Bismerthiazol Inhibits *Xanthomonas citri* subsp. *citri* Growth and Induces Differential Expression of Citrus Defense-Related Genes

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ABSTRACT

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Citrus canker, caused by *Xanthomonas citri* ssp. *citri*, is a serious disease that causes substantial economic losses to the citrus industry worldwide. The bactericide bismerthiazol has been used to control rice bacterial blight (*X. oryzae* pv. *oryzae*). In this paper, we demonstrate that bismerthiazol can effectively control citrus canker by both inhibiting the growth of *X. citri* ssp. *citri* and triggering the plant's host defense response through the expression of several pathogenesis-related genes (*PR1*, *PR2*, *CHI*, and *RpRd1*) and the

nonexpresser of PR genes (*NPR1*, *NPR2*, and *NPR3*) in 'Duncan' grapefruit, especially at early treatment times. In addition, we found that bismerthiazol induced the expression of the marker genes *CitCHS* and *CitCHI* in the flavonoid pathway and the *PAL1* (phenylalanine ammonia lyase 1) gene in the salicylic acid (SA) biosynthesis pathway at different time points. Moreover, bismerthiazol also induced the expression of the priming defense-associated gene *AZ11*. Taken together, these results indicate that the induction of the defense response in 'Duncan' grapefruit by bismerthiazol may involve the SA signaling pathway and the priming defense and that bismerthiazol may serve as an alternative to copper bactericides for the control of citrus canker.

Citrus canker, caused by *Xanthomonas citri* subsp. *citri*, is one of the most important and wide-spread bacterial diseases of citrus. It has caused extensive damage to commercial citrus cultivars in subtropical production areas of the world. In Florida, for example, an estimated \$12 million per year is spent to control it, yet it is still present and continues to spread (Pitino et al. 2015).

Citrus canker symptoms present as erumpent lesions on leaves, young stems, and fruits. The bacterium is easily dispersed by rain and can infect plants through stomata or wounds resulting from the feeding of insects. To reduce the inoculum level of citrus canker, current control measures include the use of copper-based sprays to protect the plants from infection and the eradication of infected trees (Gottwald et al. 2002). While the use of copper-based sprays allows trees to remain in production, there are many disadvantages associated with the long-term employment of copper bactericides. For example, although they are effective at reducing the bacterial population on the leaf surface, they do not have curative or systemic activity and must be applied multiple times throughout the year (Stall et al. 1981). Their effectiveness is also reduced by wind and rain and, with continual usage, the pathogen may grow resistant to copper sprays (Graham et al. 2004). In addition, accumulation of copper in soils can potentially become phytotoxic and have adverse environmental effects (Alva et al. 1995). In an effort to reduce the dependence on copper bactericides, alternative approaches to combat citrus canker are currently desired.

Utilization of host resistance is probably the most effective method to control plant diseases; however, a species of commercially grown

citrus resistant to citrus canker has yet to be identified. Nevertheless, the induction of the plant's natural defense systems to ward off pathogen attack is a popular concept that warrants further investigation in citrus. Under constant biotic stress, plants have evolved an inducible defense response to protect themselves against potentially dangerous microbes such as fungi and bacteria (Harel et al. 2014; Horváth et al. 2007). This defense response is complex and is associated with many aspects, like the induction of defense-associated gene expression, the reinforcement of the cell wall, and the activation of phytohormone signaling pathways (Bari and Jones 2009; van Loon et al. 2006). Two commonly known systemic resistance mechanisms are systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR is induced by pathogens or effectors and involves the accumulation of salicylic acid (SA), the induction of pathogenesis-related (*PR*) gene expression, and the hypersensitive response (Durrant and Dong 2004). ISR is induced by colonization with nonpathogenic, plant growth-promoting rhizobacteria and fungi and is dependent upon the plant hormones ethylene and jasmonic acid (Conrath 2011). Many studies have reported that the induced resistance triggered by the external application of chemicals broadly affects plant metabolism (Friedrich et al. 1996; Lawton et al. 1996; Ryals et al. 1996). For example, both *Arabidopsis* and tobacco treated with benzothiazole (BTH) could activate SAR, which up-regulates defense response-related genes or SA accumulation and provides protection against a diverse set of pathogen challenges. In addition to these two broad-spectrum immunity systems, plants also have a physiological state called "priming," which allows cells to respond to extremely low levels of stimulus in a stronger and more rapid manner than nonprimed cells (Conrath 2011). Low doses of the plant defense activators BTH, β -aminobutyric acid (BABA), and thiamine are capable of priming susceptible hosts (Ahn et al. 2007; Conrath et al. 2002; Kohler et al. 2002).

Because the protection provided by SAR is often broad spectrum and can last for several weeks to several months (Ward et al. 1991), chemicals that can induce this response are of value in the field.

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Some of the known SAR-inducing chemicals include SA, isonicotinic acid, and acibenzolar-*S*-methyl, in addition to neonicotinoids and bismethiazol, of which the latter two have been shown to control citrus canker (Francis et al. 2009; Graham and Myers 2011; Paranjape et al. 2014). Bismethiazol is of particular interest because of its ability to act both as a protectant as well as a curative agent (Shen and Zhou 2001).

Bismethiazol (N,N-methylene-bis [2-amino-5-mercapto-1, 3, 4-thiadiazole]), which is also known as “Saikuzuo,” “Yekuning,” and “Yeqingshuang,” was produced by the Sichuan Chemical Industry Design Academy in Sichuan, China, and is a derivative of the compound known as ATDA (2-amino-1,3,4-thiadiazole) (Zhongyin 1997). ATDA was originally developed in Japan to control bacterial blight of rice but its use was quickly forbidden because of its teratogenic effects (Beaudoin 1973). Modification of the chemical structure of ATDA into bismethiazol reduced its toxicity level (the median lethal dose when given orally to rats = 4,640 mg/kg), making it a safe alternative to ATDA. It has been in use in China for the control of bacterial blight of rice (caused by *X. oryzae* pv. *oryzae*), rice bacterial leaf streak (caused by *X. oryzae* pv. *oryzicola*), and citrus canker (caused by *X. citri* subsp. *citri*) since the 1970s, but understanding its mode of action is still in its infancy (Zhu et al. 2013). Recent studies on rice leaves infected by *X. oryzae* pv. *oryzae* have shown that bismethiazol can activate SAR-related compounds, such as defense-related gene expression, callose deposition, enhancement of H₂O₂ production, and a hypersensitive response-like cell death (Liang et al. 2015b). Additional research demonstrated that the inhibitory effects of bismethiazol on *X. oryzae* pv. *oryzae* can be seen in culture as well as in planta (Liang et al. 2015a). To date, no studies have been performed to define the effects of bismethiazol on citrus canker. In this research, we chose a technical grade of bismethiazol with a high purity (92.5%) compared with the industrial bactericide, which is wettable powder with only 20% effective components (Sun et al. 2011), to evaluate the effectiveness of bismethiazol against *X. citri* subsp. *citri* in culture and in planta. We show that the ability of bismethiazol to cure citrus canker is related not only to its capacity to affect the growth of *X. citri* subsp. *citri* but also to upregulate various defense response-associated genes in grapefruit. This not only confirms the efficacy of bismethiazol against *X. citri* subsp. *citri* but also advances our understanding of its mode of action against *Xanthomonas* species.

MATERIALS AND METHODS

Plant material. The citrus species used in this study was ‘Duncan’ grapefruit (*Citrus paradise* Macfadyen). All plants were grown under greenhouse conditions. All experiments were performed on new flushes.

Effects of bismethiazol on the growth of *X. citri* subsp. *citri* in planta and in culture. *X. citri* subsp. *citri* (A strain 3213) (Pitino et al. 2015) was used in this study to determine the bactericidal effects of bismethiazol. In-culture assays were conducted by culturing the bacteria in nutrient broth (NB) medium at 28°C until the optical density at 600 nm (OD₆₀₀) reached 0.3. Then, 20 µl of the cell suspension was added to 2 ml of NB medium to a final concentration of about 10⁶ CFU/ml. To evaluate the minimum inhibitory concentration (MIC), growth of *X. citri* subsp. *citri* in NB medium containing various concentrations of bismethiazol (30, 50, 100, 150, and 160 µg/ml) was measured as described below. For solubility purposes, the bismethiazol (purity, 92.5%; Longwan Agrichemical Co. Ltd.) was dissolved in pure N,N-dimethylformamide (DMF) (Sigma). According to a previous study, bacterial growth was not affected by a 1% (vol/vol) DMF solution (Wadhvani et al. 2009); thus, DMF was added to the various concentrations of bismethiazol until the final concentration of DMF in the NB medium reached 1% DMF. The control was cultured in a 1% DMF NB solution without the addition of bismethiazol. All cultures were grown at 28°C and the OD₆₀₀ was

measured after 24 h. The MIC was determined as the lowest concentration of bismethiazol at which bacteria failed to grow. To evaluate the minimum bactericide concentration (MBC), 20 µl of each suspension in which bacteria growth was not observed was plated in nutrient agar (NA) medium. The plates were incubated at 28°C for 24 h. The MBC is the lowest concentration at which bacteria failed to grow in NB medium with bismethiazol after subsequent transfer to NA plates (Bajpai et al. 2010). MIC and MBC evaluation experiments were performed three times.

For the in planta assays, inoculum was prepared by culturing *X. citri* subsp. *citri* in NB medium overnight. The OD₆₀₀ of the bacterium was adjusted to 0.3 with sterile tap water containing 0.1% Silwet L-77. The suspension was then diluted 1:100 with sterile tap water to give an inoculation suspension containing approximately 10⁶ CFU/ml (McCollum et al. 2011). To determine the effective concentration of the bismethiazol against *X. citri* subsp. *citri*, ‘Duncan’ grapefruit plants with new flushes were sprayed with bismethiazol suspensions of 100, 200, 300, and 400 µg/ml one day before inoculation with *X. citri* subsp. *citri*. To determine the optimum treatment time, ‘Duncan’ grapefruit plants with new flushes were sprayed with a bismethiazol suspension of 400 µg/ml 5, 3, 1 and day prior to inoculation and 1 and 3 days postinoculation. Samples were collected 14 days postinoculation from four leaves of each tree. A 7-mm-diameter disc of each leaf was removed with a No.4 cork borer. The four discs were weighed and DNA was extracted as previously described (Pitino et al. 2015). The total DNA was eluted in 100 µl of nucleic acid free water and was used for quantitative polymerase chain reaction (qPCR) directly. The primers targeting the *pthA* gene, VM3 (5'-GCATTGATGACGCCATGAC) and VM4 (5'-TCCCTGATGCCTGGAGGATA), were used for quantification of *X. citri* subsp. *citri* (Mavrodieva et al. 2004). Total DNA (2 µl) was used as template for qPCR to determine the abundance of *pthA* DNA. The qPCR conditions were 95°C for 2 min, 40 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s, followed by a melt curve analysis sequence. The equation used to convert cycle threshold (Ct) value to the copy number (CN) of *pthA* gene was CN = (38.3 – Ct)/3.56. The copy number of *pthA* gene per *X. citri* subsp. *citri* cell has been estimated to be 40; therefore, the number of *X. citri* subsp. *citri* cells is CN/40. The results were expressed as the number of cells per milligram of tissues (Stover and McCollum 2011). To determine the effect of bismethiazol on the bacterium, we calculated the inhibition rate of each treatment compared with control samples. The equation used was: Inhibition rate (%) = (cell number_(control) – cell number_(treatment))/cell number_(control). All the experiments were performed more than three times.

Relative gene expression. Citrus containing new flush tissues were sprayed with 400 µg of bismethiazol per milliliter and young leaves were collected at 0 and 6 h and 1, 3, and 5 days posttreatment (dpt). Three plants of each treatment were used as biological replicates. Total RNA was extracted using the Invitrogen RNA extraction kit according to the manufacturer’s instructions. The RNA concentration and quality were detected with the Nano drop spectrophotometer (Thermo Scientific). The RNA was treated with DNA-free DNase (Promega). RNA (1 µg) was used to synthesize cDNA via M-MLV reverse transcription (Promega) and an oligo (dT15) primer, according to the manufacturer’s instructions. Gene expression was determined by real-time PCR using SYBR Green (Applied Biosystems). SYBR Green real-time PCR amplifications were performed in a Mastercycler ep realplex (Eppendorf). The real-time PCR conditions were 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 55°C for 15 s, and 70°C for 10 s, followed by a melt curve analysis sequence. The GADPH gene was used as endogenous control. All the primers used for real-time PCR are shown in Supplementary Table S1. The 0-h posttreatment (hpt) samples were used as the reference to calculate the relative expression ratio by $\Delta\Delta Ct$ method. The upregulation was considered significant when the relative expression ratio was ≥ 2 -fold and downregulation was

considered significant when the relative expression ratio was ≤ 0.5 -fold. All the experiments were performed at least twice and showed similar results.

RESULTS

Bismethiazol inhibits the growth of *X. citri* subsp. *citri* in culture and in planta. Bismethiazol is registered in China as an effective bactericide for controlling *Xanthomonas* pathogens such as *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, and *X. citri* subsp. *citri*. To confirm the effect of bismethiazol on *X. citri* subsp. *citri* in culture, we measured the growth of *X. citri* subsp. *citri* in NB medium under various concentrations of bismethiazol. The results showed that the inhibition rates were 11.66, 21.84, 64.13, 95.44 and 99.19% when cultured in NB medium with final concentrations of 30, 50, 100, 150, and 160 μg of bismethiazol per milliliter, respectively (Fig. 1A). The OD₆₀₀ value was not detectable at concentrations of bismethiazol ≥ 170 $\mu\text{g}/\text{ml}$. We transferred cell suspensions treated with bismethiazol at 0, 200, 250, 300, and 400 $\mu\text{g}/\text{ml}$ onto agar plates and observed their growth at 24 h. We failed to observe any colony formation on agar plates for suspensions treated with bismethiazol at ≥ 300 $\mu\text{g}/\text{ml}$. Thus, the MIC and MBC of bismethiazole against *X. citri* subsp. *citri* were defined as 170 and 300 $\mu\text{g}/\text{ml}$, respectively.

We used 'Duncan' grapefruit as a host plant to investigate the effect of bismethiazol on citrus canker in planta. Using qPCR, *X. citri* subsp. *citri*-specific primers, the *X. citri* subsp. *citri* inhibition rates were quantified as 30.45, 59.32, 95.84, and 96.10% when grapefruit plants were treated with bismethiazol at 100, 200, 300, and 400 $\mu\text{g}/\text{ml}$, respectively, 1 day prior to inoculation (Fig. 1B). We also investigated if treatment time affected the effectiveness of bismethiazol in eliminating *X. citri* subsp. *citri* in grapefruit. The *X. citri* subsp. *citri* inhibition rates were 91.65, 94.40, and 96.94% when grapefruit plants were treated with bismethiazol at 400 $\mu\text{g}/\text{ml}$ for 5, 3, and 1 day prior to inoculation, respectively (Fig. 1C). Grapefruit treated with bismethiazol at 400 $\mu\text{g}/\text{ml}$ at 1 and 3 days postinoculation showed an inhibition rate of 94.90 and 61.90%, respectively.

Bismethiazol induces *PR* genes and nonexpresser of *PR* (*NPR*) gene expression in 'Duncan' grapefruit at early treatment times. Previously, it was shown that rice treated with bismethiazol upregulated defense-related genes upon *X. oryzae* pv. *oryzae* inoculation compared with noninoculated controls (Liang et al. 2015b). In order to investigate whether bismethiazol could also induce the expression of defense-associated genes in healthy citrus, we sprayed a 400- $\mu\text{g}/\text{ml}$ suspension of bismethiazol on healthy grapefruit leaves and collected young leaf tissues at 0 and 6 h and 1, 3, and 5 dpt. We then studied the following four representative *PR* genes, all of which have been reported to be induced by pathogen infection or chemical compounds: *PR1* (PR protein 1), a molecular marker for SAR that can be induced by BTH, thiamine, and SA (Ahn et al. 2007; Friedrich et al. 1996); *PR2* (β -1, 3-glucanase) and *CHI* (chitinases), which belong to the PR-2 and PR-3 families and are strongly induced when plants respond to wounding or infection by pathogens (Burketová et al. 1999; Dufour et al. 2013; Mauch et al. 1988); and *RdRp1* (RNA-dependent RNA polymerase 1), which synthesizes short cRNAs, using cellular or viral RNAs as templates, and is inducible upon SA treatment of plants (Xie et al. 2001). The results showed that the level of *PR2* gene expression increased sevenfold at 6 h and 1 dpt, while *CHI* expression increased ninefold at 6 h and 2.5-fold at 1 dpt (Fig. 2B and C). Although the expression level increased for *PR1* and *RdRp1*, the increase was not as dramatic as the other two genes, reaching about fourfold during the first day after treatment (Fig. 2A and D). As a control, it was noted that the expression level of these genes remained essentially unchanged across the same time points in plants treated with water.

Expression of the *PR* genes is dependent on the activation of *NPR* genes after SAR induction (Cao et al. 1994; Dong 2004). To identify if bismethiazol is involved in the regulation of *NPR* genes, we measured the expression level of *NPR1*, *NPR2*, and *NPR3* (homologous to *Arabidopsis NPR1*, *NPR3*, and *NPR4*) in 'Duncan' grapefruit plants. The data showed that expression of *NPR1* and *NPR2* is up-regulated approximately fourfold at 6 h but is restored to

Fig. 1. The effect of bismethiazol on *Xanthomonas citri* subsp. *citri* in culture and in planta. **A**, The inhibition rate of *X. citri* subsp. *citri* after treated with various concentration bismethiazol in culture and **B**, in planta. **C**, The inhibition rate of *X. citri* subsp. *citri* treated with 400 μg of bismethiazol per milliliter at different treatment times in planta. **D**, Symptoms caused by *X. citri* subsp. *citri* with or without bismethiazol treatment on 'Duncan' grapefruit. Inhibition rates are means $\pm$ standard error ($n = 3$). Values in a column followed by the same letter are not different according to Fisher's least significant differences test ($P = 0.05$). dbi = days before inoculation; dpi = days postinoculation.

Fig. 2. The effect of bismethiazol on the expression of pathogenesis-related (PR) genes in 'Duncan' grapefruit. The relative quantification (RQ) of gene expression levels for **A**, *PR1*; **B**, *PR2*; **C**, *CHI*; and **D**, *RdRp1* after water control (white) treatment or treatment with 400 μ g of bismethiazol per milliliter (black). The horizontal axis represents the time points posttreatment (hpt = hours posttreatment, dpt = days posttreatment). The 0 h after treatment is considered as 1. Bars are mean $\pm$ standard error ($n = 3$).

Fig. 3. The effect of bismethiazol on the expression of nonexpresser of pathogenesis-related (NPR) genes in 'Duncan' grapefruit. The relative quantification (RQ) of gene expression levels for **A**, *NPR1*; **B**, *NPR2*; and **C**, *NPR3* after water control (white) treatment or treatment with 400 μ g of bismethiazol per milliliter (black). The horizontal axis represents the time point posttreatment (hpt = hours posttreatment, dpt = days posttreatment). The 0 h after treatment is considered as 1. Bars are mean $\pm$ standard error ($n = 3$).

background levels by 1 dpt (Fig. 3A and B), whereas *NPR3* gene expression increased approximately sevenfold and remained elevated until 1 day after bismethiazol treatment (Fig. 3C). Overall, bismethiazol induced expression of both the *PR* genes and the *NPR* genes in ‘Duncan’ grapefruit, with the increased expression seen mainly in the first day posttreatment.

Effects of bismethiazol on the SA signaling pathway and the priming defense response in citrus. Many studies have reported that expression of the *NPR* genes is triggered by the accumulation of SA in a variety of plants (Dong 2004). To investigate if SA biosynthesis is regulated postbismethiazol treatment, we measured the expression of two marker genes involved in SA biosynthesis, i.e., *PAL1* (phenylalanine ammonia lyase 1) and *ICS1* (isochlorogenic acid synthase 1). The expression of *ICS1* was not up-regulated after bismethiazol treatment, but *PAL1* expression was two- to fourfold higher at 3 and 5 dpt (Fig. 4A and B). However, these results are in contrast to the expression of the *NPR* genes, since the *NPR* genes were mainly up-regulated at early treatment times, while the SA biosynthesis genes were triggered at later treatment time points. Although the expression of the *NPR* genes was not consistent with the molecular marker genes *PAL1* and *ICS1*, indicative of SA regulation in citrus, it is possible that bismethiazol triggers the transcription of other genes that indirectly influence the SA signaling pathway.

It is also possible that *PAL1* may be involved in the metabolism of other defense-related molecules. Notably, the final product of the PAL pathway, *trans*-cinnamic acid, is not only a substrate in the SA biosynthesis pathway but is also involved in the flavonoid biosynthesis pathway. Flavonoids are known to play a role in plant

defense, and we were able to detect the expression of two marker genes in the flavonoid pathway in ‘Duncan’ grapefruit: *CitCHS*, encoding chalcone synthase, and *CitCHI*, encoding chalcone isomerase. Our data showed that the expression level of *CitCHS* increased up to 10-fold at 6 h and 1 day after treatment but was restored to background levels by 3 dpt, while the expression of *CitCHI* was up-regulated 2.5-fold during this same period (Fig. 4C and D). Thus, we assumed that the bismethiazol treatment caused the induction of *CitCHS* and *CitCHI* in the flavonoid pathway in ‘Duncan’ grapefruit, which may indirectly influence the SA biosynthesis pathway and could, in turn, affect a series of genes (like the *NPR* and *PR* genes) in the SA-dependent signaling defense pathway.

The gene encoding azelaic acid-induced 1 (*AZII*) is induced by azelaic acid, which confers local and systemic immunity to pathogen infection in plants and primes the plant to accumulate higher SA levels when challenged than nonprimed plants (Jung et al. 2009). Our data showed that the expression of *AZII* was increased approximately sixfold at 6 hpt and gradually decreased to levels similar to that of the control by 3 dpt (Fig. 4E). From this, we hypothesize that bismethiazol triggers the priming defense of the plant, enhancing its resistance to various biotic or abiotic stresses.

Bismethiazol had no effect on the jasmonate (JA) and auxin signaling pathways. JA and auxin are well-known signaling molecules that induce the defense response against many biotic and abiotic stresses in plants (Horváth et al. 2007). Two important genes in the JA signaling pathway include *COI1* (coronatine-insensitive 1) and *JAR1* (JA-resistant 1), which encode for an enzyme important for the activation of JA signaling and a JA receptor, respectively

Fig. 4. The effect of bismethiazol on the expression of salicylic acid signaling pathway-related genes in ‘Duncan’ grapefruit. The relative quantification (RQ) of gene expression levels for **A**, *ICS1*; **B**, *PAL1*; **C**, *CitCHI*; **D**, *CitCHS*; and **E**, *AZII* after water control (white) treatment or treatment with 400 µg of bismethiazol per milliliter (black). The horizontal axis represents the time point posttreatment (hpt = hours posttreatment, dpt = days posttreatment). The 0 h after treatment is considered as 1. Bars are mean ± standard error ($n = 3$).

(Staswick and Tiryaki 2004; Xie et al. 1998). In this study, the expression of *COI1* and *JAR1* showed no obvious upregulation after bismethiazol treatment, as compared with the water-treated samples (Fig. 5A and B), which suggested that bismethiazol had no effect on the JA-dependent defense response.

Auxin can induce the expression of the auxin/indole-3-acetic acid (Aux/IAA) protein family, which regulates plant growth and development (Wang et al. 2005). Under a high level of auxin, the IAA protein and an auxin receptor known as transport inhibitor response 1 (TIR1) can interact with each other to regulate plant growth and development (Rodrigues et al. 2013). Here, we measured the expression of marker genes *IAA9* and *TIR1* in bismethiazol-treated plants. Although the expression of *IAA9* increased three- to fourfold throughout the entire time period following treatment, this increase also occurred in control samples and, thus, no significant difference in gene expression was found between the treated and nontreated plants (Fig. 4C). In addition, the expression of *TIR1* was not significantly different between the treated and nontreated plants (Fig. 4D). Taken together, the data suggests that the auxin signaling pathway was not triggered with bismethiazol and that the increase in *IAA9* seen in both the water control and the treated samples may have resulted from our use of young leaves for gene expression, since young tissues are in a natural state of development and expansion, which has been associated with IAA production (Ljung et al. 2001; Perrot-Rechenmann and Hagen 2002).

DISCUSSION

Bismethiazol has been widely used to control *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* infections in China. A previous study determined the EC_{50} of bismethiazol for *X. oryzae* pv. *oryzae* in culture was ~ 12 $\mu\text{g/ml}$ and ~ 18 $\mu\text{g/ml}$ for *X. oryzae* pv. *oryzicola* (Liang et al. 2015a). In this paper, we demonstrate that bismethiazol

can also inhibit the growth of *X. citri* subsp. *citri* in culture and that the half maximal effective concentration (EC_{50}) of bismethiazol on *X. citri* subsp. *citri* is 85 $\mu\text{g/ml}$ in NB medium, which is greater than the concentration for *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*. In agreement with the increased EC_{50} value for bismethiazol on *X. citri* subsp. *citri* in culture, *X. citri* subsp. *citri* also has a higher tolerance to bismethiazol in planta. ‘Duncan’ grapefruit needed concentrations of bismethiazol ≥ 300 $\mu\text{g/ml}$ in order to reduce the pathogen level by $\geq 90\%$ compared with nontreated samples, while the lesion length from *X. oryzae* pv. *oryzae* in rice is reduced by 98% after treatment with bismethiazol at 150 $\mu\text{g/ml}$ (Liang et al. 2015a; Zhu et al. 2013). The timing of the treatment is also an important factor that influences the effectiveness of bismethiazol on citrus canker, since the protective activity of bismethiazol was found to be better than the curative activity on citrus canker, which is in agreement with the studies on *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* (Zhu et al. 2013).

The main chemical control methods for citrus canker are copper-based bactericides, such as copper oxychloride, copper hydroxide, copper oxide, and basic copper sulfate. These kinds of bactericides are used to reduce the pathogen load on new leaf flushes and fruit surfaces. Research showed the use of 1,800 μg of copper oxychloride per milliliter significantly decreased the incidence of citrus canker on leaves and harvested fruits, reduced the number of prematurely dropped fruits, and increased yields in various species of citrus trees (Behlau et al. 2010). Research also showed that 500 to 1,000 ppm of streptomycin sulfate was effective when sprayed with 1% glycerine on acid lime (Rangaswami et al. 1959). Six sprays of 1,000 ppm of streptomycin sulfate along with two prunings reduced the level of canker in acid lime (Balaraman and Purushotman, 1981). Here, bismethiazol was shown to be effective at a much lower concentration (with a bismethiazol concentration of 200 $\mu\text{g/ml}$ inhibiting the growth of *X. citri* subsp. *citri* by 50% percent) and that its effectiveness was greatest when plants were treated

Fig. 5. The effect of bismethiazol on the expression of jasmonate and auxin signaling pathway-related genes in ‘Duncan’ grapefruit. The relative quantification (RQ) of gene expression levels for **A**, *COI1*; **B**, *JAR1*; **C**, *IAA9*; and **D**, *TIR1* after water control (white) treatment or treatment with 400 μg of bismethiazol per milliliter (black). The horizontal axis represents the time point posttreatment (hpt = hours posttreatment, dpt = days posttreatment). The 0 h after treatment is considered as 1. Bars are mean $\pm$ standard error ($n = 3$).

prior to pathogen infection. With the widespread and frequent use of bismethiazol to control the blight of rice, bismethiazol-resistant *X. oryzae* pv. *oryzae* have been selected for in many rice-growing areas (Zhu et al. 2013). Because of this, when promoting bismethiazol to control citrus canker, specified application times and concentrations for bismethiazol should be adhered to and treatment regimens should include a rotation of effective chemicals in an effort to avoid the emergence of resistant strains.

In addition, we showed that bismethiazol could induce the defense response in citrus during the first 24 hpt by detecting an increase in the expression of four *PR* genes. *PR* proteins are induced under specific pathological conditions and are not pathogen-specific but are determined by the type of plant reaction (van Loon 1985). Some of these proteins are antimicrobial in nature, attacking molecules in the cell wall of a bacterium or fungus, while others work as signals that spread the “news” of an infection to nearby cells (Campbell and Reece 2005). Overall, the activation of *PR* proteins defines the occurrence of a defense response in plants. In a previous study, BTH and two fungicides (azoxystrobin and fenpropimorph) induced defense-associated gene expression in wheat, showing that *PR* genes of wheat were up-regulated at early treatment times (Pasquer et al. 2005). An analysis of gene expression from cocoa during the early response to BTH showed 68 genes were up-regulated, including *PR* genes (Verica et al. 2004). A comparable gene expression analysis of BTH-treated and untreated coffee leaves resulted in the identification of 71 genes differentially regulated in the coffee leaves 16 h after BTH treatment, which included a systemic increase in *PR* proteins (Bell et al. 2006). In general, the induction of the *PR* genes in the present study is consistent with these studies regarding the timing of gene induction post-chemical treatment.

Chitinases are generally found in organisms that dissolve and digest the chitin of fungi and can be induced by chemical elicitors or by pathogen infection. Chitinases alone or in combination with glucanases can trigger a defensive response to fungal infection (van Loon et al. 2006). The *PR2* gene encoding β -1, 3-glucanase is localized in plants and has been characterized in a wide range of species. It is important for cell division, regulation of callose turnover at the plasmodesmata, and withstanding abiotic stresses. In this study, the gene encoding β -1,3-glucanase was highly expressed, while the chitinase was up-regulated at 6 hpt, which infers that bismethiazol triggers a combination of glucanase and chitinase activities to protect against pathogen infection.

In *Arabidopsis*, an *NPR1* gene mutant failed to express *PR* genes after systemic acquired resistance is induced. A recent study also found that the *NPR1* protein paralogues *NPR3* and *NPR4* act as SA receptors and modulate the interaction between SA and *NPR1* (Fu et al. 2012). *NPR3* and *NPR4* proteins bind SA with different affinities, with *NPR3* having a lower affinity for SA (micromolar range) than *NPR4* (nanomolar range), which means that the *NPR4* protein can bind SA at lower concentrations of SA in *Arabidopsis* (Moreau et al. 2012). *NPR3* and *NPR4* interact with *TGA2* and its paralogs to negatively regulate the defense response and mediate degradation of *NPR1* in *Arabidopsis* (Zhang et al. 2006). The citrus *NPR2* and *NPR3* genes are homologous to *Arabidopsis NPR3* and *NPR4* and may function similar to that reported in *Arabidopsis* (Shi et al. 2015). In this study, the expression of *NPR1*, *NPR2*, and *NPR3* genes are all up-regulated 6 h after bismethiazol treatment. It is possible that citrus also has several transcription factors that function like *TGA2* and its paralogs in *Arabidopsis* and that they influence the interaction between *NPR1*, *NPR2*, and *NPR3* and affect the defense response. But how the transcription factors in citrus regulate the interaction between *NPR1*, *NPR2*, and *NPR3* is still not clear.

NPR genes are usually induced by the accumulation of SA and, thus, follow the SA signaling pathway gene expression pattern. In general, plants have two distinct pathways for SA biosynthesis, one involving isochorismate synthase and the other involving PAL

(Mauch-Mani and Slusarenko 1996; Wildermuth et al. 2001). Although we failed to detect the regulation of *ICS1* and *PAL1* expression at the early treatment times, expression of the key enzymes and regulating factors of the flavonoid pathway (*CitCHS* and *CitCHI*) were highly induced and are known to share the same substrates as the SA biosynthesis pathway. The analysis of *CHS* and *CHI* transcripts has been used as a measure of the induced defense response in many studies (Dhawale et al. 1989; Haberer et al. 1989; Sallaud et al. 1997). *CHS* and *CHI* expression causes accumulation of flavonoid and is involved in the SA defense pathway (Dao et al. 2011). Induction of the *CHS* gene in SA-treated safflower was detected at early treatment times and was followed by the induction of the *PAL* gene at the later treatment times (Dehghan et al. 2014), which is consistent with our results for the *CitCHS* and *PAL* expression patterns. Overall, bismethiazol may be inducing the defense response in citrus by increasing the utilization of a substrate of PAL in the flavonoid pathway, which may be indirectly influencing SA biosynthesis and modulating the SA-dependent signaling pathway.

Priming is a unique physiological state in which cells respond to an extremely low level of a stimulus in a more rapid and robust manner. Primed plants have been shown to have a faster and stronger activation of the defense response when subsequently infected by pathogens (Conrath 2011). Priming has been shown to be induced by both natural and synthetic compounds, such as SA and BTH (Conrath et al. 2006). The high expression of the priming-associated gene *AZII* in this study suggests that the bismethiazol-inducible defense likely involves the priming defense response in plants.

JA is a well-known signaling molecule that induces the defense response to many biotic and abiotic stresses in various plants (Horváth et al. 2007). The accumulation of JA increases when host plants are infected by a pathogen or have tissue damage, and the application of JA can induce defense-associated gene expression (Wasternack 2007). We failed to detect any significant transcriptional modulation of the marker genes in the JA signaling pathway, which is consistent with the theory that SA and JA defense pathways are mutually antagonistic (Bari and Jones 2009). Another important hormone, auxin, which controls plant growth and developmental processes, also acts as a signaling molecule in response to pathogen infection and environmental stress (Bari and Jones 2009). We found that the expression of *IAA9* is up-regulated in both treated and nontreated samples while the expression of *TIR1* was not affected. These results indicate that the auxin signaling pathway was not involved in the bismethiazol-induced defense response and can be explained by the fact that the samples collected for gene expression were very young tissues, which would be synthesizing auxin for cell division, extension, and differentiation (Leyser 2006). Taken together, the lack of expression of marker genes in the JA and auxin signaling pathways revealed that the bismethiazol-induced defense response does not involve these hormones signaling networks.

In conclusion, this study demonstrated that bismethiazol effectively inhibited the growth of *X. citri* subsp. *citri* in medium and in citrus and induced a defense response at the early treatment times in ‘Duncan’ grapefruit plants, even without pathogen infection. The mode of action of this inducible resistance may involve the SA signaling pathway and triggering of the priming defense in plants, which explains why the defense response is stronger after pathogen infection in the bismethiazol-treated plants than in nontreated plants.

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