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ARTICLE

Isolation and Functional Characterization of the *Brassica napus* Cruciferin Gene *cru4* promoter

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Abstract The 12S globulin protein cruciferin is main seed storage protein in *Brassica napus*. To gain a better understanding of the *Bncru4* promoter function, we conducted the promoter 5' deletion analysis in transgenic *Arabidopsis*. In the β-glucuronidase (GUS) expression assay, *Bncru4* promoter was strongly active in transgenic seeds. In addition, deletion of RY-elements (–236 bp region) dramatically decreased the promoter activity in seed embryos; however, the GUS expression could be observed in seed coat. Further deletion up to −113 bp region (removed up to the CAAT and TATA box), GUS expression was completely abolished in all tissues. These results were consistent with that of the GUS activity in transgenic seeds. Therefore, we consider that RY-element is crucial to the seed-specific expression of *Bncru4* promoter.

Keywords 12S globulin · *Bncru4* promoter · *Brassica napus* · rapeseed · RY-element

Introduction

Seed storage proteins are synthesized in high abundance during seed development and serve as a nitrogen source for germinating seeds. Seeds contain large amounts of storage proteins that are classified as four major classes; (i) the water-soluble albumins, (ii) the alcohol-soluble prolamines, (iii) the salt-soluble globulins, and (iv) the acid- or alkaline-soluble glutelins (Utsumi, 1992). Dicot seeds contain mainly albumins and globulins, whereas monocot seeds contain mainly prolamines and glutelins (Li and Okita, 1993; Mandal and Mandal, 2000).

In *B. napus*, there are two main seed storage proteins, the 2S protein napin and the 12S globulin protein cruciferin. Napin is composed of two polypeptides that are linked by disulfide bridges (Crouch and Sussex, 1985). Cruciferin protein is a hexameric complex (300–50 kDa) consisted of three different groups of subunits, *cru1*, *cru2/3*, and *cru4* (Schwenke et al., 1983; Rdin and Rask, 1990; Sjdahl et al., 1991). Each subunit consists of a heavy α-chain and a light β-chain (Rdin and Rask, 1990). Both chains originate from the same precursor molecule, which is co-translationally synthesized in the late stages of seed development on the rough endoplasmic reticulum. These procruciferins are then assembled into trimers (Chrispeels et al., 1982), transported through the Golgi apparatus, and finally accumulate as mature hexamers in protein storage vacuoles of terminally differentiated cells in developing seeds (Dickinson et al., 1989; Jung et al., 1998). The cruciferin complex of *Brassica napus* has an octameric barrel-like structure, which represents a very compact building block optimized for maximal storage of amino acids within minimal space (Nietzel et al., 2013). In germinating seeds, cruciferin mRNAs are rapidly degraded (Fernandez et al., 1991).

The *cis*-acting elements involved in the regulation of expression of *napin* gene have been identified (Stålberg et al., 1993; Ellerström et al., 1996). Deletion of an overlapping E-box/abscisic-acid-responsive element (ABRE) motif abolished the activity of the *nap*A promoter. The ABRE elements are known to be involved in ABA-dependent induction of other genes and have been shown to bind G-box binding proteins belonging to the bZIP family of transcription factors (Giraudat, 1994). In addition, *nap*A promoter showed the spatial and developmental expression patterns. In cruciferin, the regulatory region of *crul* gene among three subunits was investigated (Sjödahl et al., 1995). Similarly to *nap*A promoter, *cru1* promoter also showed the spatial and developmental expression patterns. Gel retardation assays showed that a CANNTG motif (E-

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box), an SEF3 motif, an ABRE and an RY-like motif interacted specifically *in vitro* with DNA-binding proteins present in nuclear extracts from seeds of *B. napus*.

In the present study, the *cis*-acting elements of *B. napus* cruciferin gene *cru4* promoter was investigated. To this aim, a 0.75-kb fragment upstream of the translation start codon (ATG) of *Bncru4* was cloned, followed by generation of the progressive 5' deletion constructs of this promoter region, which were used to study the expression of the β-glucuronidase (GUS) reporter gene using stable transformants of *Arabidopsis thaliana*. Finally, we found that deletion of a RY-element abolished the *Bncru4* promoter activity in transgenic *A. thaliana* seeds.

Materials and Methods

RNA extraction and Northern blotting. Total RNAs were isolated from the leaf, stem, root, petal, and seeds (10, 25, 35, and 45 DAF, respectively) from *B. napus* L. cv Youngsan using Plant RNA Purification Reagent (Invitrogen, USA). For Northern hybridization, about 10 μg of total RNA was fractionated on 1.5% (w/v) formaldehyde agarose gel electrophoresis, transferred onto Hybond N^+-Nylon membranes (GE Healthcare) by capillary transfer, followed by RNA hybridization with [α-^{32}P] dCTP-labeled *Bncru4* gene [GenBank:X57848] cDNA fragment and *Bnnapin* gene [GenBank:X17542], corresponding to the entire gene open reading frame. Blots were rehybridized using a [α-^{32}P] dCTP-labeled *Bnactin* gene [GenBank:FJ529167] as an internal control.

Isolation and analysis of the *Bncru4* promoter sequence. The 5' upstream region of *cru4* gene from *B. napus* cv Youngsan was isolated using a Genome Walker™ kit (Clontech, USA). Genomic DNA was extracted from leaves using a phenol-chloroform based protocol (Ahmed et al., 2009), and digested by the four enzymes -*Dra*I, *Eco*RV, *Pvu*II and *Stu*I- to generate blunt ends. Using the GenomeWalker library as a template, primary and nested polymerase chain reactions (PCRs) were performed based on the gene-specific primers listed in Table 1 and Genome Walker adaptor primers provided in the kit. The gene-specific primers were designed on the *Bncru4* gene cDNA sequence. Nested PCR products were purified from a 1.5% (w/v) agarose gel and inserted into the pGEM-T-easy vector (Promega, USA) for sequencing. To detect putative *cis*-acting regulatory elements in the *Bncru4* promoter sequence cloned, the database PlantCARE (Lescot et al., 2002) was used.

Determination of the promoter transcription start site. Total RNA was isolated from the embryo of *B. napus* cv Youngsan using a Plant RNA purification reagent (Invitrogen, USA). The *Bncru4* transcription start site was determined by a 5'-rapid amplification of cDNA ends (RACE) PCR strategy, based on a GeneRacer® Core Kit (Invitrogen). Nested PCRs were performed using the two genes-specific primers listed in Table 1 and the adaptor primers provided in the kit. The primers (Table 1) were designed from the *Bncru4* cDNA sequence, and second round PCR products were introduced into the pGEM-T-easy vector (Promega, USA) for sequencing. The promoter transcription start site was obtained by aligning the resulting sequences with that of the promoter.

Progressive 5' deletion constructs of the *Bncru4* promoter. A series of fragments with different 5' terminal deletions were obtained by PCR amplification, using the primers described in Table 1. The size of the expected amplicons were 753, 426, 293, 236, and 113bp. An *Eco*RI restriction site was introduced into the forward primers and a *Bgl*II site was introduced into the reverse ones. Five promoter sequences of variable length were released by *Eco*RI and *Bgl*II digestion and cloned into pCAMBIA 3301 vector (CAMBIA, Australia) by replacing CaMV35S promoter upstream of the GUS reporter gene. All PCR products were entirely sequenced to avoid PCR error. *Bncru4*::GUS constructs were introduced into *Agrobacterium tumefaciens* strain EHA105 by freeze-thaw method.

***Arabidopsis* transformation.** *Arabidopsis thaliana* plants of the ecotype Columbia were transformed by *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998; Martinez-trujillo et al., 2004). The modified floral dip inoculation medium consisted of 5% sucrose and 0.05% triton-X 100. To obtain more floral buds

Table 1 Primers used in this study (The restriction sites are underlined)

Genes	Primer names	Sequence (5'-3')
GenomeWalker adaptor	AP1	GTAATACGACTCACTATAGGGC
	AP2	ACTATAGGGCACGCGTGGT
5' RACE adaptor	5'	CGACUGGAGCACGAGGACACUGA
	5'-Nested	GGACACUGACAUGGACUGAAGGAGUA
Bncru4 gene	*Bncru4*gsp1	GCCCATAGGACCACTGCTTTGAGT
	*Bncru4*gsp2	GAACTGTTGGCTTGTGGCACGTTT
Bncru4 promoter	753-F	GAATTCACAATCAAGATTTCAACTAATCGG
	426-F	GAATTCAAGTGTTTTTAACCGCTTGCTTAA
	293-F	GAATTCTTTTTTATAAAAAATATACAAAAT
	236-F	GAATTCGTTGCATGGCATGTAGACGCGGAA
	113-F	GAATTCGTTACACGTGATCTCCATGCAGAA
	Bncru4-R	AGATCTTTGCTATTTGTGTGTATGTTTTTT

per plant, primary inflorescences were clipped, relieving apical dominance and encouraging the synchronized emergence of multiple secondary bolts. Plants were inoculated when most secondary inflorescences were about 5 to 10 cm tall. Plants were inoculated by performing direct drop by drop inoculation to every flower by using a micropipette.

Histochemical and fluorometric GUS assays. The histochemical and fluorometric assays for GUS were performed essentially as described by Jefferson et al. (1987) with minor modification. The plant tissues were collected from each transgenic line at the same developmental stage. For histochemical assay, the plant materials were immersed in X-gluc buffer containing 1 mM X-gluc, 100 mM sodium phosphate buffer pH 7.0, 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide and 0.05% (v/v) triton X-100 and incubated overnight at 37°C. After staining, flowers were rinsed in 75% ethanol for 2 to 3 times, and photographed.

The GUS activity was quantified by a fluorometric assay using 4-methylumbelliferyl-β-D-glucruonic acid as a substrate. The tissue (0.1 g) was homogenized in 1 mL protein extraction buffer (50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% sodium lauryl sulphate, 10 mM β-mercaptoethanol and 0.1% triton-X 100) followed by centrifugation at 13,000 rpm for 15 min under 4°C. Protein content was measured by the Bradford (1976) method using BSA as a standard. Fifty microliters of the crude protein extract were added to 500 µL GUS assay buffer (10 mM 4-methylumbelliferyl-β-D-glucuronide trihydrate dissolved in protein extraction buffer) in a ratio of 1:10 and then incubated at 37°C for 1 h in a water bath. One hundred microliters of this mixture was transferred into a Microcentrifuge tube containing 900 µL Stop buffer (0.2 M sodium carbonate). The GUS activity was measured immediately using a Victor 3 Fluorometer system (PerkinElmer, USA) with an excitation wavelength of 355 nm and an emission wavelength of 460 nm. A calibration curve of 4-MU standards was prepared according to the manufacturer's instructions. The amount of GUS was calculated based on the established standard curve, and the specific GUS activity was expressed as µmoles 4-MU/mg/min. Data are presented as the mean (± standard error) of GUS activity from three independent determinations.

Results and Discussion

Expression patterns of the *Bncru4* gene. The expression patterns of *Bncru4* [GenBank:X57848] and *Bnnapin* [GenBank:X17542] of two main seed storage proteins in *B. napus* was observed by Northern blot analysis (Fig. 1). As expected, both cruciferin and napin were expressed only in the seeds. However, a slight difference in temporal patterns was shown; the accumulation of *cru*4 gene started at 35 DAF (days after flowering) and continued until 45 DAF, whereas the accumulation of *napin* gene started 25 DAF and continued until 45 DAF. According to previous studies (Blundy et al., 1991; Fernandez et al., 1991; Höglund et al., 1992), the synthesis of both napin and cruciferin during embryogenesis is strictly regulated with respect to time and tissue. Although Napin mRNAs started a few days earlier than that of cruciferin, both mRNAs showed similar patterns in the spatial and temporal distributions. Cruciferin mRNAs begin to accumulate in the cortex of the axis during torpedo stage of embryogeny, i.e. the first 25 DPA (days post-anthesis) and are present at high levels in the cotyledon stage (30 DPA) and maintain higher levels until mid-cotyledon stage (38 DPA). These results were in agreement with ours.

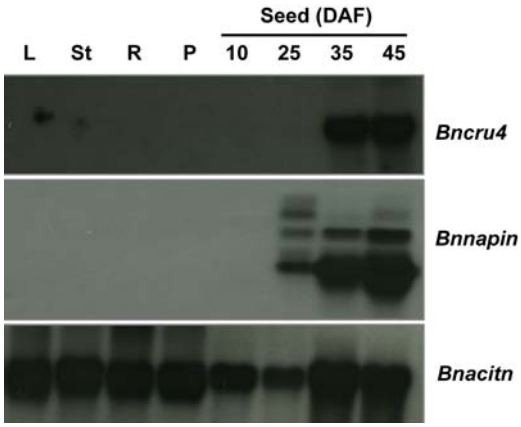

Fig. 1 *Bncru4* and *Bnnapin* expression patterns by Northern hybridization analysis in Rapeseed. *Bnactin* was used as an internal control. L, Leaf; St, Stem; R, Root; P, Petal, DAF, days after flowering. Ten micrograms of total RNA were used. GenBank accession numbers of these genes are: *Bncru4* [GenBank:X57848], *Bnnapin* [GenBank:X17542], and *Bnactin* [GenBank:FJ529167].

Isolation and analysis of the *Bncru4* promoter sequence. The promoter region of the *Bncru4* gene was isolated by the genome walking PCR using a set of walker primers and gene-specific primers in Table 1. The 753 bp sequence of the *Bncru4* promoter cloned in this study was deposited at NCBI [GenBank:GQ402149].

Analysis of the promoter sequence revealed five conserved

Table 2 List of *cis*-elements in the *Bncru4* promoter sequence predicted by database analysis

Element	Element core sequence	Function
TCT motif	TCTTAC	Light responsive element
HSE	AGAAAATTCG	Heat stress responsive element
RY element	CATGCATG	Seed specific regulation
CAAT-box	CAAT	Common cis-acting elements in promoter and enhancer region
TATA-box	TATAAAT	Core promoter element around -30 of transcription start

Fig. 2 Nucleotide sequences of the *Bncru4* promoter region. Putative regulatory *cis*-acting elements are indicated by open boxes. Translational initiation codon is underlined in bold letters. Transcription start site as determined by 5'-RACE is indicated by arrow. The endpoints of the 5' deletion derivatives are indicated below the sequence underlined. Numbers to the left of the sequence denote the position relative to the translation start site. The *Bncru4* promoter sequence has been submitted to NCBI [Genbank:GQ402149].

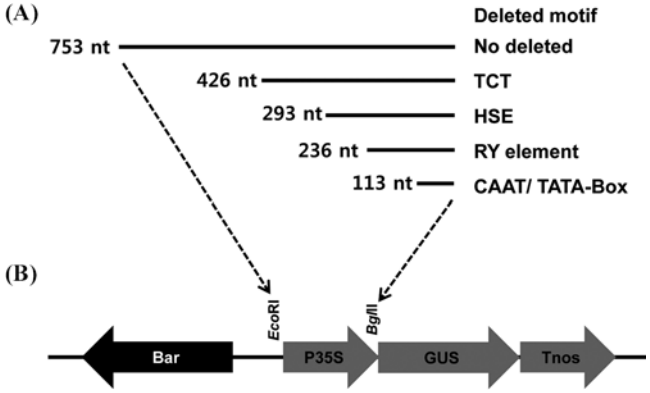

Fig. 3 Vector construction of 5' deletion derivatives. (A) Schematic drawing of the *Bncru4* promoter region deleted motif. (B) The plasmid pCAMBIA3301 vector map. The 5' deletion derivatives of the *Bncru4* promoter shown in (A) were inserted into the *EcoR*I and *Bgl*II enzyme sites. *Bar* gene was driven by the CaMV35S promoter. Tnos, Nos polyA terminator.

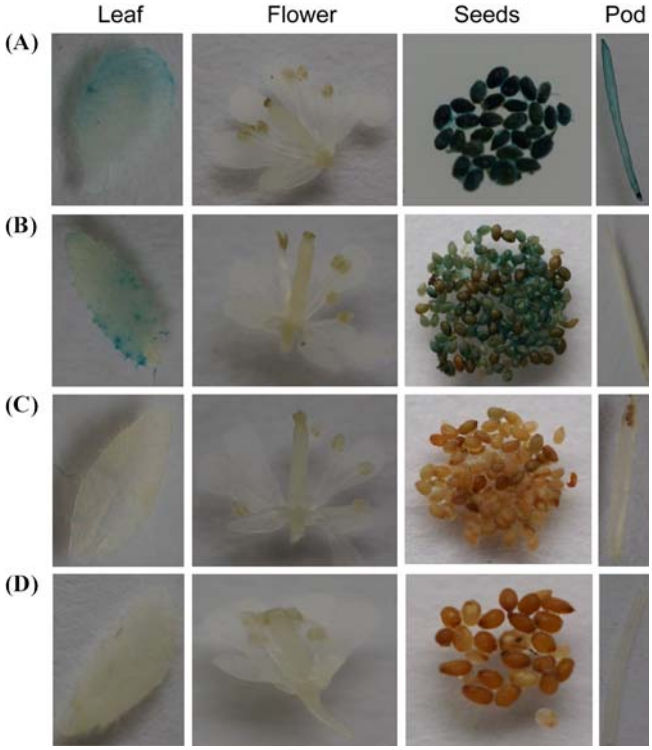

Fig. 4 Analysis of GUS expression in transgenic *Arabidopsis* (T_2) harboring the 5' deletion derivatives of the *Bncru4* promoter. (A) −753 nt of the promoter, (B) −236 nt of the promoter deleted RY element, (C) −113 nt of the promoter deleted both CAAT box and TATA box, (D) Wild type. Wild type was used as a negative control. Flower of fully expended petal stage was used. Fully expended leaf before bolting was used; Pod and seed (immature embryo) in 2 weeks after flowering was used. nt, nucleotides.

DNA motifs upstream of the *Bncru4* gene (Table 2). The promoter's transcription start site (TSS) at -45 bp upstream of the ATG codon was determined using 5'-RACE PCR based on mRNA extracted from *B. napus* embryo (Fig. 2). A potential TATA-box sequence (TATAAAT) was located at −77 bp upstream of the ATG codon and at −32 bp upstream of the TSS. In addition, a potential CAAT-box sequence (CAAT) was located at −41 bp upstream of the TATA-box sequence and at -73 bp relative to the TSS. The TCT motif (polypyrimidine initiator) is required for transcription of ribosomal protein gene promoters (Parry et al., 2014). The HSE motif is responsible for the heat-shock of the *Arabidopsis* APX1 gene *in vivo* (Storozhenko et al., 1998). RY-element (CATGCATG) is conserved in the promoter regions of the genes of legume seed storage proteins (Dickinson et al., 1988). To characterize the *Bncru4* promoter 0.75 kb region, the 5' deletion derivatives of the promoter::GUS fusion gene were constructed and then were transformed into *Agrobacterium tumefaciense* (Fig. 3).

Stable GUS expression patterns of the *Bncru4* promoter in transgenic *Arabidopsis* plants. Transgenic *Arabidopsis* plants transformed with the 5' deletion derivatives of the *Bncru4*

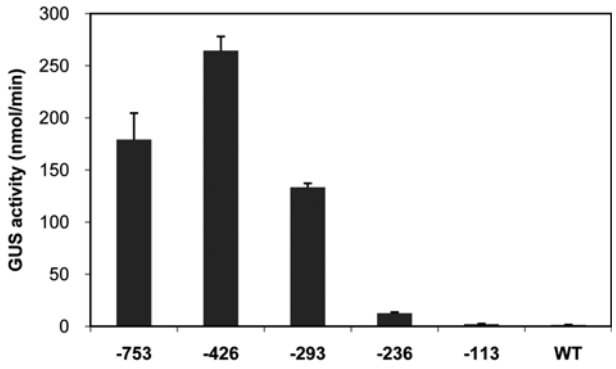

Fig. 5 GUS activities in seeds of transgenic *Arabidopsis* (T_2) harboring the 5' deletion derivatives of the *Bncru4* promoter. −753 nt of the promoter; −426 nt of the promoter deleted TCT element; −293 nt of the promoter deleted HSE element; −236 nt of the promoter deleted RY element; −113 nt of the promoter deleted both CAAT box and TATA box; WT, Wild-type plant. nt, nucleotides. Data are presented as the mean (± standard error) of GUS activity.

promoter were selected on the basis of their capacity to grow normally after BASTA (glufosinate ammonium) herbicide spraying. About 5 to 10 primary transformants of each construct were obtained. Transgenic plants were confirmed in the presence of the appropriate expression cassette by PCR using specific primers (data not shown).

To verify the GUS expression patterns driven by the 5' deletion derivatives of the *Bncru4* promoter, histochemical analyses were conducted in various tissues (leaf, flower, seed, and pod) of transgenic *Arabidopsis* plants (Fig. 4). GUS expression in transgenic *Arabidopsis* (T_2) harboring the 753 bp region of the *Bncru4* promoter was detected mainly in seeds (Fig. 4A); GUS expression in leaf and pod was observed weakly or as trace, whereas GUS expression was not detected in the flower. Furthermore, at deletion up to −293 bp region, GUS expression patterns of the transgenic *Arabidopsis* were not altered (Data not shown). However, in −236 bp region (removed up to the RY-element), GUS expression of the seed embryos was reduced dramatically (Fig. 4B); GUS expression in seed coat was observed weakly and GUS expression in leaf was observed as trace with no GUS expression in flower and pod detected. Further deletion up to −113 bp region (removed up to the CAAT and TATA box), GUS expression was completely abolished (Fig. 4C). In wild type used as a negative control, GUS expression could not be detected (Fig. 4D).

To characterize the *Bncru4* promoter isolated from *B. napus*, the GUS fluorometric assay was conducted in transgenic *Arabidopsis* seeds transformed with the 5' deletion derivatives (Fig. 5). In the 753, 426 and 293 bp regions of the *Bncru4* promoter, GUS activities were high, showing the levels at 133 to 264 nmol/min. However, at the 236 bp region the RY-element was removed, and GUS activity dropped sharply at 13 nmol/min. In the shortest (113 bp) region and a negative control (WT), GUS activity was detected at the background level.

In the present study, *Bncru4* promoter was strongly active in transgenic Arabidopsis seed. Deletion of RY-element resulted in deprivation of *Bncru4* promoter activity in seed embryos, whereas the GUS expression could be observed in seed coat. In addition, after deletion of both CAAT-box and TATA-box the promoter activities in all tissues disappeared. Furthermore, both TCT-motif and HSE-motif were shown not to affect the promoter activity. These results were consistent with those of the GUS activity in transgenic *Arabidopsis* seed. Fauteux and Strmvik (2009) reported that seed storage protein gene promoters contain conserved DNA motifs (two RY-like and one ACGT-like) in the *Brassicaceae*, *Fabaceae*, and *Poaceae*. In *Phaseolus vulgaris*, several conserved RY-elements of the DLEC2 promoter (Bobb et al., 1997) and phaseolin promoter (Chandrasekharan et al., 2003) were necessary for seed-specific expression during embryogenesis. Therefore, we consider that RY element is crucial to the seed specific expression of *Bncru4* promoter.

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