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Triacylglycerol Lipase Participates in the Formation of *n*-Hexanal in Alfalfa Seedlings

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The participation of lipase in the formation of *n*-hexanal by homogenization of alfalfa seedlings was investigated. *n*-Hexanal was increased by the addition of trilinolein, dilinolein or monolinolein to the homogenate of the seedlings. Taurodeoxycholic acid sodium salt (TDCA), an inhibitor of triacylglycerol lipase, inhibited the formation of *n*-hexanal by 36%. These findings show that the corresponding proportion of *n*-hexanal was formed through the action of triacylglycerol lipase. *n*-Hexanal was also increased by adding the total lipids of alfalfa seedlings to the homogenate prepared with TDCA. But, when the lipids removed free fatty acids from the total lipids were added to the homogenate, the increase decreased to 18% as compared with the addition of total lipids. *n*-Hexanal thus also increased through the pathway, not requiring the action of the lipase and 82% of the increased *n*-hexanal was formed from preexisting free fatty acids. The formation pathways of *n*-hexanal in the seedlings were discussed.

Keywords: alfalfa, *n*-hexanal, lipase, grassy odor

In legumes it is well known that aldehydes (*n*-hexanal, 3-*Z*-hexenal, etc.), alcohols (*n*-pentanol, *n*-heptanol, etc.), ketones (methyl butyl ketone, ethyl vinyl ketone, etc.), phenols (4-vinylphenol, etc.) and furans [*n*-pentylfuran, 2-*Z*-(1-pentenyl)furan, etc.] contribute to the grassy odor (Rackis *et al.*, 1979). Most of them are formed by enzymatic or chemical oxidation of lipids during processing or storage of legumes. Especially, C_6-aldehydes (*n*-hexanal, 3-*Z*-hexenal and 2-*E*-hexenal), which are formed by enzymatic oxidation, cause serious problems in the processing of legumes, since even a small amount of undesirable grassy odor these give off is undesirable because of the low olfactory threshold. C_6-Aldehydes have been detected not only in legumes but also in many plants such as tomato (Riley *et al.*, 1996), bell pepper (Matsui *et al.*, 1997), olive (Jose *et al.*, 1993) and tea leaf (Kitamura *et al.*, 1992) and contribute to their characteristic flavors. C_6-Aldehydes are known to be formed by the following pathway: a free unsaturated fatty acid such as linoleic acid or linolenic acid containing *Z*, *Z*-1,4 pentadiene structure is peroxidized by lipoxygenase and subsequently cleaved by hydroperoxide lyase. Free fatty acids, the starting substances in the pathway, also arise through the degradation of glycerolipids by lipase. Therefore, lipase may also participate in the formation of C_6-aldehydes, although there are few reports of the participation of lipase. In recent years, Matsui *et al.* (2000) have reported that a lipase specifically acting on galactolipids, especially on monogalactosyldiacylglycerol, is involved in the formation of C_6-aldehydes during destruction of *Arabidopsis* leaves. Moreover, Feussner *et al.* (2001) found that a lipase from cucumber exhibited a high specificity for lipids oxygenated by the action of a specific 13-lipoxygenase and released the corresponding hydroperoxy fatty acids. Therefore, there is a possibility that the lipid-hydrolyzing step by lipase plays an important role in the formation of C_6-aldehydes.

Alfalfa (*Medicago sativa* L.) is a well-known important fodder, but in Europe and North America the seedling is used frequently as a food material since it contains many nutritional constituents and is a low caloric healthy food. Also in Japan, the seedling is sold in the product forms of fresh vegetable, juice and tablet. However, the products are not popular primarily because of their undesirable grassy odor. Production of the seedlings without this grassy odor may be useful to increase consumption and improve our health.

We previously reported that *n*-hexanal, one of the C_6-aldehydes, is the main grassy odor substance in alfalfa seedlings and is formed very quickly through the destruction of the tissue (Uno *et al.*, 2002). It is presumed that there are the following four pathways in the formation of *n*-hexanal: (a) preexisting free linoleic acid is oxygenated by lipoxygenase and cleaved by hydroperoxide lyase; (b) linoleic acid released from glycerolipids by lipase is oxygenated by lipoxygenase and cleaved by hydroperoxide lyase; (c) after linoleic acid in glycerolipids is directly oxygenated by 13-lipoxygenase and hydrolyzed by lipase, the resultant 13-hydroperoxylinoleic acid is cleaved by hydroperoxide lyase; (d) *n*-hexanal is formed from glycerolipid oxygenized by 13-lipoxygenase (Fig. 5). Although two of these pathways require the action of lipase, it is unclear whether this enzyme actually takes part in the formation of *n*-hexanal. In this study, we examined the participation of lipase in the formation of *n*-hexanal in alfalfa seedlings using some inhibitors.

Materials and Methods

Plant material Alfalfa (*Medicago sativa* L., Lucerne) seeds (Sakata Seed, Kanagawa) were soaked in water for 6 h and then germinated for three days in the dark at 25°C. The seedlings were watered twice a day.

Chemicals Trilinolein (99%), 1,3-dilinolein (this may contain up to 5% 1,2-isomer), 1-monolinolein (99%), quinacrine,

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and gum arabic were purchased from Sigma Chemical (St. Louis, MO). Taurodeoxycholic acid sodium salt (TDCA), 2,4-dinitrophenylhydrazine, and sodium *N,N*-diethyldithiocarbamate trihydrate were from Nacalai Tesque (Kyoto), linoleic acid was from Tokyo Kasei Kogyo (Tokyo) and Amberlyst A26 was from ORGANO (Tokyo). All other reagents were chemical grade.

Preparation of exogenous substrates Exogenous substrates, trilinolein, dilinolein, monolinolein, and linoleic acid were emulsified at a concentration of 25 mM in 5% gum arabic by sonicating for 3 min (Matsui & Kajiwara, 1995). The emulsions were prepared immediately before use.

Determination of n-hexanal Three-day-old seedlings germinated from 0.25 g of seeds were ground using a mortar and pestle with liquid nitrogen, and then homogenized in 15 ml of 50 mM phosphate buffer (pH 6.8) with a grass homogenizer at room temperature. The homogenate was used for determination of *n*-hexanal. *n*-Hexanal was determined as the 2,4-dinitrophenylhydrazone derivative by a high performance liquid chromatograph (HPLC, L-6300 Model, HITACHI, Tokyo) according to the method of Matoba *et al.* (1985), except for the use of perchloric acid instead of phosphoric acid. The HPLC equipped with a UV-VIS detector (HITACHI L-4250) and an integrator (HITACHI D-7500) was used for determining *n*-hexanal. The column was LiChrosorb RP-18-5 (ϕ4.6×250 mm; GL Science, Tokyo) with a protective column (ϕ4×10 mm; GL Science). The derivative was eluted with acetonitrile/water/tetrahydrofuran (75 : 24 : 1, v/v/v) at a flow rate of 1.0 ml/min and detected at 350 nm.

Determination of free fatty acid contents After 3-day-old seedlings were homogenized as described above, the lipids were extracted with chloroform. The free fatty acids in the lipid extracts were converted to copper soaps and quantified using sodium *N,N*-diethyldithiocarbamate trihydrate as the color reagent (Ohtsubo *et al.*, 1987). In this method, C_8 and higher free fatty acids and the corresponding hydroperoxides and dicarboxylic acids were extracted and measured.

Removal of free fatty acids by ion exchange resin The 3-day-old seedlings germinated from 0.25 g of seeds were ground using a mortar and pestle with liquid nitrogen and the total lipids were extracted according to Bligh-Dyer method (Bligh & Dyer, 1959). The lipid extracts containing the total lipids were dried by nitrogen gas and dissolved in 15 ml of acetone/methanol (2 : 1, v/v). Free fatty acids were removed from the total lipids using an anionic exchange resin (Amberlyst A26) as described by Gandemer *et al.* (1991). After addition of the resin, the mixture was stirred using a magnetic stirrer for 30 min. Non resin-bound lipids were recovered by rinsing the resin 5 times with 15 ml of acetone/methanol (2 : 1, v/v). The recovered lipids were concentrated, redissolved in 4 ml of 50 mM phosphate buffer (pH 6.8) containing 1% Triton X-100 and used for the experiment. The removal of free fatty acids was confirmed by thin-layer chromatography (TLC). Lipids treated with resin were spotted on a thin-layer plate of silica gel 60 (Merck, Germany), and the developing solvent was diethyl ether/hexane/acetic acid (80 : 20 : 1,v/v/v). The lipids were located by 0.005% primuline and visualized under UV light.

Results

Effects of various linoleic acid substrates on the formation of n-hexanal *n*-Hexanal was increased very quickly by the homogenization of alfalfa seedlings (Uno *et al.*, 2002). After the addition of various substrates, linoleic acid, trilinolein, dilinolein or monolinolein, at a final conc. of 5 mM to the homogenate of the 3-day-old seedlings, change in the amount of *n*-hexanal was examined. When the seedlings were homogenized, *n*-hexanal in the homogenate increased rapidly for the first 3 min and then reached a plateau (Fig. 1). Therefore, each substrate was added at 10 min after the homogenization. When linoleic acid was added, the increase of *n*-hexanal was the largest, and at 20 min the amount was 11.7 times larger than that of control. *n*-Hexanal was also increased by adding glycerolipid esterified linoleic acid, i.e., trilinolein, dilinolein and monolinolein. The increase of *n*-hexanal was large in the order of monolinolein, dilinolein and trilinolein. Although the substrate specificity of lipase against these linoleic acid substrates was unclear, these results suggest that lipase may be involved in the formation of *n*-hexanal.

Participation of lipase in the formation of n-hexanal

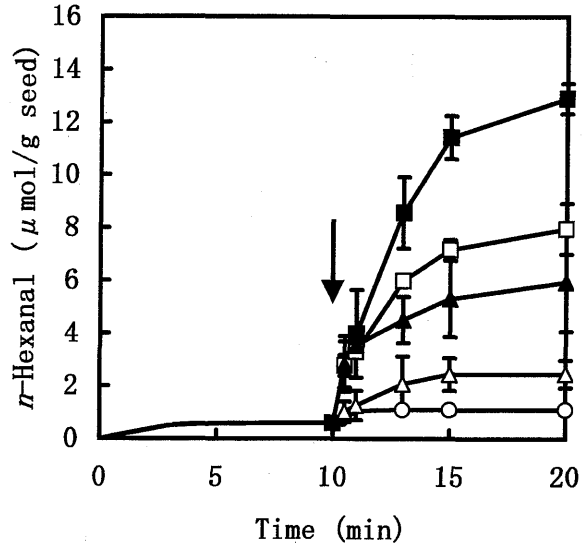

Fig. 1. Effects of various linoleic acid substrates on the formation of *n*-hexanal. After the homogenate prepared with 50 mM phosphate buffer (pH 6.8) was left for 10 min at room temperature, linoleic acid (■), monolinolein (□), dilinolein (▲), or trilinolein (△) was added (arrow, final conc. 5 mM). As a control, only 5% gum arabic was added to the homogenate (○).

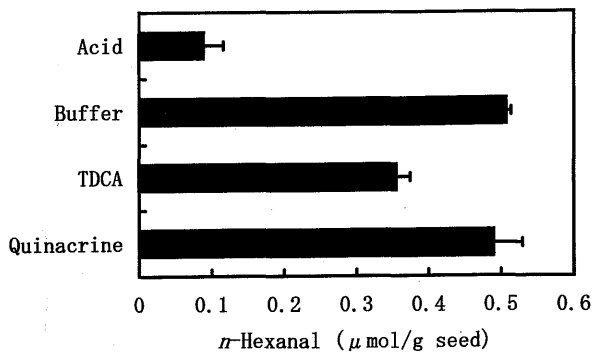

Fig. 2. Inhibition of the formation of *n*-hexanal by lipase inhibitors. After the 3-day-old seedlings were homogenized in 50 mM phosphate buffer (pH 6.8) containing or not containing 5 mM TDCA or 5 mM quinacrine, the amount of *n*-hexanal in the homogenate was measured. To determine the content of *n*-hexanal, the homogenate was prepared with 5% perchloric acid.

Effects of lipase inhibitors on the formation of *n*-hexanal were investigated by homogenizing the seedlings in 50 mM of phosphate buffer (pH 6.8) containing lipase inhibitor and then measuring the amount of *n*-hexanal in the homogenate. In this study, taurodeoxycholic acid sodium salt (TDCA) for triacylglycerol lipase and quinacrine for phospholipase A_2 were used as inhibitors (Sayari *et al.*, 2000; Matsui *et al.*, 2000). The content of *n*-hexanal in the seedlings, which was measured under enzyme-inactivated conditions by homogenizing with 5% perchloric acid, was 0.09 μmol/g seed, but *n*-hexanal was increased up to 0.51 μmol/g seed by homogenizing with 50 mM phosphate buffer (pH 6.8) (Fig. 2). The addition of TDCA and quinacrine depressed the increase to 0.36 and 0.49 μ mol/g seed, respectively. That is to say, TDCA and quinacrine inhibited 36% and 4% of the increase of *n*-hexanal, respectively, suggesting that triacylglycerol lipase participates in the formation of *n*-hexanal more than phospholipase A_2.

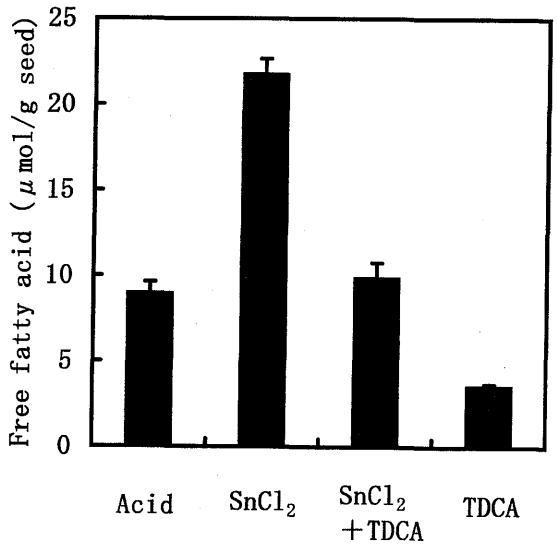

Fig. 3. Effects of inhibitors for lipoxygenase and lipase on the amount of free fatty acid in the homogenate. After the 3-day-old seedlings were homogenized in 50 mM phosphate buffer (pH 6.8) containing 5 mM $SnCl_2$, 5 mM $SnCl_2$ plus 5 mM TDCA, or 5 mM TDCA, the amount of free fatty acid in the homogenate was measured. To determine the content of free fatty acid, the homogenate was prepared with 5% perchloric acid.

Effects of TDCA on the amount of free fatty acids in the homogenate were investigated. Although the content of free fatty acids in the seedlings measured by homogenizing with 5% perchloric acid was 9.0 μmol/g seed, free fatty acids increased up to 21.8 μmol/g seed by homogenizing with 50 mM phosphate buffer (pH 6.8) containing 5 mM $SnCl_2$, a lipoxygenase inhibitor (Fig. 3). This increase is believed to occur through the action of lipase. On the other hand, when the seedlings were homogenized with the above buffer containing 5 mM $SnCl_2$ and 5 mM TDCA, the amount of free fatty acids in the homogenate was 9.9 μmol/g seed and the increase rate was only 7%. This result shows that TDCA inhibited most of the lipase activity and that triacylglycerol lipase is the main lipase in the homogenate.

Formation of n-hexanal without the action of lipase

When the seedlings were homogenized with the buffer containing 5 mM TDCA, the amount of free fatty acids in the homogenate decreased from the preexisting 9.0 μmol/g seed to 3.6 μmol/g seed (Fig. 3). This suggests the possibility that part of the preexisting free linoleic acids might be used as a substrate for the formation of *n*-hexanal (Pathway a in Fig. 5).

It is also possible that *n*-hexanal is formed from glycerolipids without the action of lipase (Pathway d in Fig. 5). Therefore, trilinolein was added to the homogenate to which TDCA had been added and the amount of *n*-hexanal in the homogenate was measured. As shown in Fig. 4A, *n*-hexanal in the homogenate was increased 2.2 fold by the addition of trilinolein. This increase indicates that there is a formation pathway of *n*-hexanal from glycerolipids, which does not require the action of lipase.

To confirm the existence of the pathway not requiring the action of lipase, the homogenate was prepared by adding TDCA

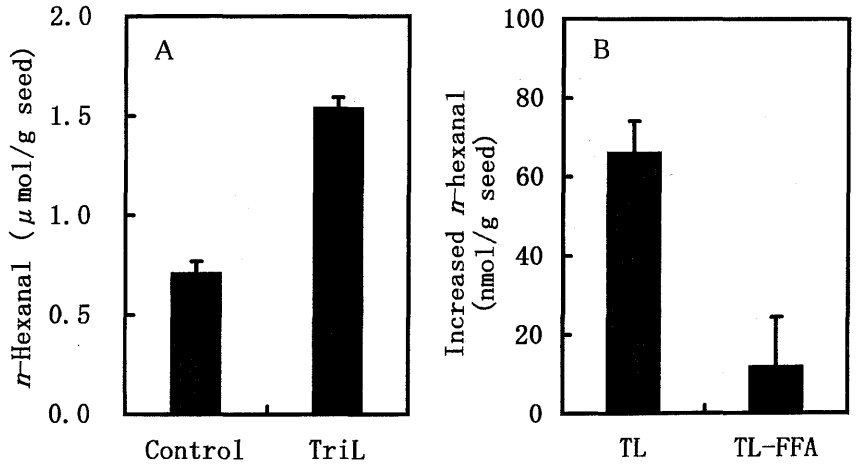

Fig. 4. (A) Effect of trilinolein on the formation of *n*-hexanal. After the 3-day-old seedlings were homogenized in 50 mM phosphate buffer (pH 6.8) containing 5 mM TDCA, 5% gum arabic (control) or trilinolein emulsified with 5% gum arabic (TriL) was added to the homogenate and the amount of *n*-hexanal was measured. (B) The increase of *n*-hexanal by the addition of the exogenous lipids extracted from the 3-day-old seedlings germinated from 0.25 g of the seeds. Total lipids (TL) and the lipids removed the free fatty acids from the total lipids (TL-FFA) were used as the exogenous lipids. After the seedlings were homogenized in 15 ml of 50 mM phosphate buffer (pH 6.8) containing 5 mM TDCA and left for 10 min, 4 ml of the exogenous lipids emulsified with 1% Triton X-100 were added to the homogenate. The increase of *n*-hexanal by the addition of the exogenous lipids was calculated by subtracting the amount of *n*-hexanal in the homogenate added only with 1% Triton X-100 from that to which the exogenous lipids had been added.

and the lipids extracted from the 3-day-old seedlings were added. With the addition of total lipids, 66 nmol/g seed of *n*-hexanal increased (Fig. 4B). On the other hand, when the lipids, which were removed free fatty acids from total lipids using an anion exchange resin were added to the homogenate, increased *n*-hexanal was 12 nmol/g seed. Thus, *n*-hexanal was actually formed from lipids of alfalfa seedlings and 82% of that formed without the action of lipase was from preexisting free fatty acids. In this experiment, the amount of increased *n*-hexanal was less than that in other experiments. This is probably because the amount of linoleic acid in the total lipids from alfalfa seedlings is considerably less than that of linoleic acid substrates added in other experiments, judging from the lipid content (about 0.2%) and the fatty acid composition (linoleic acid 53%).

Discussion

It has long been assumed that free unsaturated fatty acids are the precursors of C_6-aldehydes, and they have been demonstrated to be formed through the actions of lipoxygenase and hydroperoxide lyase (Tressl & Drawert, 1973; Stone *et al.*, 1975). However, there are some reports that the release of free fatty acids by lipase may be an initial step in the formation of C_6-aldehydes (Matsui *et al.*, 2000; Jose *et al.*, 1993; Galliard *et al.*, 1977). Therefore, we wanted to know whether lipase participates in the formation of *n*-hexanal in alfalfa seedlings, in which it is the main undesirable grassy odor substance (Uno *et al.*, 2002).

n-Hexanal was formed not only from free fatty acids but also from glycerolipid esterified linoleic acid (Fig. 1). This suggests that *n*-hexanal may be formed through the action of lipase. Therefore, the effects of lipase inhibitors, which were confirmed not to inhibit lipoxygenase or hydroperoxide lyase, on the formation of *n*-hexanal were investigated. The formation of *n*-hexanal was 36% inhibited by TDCA. TDCA inhibits triacylglycerol lipase activity by binding the enzyme molecules and reducing their availability for adsorption onto the emulsion interface as described by Momsen and Brockman (1976). However, TDCA might inhibit not only triacylglycerol lipase but also phospholipase. There is no report that any phospholipase other than phospholipase A_2 is related to the formation of *n*-hexanal, and in our work phospholipase A_2 was not closely related to *n*-hexanal formation (Fig. 2). Therefore, triacylglycerol lipase is believed to participate strongly in the formation of *n*-hexanal. Because alfalfa is an oilseed plant like sunflower and soybean, the seeds contain a large amount of storage lipids which exist mostly as triacylglycerol and are tightly packed in subcellular organelles called lipid bodies or oil bodies. Also, in the 3-day-old alfalfa seedlings, triacylglycerol was the most abundant lipid constituent (data not shown). Therefore, it is reasonable to assume that triacylglycerol lipase exists in the seedlings and acts in the homogenate to produce free linoleic acids. There are thought to be two pathways requiring the action of lipase (Pathway b and c in Fig. 5). Although the existence of pathway (c) has not been confirmed, part of *n*-hexanal is probably formed through this.

TDCA suppressed most of the action of lipase, but decreased only 36% of the formation of *n*-hexanal (Figs. 2 and 3). This is because preexisting free linoleic acids in the seedlings are used as substrate for the formation of *n*-hexanal. Actually, the amount of free fatty acids was decreased by the homogenization with TDCA (Fig. 3). These results confirmed that the traditional pathway (Pathway a in Fig. 5) also exists in alfalfa seedlings.

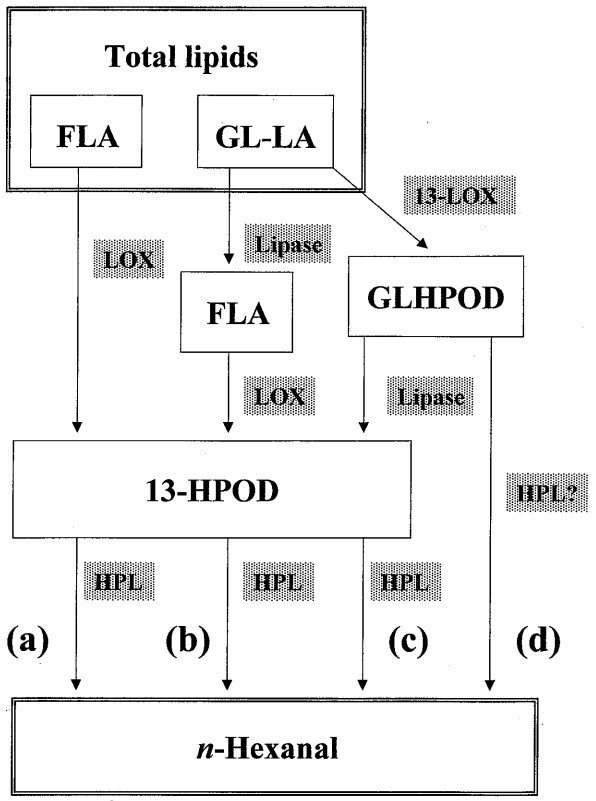

Fig. 5. Four proposed pathways for the formation of *n*-hexanal in alfalfa seedlings. FLA, free linoleic acid; GL-LA, glycerolipid esterified linoleic acid; 13-HPOD, 13-hydroperoxylinoleic acid; GLHPOD, glycerolipid esterified hydroperoxylinoleic acid; LOX, lipoxygenase; HPL, hydroperoxide lyase.

Figure 4A shows that there is a formation pathway of *n*-hexanal from glycerolipids, which does not require the action of lipase, although details of this pathway are unclear. Since the existence of 13-lipoxygenase which oxygenizes glycerolipid has been reported in other plants (Feussner *et al.*, 2001), it is presumed there is a pathway in which glycerolipid containing linoleic acid is oxygenized by the enzyme and cleaved by a hydroperoxide lyase-like enzyme to form *n*-hexanal (Pathway d in Fig. 5).

The amount of free fatty acids detected in the homogenate was about one order of magnitude larger than that of *n*-hexanal. There are three possible reasons for this difference. First, free fatty acids preexisting in the seedlings or formed by the action of lipase are not only linoleic acid. Linoleic acid accounted for only 53% of the fatty acids in alfalfa seedlings (data not shown). Second, all of the linoleic acid is not used for the formation of *n*-hexanal. *n*-Hexanal is not formed when linoleic acid is metabolized by β-oxidation or oxygenated to 9-hydroperoxylinoleic acid through the action of lipoxygenase, it is formed only from 13-hydroperoxylinoleic acid. Third, not all of the lipids in the homogenate can be used in the formation of *n*-hexanal because alfalfa lipids formed the aggregation such as a lipid body. This is supported by the result that *n*-hexanal was increased solely by the addition of 5% gum arabic to the homogenate (Fig. 1).

In this study, we showed that there are four formation pathways of *n*-hexanal in alfalfa seedlings. The proportion of *n*-hexa-

nal that is formed through each pathway by the homogenization of the seedlings was tentatively estimated. As shown in Fig. 2, the increase of *n*-hexanal was suppressed to 64% by the addition of TDCA. This means that 64% of *n*-hexanal is formed through pathways (a) and (d) which do not require the action of lipase and 36% through pathways (b) and (c), which do require it, although, of course, triacylglycerol lipase is not the only lipase that exists in the alfalfa seedlings. Further, when the lipids removed free fatty acids from the total lipids were added to the homogenate in the presence of TDCA, the increase of *n*-hexanal was only 18% as compared with the addition of total lipids (Fig. 4B). From this result, it is estimated that out of pathway (a) and (d), which account for 64% of *n*-hexanal formation, 52% is formed through pathway (a) and 12% through pathway (d).

As described above, it is estimated that 52, 36, and 12% of *n*-hexanal was formed through pathway (a), (b) plus (c), and (d), respectively. The pathway requiring the action of triacylglycerol lipase accounted for nearly 40%, showing that lipase plays an important role in the formation of *n*-hexanal in alfalfa seedlings.

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