Isolation and functional characterization of petal specific BnPI promoter from Rapeseed *(Brassica napus L.)*

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Abstract

The promoter region of rapeseed (*Brassica napus* cv. Yeongsan) seed specifically expressed genes, coding for a *PISTILLATA* protein (BnPI), was cloned and *in silico* characterized. BnPI expression was only induced in the petal. The isolated fragments are 1508 bp long and contain light responsive element motifs, such as G-Box and I-Box. Functional analysis of this promoter in transgenic Arabidopsis plants was investigated after fusing them with the beta-glucuronidase (GUS) reporter gene. Promoter deletion analyses suggest that BnPI promoter containing G-box motif (-1508 ~ -751) was expressed in petal. In this work, we demonstrate that BnPI promoter is petal specific.

Keyword: *Brassica napus*, *PISTILLATA* , Petal specific promoter, Deletion analysis

Introduction

Rape flowers are important tourism resources in a region as large-scale cultivation. However, there are only yellow rape flowers except white caused by mutation. Therefore, we have developed the petal specific *PISTILLATA* promoter to produce the rape flowers expressing various colors.

PISTILLATA is a B-class floral organ identity gene and contain a conserved protein coding domain called the MADS domain that play a major role in modulating flowering time in Arabidopsis. *PISTILLATA* expression is induced in the stage 3 flowers (early expression) and is maintained until anthesis (late expression).

In this study, we describe the isolation and characterization of *PISTILLATA* promoter from rapeseed (*Brassica napus* cv. Yeongsan). The *PISTILLATA* full-length promoter and its 5' truncated promoter regions were analyzed by transient in rapeseed flowers and stable expression systems in Arabidopsis using *GUS* as reporter gene.

Methods

Isolation of 5' flanking region of *PISTILLATA* gene from *B. napus* cv. Yeongsan was carried out following PCR-based directional genome walking method using GenomeWalker Universal Kit (Clontech labolatories, Mountain View, CA). Genomic DNA was extracted from leaves following phenol-chloroform method. The gene-specific primers used in this experiment were designed on the basis of *PISTILLATA* cDNA sequence [EMBL: NP12954195].

The 5'flanking sequence upstream to ATG of the *PISTILLATA* cDNA was searched for known transcription factor binding sites using the PLACE and PlantCARE databases. Transcription start site was predicted by using Neural Network Promoter Prediction softwares [http://www.fruitfly.org/seq tools/promoter.html].

The 1508 bp 5' upstream to ATG of the *PISTILLATA* was considered as full-length BnPI promoter in this study and was designated as PI-1.5. The 5' deletion fragments of P1-1.5 namely P2-1.25, P3-1.15 and P4-0.75 (the numbers after the hypen indicates number of nucleotides [kb] upstream to ATG present in the deletion fragment) were amplified with HD DNA polymerase (Clontech labolatories, Mountain View, CA). All the vectors were constructed according to the GateWay vector manual (Invitrogen) using the pCAMBIA3300GWGUS vector manufactured by GreenGene Bio laboratory

(Yongin, Republic of Korea), which was constructed for promoter-reporter analysis with GUS and confers BASTA resistance. DNA fragments containing the BnPI promoter region of various lengths were amplified from Genomic DNA so that they were flanked by *attB*1 and *attB*2 sites. These amplified products were used to make deletion alayses promoter entry clones and then were used to generate a GUS expression clone. The binary vectors pCAMBIA3301, P1-1.5 (full length promoter construct) and P2-1.25, P3-1.15, P4-0.75, P5-0.45, P6-0.35, and P7-0.25 (truncated constructs) were transformed into *Agrobacterium* strain EHA105 by freeze thaw method.

Transient expression analysis of BnPI promoter was carried out by Rapeseed petal bombardment method. Petals bombarded were cultured on MS basal agar medium for 2 days in dark at 25°C, and then were subjected to GUS histochemical staining as described by Jefferson (1986).

Transformation of Arabidopsis was performed following the floral dipping method by Clough and Bent (1998). The presence of the BnPI promoter::*GUS* transgene in the transgenic plants was confirmed by southern blot analysis using *GUS* gene as a probe with genomic DNA extracted from young leaves of T_1 plants as templates. Flowers, leaves, seeds and pods from transgenic plants of each construct were examined for *GUS* expression by histochemical analysis.

Results

Isolation of BnPI promoter

The mRNA levels of *PISTILLATA* gene in leaf, stem, root, petal and seed of B. napus cv. Yeongsan were determined by Northern blot analysis (Fig. 1). PISTILLATA expression was only detected in petal. The promoter region of *PISTILLATA* gene from *B. napus* cv. Yeongsan was isolated by directional genome walking PCR using a set of walker primers and gene-specific primers. Comparison of the sequence of the DNA fragment cloned from *B. napus* with the coding sequence of *PISTILLATA* coding sequence [EMBL: NP12954195] revealed the presence of 1508 bp fragment upstream to the start codon of *PISTILLATA* gene. The 1508 bp fragment upstream to the ATG codon was designated as BnPI full-length promoter (Fig. 1). The nucleotide sequence of BnPI promoter cloned in this study was deposited at NCBI [GQ402148]. The BnPI promoter sequence was analyzed for transcription start site (TSS) and potential *cis*-acting transcription factor binding sites. Neural Network Promoter Prediction identified a TSS at 206 bp 5' to ATG. PlantCARE analysis revealed a potential TATA box at 292 bp 5' to ATG and 32 bp upstream to the potential TSS. TATA box, GATA-motif, an ethylene responsive element (ERE), circadian element and light responsive elements were found in the BnPI promoter (Fig. 2)

Stable expression of BnPI promoter

To characterize the putative BnPI promoter, promoter::ß-glucuronidase (*GUS*) reporter gene fusion constructs were prepared for full length and its 5' deletion fragments, and transformed into *Agrobacterium* (Fig. 3). Functional analysis was carried out by stable expression in transgenic *Arabidopsis*. Two to eleven independent transgenic lines for each construct were screened in T_0 generation lines for each construct were screened in T_0 generation on the Southern blot analysis and histochemical GUS staining (Fig. 4). In case of P-1.5 kb P-1.5 (full-length BnPI promoter), P-1.15 and P-0.75 lines, Petals and calyx was highly stained. However, there was no GUS activity in seeds. The intensity of GUS staining was relatively similar among independent events for each construct (P-1.5 to P-0.75), though leaf and pod of P-1.5, P-1.15 and P-0.75 transgenic plant showed variation in the intensity of GUS stain. In case of P-0.45 and P-0.35 lines, there was no GUS stain in all tissues.

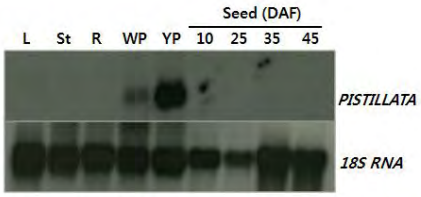

Figure 1. Expression levels of *PISTILLATA* gene in different tissues of *B. napus*. Northern blot analyses were performed with total RNA isolated from leaves, stems, roots, petals and seeds at different stages of maturation. 18S RNA was used as a internal control. L: Leaf; St: Stem; R: Root; WP: White petal; YP: Yellow petal; DAF: Days after flowering.

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-1508  AAATGTAGGGTTTGTGGAGGGAAAGGCCATAACCTAAGAACCTGTCCTAAGTCGAAACCAATGGTCACTAAAGGCATTTC
-1428  CACTAGGTATTATCAGTGTGGAAAATGCGGTGAGAGTGGTCACAATAGTAGAACATGCTAGAAGCTGGCCAGAGTAAAAC
                      Circadian
-1348  CCGCGAGGGTGGTGATGAAGATGGTGTTGGTGAGAGAGCATATGCTTGTGGGTTATGCAAGAACAAGGGATATAATGTAA
-1268  GAACATGTACAAGTAAACGAGCTTCAGACAGTGATTAATTTTTGTTTAGAGCTATAAATCCTCGACAAAACACCTAATAG
            I-box
-1188  TATAACGACCTTATACAGTGACATTTGATACTTTGGTCGGCGCCAGTATCACATATAAACGGTCTCTACCACACAAGTTG
                                G-box
-1108  TCATAAGACCACAAACTTCCAACAAGACTTCGACACGTGGCCTGTTGAAGTGAATCAGAAAACCAGAAACTTGTTTTGTT
-1028  TTCCACAACCAGTTACATTTCTCCCATACTAATTTTAGCATGATAACTCCAAAATCCCAATGGTTATACCAAAGTCTTTG
-948   GTAATTTCACTGCTTCTTTTCTTTTGTTACTTTCTTTCATATCAATCTGGATTTAACTAATCTATATTTATTTCACTCAC
                                ERE
-868   TTCTTTGTCAACATAATATCTTCATTTTTAATAATTTCAAAAGTTTGATTAATATGGGGTAGCTACGATATTCGATAAGA
-788   CCTATATATGACATAGAGTTCTTGAAATAGCAAGTCCTGGAATTACCAAAGTAAGGAACCAGTAAAAAAAGTAGTAGTCC
       CArG motif
-708   TAATTTCGACAGGGGTCATGGTTTAATCTACAAAGTTGCAATAAAAATTATGTTTCCTCTATTTAATATAGGGTTAAATT
-628   GCTATATAGACATATATGGATATTTAGTTGCAAAATGATTCATATATATGAAAATTGTTGAAGATAACTTATTTTTCCTT
                                                                    GATA-motif
-548   TTTGTTATCACTTATCAGCTTCAAATTAGTCTCAATATACAGTCCAGCCAAGAAAACATACTAAGGATAAGACCAGAGGT
                                CAAT-box
-468   TAAAACGACACATTATATGAAGCAAGTGAGATAGACAGAGATGATCAAATCAAATGAAAGCAATAAACATCAATCACAGG
-388   GAAAGAGTGTTAAGCAAATCAACTAATTTTGTTTCAACTTTATTTCATTTGTTCTACACGTTAGTTCAAAGTTTTGTTTT
                  TATA-box
-308   GTTTTCTTTTTGGTAGTGTATAAAGGAGAGAAAATAGAGTTGGCTATATGACTAAGTGAAATAAGAGAACCACAAAAACA
-228   AGCCTTCCATGACTGTGCCATCAAGAAAGTAGCTTTGTTTTCCATCCCAAACTGTCAAAGGTCTCTCTTCATCTATACAT
-148   TAATCAAACACTTTCTCTCTCTCTCATCAATGTTCCTTTGAGACAAATGCTCCTCCCTATCTTCTTCTTCATATAAACCA
-68    CATATCTTCTCCTCCATATCTTAACAATTTCATAGCAAACCCTAAAACTGAGAAGAGAGAGAAAAAAA
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Figure 2. Putative *cis*-elements identified in the *PISTILLATA* promoter from *B. napus*. The *cis*-elements are shown in boxes. Transcription start site (TSS) is indicated in bold. The 5′ upstream sequences of *PISTILLATA* gene is submitted at NCBI [GenBank accession number is GQ402148].

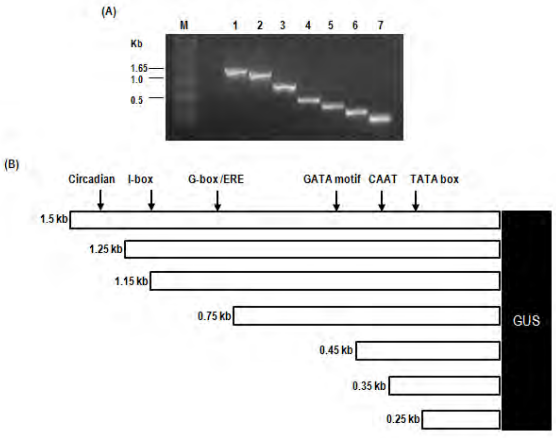

Figure 3. Cloning of BnPI full-length promoter and its deletion fragments in binary vector. (A) PCR amplification of full-length and 5′ deletion fragments of BnPI promoter. M, 1 kb plus Mol. wt. marker; Lanes 1-7, amplicons of 1.5 kb, 1.25 kb, 1.15 kb, 0.75 kb, 0.45 kb, 0.35 kb and 0.25 kb, respectively. (B) Schematic illustrations of BnPI promoter and its deletion fragments. The numbers on the left indicate the 5′ end points of the promoter fragments relative to the ATG. Binary vector pCAMBIA3301 having *GUS* gene driven by *CaMV*35S promoter was used as a positive control.

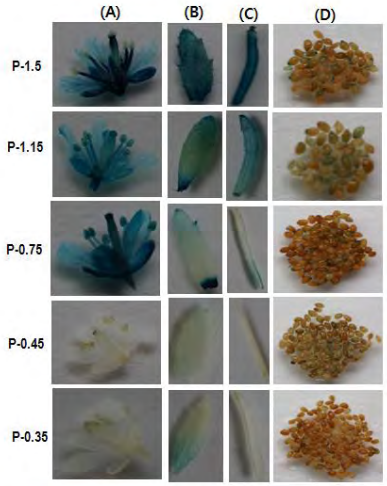

Figure 4. Histochemical analysis of BnPI full-length and its deletion fragments in transgenic *Arabidopsis*. (A) Flower, (B) Leaf, (C) Pod, and (D) Seeds. P-1.5, P-1.15, P-0.75, P-0.45, and P-0.35 represent transgenic plants carrying BnPI full-length promoter P-1.5::*GUS* and its deletion fragments P-1.15::*GUS*, P-0.75::*GUS*, P-0.45::*GUS*, and P-0.35::*GUS* respectively.