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Full Length Research Paper

Molecular cloning and characterization of a chitinase gene up-regulated in longan buds during flowering reversion

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A cDNA-amplified fragment length polymorphism (cDNA-AFLP) technique was used for differential screening of genes expressed in longan (*Dimocarpus longan* Lour.) flower buds undergoing normal development versus flowering reversion. One cDNA fragment up-regulated during flowering reversion was further cloned by rapid amplification of cDNA ends (RACE) technology. This cDNA consists of 961 nucleotides and encodes an open reading frame (ORF) of 227-amino acid residues. The nucleotides and deduced amino acid sequence were both identical against published chitinases from other species and hence this cDNA was designated as DLchi (GenBank accession No. GU177464). It has a signal peptide and glycoside hydrolase's domain. The estimated molecular weight was 24.77 kD and the isoelectric point was 5.17. This protein might be grouped as a new member of class II chitinase based on the sequences available and hypothesis discussed. DLchi might be involved in the flower bud abscission observed in longan flowering reversion.

Key words: Longan, flowering reversion, chitinase gene, cloning, sequence analysis.

INTRODUCTION

Longan (Dimocarpus longan Lour.), a tree with edible fruit, is distributed widely in tropical and subtropical regions. When longan flowering reversion occurs, flower buds cease normal development and instead form floral spikes with leaves. The floral spikes gradually shed, which result in decrease in longan fruit productivity (Chen et al., 2009).

Recently, some researchers have shown that chitinase may be involved in abscission of leaves, buds, floral organs, etc (Patterson, 2001). For example, Campillo and Lewis (1992) reported that basic chitinases accumulated to high levels in abscission zones and they serologically identified a related 33 kD protein in bean anthers and pistils during flower abscission. Coupe et al. (1997) screened two chitinases, Chia1 and Chia4, which were up-regulated in the leaflet abscission zone of *Sambucus nigra*. Four different chitinase transcripts were also identified during abscission of citrus leaves and apple fruits (Agusti et al., 2008; Zhou et al., 2008). Two chitinase genes were up-regulated in the abscission zone at 2 h after flower removal and remained highly expressed during 14 h, while in the non-abscission zone, their observed increase of expression was only transient and peaked at 2 h after flower removal and the class II chitinase was suggested as abscission zone specific gene (Meir et al., 2010). In addition, a wound-inducible class I acidic chitinase gene, win6, was reported in young undamaged poplar leaves, while a sharp increase,

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Abbreviation: cDNA-AFLP, cDNA-amplified fragment length polymorphism; **RT-PCR,** reverse transcriptase PCR; **RACE,** rapid amplification of cDNA ends; **EDTA,** ethylenediaminetertracetic acid; **DEPC,** diethyl pyrocarbonate; **ORF,** open reading frame.

predominantly in pollen, coincided with anther dehiscence in flowers (Clarke et al., 1994). An unexpected abundance (23%) of chitinase genes was found in the libraries of senescing petals of wallflower (*Erysimum linifolium*) (Price et al., 2008). Evidently, abscission involves the dissolving of cell walls or cell separation, so that dehiscence and senescence are similar processes involving cell wall disruption through the action of chitinases (Roberts et al., 2002; Lewis et al., 2006).

We identified a chitinase fragment that is up-regulated during flowering reversion of longan buds with cDNA-AFLP technique. Semi-quantitative reverse transcriptase PCR (RT-PCR) was used for further validation of these results. We proposed that this chitinase may be involved in longan flower bud abscission. To study its function in detail, we cloned the complete DLchi cDNA sequence from the screened gene using the rapid amplification of cDNA ends (RACE) method and characterized the sequence by a bioinformatics program.

MATERIALS AND METHODS

A 'Longyou' cultivar of longan (*D. longan* Lour.) growing in the orchard of the Putian Research Institute of Agricultural Sciences, Fujian Province, China was used in this study. Samples of both normal and reversion flowering buds were collected every week from March 01 to April 05, 2008. Most of the buds were immediately immersed in liquid N_2 and then stored at -80°C for later analysis.

Total RNA extraction from flower buds

1 g sample of longan flower bud tissue was ground into a fine powder in a pre-cooled pestle and mortar under liquid N_2. The powder was transferred into a centrifuge tube containing pre-heated (65°C) TB buffer (150 mM Tris Base), 575 mM H_3BO_3, 50 mM ethylenediaminetertracetic acid (EDTA) (pH 8.0), 0.5 M NaCl, 4% SDS), β-mercaptoethanol, 100% ethanol and potassium acetate (KAc) (5 M, pH 4.8) and mixed on a vortex mixer. An equal volume of chloroform : isoamyl alcohol (24:1) mixture was then added to extract the RNA. This procedure was repeated three times. The upper aqueous phases were pooled and then successively precipitated with 400 µl 9 M LiCl and 1 ml 100% ethanol at -20°C. After centrifuging, the precipitate was washed twice with 70% ethanol and dried at room temperature. The total RNA was re-suspended in diethyl pyrocarbonate (DEPC)-treated water and stored at -80°C. The RNA purity and integrity were checked by ensuring that absorbance ratios (A260/280) were between 1.8 and 2.0 and by agarose gel electrophoresis (1%).

cDNA-AFLP analysis

cDNA-AFLP was performed with normal and reversion flowering buds as described by Bachem et al. (1998). The double-stranded cDNA was synthesized using the SMART™ PCR cDNA Synthesis Kit (Clontech, USA). A 200 ng sample of each ds-cDNA was digested with *Eco*RI and *Mse*I enzymes and ligated to corresponding adapters. The sequences of the adapter were as follows: *Eco*RI up-stream adapter: 5'-CTCGTAGACTGCGTACC-3', *Eco*RI down-stream adapter: 5'-AATTGGTACGCAGTCTAC-3'; *Mse*I upstream adapter: 5'-GACGATGAGTCCTGAG-3', *Mse*I down-stream adapter: 5'-TACTCAGGACTCAT-3'. Pre-amplification and selective amplification were performed according to the protocol provided with the AFLP Kit (Dingguo, China). The primer sequences for pre-amplification were as follows: up-stream primer: 5'-GACTGCGTACCAATTCA-3'; downstream primer: 5'-GATGAGT CCTGAGTAAC-3', selective primers: up-stream: 5'-GACTGCGTA CCAATTCAAC-3'; down-stream: 5'-GATGAGTCCTGAGTAACAG-3'. PCR products were identified on a 6% polyacrylamide gel run at 70 W run until the bromophenol blue reached the bottom of the gel. Bands were then displayed by silver staining.

Cloning and sequence analysis of full-length DLchi cDNA

The target DNA fragment separated into polymorphic bands was cut and re-amplified with the same primer combinations as those used for selective amplification. After checking the amplified DNAs by 1.2% (w/v) agarose gel electrophoresis, these were cloned into a pMD18-T vector (Takara, China) and sequenced using the universal M13 and RVm-14 primers.

The flanking 5' and 3'-regions were obtained using the rapid amplifications of cDNA ends (SMART™ RACE cDNA Amplification Kit, Clontech). A pair of gene-specific primers was designed based on the sequence of the fragment screened by cDNA-AFLP. The forward primer was 5'-CTATTCGGAAGATCAATGGTGCTG-3' and the reverse primer was 5'-GAACCACAAGGCCGT CTTGAAGGCGATGG-3'. The open reading frame (ORF) was detected by DNAstar software. A similar sequence to the cDNA and its putative amino acid sequence were verified by database searching at the National Center for Biotechnology Information server using the BLAST algorithm. Multiple alignments and a phylogenetic tree were constructed using DNAMAN 2.0 software. Amino acid sequence analysis was conducted with tools available at the Expert Protein Analysis System (ExPASy).

Semi-quantitative RT-PCR analysis

2 µg sample of total RNA was used for RT-PCR with the ReverAid™ First Strand cDNA Synthesis Kit (Fermentas, Germany), according to the manufacturer's instructions. Gene-specific primers were as follows: forward primer: 5'-ATGGCCATGTTCAACTT-3' and reverse primer: 5'-TCAACAGGACAGATTCTC-3'. DLchi was amplified by 94°C for 2 min, 30 cycles of 94°C for 30 s, 44°C for 30 s and 72°C for 1 min. The longan actin gene (accession No. EU340557) was used as internal standard to normalize the amount of templates. The forward primer was 5'-TGAGGGATGCTAAGATGG-3' and the reverse primer was 5'-ATGAGTTGCCTGATGGAC-3'. All RT-PCR expression assays were performed and analyzed at least three times in independent experiments. Analysis of expression in the gel bands was performed using the Band leader software.

RESULTS

Isolation and molecular characterization of the DLchi gene

The cDNAs that were differentially expressed in normal and reversing flower buds were identified with cDNA-AFLP. One chitinase cDNA fragment was found and its up-regulation during flowering reversion was confirmed by RT-PCR (Figure 1). The full-length cDNA was completed by assembling the known partial fragment, 3' and 5' end sequences and was submitted to GenBank

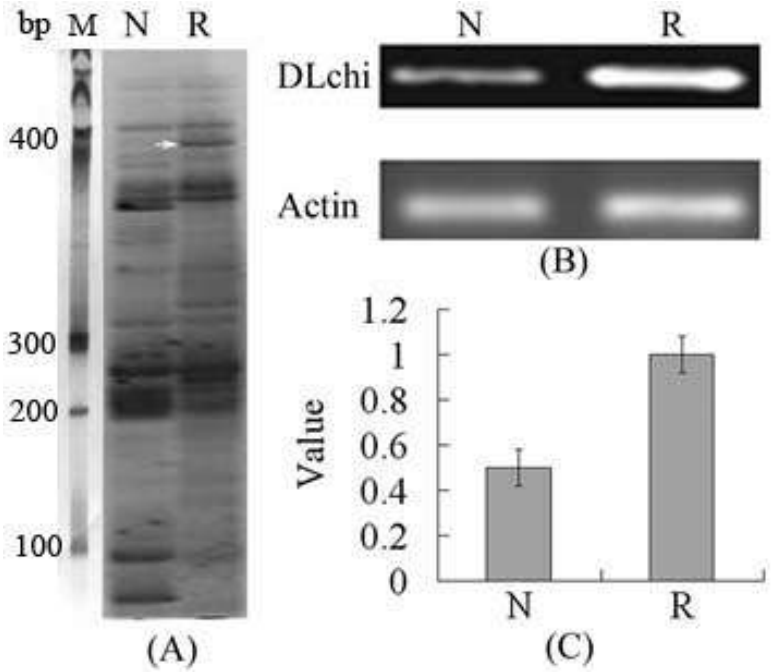

Figure 1. Isolation of a differentially expressed fragment of DLchi from reversion flower buds. A, Detection of DLchi expression using cDNA-AFLP technique from corresponding buds; M, 100 bp marker; N, normal flower buds; R, reversion flower buds. The arrow indicates the band corresponding to DLchi; B, semi-quantitative RT-PCR products were analyzed by agarose gel electrophoresis; C, relative expression profile of DLchi by Band leader software analysis. Actin was used as a standard.

(accession No. GU177464).

Comparison of the sequence with NCBI nucleotide (nt) databases revealed a close identity with the complete mRNA of several chitinases. Hence, this clone was named DLchi (*D. longan* chitinase). The known partial fragment covers 381 nt from position 367 to 626 in the full length, while the upstream 367 nt were the result of 5' RACE and the downstream 626 nt were the result of 3' RACE. The complete nucleotide sequence covers 961 bp with an open reading frame (ORF) of 684 bp, capable of encoding 227-amino-acid residues. An ATG initiation codon was found in 59 nt (5'-UTR) downstream of the 5'-start and a TGA stop codon is present in 219 nt (3'-UTR) upstream of the 3'-end. The 3'-UTR contain one AATAA motif, representing putative polyadenylation signals and 21bp polyadenylation (Figure 2). The first Met is probably the real translation initiation site since it is embedded within a sequence that conforms to the consensus for the optimal context of eukaryotic translation initiation, as defined by the motif GCC (G or A) CCAUGG (Kozak, 1991). The two most important positions in this motif- the purine at position -3 and the last G at position +4- were conserved.

The DLchi protein had a calculated molecular mass of 24.77 kD and isoelectric point (pI) of 5.17. The protein is hydrophilic with a grand average hydropathicity (GRAVY) value of -0.127. The N-terminal 25 amino acids exhibited the characteristics of a signal peptide with a highly hydrophobic core and the characteristic amino acid composition near the cleavage site (Von Heijne, 1983), with the most likely cleavage site been between S_{25} and Q_{26}. This protein showed no transmembrane signal in TMHMM analysis suggesting that it is secreted into the cytoplasm. Pfam analysis revealed that the DLchi protein has the catalytic domain of the family 19 chitinases, placing it as a member of the family 19 glycosyl hydrolases. Two chitinase family 19 signatures were found at Cys_{48} to Gly_{70} (CAGKSFYTRDGFLSAANSYAEFG) and I_{161} to M_{171} (IAFKTALWFWM). A conserved motif (NYNYG), essential to hydrolytic activity (Verburg et al., 1993), was found in the catalytic domain at position Asn_{134} to Gly_{138}. Scanning the PROSITE database also revealed a potential N-linked glycosylation site (NLSC) at the C terminal region (Asn_{224} to Cys_{227}), and one possible protein kinase C phosphorylation site [Ser_{77} to Arg_{79} (SKR)], four possible casein kinase II phosphorylation sites [Ser_{65} to Glu_{68} (SYAE), Ser_{73} to Asp_{76} (SADD), Ser_{77} to Glu_{80} (SKRE), Ser_{220} to Glu_{223} (SPGE)], four possible N-myristoylation sites [Gly_{70} to Asp_{75} (GSGSAD), Gly_{141} to Phe_{146} (GQAIGF), Gly_{191} to Gly_{196} (GAVECG), Gly_{218} to Glu_{223}(GVSPGE)], and one possible ATP/GTP-binding

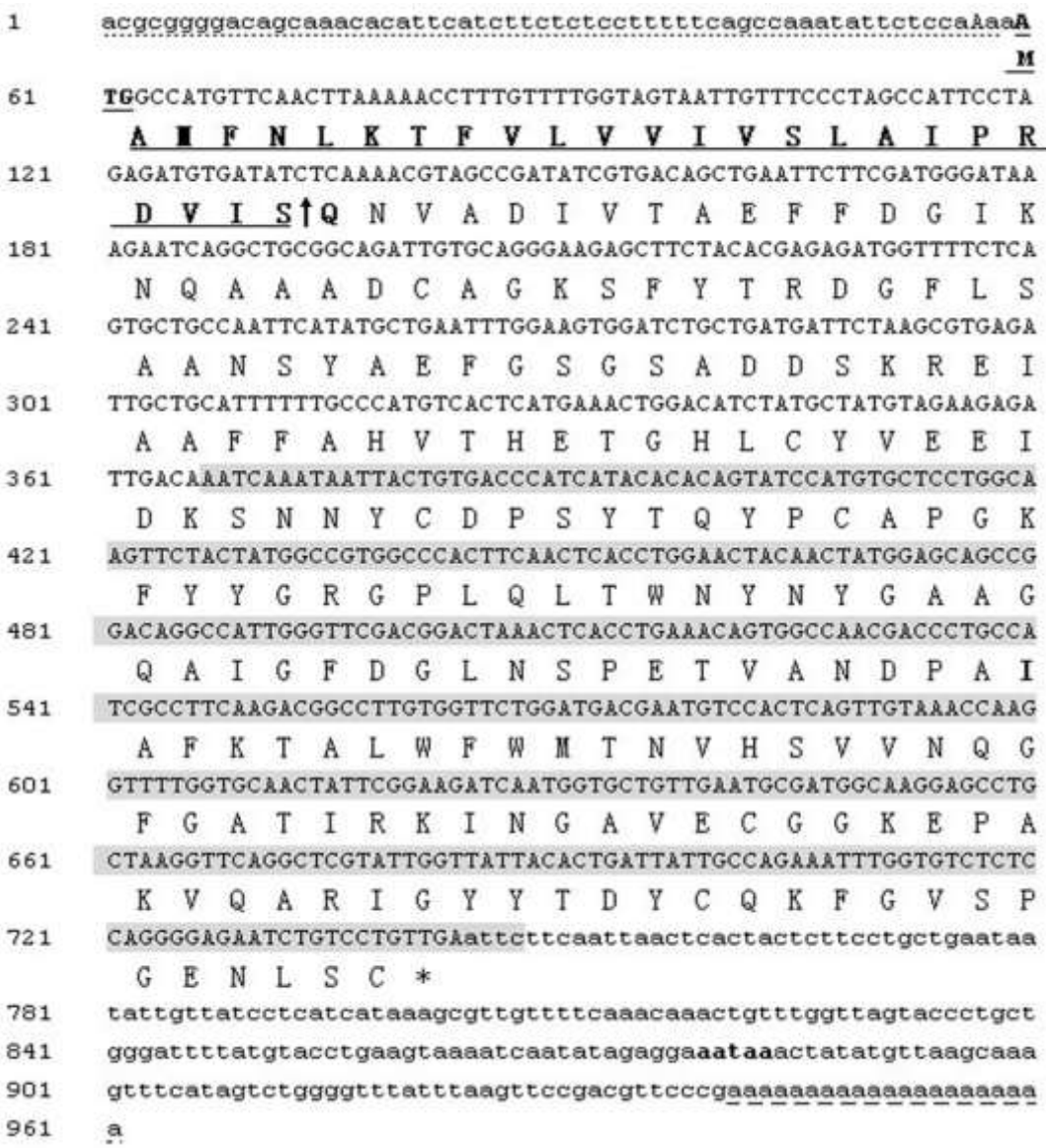

Figure 2. The full-length cDNA and deduced amino acid sequence of DLchi. The grey base indicates the cDNA fragment from cDNA-AFLP. The start codon (ATG) is underlined and an asterisk represents a termination codon. The 59 bp 5'-UTR leader sequence and the polyadenylation signal are underlined with - - - and_ _ _, respectively. The 684 bp open reading frame (from 60 to 743 bp as shown in capital letters) encodes a 227-amino acid DLchi precursor with a signal peptide of 25 amino acids. The arrow indicates the cleavage site of signal peptide between S_{25} and Q_{26}.

site motif A (P-loop) [Ala_{45} to Ser_{52} (AADCAGKS)]. These structural features suggest that this protein might have substrate affinity and enzyme activity similar to those of other plant chitinases.

Comparison of DLchi putative amino acid sequence and phylogenetic analysis

Comparison with NCBI protein databases showed that DLchi had a different identity to a number of other plant chitinases, including that of *Citrus sinensis* 1 (74%), *Pyrus pyrifolia* (71%), *Galega orientalis* (69%), *Medicago sativa* (67%), *Vitis vinifera* (68%), *Arabidopsis thaliana* (67%), *Zea mays* (65%), and *Phaseolus vulgaris* (63%), which (except for *C. sinensis* chitinase) are all of class IV chitinases. The identical region was mainly localized in the catalytic domain (Figure 3). Despite similar sequence correspondence in the catalytic region, DLchi differs from class IV chitinases by its absence of an N-terminal cysteine-rich domain. Hence, DLchi is probably a class II chitinase. Four kinds of class II chitinases were chosen

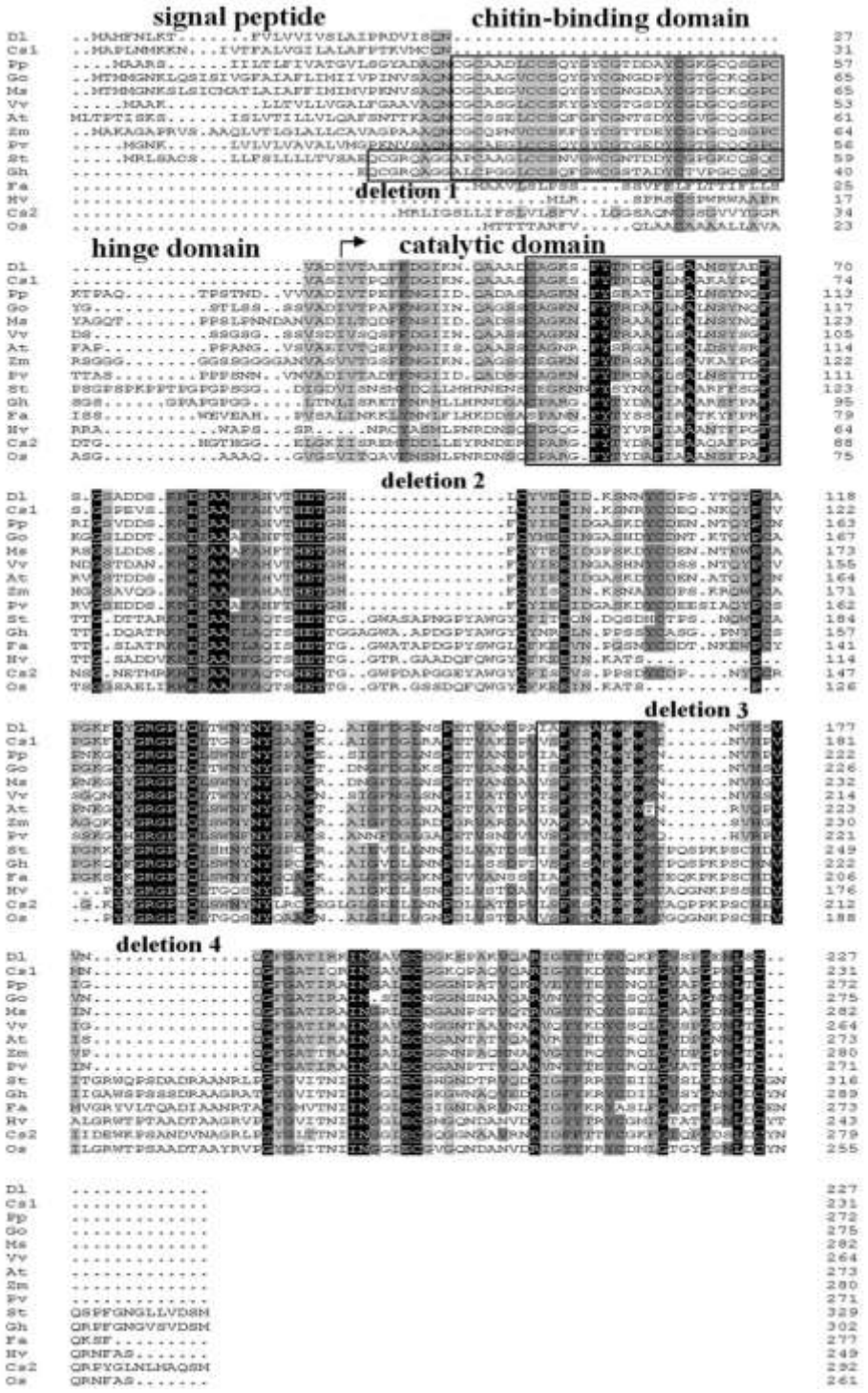

Figure 3. Alignment of amino acid sequences of DLchi with representatives of plant chitinases of class I, II and IV. Class I: St, *S. tuberosum* (AF153195.1), Gh, *G. hirsutum* (AF034566.1); Class II: Cs1, *C. sinensis* 1 (AF090336.1), Fa, *F. ananassa* (EF593027.1), Hv, *H. vulgare* (AJ276226.1), Cs2, *C. sinensis* 2 (Z70032.1), Os, *O. sativa* (L40336.1); Class IV: Pp, *P. pyrifolia* (FJ589786.1), Go, *G. orientalis* (AY253984.1), Ms, *M. sativa* (FJ487629.1), Vv, *V. vinifera* (U97521.1), At, *A. thaliana* (Y14590.1), Zm, *Z. mays* (EU724261.1), Pv, *P. vulgaris* (X57187.1). The structural domain differences are indicated. The first box represents the chitin-binding domain (cysteine-rich domain); the second box is a typical Chitinase family 19 signature 1, (C-x (4,5)-F-Y-[ST]-x (3)-[FY]-[LIVMF]-x-A-x (3)-[YF]-x (2)-F-[GSA]); the third box represents a chitinase family 19 signature 2, ([LIVM]-[GSA]-F-x-[STAG] (2)-[LIVMFY]-W-[FY]-W-[LIVM]).

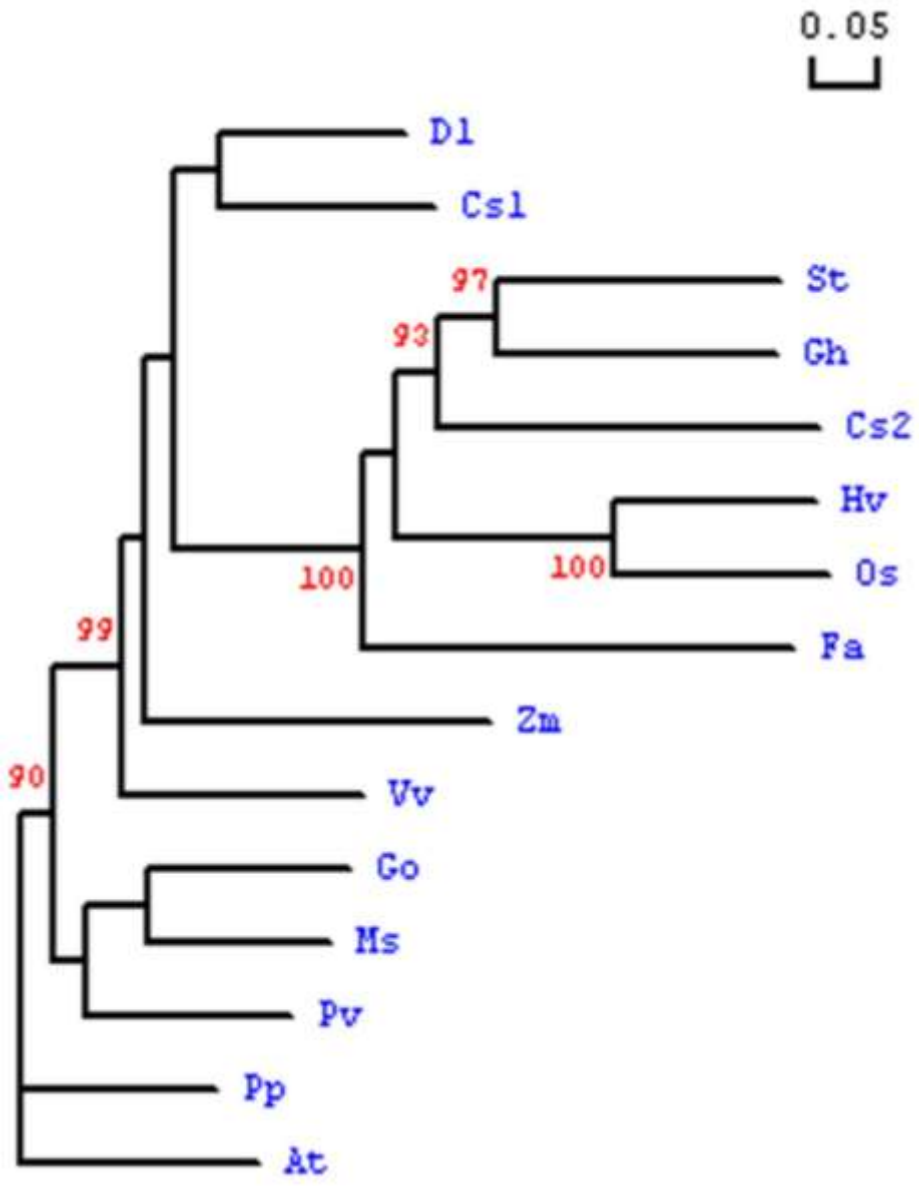

Figure 4. Phylogenetic tree of amino acid sequences of DLchi and chitinases from other species. The numbers on the branches represented bootstrap support for 1000 replicates; the phylogenetic tree was computed using standard parameters.

for further comparison, but we found lower identities with DLchi in class II chitinases from *Fragaria ananassa* (40%), *Hordeum vulgare* (39%), *Citrus sinensis* 2 (38%), and *Oryza sativa* (36%). Three deletions in catalytic domain were in accordance with the difference between class I and class IV chitinases. Two class I chitinases, from *Solanum tuberosum* (37%) and *Gossypium hirsutum* (38%), were also examined. Phylogenetic analysis (Figure 4) showed that chitinase from longan was most closely clustered with one class II (*C. sinensis* 1) chitinase. They formed the first clade, with another cluster including *S. tuberosum, G. hirsutum*, *F. ananassa, H.* vulgare, C. sinensis, O. sativa. DLchi was distantly related to class IV chitinases from *P. pyrifolia, G. orientalis, M. sativa, A. thaliana, P. vulgaris, V. vinifera,* and *Z. mays.* This suggests that DLchi might be closer to class II chitinases from the viewpoint of evolution.

DISCUSSION

We performed cDNA-AFLP to identify genes that were differentially expressed during flowering reversion. The results show that three potential genes were involved in flower reversion, namely, NIMA related protein kinases (Nek1), endo-1,4-beta-D-glucanase precursor and

chitinase (data not shown). Nek1mRNA is thought to be involved in cell cycle with an accumulation of Nek1 mRNA at the G1/S transition and throughout the G2-to-M progression (Cloutier et al., 2005). Nek1 down-expression in flower reversion may interfere with normal mitosis in flower buds. There is also no doubt that endo-1,4-beta-D-glucanase up-regulated expression plays an important role in cell wall separation in plant development (Xie et al., 2011). However, chitinase is usually suggested to be pathogenesis-related protein and exert more anti-fungal activity (Iqbal et al., 2011). The underlying role in plant development is not very clear. Therefore, we paid more attention on chitinase in this study.

The expression of the DLchi cDNA fragment was up-regulated in reversion buds. Analysis of amino acid sequences indicated similarity with class I, II, IV chitinases of other plant species. However, DLchi shared higher identities with class IV, at 63 to 76% similarity, than it did with class I, at 37 to 38%, or class II, at 36 to 40% (except for *C. sinensis* 1, which had a 76% similarity). Although class I, II and IV chitinases are all members of the family 19 glycosyl hydrolases and the sequences in the catalytic regions are highly conserved, they differ in their structural elements. Class I chitinases consist of a chitin-binding domain (CBD) and a catalytic domain (Cat), linked by a variable hinge domain (VHD); class II chitinases are structurally homologous to Cat domain of class I, but lack CBD; class IV chitinases show sequence similarity to class I, but they are smaller due to four deletions (Collinge et al., 1993). When compared with class I, II, and IV enzymes in terms of sequence, DLchi also has more similarity with class IV in structure, exhibiting three deletions in the Cat domain. However, some researchers believe that acid class II chitinase and class IV chitinase genes might have both evolved from class I chitinase genes (Wiweger et al., 2003). This model was also consistent with our phylogenetic tree results. Taken together, we suggestthat DLchi encodes a class II chitinase with the following features: (1) lack of an N-terminal cysteine-rich CBD and VHD, (2) an acidic isoelectric point and (3) a closer cluster to class II chitinase. The class II chitinase seems to be more basically associated with plant development and morphogenesis, especially in flower formation and leaf abscission (Delos Reyes et al., 2001; Meir et al., 2010).

Proscan analysis predicted that DLchi would possess many post-translational modification sites, including an N-linked glycosylation site, a protein kinase C phosphorylation site, a casein kinase II phosphorylation site, N-myristoylation sites, and an ATP/GTP-binding site motif A (P-loop). Although, the significance and exact mechanism of these motifs are not yet clear, they might be involved in signal transduction. As one strong candidate substrate for chitinase, plant arabinogalactan proteins (AGPs) (Showalter, 2001) could be hydrolyzed to generate an oligosaccharide fragment signal molecule that could regulate plant growth and development (Pilling and Höfte, 2003). Chitinase-treated AGPs were able to rescue the temperature-sensitive embryonic development in the carrot tsl1 mutant cell line (Van Hengel et al., 2001). Kim et al (2000) isolated a chitinase-related receptor-like kinase in tobacco and suggested that it might transduce a signal by binding oligosaccharides. These reports raise the possibility that chitinase may influence some physiological process in abscission process by some types of signal transduction mechanism (Stenvik, 2006). The oligosaccharide would then be ligated to a receptor for further transduction of the signal for special physiological process, but its detailed mechanism still needs further investigation. We anticipate further plant transformation in the model Arabidopsis in the coming years based on our analysis.

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