Molecular cloning and functional identification of *BnIND* gene in *Brassica napus*

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Abstract

IND (INDEHISCENT) is a transcription factor which is required for DZ (dehiscence zone) cell differentiation and pod shattering in *Arabidopsis thaliana*. A homologous gene in *Brassica napus* (designated as *BnIND*) was cloned by rapid amplification of cDNA ends (RACE). Southern blot assay showed that there were three homologous loci of *BnIND* in genome of *B. napus*. The expression of the gene was restricted to the leaves, buds and pods as shown by RT-PCR method. The RNAi vector was constructed with partial *BnIND* and transformed to *B. napus* mediated by *Agrobacterium* to suppress the endogenous expression of *BnIND*. The transgenic lines were confirmed by PCR. Phenotype assay showed that the T1 generation of transgenic lines appeared disorder in DZ development.

Key words: Oilseed rape, *Brassica napus*, *BnIND*, indehiscent, RNAi

Introduction

Oilseed rape is one of the most important oilseed crops, and its planting acreage and total yield rank the second only next to soybean. China and India have the longest history of cultivating rape, whereas the former is top in terms of planting area and yield. However, pod shattering is widespread in dicotyledonous crops bearing multi-seeded dry fruits which undergo a programmed cell-separation process, termed dehiscence, for seed dispersal, such as in the *Brassica* species(Kadkol G P 1989; Meakin and Roberts 1990). *B. napus* is a species of oilseed rape where extensive breeding activities have been made, and fully mature pods of this species are extremely sensitive to opening, resulting in seed loss (Kadkol G P 1984). Consequently, it is crucial to ravel the process for suggesting new approaches to improve rapeseed yield. In the last decade, great progress has been made on the genetic mechanisms underlying the regulation of pod dehiscence in the model plant *Arabidopsis thaliana* (Ferrandiz 2002).

Previous studies of the fruit development have identified a few of the transcriptional regulators involved in margin specification. An atypical basic helix-loop-helix (bHLH) gene *ALCATRAZ (ALC)* required for the fruit dehiscence promotes the differentiation of the separate layer (Rajani and Sundaresan 2001). The redundant *SHATTERPROOF1 (SHP1)* and *SHP2* genes both encoding MADS-box family transcription regulators are involved in the proper specification of different cell types with the valve margin and the DZ, consequently, the *shp1shp2* mutant siliques are unable to shatter after fruit dry (Liljegren, Ditta et al. 2000). Another MADS-box transcription factor FRUITFULL(FUL) is responsible for the valve cell development during the elongation of fruit(Gu, Ferrandiz et al. 1998; Liljegren, Ditta et al. 2000), but recent study has shown that FUL negatively regulates the expression of the SHP1SHP2, ALC and INDEHISCENT (IND) (mentioned below) as the expression of SHP1SHP2, ALC and IND are restricted to the valve margin from the valve side by FUL (Ferrandiz, Liljegren et al. 2000; Liljegren, Roeder et al. 2004).

The important atypical bHLH transcription factor IND is primarily involved in the differentiation of the three cell types required for fruit dehiscence and acts as the key regulator in the network of transcription factors (Liljegren, Roeder et al. 2004). Six *ind* mutant alleles reported at present which can produce indehiscent fruits have been identified (Liljegren, Roeder et al. 2004; Wu, Mori et al. 2006). The *ind-6* mutant was obtained by a transposon-enhancer-trap method with a reporter gene encoding β-glucuronidase which was expressed in a seven-layer zone in the DZ. The unequal cell division activity occurred in the seven-layer zone for the forming of distinct cell types in the wild type, whereas, ind-6 mutant lacked distinct cell types for no unequal cell division in this zone. The study showed that IND played a role in regulating the unequal cell divisions in the seven-layer zone(Wu, Mori et al. 2006). All researches stated above are mainly taken in the *Arabidopsis*. *B. napus* is a very important oil crop and its many genes have not been identified. to understand the features of the IND gene in *Brassica*, analyze the conservation in bHLH family transcription factors and compare the phylogenetic relationship of these bHLH family factors, the full-length cDNA of *BnIND* was cloned from cultivated *B. napus* using the rapid amplification of cDNA ends (RACE) method (Frohman 1993). And the function of *BnIND* was primarily identified in this study.

Materials and methods

Plant materials and DNA and RNA extractions: Oilseed rape cultivars *Ningyou12* and *You88* (*B. napus*) were used in this study. Roots, stems, leaves, buds and pods were collected at the indicate stages and stored at -70°C for RNA and DNA isolation. Total RNA was extracted with trizol reagent kit (Invetrigen) according to its manual. Mature leaves were harvested for genome DNA extraction using CTAB extraction method.

Cloning of the full-length cDNA of BnIND: 50μg total RNA from flower buds of *You88* was reverse-transcribed into

first-strand cDNAs with the PrimeScript Reverse Transcriptase (Takara, Kyoto, Japan) in a 50 μl reaction volume. For screening of the *BnIND* from *Brassica*, the cDNA sequence of *AtIND* (NM_116229) was used as a query for a Washington University -Basic Local Alignment Search Tool (WU-BLAST2) search against *Brassica* database in the TAIR (www.arabidopsis.org). A putative *BnIND* EST and a genomic DNA BAC were found to be 74% and 63%, respectively in identity with the cDNA sequence of *AtIND*. The PCR primers (IND P1, IND P2, pBnIND1, pBnIND2, pBnIND3 and pBnIND4) were designed according to the EST and genomic DNA sequences obtained above. The *BnIND*-EST and partial *BnIND* genomic DNA fragments were cloned into pMD18-T vector (Takara) and sequenced. The 3′FULL RACE Kit (Takara) was used to clone 3′ end flanking sequence of the cDNA. The PCR reaction was performed according to the protocol.

Southern hybridization analysis: Six kinds of restriction enzymes (BamHI, EcoRI, KpnI, SacI, XbaI, XhoI) were used to Southern blotting. About 10 μg of genomic DNA was digested with 20 unit of restriction enzyme in a final volume of 100 μl at 37°C for 18 h. The cleaved DNA fragments were run on a 0.8% (w/v) agarose gel and transferred to Hybond-N + membranes (Amersham Pharmacia, Little Chalfont, Buckinghamshire, England). The membranes were hybridized following the standard protocols(Chen SY 1991). The membrane was washed once with 2×SSC plus 0.1% (w/v) SDS at 65°C for 8 min and exposed to FUJI Medical X-ray film at -70°C.

Analysis of BnIND expression: 5 μg of the total RNA isolated from young roots, young stems, young leaves, mature leaves, flowers, and pods of the cultivar *You88* were reverse-transcribed respectively into first strand cDNA with the PrimeScript Reverse Transcriptase (Takara) in a 20 μl reaction volume. The first strand cDNA mix was used as template for RT-PCR. A rape β-Actin gene, amplified with primers β-Actin F and β-Actin R, was used as a control in the experiments.

Construction of vector used in RNAi: Four primers (IND^+-1, IND^+-2, IND^--1, IND^--2) introducing XhoI, KpnI, XbaI or HindIII restriction site at the 5′-end of the PCR product were designed respectively according to *BnIND*-EST sequence. A fragment was amplified by PCR using primers IND^+-1 and IND^+-2. The PCR fragment was then cloned into pMD18-T vector (Takara), and the resulting plasmid was checked by sequencing. IND^--1 and IND^--2 were used for amplification of another fragment by PCR using the fragment described above as the template. Both two PCR fragments were inserted into pKANNIBAL vector and then transformed into *E.coli* TOP10. The transformants were selected on plates with LB medium and Kanamycin (Kan 50μg/ml). A binary vector, pART27, accepting NotI restriction site fragment from pKANNIBAL, was used to express the fragment inserted into pKANNIBAL vector. The combination plasmids were selected on plates with LB medium and Kan and Spectinomycin (Spec 100μg/ml).

Transformation of the reconstruction plasmids: The resulting recombination plasmid was transformed into *Agrobacterium* (*LBA4404*). Transformants were selected on plates with LB medium and Kan, Spec and also Streptomycin (Sm 125μg/ml), and subsequently transformed into *B. napus* c.v. *Ningyou12* mediated by *Agrobacterium*. The transforming process was performed as the one described above (Cardoza and Stewart 2003).

Identification of the transgenic lines: For identifying the transgenic lines, the genome DNA was isolated from mature leaves and the NPTΠ was amplified by PCR.

Results and discussion

Cloning and structural analysis of BnIND

AtIND gene was used as a query to search against the *Brassica* database and a similar EST and a genomic DNA BAC were obtained. The putative *BnIND* EST and partial of *BnIND* genomic DNA were 360bp (AT002234.1) and 848bp (DU105139) in length, respectively, and the genomic DNA contains the EST which represents the inside sequence of *BnIND* when compared with *AtIND*. A complete open reading frame (ORF) of the genomic DNA speculated by DNAStar was 546bp which is 51bp shorter than 597bp of *AtIND* in length. The special primers were designed from the EST and BAC and anticipatory fragments were obtained from *B. napus c.v.You88*. In addition, the alignment between fragment by PCR from cDNA and that from genomic DNA in sequence indicates that *BnIND* does not contain introns as *AtIND* does (Fig1). In order to further understand the 3′-untranslated region of *BnIND*, RACE method was used. The length of sequence amplified from cDNA of *BnIND* was 788bp containing 23bp of the 5′-leader sequence and 219bp of 3′-untranslated region. The putative ORF of 546bp encoded a protein of 181 amino acids with a predicted molecular weight of 20.3 kDa and a calculated isoelectric point of 5.88 (Protparam: http:// www.expasy.ch).

Using SMRT program (Simple Modular Architecture Research Tool: http:// smart.embl-heidelberg.de), A HLH domain (amino acid 94-143) as DNA biding region was identified in the BnIND, suggesting that the protein may localize in nucleus and act as a transcription factor. However, there was no nuclear localization sequence detected, predicting that the BnIND as a small protein may be transported into nucleus by free diffusion(Garcia- Bustos, Heitman et al. 1991; Goldfarb and Michaud 1991). An alanine residue (A) was also found at site 9 within the basic region of the BnIND as well as AtIND, indicating that BnIND is an atypical bHLH protein whereas typical bHLH proteins have a glutamic acid residue (E) at that site instead (Fisher and Goding 1992; Buck and Atchley 2003; Toledo-Ortiz, Huq et al. 2003; Liljegren, Roeder et al. 2004). By comparing the BnIND with other bHLH proteins, the conservative sequence was primarily restricted to the bHLH region and the other regions showed a high degree of variation among the bHLH proteins compared (data not show). Sequence alignment of the bHLH domain from BnIND and related bHLH proteins from yeasts, plants, and animals indicated that the amino acid residues-- arginine (R) in basic region, leucine (L) in helix 1 region, and tyrosine (Y) in helix 2 region--are in common. Moreover, the bHLH domain of BnIND shared high similarities with that of AtIND and At5g09750 (an uncharacterized protein predicted from *Arabidopsis*), being 82% and 100%, respectively (rectangular box in Fig2). However, ALC, also a

bHLH transcription factor required for pod dehiscence in *Arabidopsis*, only shared 40% identity with BnIND in the bHLH domain (Fig2). The phylogenetic tree was portrayed to exhibit the distances among bHLH proteins from yeasts, plants and animals (Fig3). The phylogenetic tree obviously showed that BnIND belongs to the atypical bHLH proteins group with AtIND and At5g09750 (from the Alignment), thus, they may form similar homodimer or heterodimer with other transcription factors and further recognize and bind similar targets DNA sequence to regulate gene expressions(Heim, Jakoby et al. 2003).

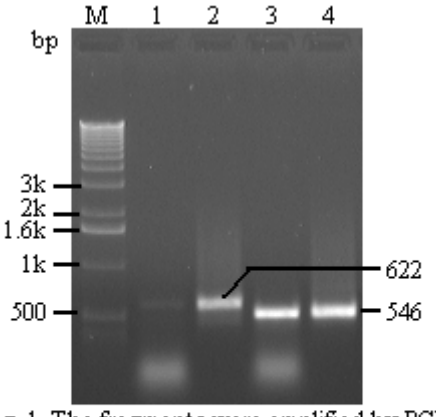

Fig. 1. The fragments were amplified by PCR with two couples of same primers from cDNA and genomic DNA of BnIND respectively. M:marker; 1 and 3: frgments amplified from cDNA of BnIND. 2 and 4: fragments amplified from genomic DNA of BnIND.

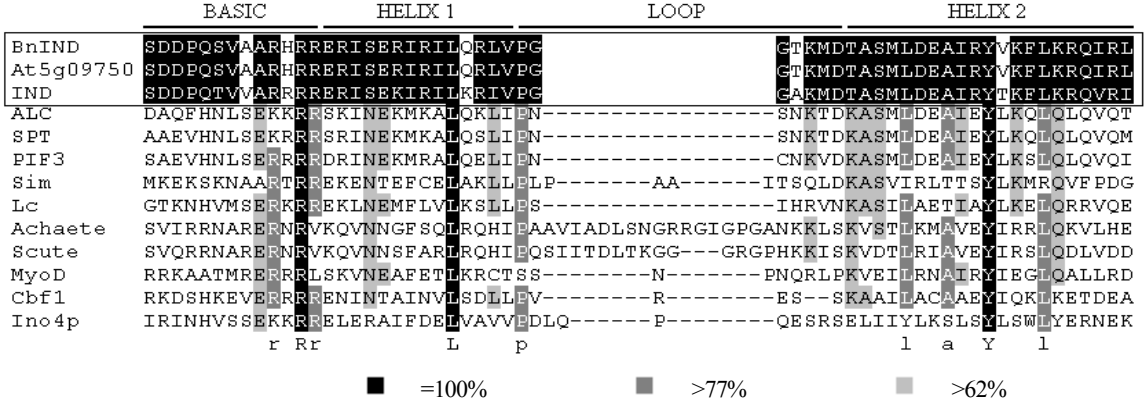

Fig.2. Multiple sequence alignment of the bHLH region from BnIND and other bHLH family proteins from plants, yeasts and animals. The proteins aligned are: INDEHISCENT(IND)(Liljegren, Roeder et al. 2004), the B-class members PHYTOCHROME INTERACTING FACTOR (PIF3), SPATULA (SPT) and ALCATRAZ (ALC) from *Arabidopsis*(Ni, Tepperman et al. 1998; Heisler, Atkinson et al. 2001; Rajani and Sundaresan 2001); centromere binding factor (Cbf1) and Ino4p from yeast(Berben, Legrain et al. 1990; Cai and Davis 1990); A-class MyoD from human(Pearson-White 1991); LC from *Zea mays*(Ludwig, Habera et al. 1989), and C-class single-minded (Sim) from fly(Nambu, Lewis et al. 1991)as well as Achaete and Scute(Villares and Cabrera 1987). IND, Sim and Uncharacterized proteins (At5g09750) all have an alanine residue that represents atypical bHLH protein at the ninth amino acid site of the basic regions like BnIND. More similar bHLH regions from BnIND, AtIND, and At5g09750 were marked with the rectangular box. Different colors showed in this fig. represent the different percentage of similarity. The alignment was performed with CLUSTALX and GENEDOC

The genomic DNA was digested by six restriction enzymes respectively and subjected to Southern analysis. The 360bp *BnIND*-EST was used as a probe. The result was illustrated in fig4. It can be seen that there were three bands (one major band and two weaker bands) in the first, second and fourth lane. The third lane had only one band but the fifth and sixth lanes had three bands with one weak band and two bright bands. The result of the Southern Blotting suggested that there were at least three duplications of *BnIND* in the genome of *B. napus* showing large significance of *BnIND* for the reason that the more the amount of duplication of one gene, the more important roles this gene may play in an organism.

Analysis of the BnIND expression

Expression profile of *BnIND* was exhibited to describe the functions and roles it may play in growth and development of *B. napus*. The result of RT-PCR showed that the expression of *BnIND* was present in young leaves rather than in roots or stems, and widely existed in mature body, most strongly in buds compared with leaves and pods. By comparison, *BnIND* expressed most strongly in young leaves. (Fig5). Flowers are abnormal organs of the leaves as well as the genetic organs of

the fruits in many plants including oilseed rape. The expression profiles of *BnIND* also showed the relationship of these three organs that BnIND may be involved in regulating the growth and development of leaves and the formation of flowers, subsequently the production of the pods in *Brassica.*

Genomic architecture of the BnIND gene

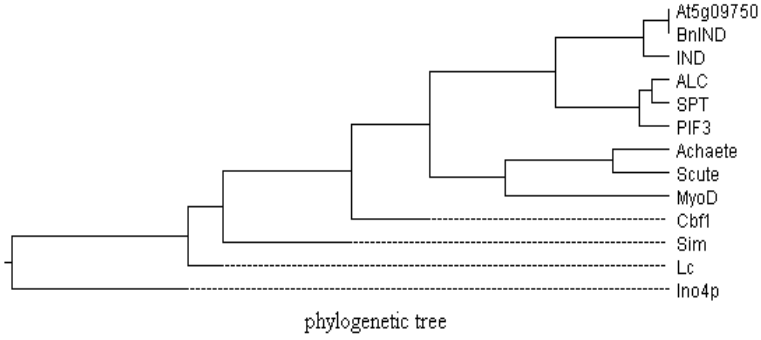

Fig.3. Phylogenetic tree of the bHLH family proteins from different species. The analysis was performed with the MegAlign program of DNAStar. Classification of bHLH proteins were shown at the right side

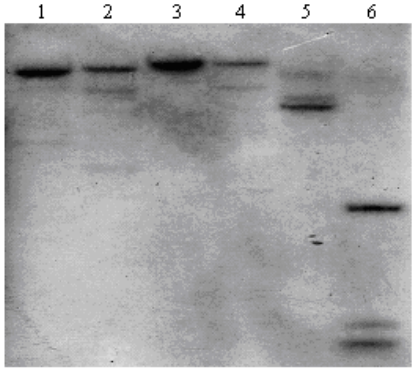

Fig. 4. The southern analysis of the BnIND gene in the genomic DNA. the restriction endonucleases used are BamHI, EcoRI, KpnI, SacI, XbaI, XhoI (from the first to sixth lane)

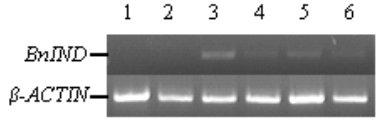

Fig. 5. Expression profile of BnIND was performed by RT-PCR using β- ACTIN as control. 1: roots; 2: stems; 3: young leaves; 4: mature leaves; 5: buds; 6: pods

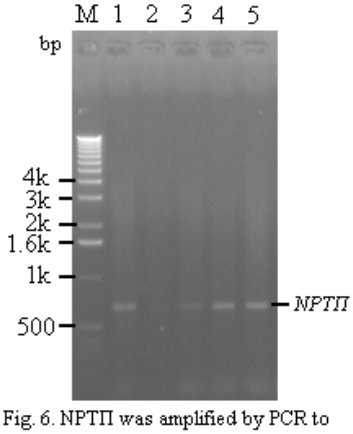

Fig. 6. NPTII was amplified by PCR to identify the transgenic lines.M: marker; 1: pKANNIBAL plasmid; 2: non-trasgenic line genome; 3,4,5: transgenic lines genome

Inhibition of the expression of BnIND by RNAi method

It is well known that AtIND is required for the formation of three cell types in the DZ and valves acting as regulator, and the pod could not open for abnormal cell division in the DZ and valves of the *ind* mutant. For identifying the function of the BnIND, the *BnIND* expression was inhibited by RNAi method and subsequent effect on the pod dehiscence was determined. NPTII was amplified by PCR to identify the transgenic lines (Fig.6). The electrophoresis showed that three lines were

successfully transformed with the *BnIND* fragment. The phenotype observation of pods from T1 transgenic lines showed a disorder in the DZ region suggesting that the expression vector loading interested gene was inserted into genome in random, hence, many distinct transgenic lines inserted from different sites were obtained, and the efficiency operated by them was also discrepant (data not show).

Conclusion

The cloning of *BnIND* gene and analysis of the characteristic in sequence suggested that *BnIND* is the homologous gene of *IND* in *B. napus*. The southern blotting showed that there were three homologous loci of *BnIND* exist in *B. napus* genome. The expression profile of *BnIND* indicated that the BnIND played very important roles in the identification and formation of specific cells of leaves, buds and pods in *B. napus*. However, the phenotype assay of pod in T1 generation of transgenic lines by RNAi method exhibited disorder. The next steps of the research would be to further identify the BnIND in the morphological and anatomical analysis of pods from transgenic lines and to screen homozygotic transgenic lines.

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