Molecular cloning and expression analyses of a putative triacylglycerol lipase gene from *Brassica napus*[1]

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Abstract

An ORF contained cDNA, designated as *TAGLP1*, was isolated from seedlings of *Brassica napus* by rapid amplification of cDNA ends (RACE). It encodes a putative triacylglycerol lipase, which involves in the triacylglycerol degradation in seed germination. The cDNA of 1.804kb contains a 1.407kb open reading frame (ORF) encoding a deduced protein of 468 amino acid residues with the isoelectric point of 6.42 and the calculated molecular mass is about 65.6 kDa. Amino acid sequence comparison analysis showed that the gene containing a serine active site had high identity with putative triacylglycerol lipases from *Arabidopsis thaliana*. Bioinformatics analysis showed that it was an membrane associated protein. Expression pattern analysis by RT-PCR at the germinating stage showed that it expressed in one to seven day-old seedlings presented as a parabola profile resembling to the expression pattern of gene related to the lipid degradation.

Key words: *Brassica napus*, triacylglycerol lipase, phylogenetic tree analysis

Introduction

As an important reserve of carbon and energy in Eukaryotes, triacylglycerols (TAGs) make up to 60% of the dry weight of oilseeds (El-Kouhen Karim, Blangy Ste′phanie, Ortiz Emilia et al., 2005). In oilseeds, massive triacylglycerols (TAGs) are hydrolyzed to provide the carbon skeletons and energy that drive postgerminative growth. The fatty acids are sent to peroxisomes through β-oxidation to yield acetyl-CoA (Graham, I.A. and Eastmond, P.J., 2002). At last most of the acetyl-CoA produced is converted to sugars by the glyoxylate cycle and gluconeogenesis. TAG lipases play a critical role in triacylglycerol hydrolysis. In germinating oilseeds TAG lipases facilitate the utilization of TAG. TAG lipases are always membrane associated and act at the oil/water interface to yield free fatty acids and glycerol (Eastmond, P.J. (2006)). These lipases can be found in the oil body, glyoxysome, or microsomal fractions of seed extracts, depending upon the species (Mukherjee, K.D, 1994). In germinating oilseeds, TAG lipases reside on the surface of the oil body may play a role in the degeneration of TAG. However, no lipase gene had been proven to play a physiological role in TAG breakdown in germinating oilseeds before lipase gene *SDP1* which has been proven to have TAG hydrolysis activity (Peter J. Eastmond, 2006).

Various lipases have been purified from plants such as corn seedlings (Lin YH and Huang AH, 1984), caster (Maeshima and Beevers, 1985; Fuchs et al., 1996), tomato (Bowsher CG, Ferrie BJ, Ghosh S, Todd J, Thompson JE, Rothstein SJ, 1992), and *Brassica napus* (Fuchs and Hansen, 1994). However, very few genes that encode these lipases have been cloned and characterized (Peter J. Eastmond, 2004). And, until now, there is still little information about genes encoding TAG lipases in *Brassica napus*. In this paper we cloned a putative lipase gene in the seedlings of *Brassica napus*, and analyzed the expression pattern of this gene in one day-old to seven day-old seedlings.

Materials and methods

Plant growth: the *Brassica napus* seeds (Ningyou12, *cv*) were soaked in water without any nutrition to support their growth, the only source of carbon and energy they obtained for germinative growth was from the triacylglycerol degradation in vivo. Rapeseeds germinated and grew in the dark at 24 °C and 50% humidity. One to seven day-old seedlings were harvested separately, and roots, stems, flowers, leaves, pods were taken from the ripe plants. All the materials were stored at -70 °C for further expression analyses.

RNA extraction and cDNA synthesis: Total RNA were extracted by using Trizol reagent (Invitrogen™.co)from seedlings, roots, stems, flowers, leaves and pods. Each step was performed following the instruction manual. cDNAs were synthesized by using RevertAid™ M-Mulv RT for RT-PCR:. Add 1μl Oligo (dT) 12-18 (500μg/ml) and 1-5μg total RNA to a nuclease-free microcentrifuge tube. Heating mixture to 70 °C for 10 min (denature) and quick chill on ice for 5 min. Collect the contents of the tube by brief centrifugation. And add: 4ul 5×M-Mulv RT Buffer, 4 μl dNTP Mixture (5 mM), 2.5μl 0.1M DTT, 1μl RevertAid™ M-Mulv RT (200U/μl) and 0.5μl RNase Inhibitor (40U/μl) to the same microcentrifuge tube. Mix contents of the tube gently and incubate at 30 °C for 10 min. Then incubate at 37 °C for 90 min. Inactive reaction by heating at 70 °C for 15 min. The cDNA was stored at -20°C. Amplification of some targets (>1kb) may require the remove of RNA complementary to the cDNA, add 1μl (20U) of E coli Rnase H and incubate 37°C for 20 min.

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Cloning of the cDNA by RACE: Blasting in NCBI (http://www.ncbi.nlm.nih.gov/BLAST/), we found the *Brassican napus* EST contained the 5′ end. So 3′-RACE had to be done to obtain the 3′ end of cDNA. The sequence of the oligonucleotide primer for reverse transcription was 3-AAP (5′ TAC TAG TCG ACG CGT GGC CTTTTTTTTTTTT 3′). The 3′-RACE ready cDNA was synthesized from 3μg of total RNA extracted from five day-old seedlings. Primers designed for 3′-RACE were as follows: 3-cap (5′ TAC TAG TCG ACG CGT GGC C 3′) as the reverse primer, S1 (5′ AAGTCAATGATCTGCCTCCG 3′) and S2 (5′ GTGGGCATTCTCAGTTCGG 3′) as the forward primers. The 3′-RACE reaction volume was 50μl, including 1 μl cDNA, 10μM dNTPs, 1×PCR buffer, 2μM Mgcl2, 1.5U *Taq* polymerase, and 10μM 3-AAP, 10μM S1 (used for the first PCR). PCR was performed using the following procedures: the cDNA was denatured at 94°C for 4 min followed by 26 cycles of amplification for first PCR (94°C for 40 s, 58°C for 40s, 72°C for 2 min) and by 7 min at 72°C. The second PCR was performed as the same procedures with the first round PCR, except using the production of the first PCR as template. The 3′ RACE product was cloned into PMD18-T vector (*TaKaRa Biotechnology Co*.) and sequenced.

Sequence analysis: ORF finder was used to predictcoding sequence and BLAST program was used to find similarity of TAGLP1 with other lipases and putative lipases in the database online (http://www.ncbi.nlm.nih.gov and http://www.ch.embnet.org). *DNAStar* and *TREEV32* were used for sequence alignment and phylogenetic analysis. Phylogenic tree was constructed by neighbor-joining method.

Tissue expression pattern analysis: Semi-quantitative two-steps RT-PCR was performed to investigate the expression of this cDNA in different days during germination period and different tissues including needles, roots, stems, flowers, leaves and pods of *Brassica napus* with the forward primer orf-F (5′ ggtaccCTTTGAGTTTCTTCTGGT 3′) and reverse primer orf-R (5′ gtcgagCTATTCTTCTTGGTCTCC 3′). The annealing temperature of this pair of primers was 48°C. RT-PCR images were captured by using a UVP transilluminator with image processed by *MultiImage™ Light Cabinet* (Alpha Innotech Corporation, Line, China).

Results and discussion

Cloning and sequencing the ORF-contained cDNA of *TAGLP*: *Arabidopsis* putative lipidase gene was used as a query to search against the *Brassica* EST database and one homologous EST was identified. The EST contained the 5′ end of the cDNA. The ORF-contained cDNA was obtained by using RT-PCR and 3′ RACE designated as *TAGLP*. The 3′ flanking sequence of 1124 bp was obtained by 3′RACE. Aligning and assembling the sequences of 3′RACE and EST, we got a cDNA of 1804bp in which there was an ORF of 1407bp (Fig. 1). The deduced protein was 468 amino acids with an theoretical isoelectric point (PI) of 6.42 and calculated molecular weight of about 65.6 kDa. The hydrophobicity profile prediction showed that LIP was a hydrophobic protein (data not shown). The richest amino acid in the deduced protein was Gly (9.8%), followed by Leu (8.8%), Asp (7.4%) and Val (7.2%).

Sequence analysis of TAGLP1: By blasting TAGLP1 in *Swisstrembl database* we found that TAGLP1 shared 86%, 74%, 67%, and 72% sequence identities with *A.. thaliana* lipase and putative lipase (GenBank Acc. No.Q9LR26, GenBank Acc. No.Q9SAC8, GenBank Acc. No. NP_172544), *Oryza sativa* lipase (GenBank Acc. No.Q6I575), *C. rugosa* (GenBank Acc. No. P20261) and shared a comparatively lower identity with lipase-like proteins from bacterium. We could hardly find homologous lipases from *Brassica napus*, Mammalians and Insects. Multiple protein sequence alignment of TAGLP1 with lipases and putative lipases from plant and bacterium revealed that the TAGLP1 protein has the highest conservation within plants and comparatively higher conservation exited among various bacterium (Fig. 2), suggesting that TAGLP1 may play an important role in oil and fat hydrolysis which can provide carbon and energy for organism growing. Analyzing by using *Motif Scan* we found that there was a lipase motif from amino acid P^{66} to D^{179}. Searching in *PIR* we found that the TAGLP1 has a LIPASE_SER pattern (I^{158}HFVGHSAGA^{167}) which has the serine active site. A signal peptide of 35 amino acids from M^{1} to A^{35} was found in *SMART* sequence analyze system. And the results from *SANGER* and *PROSITE* showed that this signal was an YSIRK signal which resides in most triacylglycerol lipases. Transmembrane regions analysis of the signal peptide by *TMpred* showed that TAGLP1 was probably a membrane associated protein (date not show). Based on the characteristics discussed above we predicted that TAGLP1 is a triacylglycerol lipase which takes the responsibility of triacylglycerol degeneration. Until now, we haven't found a protein which is homologous with TAGLP1 in *Brassica napus* database, so we inferred that it might be a new triacylglycerol lipase in *Brassica napus*. Besides *Candida rugosa* Lipase (GenBank Acc. No. 1LPP), which was a TAG lipase, found and analyzed in 1993 (Grochulski P, Pawel Grochulski and Miroslaw Cygler etc., 1994) has also shared the same active residues. The active residues in the *Candida rugosa* Lipase are Ser^{224}, Glu^{341}, and His^{464} which form triad, while in TAGLP1 the triad which takes the responsibility of the interfacial activation by changing conformation (Grochulski P, Pawel Grochulski and Miroslaw Cygler etc., 1994) are probably formed by Ser^{164}, Glu^{265}, and His^{388}.

The secondary structure of TAGLP1 predicted by *SOPMA* showed that the putative triacylglycerol lipase peptide contained 33.76 % of alpha helix, 19.02% of extended strand, 7.26% of beta turn, and 39.96 % of random coil. The alpha helix and random coil constituted interlaced domination of the main part of the secondary structure. Analyzing result of TAGLP1 from *EMBL-EBI* showed that it is a alpha/beta-Hydrolase belonging to the AB-Hydrolase superfamily.

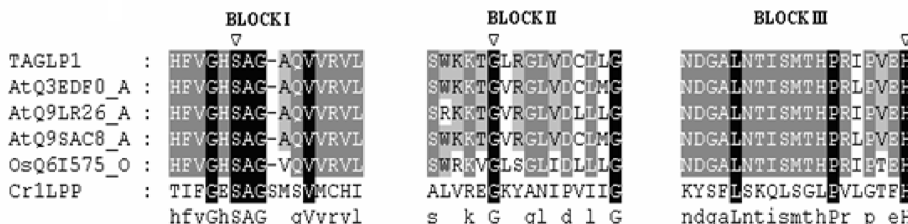

Fig.2. Multiple amino acid sequence alignment of there blocks located in TAGLP1 with the other homologous plant lipases deposited in NCBI databases and *Swisstrembl database. A. thaliana* AtQ9LR26_ARATH (GenBank Acc. No.Q9LR26), *A. thaliana* AtQ9SAC8_ARATH (GenBank Acc. No.Q9SAC8), *Oryza sativa* OsQ6I575_ORYSA (GenBank Acc. No.Q6I575), *C. rugosa* Cr1LPP (GenBank Acc. No. P20261) and *A. thaliana* AtQ3EDF0_ARATH (GenBank Acc. No. NP_172544). The amino acids (Ser^{164} in block I, Glu^{265} in block II, and His^{388} in block III) of the putative catalytic triad are indicated with up-side down triangles. The conserved residues are shaded in thick black (absolutely conserved) and in grey. The conserved blocks are marked above the alignment. Consensus amino acid residues are aligned at the bottom line.

Molecular evolution analysis: The evolutionary relationships among TAGLP1 and other lipases have been figured out as the form of a phylogenetic tree based on the deduced amino acid sequences (Fige.3). The result showed that there were two groups in Fig.2. The two groups of TAGLP1 were derived from a common ancestor in the evolution. There was only Cr1LPP (GenBank Acc. No. P20261) in the first group. The second group was a big group in which there were two small groups. There were TAGLP1, AtQ9LR26_ARATH (GenBank Acc. No.Q9LR26), AtQ9SAC8_ARATH (GenBank Acc. No.Q9SAC8), OsQ6I575_ORYSA (GenBank Acc. No.Q6I575), and AtQ3EDF0_ARATH (GenBank Acc. No. NP_172544) in the first small group. Naturally the rests including CtQ896Q1_CLOTE (GenBank Acc. No. NP_781602), BcQ4MLA6_BACCE (GenBank Acc. No. ZP_00239430), BsQ3T5M2_BACST (GenBank Acc. No. NP_172544), GbQ1L776_9BACI (GenBank Acc. No. AAY82869), GkQ5KYG5_GEOKA (GenBank Acc. No. YP_147839) and GtQ59260_9BACI (GenBank Acc. No CAA64621) formed the second small group. Our result suggests that the revolutionary relationships of lipases in bacterium and plants are relatively conserved, except for *C. rugosa*.

Gene expression analysis: Total RNAs isolated from one day-old to seven day-old seedlings, which were mentioned previously, were used as templates to detect *TAGLP1* transcript level by two-steps RT-PCR analysis. *TAGLP1* expressed in whole germinative stage of *Brassica napus* (Fig. 4). However the expression level of *TAGLP1* was different. The integrated expression pattern looks like a parabola profile. The expression of *TAGLP1* gradually increased from the third day to the fifth day. During the fifth day it reached a peak. Subsequently, *TAGLP1* expression level decreased rapidly. During the first three days the expression pattern also looks like a parabola profile. And the *TAGLP1* expression level increased greatly in the second day, then descent quickly in the third day. The *TAGLP1* expression pattern during germinative stage looks like a parabola profile, which was coincident with the character of TAG lipase.

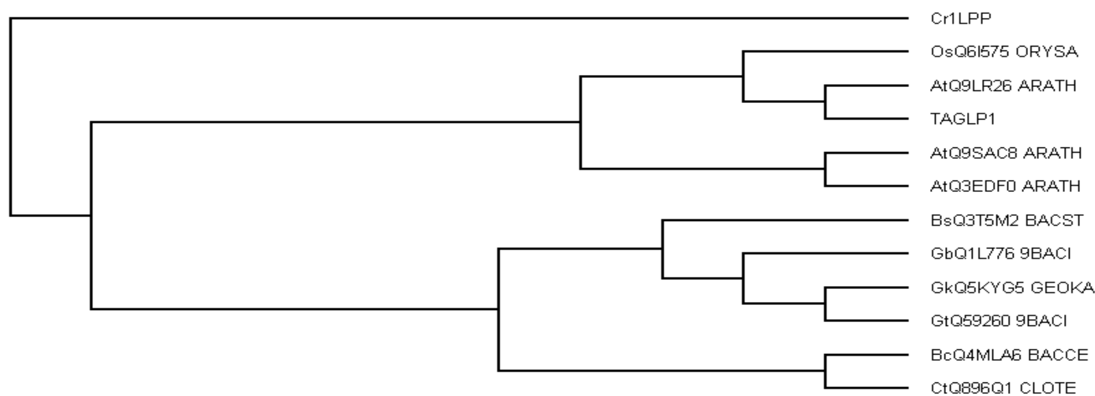

Fig.3. Phylogenetic relationships of the lipases in plants. The analysis is performed with DNAStar and Treev32, using Clustal W Method. AtQ9LR26_ARATH (*A. thaliana,* GenBank Acc. No.Q9LR26), AtQ9SAC8_ARATH (*A. thaliana*, GenBank Acc. No.Q9SAC8), OsQ6I575_ORYSA (*Oryza sativa,* GenBank Acc. No.Q6I575), Cr1LPP (*C. rugosa,* GenBank Acc. No. P20261) and AtQ3EDF0_ARATH (*A. thaliana,* GenBank Acc. No. NP_172544), BcQ4MLA6_BACCE (*Bacillus cereus* G9241, GenBank Acc. No. ZP_00239430), GkQ5KYG5_GEOKA (*Geobacillus kaustophilus,* GenBank Acc. No. YP_147839), BsQ3T5M2_BACST (*Bacillus stearothermophilus,* GenBank Acc. No. NP_172544), GbQ1L776_9BACI (*Geobacillus sp. SF1,* GenBank Acc. No. AAY82869), GtQ59260_9BACI (*Geobacillus thermocatenulatusm,* GenBank Acc. No. CAA64621), and CtQ896Q1_CLOTE (*Clostridium tetani*, GenBank Acc. No. NP_781602)

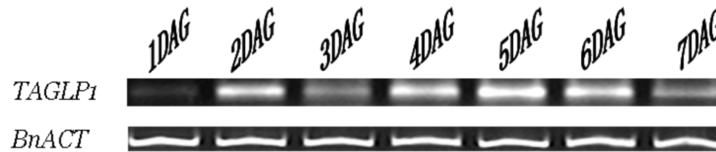

Fig.4. RT-PCR analysis of TAGLP1 expression during germinative stage. The total RNAs used as the templates for RT-PCR are isolated from the germinated seedlings of 1 DAG, 2 DAG, 2 DAG, 4 DAG, 5 DAG, 6 DAG, and 7 DAG, respectively

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