Two thiadiazole compounds promote rice defence against *Xanthomonas oryzae* pv. *oryzae* by suppressing the bacterium's production of extracellular polysaccharides

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SUMMARY

Thiazole, isothiazole, thiadiazole, and their derivatives are used to control various human, animal and plant diseases. In addition to having direct anti-microbial and anti-fungal properties, these compounds are thought to induce host defences, but the mechanism of defence induction remains poorly understood. This article reports that the thiadiazoles of zinc thiazole and bismethiazol induce H₂O₂ accumulation, up-regulation of defence-related genes, callose deposition and hypersensitive response-like cell death in rice leaves infected with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strain ZJ173, but not in non-infected leaves. These defence responses in *Xoo*-infected leaves were suppressed by the exogenous application of catalase, which reduces H₂O₂ accumulation. The application of extracellular polysaccharides (EPSs) extracted from strain ZJ173 significantly compromised rice defence against ZJ173 with or without thiadiazole treatment. The EPS-deficient *Xoo* mutant Δ *gumH* triggered a stronger defence than its parent strain ZJ173. The thiadiazole treatments reduced EPS production by strain ZJ173, but not by the thiadiazole-resistant strain 2-1-1, which is thiadiazole resistant *in vivo*, but not *in vitro*; moreover, enhanced defence was not detected in thiadiazole-treated rice inoculated with 2-1-1. Based on these data, we infer that zinc thiazole and bismethiazol promote rice defence against *Xoo* by inhibiting the production of bacterial EPS.

Keywords: bismethiazol, defence responses, extracellular polysaccharides, rice, thiadiazole, *Xanthomonas oryzae* pv. *oryzae*, zinc thiazole.

INTRODUCTION

Thiazole, isothiazole, thiadiazole and their derivatives not only have anti-microbial and anti-fungal properties, but also induce systemic acquired resistance (SAR) in host plants, and so are

widely used to reduce disease in plants and other organisms (Leoni *et al.*, 2014; Oostendorp *et al.*, 2001; Turan-Zitouni *et al.*, 2004). Although two novel thiadiazoles—zinc thiazole and bismethiazol—have been used to control bacterial leaf blight (BLB) of rice in China for several years (Chen *et al.*, 2014; Zhang *et al.*, 2005, 2013; Fig. S1, see Supporting Information), their modes of action are poorly understood.

When attacked by certain pathogens, plants may recognize the pathogens and activate complex defence responses by the initiation of appropriate signalling processes (Durrant and Dong, 2004). The defence responses mainly include reactive oxygen species (ROS) bursts, callose deposition, hypersensitive response-like cell death and the expression of defence genes (Hammond-Kosack and Jones, 1996; Lamb and Dixon, 1997). The defence genes, which mainly include those encoding pathogenesis-related (PR) proteins, play an important role in defence responses. For example, the genes *OsPR1a* and *OsPR1b* are commonly used as markers for defence responses in many plant species (Mitsuhashi *et al.*, 2008; Van Loon and Van Strien, 1999). *POX22.3*, a peroxidase gene, is expressed during resistant responses of rice to the BLB pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Chittoor *et al.*, 1997; Liu *et al.*, 2005). The induction of defence responses in rice occurs through a complex signal transduction network, which mainly involves salicylic acid (SA) and jasmonic acid (JA) (De Vleeschauwer *et al.*, 2013; Fitzgerald *et al.*, 2004; Qiu *et al.*, 2007). *OsPAL* (encoding phenylalanine ammonia-lyase, *PAL*) and *OsLOX* (encoding lipoxygenase, *LOX*) are key genes in the synthesis of SA and JA, respectively (Coquoz *et al.*, 1998; Lee *et al.*, 2004; Mase *et al.*, 2005).

As an important virulence factor of pathogenic bacteria, extracellular polysaccharide (EPS) helps pathogenic bacteria avoid and delay the activation of plant defence responses (Jahr *et al.*, 1999; Parniske *et al.*, 1994). For example, EPS cloaks *Ralstonia solanacearum* from recognition by its plant host (Milling *et al.*, 2011); EPS induces plant susceptibility to *Xanthomonas campestris* pv. *campestris* by chelating calcium and suppressing callose deposition (Aslam *et al.*, 2008; Yun *et al.*, 2006). However, the role of EPS produced by *Xoo* in its interaction with rice plants is poorly understood.

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Previous research has shown that *Xoo* isolates induced on nutrient agar (NA) medium are resistant based on growth in bismethiazol-amended medium, but are not resistant on bismethiazol-treated rice and, remarkably, show a decrease or even loss of virulence; moreover, field isolates resistant to bismethiazol based on pathogenicity are sensitive *in vitro* (Zhu *et al.*, 2013). These results indicate that resistance based on direct biocidal effects can be separated from resistance based on the induction of host defences. The mutant *Xoo* 2-1-1, which was selected on bismethiazol-treated rice inoculated with wild-type *Xoo* ZJ173, shows cross-resistance to zinc thiazole *in vivo*, but not *in vitro* (Zhu *et al.*, 2014). The mechanism by which *Xoo* 2-1-1 resists these thiadiazoles *in vivo* is unknown.

The objectives of this study were: (i) to determine whether zinc thiazole and bismethiazol affect the defence responses of rice against *Xoo*; (ii) to determine whether *Xoo* EPS is involved in *Xoo* resistance against rice defence; (iii) to investigate the relation between thiadiazole compounds, EPS and rice defence; and (iv) to study the mechanism by which *Xoo* isolates resist thiadiazole compounds.

RESULTS

Effects of zinc thiazole on H₂O₂ levels and BLB lesions in *Xoo*-inoculated rice leaves

An increase in H₂O₂ is one of the earliest defence responses in plants to pathogen infection or elicitor treatment (Orozco-Cárdenas *et al.*, 2001). In the current study, we determined whether zinc thiazole (at 200 mg/L) and *Xoo* inoculation, alone or in combination, induced the generation of extracellular H₂O₂ in rice plants. The results showed that zinc thiazole alone did not trigger the release of H₂O₂, but that zinc thiazole applied 1 day after *Xoo* inoculation triggered a sustained release of H₂O₂ (Fig. 1a). The application of zinc thiazole 1 day before *Xoo* inoculation also tended to induce H₂O₂ production, but the increase in H₂O₂ was significant only on the third day after treatment (Fig. 1a).

We also investigated the effect of zinc thiazole application time on BLB lesion expansion on rice leaves. Zinc thiazole inhibited BLB lesion expansion when applied 1 day before or 1 day after *Xoo* inoculation, but the inhibition was greater when the compound was applied before rather than after *Xoo* inoculation (Fig. 1b).

H₂O₂ levels and *Xoo* density in rice leaves are affected by zinc thiazole concentration

To determine the effect of zinc thiazole concentration on defence responses of rice against *Xoo*, we assessed the effect of zinc thiazole concentration on H₂O₂ generation and on the density of *Xoo* in rice leaves. H₂O₂ levels increased gradually as the zinc thiazole concentration increased from 0 to 200 mg/L, but then

decreased when the concentration was raised to 300 mg/L (Fig. 1c). In contrast, the density of *Xoo* in the leaves decreased steadily as the zinc thiazole concentration increased from 0 to 300 mg/L (Fig. 1d).

Effects of zinc thiazole on the expression of defence-related genes in *Xoo*-inoculated leaves

The induction of H₂O₂ by elicitors is often followed by the expression of defence genes (Apel and Hirt, 2004). Therefore, we measured the effect of zinc thiazole treatment on the expression of five defence-related genes (*OsPR1a*, *OsPR1b*, *POX22.3*, *OsPAL* and *OsLOX*) in rice. The transcription levels of all five genes were increased slightly by *Xoo* alone, but not by zinc thiazole alone (Fig. 1e). Transcription levels of *OsPR1a*, *OsPR1b*, *POX22.3* and *OsPAL*, but not *OsLOX*, were greatly increased when *Xoo* inoculation was followed by zinc thiazole treatment (Fig. 1e). PAL activity and SA levels were increased when *Xoo*-inoculated plants were treated with zinc thiazole, but not when inoculated rice plants were not treated with zinc thiazole or when non-inoculated rice plants were treated with zinc thiazole (Fig. 1f,g).

Effects of bismethiazol on defence-related gene expression and H₂O₂ levels in *Xoo*-inoculated rice leaves

We also assessed whether the expression of the five defence-related genes and the generation of H₂O₂ in plants was affected by bismethiazol application alone, *Xoo* inoculation alone or bismethiazol application 1 day after *Xoo* inoculation. The expression of *OsPR1a*, *OsPR1b*, *POX22.3* and *OsPAL*, but not *OsLOX*, was strongly increased when plants were inoculated with *Xoo* and then treated with bismethiazol; however, expression was not increased by bismethiazol alone (Fig. 2a). In addition, bismethiazol treatment triggered a sustained release of H₂O₂ in *Xoo*-inoculated rice leaves, but not in non-inoculated leaves (Fig. 2b).

Cellular defence responses of rice leaves against *Xoo* when treated with the two thiadiazoles

To determine whether zinc thiazole and bismethiazol are involved in the cellular defence responses of rice, we examined the H₂O₂ burst, callose deposition and cell death in plants that were treated with *Xoo* alone, zinc thiazole alone, bismethiazol alone, or zinc thiazole or bismethiazol 1 day before *Xoo* inoculation. The H₂O₂ burst, callose deposition and cell death were substantially greater in leaves that were treated with zinc thiazole or bismethiazol following *Xoo* inoculation than in leaves treated with *Xoo* alone or with the compounds alone (Fig. 3).

To determine whether H₂O₂ is necessary in the defence induced by zinc thiazole and bismethiazol, we used catalase

Fig. 1 Effects of zinc thiazole on H₂O₂ levels, bacterial leaf blight (BLB) lesions, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) density, expression of defence genes and signalling pathways in rice leaves. Leaves were not inoculated or were inoculated with *Xoo* on day 0 or day 2. Leaves were not treated with zinc thiazole or were treated with 200 mg/L zinc thiazole on day 1. The five treatments were as follows: CK, not inoculated with *Xoo* and not treated with zinc thiazole; T1, not treated with zinc thiazole, but inoculated with *Xoo*; T2, treated with zinc thiazole, but not inoculated with *Xoo*; T3, treated with zinc thiazole 1 day after *Xoo* inoculation; T4, treated with zinc thiazole 1 day before *Xoo* inoculation. (a) H₂O₂ levels at 2-day intervals after zinc thiazole treatment. (b) Zinc thiazole inhibition of lesion expansion on leaves; inhibition was calculated relative to the controls that were not treated with zinc thiazole, and was determined 7 days after zinc thiazole application. H₂O₂ levels (c) and *Xoo* density (d) in *Xoo*-inoculated rice leaves, 3 days after application of different concentrations of zinc thiazole. Expression of defence genes (e), phenylalanine ammonia-lyase (PAL) activity (f) and salicylic acid (SA) levels (g) were determined 3 days after *Xoo*-inoculated rice leaves had been treated with 200 mg/L zinc thiazole. Values are the means of three experiments, with three replicate plants per experiment. For (a) and (c–g), means with different letters are significantly different according to Fisher's least-significant difference test ($P < 0.05$). For (b), an asterisk indicates a significant difference ($P < 0.05$) between application time according to Student's *t*-test.

Fig. 2 Defence-related gene expression and levels of H_2O_2 in rice leaves on application of bismethiazol following *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (strain ZJ173) inoculation. Leaves were not inoculated (CK), were inoculated with *Xoo* alone (ZJ173), were treated with bismethiazol alone (Bismethiazol) or were treated with bismethiazol 1 day after *Xoo* inoculation (ZJ173 + Bismethiazol). (a) Expression of defence-related genes. (b) Changes in H_2O_2 levels over time. Values are means and standard errors (SEs) of three independent experiments (results were similar for each experiment). Means with different letters are significantly different according to Fisher's least-significant difference test ($P < 0.05$).

Fig. 3 Effects of the two thiadiazoles on the cellular defence responses against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) in rice. Rice plants were treated with zinc thiazole, bismethiazol or water. One day later, the plants were inoculated or not inoculated with *Xoo*, and the inoculated plants were immediately treated with a catalase solution at 5000 units/mL or water. '+' indicates that the plants were inoculated or treated with the indicated compounds, and '-' indicates that the plants were not inoculated or were treated with water instead of the indicated compounds. Leaves were stained and photographed 24 h after inoculation to observe the H_2O_2 burst (indicated by brown staining), callose deposition (indicated by fluorescence) and cell death (indicated by blue staining). Bar, 100 μ m.

to decompose H_2O_2 without inhibiting pathogen proliferation (Ahn *et al.*, 2007; Van Wees and Glazebrook, 2003). Catalase applied alone to plants did not induce any evident responses, whereas catalase suppressed the H_2O_2 burst, callose deposition and cell death in leaves treated with the compounds and *Xoo* (Fig. 3).

Inhibition of EPS production by the two thiadiazoles

Both thiadiazoles reduced the production of EPS by *Xoo* (Fig. 4a). The reduction in EPS production was not increased by using a higher concentration of zinc thiazole, probably because the solubility of zinc thiazole is low. In contrast, the reduction in EPS

Fig. 4 Inhibition of extracellular polysaccharide (EPS) production by the two thiadiazoles. (a) Production of EPS in thiadiazole-treated *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) cultures relative to production in non-treated cultures. (b) Effects of the two thiadiazoles on the transcription level of six EPS synthesis genes in *Xoo*. The treatments were as follows: CK, not treated with zinc thiazole and bismethiazol; a1, 45 mg/L zinc thiazole; a2, 90 mg/L zinc thiazole; b1, 6 mg/L bismethiazol; b2, 12 mg/L bismethiazol. Values are means and standard errors (SEs) of three independent experiments. Datasets marked with an asterisk are significantly different from the control as assessed by Student's *t*-test: **P* < 0.05.

production was increased by using a higher concentration of bismethiazol. The expression of EPS synthesis genes was usually suppressed by one or both of the thiadiazoles (Fig. 4b).

Effects of EPS on defence responses in rice

To determine whether EPS produced by *Xoo* suppresses rice defence responses, we treated *Xoo*-inoculated plants with EPS extracted from *Xoo* and then measured the expression of defence-related genes. The transcription levels of three *PR* genes and the *OsPAL* gene were increasingly suppressed as the EPS concentration increased, but *OsLOX* expression was not affected by EPS (Fig. 5a). In addition, EPS treatment enhanced the formation and expansion of BLB lesions on inoculated rice leaves (Fig. 5b).

We also examined the expression level of the *OsPR1b* gene in rice plants inoculated with $\Delta gumH$, which is an EPS-deficient mutant of *Xoo*. $\Delta gumH$ induced stronger expression of defence genes than ZJ173 when EPS was not added (Fig. 5c). When 200 mg/L EPS was added to the $\Delta gumH$ -inoculated rice plants, $\Delta gumH$ induced equal or even lower defence gene expression than ZJ173 (Fig. 5c).

Effect of exogenous EPS on defence responses induced by the two thiadiazoles

To determine whether exogenous EPS could reverse the defence induced by the thiadiazoles, we assessed the defence responses of rice against *Xoo* on plants treated with zinc thiazole or bismethiazol and with or without 200 mg/L EPS. The H_2O_2 production and transcription levels of three *PR* genes and the *OsPAL* gene were suppressed by EPS in both zinc thiazole- and

bismethiazol-treated plants, but *OsLOX* gene expression was not affected (Fig. 6a,b). In addition, the inhibitory effects of the two compounds on BLB lesion formation were reduced by the addition of EPS (Fig. 6c).

Effects of the two thiadiazoles on defence responses of rice leaves inoculated with the mutant 2-1-1

The *Xoo* mutant 2-1-1 is resistant to zinc thiazole and bismethiazol *in vivo*, but not *in vitro* (Zhu *et al.*, 2014), suggesting that its *in vivo* resistance involves the failure to induce host defences rather than a reduced sensitivity to the compounds. We found that EPS production by 2-1-1 was not affected by zinc thiazole or bismethiazol (Fig. 7a). Similarly, the expression of EPS synthesis genes was seldom increased by the two thiadiazoles (Fig. 7b), the expression of defence-related genes in rice inoculated with 2-1-1 was not increased by the two thiadiazoles (Fig. 7c), and lesion formation by 2-1-1 was not reduced by the two thiadiazoles (Fig. 7d).

DISCUSSION

Previous research has indicated that thiadiazole compounds protect rice plants against *Xoo* by inhibiting the pathogen's growth (Chen *et al.*, 2014; Yakushiji and Wakae, 1971; Zhang *et al.*, 2005; Zhu *et al.*, 2014). In this study, we discovered another mechanism by which thiadiazole compounds inhibit *Xoo*. Our results showed that zinc thiazole and bismethiazol enhance H_2O_2 production, cellular defence responses and defence-related gene expression in *Xoo*-inoculated rice leaves. These results indicate that zinc thiazole and bismethiazol can enhance rice defence against *Xoo*.

Fig. 5 Effects of exogenous extracellular polysaccharide (EPS) on the defence responses in rice. For CK, rice plants were not inoculated with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) or treated with EPS. For the EPS treatment, non-inoculated plants were treated with 50 mL of 200 mg/L EPS. For the ZJ173 treatment, plants were inoculated with *Xoo* (strain ZJ173). For the three other treatments, the *Xoo*-inoculated plants were treated with 50 mL of EPS (50, 100 or 200 mg/L) or water 1 day after inoculation. (a) Defence-related gene expression was measured 3 days after the treatments had been applied. (b) Lesion lengths of the leaves were measured 7 days after EPS application. (c) *OsPR1b* gene expression of EPS-treated and untreated rice leaves that were inoculated with $\Delta gumH$, which is a *Xoo* mutant that is deficient in EPS production; *OsPR1b* gene expression is relative to that in ZJ173-inoculated rice leaves. Values are means and standard errors (SEs) of three independent experiments.

Fig. 6 Effect of exogenous extracellular polysaccharide (EPS) on defence responses induced by the two thiadiazole compounds. Rice plants were inoculated with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and, 1 day later, were treated with 50 mL of zinc thiadiazole or bismethiazol and with or without 200 mg/L EPS. H_2O_2 levels (a) and defence-related gene expression (b) were measured 3 days after treatments. The lengths of bacterial leaf blight (BLB) lesions were measured 7 and 15 days after treatments, and the inhibition rate (c) was calculated. Values are the means and standard errors (SEs) of three replicates. Bars with asterisks are significantly different from the control according to Student's *t*-test at $P < 0.05$.

Fig. 7 Effect of the two thiadiazoles on extracellular polysaccharide (EPS) production of the *Xanthomonas oryzae* pv. *oryzae* (Xoo) mutant 2-1-1, and defence-related gene expression in rice leaves inoculated with 2-1-1. (a) Effect of the two thiadiazoles on EPS production of the thiadiazole-resistant 2-1-1. (b) Effect of the two thiadiazoles on the transcription level of EPS synthesis genes in the thiadiazole-resistant 2-1-1. The treatments were divided into five groups: CK, control (not treated with thiadiazole compounds); a1, treated with 45 mg/L of zinc thiazole; a2, treated with 90 mg/L of zinc thiazole; b1, treated with 6 mg/L of bismethiazol; b2, treated with 12 mg/L of bismethiazol. Datasets marked with an asterisk are significantly different from the control as assessed by Student's *t*-test: **P* < 0.05. (c) Effect of the two thiadiazoles on defence genes in thiadiazole-resistant 2-1-1-inoculated rice leaves. (d) Effect of the two thiadiazoles on the formation of bacterial leaf blight (BLB) lesions in thiadiazole-resistant 2-1-1-inoculated rice leaves. The four treatments were as follows: CK, control (not inoculated with Xoo and not treated with thiadiazoles); T1, not treated with thiadiazoles, but inoculated with Xoo 2-1-1; T2, treated with zinc thiazole 1 day after Xoo 2-1-1 inoculation; T3, treated with bismethiazol 1 day after Xoo 2-1-1 inoculation; T4, not treated with thiadiazoles, but inoculated with Xoo ZJ173; T5, treated with zinc thiazole 1 day after Xoo ZJ173 inoculation; T6, treated with bismethiazol 1 day after Xoo ZJ173 inoculation. Values are means and standard errors (SEs) of three independent experiments.

Because the rapid production and accumulation of H_2O_2 is a recognized part of various defence processes (Low and Merida, 1996; Tanaka *et al.*, 2003), we infer that the increased H_2O_2 production by thiadiazole-treated and Xoo-inoculated rice represents a defence response. In support of this inference, we found that the removal of H_2O_2 by catalase nullified the defence responses of rice induced by the two thiadiazole compounds. The results indicate that H_2O_2 acts as a trigger and mediator of thiadiazole-induced defence responses.

H_2O_2 production and inhibition were greater when zinc thiazole was applied 1 day after rather than 1 day before Xoo inoculation; moreover, when the concentration of zinc thiazole applied

was increased from 200 to 300 mg/L, H_2O_2 production decreased. These data suggest that thiadiazole-induced defence depends on the application time and concentration of thiadiazole. However, Xoo density in leaves decreased as the thiadiazole concentration increased. As an aminoglycoside used for BLB control in China, streptomycin did not enhance the defence of Xoo-inoculated rice (Xu *et al.*, 2010; Fig. S2, see Supporting Information). Overall, these findings indicate that zinc thiazole and bismethiazol enhance rice defence against Xoo and inhibit Xoo reproduction.

Some thiazole and thiadiazole compounds, such as probenazole, vitamin B1 and benzothiadiazole, can directly induce

SAR-mediated protection against BLB (Ahn *et al.*, 2007; Lin *et al.*, 2008; Mohan Babu *et al.*, 2003). Moreover, probenazole and benzothiadiazole can induce plant defence responses in rice by acting either upstream or downstream of SA (De Vleeschauwer *et al.*, 2012, 2013). Although the two thiadiazoles might work through the SA signalling pathway in *Xoo*-inoculated rice, treatment with zinc thiazole or bismethiazol only did not induce rice defence, indicating that zinc thiazole and bismethiazol failed to induce SAR in rice plants. Therefore, the mechanism of plant defence induction may differ between zinc thiazole and bismethiazol vs. probenazole, vitamin B1 and benzothiadiazole.

Because it is needed for the production of bacterial biofilms, EPS is an important virulence factor, especially for vascular pathogens (Li and Wang, 2011; Milling *et al.*, 2011; Torres *et al.*, 2006). Previous reports have shown that EPS protects *R. solanacearum* and *X. campestris* pv. *campestris* from host defences (Aslam *et al.*, 2008; Milling *et al.*, 2011; Yun *et al.*, 2006). Until now, no reports have evaluated the role of EPS in the susceptibility of rice to *Xoo*. Here, we found that treatment of rice leaves with EPS extracted from *Xoo* inhibited the expression of defence genes in *Xoo*-inoculated rice plants and enhanced the formation and expansion of BLB lesions. The EPS-deficient mutant $\Delta gumH$ induced much stronger defence than ZJ173, and the addition of EPS to rice plants decreased the defence induced by $\Delta gumH$. These results suggest that EPS produced by *Xoo* suppresses rice defence and enhances the susceptibility of rice to *Xoo*.

Several recent studies have shown that some thiazole compounds have substantial anti-biofilm activity against Gram-positive and Gram-negative bacteria (Mohammad *et al.*, 2014; More *et al.*, 2014; Rane *et al.*, 2012). Another report has indicated that bismethiazol significantly decreases EPS production (Ma *et al.*, 1997). The current study confirmed these results in that zinc thiazole and bismethiazol inhibited EPS production by *Xoo*. Interestingly, exogenous EPS did not affect the inhibitory effects of either compound *in vitro* (Fig. S3, see Supporting Information), whereas EPS addition to the plants strongly decreased the induction of defence responses. Therefore, we infer that zinc thiazole and bismethiazol promote rice defence against *Xoo* by reducing EPS production.

A previous report has described field isolates of *Xoo* that are bismethiazol resistant in the field, but bismethiazol sensitive *in vitro* (Zhu *et al.*, 2013). The mutant 2-1-1, which was screened from the wild-type *Xoo* (strain ZJ173) in bismethiazol-treated rice, is resistant to zinc thiazole and bismethiazol *in vivo*, but not *in vitro* (Zhu *et al.*, 2014). These results suggest that the resistance mechanism of *Xoo* against thiadiazoles can differ *in vivo* vs. *in vitro*. In our research with 2-1-1, we found that zinc thiazole and bismethiazol did not reduce EPS production of 2-1-1 and did not enhance the defence responses of rice leaves inoculated with 2-1-1. These findings may explain why 2-1-1 is resistant to thiadiazoles *in vivo*.

In summary, the data indicate that EPS helps *Xoo* to resist rice defences, and the two thiadiazoles promote rice defence against *Xoo* by inhibiting the production of bacterial EPS. In addition, the mechanism by which *Xoo* isolates resist thiadiazole compounds *in vivo* is related to the induction of rice defence and requires further work.

EXPERIMENTAL PROCEDURES

Bacterial strain, rice cultivar and culture conditions

The wild-type strain *Xoo* ZJ173, which is sensitive to zinc thiazole, was kindly provided by Professor Zhi-Gang Xu of Nanjing Agricultural University, Nanjing, Jiangsu Province, China. The EPS-deficient mutant $\Delta gumH$ was previously constructed on the basis of ZJ173 in our laboratory, and the mutant 2-1-1 was screened from ZJ173 on bismethiazol-treated rice leaves. *Xoo* was grown on NA containing 5 g of polypeptone, 1 g of yeast powder, 3 g of beef extract, 10 g of sucrose and 16 g of agar powder per litre. Nutrient broth (NB) contained the same ingredients, but lacked agar. Rice cultivar IR24, which is susceptible to *Xoo*, was used in this study when it had grown to the tillering stage. IR24 was grown in 500-cm³ plastic pots (three plants per pot) containing autoclaved soil in a glasshouse at 20–30 °C with ambient light and relative humidity.

Compounds

Zinc thiazole (purity, 98.7%) and bismethiazol (purity, 92.5%) were provided by Xinnong Chemical Co. Ltd., Hangzhou, Zhejiang Province, China and Longwan Agrichemical Co. Ltd., Wenzhou, Zhejiang Province, China, respectively, and were dissolved in *N,N*-dimethylformamide as stock solutions. *N,N*-Dimethylformamide was purchased from Sinopharm Chemical Reagent Co. Ltd., Shanghai, China. Tween 80 at 250 mg/L was added to the solutions when the compounds were applied to plants.

Plant treatment

Rice plants were treated with 50 mL of water or 50 mL of compounds at 200 mg/L on day 1. Rice plants were inoculated with a suspension of 10⁹ colony-forming units (CFU)/mL of *Xoo* (or with NB alone as a control) on day 0 or day 2 using the leaf clipping method (Kauffman *et al.*, 1973). Each treatment was represented by three replicate pots, and the experiment was repeated three times.

Measurement of H₂O₂ levels in rice leaves

H₂O₂ levels in *Xoo*-inoculated rice leaves were determined 1, 3, 5 and 7 days after the compounds had been applied. A 50-mg quantity of the washed leaves was ground in 750 μ L of sodium phosphate buffer (50 mM, pH 7.4) with a mortar and pestle. The homogenate was centrifuged at 10 000 *g* for 20 min at 4 °C, and the supernatants were collected for assays. H₂O₂ levels were measured with reagent kits (Nanjing Jiancheng Biology Institution, Nanjing, Jiangsu Province, China) according to the

instruction manual. H_2O_2 levels were determined by monitoring the absorbance of the titanium peroxide complex at 405 nm.

Gene expression in rice leaves

Total RNA was isolated from leaves using the TIANGEN PLANT RNA Kit (Tiangen Biotech, Beijing, China) and reverse transcribed to cDNA using the cDNA Synthesis Kit (TakaRa, Dalian, China). PCR primers were designed using Primer 5.0 (Table S1, see Supporting Information). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed with the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). 18s RNA was used as a reference gene (Kim *et al.*, 2003). qRT-PCRs were performed with SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. Each experiment was repeated at least three times with similar results.

Measurement of BLB lesions on rice leaves

The lengths of lesions on the clipped leaves were measured on day 7 or day 15. The inhibition rate (Zhu *et al.*, 2013) was calculated as follows: $1 - (\text{length of lesions treated with compounds} / \text{length of lesions not treated with compounds}) \times 100$.

Measurement of *Xoo* density in rice leaves

Rice plants were inoculated with *Xoo* or NB medium and, 1 day later, were treated with 50 mL of zinc thiazole at five concentrations (0, 25, 100, 200 and 300 mg/L). Three days after zinc thiazole treatment, the density of *Xoo* in the leaves was determined. A 50-mg quantity of washed leaves was soaked in a 5% sodium hypochlorite solution for 10 min, washed in sterile distilled water for 30 s and then homogenized in 2 mL of distilled water using a sterile mortar and pestle. The homogenate was serially diluted and 50 μL of each dilution was spread onto NA plates. After incubation for 4 days at 28 °C, the bacterial colonies on the plates were counted and the bacterial density per gram of leaf was calculated. Each treatment was represented by three replicate pots, and the experiment was repeated three times.

Observation of cellular defence responses of rice leaves

Rice plants were treated with 50 mL of water, or zinc thiazole or bismethiazol at 200 mg/L, 1 day before the upper leaves (length, 3 cm) were inoculated with *Xoo* or NB medium using a needle-less syringe (Mukoo and Yoshida, 1951). At 12 h after inoculation, the leaves of rice plants were sprayed with 50 mL of water or catalase at 5000 units/mL. Cellular defence responses (H_2O_2 burst, callose deposition and cell death) were assessed 24 h after inoculation. H_2O_2 accumulation was assessed after staining leaves with 3,3-diaminobenzidine tetrachloride and examining them under visible light with a 10 $\times$ objective (Rea *et al.*, 2004). Callose deposition was assessed by staining leaves with aniline blue and examining them under ultraviolet light with a 10 $\times$ objective (Reuber *et al.*, 1998). Leaves with symptoms of hypersensitive response-like cell death were photographed after staining with trypan blue (Peng *et al.*, 2003).

Measurement of PAL activity and SA

Apical segments (length, 3 cm) of rice leaves were removed and washed thoroughly with deionized water. A 50-mg quantity of washed leaf tissue was then homogenized with a mortar and pestle under chilled conditions in 0.65 mL of 50 mM Tris-HCl buffer (pH 8.8) containing 15 mM of β -mercaptoethanol. The homogenate was centrifuged at 10 000 *g* for 20 min at 4 °C, and the supernatant was retained as the enzyme extract. A 0.1-mL volume of the enzyme extract, plus 1 mL of the extraction buffer, 0.5 mL of 10 mM L-phenylalanine and 0.4 mL of deionized water, was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 0.5 mL of 6 M HCl, and the product was extracted with 15 mL of ethyl acetate using a rotary evaporator. The solid residue was suspended in 3 mL of 0.05 M NaOH, and the cinnamic acid concentration was measured spectrophotometrically by the absorbance at 290 nm. One unit of PAL activity was defined as 1 μmol of cinnamic acid produced per minute.

Total SA was extracted and measured as described by Verberne *et al.* (2002). The sample was dissolved in 2.4 mL of the eluent. Samples were passed through a 0.22- μm filter, and 20 μL was used for high-performance liquid chromatography. The eluent was 0.2 M sodium acetate buffer (pH 5.5) (75%) with methanol (25%) at a flow rate of 0.80 mL/min. The absorbance of the eluted samples was recorded at 295 nm, and SA concentrations were determined by comparison with SA standards.

Measurement of EPS production and expression of genes responsible for EPS synthesis in *Xoo*

EPS was extracted as described previously (Vojnov *et al.*, 1998). *Xoo* was grown in NB at 28 °C to the late logarithmic growth phase, and the suspension was diluted to approximately 10^7 CFU/mL. A 100- μL volume of *Xoo* suspension was added to 30 mL of NB in 50-mL Erlenmeyer flasks containing different concentrations of zinc thiazole (0, 45 or 90 mg/L) or bismethiazol (0, 6 or 12 mg/L). EPS production and the expression of EPS synthesis genes were determined when thiadiazole-treated and untreated bacteria grew to population densities of 10^9 CFU/mL. For the EPS production assay, the cells were removed from 20 mL of culture by centrifugation (10 000 *g* for 5 min). EPS in the supernatant was centrifuged at 10 000 *g* for 5 min at 4 °C after the addition of three volumes of ethyl alcohol. EPS production was determined with the phenol-sulfuric acid method (Dubois *et al.*, 1956).

For the determination of the expression of the genes responsible for the synthesis of EPS, total RNA was isolated from leaves using the TIANGEN BACTERIA RNA Kit (Tiangen Biotech) and was reverse transcribed to cDNA using the TaKaRa cDNA Synthesis Kit. The qRT-PCR assay was performed as described previously. The PCR primers are listed in Table S2 (see Supporting Information). The experiments were repeated three times independently with three replicates each time.

The method of purification and application of EPS

EPS was extracted from *Xoo* cultures as described earlier. To increase the purity of EPS, ethanol precipitation was performed one to three times to precipitate EPS. After centrifugation (10 000 *g* for 5 min), the resulting EPS pellet was solubilized in sterile distilled water and purified by dialysis,

using a 10 kDa molecular weight cutoff (MWCO) cellulose membrane, against cooled and stirred distilled water for 2 days. After determination of EPS concentrations, EPS was diluted to three concentrations (50, 100 and 200 mg/L) and sprayed onto the leaves of rice plants.

ACKNOWLEDGEMENTS

This study was supported by the Special Fund for Agro-scientific Research in the Public Interest (No. 201303023) and the National Science and Technology Support Program (2012BAD19B01).

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SUPPORTING INFORMATION

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

Fig. S1 Chemical structure of zinc thiazole (A) and bismethiazol (B).

Fig. S2 Defence-related gene expression and levels of H₂O₂ in rice leaves on application of streptomycin following *Xanthomonas oryzae* pv. *oryzae* (Xoo) (strain ZJ173) inoculation. Rice plants were inoculated with Xoo and then treated with 50 mL of 200 mg/L streptomycin or water. As control, rice leaves were not inoculated with Xoo and not treated with streptomycin. Gene expression was measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), 3 days after treatment. Values are means and standard errors (SEs) of three independent experiments.

Fig. S3 *In vivo* inhibitory effects of the two thiadiazoles and exogenous extracellular polysaccharide (EPS) on *Xanthomonas oryzae* pv. *oryzae* (Xoo) growth. A 100-μL Xoo suspension [10⁷ colony-forming units (CFU)/mL] was added to 30 mL of nutrient broth (NB) medium in 50-mL Erlenmeyer flasks containing serial concentrations of zinc thiazole (0, 45 mg/L) or bismethiazol (0, 6 mg/L) in the presence of EPS. When the cell density of the suspension in the control flask increased to approximately 10⁸ CFU/mL, optical density values were regressed on thiadiazole concentration to obtain the inhibition rate. Letters Z and B represent zinc thiazole and bismethiazol, respectively. Values are means and standard errors (SEs) of three independent experiments.

Table S1 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) primers for plant defence-related genes in rice.

Table S2 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) primers for extracellular polysaccharide (EPS) synthesis genes in *Xanthomonas oryzae* pv. *oryzae* (Xoo).