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Isolation and Expression Analysis of a GDSL-like Lipase Gene from *Brassica napus* L.

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As lipolytic enzymes, GDSL lipases play an important role in plant growth and development. In order to identify their functions and roles, the full-length cDNA of a GDSL lipase gene, designated *BnLIP2*, was isolated from *Brassica napus* L. *BnLIP2* was 1,300 bp long, with 1,122 bp open reading frame (ORF) encoding 373 amino acid residues. Sequence analysis indicated that BnLIP2 belonged to GDSL family. Southern blot analysis indicated that *BnLIP2* belonged to a small gene family in rapeseed genome. RT-PCR analysis revealed that *BnLIP2* was a tissue-specific expressing gene during reproductive growth and strongly expressed during seed germination. *BnLIP2* expression could not be detected until three days after germination, and it subsequently became stronger. The transcript of this gene was deficient in root of seedlings growing at different stages. When juvenile seedlings were treated by methyl jasmonate (MeJ), salicylic acid (SA) and naphthalene acetic acid (NAA), *BnLIP2* expression could not be induced in root. Our study implicates that *BnLIP2* probably plays an important role in rapeseed germination, morphogenesis, flowering, but independent of root growth and development.

Database Accession No: AY866419; AAX59709

Abbreviations: CTAB = cetyltrimethylammonium bromide, DAG = days after germination, ENOD = early nodule-specific protein, EXL = extracellular lipases, EST = expressed sequence tag, MeJ = methyl jasmonate, NAA = naphthalene acetic acid, ORF = open reading frame, RACE = rapid amplification of cDNA ends, SA = salicylic acid, UTR = untranslated region.

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Introduction

As lipolytic enzymes, GDSL lipases are an important superfamily of lipases, and active in hydrolysis and synthesis of abundant ester compounds. Unlike the GXSXG motif-containing lipases, they have a GDSL motif with the flexible active site serine located near the N-terminus (Upton and Buckley, 1995), five blocks (block I-V) containing Ser-Asp-His triad and oxyanion hole residues (Ser, Gly and Asn) (Molgaard *et al*., 2000). Generally, GDSL lipase is made up of several β-strands and α-helices arranged in alternate order, and the substrate-binding pocket between the central β-strand and long α-helix appears to be highly flexible. The flexible pocket brings conformational changes, so that the active sites expose to the solvent and easily bind to substantive substrates, conferring multi-functional character of GDSL lipases (Derewenda *et al*., 1994). Possessing multiple functions, GDSL lipases are potentially applied to food, flavor, fragrance, cosmetics, textile, pharmaceutical and detergent industry.

GDSL lipases are widely found in microbes, and much progress on their structures, functions and physiological roles has been made. A great deal of bacterial GDSL lipase genes have been cloned and characterized, and presently the crystal structures of GDSL lipases from *Escherichia coli* and *Pseudomonas fluorescens* are available (Kim *et al*., 1993; Lo *et al*., 2000; Li *et al*., 2000). The GDSL lipase TEP-I from *E. coli* strain JM109 was over-expressed. Its matured enzyme showed broad hydrolytic activity toward three kinds of

substrates including acyl-CoAs, esters, and amino acid derivatives, additionally the aryl-esterase activity of the enzyme was observed by its ability to hydrolyze several aromatic esters including α-naphthyl acetate, α-naphthyl butyrate, phenyl acetate, benzyl acetate, and eight ρ-nitrophenyl esters. Therefore, the addition of six hydrophilic His residues resulted in a change in substrate specificity of TEP-I towards more hydrophilic substrates (Ya *et al*., 1997; Lee *et al*., 1999). A GDSL-hydrolase from *Vibrio mimicus* shows thioesterase activity for benzoyl-CoA and chymotrypsin-like activity for N-carbobenzoxy-L-phenylalanine ρ-nitrophenyl ester (NBPNPE) (Chang *et al*., 1995). The *apeE* gene of *Salmonella typhimurium* encodes an outer membrane esterase that catalyzes the hydrolysis of a variety of naphthyl esters and of C_6 to C_{16} fatty acid ñ-nitrophenyl esters (Carinato *et al*., 1998). An extracellular lipase containing GDS (L)-like consensus motif from *Streptomyces rimosus* R6-554W was reported to show hydrolytic activity against ñ-nitrophenyl butyrate and palmitate (Vujaklija *et al*., 2002). Another GDSL lipase gene from *Xanthomonas vesicatoria* encodes a 63-kDa mature protein preferentially hydrolytic to short chain (up to C_8) and para-substituted nitrophenylesters as substrates, unable to hydrolyze triacylglycerols of long chain fatty acids. However, the only long-chain 1-hydroxy-pyr-ene-3, 6, 8-trisulfonic acid (HPTS)-fatty acid esters were hydrolyzed. It was also found to be active on a series of substrates with industrial interests (Talker *et al*., 2003). *Serratia liquefaciens* MG1 secrets a GDSL lipase which probably provides the cell with precursors for the biosynthesis of N-acyl-homoserine lactone (AHL) under certain growth conditions, and it is specific to hydrolysis of ρ-nitrophenyl from C_2 to C_4 esters and triacylglycerols of less than C_{10}, and no activity was obtained with nitrophenyl esters greater than C_6 (Riedel *et al*., 2003). As effective clues these advances are helpful to further understand GDSL lipases from other resources.

GDSL lipases are also found in plant species, and recently the elucidation of their properties and functions has become attractive. Several candidates from *Arabidopsis thaliana*, *Rauvolfia serpentina*, *Medicago sativa*, *Hevea brasiliensis*, *Alopecurus myosuroides* have been isolated, cloned and characterized (Brick *et al*., 1995; Pringle *et al*., 2004; Arif *et al*., 2004; Cummins *et al*., 2004; Ruppert *et al*., 2005; Oh *et al*., 2005). The recombinant Ara-1 from *Arabidopsis* showed lipolytic activity against Tween_80, additionally hydrolytic to ρ-nitrophenyl butyrate. Another *Arabidopsis* GDSL lipase GLIP1 possessed lipase and anti-microbial activities that directly disrupted fungal spore integrity, and in association with ethylene signaling it might play a key role in plant resistance to *Alternaria brassicicola*. Through a novel tobacco-virus expression system, an ajmaline pathway-specific GDSL lipase from *R. serpentina* has been functionally overexpressed in leaves of *Nicotiana benthamiana*, and its high enzymatic activity in *Nicotiana* tissues was identified. At the same time, many sequences of other GDSL lipases have been deposited in the databases. Mayfied *et al.* (2001) reported six extracellular lipases (EXL1-6) isolated from *A. thaliana* pollen coat. Anther-specific proline-rich protein genes (*APGs*) have been cloned and sequenced respectively from *A. thaliana* and *B. napus*. Another GDSL enzyme from post-germinated sunflower (*Helianthus annuus* L.) seeds was isolated, purified, and shown to have fatty acyl-ester hydrolase activity, but no detailed information about its sequence could be found (Teissere *et al*., 1995). Physiologically, these GDSL lipases that have been cloned or characterized so far are mainly involved in the regulation of plant development, morphogenesis, synthesis of secondary metabolites and defense response.

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 GDSL lipase genes from rapeseed is scarcely reported. Herein we isolated a full-length cDNA encoding a GDSL lipase BnLIP2, analyzed its expression pattern, and presented for the first time the root-deficient character of GDSL lipases in rapeseed.

Materials and methods

Plant materials. Oilseed rape (*B. napus* variety Huyou15) seeds were soaked in tap water for 24 h, and then germinated on the wet filter paper at room temperature in dark. The whole etiolated seedlings were harvested at 0, 1, 2, 3, 4, 5, 6 days after germination (DAG) respectively, which were used in RNA extraction immediately. Root, stem, leaf, flower, bud, developing pod of the mature seedlings harvested from local farm were separated and immediately used for RNA extraction. Four-week-old seedlings were used in chemical treatments.

RNA and DNA isolation. Total RNAs of different samples including germinative seed, root, stem, leaf, flower, bud and developing pod from *B. napus* L. plant were extracted using TRIzol Reagent according to the manufacturer's instruction (GIBCO BRL), and pretreated by DNase I (Takara). The genomic DNA was isolated using a CTAB-based method (Ausubel *et al.*, 1998). The quality and concentration of RNA and DNA samples were examined by ethidium bromide (EB)-stained agarose gel electrophoresis and spectrophotometer analysis.

Lipase gene cloning by RACE. The first-strand cDNA was synthesized by Superscript II (Takara). Two consensus sequences (TGRSFNGRLIIDFIEEL, QEPAKYGF) were obtained after aligning different *Arabidopsis* GDSL lipase sequences (Accession numbers: NP_973932, AAL85126, AAK76488, NP_174180, BAC43359, AAA93262). Two degenerate primers LIP1 (5'-GANTTCATTGCN GAATTCTT-3') and LIP2 (5'-CCGNATTTNGCTGGTTCTTG-3') were used to amplify the core sequence by the following procedures: the template was denatured at 95°C for 5 min, then

amplified by 30 cycles of amplification (1 min at 95°C, 1 min at 55°C and 1 min at 72°C) and finally by extension at 72°C for 10 min. The PCR product was purified, sub-cloned into pMD18-T vector (Takara) and sequenced.

The 3' RACE of the lipase cDNA from rapeseed was performed with the 3' RACE kit (Invitrogen, CA, USA). The first-strand cDNA was synthesized from 1 μg of total RNA using a cDNA synthesis primer AP (5'-GGCCACGCGTCGACTAGTAC$(T)_{16}$-3'). Utilizing primer LIP3 (5'-CAAACAAGGAAGCATACGAC-3') and the abridged universal amplification primer (AUAP, 5'-GGCCACG CGTCGACTAGTAC-3'), 3'-RACE was performed in a total volume of 50 μl containing 2 μl cDNA, 10 pmol each of primer LIP3 and AUAP, 10 μmol dNTPs, 5 μl 10 × cDNA reaction buffer and 5U *Taq* polymerase. PCR reaction was carried out under the following conditions: 94°C for 5 min, 35 cycles of amplification (1 min at 95°C, 1 min at 55°C and 1 min at 72°C), and at 72°C for 10 min. The PCR product was purified and sub-cloned into pMD18-T vector followed by sequencing.

Based on the result of assembling the core sequence and the sequence of 3' RACE PCR product, two gene specific primers LIP4 (5'-GGCTCAAGTGCCGTTGCTCC-3') and LIP5 (5'-CAAGCGG AAACCCCAAGAAT-3') were synthesized and subsequently used to amplify the 5'-end of lipase gene. The 5’ RACE was performed with the 5' RACE kit (Invitrogen, CA, USA). The first-strand cDNA for 5' RACE was synthesized followed by tailing cDNA with oligo (C). The first round of PCR was performed with LIP4 and Abridged Anchor Primer (AAP, 5'-GGCCACGCGTCGACTA GTACGGGIIGGGIIGGGIIG-3'). PCR was carried out by denaturing cDNA at 94°C for 5 min followed by 35 cycles of amplification (1 min at 95°C, 1 min at 55°C and 1 min at 72°C) and by extension at 72°C for 10 min. The PCR product was diluted 50-fold for nested PCR with the second round of amplification with LIP5 and AUAP. Under the same condition described in the first round of PCR, the 5' RACE product was purified and sub-cloned into pMD18-T vector followed by sequencing.

Sequence analysis. Sequence alignments, open reading frame (ORF) translation and molecular mass calculation of predicted protein were carried out with Vector NTI Suite 8 and DNAStar. BLAST and domain prediction were done at the NCBI server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and EBI server (http://www.ebi.ac.uk/InterProScan/), respectively.

Genomic DNA Southern blotting. Aliquots of DNA samples (20 μg/sample) were digested with *Hin*dIII, *Sac*I and *Xba*I endonucleases respectively, separated by 0.8% agarose gel electrophoresis and blotted onto a Hybond-N^+ nylon membrane (Amersham Pharmacia). The Gene Images random priming labeling module and Gene Images CDP-Star detection module were used for probe labeling, hybridization and detection procedures (GIBCO-BRL). The full coding sequence (nt20-1141) of *BnLIP2* was used as the probe in Southern blot analysis.

Analysis of the lipase gene expression profile. One microgram of total RNA was used as template for one step RT-PCR (One step RT-PCR Kit, TaKaRa, Japan) with LIP6 (5'-TTCTTTTTATCTACTCT TTTCCTACTCAC-3') and LIP7 (5'-GTTGGTGTAAGCAAAATG AATCCCT-3') as primers. The reaction was performed by reverse transcription at 50°C for 30 min, and then denatured at 95°C for 2 min followed by 25 cycles of amplification (95°C for 1 min, 55°C for 1 min, 72°C for 1 min). The RT-PCR reaction for the housekeeping gene *BnACT* (rapeseed *actin* gene, GenBank Acc. No. AF111812) using specific primers *BnA1* (5'-CGCCGCTTAACCCTAAGGCTA ACAG-3') and *BnA2* (5'-TTCTCTTTAATGTCACGGACGATTT-3') was also performed as an internal control. The PCR products (10 μl) were separated on 1.2% agarose gels stained with ethidium bromide (10 μg/ml).

Chemical treatments. Roots of rapeseed seedlings were submerged in appropriate concentrations of methyl jasmonate (50, 100 μM), salicylic acid (50, 100, 500 μM), naphthalene acetic acid (1, 10 μM) and 0.5% DMSO for 0 h, 3 h, 6 h, 12 h, 18 h, and 24 h, 36 h and 48 h, respectively (Subramanian *et al*., 2004). Then roots were separated from the treated seedlings and immediately used for total RNA extraction without frozen.

Results and discussion

Isolation of the rapeseed GDSL lipase *BnLIP2* cDNA. By the method of RT-PCR a 650 bp cDNA fragment (nt 257-906) was initially amplified as the core sequence which possessed over 80% homology with *A. thaliana* lipase gene, implying that it was probably a partial sequence of lipase gene. By 3' RACE a 578 bp cDNA fragment (nt 723-1300) was obtained with 184 bp overlap with the core sequence, and the 3'-untranslated region was 159 bp with 23 nucleotide-long poly $(A)^+$ tail. 5' RACE PCR generated a 290 bp cDNA fragment (nt 1-290) with 34 bp overlap with the core sequence, and an initiation codon ATG was located in the obtained 5' sequence. By assembling the core sequence, 3' RACE and 5' RACE products, the full-length cDNA sequence with 1300 bp of lipase from rapeseed was obtained. Confirmed by amplifying and sequencing the full-length fragment, this sequence was then submitted to GenBank (GenBank Acc. No. AY866419).

Analyzed by the program of DNAStar, the full-length cDNA was predicted to have an initiation codon ATG at the position of 20 nt, a stop codon TGA at the position of 1139 nt and a polyadenylation signal. An open reading frame (ORF) of 1122 bp was predicted to encode a protein of 373 amino acid residues (Fig. 1), with the predicted isoelectric point (pI) of 5.35 and calculated molecular mass of 41.27 kDa.

Sequence analysis of *BnLIP2* cDNA. Database search showed that *BnLIP2* cDNA shared 63.5% sequence identity with *A. thaliana* putative lipase gene (GenBank Acc. No. NM_102625). The deduced amino acid sequence (Acc. No. AAX59709) shared 77%, 58.4% and 40% sequence homology with lipase and putative GDSL-motif lipase (Acc. No. NP_174179, AAX62802, BAB09319) from *A. thaliana*, and shared a lower homology with lipase-like proteins and GDSL-motif lipases from *Oryza sativa*, *H. brasiliensis* and *M. sativa*. The lipases or lipase-related proteins from other plants, animals and microbes were not found to share remarkable

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1       tctagattctcctccacgg
20      ATGAAGAAGCTCCTTAGCTTCTTTTTATCTACTCTTTTCCTACTCACTGTCGTCAACTCT
1        M  K  K  L  L  S  F  F  L  S  T  L  F  L  L  T  V  V  N  S
80      GAGACGACTTGCCGGAACTTCAAATCGATCATCAGCTTCGGCGACTCCATTGCCGACACT
21       E  T  T  C  R  N  F  K  S  I  I  S  F  G  D  S  I  A  D  T
140     GGAAACTTGCTCGGCCTCTCTGATCCTAACAATCTTCCTAAAGTAGCGTTTCCGCCGTAC
41       G  N  L  L  G  L  S  D  P  N  N  L  P  K  V  A  F  P  P  Y
200     GGAGAAACGTTCTTCCATCATCCCACAGGCCGTTTCTCAAACGGTCGCCTCATCATCGAT
61       G  E  T  F  F  H  H  P  T  G  R  F  S  N  G  R  L  I  I  D
260     TTCATTGCGGAATTCTTGGGGTTTCCGCTTGTACCACCTTTTTACGGATCTCAAAATGCA
81       F  I  A  E  F  L  G  F  P  L  V  P  P  F  Y  G  S  Q  N  A
320     AACTTTGAGAAAGGAGTTAATTTCGCGGTCGGGGGAGCAACGGCACTTGAGCCTTCAGTT
101      N  F  E  K  G  V  N  F  A  V  G  G  A  T  A  L  E  P  S  V
380     CTCGAGGAGAGAGGGATTCATTTTGCTTACACCAACGTCAGTTTAGGAGTTCAGCTCCAG
121      L  E  E  R  G  I  H  F  A  Y  T  N  V  S  L  G  V  Q  L  Q
440     AGCTTCAAGGATAGTTTGCCTAACCTTTGTGGCTCCCCAACAGACTGCAGACATATGATC
141      S  F  K  D  S  L  P  N  L  C  G  S  P  T  D  C  R  H  M  I
500     GAAAATGCTTTAATTCTCATGGGAGAAATTGGAGGGAATGACTATAATTACCCACTCTTT
161      E  N  A  L  I  L  M  G  E  I  G  G  N  D  Y  N  Y  P  L  F
560     CTTGGCAAACCCATTGAAGAGATCAGAGAGCTGGTTCCACTTGTGATCACTACTATCCCT
181      L  G  K  P  I  E  E  I  R  E  L  V  P  L  V  I  T  T  I  P
620     TCTGCAATCACGGAGTTGATTGGTATGGGGGGAAGAACATTTCTCGTGCCAGGAGAGTTC
201      S  A  I  T  E  L  I  G  M  G  G  R  T  F  L  V  P  G  E  F
680     CCGATCGGATGCGCAGTAATCTACTTGACATTATATAAAACACCAAACAAGGAAGCATAC
221      P  I  G  C  A  V  I  Y  L  T  L  Y  K  T  P  N  K  E  A  Y
740     GACTCTTCAGGTTGTTTGAAATGGCTGAACGAGTTTGCAGTATACCACGACGACCAGCTT
241      D  S  S  G  C  L  K  W  L  N  E  F  A  V  Y  H  D  D  Q  L
800     CAGGCAGAACTCAACAAGCTCCGGAGGCTGTACCCTCATGTCAACATCATATACGCAGAC
261      Q  A  E  L  N  K  L  R  R  L  Y  P  H  V  N  I  I  Y  A  D
860     TATTACAACGCTCTGTTACGCCTTTCCCAAGAACCAACCAAATTTGGGTTCATAGACAGA
281      Y  Y  N  A  L  L  R  L  S  Q  E  P  T  K  F  G  F  I  D  R
920     GCGCTTCCTGCTTGCTGCGGTTTTGGTGAGAAGGGGATGGAATGTTGTAGTGGTCCATCA
301      A  L  P  A  C  C  G  F  G  E  K  G  M  E  C  C  S  G  P  S
980     AAGTATGTGAGTTGGGACTCTGTTCATATGACTGAGGCTGCGTACAGATTTATGGCTGAG
321      K  Y  V  S  W  D  S  V  H  M  T  E  A  A  Y  R  F  M  A  E
1040    GGGGTTCTTAAGGGACCCTATGCCATTCCACCTTTTGATTGGTCTTGCCTCAACCCTGAA
341      G  V  L  K  G  P  Y  A  I  P  P  F  D  W  S  C  L  N  P  E
1100    ATTAAGAACAGTGGATCATCTGACACAAAAAGTATCATTTGATGAACCACTGATATAGCT
361      I  K  N  S  G  S  S  D  T  K  S  I  I  *
1160    tggtcttaaaactaaaagatagaaggatggatggatactgataaaaatatgtatgttctt
1220    aagtgcttgtattggttcatgtattgtactaatagacaatttgtattcttgttatggtaa
1280    aaaaaaaaaaaaaaaaaaaa
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Fig. 1. The nucleotide and deduced amino acid sequences of *BnLIP2* from *B. napus* (GenBank Acc. No. AY866419). The start codon (ATG) is underlined in bold and the stop codon (TGA) is in bold italically.

sequence homology with BnLIP2 protein.

The deduced amino acid sequence of the BnLIP2 protein shares the following features: a putative Ser-containing active GDSL-like motif close to the N-terminus (Leu is replaced by Ile^{37}), five consensus sequences FGDSXXDTGN, TGRFSNG RXIIDFI, IGXND, LYXLGARXFXVXGTXPXGCXP and CXNPSXYVXWDGXHXTEAA in BlockI-V, Ser^{36}, Asp^{174} (or Asp^{326}) and His^{329} putatively constituting the catalytic triad SDH (Fig. 2), additionally Gly^{70} or Gly^{75} in Block II and Asn^{173} in Block III potentially acting as catalytic residues. Furthermore, GDSL-lipase domains (PF00657, PS50241) were found in the deduced amino acid sequence (http://www.ebi.ac.uk/InterProScan/). Based on the characteristics possessed by GDSL family of lipases (Upton and Buckley, 1995; Akoh *et al*., 2004) the BnLIP2 was predicted to belong to this family and the isolated *BnLIP2* cDNA putatively encoded a GDSL lipase, which was also supported by the result of phylogenetic analysis (Fig. 3).

Like GXSXG lipases, GDSL lipase family also includes large numbers of candidates. Data mining showed that 87 GDSL lipases (excluding redundant sequences) have been found in plant species especially *Arabidopsis* and rice, and they can be classified into three groups based on their protein sequences (data not shown). It indicates that more than one member of GDSL lipase family generally exists in one species, and possesses the consensus sequence (also named as block). Except for the consensus sequence, remarkable diversity of protein sequences appears among these members. For example, BnLIP2 shares less than 40% amino acid sequence homology with GDSL lipase proteins (Acc. Nos. BAC43003, CAB62065 and AAC98006) from rapeseed. In addition, the exact location of active residue Asp has not been exactly confirmed. Upton and Buckley (1995) reported that it located in Block III, while Akoh *et al.* (2004) predicted the active Asp in Block V. Furthermore, there are no published experimental evidences to support such predictions. So either Ser^{36}-Asp^{174}-His^{329} or Ser^{36}-Asp^{326}-His^{329} is potentially the catalytic triad of BnLIP2, though it should be further identified.

Southern blotting analysis. Southern blot was carried out by digesting rapeseed genomic DNA with *Hin*dIII, *Sac*I and *Xba*I respectively, followed by hybridization with the full ORF sequence of *BnLIP2* as the probe under a high stringency condition. A single band was detected in the *Hin*dIII and *Xba*I-digesting lanes, respectively, and two bands were detected in *Sac*I-digesting lane (Fig. 4). This result indicated that *BnLIP2* belonged to a small gene family.

Gene expression analysis. To investigate expression pattern of *BnLIP2* at different stages, total RNAs (1 μg/sample) (digested by RNase-free DNaseI) of the etiolated rapeseed seedlings samples and different tissue samples from mature seedlings were used as templates to detect *BnLIP2* transcript level by one-step RT-PCR analysis. The transcript level gradually increased during three to six days after germination (Fig. 5A). *BnLIP2* expression in most tissues or organs of the mature seedlings was detected, while its transcript was deficient in roots of mature seedlings (Fig. 5B), which was the same as that in roots of juvenile seedlings (data not shown). In the untreated (0 DAG), water-soaked (1 DAG) and germinated (2 DAG) samples, the transcripts of *BnLIP2* could not be detected (data not shown). Subsequently *BnLIP2* expression maintained stable until 5 DAG, and it greatly increased and reached a peak at 6 DAG. In mature seedling tissues the transcript levels of *BnLIP2* were different, and besides no detectable expression appeared in root. Exactly *BnLIP2* was expressed much more intensively in buds than that in other tissues or organs. The amounts and levels of *BnACT* transcripts were stable and detectable in all of the tested samples.

The above analysis by RT-PCR suggests that *BnLIP2* shows tissue or organ-specific expression. In dormant seeds and during the earlier stages of germination physiological activities are comparatively slow, and therefore a low level of lipid degradation is enough to sustain such status. During this period little or weak expression of *BnLIP2* is understandable. Afterwards more active degradation of lipid occurs accompanied

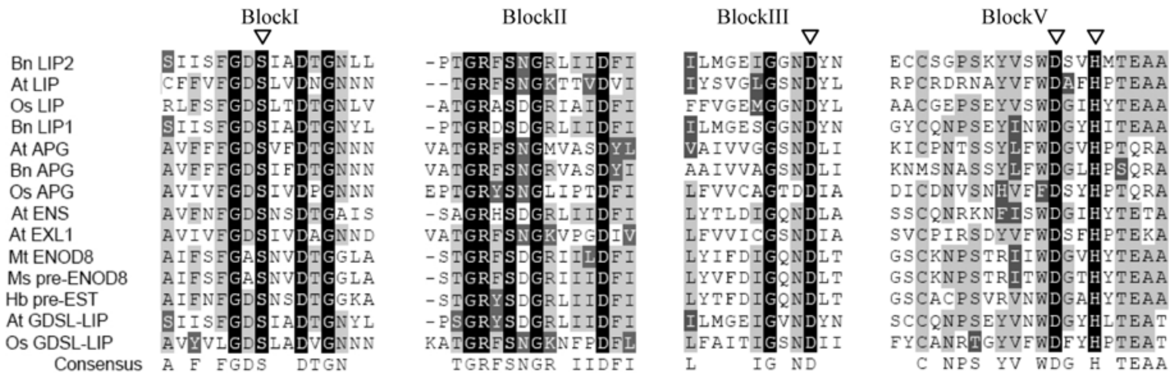

Fig. 2. Multiple amino acid sequence alignment of four blocks located in BnLIP2 with the other homologous plant lipases deposited in NCBI databases (http://www.ncbi.nlm.nih.gov/). The aligned proteins are AtLIP (*Arabidopsis thaliana* AAG51758), OsLIP (*Oryza sativa* AAM22723), BnLIP1 (*B. napus* lipase, AAX62802), AtAPG (P40602), BnAPG (P40603), OsAPG (AAP44751), AtENS (NP_189434), AtEXL1 (NP_974149), MtENOD8 (*Medicago truncatula* ENOD8, T52338), Mspre-ENOD8 (*Medicago sativa* pre-ENOD8, S59943), Hbpre-EST (*Hevea brasiliensis* lipase/ersterase precursor, Q7Y1X1), AtGDSL-LIP (NP_174185), OsGDSL-LIP (AAP05801). The amino acids (Ser^{36} in Block I, Asp^{174} in Block III/Asp^{326} in Block V, His^{329} in Block V) 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.

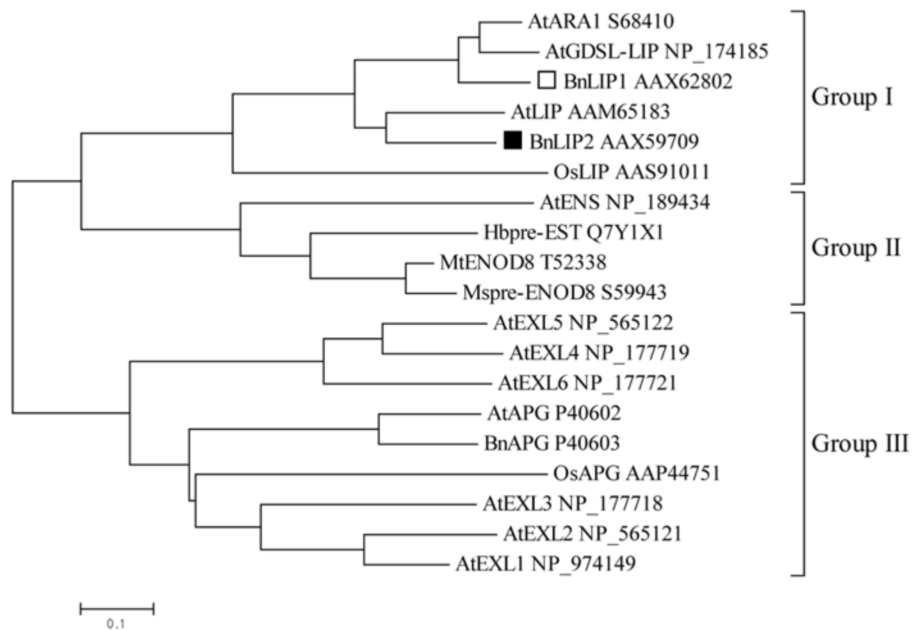

Fig. 3. Phylogenetic relationships of the lipases in plants. The analysis is performed with Clustal X and MEGA version 3.0, using Neighbor-Joining method. BnLIP2 is marked with a black filled box, and its homologous protein BnLIP1 (AAX62802) is marked with a blank box. Classification of lipases is shown at the right side. MtENO8, early nodule-specific protein 8 from *Medicago truncatula*; Mspre-ENOD8, ENOD8 precursor from *Medicago sativa*.

by rapid translation of lipases such as BnLIP2. Actually its transcription after 6 DAG is not detected, so expression at 6 DAG is indefinitely the highest. In mature seedlings transcription takes place in most tissues or organs except roots. And it can be primarily considered as a gene that is concerned with the growth and development of mature seedling tissues or organs excluding root, and involved in seed germination as well. It looks like that *BnLIP2* is a lipolytic enzyme gene with multiple physiological roles.

The exact expression pattern of *BnLIP2* differs from other GDSL lipases from plant species (Brick *et al*., 1995; Mayfield *et al*., 2001; Akoh *et al*., 2004). A GDSL lipase gene *Arab-1* from *A. thaliana* is only expressed in the etiolated shoots. Transcription from *A. thaliana EXL1* and *EXL3-6* was detected in flower buds. Another GDSL lipase was isolated from post-germinated sunflower (*H. annuus* L.) seeds, and in legume species many GDSL lipase genes were predominantly expressed in the developing/mature root nodule. Especially,

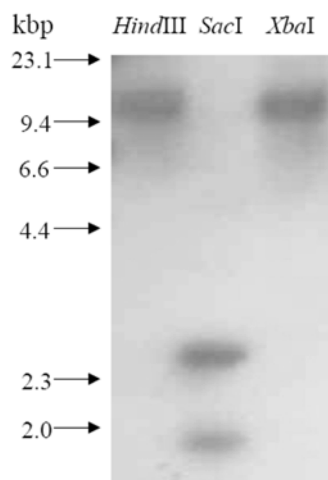

Fig. 4. Southern blot analysis of *BnLIP2*. The genomic DNA is digested with *Hin*dIII (lane 1), *Sac*I (lane 2) and *Xba*I (lane 3) respectively, followed by hybridization with the full coding sequence (nt 20-1141) of *BnLIP2* as the probe.

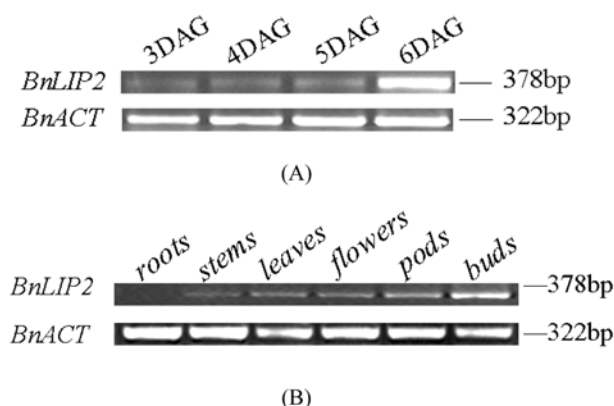

Fig. 5. RT-PCR analysis of *BnLIP2* expression. (A) *BnLIP2* expression profile during post-germinative stage. The total RNAs used as the templates (1 μg/sample) for RT-PCR are isolated from the germinated seedlings of 3 DAG, 4 DAG, 5 DAG and 6 DAG, respectively. (B) *BnLIP2* expression profile during reproductive stage. The total RNAs used as the templates (1 μg/sample) for RT-PCR are isolated from the root, stem, leaf, flower, pod and bud, respectively. Expression of *actin* gene in *B. napus* (*BnACT*) was used as an internal control. The amplified fragments of *BnLIP2* and *BnACT* are 378 bp (nt 38-415) and 322 bp (nt 380-701), respectively.

we find that another GDSL lipase gene *BnLIP1* (GenBank Acc. No. AY870270) from rapeseed expressed in all the tissues and organs of juvenile and mature seedlings, which is a little similar to *BnLIP2* (data not shown). Obviously the key difference of expression pattern between them is the transcription level in root. Transcripts of *BnLIP1* are present in root, while *BnLIP2* is deficient. That indicates that they play different physiological roles, exactly *BnLIP2* is independent of rapeseed root growth and development. The discrimination of expression is probably caused by precise regulation of 5'-upstream region, which likely contains some important *cis*-acting regulatory elements, or some other factors regulating the transcription.

***BnLIP2* is a root-deficient gene in *B. napus*.** In order to detect whether expression of *BnLIP2* was induced by chemicals in root or not, SA, NAA and MeJ were used to treat juvenile seedlings at different concentrations for appropriate time. As a result, analyzed by semi-quantitative one-step RT-PCR, none expression could be detected in all the root samples, while the expression of actin gene was detectable and stable (data not shown). This suggests that *BnLIP2* could not be induced in the roots of juvenile seedlings when treated by these chemicals at appropriate concentrations.

Undetectable expression of *BnLIP2* gene in normal and treated roots of seedlings indicates that *BnLIP2* is a root-deficient gene in rapeseed, and it is also independent of root growth and development. A *Medicago* nodule-specific gene *Enod8*-encoded protein was not found in the roots (Pringle *et al*., 2004). This property of root-deficiency is potentially caused by DNA methylation in the 5'-flanking region of *BnLIP2* gene, transcription inactivator-mediated silencing, or some transcription activators which are specifically deficient in root. Klëti *et al.* (2002) reported tissue-specific methylation of promoter region (Klëti *et al*., 2002). In fact, the exact molecular mechanism of silencing in root is not clear and should be further understood through 5'-flanking region cloning, demethylation analysis and deletion analysis of promoter specificity elements.

In conclusion, we isolated and analyzed rapeseed GDSL lipase gene *BnLIP2*. Our results revealed that *BnLIP2* was root deficient, and expressed in germinated seedlings and mature seedlings. *BnLIP2* may play multiple roles in plant physiological activities such as germination, flowering, morphogenesis, but is not involved in root growth and development. To further understand this gene, the analysis of its promoter region, over-expression *in vivo* and lipolytic activity is undergoing.

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