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Amplification of the active site of *BnLIP*3 gene of *Brassica napus* L. during germination

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Lipases are useful enzymes that are responsible for the hydrolysis of triacylglycerides and play an important role in plant growth. In this study, we report a rapid molecular method to amplify a partial sequence of the lipase class 3 family designated *BnLIP*3 gene of *Brassica napus* L. in order to follow its expression and analyze its role during seed germination. Therefore, we conceived PCR homologous primers to amplify the active site encoding region of the *BnLIP*3 family genes. Subsequently, to sequence determination of the 582 bp fragment, we deduced *BnLIP*3 specific primers for a nested RT-PCR application. The deduced 194 amino acid sequence (Genbank 1160264) was found to share 85% of identity with lipase from *Arabidopsis thaliana* class 3 family. The GxSxG consensus motif near the catalytic triad at the active serine site was also identified. The peptidic sequence showed little homology with mammalian and microbial lipases. RT-PCR analysis indicated that *BnLIP*3 gene was expressed during *B. napus* seed germination.

Key words: *Brassica napus L.*, GxSxG lipase, germination, *BnLIP*3, RT-PCR.

INTRODUCTION

Lipase (triacylglycerol hydrolase E.C.3.1.1.3) are ubiquitous enzymes which play an important role in lipid metabolism, as they catalyses hydrolysis and synthesis of triglycerides and other water insoluble esters (Patrick et al., 2008). Triacylglycerols (TAGs) are an important reserve of carbon and energy in Eukaryotes. During the germination of oil-seeds, these carbon units will eventually contribute in sugars synthesize through glyconeogenesis (El-Kouhen et al., 2005). These enzymes are distributed among higher animals, microorganisms and plants in which they play a role in the biological turnover of lipids (Schmid, 1998). They are also involved in the metabolism of intracellular lipids, and, therefore, in the functioning of biological membranes (Kazlauskas et al., 1998). Lipases activities have been studied at the biochemical level in a variety of seeds (Younghee, 2004). In most cases, the activity is only detectable upon germination and increase concomitantly with the disappearance of TAG (Peter, 2004). True lipidic substrates are insoluble in water, and lipases commonly show activation upon contact with substrate micelles or emulsions (Erick et al., 2004). This feature distinguishes lipases from esterases (EC 3.1.1.1), which act only on water-soluble substrates, such as short chain fatty acid

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esters, although, lipases show some activity on water-soluble esters (Beisson et al., 2000).

The hydrolytic activity of lipases is significantly enhanced by the presence of a lipid-water interface, a phenomena which is called interfacial activation (Schmid, 1998). In non-aqueous media, these reactions are reversed due to a hydrophobic domain (lid), covering the active site of lipases, which is made of a triad (Ser, Asp or Glu, His)(Nadia et al., 2007), whose serine residue is located in a highly-conserved Gly-X-Ser-X-Gly motif (X is His/Tyr, and sometimes the first Gly is replaced with Ala) in a turn between α/β-strand and an α-helix (Belguith et al., 2009). The GXSXG lipases are usually named "classical" lipases. Unlike the GDSL lipases have a serine-containing motif (called GDSL motif) closer to the N-terminus. They have a catalytic SDH triad and five consensus sequences, also possessing multifunctional properties such as broad substrate specificity and regiospecificity (Akoh et al., 2004). Lipases have been extensively investigated with respect to their biochemical and physiological properties, and lately for their industrial applications. These enzymes are employed in the production of pharmaceuticals, cosmetics, leathers, detergents, foods, perfume products, medical diagnostics and as well as environmental management (Ling et al., 2005). Until now, few data are available concerning molecular level of plant lipases. However, some lipases have been purified from seeds of several plants such as maize (*Zea maize*) (Lin et al., 1984), castor (*Ricinus communis*) (Fuchs et al., 1996), Cyprinus carpio (Han-liang et al., 2010) and iron weed (*Vernonia galamensis*) (Ncube et al., 1995), but none of the genes that encode these enzymes have been cloned and characterized.

Consequently, many questions remain concerning the mechanism and regulation of lipolysis. In the same way, many lipases have been purified and cloned from bacteria, (Ines Belhaj-Ben Romdhane et al., 2012) fungi and animals (Ogino et al., 2004). Their functions, primary structures and even crystal three-dimensional have been studied in detail (Kim et al., 1997). Actually, there is little homology sequence among them. Rapeseed is one of the most important oilseed crops. Large percent of vegetable lipids are stored in seeds, and during seed germination lipases turn active along with the breakdown of lipids for sustaining post-germinative growth. It is necessary to know more about the gene and biological properties in order to further understand the molecular mechanism of such biological activities. However, cloning and expression analysis of GxSxGx lipase genes from rapeseed is scarcely reported (Ling et al., 2005).

In this report, we present a recent advance in the characterization of plant lipases; molecular investigation of primers designer in order to amplify a part of lipase gene encoding the active site. Investigation of gene sequence and analysis of its expression pattern are presented for the first time during seed germination character of GxSxGx motif lipase in rapeseed.

MATERIALS AND METHODS

Plant materials

Seeds of *Brassica napus* L. (variety pactol) were cultivated in Tunisia and were kindly supplied by Dr Ben Saleh (I.N.R.A.T). Seeds were soaked in tap water for 24 h. For germination studies, the seeds were sterilized with 7% (v/v) $CaCl_2O_2$ in distilled water for 5 min. The sterilized seeds were allowed to germinate on filter paper and moistened with distilled water in darkness at 26°C. We used two lots. The first lot was the etiolated seedlings which were harvested at three days after germination (DAG), which were used in RNA extraction immediately. The second lots were root, stem, leaf, and flower developed from the mature seedlings harvested from local farm that were separated and immediately used for RNA extraction.

RNA and DNA isolation

Total RNAs of different samples including germinative seed, root, stem, leaf, and flower from *B. napus* L. plant were extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instruction, and pretreated by DNase I (Fermentas). The genomic DNA was isolated using DNA purification kit (promega). The quality and concentration of RNA and DNA samples were examined by Ethidium bromide (EB)-stained agarose gel electrophoresis and spectrophotometer analysis.

Primers design

To amplify the active site from Bnlip3, a set of primers conceived from coding region for the highly conserved domain of lipase gene from different plant species was targeted in database Genbank. Using CLUSTALW (Thompson et al., 1994), we aligned different plant species sequences obtained from Genbank. Based on the homology between the aligned sequences, we retained the most similar ones (Table 1) and used the Oligo6 software (Molecular Biology insights, Inc. CO, USA) to conceive a homologous primers pair able to target all the analyzed sequences.

Amplification of lipase gene by PCR

The PCR reaction was carried out using a reaction volume of 50 µl, containing 2 µl of genomic DNA from *B. napus* L., 10 pmol of each primer, 10 µmol deoxynucleotide triphosphates, 5 µl 10× DNA reaction buffer (1× DNA buffer consists of 20 mM Tris HCl, pH 8.4, and 50 mM KCl, Invitrogen), 5 µl 25 mM $MgCl_2$, 5 µl 0.1 M DTT and five units of Taq polymerase. Two degenerate primers BnFw1 (5'GTAAATGCAGGTTCCGAAATG-3') and lower (5'GAAAAGACAGGTTCCGAAATG-3') were used to amplify the core sequence by the following procedures: the template was denatured at 94°C for 5 min, then amplified by 35 cycles of amplification (94°C for 1 min, 55°C for 1 min, 72°C for 2 min), subsequently by extension at 72°C for 10 min. The PCR product was purified with the purlink^{tm} PCR purification kit (invitrogene), examined by agarose gel 1% electrophoresis and sequenced.

Sequence analysis

Sequence alignment and translated protein were done at the NCBI server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Table 1. Percentage identity between the lipase of Bnlip3 DNAs with other plants species.

Species	Accession number (Genbank) of lipase sequences used for the primers design	Homology with the 582 pb fragment of lipase classe3 (%)
Arabidopsis thaliana lipase class 3 family protein (AT3G14360) mRNA, complete cds	NM 112294.4	85
Arabidopsis thaliana unknown protein (At3g14360) mRNA, complete cds	AY150508.1	85
Arabidopsis thaliana genomic DNA, chromosome 3, P1 clone: MLN21	AB022220.1	85
Arabidopsis thaliana unknown protein (At3g14360) mRNA, complete	AY056160.1	85
Arabidopsis thaliana Full-length cDNA Complete sequence from clone	BX823992.1	85
Ricinus communis lipase (OBL1) mRNA, complete cds	AY360220.1	100
Arabidopsis thaliana triacylglycerol lipase (AT5G42930) mRNA, complete cds	NM123658.2	73

Reverse transcription (RT)-PCR

The total RNA (0.5 µg) was incubated with 10 units RNase-free DNase I at 37°C for 15 min, to eliminate possible DNA traces contaminating the RNA samples; the DNase was subsequently inactivated by incubation at 65°C for 15 min. The RNA was reverse transcribed in a final volume of 20 µl containing 4 µl RNA, 8. 5 µl DEPC-treated water, 4 µl of the 5X reaction buffer, the four deoxynucleotide triphosphate (dNTPs) (1 mM each); 20 units RNase inhibitor (RocheApplied Science); 20 units Moloney murine leukaemiavirus (MMLV) reverse transcriptase (RTase) (Expand RT, Roche Applied Science, Barcelona, Spain) and a 2.5 µl reverse primer (5'-GAAAAGACAGGTTCCGAAATG-3'). The reaction was carried out at 37°C for 2 h and was stopped by heating at 95°C for 10 min. Control reaction mixtures with all components except RTase were included in every experiment. PCR amplification was performed in a final volume of 50 µl, containing 4 µl of the previous RT reaction, 1 µl of dNTPs (10 mM), 12 µl of $Mgcl_2$ (10 mM), 5 µl of each primer (10 µM) upper (2) (5'-CAAGTTTGTAGTCACGGGTCACAG -3') and lower (2) (5'-CGTAAGGCAATCTCGGGACAAG3'-), 0.5 µl (1 unit) of GoTaq polymerase flexie and 10 µl of the 5X buffer (Promega). The primers used should amplify a 239 base pair (bp) fragment.

The RT-PCR reaction for the housekeeping gene BnACT (rapeseed actin gene, Genbank *B. napus* (AF111812) using specific primers Fw973: (5'-TTCCCTGGAATTGCTGACCG-3') Rv1052: (5'-ACTGTACTTCCTCTCAGGCG-3') was also performed as an internal control. The PCR programme included an initial incubation at 94°C for 2 min, then amplified by 35 cycles of amplification (30" at 94°C, 30" at 55°C and 2 min at 72°C) and finally by extension at 72°C for 10 min.

RESULTS AND DISCUSSION

PCR primer set and amplification of lipase gene

Degenerate PCR primers were designed to anneal at coding sequences for conserved amino acid residues despite degeneracy of the genetic code. Alignment of the lipase sequence is used as a guide to enable identification of conserved nucleic acid sequences of the gene to be used in the design of degenerate oligonucleotide primers for PCR. The conserved region of lipase amino acid sequences was distributed overall in coding sequences for the studied lipase. The regions of amino acids from 1 to 582 pb were especially highly conserved. The degree of similarity of lipase DNAs with other plants species are shown in Table 1. The degenerate PCR primer set successfully amplified DNA from rapeseed (Figure 1). The primer set yielded an amplified product of the predicted size of 582 bp. The amplified PCR products were sequenced. The nucleotide sequence and its deduced amino acid sequence of our isolate are shown in Figure 2. The nucleotide sequence was blasted in GenBank database and compared to all

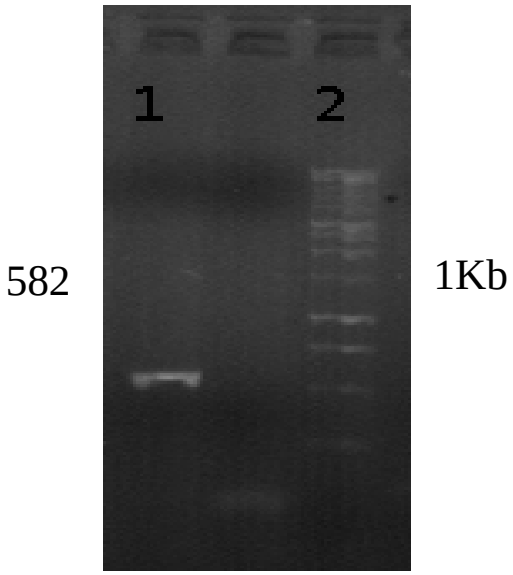

Figure 1. PCR product prior to 1% agarose gel electrophoresis (10 µg/lane). Lane 1, PCR product 582 pb; lane 2 molecular weight 1 kb.

```
1aattttgatagtgatcagcttcagaggcactgagccgtttgatgcagatgactggggaaca
   I  L  I  V  I  S  F  R  G  T  E  P  F  D  A  D  D  W  G  T
61gactttgactattcttggtatgagatccccaatgtgggtaagcttcacatgggtttcatc
   D  F  D  Y  S  W  Y  E  I  P  N  V  G  K  L  H  M  G  F  I
121gaagcaatgggtttaggtaccagagactacactcccactttcgattaccatctcgtcgag
    E  A  M  G  L  G  T  R  D  Y  T  P  T  F  D  Y  H  L  V  E
181ctaagctcctctgagaaagagaataaccaaaagaatctcccagagatggtagagagaagc
    L  S  S  S  E  K  E  N  N  Q  K  N  L  P  E  M  V  E  R  S
241gcttattacgcagttagagaaactctgaaacgtttgcttgcggagcacgcaaacgccaag
    A  Y  Y  A  V  R  E  T  L  K  R  L  L  A  E  H  A  N  A  K
301tttgtagtcacgggtcacagcttaggaggcgcgttagcgattctgtttcccacgttgctg
    F  V  V  T  G  H  S  L  G  G  A  L  A  I  L  F  P  T  L  L
361gtgttgaaagaggagacggagatgatgaggaggctgcttggggtttacactttcggacag
    V  L  K  E  E  T  E  M  M  R  R  L  L  G  V  Y  T  F  G  Q
421cctaggatcgggaacagagagattggtagtttcatgaaagctaaactgaatcaccctgta
    P  R  I  G  N  R  E  I  G  S  F  M  K  A  K  L  N  H  P  V
481gatagatactttcgtgttgtgtactgcaacgatcttgtcccgagattgccttacgacgac
    D  R  Y  F  R  V  V  Y  C  N  D  L  V  P  R  L  P  Y  D  D
541acaacgttcctctacaagcatttcggactctgtcttttaaa
    T  T  F  L  Y  K  H  F  G  L  C  L  L
```

Figure 2. Nucleic acid sequence and deduced amino acid from *Brassica napus* lip 3. The conserved consensus motif (G-x-S-x-G) around the active serine residue is bolded.

available sequences.

Homology of the lipase gene

Data base searches revealed no extensive sequence homology to mammalian and microbial lipase genes, implying that it was probably a partial sequence of lipase gene (Younghee, 2004). However, a number of plant lipases, including those of *Arabidopsis thaliana* and Castor bean, were found to be related to this enzyme. BnLip3 DNA of *B. napus* L. shared 85% sequence identity with *A. thaliana* lipase class 3 proteins (Gene Bank Acc. No. NM112294.4) and 100% with *Ricinus communis* lipase (Acc. No. AY360220.1). The deduced amino acid sequence had 92 and 63% identity to the lipase gene of class 3 family proteins of *A. thaliana* (Acc. No. NP 566484.2) and *R. communis* (Acc. No. AAV66577.1), respectively (Table 2). An alignment of some of these proteins is shown in Figure 3. Conserved residues are indicated by asterisks. The amino acid sequence of the encoded protein contains an active site seine motif GxSxG (Figure 2) (Hua ling et al., 2006). Comparison of the amino acid residues of the catalytic triad in proteases, lipases, cutinases and esterases revealed the presence of the GxSxG consensus sequence in each of these enzymes (Table 3). The only known exception to this consensus sequence is the substitution of alanine for the second glycine in subtilisins (data not shown). No such consensus sequences are present around the catalytic histidine or aspartic (glutamic) acid residues, except in enzymes from closely related species.

A recent analysis of the conformation of the pentapeptide G×S×G found that the invariant glycine residues were conserved in proteases because of conformational requirements (Derewenda and Derewenda, 1991). According to Karlsson et al. (1997), two lipase motifs are found in animal systems. One is the consensus GxSxG, the other the HG (His-Gly) dipeptide motif present in the monoglyceride lipase of mouse adipocytes. There is no HG motif in the BnLip3 (*B. napus* L.) lipase sequence. Catalytic triade surrounded the nucleophilic serine residue which embedded in a tight turn between a *β*-strand and an *α*-helix (Ollis et al.,1992), that are highly conserved among all known lipases in their deduced amino acid sequences (Zhiqiang et al., 2008), which was also supported by the result of phylogenetic analysis (Figure 5). With true lipases undergoing interfacial activation, the active site is usually sheltered from the solvent by a lid consisting of a protruding short a-helix, which is moved out upon adsorption to an interface (Kenji et al., 2004). The position of the acid member of the active-site triad is very well maintained in the sequence alignment. In most proteins of this family, the role of the acid is provided by glutamic acid.

Actually, this is the first family of enzymes containing a catalytic triad, which has Glu in this role (Cygler et al., 1993). Although, mammalian and fungal and yeast (P Emond et al., 2010). Lipases have been studied intensely, this is the first report for characterization of a true lipase of plant origin.

Analysis of the genomic lipase sequence

Genomic DNA from *B. napus L.* was isolated using kit DNA purification kit (promega). Sequence analysis revealed a single fragment of about 582 pbs, which encodes a polypeptide 194 AA residues, like lipases from other eukaryotic microorganisms, the sequences from *B. napus* L. contained the consensus motif (G-x-S-x-G) which is conserved among lipolytic enzymes (Zhiqiang et al., 2008); it is typical to α/β hydrolases such as lipases and esterases (Saxena et al., 2003).

RT-PCR analysis of *B. napus* expression

In order to detect expression of lipase gene from *B. napus* L. during germination, the total RNAs used as the templates (1 µg/sample) for RT-PCR are isolated from the germinated seedlings of three days after germination, root, stem, leaf and flower, respectively. As a result of the semi-quantitative one-step RT-PCR obtained, only seedling germinated expression was detected with a single amplified DNA fragment of the predicted size (about 239 bp); this data is confirm in mature plant tissues, even if transcript level was different, and its expression in roots, stem, leaf and flower disappears. Moreover, the levels of *B. napus* action transcripts were stable and detectable in all analyzed tissues (Figure 4). The transcript could be largely found in cotyledons. This expression profile of lipase 3 is quite similar to those of genes involved in glyoxylate cycles (Beevers, 1979), 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 b-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 b-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 (Hayashi et al., 2002), thus, the lipid hydrolyzing step is essential. Despite the importance of a lipase involved in the fat mobilization, it has not been fully studied. Our finding demonstrate that lipase 3 is a ‘true lipase’ that requires the presence of an oil-water interface for full activity, a phenomenon known as interfacial activation (Kenji et al., 2004). In addition, the GxSxG

```
brassica        ------------------------------------------------------------
Arabidopsis     MHKDNDSGSGSNPGQVSNYLIVRPHRGGYIDLFRYGVRDDQTSKAKFLEMPDNREWSTIT
[Populus        -------------------------MLDLLRYLAWADIGSGVRFLESSEEG-----I
[Ricinus        ----MAASATTSNNIAPNFLVVDPKKGRKRDIFKYLVRKDVKSGMSFLDSSEEG-----V
[Jatropha       ----MATPP-------VNFLIVNPQKGRKRDLFKYLVTKNKKSGMSFLDSSEES-----I

brassica        ------------------------------------------------------------
Arabidopsis     IDEEAEDHRWVIVVSILVRKIIRLLRTPMEFTGFVVDFFLNLFSANGGFFGLLLRLIQAK
[Populus        MGEEAADHRWIILVSIIARKIISLFGKPLEYTGFVVDFFLNLLFQNGGIMGLFLNFLQGK
[Ricinus        KGGAAVDHRWILLVSIIIRRVLALIDTPLKYLGYVIDFFLNLISQNSGFSGILNNFLHGN
[Jatropha       KGGVANDHRWILLVSIIIRRILALINTPLKYLGYVVDFILNLISQNGGISGILSNSLHGK

brassica        ------------------------------------------------------------
Arabidopsis     VVIPERGSVTFVSTIGQLDGRISLYKEWNFVEHLEGIDSVDSGRVKIELGSRGLMDLCVM
[Populus        VVIPQRDTETFISSIGHLDGRIDLYRAENLLEQIDHSVSAEK-TITEEIGNRAHMDLCIM
[Ricinus        LKIPRRGTENFISTIGQLDGRIDLYRTTILSEKVDDSVATDVNNIKAELGNRYLMDLCIM
[Jatropha       LIIPRRGSENFVSTIGQLDGRIDLYKTVSLAEKVDGNFSADANNIETHLGNRYLMDLCIM



brassica        -----------------------------------------------------ILIVISFR
Arabidopsis     ASKLAYENAKVVENVVDLHWKMNLVEFLDCWNDYQKQMSTQVFVFTDKQKDANLIVISFR
[Populus        ASKLAYENAKVVRSIV-----MHFVDFYNCWNDFQKEFSTQVFILCDKPKDANLILISFR
[Ricinus        AAKLVYENEKVAQNVVDRHWKMHFVAFYNCWNEYQKQNNTQVFICCDKPKDANLIVVSFR
[Jatropha       AAKLVYENEKVVQNVIDRYWKMHFVAFYDCWNEYQKESNTQVFICCDKPKDANLIVISFR
                                                                  **::***

brassica        GTEPFDADDWGTDFDYSWYEIPNVGKLHMGFIEAMGLGTRDYTPTFDYHLVELSS---SE
Arabidopsis     GTEPFDADDWGTDFDYSWYEVPNVGKLHMGFLEAMGLGNRDDTTTFHYNLFEQTS---SE
[Populus        GTEPFDSYDWDTDFDYSWYEIPKLGKVHMGFLEALGLGNRDDTTTFQYLLQMKNTNFNHD
[Ricinus        GTEPFNAQDWSTDFDFSWYEIPKVGKIHIGFLEALGLGNRSDATTFQTHLQRKHTGFFHL
[Jatropha       GTEPFNAQDWNTDFDFSWYEIPKVGKIHIGFLEALGLGTRRDASTFQSHLQRKRAGLFHL
                *****:: **.****:****:*::**:*:**:**:***.*  :.**.  *    :

brassica        KENNQKNLPEMVERSAYYAVRETLKRLLAEHANAKFVVTGHSLGGALAILFPTLLVLKEE
Arabidopsis     EENSKKNLLDMVERSAYYAVRVILKRLLSEHENARFVVTGHSLGGALAILFPTLLVLNEE
[Populus        YEGHKKFPPEMVKKTAYYTVRKKLKSLLVEHKNAKFIVTGHSLGGALAILFPSVLVLHQQ
[Ricinus        NGESEGNMTEWAKKSAYYAVALKLKSLLKEHRNAKFIVTGHSLGGALAILFPSILVIQE-
[Jatropha       NGQSEGTTTELSKKSAYYDVTSKLKQLLEEHKNAKFVVTGHSLGGALAILFPSVLVIQEE
                    :    :  :::*** *   ** ** ** **:*:****************::**:::

brassica        TEMMRRLLGVYTFGQPRIGNREIGSFMKAKLNHPVDRYFRVVYCNDLVPRLPYDDTTFLY
Arabidopsis     TEIMKRLLGVYTFGQPRIGNREVGLFMKAQLNQPVDRYFRVVYCNDIVPRLPYDDKTFLY
[Populus        MDVMKRLLGVYTFGQPRIGNRQLAKFMEAHLEYPVPKYFRVVYSYDLVPRLPYDDKTFLY
[Ricinus        ------------------------------------------------------------
[Jatropha       TEILRRLLNIYTFGQPRIGDVQLGNFMEAHLNYPKTRYYRVVYCNDMVPRVPFDDNVFAF

brassica        KHFGLCLL------------------------------------------------------
Arabidopsis     KHFGLCLFYDSFYNETKAEDEPDPNPYGLRYKILGHVIAVWELVRGLTMGYTHGPDYKEG
[Populus        KHFGVCLYYNSLYIEQKVDEEPDPNLYGLRNVISAHLNAIWELIRSFIIGYTHGREYKES
[Ricinus        ------------------------------------------------------------
[Jatropha       KHFGTCLYYDSRYFGRFMDEEPNKNFFGLKHIIPMRVNVLWEIFRSFLISHIHGPEYKES

brassica        ------------------------------------------------------------
Arabidopsis     WFRILFRLMGLVIPGLSDHCMTDYVNSVRLGPDNELQMSSL--------------------
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Figure 3. Alignment of the deduced amino acid sequence of the lipase (*Brassica napus* L.) with other known lipase proteins. The proteins shown are lipases from *Populus trichocarpa* XP_002303338.1, lipase class 3 family protein (*Arabidopsis* thaliana) NP_566484.2, *Ricinus communis* AAV66577. 1, *Jatropha curcas* ABN45748.1. Conserved amino acids are indicated by asterisks.

Table 2. Percentage identity between the lipase of Bnlip3 and related enzymes.

Species	Accession number (Genbank) of lipase amino acid sequences	Homology with the 193 AA of lipase class 3 (%)
Lipase class 3 family protein (*Arabidopsis thaliana*)	NP_566484.2	84
Predicted protein (*Populus trichocarpa*)	XP_002303338.1	69
Lipase (*Ricinus communis*)	AAV66577.1	60
Lipase (*Jatropha curcas*)	ABN45748.1	63
Lipase (*Oryza sativa*)	BAA94239	78

Table 3. Sequences around the catalytic serines.

Enzyme class	Enzyme	Local sequence	Reference
Proteases	Trypsin (bovine)	* * *	Dayhoff et al. (1972)
	Elastase (pig)	Q-G-D-S-G-G-P	
	Plasmin (human)	Q-G-D-S-A-G-G	
	Protease-A (*S. griseus*)	P-G-D-S-G-G-S	
	Pancreatic lipase (human)	I-G-H-S-L-G-A	Lowe et al. (1989)
Lipases	Lipase (*R. miehei*)	T-G-H-S-L-G-G	Boel et al. (1988)
	Lipase (*G. candidum*)	F-G-H-S-A-G-A	Shimada et al. (1989)
	Lingual lipase (rat)	V-G-H-S-Q-G-T	Docherty et al. (1985)
	Lipase (*Oryza sativa*)	Y-G-T-S-N-G-A	
Cutinases	Cutinase (*P. mendocina*)	S-G-H-S-Q-G-G	Poulose et al. (1990)
	Cutinase (*F. solani*)	G-G-Y-S-Q-G-A	Soliday et al. (1983)
	Cutinase (*C. capsici*)	G-G-Y-S-Q-G-T	Ettinger et al. (1987)
Esterases	Butyrylcholineesterase (human)	F-G-E-S-A-G-A	Arpagaus et al. (1990)
	Carboxylesterase (murine)	F-G-E-S-S-G-G	Ovnic et al. (1991)
	Acetylcholine esterase (*T. californica*)	F-G-E-S-A-G-G	Schumacher et al. (1986)
	Thioesterase (duck)	F-G-H-S-F-G-S	Poulose et al. (1985)

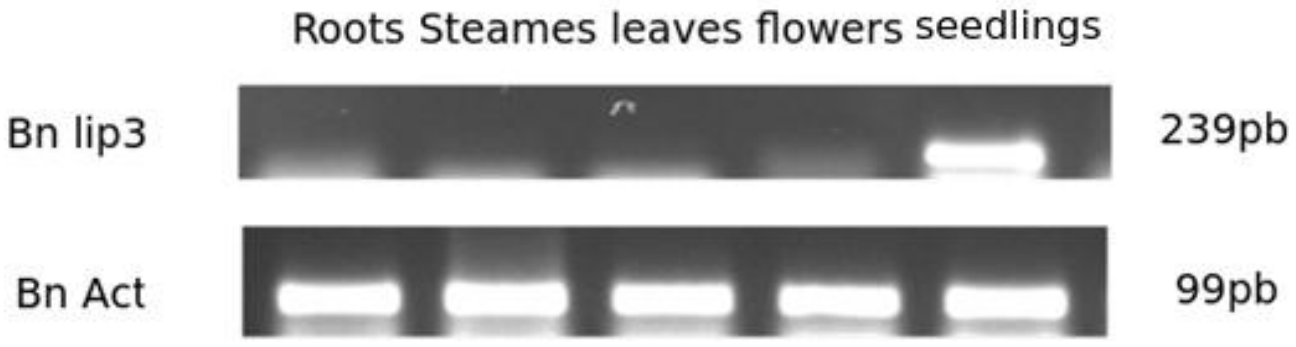

Figure 4. RT-PCR analysis of BnLIP3 expression. The total RNAs used as the templates (1 µg/sample) for RT-PCR were isolated from the germinated seedlings of 3 DAG; BnLIP3 expression profile during the reproductive stage. The total RNAs used as the templates (1 µg/sample) for RT-PCR were isolated from the roots, stems, leafs and flowers, respectively. Expression of actin gene in *B. napus* (BnACT) was used as an internal control. The amplified fragment of BnLIP3 was 239 bp and BnACT was 99 pb.

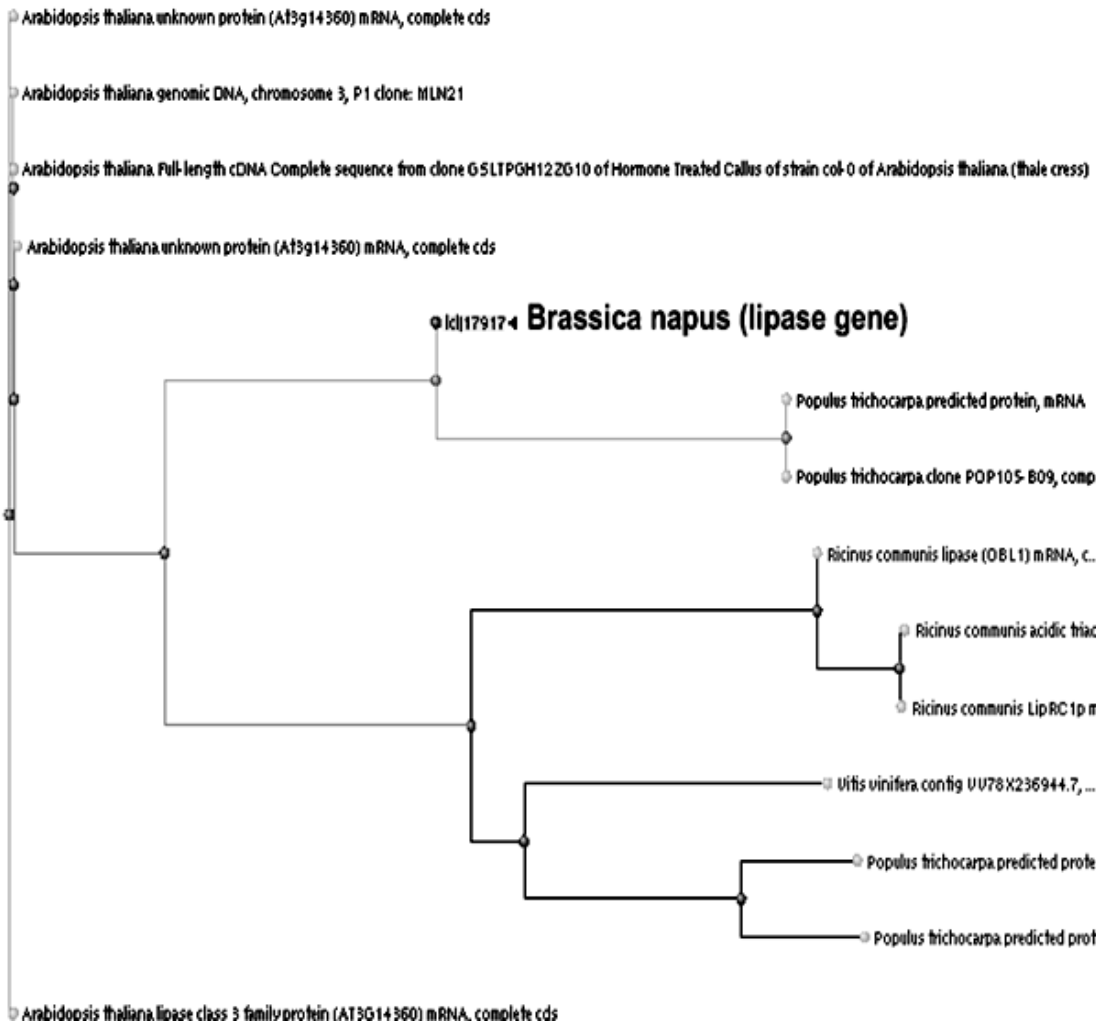

Figure 5. Phylogenetic relationships of lipases in plants.

lipase family includes large numbers of candidates. Data mining showed that GxSxG lipases have been found in plant species especially *Arabidopsis*, rice and tomato, which suggested that one lipase genes encoding lipase proteins exist in oilseed rape (Ling et al., 2005). Therefore, BnLIP1 and the previously reported 55-kD lipase are different members of lipase family in oilseed rape (Belguith et al., 2001).

Conclusion

In conclusion, we isolated and analysed a part of rapeseed lipase 3 family gene (lip 3). Our results revealed that lip 3 was expressed in germinated seedlings; the encoded protein is predicted to be a lipase containing motif GxSxG. Lip 3 may play multiple roles in plant physiological activities such as germination. Lip 3 encodes a true lipase, and from its expression profile suggest that lip 3 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 RNA and isolate the full-length cDNA of gene *B. napus* L. are needed.

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