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Accumulation of HDMBOA-Glc is induced by biotic stresses prior to the release of MBOA in maize leaves

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Abstract

The effects of biotic stresses on the contents of benzoxazinones (Bxs) were investigated in maize leaves. When the causal agent of southern corn leaf blight, *Bipolaris maydis*, was inoculated on the third leaf, the amount of 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside (HDMBOA-Glc) increased, reaching a maximum level 48 h after inoculation. The inoculation of weakly pathogenic *Curvularia lunata* and non-pathogenic *Alternaria alternata* also resulted in accumulation of HDMBOA-Glc, and filtrates of the cultures of *B. maydis*, *C. lunata* and *A. alternata* also showed the accumulation of elicitor-active compounds by the fungi. Furthermore the infection of *B. maydis* induced formation of dark brown lesions, where most abundant Bx-related compound was 6-methoxy-2-benzoxazolinone (MBOA). The later is formed by degradation of DIMBOA and HDMBOA, whereas HDMBOA-Glc was most abundant in the surrounding green tissues. Among the Bx-related compounds, MBOA exhibited the strongest inhibition of the germination of the conidia and of the growth of germ tubes of *B. maydis*, *C. lunata* and *A. alternata*. In addition to fungal infection, the feeding by rice armyworm larvae resulted in HDMBOA-Glc accumulation. These findings are discussed in relation to the possible ecological relevance of the conversion of DIMBOA-Glc into HDMBOA-Glc.

Keywords: *Zea mays*; Gramincae: benzoxazinone; Armyworm; Elicitor; Infection by pathogens; HDMBOA-Glc; MBOA

1. Introduction

Benzoxazinones (Bxs) are the major secondary metabolites found in poaceous plants, such as maize, wheat and rye, and are involved in the defense response against pathogens and insects (Niemeyer, 1988). The main Bx in maize and wheat is 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside (DIMBOA-Glc, **1**). Bxs are sequestered in the vacuole as glucosides, and when the tissue is damaged by pathogen infection or feeding by herbivores, Bx glucosides react with β-glucosidase that is present in the plastid. Consequently, Bx glucosides are hydrolyzed and release aglycones, which have strong antifungal and antifeeding activities (Esen, 1992; Oikawa et al., 1999; Sue et al., 2000a,b).

2-Hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside (HDMBOA-Glc, **3**) has been identified in maize as a minor Bx (Hofman et al., 1970). Its aglycone, HDMBOA, has not been isolated following enzymatic hydrolysis of the glucoside because of its instability (Grambow et al., 1986). Recently, **3** was found to accumulate in maize leaves treated with chitooligosaccharides, $CuCl_2$ and jasmonic acid (Oikawa et al., 2001), and in wheat leaves treated with $CuCl_2$ and jasmonic

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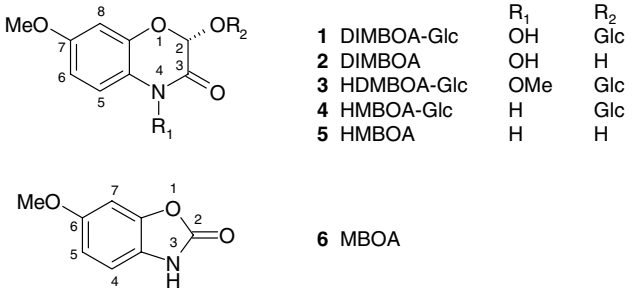
acid (Oikawa et al., 2002). The accumulation of **3** was accompanied by a decrease in constitutively present **1**. The labeled methyl group of [Me–$^{2}H_3$]L-methionine exogenously applied was efficiently incorporated into **3** in jasmonate-treated maize leaves (Oikawa et al., 2001). In addition, jasmonic acid induced DIMBOA-Glc 4-*O*-methyltransferase activity in wheat leaves (Oikawa et al., 2002). These lines of evidence indicate the induced conversion of **1** into **3**.

Jasmonic acid acts as a signal mediator in response to various stresses and elicits defense reactions (Sembdner and Parthier, 1993; Creelman and Mullet, 1997), and chitooligosaccharides (Bordin et al., 1991) and $CuCl_2$ (Kodama et al., 1988; Rouxel et al., 1991) also elicit defense reactions. Accordingly, the accumulation of **3** has been suggested to be part of an inducible defense reaction in Bx-accumulating plants. However, the role of **3** has not been clarified in plants attacked by parasites and herbivores even though **3** accumulates in wheat leaves infected by stem rust fungus in moderately resistant cultivars (Bücker and Grambow, 1990). In the present study, we investigated the induction of accumulation of **3** in maize leaves by biotic stresses including infection of phytopathogenic fungi and feeding by caterpillars. In addition, after analyzing the composition of Bxs in and around the site of infection, we examined the antifungal activity of Bxs and degradation product of Bxs, 6-methoxy-2-benzoxazolinone (MBOA, **6**), to address the ecological significance of the conversion of **1** into **3**. The structures of the Bxs and **6** studied are shown in Fig. 1.



		R_1	R_2
1	DIMBOA-Glc	OH	Glc
2	DIMBOA	OH	H
3	HDMBOA-Glc	OMe	Glc
4	HMBOA-Glc	H	Glc
5	HMBOA	H	H

6 MBOA

Fig. 1. The structures of Bxs and MBOA (**6**).

2. Results

2.1. Induction of 3 accumulation by inoculation with pathogenic fungi

To examine the effect of fungal infection on **3** accumulation, the third leaf segments of maize were inoculated with hyphal suspensions of *B. maydis*, *C. lunata* and *A. alternata* (Fig. 3). In the leaves inoculated with *B. maydis*, which is strongly pathogenic toward maize, the concentration of **3** rapidly increased and reached a maximum of 6 μmol/g fr. wt 48 h after infection. The level of **3** remained high for up to 96 h. Inoculation of *C. lunata*, which is weakly pathogenic toward maize, also caused increased accumulation of **3**, although it was delayed by 48 h. The maximum amount of **3** was close to the amount in the leaves inoculated with *B. maydis*. In the leaves inoculated with *A. alternata*, which is not pathogenic toward maize, **3** increased in a similar manner. In the leaves treated with water, the concentration of **3** remained at a constant and low level after 48 h, and gradually increased thereafter to 4.1 μmol/g fr. wt 96 h after infection. A slight increase in the concentration of **3** was also detected in the control leaves, probably due to aging (Cambier et al., 2000). The accumulation of **3** was always accompanied by an equivalent decrease in **1** independent of the species of inoculated fungi (data not shown), indicating the conversion of **1**–**3** as

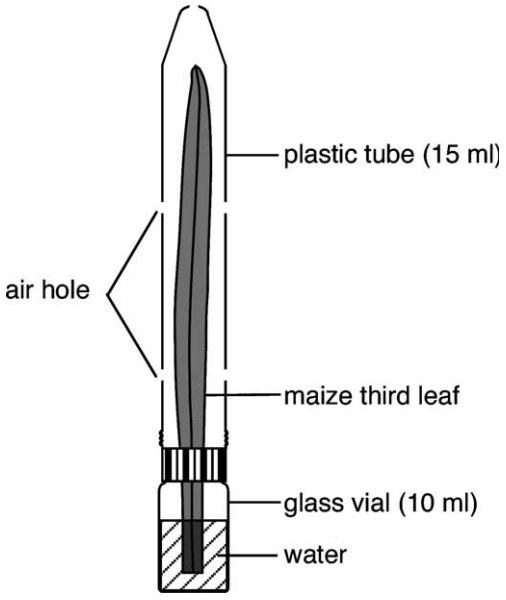


Fig. 2. The instrument used for feeding by the armyworm. A plastic tube and glass vial filled with water were fixed by scotch tape.

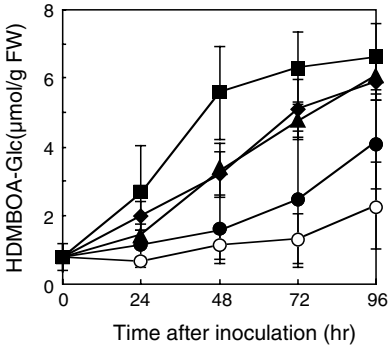


Fig. 3. Induction of the accumulation of HDMBOA-Glc (**3**) after inoculation with *B. maydis* (■), *C. lunata* (◆), and *A. alternata* (▲). The amounts of **3** in the leaves treated with distilled water (●) and intact leaves (○) were determined as controls. The error bars indicate the standard deviations of five replicates.

observed for the leaves treated with chitiooligosaccharides, $CuCl_2$ or jasmonic acid (Oikawa et al., 2001).

The strong pathogenicity of *B. maydis* was obvious from the necrotic lesions that developed at the sites of inoculation, which covered approximately 40% of the leaf area 48 h after inoculation. No lesion formed on the leaves inoculated with *C. lunata* or *A. alternata* throughout the experimental period.

2.2. Induction of accumulation of 3 by treatment with culture filtrates

The concentrated culture filtrates from all three species of fungi were active for the induction of **3** accumulation, with the accumulated amounts of **3** comparable to the quantities in leaf segments inoculated with the fungi (Fig. 4). Boiling the filtrates reduced the activity somewhat, suggesting the presence of heat-labile active components in the filtrates. Treatment of maize leaves with the concentrated filtrate from the culture of *B. maydis* resulted in the development of necrotic lesions regardless of heat treatment, with approximately 30% of the leaf segments being covered by lesions within 48 h of incubation. The concentrated filtrates from the cultures of *C. lunata* and *A. alternata* did not induce formation of necrotic lesions on leaf segments.

2.3. Composition of Bx-related compounds in and around the infection sites

The leaf segments infected with *B. maydis* were divided into the lesion and surrounding tissues 48 h after inoculation, and the Bxs composition in each part was determined. As shown in Fig. 5, the main detectable compound in the lesion was **6**, comprising 80% of the total of Bxs and benzoxazolinone. 2-Hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA, **5**) and **3** were also detected in the lesion, but their percentages were 15% and 7%, respectively. Since **5** released from its glucoside (HMBOA-Glc, **4**) does not spontaneously degrade under physiological conditions into **6** (Grambow et al., 1986), the compound appeared to remain intact in the lesions. In the surrounding tissues, the main Bx was **3** as expected from the time-course experiment (Fig. 3), comprising about 60% of the total Bxs found. The total concentration of Bx-related compounds in the lesion was much lower than that in the surrounding tissues; the concentration in the lesion area was on average 1.9 μmol/g fr. wt, whereas it was 14.7 μmol/g fr. wt in the surrounding tissues.

2.4. Antifungal activity of Bxs

The conidia of *B. maydis*, *C. lunata* and *A. alternata* were suspended in solutions of **1**, **2**, **3** and **6**. After 24-h of incubation, the number of germinated conidia

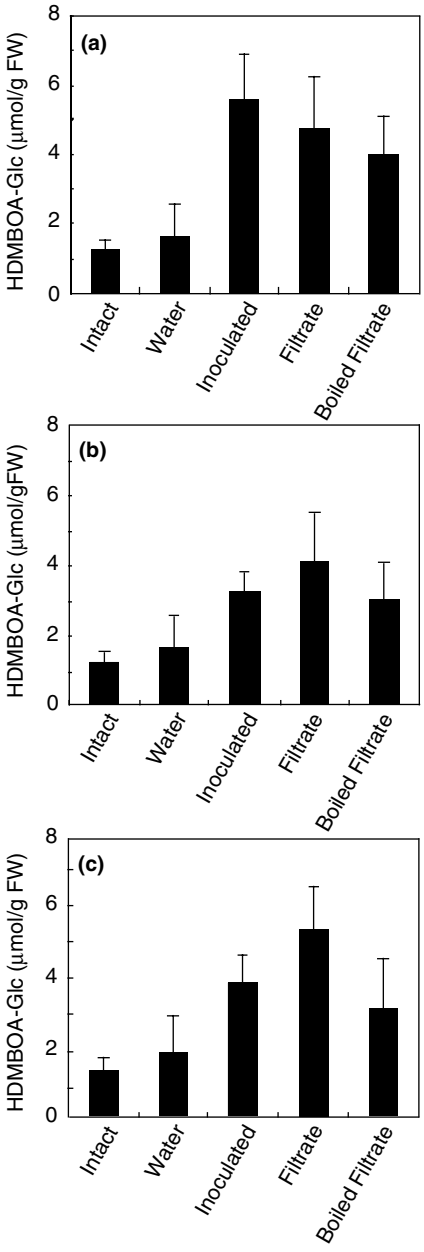


Fig. 4. Induction of the accumulation of HDMBOA-Glc (**3**) by treatment with culture filtrates of *B. maydis* (a), *C. lunata* (b) and *A. alternata* (c). The amounts of **3** were determined 48 h after treatment, and the quantities of **3** were determined in intact leaves (Intact), leaves treated water (Water) and leaves inoculated with fungi (Inoculation) as controls. The error bars indicate the standard deviations of five replicates.

was counted to assess the antifungal activity of Bxs. **1** and **3** had no inhibitory activity toward the germination of conidia even at 10 mM (Table 1), and **2** did not affect the germination rate but inhibited the growth of germ tubes at 10 mM. The average lengths of the germ tubes of *B. maydis* and *C. lunata* were approximately 60% and 40% of that of the control, respectively. **6** exhibited strong activity toward the inhibition of

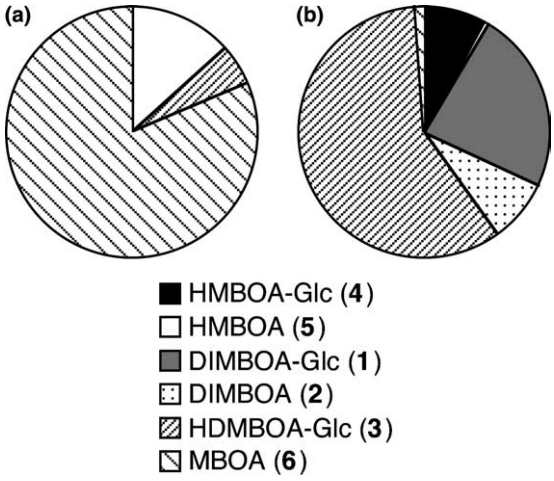


Fig. 5. The composition of Bxs and MBOA (**6**) in lesion areas (a) and peripheries (b) in leaves infected with *B. maydis*. Leaves were divided into the lesion area and the periphery 48 h after inoculation, and the amounts of Bxs and **6** were determined in each part by HPLC.

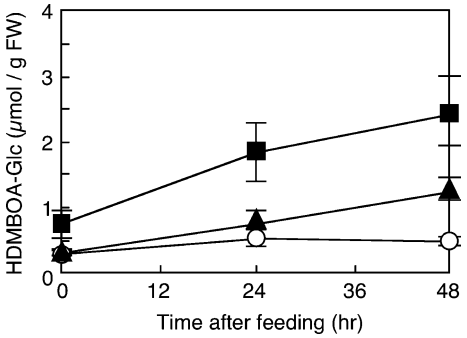


Fig. 6. Induction of the accumulation of HDMBOA-Glc (**3**) by rice armyworm larvae feeding. Larvae (second or third instar) were allowed to feed on the third leaf for 12 h, and the changes in **3** were analyzed thereafter (■). Leaves mechanically wounded by a razor blade (▲) and intact leaves (○) were used as controls. The error bars indicate the standard deviations of five replicates.

conidia germination, the respective germination rates of *B. maydis*, *C. lunata* and *A. alternata* being 9%, 5% and 40% at 10 mM, while the average length of the germ tubes was less than 30% of that of the controls for all fungi. The inhibition of germination was weak for all fungi at 1 mM, but the germ tube growth of *C. lunata* was significantly suppressed.

2.5. Accumulation of *3* following feeding by caterpillars

The larvae were allowed to feed on the leaves for 12 h, and thereafter the changes in **3** content were determined (Fig. 6). The level of **3** in the leaves damaged by feeding gradually increased after the removal of the larvae, and reached 2.4 μmol/g fr. wt by 48 h. Although a slight increase in **3** was also detected in leaves mechanically wounded by a razor blade, the level of **3** was less than half that of leaves damaged by insect feeding. In intact leaves, the level of **3** hardly changed.

3. Discussion

The accumulation of **3** was induced by inoculation of all three species of fungi with different pathogenicities, indicating that accumulation of **3** is a non-specific reaction. However, the rate of accumulation was different, which

Table 1
Inhibition of germination of conidia of *B. maydis*, *C. lunata* and *A. alternata* by Bx-related compounds[a]

Compound	Concentration (mM)	*B. maydis*	*C. lunata*	*A.alternata*
Water		100(0)	94(2.5)	89(2.0)
DIMBOA-Glc (**1**)	0.1	100(0)	93(2.0)	91(1.2)
	1	100(0)	91(2.6)	89(2.1)
	10	100(0)	93(0.6)	90(2.1)
HDMBOA-Glc (**3**)	0.1	100(0)	95(1.0)	92(1.2)
	1	100(0)	90(3.2)	88(1.0)
	10	100(0)	94(0.6)	89(2.1)
DIMBOA (**2**)	0.1	100(0)	92(1.0)	89(0.6)
	1	99(0.6)	93(1.2)	90(0.6)
	10	95[b](1.7)	90[c](4.7)	87(3.6)
MBOA (**6**)	0.1	91(2.0)	95(2.1)	88(4.0)
	1	83(3.2)	89[c](2.5)	81(3.1)
	10	9[d](2.1)	5[c](4.4)	40[c](4.2)

The conidia of fungi were suspended in solutions of DIMBOA-Glc (**1**), HDMBOA-Glc (**3**), DIMBOA (**2**) and MBOA (**6**). After a 24-h incubation period, at least one hundred conidia were observed under a microscope.

[a] Inhibition is represented by average percentages of three replicates of the water control, and standard deviations are indicated in parentheses.

[b] The average length of germ tubes was 80–50% of that of the control.

[c] The average length of germ tubes was 50–20% of that of the control.

[d] The average length of germ tubes was less than 20% of that of the control.

is probably attributable to the rapid colonization of leaves by *B. maydis*. Bücker and Grambow (1990) reported that **3** accumulates only in moderately resistant combinations in interactions between wheat cultivars and stem rust races. A specific recognition system may be involved in induction of **3** accumulation in the wheat's stem rust interaction because gene-for-gene relationships exist between wheat and its biotrophic pathogens (Day, 1974).

The induction of **3** by culture filtrates indicates the presence of elicitor active compounds. The filtrates largely retained their activity after boiling, suggesting that the elicitors present were mostly heat stable. Since chitooligosaccharides are active in inducing accumulation of **3** (Oikawa et al., 2001), the degradation products of fungal cell walls may served as heat-stable elicitors. The elicitor activity of the filtrates was slightly reduced by heat treatment, implying the involvement of proteinaceous components in the induction of **3**. Various proteins and peptides are recognized as elicitors by plants (Hahn, 1996).

Only the filtrate of *B. maydis* induced formation of necrotic lesions. This indicates that induction of **3** accumulation is not the result of necrosis. In these lesions, the main Bx-related compound was **6**. The plant tissues are severely damaged in the lesions, and the integrity of the membranes is lost. Thus, benzoxazinone glucosides come into contact with constitutive β-glucosidases in lesions, which result in the release of aglycones. **2** and HDMBOA chemically degrade into **6** (Grambow et al., 1986). The presence of **6** in the lesions of leaves inoculated with *B. maydis* indicates that this process occurred in the plant tissues although the possibility of involvement of enzymes from the pathogen can not be excluded. The total concentration of Bxs in the lesion area was 13% of that in the surrounding tissues. This is probably because degradation of HDMBOA into **6** does not necessarily proceed in a quantitative fashion (Woodward et al., 1978). Additionally, **6** may have been degraded by the fungi.

The antimicrobial activity of Bxs and benzoxazolinones has been studied in numerous species of bacterial and fungal pathogens (Virtanen et al., 1957; Elnaghy and Linko, 1962; Nakagawa et al., 1995), but a general conclusion has not been reached regarding the differing toxicities of Bxs and benzoxazolinones. In this study, **6** was more effective than **1**–**3**, at least for inhibiting the germination of conidia and growth of germ tubes of three species of fungi, although **6** is less toxic than **2** for *Erwinia* spp. (Corcuera et al., 1978). A positive correlation between pathogenicity and the ability to degrade **6** exists (Friebe et al., 1998; Yue et al., 1998), suggesting the importance of **6** formation. Among the species of fungi tested, *B. maydis* showed the strongest pathogenicity against maize. This may indicate that the fungus is tolerant toward **6** in the plant tissue, although the germination of conidia was inhibited by **6** in vitro.

Herbivorous insects as well as phytopathogenic fungi are critical objects of the defense reactions evoked by plants. Bxs have toxic and antifeeding effects on herbivorous insects (Argandoña et al., 1980; Xie et al., 1992; Hedin et al., 1993; Yan et al., 1999), and a correlation between resistance and the Bx level has been demonstrated in various interactions between Bx-accumulating plants and insects (Niemeyer, 1988). We found that the accumulation of **3** was induced by armyworm feeding, implying that the accumulation of **3** may play a role in the defense response against insects as well. Analogous to the case of infection, some elicitor-active molecules appear to be formed to induce **3** accumulation.

The conversion of **1** into **3** occurs in leaves treated with abiotic elicitors and jasmonic acid (Oikawa et al., 2001, 2002). In this study, we found that the process was induced in leaves inoculated with fungi and in leaves infested by rice armyworms as well, proving that it actually operates against biotic stresses. It has been believed that **2** is released from constitutively present **1** by contact with β-glucosidase stored in the plastid when leaves are infected by pathogens or attacked by insects. This is probably true during the initial phase of the interaction between Bx-accumulating plants and parasites that mechanically damage plant tissues. However, we found

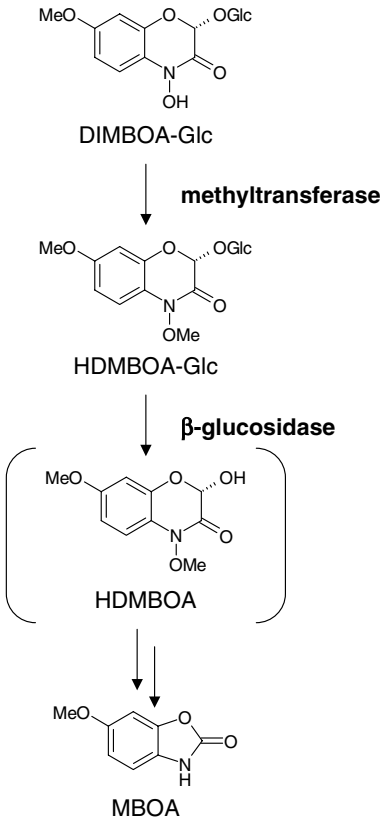


Fig. 7. Schematic representation of a hypothetical pathway leading to the release of MBOA (**6**) in maize leaves infected by pathogens.

that in leaves inoculated with phytopathogenic fungi, **1** was converted into **3** before disintegration of the tissues. This indicates that the compound formed upon cell-destruction is HDMBOA rather than **2** when the plants are continuously challenged by pathogens (Fig. 7).

Grambow et al. (1986) failed to isolate HDMBOA after hydrolysis of its glucoside with β-glucosidase because HDMBOA is so easily chemically decomposed. HDMBOA was degraded into **6** more quickly than **2** in our own experiments as well when their glucosides were mixed with β-glucosidase extracted from maize (data not shown). It is thus conceivable that the induced conversion of **1** into **3** is a mechanism that accelerates the formation of **6** upon infection by pathogens.

4. Experimental

4.1. Plant materials

Maize (*Zea mays* cv. Snowdent 108, Yukijirushi Seeds and Plants Co., Sapporo, Japan) seeds were sown according to the method described previously (Oikawa et al., 2001). The seeds germinated about 24 h after sowing, and the third leaves of 14-day-old seedlings were used for the experiments.

4.2. Phytopathogenic fungi and armyworms

Thee fungal pathogens and a herbivorous insect were utilized. The fungal pathogen *Biporalis maydis* race O strain HITO771 is highly pathogenic toward *Zea mays* cv. Snowdent 108, and causes elongated tan to dark brown colored lesions (2–3 mm × 5–10 mm) between the veins of the leaves after inoculation. *Curvularia lunata* strain SGC72156 is a weak or opportunistic pathogen of maize. Large lesions are formed from the coalesced spots on decaying leaves, while the spots remain small on young leaves. *Alternaria alternata* apple pathotype strain AMKT is non-pathogenic toward maize. The fungus forms appressoria at the hyphal tips, but infection is unsuccessful due to host hypersensitive reactions. These fungal strains were obtained from a stock culture of the Pesticide Research Institute at the Faculty of Agriculture of Kyoto University. The herbivorous rice armyworm *Leucania separata* Walker (Noctuidae) was reared on an artificial diet (Insecta-LFS, Nihon Nosan Kougyo Ltd. Yokohama, Japan) and the larvae were maintained under a 16:8 LD cycle at 23–25 °C.

4.3. Chemicals

Bxs and **6** were prepared by the method described previously (Oikawa et al., 2001). Tryptone peptone and yeast extract were obtained from Difco, sorbose was acquired from Tokyo Kasei Kogyo (Tokyo, Japan), and all other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

4.4. Inoculation of fungi

All fungi were cultured in liquid CM medium (0.1% tryptone peptone, 0.1% yeast extract, 1% glucose, 0.15% $Ca(NO_3)_2 \cdot 4H_2O$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.04% KH_2PO_4 and 0.003% K_2HPO_4) at 25 °C. After a 2-week incubation period, the hyphae were cut into fragments using a Polytron, and the resulting suspension was centrifuged for 10 min at 1000*g*. The supernatant was discarded, and the hyphal fragments were washed by sterile distilled water followed by centrifugation (10 min, 1000*g*). The fragments were resuspended in 100 ml of sterile distilled water and used as an inoculum. The colony forming unit (CFU) of the inoculum was determined by counting the number of colonies 3 days after spreading the diluted hyphal suspension on a CM sorbose plate (reduced glucose in CM to 0.01% and added sorbose to 1%). The CFU of the hyphae suspension used for inoculation of *B. maydis*, *C. lunata* and *A. alternata* inocula were 1.3×10^5, 1.5×10^5 and 1.6×10^5 cm^{-3}, respectively. Maize leaf segments (6 cm) were taken 7–17 cm from the leaf tip, and the upper surface of the segments was depressed in 5 points with punches at 1 cm intervals. The hyphal suspension (10 μl) was placed on each depressed point, and droplets of distilled water were placed on the leaf segments as controls. After incubation on wet filter paper at 25 °C with a 12-h period of illumination under fluorescent lamps, the segments were extracted with methanol, and the amounts of Bxs and **6** were determined by reversed-phase HPLC analysis as described previously (Oikawa et al., 2002).

4.5. Treatment of maize leaves with culture filtrates of fungi

The suspension of hyphae prepared by the method described above was incubated for 24 h at 25 °C and centrifuged for 10 min at 1000*g*. The supernatant (35 ml) was sterilized by filtration though a Millex-GV filter (Millipore), concentrated in vacuo, and the residue was dissolved in distilled water (1 ml). The resulting solution was boiled for 20 min to examine the effect of heat treatment. A 10-μl droplet of the solution was placed on the depressed points of the leaf segments, similar to the inoculation of the fungi, and droplets of distilled water were used as controls. After 48-h of incubation, the leaf segments were extracted with methanol, and their Bx contents were determined.

4.6. Feeding by caterpillars

Three armyworms (second or third instar) were put into a feeding tube assembled as shown in Fig. 2. The

tube was incubated in the dark for 12 h at 25 °C, after which the armyworms were removed. After additional incubation for 0, 24 and 48 h, the leaves were extracted. Leaves incubated without feeding by armyworms were used as controls. To examine the effect of mechanical wounding, the parts of leaves about the same in size and position as those fed on by the armyworms were cut off by a razor blade, and the remaining leaves were extracted after incubation for 0, 24 and 48 h.

4.7. Inhibition of conidia germination by Bxs and **6**

To obtain the conidia, *B. maydis*, *C. lunata* and *A. alternat* a were cultured on CM plates under continuous black light irradiation at 25 °C for 3 weeks. The conidia were then collected and moderately suspended in distilled water. Solutions of Bxs and **6** were added to the droplets of the suspension of conidia on a glass plate, and the final concentrations of Bxs and **6** were adjusted to 0.1, 1 and 10 mM. The plate was incubated at 25 °C on wet filter paper in a plastic box. The number of germinated spores was then counted under a microscope 24 h after the start of incubation.

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