

Full Length Research Paper

# Expression analysis of four flower-specific promoters of *Brassica* spp. in the heterogeneous host tobacco

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The 5'-flanking region of ca. 1200 bp upstream of the translation start site (TSS) of a putative cell wall protein gene was cloned from *Brassica campestris*, *B. chinensis*, *B. napus* and *B. oleracea*, and transferred to tobacco via *Agrobacterium*-mediation after fused to promoter-less beta-glucuronidase (GUS) reporter gene. Histochemical GUS staining and fluorometric quantification of the transgenic tobacco showed that all four promoters conferred GUS expression in petal, anther, pollen and stigma of the flower, not in any vegetative organs or tissues of the plants. A series of 5'-end deletion of the promoter from *B. napus* disclosed that the region -104 to -17 relative to TSS was sufficient to confer flower-specific expression, and the region -181 to -161 played a key role in maintaining strong driving power of the promoter. Besides, several enhancer and suppressor regions were also identified in the promoter.

**Key words:** Flower-specific promoter, floral-specific promoter, *Brassica campestris*, *Brassica chinensis*, *Brassica napus*, *Brassica oleracea*, minimal promoter, enhancer, suppressor, coordinated expression.

## INTRODUCTION

Genetic engineering of plants does require not only appropriate target gene but also highly specific promoter with regard to specific spatial and/or temporal expression of the target gene and decrement of the burden of plant growth and biosafety debate. A grand body of organ- and/or tissue-specific promoters, therefore, were cloned, characterized and explored, such as leaf-specific promoter (Gowik et al., 2004), phloem-specific promoter (Husebye et al., 2002), root-specific promoter (Yamamoto et al., 1991), fruit-specific promoter (Pear et al., 1989), pollen-specific promoter (Rogers et al., 2001) and flower-specific promoter (van Tunen et al., 1988). Among organ- and/or tissue-specific promoters, the flower-specific promoter and floral organ-specific promoter have been extensively studied and their cis-acting elements dissected, because that flower serves sexual reproduction and has great market in horticulture worldwide.

The flower has a complex structure consisting of diffe-

rent floral organs such as sepal, petal, stamen and carpel in flowering plants. Almost all floral organs have been subject of floral bioengineering for extending the shelf life, developing new fragrances, breaking color barrier, modifying male and/or female fertility and improving resistance etc. and the bioengineering depends upon, in great extent, flower-specific and/or floral organ-specific promoters and their key cis-acting elements. van der Meer et al. (1990) reported that a 67 bp promoter region of petunia chalcone synthase (*chsA*) gene could direct flower-specific expression and the TACPyAT repeats in the region was important in the specific regulation of the gene in petunia. For bean *chs15* promoter, presence of two cis-acting elements in close proximity to the TATA box was essential for high petal-specific expression of the promoter in transgenic tobacco and point mutation in the H-box element (CCTACC) and G-box element (CACGTG) dramatically decreased *chs15::GUS* fusion gene expression in the floral tissue (Faktor et al., 1996). The region between -1800 bp and -800 bp of the promoter of petunia *EPSPS* (5-enolpyruvylshikimate-3-phosphate synthase) gene was sufficient for petal-specific expression in petunia, but it showed very low expression in the petals of transgenic tobacco (Benfey and Chua, 1989). In

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**Table 1.** Oligonucleotide primers used in promoter cloning and 5' deletion of the promoter\*.

Primer name	Primer sequence
ptlFW	5'- <u>AAGCTT</u> AGCAGCACGAATGAAGTTC-3'
ptlRW	5'- <u>GGATTC</u> TTGTAGTGAGAAAACTCGGGGAA-3'
ptlF1	5'- <u>AAGCTT</u> TCATCATGTCGGAATGAT-3'
ptlF2	5'- <u>AAGCTT</u> AAATTACCCTGTGTATG-3'
ptlF3	5'- <u>AAGCTT</u> GTCGACTAAATTGAAACAG-3'
ptlF4	5'- <u>AAGCTT</u> ACCGCATTTCAGACTGCC-3'
ptlF5	5'- <u>AAGCTT</u> AAGGGCACGTACATACGTTG-3'
ptlF6	5'- <u>AAGCTT</u> TGTTGGCTCCACCAAAC-3'
ptlF7	5'- <u>AAGCTT</u> CTCTAATAATTAGCACAC-3'
ptlF8	5'- <u>AAGCTT</u> CACCTTCTATTTTCAACC-3'
ptlF9	5'- <u>AAGCTT</u> ATCTCACCTTTTTTAAATG-3'
ptlF10	5'- <u>AAGCTT</u> CTCAGTAAATTCAC-3'
pD1FW	5'-CACACCTTCTATTTTCAACCATTTCATC-3'
pD1RW	5'-GAGTGCTAATTATTAGAGAGCCTGG-3'

\*: *Hind* III and *Bam*HI sites added for cloning were underlined.

*Arabidopsis APETALA3* (AP3) promoter, the *cis*-element required for petal-specific expression was located in the region from -224 to -83 while two other regions, from -556 to -374 and from -328 to -83 were both required to confer the full spectrum of stamen-specific AP3 expression (Hill et al., 1998). An "anther box" (GTGA) was discovered in pollen-specific promoters of tomato and tobacco (Twell et al., 1991; Rogers et al., 2001) and it could confer pollen-specific expression of the chimerical promoter in petunia when fused in copies to CaMV 35S promoter (van der Meer et al., 1992). Mariani et al. (1990, 1992) used tobacco anther tapetum-specific promoter TA29 to drive *Barnase* and *Barstar* genes separately and obtained male sterile plants and their restorer lines respectively. These examples demonstrated the importance of floral organ-specific promoters and/or their key *cis*-acting elements in single floral organ improvement. For entire flower improvement, the flower-specific promoter that co-ordinately expressed exclusively in different floral organs (such as sepal, petal, stamen and carpel) may be more interesting.

Here we reported cloning and characterization of flower-specific promoters from 4 species of the genus *Brassica*. The cloned flower-specific promoter co-ordinately directed the expression of GUS reporter gene exclusively in the petal, anther, pollen and stigma of transgenic tobacco flowers.

## MATERIALS AND METHODS

### Plant material

Tobacco plantlets (*Nicotiana tabacum* var. NC89) used for transformation were grown in MS medium (Murashige and Skoog, 1962). Cabbage (*Brassica oleracea* L), Napa Chinese cabbage (*B. campestris* L), Pak choy Chinese cabbage (*B. chinensis* L) and oil-seed

rape (*B. napus* L) materials used for isolation of genomic DNA were grown in the greenhouse.

### Amplification of 5'-flanking sequences

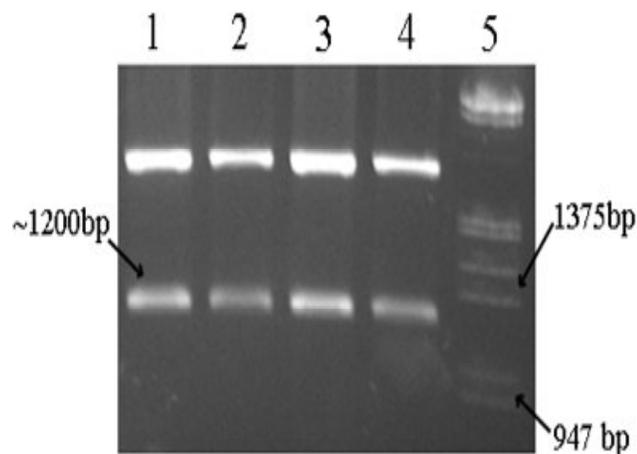
A search of genomic sequence databases using both anther- and/or flower-specific and/or -preferred motifs of promoters identified an oil-seed rape gene coding for putative cell wall protein (GenBank accession number AF213504). The 5'-flanking region upstream of -17 bp relative to the translation start site (SST) of this gene was PCR-cloned with forward primer 5'-AAGCTTAGCAGCACGAATGAA GTTC-3' (ptlFW, *Hind*III site added was underlined) and reward primer 5'-GGATCCTTGTAGTGAGAAAACTCGGGGAA-3' (ptlRW, *Bam*HI added was underlined) from the genomic DNAs extracted from young leaves of cabbage, Napa Chinese cabbage, Pak choy Chinese cabbage and oil-seed rape with CTAB method (Doyle and Doyle, 1987). The PCR product was cloned into pUCm-T vector (Shanghai Shenggong, China) and sequenced to check the identity.

### Construction of promoter::gus fusions

DNA manipulation, including restriction digests, agarose gel electrophoresis, ligation and transformation to *Escherichia coli* strain DH5a were carried out according to Sambrook and Russell (2001). Restriction endonucleases and T4 DNA ligase were purchased from Promega (Madison, WI, USA).

The sequencing-verified 5'-flanking regions were cut from the pUCm-T vector with *Hind*III and *Bam*HI and separately inserted into *Hind*III-*Bam*HI double-cut binary vector pRD410 (Datla et al., 1992) that contains a promoter-less  $\beta$ -glucuronidase (GUS) reporter gene and a NptII selectable marker flanked by the T-DNA border sequence. This gave rise to 4 recombinant plant expression vectors (pBnfs, pBofs, pBpfs and pBcfs) which were verified by enzyme-cut and sequencing.

A series of 5'-deletion of cloned oil-seed rape promoter region (bnfs) were conducted by using PCR with the reward primer described above and 10 forward primers newly synthesized (Table 1). As described above, a *Hind* III site was added to the 5'-end of the forward primer. The PCR products were sequenced and linked into



**Figure 1.** Determination of bnfs, bofs, bpfs, bcfs on the cloning vector by restriction digestion. All vectors containing cloned fragments were digested with *Hind*III and *Bam*HI. Lane 1: bnfs; lane 2: bofs; lane 3: bpfs; lane 4: bcfs; lane 5: DNA marker ( $\lambda$ DNA/*Eco*RI + *Hind*III). bnfs, bofs, bpfs and bcfs: the promoters cloned from *Brassica napus*, *B. oleracea*, *B. campestris* and *B. chinensis*, respectively.

pRD410 as described above to produce 10 plant expression vectors (p769, p469, p340, p294, p245, p227, p199, p159, p136 and p104) which were also enzyme-cut and sequencing-verified.

In order to study the function of a predicted anther-preferred cis-acting element, one internal deletion promoter was constructed by PCR from pUCm294 using primer pair pD1FW and pD1RW (Table 1). The PCR product was autoligated. From the autoligated PCR product, the internal deletion promoter was taken out by *Hind* III-*Bam*HI double-cutting and inserted into pRD410 as described above. This promoter, named pD1, had a region -181/-161 deleted in comparison with p294.

### Plant transformation

All plant expression vectors constructed were separately introduced into *Agrobacterium tumefaciens* strain LBA4404 using a freeze-thaw method (Komari et al., 1996). The transformed *Agrobacterium* were selected on YEB-agar plates with 50  $\mu$ g/ml of kanamycin (Kan) and 100  $\mu$ g/ml of streptomycin (Str), used to infect tobacco leaf discs according to Hirsch et al. (1985). The putative transformants were selected on solid MS with Kan (50  $\mu$ g /ml) and Carbenicillin (100  $\mu$ g /ml) and then PCR-identified. The PCR positive transgenic plants were grown in a growth chamber for rooting at 25- $^{\circ}$ C under fluorescent white light in a 16/8 h light/dark cycle.

Rooted transformants were transplanted in pots in greenhouse and the seeds from at least 11 independent and strong-GUS expressed transgenic plants in each of the constructs were sowed in pots in greenhouse for producing T1 plants.

### Histochemical GUS staining

Vegetative tissues and floral tissues (petal, sepal, anther, pollen, style and ovary) of different developmental stages were sampled from primary transgenic plants, T1 and wild-type plants and were GUS-stained according to Jefferson et al. (1987). After staining, the samples were then cleared of chlorophyll by using 70% ethanol and used for imaging.

### GUS-activity assay

Anthers and petals were ground in a cold mortar with liquid nitrogen. The frozen tissue powder (~100 mg) was supplied with protein extraction buffer (50 mM  $\text{NaH}_2\text{PO}_4$ , pH 7.0, 0.01% SDS, 0.01 M EDTA, pH 8.0, 20% methanol, 0.1% Triton X-100 and 10 mM  $\beta$ -mercaptoethanol) and vortexed to homogeneity. The homogenate was centrifuged at 11,000 g for 15 min at 4 $^{\circ}$ C and the supernatant was used for protein quantification following Bradford (1976) and then for fluorometric assay.

Fluorometric assay of GUS activity was performed with the method of Jefferson et al. (1987) in a reaction volume of 500  $\mu$ l. The reaction was incubated at 37 $^{\circ}$ C. At zero time, an aliquot of 50  $\mu$ l reaction solution was taken out and added to 950  $\mu$ l 0.2 M  $\text{Na}_2\text{CO}_3$  and the same manipulation was performed at subsequent times 10, 20, 30, 45 and 60 min. The GUS Activity was detected in HITACHI F-4500 spectrofluorometer with excitation at 365 nm and emission at 455 nm and expressed as nmol of 4-methylumbelliferone (MU) produced per min per mg protein.

## RESULTS

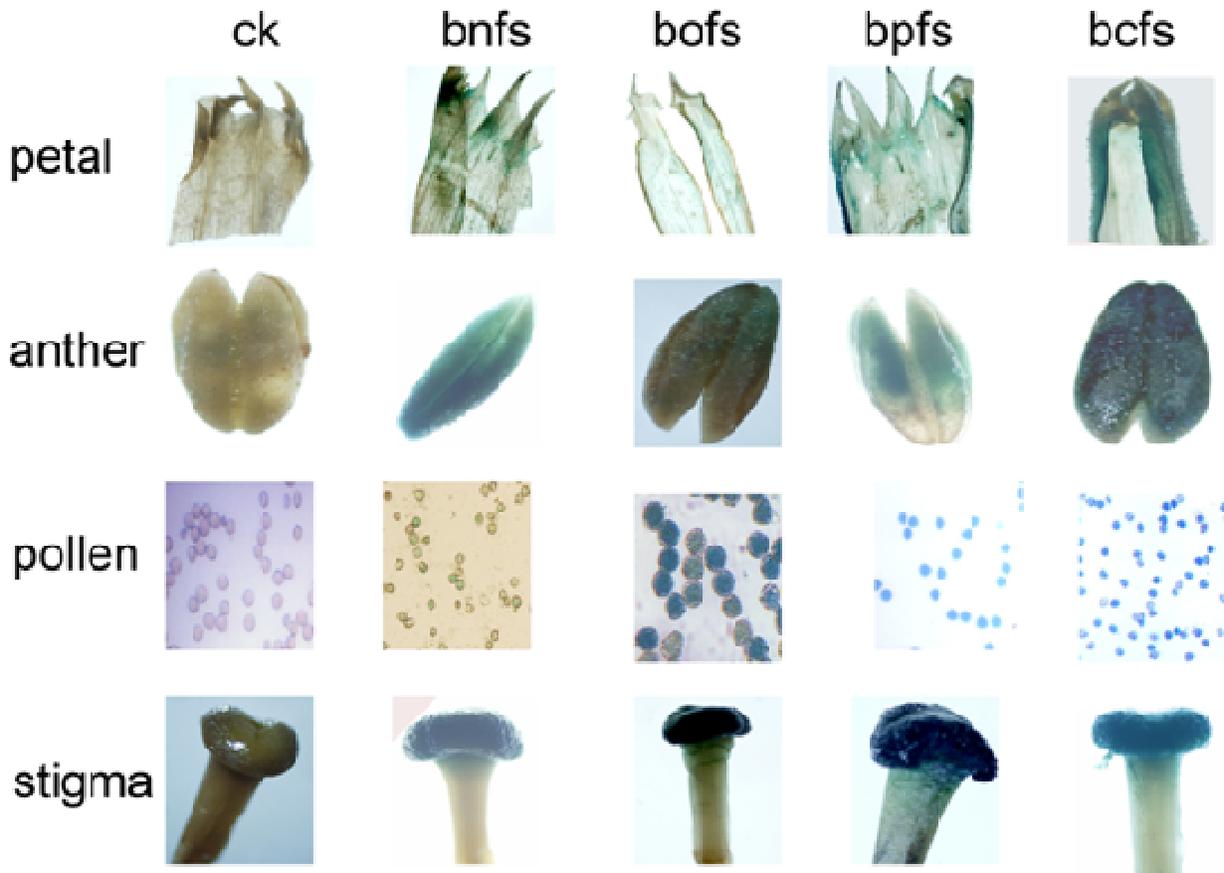
### Cloning and analysis of the 5'-flanking regulatory regions

Four PCR-cloned 5'-flanking regions upstream of -17 relative to the translation start site of the putative cell wall protein gene from *Brassica campestris*, *B. chinensis*, *B. napus* and *B. oleracea* were respectively 1210, 1220, 1221 and 1210 bp in length with a DNA sequence similarity of 96.9% (Xiao et al., 2007), and named bpfs, bnfs and bofs, respectively (Figure 1). The DNA identity between oil-seed rape (bnfs) and cabbage (bofs) was 99.35%, while between two types of Chinese cabbage, bpfs and bcfs, 99.8%. In all 4 fragments the TATA-box, CAAT-box and a number of potential regulatory motifs predicted for promoter regulatory elements with PLACE (Higo et al., 1999) and Plant CARE (Lescot et al., 2002) were present. The putative transcription start site was also predictably located although the actual one was not determined experimentally in each of the putative promoter.

### Construction of plant expression vector and obtaining of transgenic tobacco plants

Ligation of bcfs, bnfs, bofs or bpfs to pRD410 at *Hind*III-*Bam*HI site formed 4 recombinant plant expression vectors, pBcfs, pBnfs, pBofs and pBpfs, respectively. As expected, ligation of series of 5'-deleted bnfs to pRD410 at *Hind*III-*Bam*HI site gave rise to 11 recombinant plant expression vectors, p769, p469, p340, p294, p245, p227, p199, p159, p136, p104 and pD1, respectively. The promoter::GUS expression boxes of these vectors were in frame verified by enzyme-cut and sequencing (data not shown).

More than 20 independent transgenic tobacco plants were obtained in each of the constructs and the transformants were PCR- and Southern blot-confirmed (data not shown).



**Figure 2.** GUS expression in different floral organs of the flowers of transgenic tobacco transformed with each of 4 full-length promoters. The floral organs of the transgenic tobacco plants harboring *bxfs::GUS* chemiric gene (where x = n, o, p or c ) were GUS-stained. *bnfs*, *bofs*, *bpfs* and *bcfs*: the full-length promoters cloned from *Brassica napus*, *B. oleracea*, *B. campestris* and *B. chinensis*, respectively.

### Histochemical GUS staining of *pBcfs*, *pBnfs*, *pBofs* or *pBpfs* -transgenic plants

Independent T0 and T1 transgenic plants of *pBcfs*, *pBnfs*, *pBofs* or *pBpfs* were histochemically assayed for GUS staining at stage of 2 - 4 true leaves and at different flower developmental stages with at least 10 lines for each construct. In all tested transgenic plants of 4 constructs, no visible GUS staining was observed in the vegetative tissues such as leaf, stem and root (data not shown), whereas the petal, anther, pollen and stigma were GUS blue-stained in all 4 constructs (Figure 2), indicating that 4 promoters cloned had highly similar expression pattern and were flower-specific.

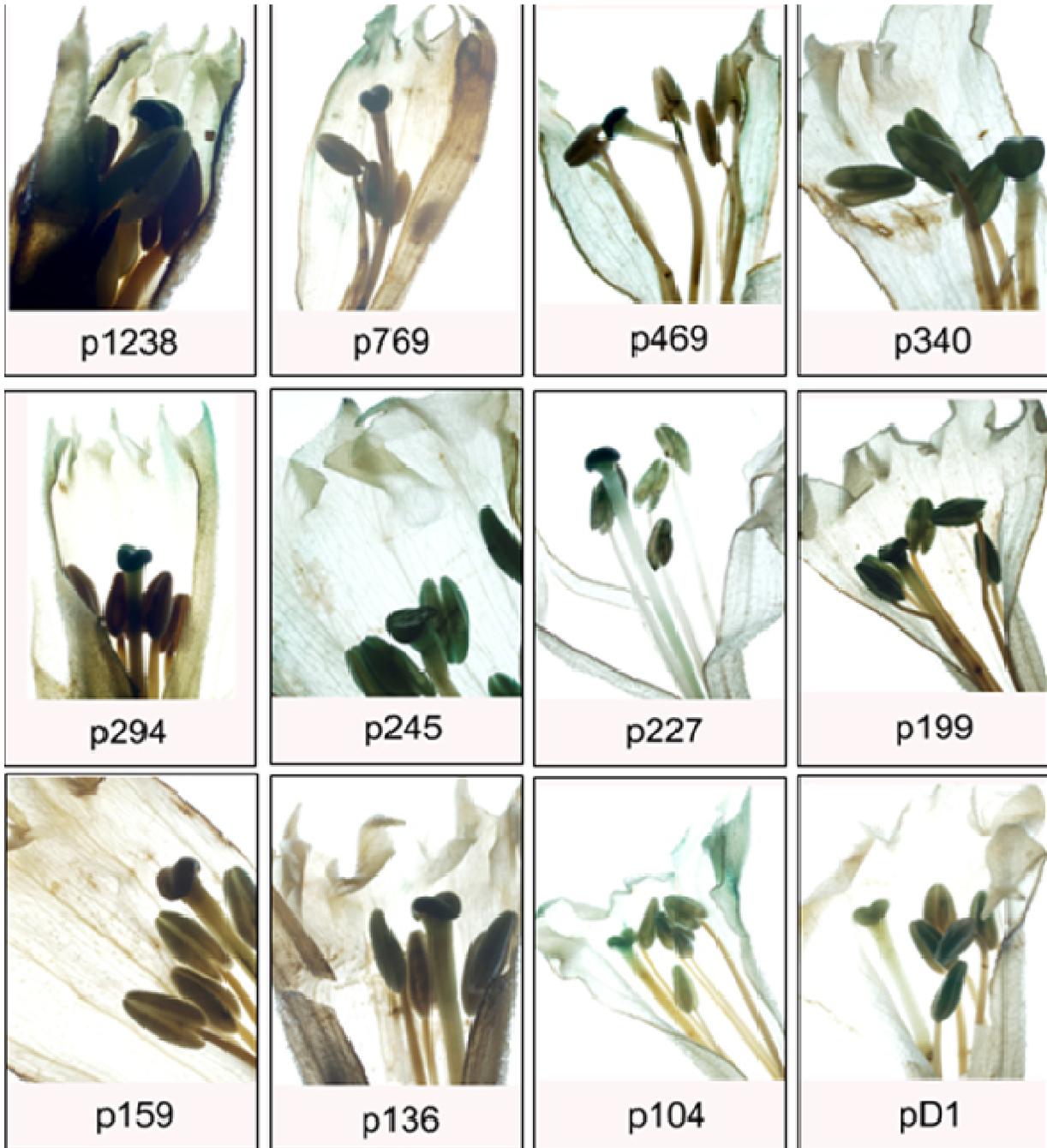
### GUS analysis of 5'-deleted promoter from *B. napus*

Only the promoter from *B. napus*, *bnfs*, -1221 to -17 relative to SST was chosen for 5'-deletion, because of high similarity both in DNA sequence and in expression pattern among the 4 promoters.

First series of 5'-deletion at position -769, -469, -340, and -294 did not change expression pattern of the promoter and removal of 5'-end to -294 remained GUS expression exclusively in petal, stigma, anther and pollen (Figure 3), but not in any vegetative tissues (data not shown) as full-length *bnfs*.

In order to define a minimal flower-specific promoter and to determine putative cis-acting elements in the region -294/-17, the second series of 5'-end deletion were performed at position -245, -227, -199, -159, -136 and -104. In addition, an internal deletion of -294 was also performed. GUS staining of the transgenic plants showed all deleted promoters remained GUS positive in the anther, pollen and stigma, although the staining strength changed (Figure 3). For petal expression, removal of 5'-end to -199 still maintained GUS expression, whereas no GUS expression was detectable when the promoter was further deleted to -159 and to -136. Interestingly, when the promoter was 5'-deleted to -104, GUS staining appeared again in the petal (Figure 3).

Fluorometric quantification of GUS activity in the petal and anther of T0 transgenic plants obtained from the

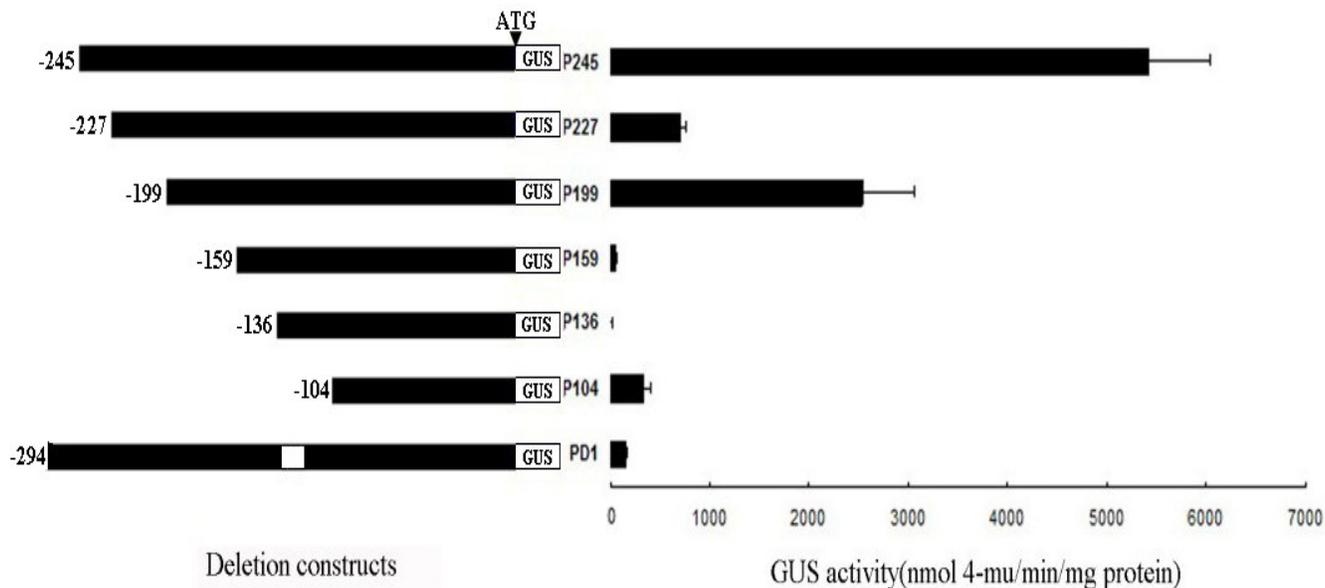


**Figure 3.** GUS expression in different floral organs of the flowers of transgenic tobacco transformed with 5'-deleted or internal-deleted promoters of bnfs. The floral organs of the transgenic tobacco plants harboring deleted- bnfs::GUS chimera gene were GUS-stained. bnfs: the promoter cloned from *Brassica napus*; p1238, full-length bnfs starting from the translation start site; p769-p104: 5'-deleted bnfs starting from -17 relative to the translation start site; pD1: derived from p294 with an internal region -181/-161 deleted.

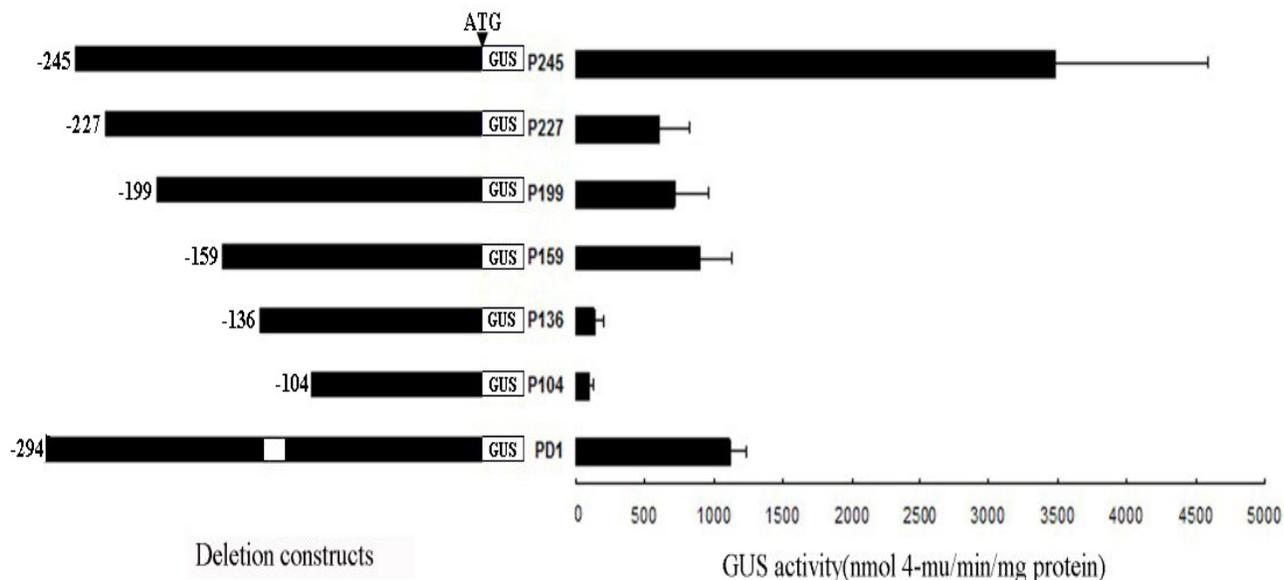
second series of 5' deletion promoters was determined when the flower bud reached 30 mm.

In petal, GUS activity varied with promoter's length (Figure 4). After deletion from -245 to -227, GUS activity was dramatically decreased. Further deletion from -227 to -199, the enzyme activity, however, was increased

about 4 times compared to -227. Deletion from -199 to -159, GUS activity was decreased dramatically again. No obvious difference was detected between -159 and -136. Interestingly, further deletion from -136 to -104 increased the activity of the enzyme. The GUS activity of the construct pD1 was very low.



**Figure 4.** Quantification of GUS activity in the petal of transformed tobacco carrying the 5'-deletion construct or internal deletion construct. The left panel shows a schematic map of 5'-deletion constructions. The white box in p294 represents an internal deletion. The right panel indicates the corresponding GUS activities of each construct. The values are the average of 10 independent transgenic lines for each construct, with triple independent quantifications. The error bars represent standard deviation of the mean.



**Figure 5.** Quantification of GUS activity in the anther of transformed tobacco carrying the 5'-deletion construct or internal deletion construct. The left panel shows a schematic map of 5'-deletion constructions. The white box in p294 represents an internal deletion. The right panel indicates the corresponding GUS activities of each construct. The values are the average of 10 independent transgenic lines for each construct, with triple independent quantifications. The error bars represent standard deviation of the mean.

In anther, GUS activity was a little different from that in the petal (Figure 5). When the promoter was deleted from -245 to -227, GUS activity was decreased about 6 times. Further deletion from -227 to -199 to -159 did not give rise to significant difference in enzyme activity. After dele-

tion from -159 to -136 to -104, the GUS activity was decreased ca. 6-fold, while no visible difference was detected between -136 and -104. The pD1, an internal deletion of p294, remained strong GUS activity in anther which is higher than that from -227 to -104, although 6-

fold less than -245.

## DISCUSSION

In this work, we isolated and functionally analyzed 5'-flanking fragment of ca. 1200 bp upstream -17 relative to the translation start site of a putative cell wall protein gene in 4 species of the genus *Brassica* L. (*B. campestris*, *B. chinensis*, *B. napus* and *B. oleracea*). Although they were highly similar in DNA sequence (96.9%), the fragments could be clustered into 2 groups: *B. campestris* (bpfs) with *B. chinensis* (bcfs) and *B. napus* (bnfs) with *B. oleracea* (bofs), which is consistent with traditional classification of the species. All four fragments had the characteristics and function of promoter predicted and experiment-confirmed and they demonstrated similar expression pattern-flower-specific expression (Figure 2). Each of the promoters coordinately directed *GUS* reporter gene expressed in the petal, anther, pollen and stigma of the flower but not in any vegetative tissues in transgenic tobacco (Figure 2). This implied that the promoter cloned together with its native gene might be one of the rudimentary bases of A, B and C function genes in Meyerowitz's ABC model of flower development (Meyerowitz et al., 1991) and therefore one of the rudimentary bases of AP1-AP3/PI-AG in Ma's model (Ma, 1994) as well as in Levin and Meyerowitz's model (Levin and Meyerowitz, 1995).

A series of 5' deletion analysis of 1221 bp promoter of *B. napus* (bnfs) showed that removal of 5'-end until position -245 did not affect the flower-specificity, nor the expression strength of the promoter (Figure 3). Further deletion to position -104 decreased significantly the expression strength but did not change the expression pattern of the promoter (Figures 3 - 5). This indicated that the fragment -245/-17 had full function of 1221 bp promoter, while -104/-17 was the minimal promoter that starts from 11 nucleotides upstream of the TATA-box.

Sequence analysis with Plant CARE and PLACE showed that several copies of putative pollen/anther-specific motifs were located in the region -245 to -17. The motifs "GTGA" which can enhance pollen specific expression (Rogers et al., 2001) were located at -132, -93 and -25. The sequence "AGAAA", a *cis*-regulatory element required for pollen-specific transcription (Bate and Twell, 1998; Rogers et al., 2001), was located at -29. In consideration of our results that -104/-17 fragment of the bnfs was sufficient for anther-preferred expression, we postulate that the motifs GTGA and AGAAA together may be essential for high-level anther-preferred expression of the promoter bnfs.

Deletion from -245 to -227 resulted in very significant reduction of *GUS* activity in petal and in anther (Figures 4 and 5), which means existence of important enhancer (s) that may confer high-level expression in petal and anther within the 18 bp (AAGGGCACGTACATACGT) region

between -245 and -227. A search of the PLACE database for potential *cis*-element in the 18 bp region revealed a *cis*-element "AAAG" core sequence located at site -246 to -243 and this element was reported to be the target site for different Dof proteins (Dof 1-3 and PBF) in maize (Yanagisawa and Schmidt, 1999). Motif "GTGCCCTT (-)" located at site -245 to -238 was the binding site for "MNF1", later called Dof1 (Yanagisawa and Izui, 1993).

Deletion from -227 to -199 resulted in marketable increment of *GUS* activity in the petal but only slight one in the anther (Figures 4 and 5), which suggested that there might be some suppressors, in particular that for petal-preferred expression in the region between -227 and -199, that is, TGTTGGCTTCCACCAACTTTGG AGGCT. This fragment contained at least four reported *cis*-elements: T-box "ACTTTG" responding to light induction (Chan et al., 2001), RAV1 binding motif "(-) CAACA" of *Arabidopsis* (Kagaya et al., 1999), Dof binding motif "(-)AAAG" and P-box "MACCWAMC" which is a plant MYB binding site. The function of these elements and those unidentified in the suppression of petal-preferred expression of the promoter needs further investigation.

Deletion from -199 to -159 decreased significantly the *GUS* activity in petal, but slightly increased the enzyme activity in anther (Figures 4 - 5). This suggested presence of strong petal-preferred enhancer(s) and of weak anther-preferred suppressor(s) at same time in the 40 bp region between -199 and -159.

Elucidation of the enhancer and suppressor in this 40 bp region is in progress.

It was notable that an internal deletion of a 20 bp fragment (-181 to -161) from -294 promoter greatly decreased *GUS* activity both in anther and in petal (Fig. 4-5) and the enzyme activity in petal was almost abolished. That showed a key role of this 20 bp in promoter's driving power, which was also supported by deletion from -199 until -104. Sequence elucidation of this 20 bp fragment disclosed an E-box motif (CANNTG), also named MYC recognition site, was present in the regions from -170 to -165. E-Box is recognized by the transcription factor bHLH and presented also in some other flower-specific promoters such as *Arabidopsis chs* gene promoter (Hartmann et al., 2005). A MYB motif "TAACAA" was also located at the position -168 to -163. The cooperation of bHLH with MYB could mediate activation of the *CHS* minimal promoter in *Arabidopsis* (Hartmann et al., 2005). Therefore, the cooperation of bHLH with MYB in the 20 bp region -181/-161 might guarantee the strong driving power of the promoter.

In conclusion, we cloned and characterized 4 flower-specific promoters from the genus *Brassica* and identified several enhancer and suppressor regions in the bnfs, a flower-specific promoter from *B. napus*. The promoters cloned and the minimal one elucidated would benefit engineering improvement of flowers via native promoter, and the enhancers and suppressors preliminarily identified, if further elucidated, would be used for construction

of chimeric promoters to overcome the limit of native ones.

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