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# Biosynthesis of fatty acid derived aldehydes is induced upon mechanical wounding and its products show fungicidal activities in cucumber

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**Abstract**

Fatty acid 9/13-hydroperoxide lyase (9/13-HPL) in cucumber is an enzyme that can cleave either 9- or 13-hydroperoxides of polyunsaturated fatty acids to form C9- or C6-aldehydes, respectively, as products. In order to reveal the physiological function of 9/13-HPL, its expression profiles were analyzed, and it was found that 9/13-HPL expression was developmentally regulated and high in the hypocotyls, female flowers and mature fruits. However, its transcript as well as its activity was only induced by mechanical wounding in mature leaves. To analyze the biosynthesis of HPL-derived aldehydes in more detail we isolated and characterized the yet missing 9-lipoxygenase (LOX) that is mainly expressed in hypocotyls, cotyledons and flowers and that may provide HPL with fatty acid 9-hydroperoxides as substrates. As in the case with C6-aldehydes in most plant species, C9-aldehydes were also formed rapidly after disruption of the tissues. C9-aldehydes had fungicidal activities against fungal pathogens, *Botrytis cinerea* and *Fusarium oxysporum*. Because the concentration needed to cause toxic effect on the pathogens was almost equivalent to that found in disrupted tissues, the C9-aldehydes thus formed could be helpful to sterilize the wounds since they are less volatile in comparison to C6-aldehydes.


*Keywords:* *Cucumis sativus* L.; Cucurbitaceae; Cucumber; Fungicidal activity; Fatty acid 9-hydroperoxide lyase; 9-Lipoxygenase

## 1. Introduction

Fatty acid hydroperoxide lyase (HPL) is an enzyme that cleaves a fatty acid hydroperoxide (HPO) derived from the activity of a lipoxygenase (LOX) into two carbonyl compounds (Feussner and Wasternack, 2002; Noordermeer et al., 2001). LOXes introduce molecular oxygen at either carbon atom C-9 or C-13 position of linoleic acid or linolenic acid. Depending on the substrate specificities HPLs can be classified into three groups; 13-HPL that specifically cleaves fatty acid 13-HPO to form C6-aldehydes (*n*-hexanal (**1**) or (*Z*)-3-hexenal (**2**)) and 12-oxo-(*Z*)-9-dodecenoic acid (Matsui et al., 1991), 9/13-HPL that can cleave both 13-HPO and 9-HPO in almost the same efficiency (Matsui et al., 2000), and 9-HPL that specifically cleaves 9-HPO (Mita et al., 2005). From 9-HPO of C18 fatty acids, C9-aldehydes ((*Z*)-3-nonenal (**3**) or (*Z*,*Z*)-3,6-nonadienal (**4**)) and 9-oxo-nonanoic acid are formed. In some plants, part

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of the β,γ-unsaturated carbonyls are enzymatically or nonenzymatically isomerized to form corresponding α,β-unsaturated carbonyls, such as (*E*)-2-hexenal (**5**), (*E*)-2-nonenal (**6**), or (*E*,*Z*)-2,6-nonadienal (**7**). They are often further converted to the corresponding alcohols and esters (Arimura et al., 2005). 13-HPL is widespread in the plant kingdom, and almost every plant examined so far has the enzymatic activity. On the contrary, 9/13-HPL activity could not be detected in every plant. 9/13-HPL is abundant in a family of Cucurbitaceae among others; therefore, C9-aldehydes are important flavor impact compounds in cucumbers or melons (Schieberle et al., 1990).

It has been reported that 13-HPL is involved in defense responses of plants against herbivore and pathogen attacks (Arimura et al., 2005; Vancanneyt et al., 2001; Croft et al., 1993). On the contrary, the physiological function of 9/13-HPL has not been fully elucidated yet. Other than their importance as flavor compounds, C9-aldehydes have antibacterial (Cho et al., 2004) and antifungal activities (Hamilton-Kemp et al., 1992), from which it has been assumed that 9/13-HPL is also involved in defense responses of plants. In this study, we examined expression profiles of 9/13-HPL in cucumber, and addressed whether 9/13-HPL in cucumber is involved in defense responses or not.

## 2. Results and discussion

### 2.1. HPL activity in cucumber plants

In order to determine the distribution of 9- and 13-HPL activities in cucumber plants, those in the organs from 9, 30, and 120 day old cucumber plants were analyzed (Fig. 1). In the 9 day old seedlings, the hypocotyls showed the highest 9-HPL activity. This was also the case with 30 day old plants and the hypocotyls had the highest activity. The activity in the petiole was also high. 9-HPL activity in the hypocotyls increased gradually at least until 9 days after germination. This developmental change in the activity was little affected by light, and was almost the same either with the etiolated or green seedlings (data not shown). In the female flowers high 9-HPL activity could be found as well; however, in the male flowers, it was almost one-third of that found in the female flowers. During fruit maturation the activity gradually decreased, but it increased again in the fully matured cucumber fruits of stage IV. Highest 13-HPL activity could be found in the roots of 9 day old seedlings. Both the female and male flowers showed high 13-HPL activity with almost the same level. 13-HPL activity decreased during maturation of the fruits until stage IV. Apparently, the ratio between 9- and 13-HPL activities differed depending on the organs and developmental stages although 9/13-HPL in cucumber has both activities to almost the same extent, which suggested that beside a 9/13-HPL an additional 13-HPL exists. The 13-HPL and 9/13-HPL seem to be independently regulated. When a crude extract was prepared from the hypocotyls of 9 day old seedlings, ca. 85% of 9-HPL activity could be found in the microsomal fraction, while only 50% of 13-HPL activity could be found in the fraction. Again, this suggested that there must be at least two HPLs in cucumber plants that have distinct substrate/product specificities as reported previously (Matsui et al., 1989). The 13-HPL might be the one that was local-

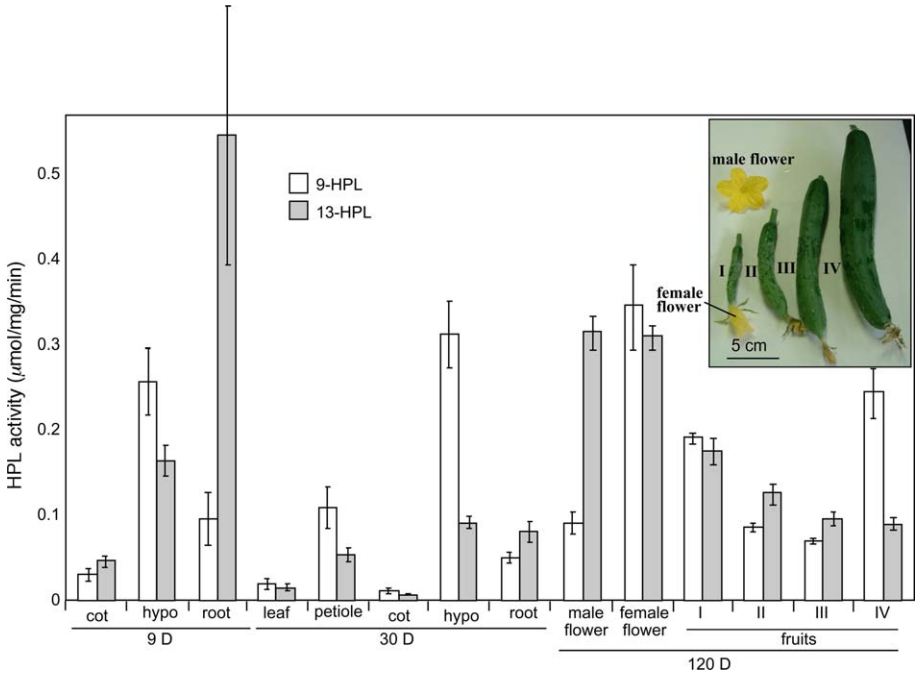


Fig. 1. Developmental changes of 9-HPL activity (open bars) and 13-HPL activity (shaded bar) in cucumber plants. The means (±SE) of three replicates are shown. Inset shows the size of cucumber fruits used.

ized in the membranes of lipid bodies (Weichert et al., 2002). We reported that cucumber has a pseudogene that might originally encode 13-HPL, and interestingly, this pseudogene was transcribed into mRNA (Matsui et al., 2000). Apparently, there must be another 13-HPL gene in cucumber. However, an extensive effort to find a gene encoding an active 13-HPL based on the sequences conserved among 13-HPL in plants has failed till today. Cucumber 13-HPL gene might have a peculiar sequence, or the pseudogene might undergo a post-transcriptional modification to encode an active 13-HPL.

### 2.2. Isolation and characterization of a 9-LOX from cucumber

It has been reported that one of the LOX isozymes was a predominant protein in the phloem sap of cucumber plants (Avdiushko et al., 1994). High 9-HPL activity could be found in the hypocotyls and the petioles where the phloem sap was abundant, and this LOX may provide the substrates for the 9-HPL activity. Thus, it was expected that the HPL coexisted in the phloem with a LOX. When the phloem sap was collected from cut hypocotyls or cut petioles and subjected to SDS-PAGE analyses, three major protein bands of 100, 55, and 45 kDa could be found. The one of 100 kDa might correspond to the LOX (Avdiushko et al., 1994). However, 9-HPL activity was low in this preparation. The amounts of C9-aldehydes were also scarce. This was also the case with 13-HPL activity and with the amount of C6-aldehydes. Thus, it could be concluded that high HPL activities in the hypocotyls and petioles were not directly associated with accumulation of the enzyme in the phloem.

It has been reported that cucumber has several LOX isozymes (Feussner and Kindl, 1994). The LOXes that were extensively characterized so far were 13-LOXes from cotyledons and roots (Höhne et al., 1996; Matsui et al., 1999a), although there was no report of the molecular characterization of 9-LOX in cucumber. Therefore, we isolated the 9-LOX from cucumber by screening a cDNA library of 4 day old etiolated cucumber cotyledons (Ahnert et al., 1996). Of ca. $1 \times 10^6$ plaques screened 5 positives were isolated. Partial sequencing of the clones demonstrated that all were derived from the same mRNA. The longest insert sequenced had a length of 3166 bp including a part of its poly(A) (accession no. AJ271161). The coding sequence started at position 273 and ended at position 2918 of the isolated cDNA fragment. The deduced protein consists of 881 amino acids and has a predicted molecular mass of ca. 100.7 kDa (accession no. CAB83038). The amino acid sequence was most similar to that of LOX A from tomato (80%; accession no. P38415) and LOX1 from potato (79%; accession no. P37831). Thus, this clone codes for a third LOX beside the already published two 13-LOXes from roots and cotyledons probably encoding a 9-LOX as indicated by its highest sequence similarity to plant 9-LOXes (Höhne et al., 1996; Matsui et al., 1999a). To analyze the

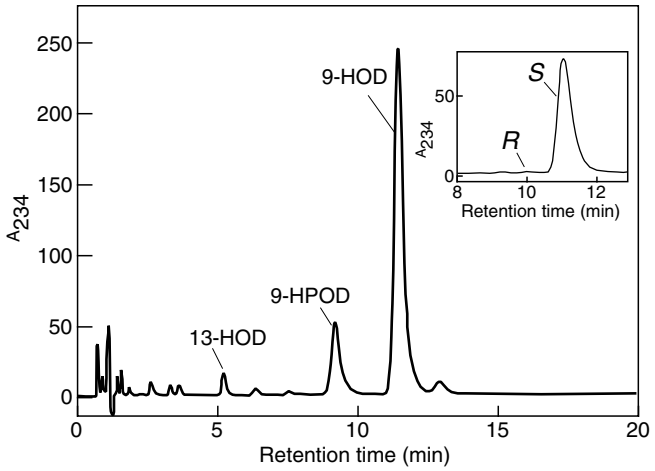


Fig. 2. HPLC analysis of hydroxyl fatty acids formed from linoleic acid by LOX1:Cs4. Crude cell lysate of LOX1:Cs4 was incubated with linoleic acid, the produced hydroperoxides reduced with sodium borohydride and after acidification subjected to MeOH/$CH_3Cl$ extraction. Oxygenated fatty acid derivatives were isolated by reversed phase HPLC and positional isomer were analyzed by normal phase HPLC. Ratio of *S* and *R* of the 9-hydroxide was determined by chiral phase HPLC (*Inset*). HOD, hydroxyoctadecadienoate; HPOD, hydroperoxyoctadecadienoate.

biochemical properties of this LOX the full-length protein was expressed in *Escherichia coli*. After incubation of bacterial cell lysate with linoleic acid the major reaction product was identified as (9*S*)-hydroperoxy linoleic acid (**10**), which indicated that this LOX was 9-LOX (Fig. 2).

### 2.3. Expression of 9/13-HPL and 9-LOX in cucumber plants

When Northern blot analyses were performed, high expression of 9/13-HPL gene could be found in the

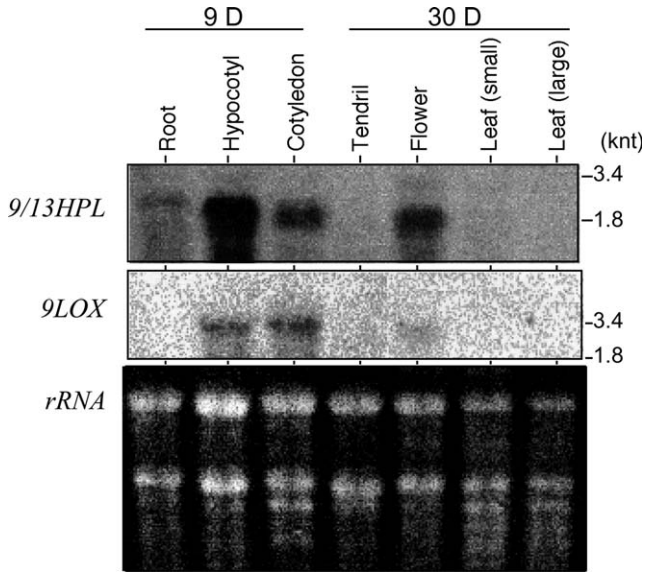


Fig. 3. Expression of *9/13-HPL* and *9-LOX* in various organs of cucumber. To prove equal loading of the total RNA, the ethidium bromide-stained RNA is shown at the bottom. The 9/13-HPL transcripts have a size of ca. 1.8 kb and the 9-LOX transcripts have a size of ca. 3.3 kb.

hypocotyls of 9 day old seedlings (Fig. 3). Next to the organ, the cotyledons of 9 day old seedlings and the flowers of 30 day old plants showed high transcript levels. Low expression could be found in the roots of 9 day old seedlings; however, the transcripts were hardly detected in either leaves or tendrils. The expression profile was almost similar to 9-LOX, i.e., high in the hypocotyls and cotyledons, and moderate in the flowers. From this it might be assumed that the expression of both 9/13-HPL and 9-LOX was cooperatively regulated. Thus, we propose that this 9-LOX may provide the substrate for the formation of C9-aldehydes by 9/13-HPL.

### 2.4. Induction of 9/13-HPL activity and 9/13-HPL and 9-LOX transcripts after wounding

It has been known that 13-HPL has a physiological role during plant responses against insect feeding and mechanical wounding (Arimura et al., 2005; Vancanneyt et al., 2001; Croft et al., 1993). In order to identify whether 9/13-HPL is involved in plant responses against wounding as well, the leaves of 30 day old cucumber plants were mechanically wounded, and the activities of 9- and 13-HPLs were determined (Fig. 4A). Six hours after wounding, 9-HPL activity was specifically induced and after 24 h it returned to its original level. Despite the wound-induction of 13-HPL activity in other plant species (Matsui et al., 1999; Noordermeer et al., 2001), the cucumber 13-HPL activity was little changed after wounding. The wound-induction of 9-HPL activity could be found only with the local leaves, with no change in activity found in the systemic leaves (unwounded leaves of the wounded plants). When transcripts of 9/13-HPL gene were quantified by semi-quantitative real time RT-PCR, a slight increase in their amounts could be found (Fig. 4B). Unexpectedly, the transcript levels increased until 24 h after wounding, which apparently disagreed with changes in activity. This discrepancy between activity and transcript level of 13-HPL has been reported in potato plants (Vancanneyt et al., 2001). When we compared the observed increase in 9/13-HPL transcripts with those of 9-LOX, we observed no changes in the transcripts of 9-LOX. When the amounts of transcript encoding ACC oxidase that are known to be induced after wounding in cucumber were analyzed (Fig. 4B, *ACO*; Watanabe et al., 2001), a distinct induction could be found, which suggested that the wound treatment performed in this study appropriately induced wound responses in the cucumber leaves.

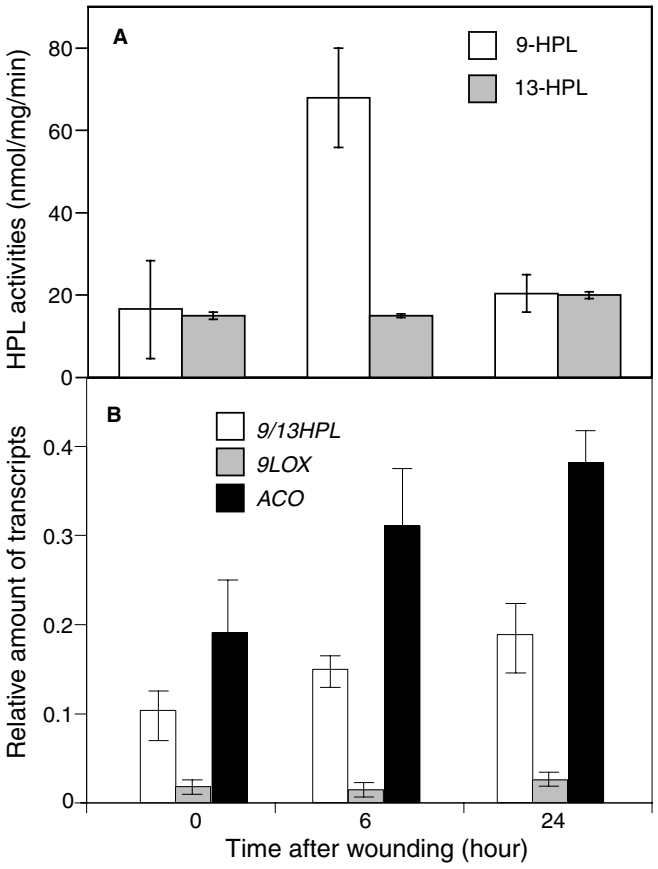


Fig. 4. Effect of mechanical wounding on 9- and 13-HPL activities (A) and on the expression of *9/13-HPL*, *9-LOX*, and ACC oxidase (*ACO*) (B). The relative amounts of the transcript against that of actin are shown in panel B. Error bars indicate ±SE ($n = 3$).

It has been reported that jasmonates are involved in plant responses against wounding and herbivore attacks (Arimura et al., 2005). For example, in Arabidopsis, jasmonates induced expression of 13-HPL (Matsui et al., 1999), and in cucumber, the amount of C6-aldehydes increased after treating the plants with jasmonates (Avdiushko et al., 1995). When 30 day old cucumber plants were treated with vapor of methyl jasmonate, no change in either 9-HPL activity or in expression level of 9/13-HPL gene could be found (data not shown). On the contrary, 13-HPL activity was induced after methyl jasmonate treatment. This again suggested that 9/13-HPL and 13-HPL activity were regulated independently. In order to find out a component that might be involved in the regulation of 9-HPL activity, other compounds, such as methyl salicylate, ethephone, cytokinine, gibberellins, and auxin, were used to treat cucumber plants. However, 9-HPL showed no significant change in activity level with any of the compounds examined.

### 2.5. The levels of HPL products after tissue disruption

In most plants, the amounts of HPL products increase after disruption of their tissues (Noordermeer et al., 2001). Such rapid formation of C9-aldehydes after tissue disruption has been also reported with cucumber fruits (Galliard and Phillips, 1976), but not with its other organs. When the amounts of C6- and C9-aldehydes in the intact leaves of 30 day old cucumber plants were analyzed, **2** was found to be the main aldehyde (Fig. 5, open columns). This is in contrast to etiolated cotyledons where **1** was the main aldehyde (Weichert et al., 2002). After disruption of the leaf tissues, the amounts of C6- and C9-aldehydes

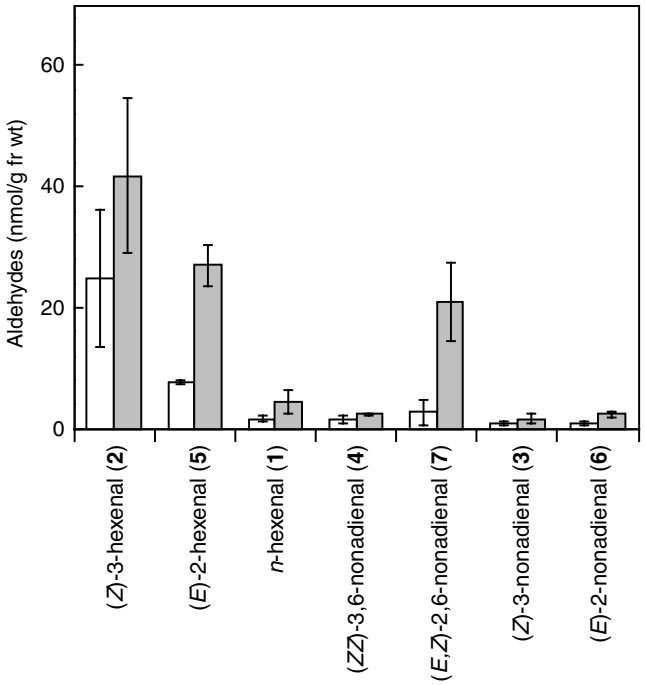


Fig. 5. Formation of C6- and C9-aldehydes after disruption of cucumber leaves. The amounts of aldehydes in the intact hypocotyls (open bars) and in the disrupted hypocotyls (shaded bars) are shown. The means (±SE) of three replicates are shown.

increased rapidly (Fig. 5, shaded columns). This indicated that the formation of C9-aldehydes was also accelerated by disruption of the cucumber leaves as well as that of C6-aldehydes. Among C9-aldehydes, **7** increased significantly. Because the primary product of cucumber 9/13-HPL was **4** (Matsui et al., 2000), this aldehyde must be formed through 9/13-HPL reaction and a subsequent isomerase reaction that is known to occur in cucumber (Noordermeer et al., 2001). A rough estimation implies that the concentration of C9-aldehydes after tissue disruption might reach approximately more than 20 μM in the tissue.

By using a transgenic tomato over-expressing cucumber 9/13-HPL, it has been shown that the metabolic flow of C6- and C9-aldehyde forming pathways are independently regulated (Matsui et al., 2001). When a crude extract prepared from hypocotyls was incubated with 13-hydroperoxy- or 9-hydroperoxy linolenic acid, almost the same amounts of **5** and **7**, respectively, were formed. This may indicate that the 9- and 13-HPL activities were almost same in this organ. Almost the same amounts of C6- and C9-aldehydes could be also found when linolenic acid was added to the crude extract. This indicated that the capacities to form C6- and C9-aldehydes from fatty acids are comparable, and there is no specific preference between the two metabolic pathways.

### 2.6. Fungicidal activities of C9- and C6-compounds

It has been reported that C6- and C9-aldehydes have bactericidal activities against a wide spectrum of bacterial species (Nakamura and Hatanaka, 2002; Cho et al., 2004). In this study, fungicidal activities of a subset of C9- and C6-compounds were estimated by using pathogenic fungi, *B. cinerea* and *Fusarium oxysporum*. *B. cinerea* is a typical necrotrophic pathogen, and causes necrotic lesions on leaves of a wide variety of plants including cucumber (Kishimoto et al., 2002). *F. oxysporum* is a soil borne fungus and causes vascular wilt disease in many plant species (Kannangara et al., 2000). The spores of fungi were collected, and mixed with the test compound with a given concentration, and thereafter, germination rates were examined (Figs. 6 and 7). In the absence of the compound, the spores germinated and the hyphae grew vigorously. When **6** was added to the spore suspension of *B. cinerea*, the germination was extensively inhibited, and with 0.1 mM of the chemical no germination could be observed (Fig. 7A). Almost the same fungicidal activity occurred with the saturated C9-aldehyde, *n*-nonanal (**8**). From this finding, the α,β-unsaturated functionality is not an essential factor to make the compound fungicidal. The corresponding α,β-unsaturated alcohol, (*E*)-2-nonenol (**9**), also showed fungicidal activity against *B. cinerea*, however,

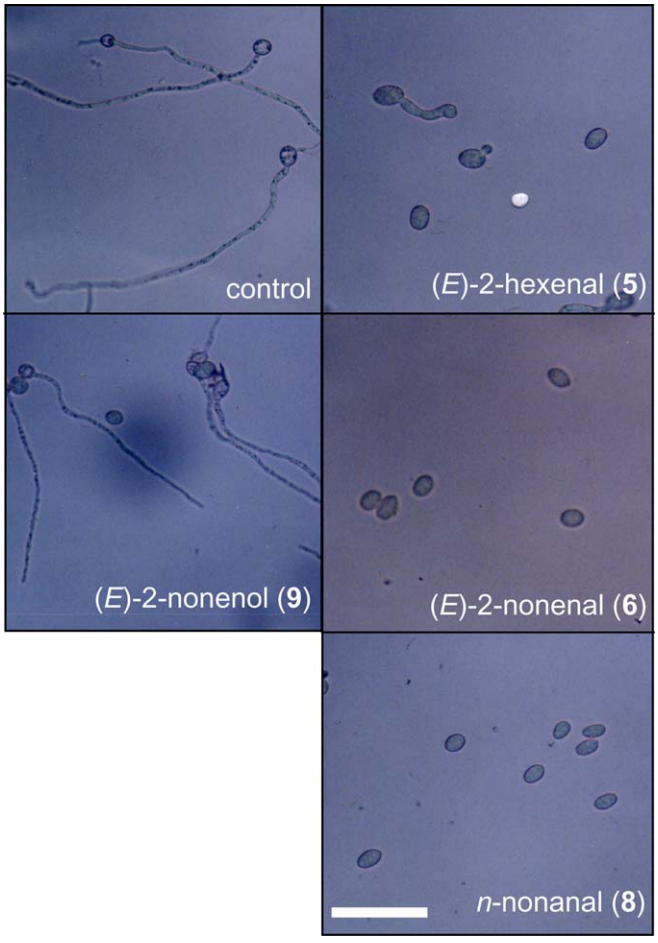


Fig. 6. Effect of C6- and C9-compounds on spore germination of *Botrytis cinerea*. The conidia were mixed with 0.1 mM of the compound and incubated under darkness for 24 h. The bar represents 60 μm.

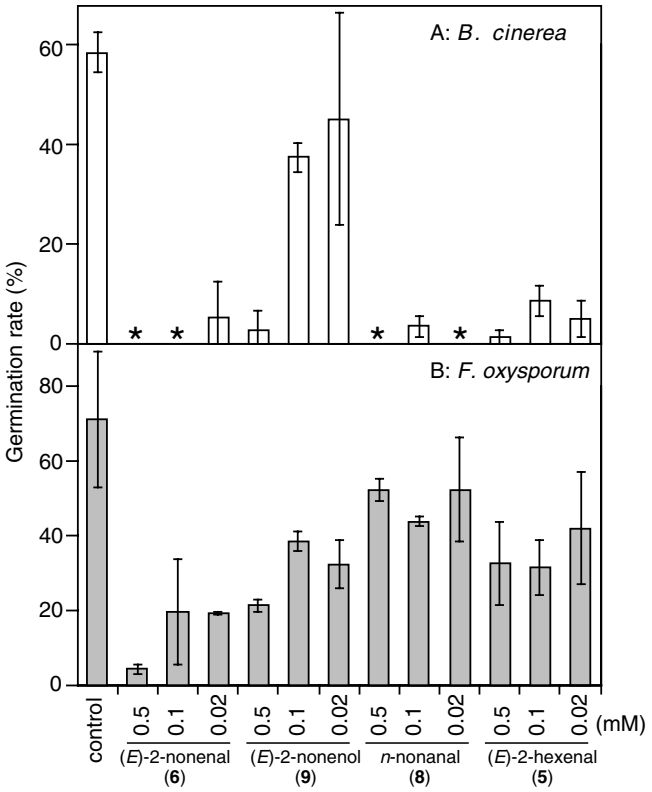


Fig. 7. Effect of C6- and C9-compounds on spore germination of *Botrytis cinerea* (A) and *Fusarium oxysporum* (B). The conidia were mixed with a given concentration of compound, then incubated under darkness for 24 h. The germination rate was counted under a microscope. *, no germination could be observed. Error bars indicate ±SE ($n = 3$).

the activity was much less than that with **6**. Thus, the oxo group must be an important requisite for the activity. With **5**, again high fungicidal activity was observed. The spores of *F. oxysporum*, however, were slightly more resistant against the chemicals used here (Fig. 7B). Among the compounds, **6** was most active, and with 0.5 mM only 5% of the spores germinated. Interestingly, and apart from the results obtained with *B. cinerea*, the corresponding alcohol showed moderately high fungicidal activity, whereas the fungicidal activities of *n*-nonanal and **5** were not as high. A comparison of fungicidal activities of **6** and **5** suggested that the chain length of the compounds might be important for fungicidal activity. In other words, especially in the case with *F. oxysporum*, it was suggested that the net hydrophobicity of a substance was important to display fungicidal activity. Probably, the more hydrophobic compounds could penetrate into the spore cells more easily, which might result in this difference. The concentrations of C6- and C9-volatiles that can cause inhibition of spore germination was mostly comparable to their concentration in the disrupted tissues (Fig. 5). When plant tissues are mechanically wounded, the wound could also be a suitable site for pathogens to invade into the tissues. The cells disrupted at the wounds could form the C6- and C9-volatiles to the concentrations equivalent to those found in the disrupted tissues. Thus, the fungal pathogens might fail to germinate efficiently on the wounds of plants because of the toxicities of the C9- and C6-aldehydes accumulated at that limited area.

## 3. Concluding remarks

By surveying the expression profiles of cucumber 9/13-HPL, it was found that 9/13-HPL expression was developmentally regulated. The expression level showed little change after treating the plants with a variety of phytohormones; however, mechanical wounding caused enhancement of the activity. This enhancement might be caused by transcriptional regulation; however, there could be another component that regulates the activity because the transcript level was not always in accordance with that of the activity. As in the case with C6-aldehydes in most plant species, C9-aldehydes were also formed rapidly after disruption of the tissues. C9-aldehydes had fungicidal activities against fungal pathogens, *B. cinerea* and *F. oxysporum*. Because the concentration needed to cause toxic effects on the pathogens was almost equivalent to that found in disrupted tissues, the C9-aldehydes thus formed could be helpful to sterilization of the wounds.

## 4. Methods and materials

### 4.1. Preparation of cDNA libraries

Total RNA was isolated from etiolated cotyledons at day 4 of germination and poly(A)-enriched RNA was prepared using oligo(dT)-cellulose. The Superscript synthesis and cloning system from Invitrogen Life Technologies (Carlsbad, CA, USA) was used to prepare a cDNA library according to the manufacturer. The cDNA was size fractionated and ligated into the *SalI*/*NotI* site of the pSPORT-1 vector. The cDNA library was transformed into *E. coli* strain DH5α.

### 4.2. Isolation of lipoxygenase cDNA and expression in E. coli

The screening with an oligonucleotide (5′-A/GAAT/CTCT/CTCA/GTCIGTCATCCA-3′) synthesized according to a highly conserved region in soybean and pea LOXs (WMTDEEF) yielded 5 clones. Four of the 5 clones contained inserts ranging from 2.0 to 2.6 kb whereas one clone contained an insert of 3.1 kb (GenBank/EMBL accession no. AJ271161, deduced protein sequence;CAB83038). This clone was named pSport-LOX1:Cs4. For bacterial expression the ORF of LOX1:Cs4 was cloned into the pQE30-expression vector (Qiagen, Hildesheim) using pSport-LOX1:Cs4-clone as template for PCR, resulting in pQE-LOX1:Cs4 and transformed into *E. coli* strain M15 (pREP4). Expression was done as described (Feussner et al., 1998). Cells from 200 ml culture were resuspended

in 10 ml of lysis buffer (50 mM Tris–HCl, pH 7.5, containing 10% glycerol, 0.1% Tween 20, 0.5 M NaCl) and disrupted by using a sonifier tip with five pulses each of 30 s, cellular debris was pelleted by centrifugation and the crude cell lysate was used for further analysis.

### 4.3. LOX assay

For product analysis 0.9 ml cell lysate was incubated with 250 μg linoleic acid for 30 min at room temperature. Hydroperoxides were reduced to their corresponding hydroxides with sodium borohydride. After acidification to pH 3, the fatty acids were extracted with $CHCl_3$ and MeOH (Bligh and Dyer, 1959). After removing the solvent, the residues were individually reconstituted in MeOH and subjected to HPLC analysis. HPLC analysis was performed with an Agilent 1100 HPLC system coupled to a diode array detector. Hydroxy fatty acids were separated from fatty acids by reversed phase chromatography (EC 250/4 Nucleosil 120-5 C18, Macherey-Nagel, Düren, Germany) eluted with a solvent system of MeOH/$H_2O$/AcOH (85:15:0.1) at a flow rate of 1 ml/min. The absorbances at 234 nm (conjugated diene system of the hydroxyl fatty acids) and 210 nm (polyenoic fatty acids) were recorded simultaneously. Normal phase HPLC (SP-HPLC) of hydroxyl fatty acid isomers was carried out on a Zorbax SIL column (250/4.6, 5 μm particle size, Daicel Chemical Industries, distributed by Baker) eluted with a solvent system of hexane/2-proOH/AcOH (100:5:0.1) at a flow rate of 1 ml/min.

### 4.4. Plant materials

Cucumber (*Cucumis sativus* L. cv. Suyo) seeds were soaked in tap water for 12 h and sown on moistened vermiculite. Seedlings were grown in the darkness or under the continuous light at 25 °C. When cucumber was grown for a long period (30 days), each seedling was transplanted to a pot with vermiculite and grown under continuous light at 25 °C. As nutrients, Hyponex (Hyponex Japan, Tokyo, Japan) was added periodically. In some cases cucumber plants were grown in the experimental farm in Yamaguchi University from spring to summer in 2003. Mechanical wounding was accomplished by pinching the first leaves of 30 day old plants across the midvein four times by forceps. Ca. 25% of the leaves were wounded.

### 4.5. HPL assay

Crude enzyme solution was prepared by homogenizing the organ with four volumes of 0.1 M potassium phosphate buffer, pH 6.5. The homogenate was filtered through four layers of cheesecloth and centrifuged at 3000*g* for 10 min at 4 °C. The resulting supernatant was collected and immediately used for the HPL assay. The phloem sap was also used for HPL assay. The stems of cucumber plants were cut with a razor blade at just above the first leaf and 2–3 cm below the second internode. The cut surfaces were immediately soaked in 5 ml of 20 mM EDTA, pH 7.0, and the sap was allowed to exude for 3 h at 25 °C (Avdiushko et al., 1994). The collected solution was centrifuged at 10,000*g* (RPR-20-2 rotor, Hitachi, Tokyo, Japan) for 10 min at 4 °C, with the resulting supernatant used for the HPL assay. The substrate (2 μl of 0.1 M **10** or 0.1 M 13-hydroperoxy-(*Z*,*E*)-9,11-octadecadienoic acid (**11**) dissolved in EtOH) and the crude enzyme solution (100 μl) were mixed with 0.1 M potassium phosphate buffer, pH 6.5 (2.4 ml), and incubated at 25 °C for 30 min. Then, an internal standard (*n*-heptanal, 10 nmol/10 μl in ethanol) and 1 ml of 0.2% 2,4-dinitrophenylhydrazine (in ethanol containing 0.5 M acetic acid) was added to stop the reaction. The hydrazones were extracted with *n*-hexane, and subjected to the HPLC analyses after concentration. HPLC was performed with Mightysil RP-18 (4.6 mm × 250 mm, Kanto Kagaku Kogyo, Tokyo, Japan) with $CH_3CN$/THF/$H_2O$ (74/1/25, v/v) as eluant at a flow rate of 1.0 ml/min and monitoring at $A_{350}$. In order to quantify each aldehyde, a calibration curve was constructed, respectively. The experiments were repeated at least three times with different plants. In this article, the activity to form C9-aldehydes from **10** was termed as 9-HPL activity and that to form C6-aldehydes from **11** was termed as 13-HPL activity.

### 4.6. Quantification of aldehydes formed after tissue disruption

Cucumber hypocotyls (0.5 g fr wt) was homogenized with 2.0 ml of 0.1 M potassium phosphate buffer, pH 6.5, and incubated for 30 min at 25 °C. Then, MeOH (2.0 ml) was added to the mixture and heated at 60 °C for 30 min to stop the reaction and to extract aldehydes formed. In order to quantify the amounts of aldehydes in intact organs, freshly cut hypocotyls were heated at 60 °C for 30 min with MeOH. The resulting extract was mixed with 2,4-dinitrophenylhydrazine and internal standard (10 nmol of *n*-heptanal), with the resulting hydrazones extracted with hexane as described above. The hexane extracts were partially purified by using preparative TLC (Silicagel 60, Merck, Darmstadt, Germany, hexane/EtOAc 2/1, v/v). The spot containing the hydrazones of short chain aldehydes were excised, with the hydrazones extracted with $Et_2O$, then, subjected to HPLC analyses after concentration as described above.

### 4.7. Northern and RT-PCR analyses

Total RNA was isolated from cucumber organs by using TRIZOL reagent (Invitrogen). Total RNA (20 μg) was separated on 1.2% denaturing agarose gel containing formaldehyde, and the gels were capillary-blotted onto the nylon membrane (Hybond-N, Amersham Biosciences, Piscataway, NJ, USA) with 20 × SSC. The membrane was baked and blocked according to the instruction manual, and subsequently incubated with a $^{32}$P-labelled DNA

probe corresponding to *Eco*RI fragment of cucumber 9/13-HPL cDNA (Matsui et al., 2000) at 65 °C for 24 h. The membrane was washed under high stringency conditions, and autoradiograms were obtained with pre-flashed X-ray film (New RX, Fuji Photo Film, Tokyo, Japan). For RT-PCR analyses, total RNA was reverse-transcribed with ThermoScript RT-PCR System (Invitrogen), and remaining DNA was degraded by DNA-Free Kit (Ambion, Austin, TX, USA). PCR was performed with primers designed with Primer Express (Ver. 1.0, PE Applied Biosystems, Foster City, CA, USA) by using SYBR Green PCR Core Reagents with a GeneAmp 9600 PCR thermalcycler. GeneAmp5700 Sequence Detection System (Ver.1.3, PE Applied Biosystems) was used to process the data. The amplified products were confirmed by sequencing. Every time after amplification, the specificity was evaluated by checking the melting curve of the amplified products. Primers used were: 9/13-HPL sense; TCATCTCCTCCGATTCCAGAGT, antisense; CTTTGGTGGTGTCGAAGAGGAT, actin sense; GAGACATTCAATGTGCCTGCTATG, antisense; CACGATACCAGTGGTACGTCCA, aminocyclopropane carboxylate (ACC) oxidase sense; GGCATCATCCTCCTCTTCCAA, antisense; TCGATCCAGTCGCCATCTTT. The relative amounts of transcripts were estimated by dividing each value with that obtained with actin.

### 4.8. Fungal pathogens

*F. oxysporum* f. sp. *Raphani* was grown at 25 °C for 2 days in potato–sucrose medium. The culture was filtered with two layers of sterile Kimwipes, and centrifuged at 1000*g* (R10A2 rotor, Hitachi) for 5 min at 4 °C to collect the conidia. After washing with sterile water once, the conidia were suspended with 2.5% glucose to be $2 \times 10^5$ cfu/ml. *B. cinerea* (Kishimoto et al., 2002) was grown at 20 °C for one week on potato–sucrose–agar medium, and exposed to illumination of BLB lamp (20 W, Toshiba, Tokyo, Japan) for 3 days to facilitate germination of conidia. Sterile water was poured onto the plate, and the conidia were gently collected by using a brush. The suspension was collected and filtered with two layers of sterile Kimwipes. To the filtered suspension, glucose was added to be 2.5% and the density of the conidia was adjusted to $2 \times 10^5$ cfu/ml with 2.5% glucose. **5**, **6**, **8**, or **9** was added to the suspension of conidia to be 0.02, 0.1 or 0.5 mM, and a drop of the mixture was spotted on a glass slide. The suspension was sealed with a cover slip and incubated under the darkness at 20 °C in a moistened box, then, the germination of conidia was investigated with a microscope after 24 h.

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