A tomato *lipase homologous to DAD1* (*LeLID1*) is induced in post-germinative growing stage and encodes a triacylglycerol lipase

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Abstract A tomato lipase gene homologous to *Arabidopsis DAD1* (*lipase homologous to DAD1*; *LeLID1*) was cloned and characterized. The corresponding transcript increased rapidly during germination of the seeds and reached a maximum level at four days after germination. Thereafter, it decreased rapidly. Little expression could be found in flowers or fruits. Immunoblot analyses showed that the gene products could be found in the cotyledons and hypocotyls, but not in the roots. In the cotyledons most LeLID1 could be recovered in a soluble fraction. The recombinant LeLID1 protein showed maximum lipase activity at pH 8.0. It showed high activity against triacylglycerols (TAGs) with long acyl chains, but little activity with phosphatidylcholine or monogalactosyldiacylglycerol. TAGs composed of short acyl chains could not be a substrate for the enzyme. A possible involvement of LeLID1 in fat mobilization during seed germination is discussed.

Keywords: Triacylglycerol lipase; DAD1; Fat mobilization; Oil body; Tomato (*Lycopersicon esculentum*)

1. Introduction

In most plants, especially those storing fats as major food reserves in seeds (oilseed plants), triacylglycerols (TAGs) are deposited into small organelles called oil bodies. A half-unit membrane of one phopholipid layer surrounds oil bodies. Usually amphipathic proteins called oleosines cover the surface of oil bodies in order to avoid aggregation of the organelles during seed dehydration [1]. During seed germination and seedling growth, TAGs are hydrolyzed to glycerol and free fatty acids. Free fatty acids thus formed are further degraded by β-oxidation system in glyoxysomes, and then, glyoxylate cycle and gluconeogenesis affords carbohydrates for the growth of the seedlings [2]. In some plants, it has been postulated that TAGs with linoleic acid are converted to hydroperoxides by a lipoxygenase, and then, the oxygenated acyl groups are hydrolyzed to form free fatty acid hydroperoxides, which undergo β-oxidation and the following metabolism to afford carbohydrates [3,4]. The oxygenation step is thought to be a trigger for the fat mobilization [5]. This fat mobilization is critical for plants to sustain their seedling growth until they can start photosynthesis. The lipid hydrolyzing step, probably caused by a TAG lipase, must be essential for the fat mobilization, because fatty acids must be converted into acyl-CoAs to enter into the β-oxidation system. Lipases induced during post-germinative growth had been reported from several plant species [1]. For example, a lipase purified from the scutellum of maize kernel associates with oil bodies and is induced specifically during germinating stage, from which it has been suggested that this lipase is involved in fat mobilization [6]. Lipases induced during post-germinative growth are strong candidates for the lipases essential to fat mobilization, however, they have not been characterized at a molecular level, and direct evidence to support their involvements in fat mobilization has not been reported. Our knowledge on the lipase is still immature.

Recently, a lipase gene, *AtDAD1*, that catalyzes liberation of free linolenic acid to serve a substrate for jasmonate synthesis in flower organs has been isolated and characterized in *Arabidopsis* [7]. A recombinant AtDAD1 expressed in *Escherichia coli* showed phospholipase A1 activity although its amino acid sequence has significant similarity with fungal lipases that are known to be TAG lipases [7,8]. In carnation petals a lipase induced during senescence of the petals has been cloned, and later, it was found to be AtDAD1-homologous lipase [9]. A recombinant carnation lipase can act on TAG. Database search showed that there are many genes that show significant homology both with AtDAD1 and fungal lipases in various plant species. Other than the two shown above, the function and/or enzymatic properties of products of any genes homologous to *AtDAD1* have not been studied so far. When tomato EST database in TIGR was analyzed, we found that one of the lipase genes homologous to *AtDAD1* (*LeLID1*, *Lycopersicon esculentum* lipase homologous to DAD1) expresses strongly in germinating seedlings. Because LIDs have high sequence similarity to fungal TAG lipases, we assumed that the tomato *LID* gene might be involved in fat mobilization during post-germinative growth of the seedlings as a TAG lipase. In this study, we obtained full-length cDNA for the gene, and expression profile of the gene was analyzed. Furthermore, we succeeded to establish a heterologous expression system to express an active enzyme with the gene and found that it encoded a TAG lipase. Possible involvement of the gene product in fat mobilization is discussed.

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Abbreviation: TAG, triacylglycerol

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2. Materials and methods

2.1. Plant materials

Tomato (*Lycopersicon esculentum* Mill. cv Momotarou) seeds were soaked for 12 h with tap water and sown on a pile of moisten paper towel. They were grown at 25 °C under 14 h light/10 h dark photo regime.

2.2. RT-PCR

Total RNA was isolated by using TRIzol reagent and reverse-transcribed with ThermoScript RT-PCR System (both from Gibco-BRL) to afford single stranded cDNA. PCR was carried out with GeneAmp 5700 by using SYBR Green PCR Core Reagent Kit (Applied Biosystems). In order to semi-quantify the transcript, tomato actin gene (Accession No. U60480) was used as an internal reference. The primers were designed with Primer Express (version 1.0, Applied Biosystems). The primers used were; *LeLID1*: sense, 5′-GGTTGGACTTGACCCAAAAAAG-3′, and antisense, 5′-GCTTCCCTTGACAATGATTTCG-3′; *actin*: sense, 5′-CCACCAGAGAGGAAATACAGTGTCT-3′; antisense, 5′-AATGCAAACCTGCTGGAAGG-3′.

2.3. Purification of the recombinant LeLID1

The open reading frame of *LeLID1* was inserted into downstream of the His-tag sequence of pQE30 vector and the resultant construct was used to transform *E. coli* SG13009 [pREP4] strain (Qiagen). The other host strains such as M15 [pREP4] (Qiagen), Y1090, or Origami (DE3) (Novagen) were also used. The overnight culture of the cells (0.5 ml) was inoculated to fresh LB medium (50 ml) and the cultivation was continued for approximately 3 h until the OD600 reached to 0.8. Thereafter, the culture was chilled to 14 °C and isopropyl β-thiogalactopyranoside was added to be 1 mM. The cells were further grown at 14 °C overnight and then harvested with centrifugation at 3000 rpm (Hitachi, RPR-20-2 rotor) for 5 min at 4 °C. The collected cells were resuspended with 5 ml of 50 mM Tris–Cl, pH 7.5, containing 100 mM NaCl and disrupted with a tip-type sonicator (model UD-201, Tomy Co. Tokyo) to prepare a crude lysate. The lysate was centrifuged at 12 000 rpm for 20 min at 4 °C to remove cell debris and the resultant supernatant was mixed with TALON resin (corresponding to 1 ml of the bed size, BD Biosciences) equilibrated with 10 ml of 50 mM K-phosphate, pH 8.0, containing 100 mM NaCl. The mixture was gently shaken at ambient temperature for 20 min and then centrifuged at $700 \times g$ for 5 min. The resin was washed once with the same buffer. The recombinant LeLID1 was desorbed from the resin with the same buffer containing 150 mM imidazol.

2.4. Enzyme assay

Triolein (Wako Pure Chemicals, Osaka, Japan) was emulsified with 5% Gum Arabic to be 50 mg/ml by the tip-type sonicater. The emulsified substrate (5 μl) was mixed with 50 mM K-phosphate, pH 7.5, and the purified enzyme to make up to 100 μl. The mixture was incubated at 25 °C for 60 min and then a portion (5 μl) was withdrawn to determine the amount of free fatty acids liberated by using NEFA C-test kit (Wako Pure Chemicals) [7]. Oleic acid was used to construct a calibration curve. All TAGs and monogalactosyldiacylglycerol (MGDG) (from spinach) used in this study were from Wako Pure Chemicals. Dilinoleylphosphatidylcholine was from Sigma. They were suspended with 5% gum arabic as described above for the assay.

2.5. Fractionation of tomato seedlings

The seedlings grown for 5 days were divided into cotyledons, hypocotyls, and roots. Each organ was homogenized with 0.15 M Tris–Cl, pH 7.5, containing 15% sucrose, 10 mM KCl, 0.1 mM $MgSO_4$, 5 mM dithioerythritol, and 10 mM sodium ascorbate. After centrifugation at 1500 rpm (RPR-20-2 rotor) for 3 min at 4 °C, the supernatant was taken as a crude extract. The crude extract was centrifuged at 70 000 rpm (S100AT5 rotor, Hitachi) for 1 h at 4 °C. The upper layer was carefully taken as an oil body fraction and the pellet as a membrane fraction. The clear supernatant was used as a soluble fraction. The membrane fraction was suspended with the same buffer used for homogenization. The oil body fraction was suspended with 0.1 M $NaHCO_3$ and centrifuged as above to remove non-specifically bound proteins. For immunoblot analyses, each fraction corresponding to 2.7 mg fr wt of the organ was electrophoresed. Immunoblot was performed with nitrocellulose membrane (Schleicher&Schell, BA85). *E. coli*-expressed recombinant LeLID1 was used to raise an antibody with rabbits by using adjuvants. The antiserum (after diluted 5000-fold) was used as the primary antibody and goat anti-rabbit IgG conjugated to horseradish peroxidase (Jackson Immunoresearch, Co.) was used as the secondary antibody according to the instruction manual. The band was detected by using Super Signal West Pico Chemiluminescent Kit (Pierce).

3. Results

3.1. Molecular cloning of LeLID1

When BLAST search for TIGR tomato gene indices was performed with the amino acid sequence of AtDAD1, twelve TC (tentative consensus) sequences could be identified. One of them, TC124040, was chosen for further analyses because the largest number of EST clones corresponding to the sequence could be found in the database. An EST clone distributed by Clemson University Gene Initiative (cLEI04K06) was found to be the longest one, thus, the sequence of it was fully determined. It was 1421 nucleotides long with an open reading frame encoding 398 amino acids. In its 42 nucleotide stretch of 5′ non-coding sequence, an in-frame stop codon was found at position −33, and the 3′ non-coding sequence had 51 nucleotide-long poly(A)$^+$ tail. Therefore, this cDNA was thought to be almost full-length. The deduced amino acid sequence showed 31.9% homology with AtDAD1 ([7], At2g44810, NP_182008) and 34.4% with senescence-inducible carnation lipase ([9], AAD01804) (Fig. 1). With *Rhizomucor miehei* lipase (CAA00250), the tomato protein showed 28.0% homology. According to the Conserved Domain Database at NCBI, the tomato sequence has a lipase 3 (pfam01764.11) domain with 126 bits of score. In fact, a consensus sequence of lipases, GHSLG, could be found at position 220 (Fig. 1). Other than the serine residue, the other members of the catalytic triad essential to most serine hydrolases could be found with D286 and H330, and both of them are conserved within the plant lipases. From these features, the gene is tentatively named *LeLID1* (lipase – homologous to DAD1). With TargetP analysis (http://www.cbs.dtu.dk/services/TargetP/), the protein is expected to have no N-terminal presequences. With SOSUI program (http://sosui.proteome.bio.tuat.ac.jp/), the protein is expected to be soluble.

Within the EST database in TIGR, the clones corresponding to *LeLID1* could be found dominantly in a library constructed from early germinating seedlings/cotyledons (cLEI library). Other than the library, only one clone corresponding to *LeLID1* could be found with the library constructed from radices. Library expression search (TIGR) indicated that the clones corresponding to *LeLID1* consisted of as much as 2.75% of total clones in the cLEI library (108 out of 4000). The library seems to be enriched with genes essential to the glyoxylate cycle, and 33 clones for isocitrate lyase, 28 clones for acetyl-CoA acyltransferase, and 26 clones for malate synthase are found.

3.2. Expression of LeLID1

Expression profiles of *LeLID1* during early germination stage of tomato were examined by using semi-quantitative RT-PCR technique (Fig. 2). In the water-imbibed tomato seeds (day 1), the transcripts of *LeLID1* could be little detected, while its amount increased significantly after 2 days when the radices emerged from the seed coats. At day 3, the cotyledons, hypocotyls and roots could be separated, and it was revealed

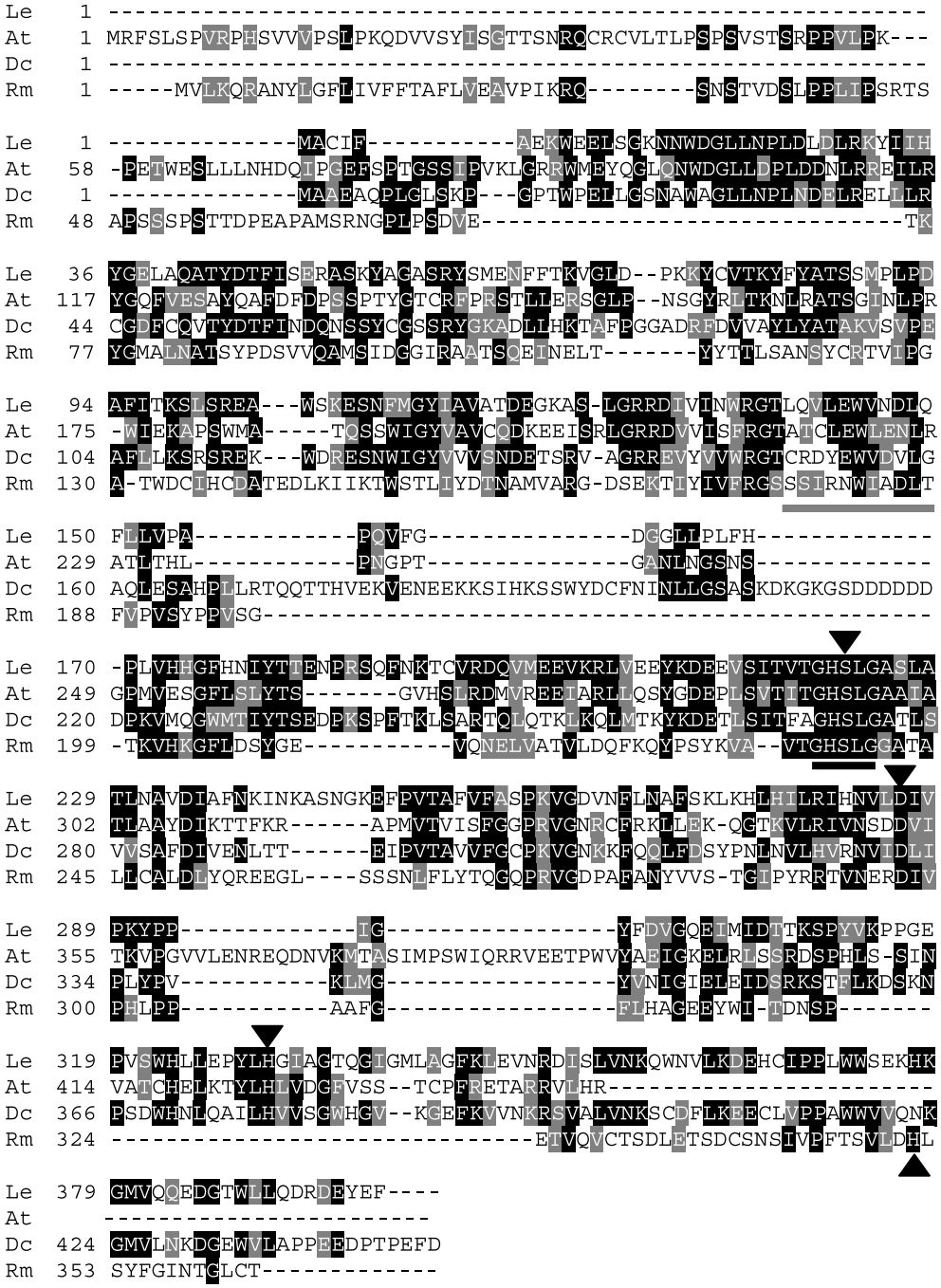

Fig. 1. Amino acid sequence comparison of LeLID1 (Le), AtDAD1 (At, GenBank Accession No. BAB69954), carnation senescence induced lipase (Dc, AAD01804) and *Rhizomucor miehei* lipase (Rm, CAA00250). The amino acids possibly composing of the catalytic triad are indicated with triangles and the lipase motif is underlined. The possible lid for the substrate recognition site is underlined with a gray line.

that cotyledons had highest amounts of the transcript. Hypocotyls followed, and in roots, its amount was very low. The level of transcript in cotyledons increased thereafter and met a peak at day 4 when the cotyledons started to break the seed coats and expanded rapidly. The level was still high at day 5, but was lowered thereafter and only very low level of the transcript could be found at day 6 and 7. In hypocotyls, the maximum level could be found at day 3 and then it decreased gradually. At day 6 it was almost undetectable. It is known that expression of *AtDAD1* is highly restricted to the flowers of *Arabidopsis*, where the gene product is involved in the biosynthesis of jasmonates. When RT-PCR analyses of *LeLID1* were carried out with reproductive organs (buds, flowers, or immature and mature fruits) of tomato, its transcript could be hardly detected; the expression ratios were less than 0.2. The expression level is also under the detection limit with mature leaves.

3.3. Localization in the cells

When immunoblot analyses were performed with crude extract prepared from cotyledons, hypocotyls and roots of

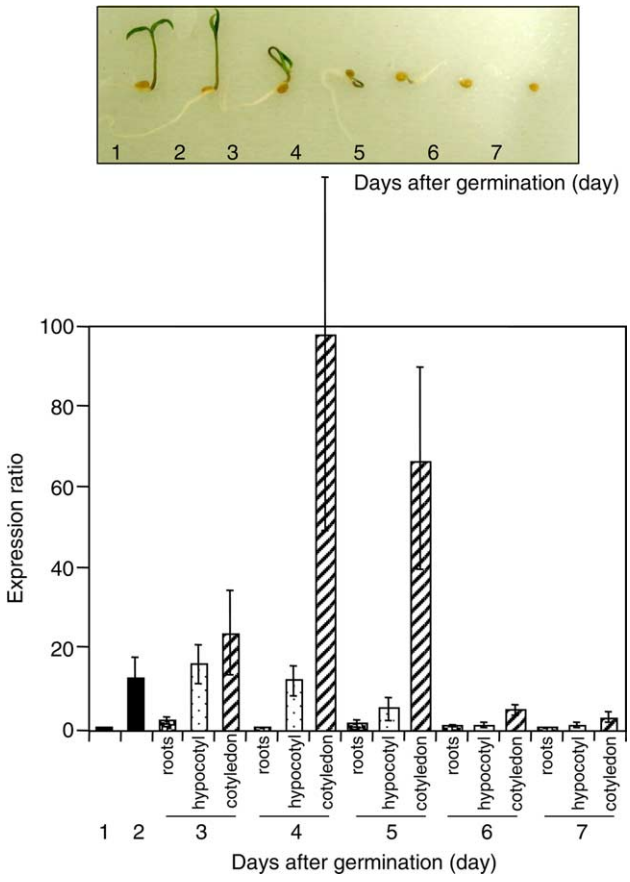

Fig. 2. Levels of *LeLID1* transcripts during germination of tomato seeds. The amounts of *LeLID1* transcripts were semi-quantified with RT-PCR with tomato *actin* gene as a control. The transcript level of *LeLID1* is shown as a relative ratio to the level of *actin* gene transcript. The growth of tomato seedlings is shown in the upper panel.

tomato seedlings of day 5, a major band of about 45 kDa could be detected with cotyledons and hypocotyls (Fig. 3). The size of protein is in good accordance to that expected from the deduced amino acid sequence of *LeLID1* (45.3 kDa). The amount of LeLID1 in cotyledons and hypocotyls was almost equivalent, but no band could be detected with roots. When crude extract prepared from cotyledons was fractionated into soluble, membranous or oil body-enriched fraction, most of LeLID1 was recovered in the soluble fraction. A faint band could be seen with membranous fraction; however, no signal could be detected with oil body fraction.

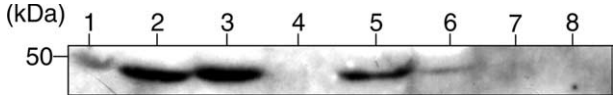

Fig. 3. Immunochemical detection of LeLID1. Recombinant LeLID1 expressed in *E. coli* (lane 1), and crude homogenates (corresponding to 2.7 mg fr wt of each organ) prepared from cotyledons (lane 2), hypocotyls (lane 3), and roots (lane 4) were subjected to immunoblot analyses. From crude homogenate of cotyledons, soluble fraction (lane 5), membrane fraction (lane 6), oil body fraction (lane 7) and $NaHCO_3$-washed oil body fraction (lane 8) were prepared and subjected to immunoblot analyses.

3.4. *LeLID1 encodes TAG lipase*

In order to characterize the enzymatic properties of LeLID1, a recombinant protein fused with the Hisx6-tag sequence was expressed with *E. coli*. In order to get highest activity, we tried to use different strains of *E. coli.* Among the strains we tested (M15 [pREP4], SG13009 [pREP4], Origami (DE3) and Y1090), SG13009 [pREP4] and Origami (DE3) were shown to be better hosts for the efficient expression of LeLID1. Because of faster growth of SG13009 [pREP4], further experiment was carried out with the expression system with the strain. A lipase activity could be found in the soluble fraction of the cell lysate and the corresponding enzyme could be purified to the apparently homogenous state by using Co^{2+}-affinity chromatography. M_r of the purified recombinant LeLID1 was estimated to be 47.5 kDa, which corresponded well with the calculated value from the sequence. When triolein suspended with gum arabic was used as a substrate, significant activity to form free oleic acid could be found, and the reaction proceeded almost linearly for 60 min (Fig. 4A). The pH-activity profile

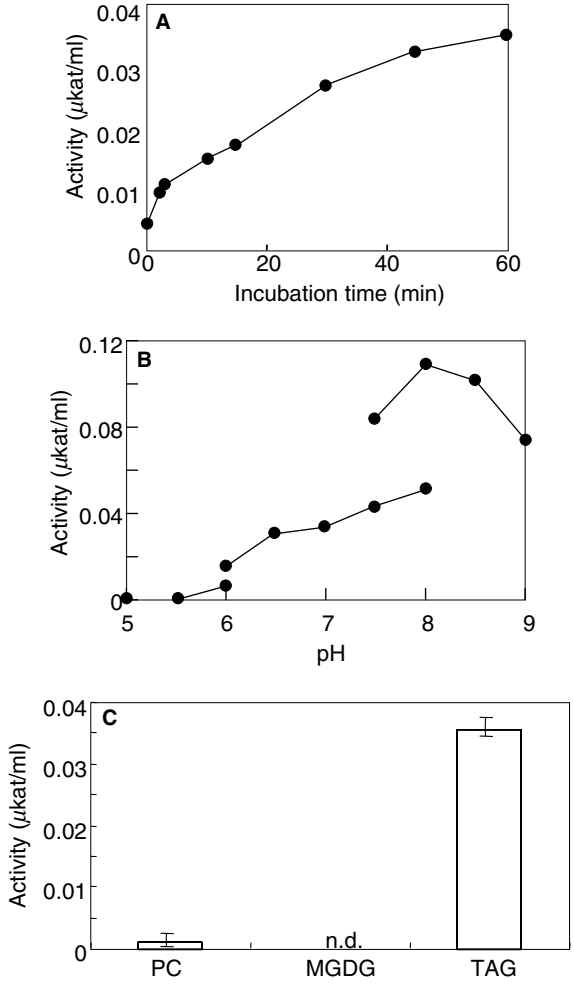

Fig. 4. Lipase activity of recombinant LeLID1 protein. (A) Time dependence of LeLID1 with triolein under a standard reaction condition. (B) pH dependence of LeLID1 activity. The buffers used were 0.1 M MES–KOH (pH 5.0–6.0), 0.1 M sodium phosphate (pH 6.0–8.0) and 0.1 M Tris–HCl (pH 7.5–9.0). (C) Substrate specificity of LeLID1. The activity was determined with dilinoleoylphosphatidylcholine (PC), MGDG from spinach, or triolein (TAG).

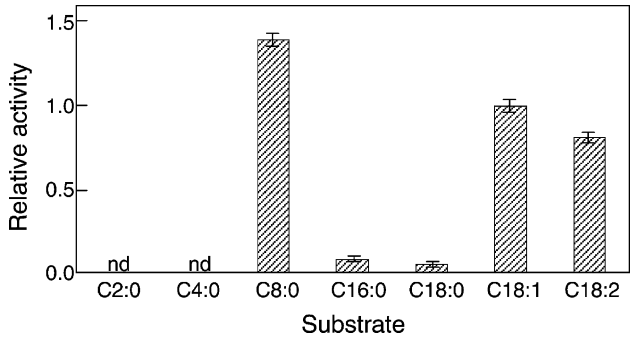

Fig. 5. Substrate specificity of recombinant LeLID1. The activity was determined with TAG with acyl groups of different length from acetyl (C2:0), butyl (C4:0), octyl (C8:0), palmitoyl (C16:0), stearoyl (C18:0), oleyl (C18:1) or linolenoyl (C18:2). The activity for triolein (C18:1) was assigned to 1.0 and relative values are shown.

showed that the optimum pH for the activity is 8.0 (Fig. 4B). At the same pH of 7.5 and 8.0, the recombinant LeLID1 preferred Tris buffer rather than sodium phosphate buffer. With soybean phosphatidylcholine, only trace activity could be found and with MGDG (from spinach), the activity could be hardly detected (Fig. 4C). When TAGs composed of different chain-length of acyl groups were used, the recombinant LeLID1 showed highest activity with the tricaprylin (C8:0). TAGs with unsaturated acyl groups, such as triolein (C18:1) and trilinolein (C18:2) showed moderate activity, however, those with saturated acyl groups, such as tripalmitin (C16:0) and tristearin (C18:0) showed only low activities. With TAGs of short acyl chain, i.e., triacetin (C2:0) and tributyrin (C4:0), no activity could be detected (see Fig. 5).

4. Discussion

In this study, we characterized an *AtDAD1*-homologous gene, *LeLID1* (lipase homologous to DAD1), which was abundantly and specifically expressed in germinating tomato seedlings, and found that it encoded a TAG lipase. According to the substrate specificity of recombinant LeLID1 shown in this study, we can assume that LeLID1 is a 'true lipase' that requires the presence of an oil–water interface for full activity, a phenomenon known as interfacial activation [10]. Although mammalian and fungal lipases have been studied intensely, this is the first report on the cloning and characterization of a true lipase of plant origin. With lipases undergoing interfacial activation, the active site is usually sheltered from the solvent by a lid consisting of a protruding short α-helix, which is moved out upon adsorption to an interface [11]. A stretch ranging from L141 to D147 and its vicinal regions in LeLID1 shows moderate similarity with the region composing the lid in *Rhizomucor miehei* lipase [12]. The secondary structure prediction (SSPro, [Pollastri]) indicates that the stretch in LeLID1 is α-helix, thus, it can be possible to assume that the stretch functions as a lid to evoke interfacial activation, which functions to discriminate TAGs with short acyl chains and those with long acyl chains as shown in this study.

As genome analyses are going on, the number of *LID* genes in the genome and EST databases derived from various plant species is increasing. In *Arabidopsis* genome, at least 13 genes could be identified as members of *LID* family [7], and in tomato 12 different EST clones corresponding to them could be found (TIGR gene indices). Even in rice plants, more than 10 *LID*s could be found in the genome sequence. These cumulative data suggest that *LID*s are widespread in the plant kingdom and that one plant species generally has several members of *LID*. Based on the presence of N-terminal stretches, *LID*s could be classified into three classes: class I that targets chloroplasts, class II that lacks the stretches and probably targets cytosol, and class III that targets mitochondria [7]. By now, enzymatic properties of LIDs have been analyzed with only two proteins. AtDAD1 is a member of chloroplast-localized LID (class I) and is reported to be an essential enzyme to form free linolenic acid for jasmonate formation in flower organs in *Arabidopsis*. Recombinant AtDAD1 expressed in *E. coli* showed highest activity to phosphatidylcholine (PC). MGDG and especially TAG were not good substrates. Because the sn1-position of PC was preferably hydrolyzed, AtDAD1 was categorized as a phospholipase A1. A senescence-induced lipase from carnation flowers is a member of class II LID [9]. The recombinant carnation LID can act on TAG and PC with almost the same efficiency, and it can also act on soluble substrates such as Tween 40 or Tween 80. These enzymatic features are different from those with LeLID1, which is also a member of class II LID. The tomato enzyme could act essentially only on TAG with long acyl chains and showed no activity against soluble substrates such as triacetin or tributyrin. Therefore, LeLID1 can be categorized as a 'true TAG lipase', which needs an oil–water interface to exert its catalytic activity. From these observations, it can be assumed that there might be various types of lipases in LID family in terms of their enzymatic properties and probably in terms of their physiological functions although they share high sequence similarity to fungal lipases. Each member in the LID family functions in a different manner through different catalytic activity and through different subcellular localization. Comparisons of the sequences of LeLID1, AtDAD1, and carnation senescence induced lipase showed that LeLID1 is the smallest among them, and AtDAD1 has an intervening stretch in its C-terminal region (ranging from 360 to 391) other than the chloroplast targeting N-terminal stretch, and the carnation lipase has an intervening stretch in its central region (from 167 to 219) (Fig. 1). These regions might be responsible for the differences in their substrate specificities.

The transcript level of *LeLID1* rapidly increased upon germination of tomato seeds and reached to the maximum level just before cotyledon openings, and then it decreased rapidly thereafter. The transcript could be largely found in cotyledons. This expression profile of *LeLID1* is quite similar to those of genes involved in glyoxylate cycles [2], which are involved in fat mobilization during early germination stage of plants. During seed germination, the TAGs stored in lipid bodies are rapidly degraded and transported to glyoxysomes where they undergo the β-oxidation, glyoxylate cycle and gluconeogenesis successively to form glucose. The glucose thus formed is translocated to the organs where it is needed for growth and development until plants can acquire the photosynthetic machinery. To enter the β-oxidation pathway, the acyl groups in TAGs must be hydrolyzed to form free fatty acids, then they need to be activated to acyl-coenzyme As by acyl-CoA synthetase [13], thus, the lipid hydrolyzing step is essential. Despite this importance of a lipase involved in the fat mobilization, it has not been fully studied. One of the possible

candidates for the lipase involved in fat mobilization was isolated from the scutella of maize [14]. In this tissue, lipase activity starts to appear two days after imbibition and reaches a maximum at about day 5–6 when the amount of total lipids decreases rapidly. The enzyme has an optimal activity at pH 7.5. These features are similar with those found with LeLID1, which would make the possibility of the tomato enzyme as a lipase involved in fat mobilization strong. Linoelic acid consists more than 50% of total fatty acids in tomato seed oil and oleic acid follows [15]. The substrate specificity of recombinant LeLID1 is in good agreement of the fatty acid composition of tomato seed oil, which again might strengthen the possibility. The lipase of maize was largely recovered in lipid body fraction, while LeLID1 was recovered in soluble fraction and essentially not recovered in lipid body fraction. This result seems to be inconsistent with the possible function of LeLID1 as a fat mobilizing lipase, however, even a soluble lipase can act on the TAGs in oil bodies [16]. For example, in cotton, most of the lipase activity found in germinating seeds was recovered in the soluble fraction [1]. In this case, it is presumed that the cotton lipase is associated with the oil bodies only transiently or loosely during catalysis. It is also known that an oil body and a glyoxysome contact physically in a cell during fat mobilization [17]. If this is the case with tomato cotyledons, the lipase must be confined to the contacting area. Precise subcellular localization of LeDIL1 in tomato cotyledons should be essential to confirm its function.

In summary, *LeLID1* encodes a true lipase, and from its expression profile it can be assumed that it is involved in fat mobilization as a lipase to liberate free fatty acids from TAGs stored in oil bodies. To confirm this possibility, further works to know the subcellular localization and to configure its physiological significance through analyzing RNAi suppressed transgenic tomatoes are now underway.

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