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Direct fungicidal activities of C6-aldehydes are important constituents for defense responses in Arabidopsis against *Botrytis cinerea*

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ABSTRACT

C6-aldehydes, such as (*Z*)-3-hexenal, (*E*)-2-hexenal, and *n*-hexanal, are volatile compounds formed by hydroperoxide lyase (HPL) and found in most terrestrial plants. They are fungicidal and bactericidal compounds, and are also signaling compounds to induce defense responses in plants. Transgenic plants having overexpressed or suppressed HPL activity (SH or ASH, respectively) showed lower or higher susceptibility against a necrotrophic fungal pathogen, *Botrytis cinerea*. In this study, we examined whether the modulated susceptibility was accountable to the direct fungicidal activity or to the signaling potency of C6-aldehydes. When wild-type Arabidopsis leaves were inoculated with *B. cinerea*, HPL expression was upregulated, and concomitantly, the amounts of C6-aldehydes increased. Higher amounts of C6-aldehydes found in inoculated SH plants inhibited growth of *B. cinerea in vitro*, while lower amounts found in ASH plants caused no inhibitory effect on the fungi. Thus, it was suggested that direct fungicidal activity of C6-aldehydes accounted for the modulated susceptibility. With SH plants higher amounts of camalexin could be found, but with the ASH plants no difference from wild-type plants could be found. Surplus amounts of C6-aldehydes could induce formation of camalexin as signaling compounds; however, this was not the case with wild-type and ASH plants. Accordingly, it could be assumed that direct fungicidal activity of C6-aldehydes were prominently responsible to the defense against *B. cinerea* but their signaling roles could be little responsible if any.

1. Introduction

C6-aldehydes, such as (*Z*)-3-hexenal, (*E*)-2-hexenal, and *n*-hexanal, are volatile compounds found in most terrestrial plants. They are formed by hydroperoxide lyase (HPL) catalyzing cleavage of 13-hydroperoxides of linoleic and linolenic acids to yield *n*-hexanal and (*Z*)-3-hexenal, respectively (Matsui, 2006). (*Z*)-3-Hexenal can be converted into its isomer, (*E*)-2-hexenal, enzymatically or non-enzymatically. In intact, healthy plant tissues, the amounts of C6-aldehydes are usually low; however, mechanical or herbivore-induced damage on the tissues causes rapid formation of them (Fall et al., 1999; D'Auria et al., 2006). Induction and systemic release have also been reported (Rose et al., 1996; Mithöfer et al., 2005). C6-aldehydes can be insect-repelling and -attracting compounds depending on insect species, and several lines of evidence showed that C6-aldehydes can modulate plant–insect interaction both directly and indirectly (Vancanneyt et al., 2001; Shiojiri et al., 2006a). C6-aldehydes might also regulate plant defense by activating wound- and herbivore-induced defense responses. For instance, treating cotton balls or Arabidopsis leaves with C6-aldehydes caused formation of their phytoalexines (Zeringue, 1992; Kishimoto et al., 2006). With Arabidopsis, lima bean, maize, citrus, or *Nicotiana attenuata* C6-aldehyde-treatment induced a subset of defense genes (Bate and Rothstein, 1998; Arimura et al., 2000; Gomi et al., 2003; Farag et al., 2005; Kishimoto et al., 2005; Paschold et al., 2006).

It has also been reported that C6-aldehydes can be bactericidal and fungicidal compounds (Hamilton-Kemp et al., 1992; Nakamura and Hatanaka, 2002; Prost et al., 2005; Myung et al., 2007). Thus, involvement of C6-aldehydes in plant–pathogen interactions has also been expected. Induction of HPL expressions or enhancement of C6-aldehyde formation in several plants after infection by bacterial and fungal pathogens and concomitant higher resistance to pathogens were reported (Croft et al., 1993; Shiojiri et al., 2006b).

Previously, we found that overexpression of *HPL* in Arabidopsis resulted in higher resistance of the transgenic plants against a necrotrophic fungal pathogen, *Botrytis cinerea*. On the contrary,

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suppression of *HPL* caused higher susceptibility against the pathogen (Shiojiri et al., 2006b). This might be caused by direct effect of higher or lower concentration of C6-aldehydes needed to limit the growth of the fungi, or by signaling effect of C6-aldehydes as signaling molecules to enhance defense responses in Arabidopsis. In this study, we examined whether the direct toxic effects of C6-aldehydes or their signaling effect, or both, would be responsible to the modulation of susceptibility of the transgenic Arabidopsis.

2. Results

2.1. Upregulation of AtHPL expression

In order to investigate whether the increase in HPL activity observed after *B. cinerea* infection (Shiojiri et al., 2006b) was caused by upregulation of the expression of *AtHPL*, the transcript level of *AtHPL* after inoculation of *B. cinerea* on Arabidopsis (ecotype *No-0*) leaves was examined by semi-quantitative RT-PCR (Fig. 1). Upregulation of *HPL* expression could be observed as fast as 4 h after inoculation, and reached its maximum level after 24 h when necrotic lesions caused by the fungi started to appear. The high transcript level was kept until 48 h after inoculation.

2.2. Time course of C6-aldehydes accumulation

In uninfected, healthy leaves of wild-type (WT) Arabidopsis, (*Z*)-3-hexenal was most abundant among the C6-aldehydes, and the amounts of (*E*)-2-hexenal and *n*-hexanal were almost one-third of that of (*Z*)-3-hexenal. After infection with *B. cinerea*, the amounts of them started to increase within 6 h, and continuously increased until 48 h after infection (Fig. 2). During the course of increase, the composition of C6-aldehydes was almost constant. The time course of increase in the amounts of C6-aldehydes was mostly correlated with that of *AtHPL* transcript.

With the uninfected, healthy leaves of transgenic Arabidopsis overexpressing bell pepper HPL (SH), the amounts of C6-aldehydes were similar with those found with healthy WT leaves even though higher HPL activity was evident in SH leaves as reported previously (Shiojiri et al., 2006b). This might be caused by limited substrate flux. After inoculation, the amounts of all the three C6-aldehydes increased more extensively in SH plants, and higher amounts of them could be found 6–24 h after inoculation. Thereafter, the increase rate slowed down and after 48 h, almost the same amounts of the three C6-aldehydes could be found with WT and SH. On the contrary, the original level of C6-aldehydes in the leaves of transgenic Arabidopsis having reduced level of HPL (ASH) was significantly lower than that of WT. After infection with *B. cinerea*, their amounts slightly increased, however, only about one-fourth of those found in WT could be detected throughout the period examined.

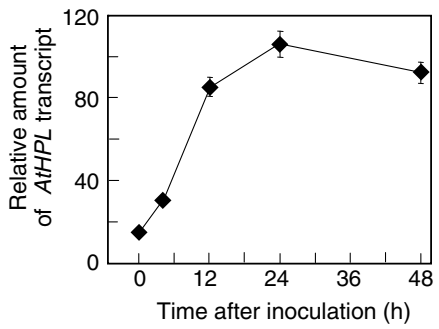

Fig. 1. Induction of *AtHPL* in Arabidopsis leaves after *B. cinerea* inoculation. RT-PCR was performed to examine transcript level of *AtHPL* (At4g15440) after infection. Relative amounts of the transcript to that of actin are shown (mean ± SD, $n = 3$).

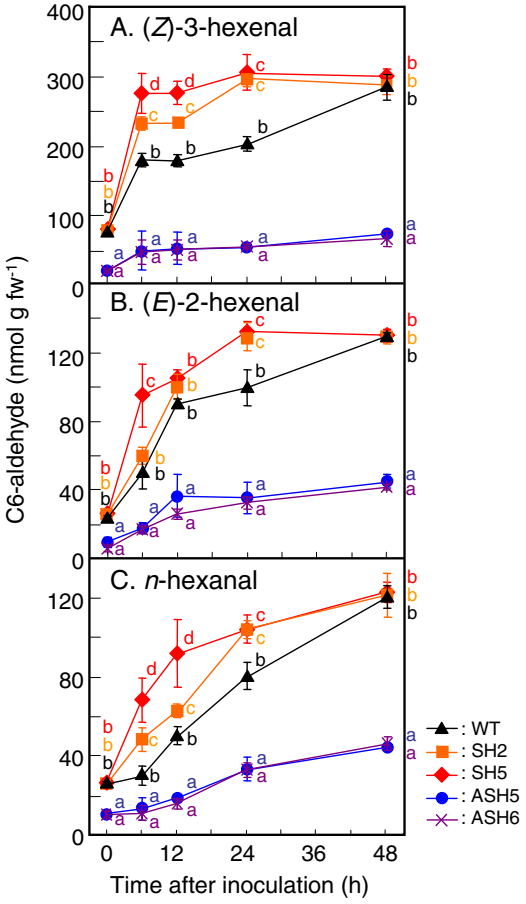

Fig. 2. Accumulation of C6-aldehydes in Arabidopsis leaves after *B. cinerea* inoculation. The amounts of (*Z*)-3-hexenal (A), (*E*)-2-hexenal (B), or *n*-hexanal (C) in the leaves of Arabidopsis plants were determined after 0, 6, 12, 24, 48 h of inoculation with *B. cinerea* (mean ± SD, $n = 3$). Different letters at a given time in each panel indicate significant differences at $P < 0.05$ (Tukey's-test).

2.3. Fungitoxic activities of C6-aldehydes of the concentration found in diseased Arabidopsis leaves

It has been reported that C6-aldehydes, either in the form of vapor or in the form of solution, could be toxic against various bacterial and fungal pathogens (Hamilton-Kemp et al., 1992; Croft et al., 1993; Andersen et al., 1994; Nakamura and Hatanaka, 2002; Gomi et al., 2003; Prost et al., 2005; Myung et al., 2007; Arroyo et al., 2007). In this study, we examined the effect of an artificial blend of C6-aldehydes compounded based on the amounts found in the whole leaves of WT, SH, or ASH at 6 h after infection. Under control conditions, where no C6-aldehydes were added, about 65% of the conidia germinated, and their hyphae grew to 120 μm after 24 h. When a blend of (*Z*)-3-hexenal, (*E*)-2-hexenal, and *n*-hexanal was added to the conidial suspension at the concentration and composition found in infected WT leaves, both the germination rate and hyphal length significantly decreased (Fig. 3). Higher growth-inhibition effect could be observed with higher concentration of C6-aldehydes mimicking those found in SH plants. On the contrary, the conidia could germinate and grow more vigorously under the presence of C6-blend of the concentration found in ASH leaves. In fact, the ASH blend rather enhanced germination of the conidia. Such germination-stimulating effects of low concentration of C6-volatiles have been reported (Fallik et al., 1998).

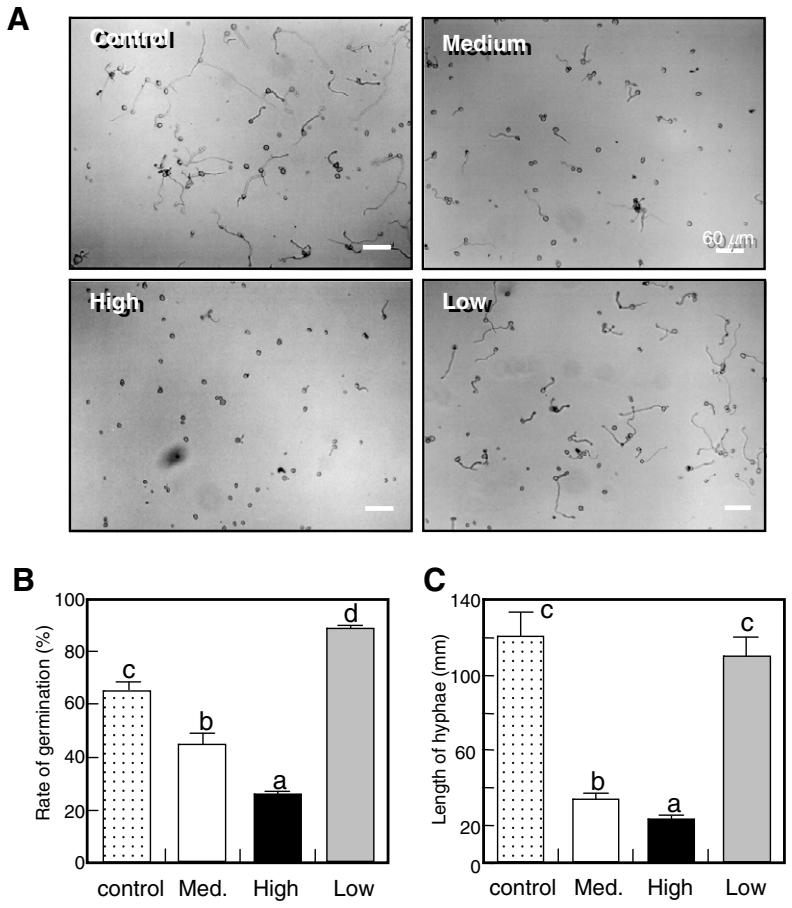

Fig. 3. Fungicidal activity of the C6-aldehyde mixture compounded according to their contents in the WT, SH, or ASH Arabidopsis leaves 6 h after *B. cinerea* infection (medium, high, or low, respectively). *B. cinerea* conidia was mixed with the respective C6-aldehyde blend, then incubated under the darkness for 24 h (A, scale bar = 60 μm). The rate of the germination (B) and length of the hyphae (C) were also determined (mean ± SD, $n = 3$). Different letters indicate significant differences at $P < 0.05$ (Tukey's-test; on arc-sine transformed data).

2.4. Accumulation of camalexin

C6-aldehydes could function as signal molecules. We also observed enhanced formation of camalexin, an Arabidopsis phytoalexin, after exposing Arabidopsis seedlings to the vapor of C6-aldehydes (Kishimoto et al., 2006). Camalexin is one of important components of defense in Arabidopsis against *B. cinerea* (Ferrari et al., 2003), thus, it was expected that enhanced formation of C6-aldehydes after inoculation might result in upregulation of the camalexin forming system to yield higher amounts of camalexin.

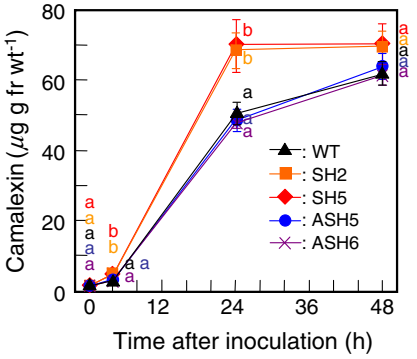

Fig. 4. Accumulation of camalexin in Arabidopsis leaves 0, 4, 24, and 48 h after *B. cinerea* inoculation (mean ± SD, $n = 3$). Different letters at a given time indicate significant differences at $P < 0.05$ (Tukey's-test).

With WT leaves, camalexin accumulation could be observed as fast as 4 h after inoculation, and its amount increased until 48 h (Fig. 4). This rapid formation of camalexin could be found with SH plants but in more vigorous manner. Even though the basal level of camalexin in the healthy WT and SH plants were equivalent each other, significantly higher amount of it could be detected in SH leaves after 4 h of inoculation. Higher amount of camalexin could be observed until 24 h, then, its increase mostly ceased to become the same level as found in WT leaves after 48 h. This profile observed in the camalexin content in WT and SH plants resembled with that found with C6-aldehydes. On the contrary, its level in ASH plants was almost the same with that found in WT plants throughout the experimental period even though significantly lower amounts of C6-aldehydes could be detected in ASH leaves.

3. Discussion

It has been well documented that *HPL* expression is upregulated after mechanical wounding, or herbivore attacks (Bate et al., 1998; Matsui et al., 1999; Vancanneyt et al., 2001; Van Poecke et al., 2001). HPL products, namely, C6-aldehydes, could be repelling and attracting compounds for various arthropods (Noordermeer et al., 2001; Matsui, 2006). From these, physiological significance of HPL is widely discussed in correlation with plant–insect interaction. Involvement of HPL in plant–microbe interaction has also been reported. Extensive evolution of C6-volatiles, such as (*E*)-2-hexenal and (*Z*)-3-hexen-1-ol, during a hypersensitive resistance response of beans (*Phaseolus vulgaris*) caused by infection of

Pseudomonas syringae was reported (Croft et al., 1993). In rough lemons (*Citrus jambhiri*), both pathogenic and nonpathogenic *Alternaria alternata* induced expression of *HPL* (Gomi et al., 2003). A nonpathogenic plant growth-promoting rhizofungi, *Trichoderma asperellum*, also induced expression of *HPL* as well as *phenylalanine ammonia lyase* in cucumber, resulting in induction of systemic resistance to pathogenic*P. syringae* (Yedidia et al., 2003). (*Z*)-3-Hexenal formation was stimulated in beans (*P. vulgaris*) after treating them with plant growth-promoting rhizobacteria (*Pseudomonasn putida* BTP1) (Ongena et al., 2004). These cumulating forms of evidence suggest that HPL is one of the defense-related genes induced by a wide-spectrum of biotic attacks. In this study, we also observed significant upregulation of *AtHPL* after inoculation of *B. cinerea*, which resulted in accumulation of higher amounts of C6-aldehydes. Through examining susceptibilities of transgenic Arabidopsis with higher and lower activities of HPL, we found that *AtHPL* was one of components to invest higher resistance in Arabidopsis against *B. cinerea* (Shiojiri et al., 2006b). C6-aldehydes are bactericidal and fungicidal compounds (Hamilton-Kemp et al., 1992; Nakamura and Hatanaka, 2002; Prost et al., 2005; Myung et al., 2007), thus, it has been assumed that C6-aldehydes accumulated after pathogen invasion can exert their toxic effects on the pathogen. Also, they could be signaling molecules to enhance defense responses, such as formation of phytoalexin, in plants (Zeringue, 1992; Kishimoto et al., 2005). This study was performed to examine whether the direct toxic effects of C6-aldehydes or their signaling potency, or both, would be responsible to the modulated susceptibility observed with the transgenic Arabidopsis.

We first examined whether the concentrations of C6-aldehydes accumulated in pathogen-attacked plant tissues are enough to exert their fungicidal effects. This study indicated that the growth-inhibitory effect of the C6-aldehyde-blend became to be observable at the concentration higher than those found in the whole leaves of ASH plants 6 h after infection with *B. cinerea*. The concentrations equivalent to those found in the infected WT and SH plants were effective in a concentration-dependent manner. Thus, the concentration range of C6-aldehydes observed with whole leaves of WT, SH, or ASH plants after *B. cinerea* infection was critical to the germination of conidia and to the growth of mycelia. Possibly, the C6-aldehydes were formed and accumulated locally at the infected sites, and concomitantly, *B. cinerea* would encounter higher concentration of C6-aldehydes *in planta*. If this is the case, the direct effect of C6-aldehydes formed after infection could be more pronounced. Taken together, it was suggested that the direct, antifungal effect of HPL products was responsible to the modulated susceptibilities of transgenic Arabidopsis. Prost et al. (2005) reported that (*Z*)-3-hexenal at 100 μM showed little effect on mycelial growth of *B. cinerea*. The possible reasons why we observed the growth-inhibitory effects would be (1) we used higher concentration and (2) we used a blend of C6-aldehydes consisting of much more reactive (*E*)-2-hexenal that showed 100% growth-inhibition against pathogenic bacteria at 100 μM while (*Z*)-3-hexenal showed modest inhibitory activity against them at the same concentration (Prost et al., 2005).

As a diagnosis for signaling functions of C6-aldehydes, we examined accumulation of camalexin after infection. Higher camalexin content could be observed in SH plants after infection, which suggested that surplus C6-aldehydes could enhance the formation of camalexin. The concentration of camalexin found after infection was enough to exert its antifungal activity *in vitro* (Ferrari et al., 2003). Thus, it could be expected that not only the higher amounts of C6-aldehydes but also higher amount of camalexin was at least partly accountable to the higher resistance against *B. cinerea* found in SH plants. On the contrary, with ASH plants camalexin was formed almost in the same manner after infection with WT plants even though ASH plants could form significantly lower amounts of C6-aldehydes. From this, it was obvious that C6-aldehydes was little accountable to the formation of camalexin in WT Arabidopsis, and the other signaling molecules, such as jasmonates, should be mainly accountable (Thomma et al., 1998). *B. cinerea* infection also induced expression of *PR* genes (Zheng et al., 2006) and exogenous addition of C6-aldehydes induced *PR-3* expression (Kishimoto et al., 2006). We observed rapid induction of *PR-1*, *-2*, and *-3* genes after *B. cinerea* infection, but the induction profiles were almost similar among WT, SH and ASH plants (data not shown), which again suggested that function of C6-aldehydes as signaling molecules was not so important for the modulation of susceptibilities of the SH and ASH plants against *B. cinerea*. In our previous works (Kishimoto et al., 2005, 2006), we used Arabidopsis ecotype *Col-0* that has no HPL activity (Duan et al., 2005). Because of the lack of endogenous C6-aldehydes in *Col-0*, signaling effect of exogenously supplied C6-aldehydes might be emphasized.

Before pathogen infection, the amounts of C6-aldehydes in SH plants were almost same with those in WT plants; while those in ASH plants were significantly lower. In healthy leaves, the substrate supply should be just enough to the intrinsic HPL activity, thus, additional HPL activity brought by overexpression of *CaHPL* could cause little effect on its products, but suppression of the activity could make the HPL step rate-limiting. On the contrary, higher amounts of C6-aldehydes found 6–24 h after inoculation in SH leaves could be accountable to the higher activity of HPL brought by overexpression of *CaHPL*. This suggested that substrate supply to HPL was sufficient probably through activation of lipoxygenase and other upstream enzymes after infection. Little difference in the amounts after 48 h indicated that endogenous AtHPL activity increased sufficiently enough to consume the enhanced amounts of substrates. In *N. attenuata*, when regurgitant of *Manduca sexta* was supplied to the mechanically wounded leaves of *N. attenuata*, higher C6-aldehyde release was observed while no apparent increase of *NaHPL* was observed, suggesting flux also controls C6-aldehyde biosynthesis (Halitschke et al., 2004). Taken together, it is apparent that both the substrate supply and HPL activity are regulated in order to meet the demand of higher production of C6-aldehydes under biotic stress.

4. Experimental

4.1. Plant materials and fungal inoculation

Arabidopsis thaliana (ecotype *Nossen-0*) harboring full-length bell pepper (*Capsicum annuum* L.) *HPL* (*CaHPL*) cDNA (SH) or antisense sequence of full-length Arabidopsis *HPL* (*AtHPL*) cDNA (ASH) under a cauliflower mosaic virus *35S* promoter was as described previously (Shiojiri et al., 2006). Two lines each of SH and ASH plants (SH2, SH5, ASH5 and ASH6) were used in this study. Wild-type (WT, *Nossen-0*), SH, and ASH seeds were grown on soil (Metro-Mix, SunGro Horticulture Distribution Inc., Bellevue, WA, USA) in pots in a chamber at 22 °C with light from fluorescent lights (16 h light/8 h dark, 70–80 μmol m^{-2} s^{-1}). Plants grown for 5 weeks were used. For inoculation of *B. cinerea* (strain *IuRy-1*; Kishimoto et al., 2002), the suspension of conidia (5 μl, 10^5 cfu ml^{-1} in 2.5% glucose) was placed on the upper surface of Arabidopsis leaves, and the plants were covered with plastic sheet in order to keep humidity. The plants were placed under the darkness at 22 °C.

4.2. Detection of HPL gene

Extraction of total RNA and the synthesis of cDNA were performed as previously described (Kishimoto et al., 2005). Primers for *AtHPL* (At4g15440) were 5′-AACCCTAACATCGTCGCCGTTCT-3′

(forward primer) and 5′-ATTTTGAATAGCCTCATCTCGGG-3′ (reversed primer). The PCR was carried out on a Perkin–Elmer 9700: 94 °C for 2 min followed by 94 °C for 45 s, 55 °C for 45 s and 72 °C for 45 s for 23–27 cycles, followed by a final 72 °C extension for 8 min.

4.3. Detection of C6-aldehydes and camalexin

Arabidopsis leaves (0.5 g) were soaked in MeOH–H_2O (5 ml, 4:1, v/v) containing 5 nmol of *n*-heptanal (as an internal standard), and incubated at 60 °C for 1 h. The MeOH solution was mixed with 2.5 ml of 0.1% 2,4-dinitrophenylhydrazine (in EtOH containing 2% HOAc) and incubated at 25 °C for 1 h. Hydrazones were extracted with *n*-hexane (2.5 ml). After solvent removed, the residue was reconstituted with CH_3CN (50 μl) for HPLC analyses (Matsui et al., 2006). Camalexin was isolated from Arabidopsis leaves (100 mg) by the methods of Glazebrook and Ausubel (1994). The concentration of camalexin was determined fluorometrically according to the method of Kishimoto et al. (2006) by using a standard curve constructed with purified camalexin kindly provided by Dr. Kazuhiro Toyoda (Okayama University, Japan).

4.4. Antifungal activity assay

(*Z*)-3-Hexenal, (ca. 99%; kindly provided by Zeon Cooperation, Tokyo, Japan), (*E*)-2-hexenal (ca. 97%; Wako Pure Chemicals, Osaka, Japan), *n*-hexanal (ca. 95%; Wako Pure Chemicals), were used to make an artificial blend based on the concentration of C6-aldehydes found in WT, SH, or ASH Arabidopsis leaves at 6 h after *B. cinerea* inoculation. High C6-aldehyde mixture mimicking those found in SH leaves contained 300, 100 and 70 nmol ml^{-1} of (*Z*)-3-hexenal, (*E*)-2-hexenal, and *n*-hexanal, respectively. Accordingly, low C6-aldehyde mixture corresponding to ASH leaves contained 50, 20 and 10 nmol ml^{-1}, and medium C6-aldehyde mixture corresponding to WT leaves contained 200, 50 and 30 nmol ml^{-1} of (*Z*)-3-hexenal, (*E*)-2-hexenal, and *n*-hexanal, respectively. These mixtures also contained 2.5% glucose and 0.5% EtOH. Control mixture contained 2.5% glucose and 0.5% EtOH. The mixtures (10 μl) were mixed with conidia (1.0×10^3 conidia) of *B. cinerea* on glass slide. The mixture was covered with a glass coverslip and incubated at 22 °C in the dark for 24 h. The fungal growth was then observed by microscopy.

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