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Analysis of defensive responses activated by volatile *allo*-ocimene treatment in *Arabidopsis thaliana*

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Abstract

Since volatile *allo*-ocimene enhances resistance of *Arabidopsis thaliana* against *Botrytis cinerea*, we attempted to dissect the factors involved in this induced resistance. The penetration of *B. cinerea* hyphae into *Arabidopsis* epidermis and the growth of hyphae after penetration were suppressed on *allo*-ocimene-treated leaves. *allo*-Ocimene also induced lignification on cell walls and veins of the leaves. The treatment induced accumulation of antifungal substances including the *Arabidopsis* phytoalexin, camalexin. Induction of lignification and accumulation of camalexin elicited by *B. cinerea* infection on *Arabidopsis* leaves after treating with *allo*-ocimene was faster and more intense than that observed with the leaves that had not been treated with this volatile. This suggested that *allo*-ocimene could prime defensive responses in *Arabidopsis*. *allo*-Ocimene enhanced resistant against *B. cinerea* in an ethylene resistant mutant (*etr1-1*), a jasmonic acid resistant mutant (*jar1-1*) and a salicylic acid resistant mutant (*npr1-1*). Thus, it is suggested that a signaling pathway independent for ETR1, JAR1 and NPR1 was operative to induce the resistance. The series of responses observed after *allo*-ocimene-treatment was mostly similar to that observed after C6-aldehyde-treatment. The effect of C6-aldehyde-treatment has been largely accounted to the chemical reactivities of the compounds; however, from this result it can be suggested that resistance responses of *Arabidopsis* could be induced by the volatiles mostly independent on their reactivities and that a common signaling pathway unaffected by the reactivities of compound was activated by the volatiles.

Keywords: *Arabidopsis thaliana*; Cruciferae; Defensive response; *Botrytis cinerea*; Camalexin; Lignification; *allo*-Ocimene

1. Introduction

Monoterpenes are a C_{10} class of isoprenoids, encompassing nearly 1000 colorless, lipophilic and volatile metabolites. Found throughout nature, these substances are best known as constituents of essential oils and floral scents in aromatic plants, to which they impart their characteristic aromas and flavors (Mahmoud and Croteau, 2002). Monoterpenes are reported to function as pollinator attractant or defenses in plants (Pichersky and Gershenzon, 2002). Genes for key enzymes in the biosynthesis of monoterpenes are known to be induced during development of floral organs (Chen et al., 2003) or after herbivore attack in leaves (Arimura et al., 2004). Monoterpenes were also suggested to function as signal compounds for defense responses against herbivores in plants. For example, gerbera (*Gerbera jamesonii*) or lima bean (*Phaseolus lunatus* cv. Sieva) actively emits volatile monoterpenes such as (*E*)-β-ocimene-**2** (Fig. 1) or limonene when plants are attacked by spider mites (*Tetranychus urticae*) (Ozawa et al., 2000; Krips et al., 2001). The emitted monoterpenes are thought to attract natural enemies against the spider mites.

While the amounts of (*E*)-β-ocimene-**2** in intact *Arabidopsis* plants are not high, they are dramatically increased after mechanical wounding or insect damage (Fäldt et al., 2003). In addition, exogenous (*E*)-β-ocimene-**2** treatment induced defense genes, such as basic *pathogenesis related*

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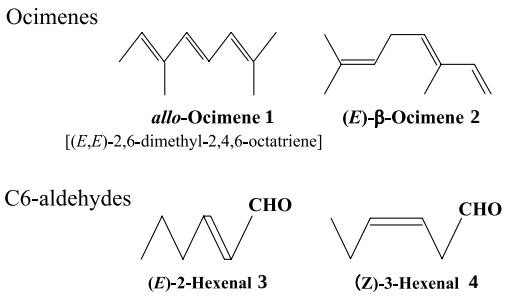


Fig. 1. Structure of volatile compounds **1**–**4**.

protein (*PRs*) or *phenylalanine ammonia lyase* (*PAL*) in lima beans (Arimura et al., 2000). We found that treatment of *Arabidopsis* plants with a structural isomer of (*E*)−β-ocimene, *allo*-ocimene-1 (Fig. 1), induced a subset of defense genes, such as *chalcone synthase* (*CHS*), *caffeic acid O-methyltransferase* (*COMT*), *diacylglycerol kinase 1* (*DGK1*), *glutathione S-transferase 1* (*GST1*) and *lipoxygenase 2* (*LOX2*). This enhanced resistance against *Botrytis cinerea*, and the gene-inductions were also partly dependent on the jasmonic acid (JA) and ethylene (ET) signaling pathways (Kishimoto et al., 2005). *allo*-Ocimene-1 (2,6-dimethyl-2,4,6-octatriene) is a monoterpene found in nature (Figueiredo et al., 2005), and a constituent of the essential oil of marigold (*Tagetes patula* L.) (Romagnoil et al., 2005) and tarragon (*Artemisia dracunculus* L.) (Sayyah et al., 2004).

Treatment of *Arabidopsis* with vaporized C6-aldehydes, such as (*Z*)-3-hexenal-4 or (*E*)-2-hexenal-3 (Fig. 1) also enhanced resistance against *B. cinerea* (Kishimoto et al., 2005). This enhancement was shown to be largely due to induced lignification of epidermal cells and vascular tissues, and to accumulation of antifungal substances including camalexin (Kishimoto et al., 2006). These C6-aldehydes are chemically reactive, and (*E*)-2-hexenal-3 readily reacts with amine and sulfhydryl residues of biological compounds because of its reactive α,β-unsaturated carbonyl moiety (Janzowski et al., 2003). Thus, it has been postulated that C6-aldehydes can activate defense responses of *Arabidopsis* through their electrophilic reactivities (Alméras et al., 2003). By contrast, *allo*-ocimene-1 does not have any chemically reactive functional group, except for its conjugated double bonds. Nonetheless, *allo*-ocimene-1 treatment brought about resistance responses similar to C6-aldehydes, although little is known about the mechanism. In this study, we attempted to dissect factors involved in resistance induced by *allo*-ocimene-1 in *Arabidopsis* by comparison to those of the C6-aldehydes.

2. Results

2.1. Infection behavior of *B. cinerea* on allo-ocimene-treated *Arabidopsis* leaves

We have already reported that *allo*-ocimene-1-treatment of *Arabidopsis* enhanced its resistance against *B. cinerea* (Kishimoto et al., 2005). In order to dissect the components essential to this enhancement, the growth of *B. cinerea* on leaves was examined with a microscope after staining the pathogen with methyl blue (Fig. 2). The conidia placed on the leaves germinated at the same frequency independent of the treatment. The second appressorium was also formed at a similar rate. Thus, the growth of *B. cinerea* on the surface of leaves was unaffected with *allo*-ocimene-1 pretreatment. On the contrary, the number of hyphae penetrated into the plant cells pre-treated with *allo*-ocimene-1 (distinguishable by the pale staining, Kishimoto et al., 2006) was significantly lower than that of the control leaves. The penetrated hyphae on the control leaves vigorously grew, and about half of the penetrated hyphae extended through more than two epidermal cells. On the other hand, the growth of hyphae in the epidermal cells of *allo*-ocimene-1-treated leaves was significantly repressed and less than 30% of the penetrated hyphae could grow into the adjacent cells (Fig. 1, lower inset).

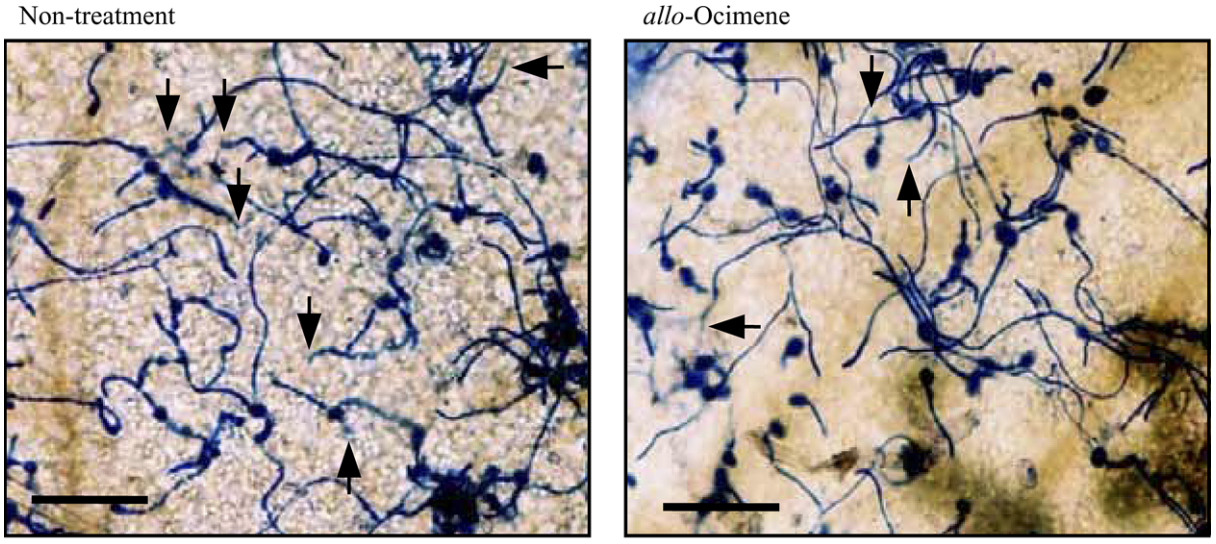
2.2. *allo*-Ocimene-induced lignification

When *Arabidopsis* leaves were stained with phloroglucinol/HCl to visualize lignification, leaf veins and cell walls of the *allo*-ocimene-treated leaves stained stronger than those of the control leaves (Fig. 3). Leaf veins, in particular, appeared to be more heavily lignified by *allo*-ocimene-1 treatment. The veins were also significantly lignified 24 h after inoculation with *B. cinerea*, while the lignification level in the epidermal cells was unaffected only with pathogen infection. However, a significant increase in the degree of lignification of the epidermal cells was observed when *Arabidopsis* previously exposed to *allo*-ocimene-1 was infected with *B. cinerea*. The degree of lignification after infection with *B. cinerea* in the epidermal cells of *allo*-ocimene-1-pretreated leaves was highest among those of other treatments, and was about three times higher than that observed after inoculation by *B. cinerea* on *Arabidopsis* that had not been treated with *allo*-ocimene-1.

2.3. Accumulation of antifungal substances in the treated leaves

When *B. cinerea* conidia were treated with HF and LF prepared from the control leaves, most germinated and vigorously developed their hyphae (Fig. 4). On the other hand, the growth of conidia in the presence of HF and LF prepared from *allo*-ocimene-1-treated leaves was strongly inhibited (Fig. 4). The growth of conidia in LF prepared from *allo*-ocimene-1-treated leaves was significantly slower than that in HF prepared from the same leaves. With either fraction, even those from *allo*-ocimene-1-treated leaves, direct, visible, effects on conidia could hardly be detected (data not shown).

After treating the leaves with *allo*-ocimene-1 for 4 h, the levels of camalexin, an *Arabidopsis* phytoalexin, started increasing until 24 h (Fig. 5A). Almost the same time



	Rate of germination (%)	Formation of second appressorium (%)	Penetration of hyphae (%)	Hyphae penetrated into more than two epidermal cells (%)
Control	90.5±1.5 a	18.8±1.5 a	7.6±1.5 a	3.7±1.3 a
allo-Ocimene	91.0±3 a	19.8±3.1 a	3.5±0.5 b	1.0 ±1 b

Fig. 2. Infection behavior of *Botrytis cinerea* on *Arabidopsis* leaves treated with/without *allo*-ocimene-1. *Arabidopsis* plants were treated with/without the vapor of *allo*-ocimene for 24 h, then, 3 μl of the conidial suspension (1×10^5 cfu ml^{-1}) was spotted onto the leaves. One day after inoculation, the hyphae on the leaves were stained with methyl blue and were observed with a microscope (upper panel). Arrows indicate penetrated hyphae. Bars = 60 μm. A summary of infection behavior is shown in the lower panel. Different letters indicate significant differences at $P < 0.05$ (Tukey's-test).

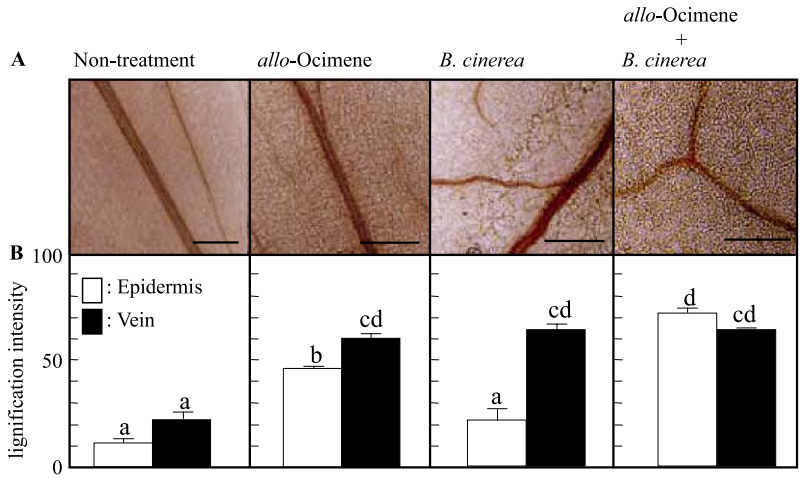


Fig. 3. Lignification on *allo*-ocimene-1-treated *Arabidopsis* leaves. *Arabidopsis* plants were treated with/without *allo*-ocimene-1 for 24 h, then, lignification was detected with phloroglucinol/HCl test (Panel A). Bars = 140 μm. Lignification intensities were analyzed by using NIH-image 1.62f and the raw data are shown in panel B. Error bars indicate ±SE ($n = 3$). Different letters is in each panel indicate significant difference at $P < 0.05$ (Tukey's-test).

course of accumulation of camalexin could be observed when the plants were infected with *B. cinerea*. However, the content of camalexin found 24 h after *B. cinerea* infection was about two times as much as that found 24 h after *allo*-ocimene-1 treatment. When *Arabidopsis* plants previously exposed to *allo*-ocimene-1 for 24 h were infected with *B. cinerea*, rapid formation of camalexin could be found, and the amount found 4 h after infection on exposed plants reached the level observed 24 h after infection on non-exposed plants.

In *Arabidopsis*, plant defensin 1.2 (PDF1.2) is thought to be one of its antifungal substances, and its formation is induced by infection of *B. cinerea* (Thomma et al., 2002). When the transcriptional level of *PDF1.2* was estimated

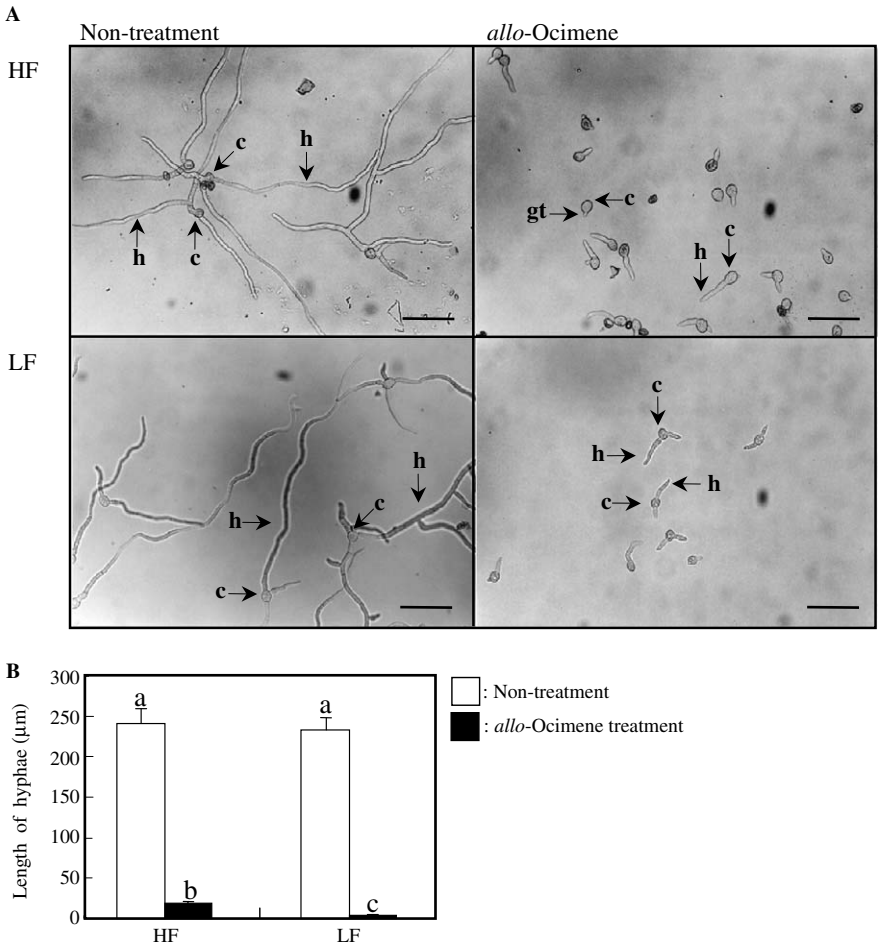


Fig. 4. Antifungal activity of leaf extracts prepared from *allo*-ocimene-treated *Arabidopsis*. *Arabidopsis* plants were treated with *allo*-ocimene for 24 h, then, crude leaf extract was prepared and fractionated into high molecular weight fractions (HF) (>12,000) and low molecular weight fraction (LF) (<12,000). The conidia were incubated with HF or LF for two days at 22 °C (Panel A). c; conidia, h; hyphae, gt; germ tube. Bars = 60 μm. The length of *Botrytis cinerea* hyphae is shown in panel B. Error bars indicate ±SE ($n = 10$). Different letters in each treatment indicate significant differences at $P < 0.05$ (Tukey's-test).

by real time RT-PCR, this was little of an increase of the level observed 4 h after *allo*-ocimene-1-treatment; however, after 12 h, the level significantly increased (Fig. 5B). The high transcript level persisted at least until 24 h after the onset of treatment.

2.4. Induction of defense gene

In order to gain insight into the signaling pathway activated by *allo*-ocimene-1-treatment, we first analyzed the expression profile of some of genes established as markers for the signaling pathways and compared it with that caused by MeJA or MeSA. *AOS* and *HPL* are key enzymes in phytooxylipin pathway, and are induced with mechanical wounding in *Arabidopsis* (Bate et al., 1998; Kubigsteltig et al., 1999). *PR-1* and *PR-2* were molecular marker genes of salicylic acid (SA) signaling in *Arabidopsis* (Thomma et al., 1998). *PR-3* and *VSP1* are molecular marker genes of ET/JA signaling and JA signaling, respectively, in *Arabidopsis* (Solano et al., 1998; Ellis and Turner, 2001). When *Arabidopsis* plants were exposed to the vapor of MeJA, *AOS*, *PR-3* and *VSP1* were upregulated, while *PR-1* and *PR-2* were not (Fig. 6). Treatment with volatile MeSA resulted in inductions of *PR-1* and *PR-2*. These results indicated that the experimental procedures employed here could monitor activation of JA or SA signaling. *allo*-Ocimene-1-treatment induced *AOS*, *HPL*, *PR-3* and *VSP1* expressions in *Arabidopsis* (Fig. 6). Induction of *PR-3* and *VSP1* could be observed as fast as 4 h after the treatment while that of *AOS* and *HPL* was visible only after 12 h. This time course was similar to that found after JA-treatment. On the other hand, *PR-1* and *PR-2* were not induced by *allo*-ocimene-1-treatment.

2.5. Enhancement of resistance against B. cinerea

We compared the susceptibilities to *B. cinerea* with an ET resistant mutant (*etr1-1*; Schaller and Bleecker, 1995), a JA resistant mutant (*jar1-1*; Staswick et al., 2002), a SA resistant mutant (*npr1-1*; Mou et al., 2003), after treating them with *allo*-ocimene-1 (Fig. 7). The inherent resistances of *jar1-1* and *npr1-1* were equivalent to that of WT, but

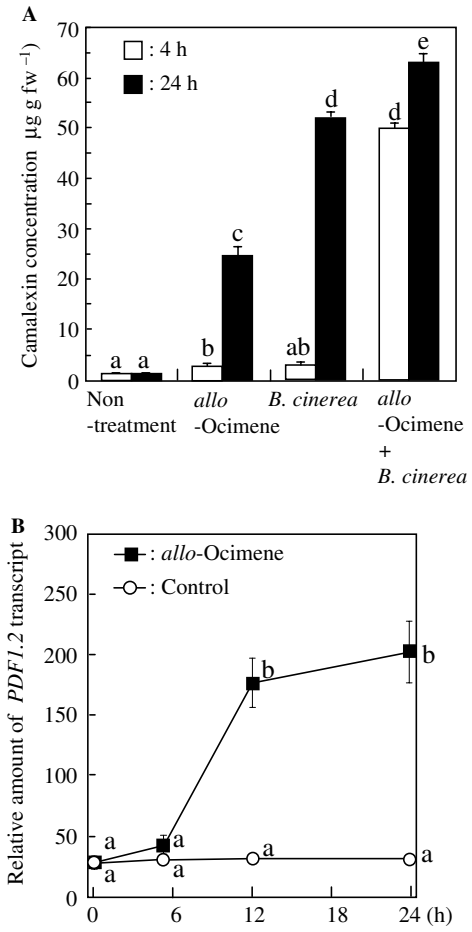


Fig. 5. Accumulation of camalexin and *plant defensin* (*PDF1.2*) transcript in the *allo*-ocimene-treated *Arabidopsis*. *Arabidopsis* plants were treated with *allo*-ocimene or with conidial suspension of *B. cinerea* for 4 and 24 h, or were pretreated with *allo*-ocimene for 24 h, then, treated with the conidial suspension for 4 and 24 h. Thereafter, camalexin was extracted from the leaves with 80% methanol, then, quantified (panel A). *Arabidopsis* plants were treated with *all*-ocimene (squares) or without volatile (circles) for 4, 12 and 24 h. RNA was extracted and subjected to semi-quantitative RT-PCR analysis. The relative amounts of *PDF1.2* transcript against that of actin (*AAc1*) are shown (panel B). Error bars indicate ±SE ($n = 3$). Different letters in each time point indicate significant differences at $P < 0.05$ (Tukey's-test).

that of *etr1-1* was significantly lower than the other genotypes. When WT was exposed to volatile *allo*-ocimene-1, enhancement of resistance against *B. cinerea* was observed. The resistance of *jar1-1* and *npr1-1* after the treatment was also induced and it was statistically identical to that of WT after the treatment. The resistance of *etr1-1* after the treatment was also significantly enhanced even though the resistance after the treatment was significantly weaker than those of WT, *jar1-1* and *npr1-1*.

3. Discussion

We previously reported that treatment of *Arabidopsis* with vaporized C6-aldehydes enhanced resistance against *B. cinerea* (Kishimoto et al., 2005) largely due to induced lignification of epidermal cells and vascular tissues, and to accumulation of antifungal substances including camalexin (Kishimoto et al., 2006). We also reported that *allo*-ocimene-1 could enhance resistance of *Arabidopsis* against the pathogen; however, the molecular mechanism of this enhancement has been little studied. Because the structures of the C6-aldehydes-3 and -4 and *allo*-ocimene-1 are different from each other, it was thought to be important to dissect the molecular mechanism evoked after *allo*-ocimene-1-treatment by comparing it with that evoked by C6-aldehydes-3 and -4.

When infection behavior of *B. cinerea* was examined under the microscope, it was found that *allo*-ocimene-1 pretreatment resulted in retarded penetration of *B. cinerea* hyphae into the *Arabidopsis* epidermis and in slower growth of its hyphae in the epidermal cells. Enhanced lignification of the epidermal cells and vascular tissues were evident after *allo*-ocimene-1-treatment of *Arabidopsis*. Lignification might account to the inhibition of the penetration of the fungal hyphae (Lewis and Yamamoto, 1990) as has been suggested with C6-aldehyde-treatment (Kishimoto et al., 2006). Accumulation of antifungal substances including camalexin, and defense proteins such as PDF1.2 or PR-3, which encodes a basic chitinase (Thomma et al., 1998), should also be accountable for inhibition of growth of the hyphae. All the responses observed after *allo*-ocimene-1-treatment were largely similar to that observed when *Arabidopsis* plants were exposed to C6-aldehydes (Kishimoto et al., 2006). Taken together, the molecular mechanism accountable to enhanced resistance against *B. cinerea* elicited by *allo*-ocimene-1-treatment could be largely similar to that caused by C6-aldehyde-3 and -4-treatment.

This conclusion could be further confirmed by analyzing induction profiles of a subset of defense genes. *allo*-Ocimene-1 could induce molecular marker genes for JA signaling and wound signaling, thus, it could be implied that *allo*-ocimene-1 treatment could simultaneously activate these signaling pathways in *Arabidopsis*. This is also the case with C6-aldehyde-treatment, and we have already reported an involvement of JAR1-dependent JA signaling in responses against C6-aldehydes-3 and -4 and *allo*-ocimene-1 by using *jar1-1* mutant plants (Kishimoto et al., 2005). Involvement of ETR1-dependent ET signaling in responses against C6-aldehydes-3 and -4 and *allo*-ocimene-1 has also been reported, while involvement of SA-signaling could be largely excluded because of no upregulation of *PR-1* and *PR-2*.

(*E*)-2-Hexenal-3 has an α,β-unsaturated carbonyl moiety that has high electrophilicity to react with various biological substances (Alméras et al., 2003; Janzowski et al., 2003). (*Z*)-3-Hexenal-4 is also a reactive compound because of its carbonyl moiety. Therefore, the activities of C6-aldehydes to induce defense responses in plants have been largely attributed to their chemical reactivities (Bate and Rothstein, 1998; Farag and Paré, 2002). On the

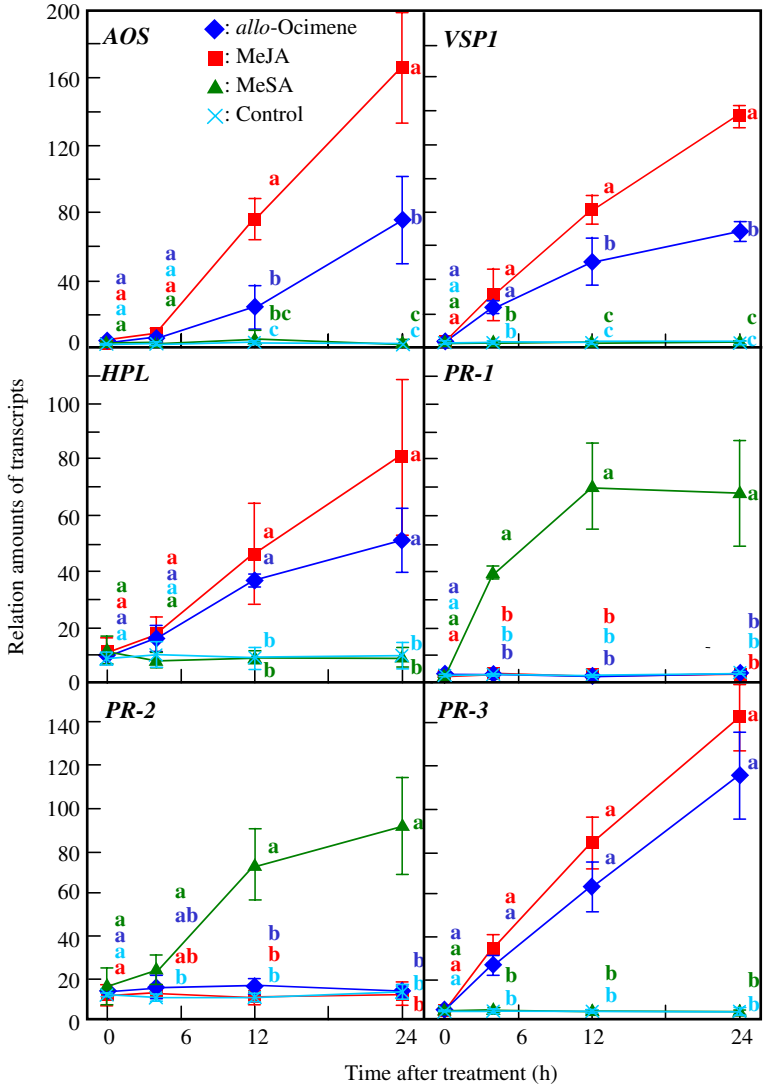


Fig. 6. Comparison of defense gene inductions with *all*-ocimene-1, methyl jasmonate (MeJA) or methyl salicylate (MeSA). *Arabidopsis* wild type plants were treated with *allo*-ocimene, MeJA or MeSA for 0, 4, 12, or 24 h. RNA were extracted from the leaves after treatment and subjected to RT-PCR. Details of defense genes and specific primers are shown in Table 1. The relative amounts of the transcripts against that of actin (*ACT2*) are shown. Error bars indicate ±SE ($n = 3$). Different letters in each time indicate significant difference at $P < 0.05$ (Tukey's-test).

contrary, *allo*-ocimene-1 has no such reactive moiety other than unsaturated bonds. The results that *allo*-ocimene-1 can induce almost the same set of defense responses in almost the same way as C6-aldehydes-3/-4 suggests that at least part of the defense responses caused by *allo*-ocimene-1 and C6-aldehydes-3/-4 is largely unrelated to the chemical reactivities of these substances. Maffei et al. (2004) reported that SDS could mimic the effect of volicitin, an elicitor isolated from oral secretion of a herbivore, on membrane potential of lima bean leaves, which might lead to elicitation of volatile biosynthesis in the plants. From this result, they proposed that an amphiphilic nature of a compound was important to cause depolarization of membrane potential. In this context, hydrophobic nature of *allo*-ocimene-1 and C6-aldehydes-3/-4 could be a factor to evoke defense responses in *Arabidopsis*. It has been reported that camalexin formation and liginification are regulated by reactive oxygen species in *Arabidopsis* (Tierens et al., 2002; Bohman et al., 2004; Schützendübel and Polle, 2002). *GST1*, whose expression is reported to be dependent on hydrogen peroxide (Rentel and Knight, 2004), was also induced by *allo*-ocimene-1 and C6-aldehydes-3/-4 (Kishimoto et al., 2005). Thus, an oxidative burst might be elicited after the volatile treatment. Recently, it has been reported that treating *Arabidopsis* with volatile compounds resulted in transient Ca^{2+} influx and generation of reactive oxygen species (Asai et al., 2006). Again, SDS could mimic volicitin's action to cause transient Ca^{2+} influx (Maffei et al., 2004), which was inconsistent with the hypothesis that hydrophobicity of a compound was one of important prerequisites.

allo-Ocimene-1 treatment could induce resistance of WT, *jar1-1*, and *npr1-1* against *B. cinerea* significantly. The effect of the treatment was not significantly different

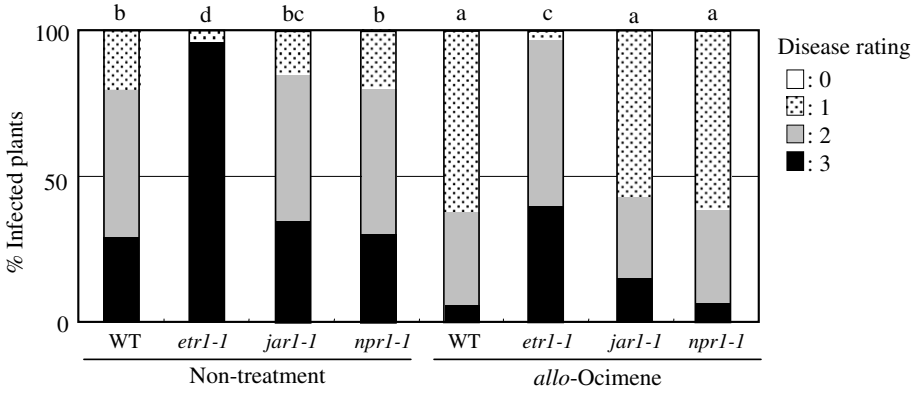


Fig. 7. The summary of symptoms caused by *B. cinerea* 2 days after inoculation of *B. cinerea*. *Arabidopsis* mutants (*etr1-1*, *jar1-1* and *npr1-1*) and wild type plants (WT) were treated with *allo*-ocimene for 24 h. Then, the plants (about 60 plants × 2) were sprayed with 3 ml of conidial suspension (1×10^5 cfu ml^{-1}) of *B. cinerea*. After 48 h, the disease symptoms were scored from no symptom to fully dead: 0, no symptom; 1, slight symptom (1–4 leaves of a plant showed necrosis); 2, serious symptom (5–10 leaves showed necrosis); 3, decayed (fully dead). We adopted Kruskal–Wallis non-parametric analysis of variance to compare different disease symptom among all 8 groups, which were WT, *etr1-1*, *jar1-1* and *npr1-1* with *allo*-ocimene treatment or non-treatment. If a level of significance less than 0.05 was observed, Scheffe's method of multiple comparisons was used to determine differences among groups. There is a significant difference between groups followed by different letters in $n = 3$ at $P < 0.05$ (Scheffe's test).

from each other, thus, it could be concluded that JAR1-dependent JA signaling and NPR1-dependent SA signaling were not essential for *Arabidopsis* to enhance resistance against the fungal pathogen in response to *allo*-ocimene-1 treatment. Even *etr1-1*, that showed higher susceptibility than WT when their inherent susceptibilities were evaluated, could significantly enhance the resistance after *allo*-ocimene-1 treatment. This showed that the components accountable to the induction of the resistance by *allo*-ocimene-1 treatment were, at least partly, independent of the components accountable to the higher susceptibility of *etr1-1*. Taken together, the defense responses evoked by signalings independent on JAR1 and ETR1 might operate to enhance the resistance. Further study on the involvement of oxidative burst in the defense responses elicited by *allo*-ocimene-1 is now underway.

Intriguingly, the *allo*-ocimene-1-pretreated *Arabidopsis* could induce lignification and accumulation of camalexin after *B. cinerea* inoculation much faster and more strongly than non-treated *Arabidopsis*. Exogenous pretreatment of plants with benzothiadiazole or β-aminobutyric acid could enhance stronger and faster defense response upon pathogen infection, which is known as 'priming' (Conrath et al., 2002). Engelberth et al. (2004) reported 'priming' caused by green leaf volatiles (GLV). They reported that corn seedlings previously exposed to GLV formed more JA and sesquiterpenes when treated with elicitors derived from herbivore regurgitant than the seedlings not exposed to GLV. *allo*-Ocimene-1 might also prime the plants for lignification and accumulation of camalexin. Then, the pre-treated *Arabidopsis* could respond more quickly and strongly to *B. cinerea* inoculated thereafter. In the case of priming against bacterial pathogens, involvement of SA has been postulated, however, priming caused by *allo*-ocimene-1 might be independent on SA because *allo*-ocimene-1 could enhance resistance against *B. cinerea* even with the SA-insensitive *npr1* mutant as with WT. The molecular mechanism underlying priming has not been revealed yet. However, the finding that *allo*-ocimene-1 could cause priming might be a representative experimental system to examine the molecular mechanism of priming.

4. Experimental

4.1. Materials

Arabidopsis thaliana WT, *etr1-1*, *jar1-1*, or *npr1-1* seeds, with ecotype *Col-0* background, supplied by the *Arabidopsis* Biological Resource Center, were surface sterilized, incubated at 4 °C for 48 h in 0.1% agar, and dispersed onto a MS plate containing 2% sucrose. Plants were grown in a chamber at 22 °C with fluorescent light (70–80 μmol m^{-2} s^{-1}, 16 h light/8 h dark). Unless otherwise stated, plants were grown for 3 weeks. For some experiments, the seedling were removed to soil (Metro-Mix, Sun-Gro Horticulture Distribution Inc., Bellevue, WA, USA) in pots, and were grown for further 3 weeks under the same condition.

allo-Ocimene-1 (ca. 94%) and methyl salicylate (MeSA; ca. 98%) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Methyl jasmonate (MeJA; ca. 90%) was purchased from Wako Pure Chemicals (Osaka, Japan).

4.2. Volatile treatment

The seedlings grown for 3 weeks in a petri dish (60 plants per dish) or the plants in a pot for 6 weeks (one plant per pot) were placed into a glass separable flask (1 l) and was incubated as shown above for one day. The volatile compounds were diluted to 0.1 M in dichloromethane

and 10 μl of the cold solution were applied onto the top of a sterile cotton swab hanged from the glass lid of the flask where the plants had been placed. Control plants were treated with only CH_2Cl_2.

4.3. Fungal inoculation

After volatile treatment for 24 h, the soil-grown plants in pots were moved to clean separable flasks, and 3 μl of conidial suspension (1×10^5 cfu ml^{-1} in 2.5% glucose) of *B. cinerea* (strain IuRy-1) (Kishimoto et al., 2005) was placed onto the leaves. One day after inoculation, the leaves were fixed and decolorized in formalin–EtOH–AcOH solution (1:1:1, v/v), then, the hyphae were stained with 0.5% methyl blue solution according to the methods previously reported (Kishimoto et al., 2002). After staining, number of germinated conidia, second appressorium, and hyphae penetrated into plant epidermal cells were counted under a microscope. Methyl blue hardly infiltrates into the plant tissue, thus, the hyphae on the surface of plant epidermis are strongly stained with methyl blue, however, the hyphae in the epidermal cells are stained weakly. Thus, the hyphae on the surface of plant cells and those in the cells could be distinguishable based on the intensity of staining (Kishimoto et al., 2006).

After the treatment with volatiles for 24 h, the plants in a petri dish were also moved to a new separable flask, and 1.5 ml of the conidial suspension was sprayed onto the plants. The inoculated plants were incubated further for 48 h under the growth condition described above. Disease symptoms of 316–462 plants were recorded according to the method of Berrocal-Lobo et al. (2002). We adopted Kruskal–Wallis non-parametric analysis of variance to compare different disease symptom among all 8 groups, those are, WT, *etr1-1*, *jar1-1* and *npr1-1* with/without *allo*-ocimene-1 treatment. If a level of significance less than 0.05 was observed, Scheffe's method of multiple comparisons was used to determine differences among groups.

4.4. Detection of lignification

The degree of lignification in *Arabidopsis* leaves was estimated by the phloroglucinol/HCl test (Mauch-Mani and Slusarenko, 1996). After the *allo*-ocimene-1-treatment for 24 h or after the *B. cinerea*-inoculation for 24 h, the leaves were incubated in a solution of 1% phloroglucinol in 70% EtOH-H_2O until photosynthetic pigments were totally extracted from the tissues. A leaf was then mounted on a slide, and covered with a few drops of concentrated HCl. The leaf was covered with a coverslip. After incubation for 5 min, lignified structure turned browny red. The intensity of color change after phloroglucinol/HCl test was quantitated with NIH-image 1.62f (http://rsb.info.nih.gov/nih-image/). The intensity was evaluated after subtracting the background intensity of the leaves before color development.

4.5. Detection of camalexin

After the *allo*-ocimene-1-treatment or the *B. cinerea*-inoculation for 4 and 24 h, camalexin was isolated from the *Arabidopsis* leaves by the methods of Glazebrook and Ausbel (1994). The concentration of camalexin was determined fluorometrically with emission at 385 nm and excitation at 315 nm with a spectrofluorometer RF1500 (Shimadzu, Kyoto, Japan). The concentration was calculated by using a standard curve constructed with purified camalexin kindly provided by Dr. Kazuhiro Toyoda (Okayama University, Japan).

4.6. RT-PCR

Total RNA was extracted from *Arabidopsis* leaves (100 mg) with TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) after treating with the volatile for a given time, and the remaining DNA was degraded by treating with DNA-free kit (Ambion, Austin, CA, USA). cDNA was synthesized from the total RNA (4 μg) by Thermoscript RT-PCR System (Invitrogen) with oligo $(dT)_{20}$ as a primer according to the manufacturer's instructions. RT-PCR was performed by using Takara ExTaq polymerase (Takara Biomedicals, Otsu, Japan). The reaction mixture contained 0.1 μg of cDNA, 500 nM forward and reverse primer, 1 × ExTaq buffer, 200 nM dNTPs and 0.5 U of ExTaq polymerase in a 20 μl solution. Primers of each gene were shown in Table 1. The PCR was carried out on a Perkin–Elmer 9700 (Perkin–Elmer Applied Biosystems, Foster City, CA, USA): 94 °C for 2 min followed by 94 °C for 45 s, 57 °C for 45 s and 72 °C for 45 s for 20–27 cycles, followed by a final 72 °C extension for 8 min.

Semi-quantitative RT-PCR was performed by using SYBR Green PCR core reagents kit (Perkin–Elmer Applied Biosystems). The reaction mixture contained 10 ng cDNA, 50 nM forward and reverse primers, 1 × SYBR Green PCR Buffer, 800 nM $MgCl_2$, 200 nM dNTPs, 0.4 units Ampli Taq Gold and 0.16 units AmpErase UNG in a 50 μl solution. PCR (95 °C for 15 s, 60 °C for 1 min) for 40 cycles were performed by a GeneAmp 9600 PCR thermalcycler (Perkin–Elmer Applied Biosystems), and the data were processed by using GeneAmp 5700 Sequence Detection System version 1.3 (Perkin–Elmer Applied Biosystems). Primers for *PDF1.2* and *AAc1* were described as Kishimoto et al. (2006).

4.7. Antifungal activity assay

Antifungal activity of the leaf extract was examined according to the methods of Kishimoto et al. (2006). After the *allo*-ocimene treatment for 24 h, crude extract was prepared, and fractionated into a high molecular weight fraction (HF; >12,000) and low molecular weight fraction (LF; <12,000) by limited dialysis using Spectra/Por4 Membranes (Funakoshi, Tokyo, Japan). Each fraction (5 μl) was mixed with an equal volume of a suspension

Table 1
The primers used for RT-PCR

MIPS code	Gene name	Description[a]	Primer
At3g18780	*ACT2*	Structural protein of actin	5′-CTAACCTCTCTCAAGATCAAAGGCT-3′ 5′-ACTAAAACGCAAAACGAAAGCGG-3′
At5g42650	*AOS*	Allene oxide synthase (Lipid, fatty-acid and isoprenoid metabolism. Jasmonic acid biosynthesis)	5′-ATGAAGATTTTGTTTCCGAATAT-3′ 5′-TTCCTAACGGCGACGTACCAACC-3′
At4g15440	*HPL*	Hydroperoxide lyase (Lipid, fatty-acid and isoprenoid metabolism. C6-aldehydes biosynthesis)	5′-AACCCTAACATCGTCGCCGTTCT-3′ 5′-ATTTTGAATAGCCTCATCTCGGG-3′
At5g24780	*VSP1*	Vegetative storage protein (Nutritional roles during plant development and stress adaptation)	5′-CAAACAGTACCAATACGACTC-3′ 5′-CTTCCATTTTGTTACACCAAC-3′
At2g19990	*PR-1*	Pathogenesis-related protein	5′-AACCGCC AAA AGCAAACGCAA-3′ 5′-TCACGGAGGCACAACCAAGTC-3′
At3g57260	*PR-2*	Pathogenesis-related protein (β-1,3-Glucanase and antifungal activity)	5′-CAAGGAGCTTAGCCTCACCACCA-3′ 5′-GGCCGTGTCTCCCATGTAGCTGA-3′
At3g12500	*PR*-3	Pathogenesis-related protein (chitinase and antifungal activity)	5′-TGCGGTAACACCGAACCATAC-3′ 5′-CGACAGTTGCATTGGTCCTCT-3′

[a] According to MATDB (http://mips.gsf.de/proj/thal/db/index.html).

(1×10^5 cfu ml^{-1}) of *B. cinerea* conidia in 5% glucose on a glass slide. The mixture was covered with a glass coverslip and incubated at 22 °C in the dark for 48 h. The fungal growth was then observed by microscopy.

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