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Volatile C6-aldehydes and Allo-ocimene Activate Defense Genes and Induce Resistance against *Botrytis cinerea* in *Arabidopsis thaliana*

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Green leafy volatiles or isoprenoids are produced after mechanical wounding or pathogen/herbivore attacks in higher plants. We monitored expression profiles of the genes involved in defense responses upon exposing *Arabidopsis thaliana* to the volatiles. Among the genes investigated, those known to be induced by mechanical wounding and/or jasmonate application, such as *chalcone synthase* (*CHS*), *caffeic acid-O-methyltransferase* (*COMT*), *diacylglycerol kinase1* (*DGK1*), *glutathione-S-transferase1* (*GST1*) and *lipoxygenase2* (*LOX2*), were shown to be induced with (*E*)-2-hexenal, (*Z*)-3-hexenal, (*Z*)-3-hexenol or allo-ocimene (2,6-dimethyl-2,4,6-octatriene). A salicylic acid-responsive gene, *pathogenesis-related protein2* (*PR2*), was not induced by the volatiles. Detailed analyses of the expression profiles showed that the manner of induction varied depending on either the gene monitored or the volatile used. A chemically inert compound, (*Z*)-3-hexenol, was also potent, which suggested that chemical reactivity was not the sole requisite for the inducing activity. With a jasmonate-insensitive mutant (*jar1*), the induction by the volatiles was mostly suppressed, however, that of *LOX2* was unaltered. An ethylene-insensitive mutant (*etr1*) showed responses almost identical to the wild type, with minor exceptions. From these observations, it was suggested that both the jasmonate-dependent and -independent pathways were operative upon perception of the volatiles, while the ETR1-dependent pathway was not directly involved. When *Botrytis cinerea* was inoculated after the volatile treatment, retardation of disease development could be seen. It appears that volatile treatment could make the plants more resistant against the fungal disease.

Keywords: *Arabidopsis thaliana* — *Botrytis cinerea* — Defense-related gene — Green leafy volatiles — Isoprenoids — Volatile organic compounds.

Abbreviations: AAc1, *Arabidopsis* actin 1; AOS, allene oxide synthase; CHS, chalcone synthase; COMT, caffeic acid-*O*-methyltransferase; DGK1, diacylglycerol kinase 1; ET, ethylene; GST1, glutathione-*S*-transferase 1; HPL, fatty acid hydroperoxide lyase; JA, jasmonic acid; LOX2, lipoxygenase 2; OPDA, oxophytodienoic acid; PAL, phenylalanine ammonia lyase; PR2, pathogenesis-related protein 2; RT–PCR, reverse transcription–polymerase chain reaction; SA, salicylic acid.

Introduction

It has been reported that various biotic and abiotic stresses on plants cause the formation of a wide array of volatile compounds, such as jasmonates (JAs), salicylates (SAs), green leafy volatiles (GLVs), isoprenoids, and so on (Pichersky and Gershenzon 2002). The volatiles released from leaves upon herbivore attack help plants deter herbivores or attract their predators (Takabayashi and Dicke 1996, Kessler and Baldwin 2001). It has also been expected that plant volatiles play a role in the control of defense gene expression, probably as a signal mediator within and between plants (Farmer 2001), as shown in *Arabidopsis thaliana* (Seo et al. 2001), lima beans (Ozawa et al. 2000, Arimura et al. 2002), tomatoes (Farmer and Ryan 1990) and *Citrus* (Gomi et al. 2003). It has been proposed that plants can 'talk' to each other in a field condition (Dolch and Tscharntke 2000, Karban et al. 2003) and that volatile compounds are involved in this 'plant talk' (Dicke et al. 2003).

GLVs, including (*E*)-2-hexenal, (*Z*)-3-hexenal, *n*-hexanal and their corresponding alcohols or esters, are produced from damaged plant tissues, as the products of fatty acid hydroperoxide lyase (HPL) from 13-hydroperoxides of linoleic or linolenic acid, as one of the branches of the phytooxylipin pathway (Hatanaka 1993). GLVs were also formed during the hypersensitive response against pathogens (Croft et al. 1993) or by exogenous application of JA (Avdiushko et al. 1995). Engelberth et al. (2004) showed that GLVs emitted from herbivore-infested corn plants can prime intact plants against insect herbivore attacks. Bate and Rothstein (1998) showed that (*E*)-2-hexenal induced several defense-related genes in *A. thaliana*, such as *chalcone synthase* (*CHS*), *allene oxide synthase* (*AOS*) and *lipoxygenase 2* (*LOX2*). Arimura et al. (2001) also showed that (*E*)-2-hexenal, (*Z*)-3-hexenol or (*Z*)-3-hexenyl acetate could induce genes encoding basic pathogenesis-related proteins (PRs), LOX or phenylalanine ammonia lyase (PAL) in lima beans. With *Citrus*, Gomi et al. (2003) reported that (*E*)-2-hexenal induced genes encoding AOS, LOX and HPL. These findings suggested that GLVs function as a signal to facilitate defense responses.

Isoprenoids are the largest group of plant volatiles and serve to attract pollinators, fruit-dispersing animals and enemies of herbivorous arthropods (Pichersky and Gershenzon

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Table 1 The primers used for semi-quantitative real-time RT-PCR

MIPS code	Gene name	Description [a]	Primer
At2g37620	*AAc1*	Structural protein of actin	5′-TGTATGTGGCTATTCAGGCTGTTC-3′ 5′-CATAAGGACACAAAAGCGCTACAC-5′
At5g13930	*CHS*	Chalcone synthase (phenylpropanoid and isoprenoid biosynthesis)	5′-TGGCTATTGGCACTGCTAACC-3′ 5′-GTGATGCGGAAGTAGTAGTCAGGA-3′
At5g45160	*COMT*	Caffeic acid-*O*-methyltransferase (phenylpropanoid biosynthesis)	5′-CCGTCTTCTTACGTCTTACTCCG-3′ 5′-AAGCCCGTAAATCCGTTCAA-3′
At3g45140	*LOX2*	Lipoxygenase (lipid, fatty acid and isoprenoid metabolism)	5′-GATCACATCGGACGAGGAACTC-3′ 5′-TCTTTCTTGTCTCCGTGGCCT-3′
At1g02930	*GST1*	Glutathione-*S*-transferase (detoxification and organization of cytoplasm)	5′-GAAGATCGACCAAAGTGAAGTGG-3′ 5′-GAAGATCGACCAAAGTGAAGTGG-3′
At5g07920	*DGK1*	Diacylglycerol kinase (phospholipid biosynthesis)	5′-AACTTTGTGCGCCTTTACCAGT-3′ 5′-CATGGTGCGATATGGTTAAGGTAC-3′
At3g57260	*PR2*	β-1,3-glucanase (pathogenesis-related protein and antifungal activity)	5′-CAGTGATAGGTTTCTTGGCA-3′ 5′-CGTGTCTCCCATGTAGCTGA-3′

[a] According to MATDB.

2002). As low molecular weight monoterpenes, myrcene and β-ocimene are formed by *A. thaliana* (Faldt et al. 2003). These isoprenoids are produced from damaged plant leaves (Loughrin et al. 1994, Röse et al. 1996). (*E*)-β-Ocimene and two related isoprenoids were also shown to activate the expression of a number of defense-related genes such as *PR2* and *PAL* in lima beans (Arimura et al. 2000).

These findings indicate that phytogenic volatile compounds such as GLVs and isoprenoids can induce a wide variety of defense-related genes. However, the mechanism(s) for how plants can perceive volatiles have not been fully elucidated. In order to address this question, we monitored expression profiles of several defense genes. Detailed analyses of the induction profiles concomitant with those along with mutants deficient in the signaling pathways brought insight into the mechanism. It has also been reported that treatment of plants with volatile compounds, such as JA, ethylene (ET), GLVs or 2,3-butanediol, can induce resistance against pathogens (Hoffman et al. 1999, Gomi et al. 2003, Ryu et al. 2004). We also found that the treatment of *A. thaliana* with GLVs or an isoprenoid could confer higher resistance against a necrotrophic fungal pathogen, *Botrytis cinerea*.

Results

Volatile compounds can induce expression of defense genes

Arabidopsis thaliana plantlets were exposed to the vapor of (*E*)-2-hexenal, (*Z*)-3-hexenal or allo-ocimene (their structures are shown in Fig. 1) for 24 h. After 24 h, no visible change could be detected with the plantlets treated with the chemicals. The amounts of transcripts for defense-related genes listed in Table 1 were semi-quantified by real-time reverse transcription–polymerase chain reaction (RT–PCR) using the actin gene (*AAc1*) as the reference (Fig. 2). With *CHS*, *diacylglycerol kinase* (*DGK1*), *caffeic acid-O-methyltransferase* (*COMT*), *glutathione-S-transferase 1* (*GST1*) and *LOX2*, treatment of *A. thaliana* with C6-aldehydes and allo-ocimene resulted in the induction of their expression. On the other hand, the compounds did not induce the expression of *pathogenesis-related protein 2* (*PR2*). The induction of the genes could be observed as soon as 4 h after the treatment. It must be noticed that the induction profile of each gene was different. For example, (*E*)-2-hexenal and allo-ocimene were most potent in the induction of the expression of *CHS*, *COMT* and *DGK1*, while for *GST1* these two compounds were slightly less active than (*Z*)-3-hexenal. *LOX2* expression was induced in almost the same way irrespective of the structure of the compound used.

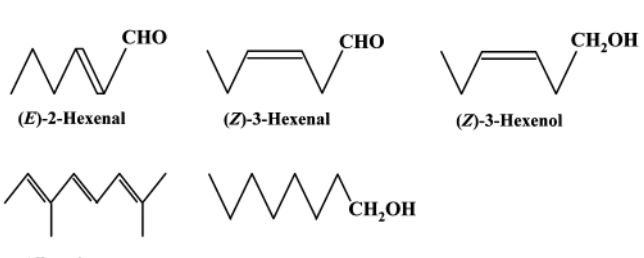

Fig. 1 Volatile compounds used in this study.

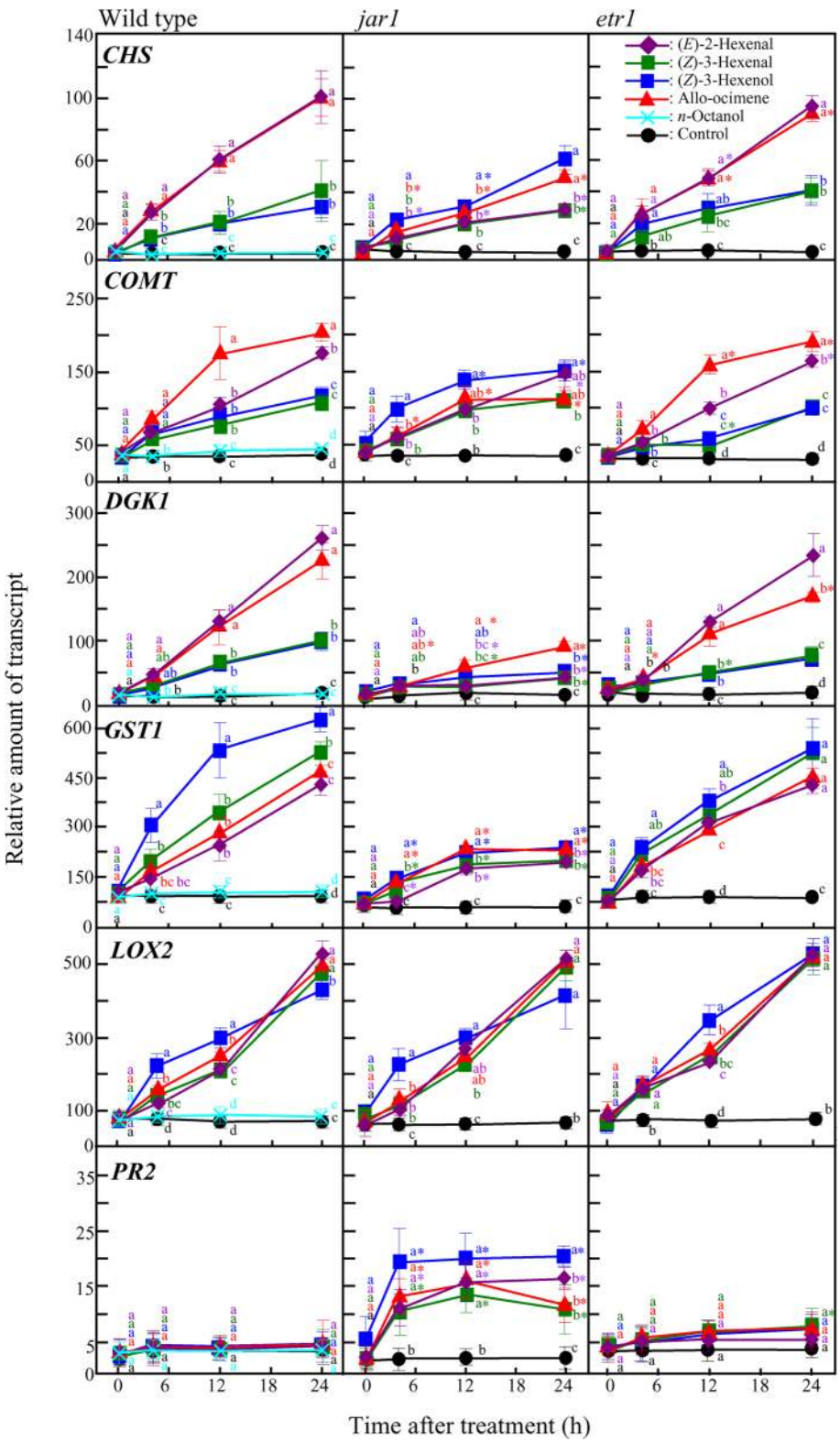

Fig. 2 Volatile compounds induced the expression of defense genes. *Arabidopsis* plants were treated with (*E*)-2-hexenal, (*Z*)-3-hexenal, allo-ocimene, (*Z*)-3-hexenol or *n*-octanol, and the leaves were harvested at a given time to extract RNA. The relative amount of each transcript against the amount of *AAc1* was estimated by semi-quantitative RT–PCR. Different letters at a given time in each panel indicate significant differences at $P < 0.05$ (Tukey's test, $n = 3$). Asterisks given with *jar1* and *etr1* indicate that the values are significantly different from the corresponding values of the wild-type plants at $P < 0.05$ (Dunnett's test, $n = 3$).

The induction profiles caused by allo-ocimene were similar to those caused by (*E*)-2-hexenal although this is not the case with *COMT*. When a chemically inert volatile, namely *n*-octanol, was used, no induction could be found with any genes investigated here (Fig. 2). Induction of *CHS*, *GST1* and *LOX2* with (*E*)-2-hexenal, (*Z*)-3-hexenal or allo-ocimene could also be

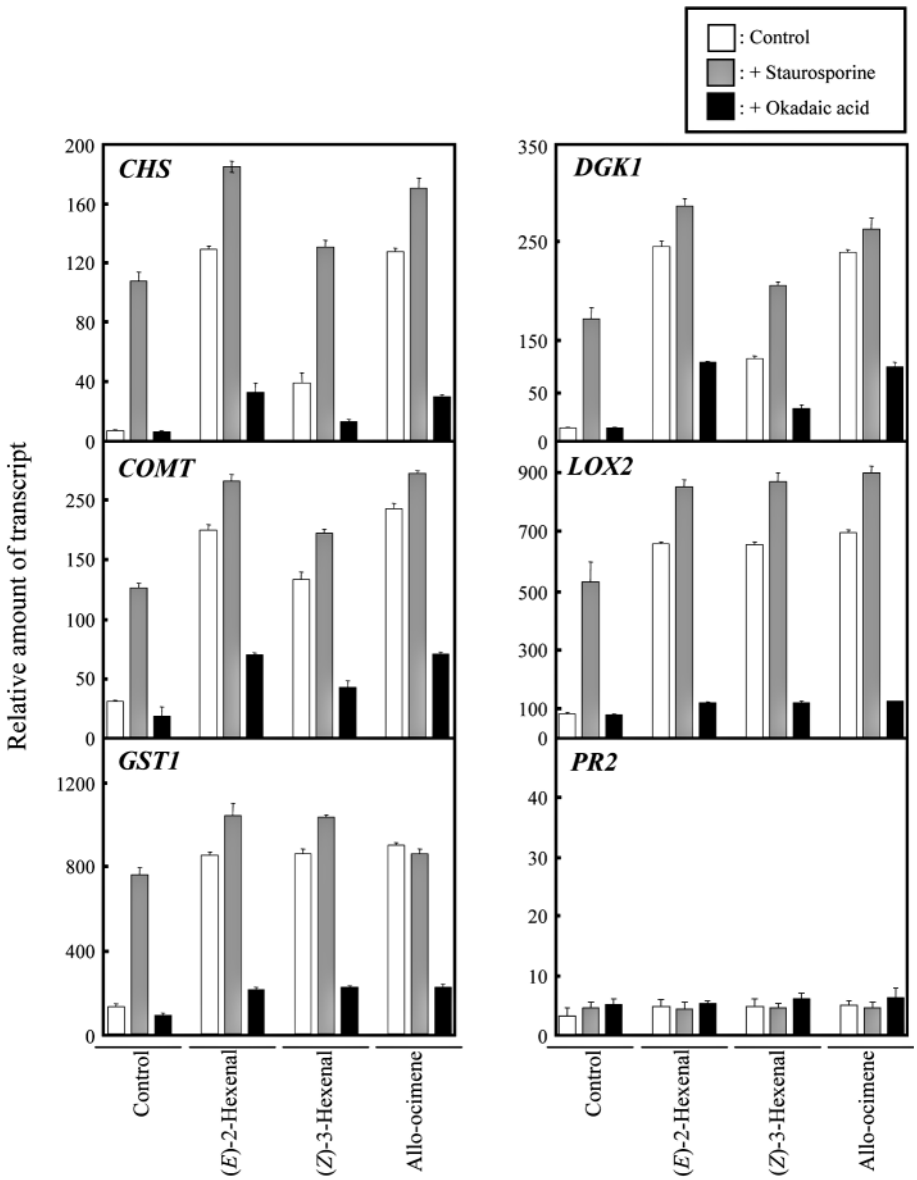

Fig. 3 Effect of staurosporine and okadaic acid on the induction profiles of defense genes. *Arabidopsis* plants were transplanted to a liquid medium (0.5× MS). After pre-conditioning for 2 d, staurosporine (final concentration, 1 μM) or okadaic acid (0.5 μM) was added to the medium, and then the plants were treated with (*E*)-2-hexenal, (*Z*)-3-hexenal or allo-ocimene as in Fig. 2. After treatment for 24 h, total RNA was extracted for semi-quantitative RT–PCR. Error bars indicate ±SE ($n = 3$).

observed with *Arabidopsis* plants grown on soil (see Fig. 5a). Again, *PR2* was not induced.

When 10-fold amounts of the chemicals were used, almost the same induction profiles could be observed. However, with this amount of chemicals (i.e. 10 μmol l^{-1}), deteriorative effects on plants, such as wilting of leaves, were evident (data not shown). The deteriorative effect was most severe with (*E*)-2-hexenal. When 0.1 μmol l^{-1} of each compound was used, little induction of defense gene expression could be seen (data not shown).

We treated *A. thaliana* with (*Z*)-3-hexenol, which is chemically less reactive than the corresponding aldehyde, and followed the changes in the expression level of the genes. Interestingly, activation of the gene expression could still be observed with the alcohol, while the expression of *PR2* was not induced (Fig. 2, WT). The induction profiles of *CHS*, *DGK1* and *COMT* with the alcohol were almost same as those with the corresponding aldehyde, (*Z*)-3-hexenal. The ability of (*Z*)-3-hexenol to induce *GST1* expression was highest among the volatiles used in this study. *LOX2* was induced with (*Z*)-3-hexenol in a quicker manner than with the other volatile compounds.

Effects of volatile compounds on jar1 and etr1

It has been well established that JA and ET play key roles in plant responses against mechanical wounding, herbivore and pathogen attacks (Thomma et al. 1998, Lorenzo et al. 2003). In this context, it can be expected that JA and/or ET would be involved in the activation of gene expression by the volatile compounds. In order to address this hypothesis, we used two mutants, *jar1* and *etr1*, for the exposure experiments (Fig. 2).

Table 2 The summary of disease symptoms caused by *B. cinerea* after 2 d of inoculation

Treatment	Infected plants (%, means ± SD, n = 3) [a]			
	No symptoms	Slight symptoms	Serious symptoms	Decayed
(*E*)-2-Hexenal	0	75 ± 3.6 c	18 ± 4.6 bc	7 ± 1.0 b
(*Z*)-3-Hexenal	0	51 ± 6.6 b	42 ± 4.6 ab	7 ± 2.0 b
Allo-ocimene	0	62 ± 6.9 b	32 ± 5.9 b	6 ± 1.5 b
Control	0	21 ± 5.0 a	50 ± 7.0 a	29 ± 3.5 a

Arabidopsis thaliana were treated with the volatiles as in Fig. 2 for 24 h, and then, the plants were sprayed with 3 ml of 1×10^5 conidia of *B. cinerea* suspended in 2.5% glucose. Means followed by different letters are significantly different at $P < 0.05$ (Tukey's test).

[a] The disease symptoms were scored with 120 plants from no symptom, slight symptoms (1–4 leaves of a plant showed some necrosis), serious symptoms (5–10 leaves showed necrosis) and decayed (fully dead).

JAR1 encodes an enzyme catalyzing acyl-adenylation of JA (Staswick et al. 2002). Although the function of the enzyme in JA signaling has not been fully established, a mutation in the gene showed deficiencies in JA signaling (Rojo et al. 1999). *ETR1* encodes an ET receptor (Schaller and Bleecker 1995), and a mutation in the gene led to a loss of physiological sensitivity to ET (Hua and Meyerowitz 1998). The induction of *CHS*, *COMT*, *DGK1* and *GST1*, with (*E*)-2-hexenal, (*Z*)-3-hexenal or allo-ocimene, especially at 12 and 24 h after the treatment, was significantly suppressed with *jar1*. On the contrary, the induction of *LOX2* expression was unaltered with the JA-resistant mutant. The ability of allo-ocimene to induce expression of *COMT* was also suppressed in *jar1* although the induction profile of the gene with (*Z*)-3-hexenal was little modulated. Induction of *DGK1* and *GST1* with (*Z*)-3-hexenol was also suppressed with *jar1*; however, slightly higher induction of *CHS* and *COMT* with the alcohol was observed in *jar1*. *LOX2* induction with (*Z*)-3-hexenol was similar between wild type and *jar1*. When the expression level of *PR2* was monitored after treating *jar1* with the volatiles, induction of its expression with (*E*)-2-hexenal, (*Z*)-3-hexenal, (*Z*)-3-hexenol and allo-ocimene was observed. The increases were evident by 4 h after the treatment, and, thereafter, the expression levels were constant.

When plantlets of *etr1* were exposed to the volatile compounds, the expression pattern of each gene except *PR2* was almost similar to those of the wild type (Fig. 2). Slight suppression of the induction could be found with *CHS*, *COMT* and *DGK1* at 24 h after treating with allo-ocimene; however, the degree of suppression was small.

Effect of staurosporine and okadaic acid

We investigated the effects of staurosporine, a protein kinase inhibitor, and okadaic acid, a protein phosphatase inhibitor, on the gene-inducing abilities of (*E*)-2-hexenal, (*Z*)-3-hexenal and allo-ocimene. Treating the plantlets only with staurosporine increased the amounts of transcripts of *CHS*, *COMT*, *GST1*, *DGK1* and *LOX2*, but not that of *PR2* (Fig. 3). Simultaneous treatment with the volatile compounds and staurosporine resulted in stronger expression of *CHS*, *COMT*, *DGK1* and *LOX2* than that of the corresponding single treatment; however, the induction of *GST1* by allo-ocimene was not enhanced by the addition of staurosporine. When plants were treated with okadaic acid alone, no significant change in the expression levels of these defense genes could be observed. When volatiles and okadaic acid were used simultaneously, the accumulations of transcripts were significantly inhibited in any genes investigated here. Again, the expression profile of *PR2* was exceptional, and no change could be seen with okadaic acid treatment in either the presence or absence of the volatile compounds.

Effect of volatile treatment on susceptibility to Botrytis cinerea

(*E*)-2-Hexenal, (*Z*)-3-hexenal and allo-ocimene induced the expression of some defense-related genes in *A. thaliana*. Thus, it was assumed that the volatile treatment would render *A. thaliana* resistant against biotic stress. *Botrytis cinerea* is a necrotrophic pathogen that attacks many plant species including *A. thaliana* (Govrin and Levine 2002). After *Arabidopsis* plantlets grown on agar plates were treated for 24 h with each chemical, the chemical was removed and *B. cinerea* conidia were inoculated onto the leaves. Under the inoculation procedure employed here, severe disease symptoms were observed with the plants that had not been treated with the compounds; however, the volatile treatments apparently retarded the development of the disease symptoms (Fig. 4). When the disease symptoms were scored according to Berrocal-Lobo et al. (Berrocal-Lobo et al. 2002; Table 2), it was clearly shown that the proportions of decayed plants among the volatile-treated plants were far less than that with the control plants. The proportions of plants showing serious symptoms were significantly smaller in plants treated with (*E*)-2-hexenal and allo-ocimene.

Effects of volatiles on soil-grown plants

We also investigated whether soil-grown *Arabidopsis* plants could induce defense genes and acquire resistance against *B. cinerea* after the volatile treatments (Fig. 5).

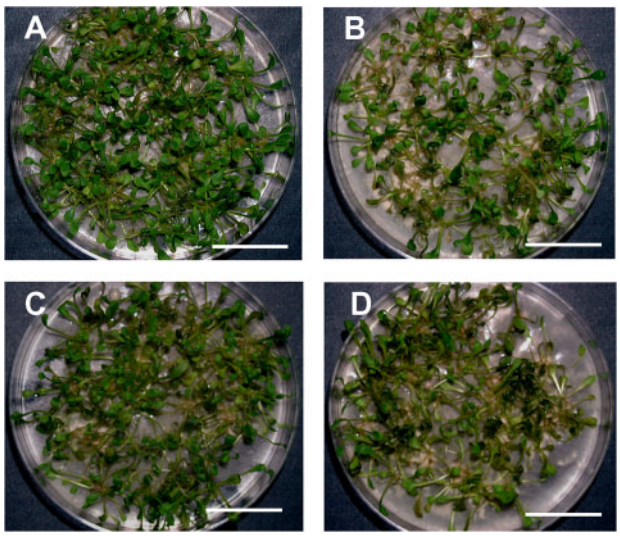

Fig. 4 Effect of volatile treatment on disease symptoms of *Botrytis cinerea*. Disease symptoms caused by *B. cinerea* were observed 2 d after inoculation. *Arabidopsis* seedlings grown on MS-agar were treated with (*E*)-2-hexenal (A), (*Z*)-3-hexenal (B), allo-ocimene (C) or without volatiles (D) as in Fig. 2 for 24 h, and the plants were sprayed with *B. cinerea* conidial suspension. Scale bar = 3 cm.

Arabidopsis plantlets grown on soil were treated with (*E*)-2-hexenal, (*Z*)-3-hexenal and allo-ocimene in the same manner, and, the leaves were harvested at a given time for RT–PCR analysis. Apparently, the volatiles could induce expression of *CHS*, *GST1* and *LOX2*, but not *PR2*, which mostly coincided with the results obtained with agar-grown plants (Fig. 5a). In addition, treating plants with (*E*)-2-hexenal, (*Z*)-3-hexenal or allo-ocimene could retard the disease symptom of the gray mold (Fig. 5b). When the sizes of the necrotic lesions on leaves were measured, smaller necrotic lesions were found with volatile-treated plants (Fig. 5c).

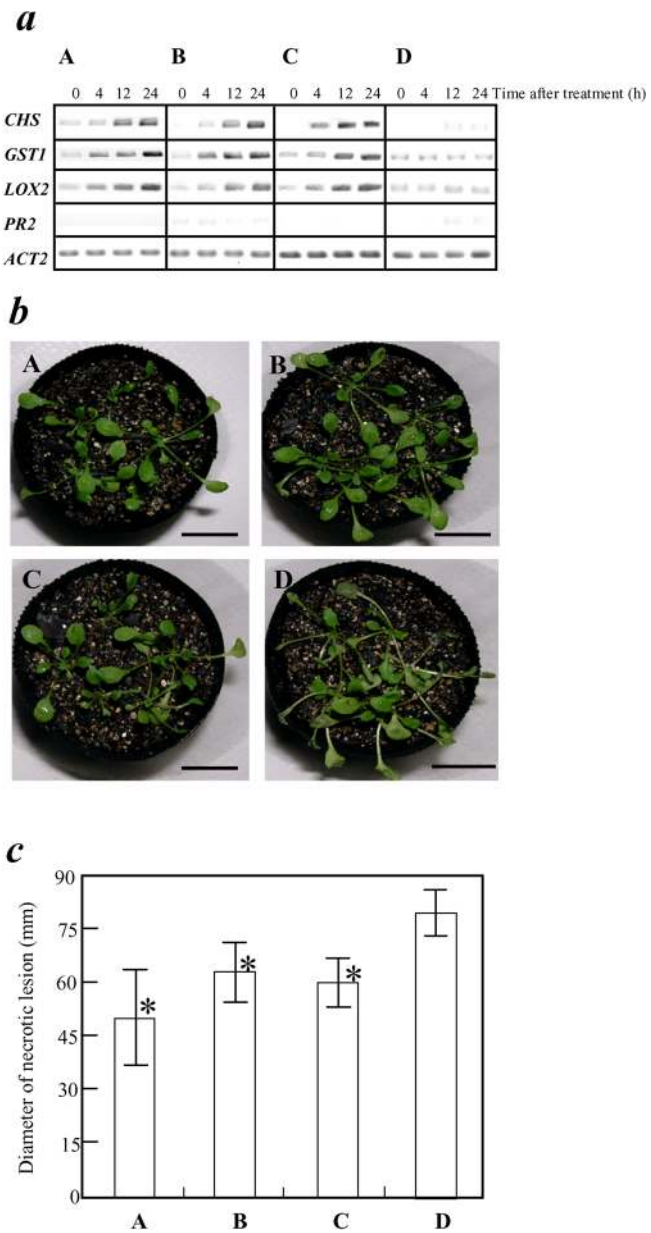

Fig. 5 The effect of volatiles on soil-grown *Arabidopsis*. (a) *Arabidopsis* seedlings grown on soil were treated with volatile (*E*)-2-hexenal (A), (*Z*)-3-hexenal (B), allo-ocimene (C), or without volatiles (D) in a separate flask, and then the leaves were harvested for RT–PCR analyses to monitor expression levels of *CHS*, *GST1*, *LOX2*, *PR2* and *ACT2* (as a control). (b) The plants treated with each volatile for 24 h were inoculated with *B. cinerea* by placing droplets of the conidial suspension on each leaf. The disease symptoms were observed 2 d after inoculation. Scale bar = 3 cm. (c) The diameters (mm) of necrotic lesions on the leaves at 2 d after inoculation. Asterisks indicate a significant difference at $P < 0.05$ ($n = 3$) against control. Error bars indicate ±SE.

Discussion

Volatile compounds induce defense genes

In this study, it was revealed that treating *A. thaliana* with (*E*)-2-hexenal, (*Z*)-3-hexenal, (*Z*)-3-hexenol or allo-ocimene induced expression of *CHS*, *DGK1*, *COMT*, *GST1* and *LOX2*, all of which are known to be involved in defense responses. This response could be found either with agar-grown or with soil-grown plants. Generally, the amounts of GLVs and volatile isoprenoids are low in intact plant tissues; however, these volatiles are formed rapidly after wounding, pathogen or herbivore attack (Croft et al. 1993, Loughrin et al. 1994, Arimura et al. 2000). The volatile organic compounds thus formed can be signal molecules in two ways, i.e. those within a plant and those between plants. The amount of (*Z*)-3-hexenal in *Arabidopsis* leaves reached 0.5 μmol g FW^{-1} after disruption (Matsui et al. 2000). This value is comparable with the amount of volatiles used in this study, with the assumption that the plants would take up a portion of the chemical. In this context, the results shown in this study may indicate that the volatile

compounds formed after tissue disruption caused by herbivore attack or mechanical damage can function as gene-inducing molecules within a plant. A transgenic *Nicotiana attenuata*, which had lower HPL activity through antisense suppression, showed a deficiency of induced defense genes, such as *xyloglucan endotransglucosylase/hydrolase* or *proteinase inhibitor*, after herbivore attack (Halitschke et al. 2004), which also indicates that GLVs can be signal molecules within a plant.

The second possibility is that the volatiles function as airborne signal molecules between plants. In this experimental condition, 1 μmol l^{-1} of volatiles induced defense genes in *Arabidopsis*; however, little induction could be observed with 0.1 μmol l^{-1}. From six glanded cotton plantlets, about 2–90 nmol of GLV were released within 3 h after being fed on by *Spodoptera exigua* larvae (McAuslane and Alborn 1998). From two leaves of a French bean plant, about 50 and 200 nmol of (*E*)-2-hexenal and (*Z*)-3-hexenol, respectively, were emitted to the atmosphere after the bacterial infection (Croft et al. 1993). Although direct comparison is impossible, these amounts seem to be much lower than those used in this study. Therefore, it was suggested that the volatiles used in this study hardly function as airborne signals in *Arabidopsis*, at least when they were used individually and/or when they were used under the conditions employed here. It might be possible that a synergistic effect caused by a complex mixture of volatiles is needed. The amounts of GLVs formed differ greatly among plant species (Hatanaka 1993), thus, if a plant would grow alongside plants that can emit high amounts of volatiles, it would be still possible even for *Arabidopsis* to induce defense genes. Alternatively, local attack by herbivores might lead to substantial release of a blend of volatiles that together could be sufficient to, at least locally, induce defense-related genes. These possibilities must be evaluated through further study.

Possible mechanisms involved in the perception of volatiles by plants

Among the volatiles used here, only (*E*)-2-hexenal falls into the category of reactive electrophilic species defined by Alméras et al. (2003) because of its α,β-unsaturated carbonyl moiety. At least part of the ability of (*E*)-2-hexenal to induce defense gene expression can be attributed to the chemical reactivity of this compound. The aldehyde with a β,γ-unsaturated carbonyl moiety, (*Z*)-3-hexenal, was also a potent inducer of the defense genes. In some plants, an enzyme that catalyzes the isomerization of the β,γ-unsaturated carbonyl to the corresponding α,β-form occurs (Hatanaka 1993). Thus, a portion of (*Z*)-3-hexenal might be converted into (*E*)-2-hexenal in plants, which in turn might induce the defense genes. The chemical reactivity of (*Z*)-3-hexenol is even lower; however, the ability of the alcohol to induce defense gene expression was comparable with those of (*E*)-2- or (*Z*)-3-hexenal. Especially with *GST1*, the alcohol was most potent in inducing its expression. Farag et al. (2005) also showed the high ability of (*Z*)-3-hexenol to induce defense genes in maize. These findings support that the chemical reactivity of a volatile compound is not the sole factor in activation of defense genes. Bate and Rothstein (1998) reported that (*Z*)-3-hexenol, abundant in wounded plant tissues, showed a higher ability to induce *LOX* expression than (*E*)-2-hexenol, which is scarce in nature. Therefore, machinery that preferably recognizes (*Z*)-3-hexenol should operate in *A. thaliana*. Allo-ocimene has no functional group other than the double bonds; even so, the potential of allo-ocimene to activate gene expression was high. From this result, again, it is suggested that machinery that can specifically recognize a compound operates in *A. thaliana*.

JAR1-dependent and JAR1-independent pathways are operative

When *jar1* was used, induction of *CHS*, *DGK1* and *GST1* by the volatile compounds was extensively suppressed. This indicated that the GLVs and the isoprenoid activated the JAR1-dependent signaling pathway. *JAR1* encodes an acyl-adenylating enzyme that is highly specific to JA (Staswick et al. 2002). On the contrary, the induction profiles of *LOX2* expression by the volatiles were little modified in *jar1* plants. Thus, it is suggested that *LOX2* was induced in the JAR1-independent pathway. Induction of *COMT* by allo-ocimene was suppressed with *jar1* but that by (*Z*)-3-hexenal was not, which suggested that *COMT* was induced by the volatile compounds either in the JAR1-dependent or -independent pathway, depending on the compound used. It has been indicated that oxophytodienoic acid (OPDA) is the regulator of wound induction of a subset of defense genes, such as *COMT* and *LOX2* (Stintzi et al. 2001, Park et al. 2002). OPDA cannot be a substrate for JAR1 (Staswick et al. 2002). It has been reported that the level of OPDA was elevated after addition of a volatile aldehyde, acrolein (Alméras et al. 2003). Taken together, one of the JAR1-independent pathways might be OPDA dependent.

With the wild-type plants, the volatile compounds failed to induce *PR2*; however, with *jar1*, it was clearly induced by any volatile compounds used in this study. It has been suggested that the JA signaling pathway and SA signaling pathway cross-talk with each other in a suppressing way (Glazebrook et al. 2003). Therefore, it can be assumed that the volatiles could activate the SA signaling pathway at least in the absence of functional JAR1, although the other possibilities cannot be ruled out at this moment. When an ET-resistant mutant, *etr1*, was used instead, the induction profiles were similar to those found with wild-type plants. From this, it can be assumed that ETR1-mediated signaling was little involved in defense gene induction by the volatile compounds.

A protein phosphatase inhibitor, okadaic acid, efficiently suppressed the induction of defense genes by the volatile compounds. These results suggested that protein dephosphorylation was involved in the induction of the defense genes by the volatile compounds, and that the dephosphorylated state of a protein was important for the induction. It has been reported that okadaic acid blocked JA-inducible expression of a subset of JA-responsive genes (Dammann et al. 1997, Rojo et al.

1998, Jensen et al. 2002), from which it is indicated that a protein phosphatase is a component of the JA signaling pathway. Although *LOX2* was induced in the JAR1-independent pathway by the volatile compounds, the induction of *LOX2* was also inhibited by okadaic acid. Thus, an involvement of a protein phosphatase in the signaling pathway to induce *LOX2* could be suggested. From the results with staurosporine, it could be assumed that a protein kinase might be involved in the defense gene induction. It has been reported that staurosporine treatment on *Arabidopsis* resulted in induction of a JA-related defense gene, such as *CHS* or *LOX2* (Rojo et al. 1998, Jensen et al. 2002). However, a side effect and/or indirect effect of staurosporine could not be ruled out especially because it enhanced the expression of defense genes even in the absence of the volatiles.

Exposure to volatiles confers resistance against B. cinerea

The volatile treatment on *A. thaliana* retarded the development of disease symptoms caused by the *B. cinerea* infection in both agar-grown and soil-grown plants. It might be possible that the chemicals bound on the surface of the leaves directly exerted their antifungal effects because C6-aldehydes are fungicidal (Wright et al. 2000, Gomi et al. 2003). When a high molecular weight fraction (>12,000 Da) was prepared from the volatile-treated plants, it showed high fungicidal activity, and its activity disappeared after boiling (K. Kishimoto et al. unpublished). Therefore, the higher resistance to *B. cinerea* caused by the volatile treatment might be at least partly attributable to the accumulation of fungicidal high molecular weight compounds. Gomi et al. (2003) also found that treating *Citrus* plants with (*E*)-2-hexenol enhanced resistance against a necrotrophic fungal pathogen, *Alternaria alternata*. Unexpectedly, in their case, the enhancing activity could be found only with (*E*)-2-hexenol that is scarce in nature, but not with (*Z*)-3-hexenol that is abundantly formed by most plants. In this study, it was shown for the first time that volatiles commonly found in nature enhanced the resistance of *Arabidopsis* against *B. cinerea*. Induction of *CHS* may result in formation of fungicidal flavonoids as reported with soybean (Juergen et al. 1984), while induction of *COMT* may result in formation of lignin to prevent invasion of the fungi. Induction of the genes other than those investigated here, or alteration of metabolism in plants after volatile treatment (Farag et al. 2005) might also cause a higher resistance against the fungus. The precise mechanism attributable to the high resistance is now under study.

Materials and Methods

Plant material

Arabidopsis thaliana (ecotype *Col-0*) seeds were surface sterilized, incubated at 4°C for 48 h in the dark in 0.1% agar, and dispersed onto an MS plate containing 2% sucrose. Plants were grown in a chamber at 22°C with light from fluorescent lights (70–80 μmol m^{-2} s^{-1}). The light/dark condition used was 16 h light/8 h dark. Unless otherwise stated, plants were grown for 21 d. In some cases, plants were removed to soil (Metro-Mix, SunGro Horticulture Distribution Inc., Bellevue, WA, USA) in pots, and were grown further (for 9 d) under the same condition. To pre-treat the agar-grown plants, the lids were removed and the plants were placed inside a separate flask (1 liter). They were incubated as above for 24 h before treatment. When the soil-grown plants in pots were used, they were also placed inside the flask and incubated for 24 h prior to the volatile treatment. (*E*)-2-Hexenal (>97%), (*Z*)-3-hexenol (>99%) and *n*-octanol (>98%) were purchased from Wako Pure Chemicals (Osaka, Japan) and allo-ocimene (2,6-dimethyl-2,4,6-octatriene; >94%) was from Tokyo Kasei (Tokyo, Japan). (*Z*)-3-Hexenal (> 92%) was synthesized through oxidation of (*Z*)-3-hexenol. The volatiles 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 hanging from the glass lid of the flask where the agar-grown or the soil-grown *Arabidopsis* plants had been placed. Control plants were treated only with dichloromethane.

Stock solutions of 1 mM staurosporine and 0.5 mM okadaic acid (both from Wako Pure Chemicals) were prepared in dimethylsulfoxide. The plants grown for 20 d were removed from the agar plates and the roots were placed into 0.5× MS liquid medium. The plants were incubated in a separate flask (1 liter). After 2 d, the chemical was added to the medium. The final concentration of staurosporine and okadaic acid was 1 and 0.5 μM, respectively. At the same time, the plants were treated with the volatiles.

RT–PCR

Total RNA was extracted from leaves (100 mg FW) of treated plants at a given time after the volatile treatment by TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) and treated with a DNA-free kit (Ambion, Austin, TX, USA). cDNA was synthesized from the total RNA (4 μg) by the Thermoscript RT–PCR System with oligo $(dT)_{20}$ as a primer (Invitrogen). Semi-quantitative real-time RT–PCR was performed by using SYBR Green PCR core reagents kit (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instruction. Primers of each gene, shown in Table 1, were designed by Primer Express Software version 1.0 (Perkin-Elmer Applied Biosystems). The reaction mixture contained 10 ng of cDNA, 50 nM each of forward and reverse primer, 1× SYBR Green PCR Buffer, 800 nM $MgCl_2$, 200 nM dNTPs, 0.4 U of Ampli Taq Gold and 0.16 U of AmpErase UNG in a 50 μl solution. PCR (95°C for 15 s, 60°C for 1 min) for 40 cycles was performed by a GeneAmp 9600 PCR thermocycler, and the data were processed by using GeneAmp 5700 Sequence Detection System version 1.3 (Perkin-Elmer Applied Biosystems). The sequence of each amplified DNA was verified, and every time after PCR amplification, a heat dissociation curve of the amplified products was examined in order to check the specificity of amplification. Expression levels of the respective gene are shown as amounts relative to the transcripts of *AAc1*. The experiment was repeated at least three times with different plants.

In some cases, the transcript levels were estimated by agarose gel electrophoreses of the amplified DNA fragments. RNA was extracted and purified, and converted to cDNA as shown above. RT–PCR was performed by using TaKaRa ExTaq polymerase (Takara Biomedicals, Japan). Primers for *ACT2* (At3g18780) were 5′-CTAACCTCTCTCAAGATCAAAGGCT-3′ (forward) and 5′-ACTAAAACGCAAAACGAAAGCGG-5′ (reverse); for *CHS* they were 5′-GCAGGCATCTTGGCTATTGGCAC-3′ and 5′-AGTCTGGAAGGATGGTCTGAGCG-3′; for *GST1* they were 5′-CGGTCACCCAGCTTCCACAGCCA-3′ and 5′-TGTCAGCAACCCAAGCACTCACA-3′; for *LOX2* they were 5′-GCATCCTCATTTCCGCTACACCA-3′ and 5′-TCCGCACTTCACTCCACCATCCT-3′; and for *PR2* they were 5′-CAAGGAGCTTAGCCTCACCACCA-3′ and 5′-GGCCGTGTCTCCCATGTAGCTGA-3′. The reaction mixture contained 0.1 μg of cDNA, 0.5 μM each of

forward and reverse primer, 1× ExTaq Buffer, 250 nM dNTPs and 0.5 U of ExTaq polymerase in a 20 µl solution. The PCR was carried out on a Perkin-Elmer 9700 with 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.

Plant infection with pathogens

After the volatile treatment for 24 h, the plants were placed in a fresh, clean flask. The seedlings (60) in a Petri dish were sprayed with 1.5 ml of *B. cinerea* conidial suspension (1×10^5 c.f.u. ml^{-1}) in 2.5% glucose, or 3 µl of the conidial suspension was placed on the upper surface of leaves grown on soil in a pot. The inoculated *Arabidopsis* plants were incubated in an environmental growth chamber at 22°C with a 16 h/8 h photoperiod. After inoculation (48 h), disease symptoms of the plants in a Petri dish were recorded according to the method of Berrocal-Lobo et al. (2002). With the soil-grown plants, diameters of necrotic lesions were measured.

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