controls. There were also treatments (HT plus A23187) that caused reduced disease incidence and also slightly delayed ripening (Figs. 17 and 18), and treatments (BTH and MeJA) that slightly enhanced ripening but reduced disease incidence (Figs. 5B and 6). In addition, there were treatments (BTH, MeJA, and HT plus CaCl₂) that led to little or no difference in ripening, but significant differences in disease severity (Figs. 5B, 6, 13, and 16). These results suggest that treatment-inhibited or treatment-delayed ripening does not explain the reduced disease severity. Despite the ripening differences, the treatments that induced chitinase gene expression also reduced disease incidence.

Heat treatment alone did not obviously induce higher expression of chitinase than in the control, but it did induce

Fig. 18. Effects of heat treatment (HT) and the combination of heat treatment (HT) and A23187 on change in ripening index during storage. For the HT plus 23187 treatment, banana fruit were dipped in water for 3 min at 52 °C, cooled at room temperature for 3 h, and then sprayed with A23187 (5 μmol·L⁻¹). For the A23187 plus HT treatment, bananas were first sprayed with A23187 (5 μmol·L⁻¹), held for 3 h, and then exposed to HT. After treatment, bananas were placed into plastic bags and stored at 22 °C. The ripening stages were determined using the 1 to 7 scale of Kader (2005), where stage 1 = hard and green; stage 2 = green with a trace of yellow; stage 3 = more green than yellow; stage 4 = yellow with a green hint; stage 5 = all yellow with a green tip on the crown; stage 6 = all yellow; stage 7 = yellow with brown “sugar spots.” Significance of differences is indicated by letters above the bars (*P* < 0.05). Standard errors are also shown. The experiments were repeated two times with similar result.

Fig. 19. Northern blots showing effects of heat treatment (HT) and the combination of heat treatment (HT) and A23187 on chitinase (*MaChit*) gene expression in banana peel. For the HT plus 23187 treatment, banana fruit were dipped in water for 3 min at 52 °C, cooled at room temperature for 3 h, and then sprayed with A23187 (5 μmol·L⁻¹). For the A23187 plus HT treatment, bananas were first sprayed with A23187 (5 μmol·L⁻¹), held for 3 h, and then exposed to HT. After treatment, bananas were placed into plastic bags and stored at 22 °C. RNA gel-blot analysis was performed with a ³²P-labeled probe for *MaChit*. The experiments were repeated two times with similar results.

higher disease resistance. We suggest that in this instance, heat acted as physical elicitor, and therefore that heat treatment induced disease resistance using a mechanism that was different from SAR. It could be argued that direct physical inhibition of fungal growth was the mechanism of the HT effect, but clearly, in combination with CaCl₂ (Fig. 15), gene induction indicates a biochemical mode of defense.

Ca²⁺-related signaling in plant defense involves changes in cellular Ca²⁺ concentrations (Lecourieux et al., 2006), and Ca²⁺ is likely to function in concert with other molecules, such as H₂O₂, NADPH oxidases, and Ca²⁺ channels. Where BTH, in particular, was effective in disease defense, we could not determine whether the mechanism involved regulation of Ca²⁺ fluxes from the present studies. To achieve this objective, monitoring of Ca²⁺ fluxes at the cellular level is needed. However, we have found the treatments HT plus Ca²⁺, H₂O₂, and HT plus A23187 provide good control of disease symptoms and show high expression of the chitinase gene. Therefore, we contend that despite the limitations of the approach we have used, such as the gross application of chemicals to whole fruit, the results suggest Ca²⁺-mediated signaling plays a significant role in the induction of defense against *C. musae*, the causal organism of anthracnose in bananas.

Fig. 20. Effects of heat treatment (HT) and the combination of heat treatment (HT) and A23187 on disease percentage of bananas during storage (D). For the HT plus 23187 treatment, banana fruit were dipped in water for 3 min at 52 °C, cooled at room temperature for 3 h, and then sprayed with A23187 (5 μmol·L⁻¹). For the A23187 plus HT treatment, bananas were first sprayed with A23187 (5 μmol·L⁻¹), held for 3 h, and then exposed to HT. After treatment, bananas were placed into plastic bags and stored at 22 °C. Data were from evaluation 10 d following treatment. Significance of differences is indicated by letters above the bars (*P* < 0.05). Standard errors are also shown. The experiments were repeated two times with similar results.

In conclusion, there is a relationship between chitinase gene expression and disease resistance in harvested bananas. Although chitinase may not be the sole PR protein involved, disease resistance of green bananas could be related to high and prolonged levels of chitinase gene expression, and increased disease severity in ripe bananas could be related to reduced chitinase gene expression. Upregulation of chitinase gene expression strengthens resistance to natural infections of *C. musae* in harvested banana fruit, and H₂O₂ and Ca²⁺-related signaling plays a role in the activation of this resistance.

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