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DEVELOPMENTAL CHANGES OF LIPOXYGENASE AND FATTY ACID HYDROPEROXIDE LYASE ACTIVITIES IN CULTURED CELLS OF *MARCHANTIA POLYMORPHA*

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Key Word Index—*Marchantia polymorpha*; Marchantiaceae; liverwort; developmental changes; lipoxygenase; fatty acid hydroperoxide lyase.

Abstract—Lipoxygenase activity in *Marchantia polymorpha* cells rapidly increased during the lag phase of cell growth. In the logarithmically-growing cells activity tended to decrease, but again increased in the stationary phase. The two lipoxygenases enhanced in the lag and the stationary phases were purifed. Then could not be differentiated from each other by their enzymatic properties, such as pH-activity profiles, substrate and product specificities. Furthermore, antibody raised against lipoxygenase purified from the cells in the stationary phase reacted with the enzyme induced in the lag phase with almost the same reactivity. From these observations and Western immunoblot analysis, it was revealed that the developmental change in the activity during cell growth is caused by *de novo* synthesis and degradation of essentially the same lipoxygenase. Enzyme activity which degrades fatty acid hydroperoxides, products of lipoxygenase, was also detected in the cells. *n*-Hexanal and 12-oxo-(9*Z*)-dodeceonoic acid were identified as the products formed from linoleic acid 13-hydroperoxide by the degradation enzyme; thus, it was revealed to be a fatty acid hydroperoxide lyase. The lyase activity was also enhanced synchronously with lipoxygenase activity in the lag phase of cell growth, but no induction was observed during the stationary phase. Addition of linoleic acid to the cells caused little increase in the amount of lipid peroxide at the lag phase, while at the stationary phase, the addition doubled the amount. Furthermore, higher amounts of C_6-aldehydes were detected in the cells in the lag phase than those in the stationary phase. These observations suggest that lipoxygenase in the lag phase is involved in C_6-aldehyde formation in cooperation with fatty acid hydroperoxide lyase, while lipoxygenase in the stationary phase might exert its role by forming lipid peroxides.

INTRODUCTION

Lipoxygenase (LOX, EC 1.13.1.13) catalyses the oxidation of linoleic acid and certain other polyunsaturated fatty acids, to form conjugated diene hydroperoxides [1]. Fatty acid hydroperoxides (HPOs) thus formed are further converted to various lipid-breakdown compounds in plant tissues [2]. Fatty acid hydroperoxide lyase (HPO lyase) cleaves the HPOs to form C_6-aldehydes and C_{12}-oxo acids [3, 4]. Involvement of C_6-aldehyde formation in hypersensitive resistance responses has been proposed [5], whereas oxo acids are known to be a growth stimulant for mushrooms [6]. Allene oxide synthase [7], has been attracting much attention because its product is ultimately converted to an important signal transducer, jasmonic acid [8]. HPO-dependent peroxygenase catalyses a highly efficient and stereoselective epoxidation of unsaturated fatty acids strictly depending on fatty acid HPOs. Its involvement in biosynthesis of cutin monomers is proposed [9]. On the other hand, LOX is known to be regulated developmentally in some plant tissues [1]. In *Arabidopsis* seedlings [10] and cucumber cotyledons [11], LOX transiently accumulates in large amounts. In tomato fruit pericarp, rapid induction of LOX activity just prior to the breaker stage is observed [12]. From these results it is also predicted that LOX in some plant tissues is involved in growth and development of the tissues [1].

It has been reported that not only higher plants but lower plants, such as green algae [13], red algae [2] and cyanobacteria [14], also have a lipid-breakdown pathway. However, little study has been made of the physiological significance of the pathway. Previously, we found that cultured cells of *Marchantia polymorpha* contain LOX activity [15] but a LOX-mediated lipid-breakdown pathway in the cells had not been clarified. In the present study, we intended to reveal the pathway and found that *M. polymorpha* cells contain HPO lyase. This is the first report on the isolation and partial characterization of

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HPO lyase from bryophytes. Additionally, we noted that the activities of both enzymes varied greatly during cell culture. Because *M. polymorpha* cells form small cell aggregates, the growth phase of the cells is relatively uniform. Thus, precise analyses on the developmental changes of LOX activity concomitant with the other enzymes involved in the lipid-breakdown pathway in the cells should shed light on the physiological function of the pathway in the cells. Furthermore, we took advantage of the cultured cells which can easily incorporate fatty acids added exogeneously [16] to speculate about *in vivo* metabolism of the pathway in the cells.

RESULTS AND DISCUSSION

Under the culture condition used *M. polymorpha* cells grew logarithmically after about four days of the lag phase (Fig. 1A). The doubling time was calculated to be *ca* 48 hr from the increase in the dry weight at the log phase. Eight days after transplanting, no increase in fresh or dry weights was observed. LOX activity was low just after transplanting (Fig. 1B), but it increased till five days after transplanting just when the cells started to grow logarithmically. In the logarithmically growing cells LOX activity decreased to the original level when the growth phase of the cells turned to the stationary phase. After that time, LOX activity again rapidly increased to meet the second activity peak at 14 days, then gradually decreased thereafter. Previously we reported a U-shaped profile of the growth-activity curve [15]. This discrepancy may be due to the difference in the culture conditions. Previously we transplanted cells every 14 days rather than every seven days. As shown in this experiment, cells cultured for 14 days had high LOX activity. Thus, the first peak of the activity at five days would have been hidden by the carried-over activity.

It is wel known that most plant species have several LOX isoenzymes [1]. In order to elucidate whether the activities in the two phases stem from the same LOX or from two or more LOX isoenzymes, each LOX was partially purified from the cells in the respective phase, and their enzymatic properties compared. When linoleic acid was used as a substrate, both LOXs showed almost identical pH-activity profiles with the highest activity at pH 9.0. They also showed the same substrate specificities, i.e., activities against γ-linolenic acid were twice those against linoleic acid, while those against α-linolenic acid were only one-third (e.g., [15]). Esterified fatty acids were not good substrates for both LOXs. Furthermore, both of them introduced molecular oxygen predominantly to the C_{13}-position of linoleic acid to give 13-hydroperoxy-(9*Z*, 12*E*)-octadecadienoic acid. Taken together, these two LOXs could not be differentiated from each other with respect to their enzymatic properties.

Further study was conducted with an antibody raised against *M. polymorpha* LOX highly purified from the cells in the stationary phase. With the antibody, only one band of 100 kDa could be detected in both the crude enzyme extracts which indicated that they had the same subunit sizes. Furthermore, the antibody reacted with

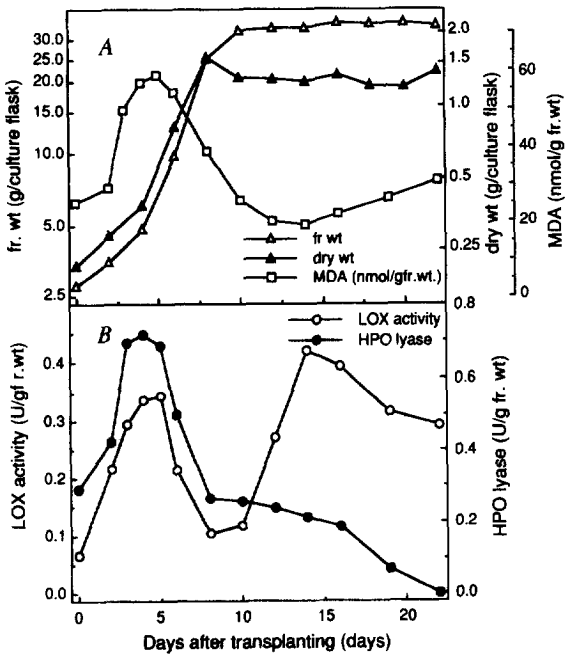

Fig. 1. Time-course of growth and MDA value (A), and LOX and fatty acid HPO lyase activities (B) of *M. polymorpha* cultured cells. After transplanting cells to fresh culture medium, cells were harvested at the time given and fr. wt (△) and dry wt (▲) per 100 ml of the culture medium, MDA value (□), LOX (○) and HPO lyase (●) activities determined.

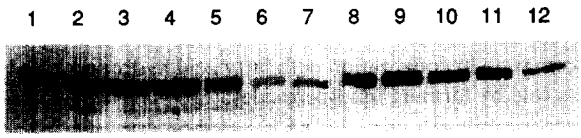

Fig. 2. Western immuno-blot analysis of crude extracts prepared from the cells at different days after transplanting. Crude extracts prepared from cells at 0, 2, 3, 4, 6, 8, 10, 12, 14, 16, 19 and 22 days after transplanting were loaded to lanes 1–12, respectively. In each lane, the extract corresponds to 18.8 mg fr. wt.

both the extracts with almost the same reactivities when the respective amount of the extract corresponding to the same LOX activity was applied (data not shown). This indicates that both LOXs are immunologically equivalent. Immuno-detection of LOX in the crude homogenate prepared from the cells in a given developmental stage showed that the band intensities highly correlated with the developmental change of LOX activity (Fig. 2). Thus, the change of activity during cell growth must be essentially caused by *de novo* synthesis and degradation of LOX protein. In summary, these results indicated that quite similar LOXs are synthesized twice during the growth of the cells.

It is well known that some enzyme activities, such as phenylalanine ammonia lyase, are induced rapidly after transplanting [17]. The phenomenon is known as the 'transfer' or 'dilution' effect. The effect is thought to be

caused by mechanical stress during transplanting and/or by metabolic stress through drastic changes of the composition of culture medium. If one assumes that induction of LOX activity in the lag phase of *M. polymorpha* cells is caused by the transfer effect, the induction should occur independently of the development of the cells. In order to elucidate whether the induction was caused by the transfer effect or by the developmental effect, different amounts of cells were transplanted to fresh culture medium, then their growth-activity profiles were compared. As shown in Fig. 3, long lag phase extending up to 90 hr could be seen when only 1 g of cells was transplanted to the fresh medium. When 3 g of the cells were transplanted, the lag phase was shortened. LOX activity started to increase at almost the same time, 24 hr after transplanting in both cases. However, when cell growth was retarded, the increasing rate of LOX activity was lowered and appearance of the activity peak was delayed. As a result, the activity peak was always observed just when the cells started to grow logarithmically irrespective of the length of the lag phase. LOX activity was not induced by treatment of the cells with ethephon or salicylic acid (data not shown). These results suggest that the induction of LOX activity is not a result of the transfer effect but is essentially regulated developmentally.

Because products of LOX, fatty acid HPOs, are highly toxic to cells, the existence of an enzyme activity to degrade the HPOs is expected. With crude homogenate of cells, activity of a fatty acid HPO-degrading enzyme could be detected. After differential centrifugation of the cell homogenate, most activity was detected in the membrane fraction. The activity was partially purified. Only one decomposing activity was observed during the purification steps. Products formed by the purified enzyme from linoleic acid 13-HPO were elucidated to be *n*-hexanal and 12-oxo-(9*Z*)-dodecenoic acid by reverse-phase HPLC analysis. This indicates that the enzyme was a fatty acid HPO lyase. HPO-dependent peroxygenase activity could not be detected in the cells. Furthermore, jasmonic acid and its related compounds could not be found in lipid fractions or in essential oils prepared from the cells. Thus, HPO lyase is thought to be a major enzyme activity in the cells which degrades fatty acid HPO. The lyase was most active at pH 5.0 with the half maximum activity at pH 3.5 and 6.5. These values are comparable to those of lyases purified from tea leaves [3] or green bell pepper fruits [4]. On the contrary, substrate specificity of *M. polymorpha* lyase was relatively broad (Table 1) when compared with those in higher plants [3, 4]. *M. polymorpha* lyase was most active against linoleic acid 13-HPO. Although tea leaf and bell pepper fruit lyases act on α-linolenic acid 13-HPO ten times more efficiently than on linoleic acid 13-HPO [3, 4], the activity of *M. polymorpha* lyase against α-linolenic acid 13-HPO was only one-third of that with linoleic acid 13-HPO.

Lyase activity increased for four days after transplanting, then rapidly decreased to the original level within the next four days (Fig. 1B). During the stationary phase, the activity decreased gradually and after 22 days it was not detectable. The developmental change of HPO lyase activity in the lag phase highly correlated with that of LOX. This suggests that LOX and HPO lyase play their roles cooperatively in cells at the lag phase. On the contrary, lyase activity was not enhanced in the stationary phase when LOX activity was. This indicates that LOX in the stationary phase has a different role from that in the lag phase.

In order to confirm whether LOX induced once in the lag phase and once in the stationary phase catalyse their reactions *in vivo*, the amount of lipid peroxide in the cells was estimated after exogenous administration of fatty acids to the cells. During the lag to log phase of cell growth the malondialdehyde (MDA) value changed correlating with LOX and HPO lyase activities; however, the MDA value during the stationary phase was low and showed no correlation with that of LOX (Fig. 1A). The

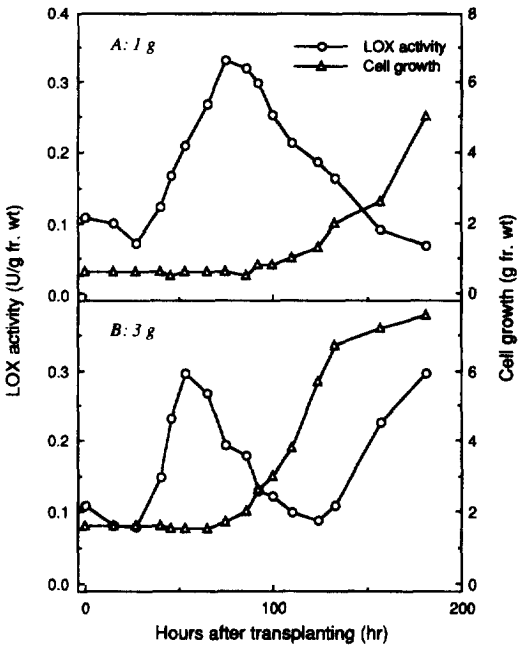

Fig. 3. Effect of amount of initial sample size to be transplanted on time-course of growth and LOX activities. One (A) or three (B) g fr. wt of the cells were transplanted to 100 ml of fresh culture medium, and fr. wt (△) and LOX activities (○) were determined at each time.

Table 1. Substrate specificity of *M. polymorpha* HPO lyase

Substrate	Relative activity (%)
Linoleic acid 13-hydroperoxide	100
α-Linolenic acid 13-hydroperoxide	35.4
γ-Linolenic acid 13-hydroperoxide	65.2
Linoleic acid 9-hydroperoxide	41.3
Eicosatetraenoic acid 15-hydroperoxide	48.4
Eicosapentaenoic acid 15-hydroperoxide	23.0

amount of lipid peroxide changed little after incubating the cells for 3 hr in fresh medium without any additives (Fig. 4). Addition of linoleic acid caused only a little increase of MDA value in cells of the lag phase. However, addition of linoleic acid into the cells in the stationary phase doubled the value. The increase was enhanced by the concomitant addition of Triton X-100 probably because the detergent facilitated the incorporation of linoleic acid into the cells. An increase of MDA value was not observed when linoleic acid was added with a LOX inhibitor, nordihydroguaiaretic acid, or when oleic acid, which is not a substrate of LOX, was added instead of linoleic acid. Thus, the increase in MDA value in the stationary phase must be mainly due to LOX activity resulting in the formation and accumulation of fatty acid HPO. This result is in good accordance with the developmental changes of LOX and HPO lyase activities. In the cells of the lag phase, fatty acid HPOs formed by LOX should be cleaved into aldehydes by concomitantly existing HPO lyase, while in the stationary phase, fatty acid HPOs formed by LOX would accumulate because of the low HPO lyase activity. This is confirmed by the result shown in Table 2. The total amount of C_6-aldehydes was *ca.* twice that in cells in the lag phase than those in the stationary phase. These results indicated that *M. polymorpha* LOX in the stationary phase does not catalyse its oxygenation reaction *in vivo*. Because *M. polymorpha* LOX showed little activity against esterified fatty acids [15], liberation of free fatty acids is essential for *M. polymorpha* LOX to exert its catalytic activity. Thus, lipolytic enzymes which form free fatty acids from membrane lipids may be rate-limiting for the LOX-pathway. As proposed by Farmer and Ryan [18], free fatty acids are released in response to some signals, such as pathogen attack. On the other hand, accumulation of lipid peroxide upon elicitation has been reported [19]. The reason why *M. polymorpha* cells accumulate LOX, which would be harmful to the cells, if it works, is not clear. One possible explanation is that fatty acid HPOs which would be formed upon activation of lipolytic enzyme through LOX catalysis might be involved in response to some stresses. Involvement of active oxygen species in disease-resistance response is reported [20, 21].

On the other hand, in the lag phase the enzyme system forming C_6-aldehyde is regulated developmentally. Developmental and cooperative induction of LOX and HPO lyase activities has been reported in watermelon [22] and soybean seedlings [23]. Furthermore, Sekiya *et al.* [24] showed that young bean leaves had relatively high activities of both the enzymes but their activities decreased gradually with leaf expansion, suggesting that the enzyme system plays a role in the early stage of plant growth. In the case of *M. polymorpha* cells, the activities were developmentally regulated and high when the cells start to grow rapidly. Thus, *M. polymorpha* LOX in the growing period may also play the same role in the early stage of growth. The physiological roles of the aldehydes and oxo acids, however, are not well understood, but 12-oxo-(10*E*)-dodecenoic acid is known as the active component of the wound harmone, traumatin [25], which stimulates cell growth. An analogous compound, 10-oxo-(8*E*)-decenoic acid, is also known as a growth stimulant to mushrooms [6]. The C_{12}-oxo acid may play a role in the regulation of cell growth at this period.

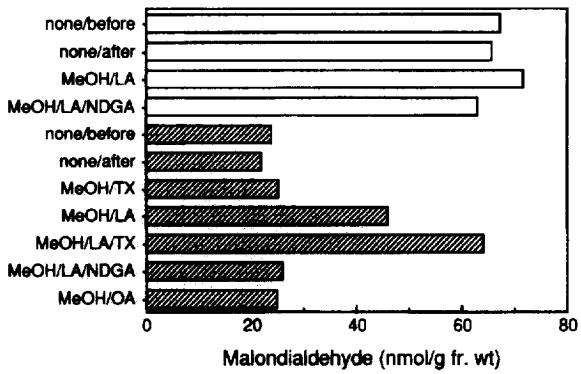

Fig. 4. Effect of administration of fatty acids on MDA values. Cells (2 g fr. wt) cultured for 4 (□) or 14 (■) days were transplanted to 20 ml of fresh medium containing fatty acid and/or other compounds. After incubation for 3 hr, MDA values in cells were determined. For comparison, MDA values before incubation (none/before) and those after incubation without any additives (none/after) are also shown. Abbreviations and final concentrations LA linoleic acid (1 mM); OA oleic acid (1 mM); NDGA nordihydroguaiaretic acid (1 mM); TX Triton X-100 (0.05%); MeOH methanol (0.1%).

Table 2. Amounts of C_6 aldehyde in *M. polymorpha* cells cultured for 4 or 14 days

Compound	C_6-Aldehyde (nmol/g fr. wt)	
	4 days	14 days
n-Hexanal	2.7	1.4
(3*Z*)-Hexenal	12.7	6.8
(2*E*)-Hexenal	1.6	1.1
Total	17.0	9.3

EXPERIMENTAL

Materials. Marchantia polymorpha cells [26] were kindly provided by Dr K. Ohyama. Cells were grown under continuous fluorescent light at 25° in 100 ml of 1M51C medium [26] in a 300 ml flask with shaking at 120 rpm. Stock cultures were subcultured every 7 days. Fr. wt of the cells was determined after filtration through filter paper under red. pres. for 5 min. Dry wt of the cells was determined after drying at 60° for 72 hr. Fatty acids (> 98% purity) were purchased from Sigma. Linoleic acid 13-HPO, 9-HPO, α-linolenic 13-HPO, γ-linolenic 13-HPO were prepd as described in ref. [4]. Eicosatetraenoic acid 15-HPO and eicosapentaenoic acid 15-HPO were purchased from Funakoshi (Japan). Other chemicals were from Wako Pure Chemicals (Japan).

Enzyme assays. Cells were homogenized with an equal vol. of 1/4 strength McIlvaine's buffer, pH 8 in an ice-cold mortar. The homogenate was centrifuged at 20000 *g* for 10 min and the resulting supernatant was carefully collected to remove cell debris and the fatty layer, and immediately used for the assay. The reaction was initiated by addition of 8.3 μl of the substrate soln (10 mM linoleic acid in 0.2% Tween 20) into 50 mM Na-borate, pH 9 containing an appropriate amount of the homogenate (final vol. 1 ml). Formation of linoleic acid HPO was followed by an increase of *A* at 234 nm ($\varepsilon = 2.5 \times 10^4\ M^{-1}\ cm^{-1}$) at 25°. Product specificity of LOX was determined as described previously [15].

For HPO lyase, cells were homogenized with an equal vol. of Tris-Cl, pH 8.0 in an ice-cold mortar. After filtration through four layers of cheesecloth, the homogenate was centrifuged at 500 *g* for 5 min and the resulting supernatant immediately used for assay. The reaction was initiated by the addition of 2 μl of the substrate soln (10 mM linoleic acid 13-HPO in EtOH) into 50 mM NaPi, pH 6 containing an appropriate amount of homogenate (final vol. 1 ml). The decomposition of HPO was followed spectrophotometrically at 234 nm at 25°.

In both cases, one unit of the enzyme activity is defined as the enzyme forming (or consuming) 1 μmol of product (or substrate) per min. HPO-dependent peroxygenase activity was determined as described in ref. [27] with linoleic acid 13-HPO and indole as substrates.

Enzyme purification. LOX in stationary phase cells was purified 531-fold (sp. act. 15.3 $U\ mg^{-1}$) from the cells cultured for 14 days essentially as described in ref. [15]. The enzyme in the log phase was purified from cells cultured for 5 days in the same way, but the ion-exchange and gel-filtration steps were omitted. It was purified 30.6-fold to a sp. act. of 1.1 $U\ mg^{-1}$. HPO lyase was purified from cells cultured for 5 days. Cells were homogenized with an equal vol. of 50 mM Tris-Cl, pH 8 in an ice-cold mortar and filtered through four layers of cheesecloth. The filtrate was centrifuged at 500 *g* for 5 min and the supernatant centrifuged at 100000 *g* for 60 min to give a membrane fr. The membrane fr. was suspended in 20 mM Tris-Cl, pH 8 containing 0.5% Triton X-100 adjusting its chlorophyll concn. to 20–30 $\mu g\ ml^{-1}$, then incubated on ice for 1 hr with gentle stirring. Solubilized enzyme soln was obtained after centrifugation at 30000 *g* for 90 min and was then applied to a DEAE-Cellulofine A-500 column (10 mm i.d. × 100 mm) equilibrated with 20 mM Tris-Cl, pH 8 containing 0.1% Triton X-100. The column was washed with the same buffer and the activity was eluted with a linear gradient of KCl (0–0.03 M) formed with the same buffer.

Immunological studies. Anti-LOX antiserum was prepared with *M. polymorpha* LOX purified from cells in the stationary phase. SDS-PAGE and immunoblotting were performed as described previously [11].

Analyses of aldehyde and fatty acid compositions. Cells (10 g fr. wt) were homogenized with 37.5 ml of $CHCl_3$–MeOH (1:2). The residues collected by filtration were re-extracted with $CHCl_3$–MeOH–H_2O (1:2:0.8) × 2. The extracts were comb. and H_2O and $CHCl_3$ added. The organic layer was concd *in vacuo* and the residue dissolved in $CHCl_3$ containing 0.1% butylated hydroxytoluene. Crude lipids (3 mg) were transmethylated with 5% HCl in MeOH at 100° for 3 hr with heptadecanoic acid as int. standard. Resultant Me esters were analysed by FID-GC (on a glass column (3 min × 3 m) packed with 2% Silar 5CP. The column temp. was increased from 180 to 210° at 2° min^{-1}. Carrier gas was N_2 at 30 $ml\ min^{-1}$. Each peak was identified by comparison of its R_t with that of authentic compounds and quantified using an integrator, on the basis of the int. standard. For quantification of aldehydes, cells (4 gr fr. wt) were incubated for 30 min with EtOH at 80° in an air-tight test tube, then homogenized thoroughly with a Polytron mixer. *n*-Heptanal was added to the homogenate as an int. standard, then 20 ml of 0.5% 2,4-dinitrophenylhydrazine in EtOH supplemented with 0.5 M AcOH. The mixt. was incubated for 2 hr and the resultant hydrazones extracted into hexane. The extracts were applied to a silica gel 60 prep. TLC plate and developed with hexane–EtOAc (2:1). Hydrazones thus obtained were separated by reverse-phase HPLC on a Wakosil 5C18 column (4.6 mm × 250 mm) using MeCN–H_2O–THF (2:1:0.01) at 1 ml min^{-1}. Detection was at 350 nm. Volatiles in cells were collected by stream-distillation [28] and essential oils analysed by capillary GC on SF-96 [28].

Administration of fatty acid. Cells (2 g fr. wt) cultured for 4 or 14 days were harvested and transplanted to 20 ml of fr. 1M51C medium containing test compounds. Cells were incubated for 3 hr at 25° with shaking under fluorescent light, then MDA value of cells determined as follows [29]. Cells were homogenized with 0.1% TCA and 1% SDS, and centrifuged at 1000 *g* for 15 min. The supernatant (0.5 ml) was mixed with 1.5 ml of 10% OHAc containing 0.4% thiobarbituric acid (pH 3.5) and incubated for 1 hr at 95°. After cooling to ambient, 0.5 ml of H_2O and 2.5 ml of *n*-BuOH–pyridine (1:1) were added and mixed thoroughly. The organic layer was collected and A at 532 nm read. Calibration curves were constructed with 1,1,3,3-tetraethoxypropane as standard.

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