Isolation, characterization and expression of a gene from *Brassica napus* encoding a LIM-domain protein

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Abstract

LIM proteins are important eucaryotic developmental regulators and also involved in the transcriptional control of lignification. In this research a LIM-domain protein (*BnLIM*) was obtained by RT-PCR from seeds of *B. napus*. The deduced BnLIM protein is 189 amino acids long and contains two LIM domains separated. It is highly identical to the LIM-domain protein of *NtLIM1* from *Nicotiana tabacum* L.. A genomic copy of the gene has also been isolated and sequenced; it is split by four short, AT-rich introns. *BnLIM* transcript was detected by RT-PCR in all vegetative and reproductive organs. The expression level of *BnLIM* changed during seed development, which increased in early stage, peaked at 23-29 DAA during seed filling up to a maximal level and reduced slightly 32 DAA. The expression profile of *BnLIM* was highly similar to that of Cinnamoyl-CoA reductase (CCR, EC 1.2.1.44), a key lignin biosynthesis gene. Transient expression of BnLIM-GFP fusion protein in onion (*Allium cepa*) epidermal cells demonstrated that BnLIM exhibited a dual subcellular localization, i.e. in the nucleus and cytoplasm. *BnLIM* mRNA accumulation in response to sucrose treatment was also investigated. All these results proposed that BnLIM plays some roles in lignin biosynthesis and its expression is developmentally regulated.

Key words: *Brassica napus*; LIM-domain protein; expression characterization, lignin biosynthesis, seed development

Introduction

Lignin is an aromatic heteropolymer mainly present in secondary thickened plant cells, where it provides rigidity and impermeability to the cell walls (Rogers, 2004). Lignin is one of the most abundant biopolymers on the earth, and represented a significant carbon sink in the biosphere (Amthor, 2003; Boudet, 2003). The seeds of canola contained 8.6% DM lignin (Slominski, 1999). Due to the existence of shared precursors of fatty acid with lignin and pigment biosynthesis, the lower lignin content yellow rapeseeds exhibit an increased oil and/or protein content in comparison to black seeds (Rahman. 2001). Current data suggest that the genes encoding lignin biosynthetic enzymes are likely to be under some forms of coordinate control (Harmer, 2000). NtLIM1, a member of the LIM family of transcriptional regulators, has the capacity to regulate the expression of some lignin biosynthetic genes (Kawaoka, 2000). NtLIM1 has a specific DNA-binding ability to an AC element in the upstream region of the genes encoding lignin biosynthetic enzymes. The transgenic tobacco plants with antisense NtLIM1 showed lower levels of transcripts of some key phenylpropanoid pathway genes and more than 20% reduction of lignin content.

The results of microarray analysis of developing *Arabidopsis* seeds indicated that expression level of some lignin biosynthesis related genes changed more than 2-fold during seed development (Ruuska, 2002). However, neither any isolation of LIM-domain containing proteins homology from *Brassica*, nor its expression in plant seeds had been reported. Here, we present our studies on isolation of such a cDNA encoding a LIM-domain protein from developing seeds of rape and its expression characters.

Material and methods

Plant material: For analysis of gene expression in various tissues, cauline leaves, stems and roots were collected from 10-week-old plants. Cotyledons were taken from 10-day-old seedlings. Silique walls were taken from 20 days after anthesis (DAA) siliques. To harvest seeds at different development stages, individual flowers were tagged on the day of flowering. Only primary shoots were used. The seeds were collected at nine different stages from 8DAA to 32DAA.

Cloning of cDNA and *genomic DNA sequence of B. napus Lim-domain containing protein:* Genomic DNA was extracted using the CTAB method. Total RNA was isolated using TRIzol reagent (Invitrogen). The NCBI EST database was screened using the mRNA sequence of *NtWLIM1*. Several ESTs and genomic sequences were identified and used to design primers. Sequence of the forward primer named BnLIMF is: 5′-CAA ATG GCG TTC GCA GGA AC-3′, and sequence of the reverse primer named BnLIMR is: 5′-CTT CAG GGT CAA AAT GAA CTG-3′. The first strand cDNA was synthesized using 2μg total RNA from 20DAA seeds, oligo-d(T)$_{18}$ primer, 18S rRNA reverse primer P18SR (5′ –AAG AAG CTG GCC GCG AAG

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GGA TAC-3′). This reverse transcription product was used as template. RT-PCR reaction products were cloned into pMD18-T vector (TaKaRa). Genomic PCR was performed using genomic DNA of *B.napus* with the primer set identical to that used for cDNA sequence cloning. The positive cDNA and genomic DNA clones were sequenced in the Shanghai Sangon Sequencing Center .

Semiquantitative RT-PCR analyses: Semiquantitative RT-PCR analyses were employed to study *BnLIM* transcription levels in various tissues. Gene specific primers, BnLIMF and BnLIMR were used for RT-PCR, which were performed using total RNA isolated from various tissues. The expression profiling of a key lignin biosynthesis gene *CCR* in *B. napus* had also been analyzed and the amplification primers were CCR5′ (5′-ATG AGC TCG CTG GTG GAT ACA TCG CTT CT-3′) and CCR3′ (5′-ATG AGC TCA GGA GAA GCC GTG TGA AAG AC-3′). cDNAs used for semiquantitative RT-PCR were normalized based on the intensity of PCR-amplified 18S rRNA fragments obtained by using primers P18SF (5′-CAT GGC CGT TCT TAG TTG GTG GAG-3′) and P18SR (5′–AAG AAG CTG GCC GCG AAG GGA TAC-3′). For sucrose treatments, 12-d-old seedlings grown on 1×MS basal salt mixture supplemented with B5 Vitamins were transferred to 1×MS media with 2% sucrose treated for 6h and 12 h separately.

Subcellular localization of BnLIM-GFP fusion protein: The GFP report gene was amplified with gene-specific primers GFPF-XhoI-NcoI(5′-CTC GAG CCA TGG TGA GCA AGG GCG A-3′) and GFPR-BamHI(5′-TTA GGA TCC CTT GTA CAG CTC GTC CA-3′) from the Plasmid pEGFP-CI (Clontech, Palo Alto, CA). The resulting DNA fragments were digested with restriction enzymes of *XhoI* and *BamHI* and ligated with the plasmid pFGP5941 precut with the same restriction enzymes. The generated plasmid pGFP-FGC5941 containing a 35S:: GFP-T_{OCS} construction. The *BnLIM* coding cDNA fragment without the stop codon were amplified with gene-spesific primer pairs BnLIMF-cDNA-XhoI (5′-TGC TCG AGA TGG CGT TCG CAG GAA C-3′)/BnLIMF-cDNA-NcoI (5′-CAC CAT GGC AGC GTC CAC TTT GTC CTT-3′). The resulting DNA fragments were subcloned into the pGFP-FGC5941 before the start codon of the GFP coding sequence, resulting a 35S::BnLIM-GFP-T_{OCS} construct. The plasmid with the BnLIM-GFP gene fusions was introduced into onion (*Allium cepa*) epidermal cells by particle bombardment. After incubation on MS medium for 24 h, the onion cells were subjected to fluorescence microscopy analysis.

Results and discussion

Isolation of a Brassica napus cDNA Encoding a LIM-domain Protein: A fragment corresponding to the expected size was amplified from cDNA of seeds of rape. This cDNA contained a 570 bp open reading frame (ORF) and encoding a polypeptide of 189 amino acids with a calculated molecular mass of 20.99 kD and a theoretical isoelectric point of 9.02.

To analyze the genomic structure of the *BnLIM*, a fragment of 1127 bp had been amplified from genomic DNA of rape. Comparison between the cDNA and genomic sequences indicated that the *BnLIM* gene consists of five exons and four introns. The four AT-rich introns were at conserved positions of LIM-domain protein as that in other plants: one in the first LIM domain, two in the inter-LIM spacer region, and one in the second LIM domain.

Homology and Structure Analysis of BnLIM: The deduced amino acid sequence of *BnLIM* was compared with those of other known plant LIM-domain proteins. These data suggested that *BnLIM* had a higher homology with LIM-domain proteins from woody plants (*Eglim1* and *PkLIM1*) and *NtLIM1*, whereas it had a lower homology with those from pollen and ovule such as *AtPLIM2* and *GhLIM1* (Table. 1).

Previously, Kawaoka (2000) indicated that the NtLIM1 protein is able to bind to the Pal-box sequence and regulate transcription of the phenylpropanoid biosynthesis genes. Each LIM domain of *NtLIM1* is sufficient for DNA binding *in vivo* and *in vitro.* Secondary and stereostructural analysis revealed that the two LIM-domains of *BnLIM*, which were 92% and 86% identical to those of *NtLIM1* respectively. Besides the two LIM-domains, the acidic domain of *NtLIM1* was also definitely required for its transcriptional activity. Although the isoelectric point (pI) of the entire BnLIM protein is 9.02, the pI of the C-terminal region (amino acids 163-189) is 4.86. The high degree of sequence and structure similarity between *BnLIM* and *NtLIM1* suggests that these proteins participate in related functions.

Table 1. Comparison of the degree of sequence identity (in %) between the BnLIM protein and other plant LIM-domain proteins

Protein Name	Protein ID	Score
BnLIM	ABB51648.1	100
EgLIM1	BAD91878.1	81
PkLIM1	BAB84581.1	81
NtLIM1	AAD56948.1	78
HaWLIM1	AAD56959.1	78
pNtPLIM1a	AAF13231.1	75
HaPLIM1a	AAD56958.1	66
AtWLIM2	AAB95275.1	54
GhLIM1	AAL38006.1	53
NtWLIM2	CAA71891.1	51
NtPLIM2	AAF75828.1	50
HaPLIM2	AAD15745.1	47
AtPLIM2	AAC28544.1	47

The Expression of BnLIM is Conformed to the Time Course of Seed Filling and Induced with Sucrose: RT-PCR analysis indicated that *BnLIM* was expressed in all tissues tested, which was most abundantly in stem, cotyledon, root and leaf

(Figure.1A, B). The expression of *BnLIM* was not limited to the lignified organs, i.e. root and stem tissues. This kind of expression feature had been detected in other lignin biosynthesis related genes *EuCCR* and *EuCAD* whose expressions were found in leaves of Eucalyptus gunnii, suggests that monolignols may be precursors of end products other than lignin.

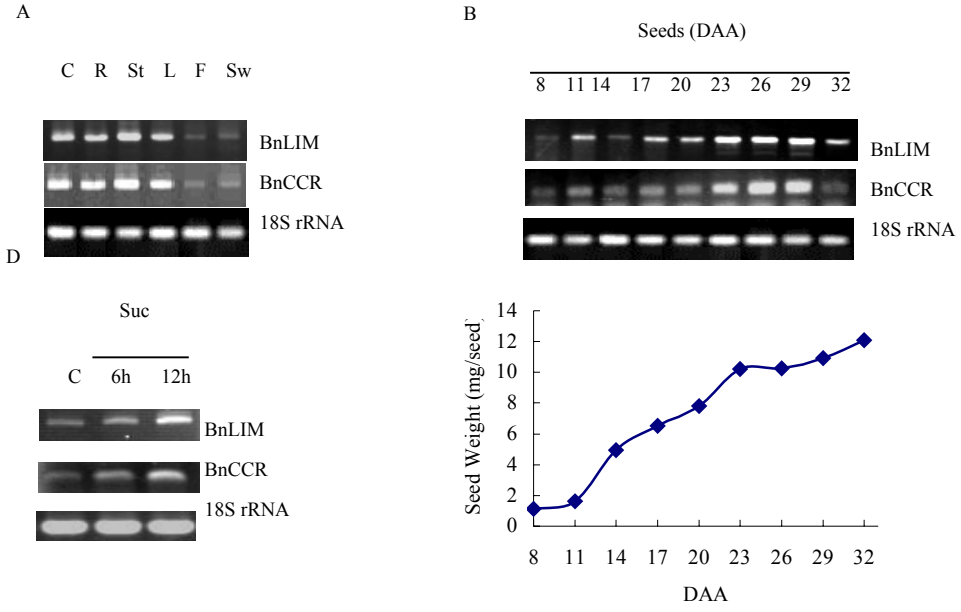

Figure.1 Expression of *BnLIM* gene in *B. nupus.* (A) *BnLIM* expression was determined by semiquantitative RT-PCR in a variety of tissues: C-cotyledon; R-root; St-stem; L-leaf; F-flower; Sw-silique wall. (B) The expression of *BnLIM* and *CCR* in developing seeds. (C) The time course of seed filling. (D) *BnLIM* response to sucrose treatment. C, untreated control; 6h, 12h: 2% (w/v) treatment for 6h and 12h respectively. Suc, sucrose.

In this research, only lower level expression of *BnLIM* had been detected in flower and silique wall. As *AtWLIM2* was reported to strongly express in developing silique (Rider, 2003), we further focused on expression of *BnLIM* in developing seeds removed from the silique walls. The expression of *BnLIM* gene during seed development conformed to the time course of seed filling (Figure 1B, C), which was lower in the early stage, highest in 23-29 DAA and reduced slightly 32 DAA when seed weight reached a maximum. The high level expression of *BnLIM* possibly reflects the need of a large supply of LIM-domain protein during the seed development of rapeseed. The expression pattern of *BnLIM* gene was similar to that of cinnamoyl-CoA reductase (CCR, EC 1.2.1.44), which catalyzed the first specific step in the synthesis of the lignin monomers. These results suggest a possible role of BnLIM in lignin biosynthesis and seed development. Increased evidence suggests that carbohydrate modulates transcript accumulation of lignin biosynthetic genes (Rogers, 2005). To examine if the expression of *BnLIM* was also sucrose regulated, we followed the transcript accumulation of *BnLIM* gene in 12-d-old rapeseed seedlings transferred to 2% ($w.v^{-1}$) sucrose containing liquid media by semiquantitative RT-PCR. As shown in Figure 1D, the expressions of *BnLIM* and *BnCCR* were increased when seedlings treated with 2% ($w.v^{-1}$) sucrose for 6h and 12h.

BnLIM Localizes to the Nucleus and the cytoplasm: To investigate the subcellular localization of BnLIM, a BnLIM-green fluorescent protein (GFP) fusion protein was transiently expressed in onion (*Allium cepa*) epidermal cells under the control of the cauliflower mosaic virus 35S promoter. The BnLIM-GFP fusion protein was localized in both the cytoplasm and the nucleus wherase free GFP was uniformly distributed throughout the cells. The dual cytoplasmic and nuclear localization feature of BnLIM is shared by other plant LIM proteins and the animal CRP-type LIM proteins (Eliasson. 2000). The detection of the BnLIM-GFP fusion in the nucleus suggested that this protein also participates in the transcription of genes.

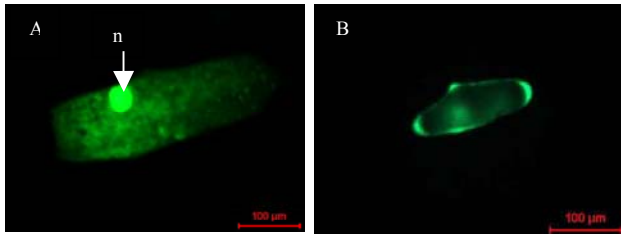

Figure.2 BnLIM localize to both the nucleus and cytoplasm. (A) BnLIM-GFP fusion structure transient transformation of onion epidermal cells. (B)Empty vector pEGFP-FGC5941 was used as control. n, nucleus.

Conclusion

A gene encoding LIM-domain protein was isolated from *Brassica napus* and it was also the first cloned transcription

factor involved in lignin biosynthesis pathway in *B. napus*. The full-length cDNA of *BnLIM* contained a 570 bp ORF encoding a protein of 189 amino acids. *BnLIM* was found to have an extensive homology with *NtLIM1*, a transcription factor involved in lignin biosynthesis. All three domains definitely required for NtLIM1 transactivation activity *in vivo*, were also found in BnLIM, implying BnLIM may have similar functions as NtLIM1. Alignment analysis revealed that BnLIM had a closer relationship with LIM-domain protein from woody plants (Eglim1 and PkLIM1) and NtLIM1, whereas had a lower homology with LIM-domain proteins from pollen and ovule. The expression profile of *BnLIM* was highly similar to that of *CCR*, and the accumulation of *BnLIM* mRNA is fitted with the seed development and up regulated by sucrose treatment. The subcellular localization of BnLIM with BnLIM-GFP fusion protein indicated its dual cytoplasmic and nuclear location feature. It was hypothesized that BnLIM may function as a transcription factor related to lignin biosynthesis and seed development.

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