Transgenic Research **13:** 559–566, 2004.

Characterization and activity enhancement of the phloem-specific pumpkin *PP2* gene promoter

Hongnian Guo†, Xiaoying Chen†, Haili Zhang, Rongxiang Fang, Zhengqiang Yuan, Zhenshan Zhang &Yingchuan Tian*

National Key Laboratory of Plant Genomics, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, China

Received 22 November 2003; accepted 15 June 2004

Key words: *cucurbita moschata*, deletion analysis, enhanced promoter, phloem-specificity, *PP2* promoter

Abstract

The promoter of the pumpkin (*Cucurbita moschata*) *PP2* gene (designated NP) was isolated from the restriction enzyme-digested genomic DNA pool by genome walking and its activity and phloem specificity were examined in transgenic tobacco plants by using GUS as a reporter. Deletion analysis of the promoter revealed that the 473-bp fragment (−465 to +8 relative to the transcription start site; designated as NPII) exhibited similar activity as the full-length NP promoter and retained its phloem specificity. Furthermore, the sequence from −465 to −171 was shown to contain positive regulatory cis-elements for the promoter activity. An enhanced NP promoter was constructed by duplicating the sequence −465 to −85, and its activity in phloem tissue was shown to be higher than that of the Commelina Yellow Mottle Virus (CoYMV) promoter or a chimeric promoter consisting of the double enhancer sequence from the Cauliflower Mosaic Virus (CaMV) 35S promoter fused upstream to the NPII fragment.

Abbreviations: GUS – β-glucuronidase; LUC – Luciferase; NosT – Transcriptional termination sequence of the nopaline synthase gene; NPTII – Neomycin phosphotransferase II; NP – Pumpkin (Nangua in Chinese) *PP2* gene; PCR – Polymerase chain reaction; PP2–Phloem protein 2; RT-PCR – Reverse transcription polymerase chain reaction; 35SP – CaMV 35S promoter

Introduction

Constitutive promoters are widely used in plant genetic engineering to express genes throughout the plant. However, constitutive expression of foreign proteins in transgenic plants may cause some adverse effects, such as the metabolic burden imposed on plants for constant synthesis of foreign gene products and development of resistance in target organisms for example insects, against the *Bacillus thuringiensis* insecticidal crystal proteins produced in transgenic plants (Shelton et al., 2002). It is therefore, in certain circumstances, desirable to use promoters which only express the foreign gene in specific plant tissues or organs. In this regard, phloem-specific promoters have particular advantages to express defensive proteins for several biotechnological applications, e.g., to confer resistance to bacterial pathogens and insect pests, since they usually attack the vascular tissues of host plants.

In the phloem exudates of cucurbitaceous plants, there are more than 100 phloem proteins in which the phloem protein 1 (PP1) and phloem protein 2 (PP2) are predominant. PP1, a 96 kDa protein, is the primary structural

†These two authors contributed equally to this work
*Author for correspondence
E-mail: tianyc@sun.im.ac.cn

protein of phloem filaments and is involved in the formation of slime plugs at the wounded site of vascular tissue (Kleinig et al., 1975), while *PP2* is a 49 kDa poly-GlcNAc-binding lectin and may covalently link to the filaments via disulfide bridges as revealed by *in vitro* studies (Read and Northcote, 1983). Other intriguing functions of PP2 or PP2-like lectins, e.g., in the plant defense system, in intracellular and/or intercellular signal transduction and macromolecule translocation, and in interaction with micro-organisms have been proposed and/or verified (Read & Northcote, 1983; Balachandran et al., 1997; Owens et al., 2001; Gomez & Pallas, 2001; Van Damme et al., 2004). A cDNA clone encoding *Cucurbita maxima* PP2 and the corresponding genomic clone encompassing the upstream sequence and introns were isolated (Bostwick & Thompson, 1993; Bostwick et al., 1994). Analysis of the diversity of PP2 proteins in vascular plants has led to the identification of *PP2*-like genes in species from 17 angiosperm and gymnosperm genera (Dinant et al., 2003). Regarding to the regulation of *PP2* expression, an 1151bp DNA fragment upstream of the PP2 protein coding region from *C. maxima* was identified as a phloem-specific promoter (Thompson & Larkins, 1996). The tissue specific expression of promoters of *PP2*-like genes from Arab idopsis AtPP2-A1 and -A2 has recently been demonstrated in transgenic Arabidopsis and tobacco plants (Dinant et al., 2003). We have previously shown that the *C. maxima PP2* promoter could drive the expression of a reporter gene in the phloem of transgenic tobacco plants albeit with low activity based on the observation of the intensity of GUS staining (Jiang et al., 1999). However, to our knowledge detailed analysis of *PP2* promoters from cucurbitaceous plants has not been reported. We have cloned the cDNA sequence of pumpkin (*Cucurbita moschata*) *PP2* gene (GenBank Accession No. AF150627) by RT-PCR and recently isolated the promoter of this gene. Although the sequence homology of cDNAs or the encoded proteins among *PP2* genes of different *Cucurbita* species [*C.maxima* (Bostwich et al., 1994), *C. pepo* (Wang et al., 1994) and *C. maschata*] is more than 90%, significant differences in their regulatory sequences exist, which probably imply diversity in the regulation of gene expression. In this paper we report the cloning and characterization of the pumpkin *PP2* gene (NP) promoter and show that a reconstituted promoter consisting of the NPII fragment (a deleted form of the NP promoter) with the sequence between −465 and −85 duplicated has an enhanced activity without altering its phloem specificity.

Materials and methods

Cloning of the NP *promoter fragment*

Pumpkin (*Cucurbita maschata* cv. Dunzi) genomic DNA was extracted essentially as described (Paterson et al., 1993). About 0.5 μg genomic DNA was digested with *EcoRI*, *SalI*, *StuI*, *DraI* and *PvuII*, respectively, extracted with chloroform, precipitated by ethanol and then re-suspended in 15 μl TE buffer. The digested DNAs were saved separately as genomic DNA pools. Genomic DNA walking and suppressed PCR were carried out as described (Siebert et al., 1995). The sequences of the DNA adaptors are 5′-TCCTAATACGACT CACTATAGGGCTCGAGCGGCCGCCCGGG CAGGT-3′ (forward), and 5′-ACCTGCCC-3′ (reverse). The NP promoter fragment was amplified using the gene-specific primers GSP-1 (5′-G GCCAAGCTTCACTTCTCCTCC-3′) and GSP-2 (5′-CTCTGGCTTCCCTCT CTTGTTGTC-3′) in combination with a primer of the adaptor sequence, respectively. After gel purification, the PCR product was inserted into pBluescriptII SK^+ vector (Stratagene) and then cloned in *E. coli* DH5α (Gibco-BRL). The positive clones were subjected to DNA sequencing.

Construction of plant expression vectors

A NP promoter fragment from −833 to +8 relative to the transcriptional initiation site (named NPO) with additional restriction sites (a *Hin*dIII site at the 5′ end and a *Bam*HI site at the 3′ end) was obtained by PCR using the clone containing the *C. maschata PP2* gene promoter sequence as a template. A plant expression vector pBLNG which contains two reporter genes was generated by fusing NPO to the β-glucuronidase gene (*gusA*) and the CaMV 35S promoter to the luciferase gene (*luc*). The recombinant plasmid pBLCG (Yuan et al., 2002), as a positive control vector in this study, is similar to pBLNG except that NPO was replaced with the Commelina Yellow Mottle Virus

(CoYMV) promoter which is a strong phloem-specific promoter (Medberry et al., 1992).

Construction of expression vectors with deleted NP promoter fragments

Five NP promoter fragments with progressive 5′ deletions were obtained through PCR amplification and then inserted into the *Hind*III and *Bam*HI sites of pBI121 (Clontech), respectively, by replacing the CaMV35S promoter to form pBNO (containing NPO, −833/+8), pBNI (containing the fragment NPI, −608/+8), pBNII (containing the fragment NPII, −465/+8), pBNIII (containing the fragment NPIII, −270/+8) and pBNIV (containing the fragment NPIV, −171/+8).

Construction of the enhanced NP promoter

Based on the results from the 5′ deletion analysis, recombinant plasmids pBdENP and pBENII were generated as follows. The former contains a recombined NP promoter constructed by doubling the fragment −465/−85 of the promoter fragment NPII, and the latter as a control, contains a chimeric promoter constructed by fusing the doubled CaMV 35S enhancer sequence, a 565-bp *Hin*dIII–*Eco*RV fragment containing doubled −92/−344 sequence of the 35S promoter isolated from pBin438 (Li et al., 1985), to the upstream of NPII.

Plant transformation

Transformation of tobacco (*Nicotiana tabacum* cv. K326) plants mediated by *Agrobacterium tumefaciens* was carried out as described (Horsch et al., 1985).

GUS and LUC activity analysis and GUS histochemical staining of transformed tobacco plants

Total soluble proteins of leaves (with main-vein), leaf blade (without main-vein) and main leaf-vein (mv) were isolated and protein concentration was determined using the method described by Bradford (1976). Analysis for GUS activity by the fluorometric method was performed as described (Jefferson et al., 1987) and LUC activity was assayed as previously described (Yuan et al., 2002). Normalization of GUS activity with LUC activity was performed using the formula shown in the footnote of Table 1.

The GUS activities of transgenic plants transformed with pBNO–pBNIV, pBdENP and pBENII vectors containing only one reporter gene were determined using whole leaf tissues including the main vein. Data of GUS activity were analyzed using the program SPSS12.0.

Histochemical localization of GUS in cross-sections of transgenic plants was carried out as described (Jefferson et al., 1987).

Results

Cloning of the upstream regulatory sequence of NP *promoter and construction of plant expression vector*

Five genomic DNA fragment pools were established by digesting pumpkin (*C. maschata*)

Table 1. Analysis of GUS activity of transgenic tobacco plants.

Transgenic Plant[a]	GUS activity[b]		GUS activity ratio (mv/leaf)	LUC activity[b] (mv)	$\|GUS_i\|$[c]
	leaf	mv			
CP1	2.384	11.920	5.0	1.319	5.350
CP2	2.038	11.309	4.9	1.040	6.437
CP3	0.671	5.368	8.0	0.780	4.074
CP4	1.919	4.798	2.5	0.665	4.271
CP5	0.126	0.563	4.5	0.087	3.831
NP1	4.312	10.78	2.5	0.980	6.512
NP2	0.553	1.991	3.6	0.370	3.186
NP3	0.641	1.795	2.8	0.300	3.542
NP4	0.374	1.384	3.7	0.270	3.034
NP5	0.113	0.441	3.9	0.110	2.373

[a]CP1-5 and NP1-5 are five transgenic tobacco plants of pBLCG (containing expression cassettes NosT-*luc*-35SP – CoYMV promoter-*gus*-NosT) and pBLNG (NosT-*luc*-35SP – NP promoter(−833/+8)-*gus*-NosT), respectively.

[b]GUS activity unit: pmol 4MU/min μg total soluble protein; LUC activity unit: counts/min pg total soluble protein of main vein. mv: the main vein; leaf: the leaf blade without the main vein.

[c]$|GUS_i|$ represents the normalized GUS activity in the main vein of transgenic plant *i*, as calculated by the formula $|GUS_i| = GUS_i \times LUCa/LUC_i$, where GUS_i and LUC_i represent the GUS and LUC activity of plant *i*, respectively, LUCa represents the average LUC activity of all plants determined.

genomic DNA with *Eco*RI, *Sca*I, *Stu*I, *Dra*I and *Pvu*II, respectively. A 1.2-kb DNA fragment was obtained from the *Pvu*II-digested pool by suppressed PCR and then cloned into pBluescriptIISK(+). Sequencing of three clones showed that they all carried the same 1115-bp fragment (GenBank Accession No. AY312402), which contained the *C. maschata* PP2 gene-specific 5′ upstream sequence at the 3′ end and the adaptor sequence used in the suppressed PCR at the 5′ end. A putative 'TATA' box motif 'TATATATA' was found at 65-bp upstream of the initiation codon ATG of the *PP2* open reading frame. This fragment is therefore considered to contain the promoter region of the NP gene. Further experiments by 5′ RACE (rapid amplification of cDNA ends) identified the transcription start site at 37 bp upstream of ATG or 27 bp downstream of the TATA box (data not shown).

In order to analyze the expression properties of the NP promoter, a fragment spanning −833 to +8 relative to the transcription start site was PCR-amplified and inserted into pBI121 upstream of *gusA* to replace the CaMV 35S promoter for the construction of plant expression vector pBLNG. A 35SP-LUC expression cassette was also included in pBLNG to serve as an internal reference marker. The structure of the T-DNA region in pBLNG is briefly indicated in the footnote of Table 1.

Expression of NP promoter-gusA *in transgenic tobacco plants*

Nicotiana tabacum cv. K326 plant was transformed with *Agrobacterium tumefaciens* carrying either pBLNG (containing NPO-*gus* and 35SP-*luc*) or pBLCG (containing CoYMV promoter-*gus* and 35SP-*luc*). For each transformation event, more than 10 transformed plants were first detected by PCR and GUS-histochemical staining, from which five PCR- and GUS staining-positive plants were further analyzed by the fluorometric assays for GUS activity and LUC activity. From the results shown in Table 1, the average GUS activity ratio of main vein (mv) to leaf (without the main vein) in pBLNG-transformed plants was 3.3 ± 0.61 and that in pBLCG-transformed plants was 5.0 ± 1.9, indicating that the NP promoter expressed preferentially in the phloem tissues. Therefore, the NP promoter, like the CoYMV promoter, is also phloem-specific. The normalized GUS activity in mv driven by the NP promoter was about 77.8% of that driven by the CoYMV promoter. To make the NP promoter stronger, further analysis and modification of this regulatory sequence was carried out.

Deletion Analysis of the NP promoter

To study the contribution of different regions of the NP promoter to its expression activity, the NP promoter was progressively truncated from the 5′ end. Five expression vectors containing different promoter fragments (NPO,−833/+8; NPI, −608/+8; NPII, −465/+8; NPIII, −270/+8; and NPIV, −171/+8) fused with the *gusA* reporter gene were constructed respectively (Figure 1a). To ascertain the effects of transgene copy number and insertion position on the resultant average GUS activity, at least 40 PCR-positive transgenic tobacco plants for each of these five vectors were tested for GUS activity as described in Materials and methods. As shown in Figure 1b, the GUS activities were not significantly changed when the NP promoter was deleted from −833 to −465 (pBNO to pBNII) as judged by t-test ($|t| < t0.05$). However, the differences in GUS activity between pBNII-, pBNIII- and pBNIV-transgenic plants were significant. The promoter activity decreased significantly with further deletion from −465 and the 179-bp. This promoter fragment (NPIV) displayed the lowest activity. Clearly, these results suggest that the region between −833 and −465 is not critical for the promoter activity, while the region between −465 and −171 may contain positive regulatory elements. Histochemical GUS staining with these transgenic plants showed that the −465/+8 fragment could still drive phloem-specific expression (data not shown). All these results provided strong basis for our further study of constructing an enhanced NP promoter.

Construction and the activity of the reconstituted NP promoter

Deletion analysis of the NP promoter suggested the presence of positive regulatory elements between −465 and −171. To study the function of this positive regulatory region in enhancing

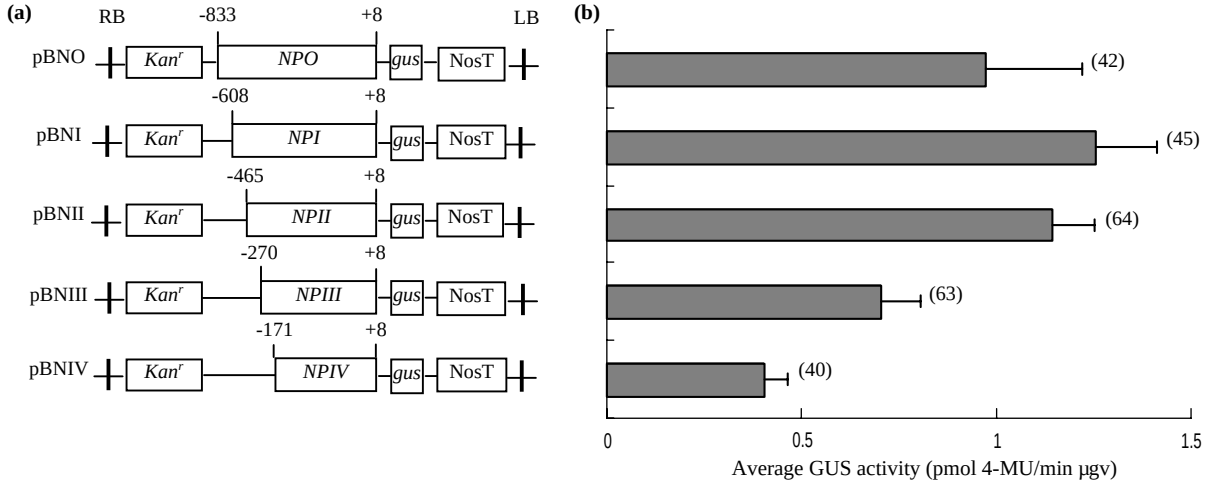

Figure 1. Schematic maps of plant expression vectors with NP promoter fragments of different 5′-end point (a), and the average GUS activity in each group of transgenic tobacco plants (b). The T-DNA right and left border (RB and LB) sequences were represented by thick vertical bars. Kan^r represents the NosP-NPTII-NosT expression cassette which confers kanamycin-resistance in transgenic plants. Horizontal thin lines in (b) represent the standard error of the data for each group analyzed by the program SSPS12.0. tsp: Total leaf (the whole leaf including main-vein) soluble protein. The figures in parentheses in (b) represent the numbers of plants assayed.

the NP promoter activity, an expression vector, pBdENP, was constructed, in which the promoter fused to *gusA* was composed of NPII with the −465/−85 region (NPE) duplicated (Figure 2a). For comparison, another vector pBENII was constructed by inserting the doubled CaMV 35S enhancer (35SDE) upstream of NPII (Figure 2a). Tobacco plants were transformed with these two vectors respectively, as well as with pBI121, pBNO and pBCod1 which is the same as pBI121 except the CaMV 35S promoter was substituted by the −870/+12 fragment of CoYMV promoter (Wu et al., 1999). To obtain a statistically convincing evaluation on the strength of the enhanced NP promoter, more than 32 PCR-positive transgenic tobacco plants for each expression vector were analyzed for GUS activity in leaves by the fluorometric method and the tissue-specificity of GUS expression in each group of plants was examined by histochemical staining. The results of these experiments were shown in Figure 2b and Figure 3, respectively.

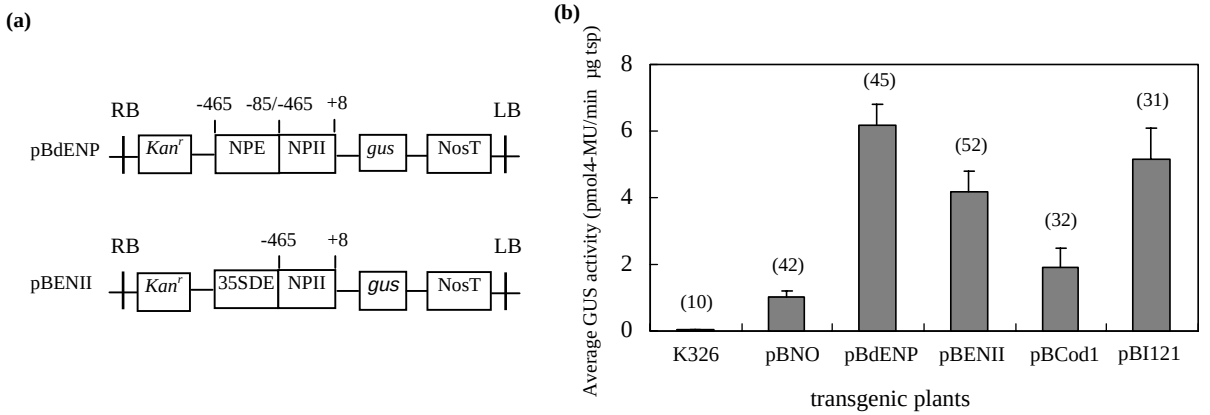

Figure 2. The T-DNA structure of enhanced NPII promoter expression vectors (a) and GUS activity in leaves of five groups of transgenic tobacco plants (b). 35SDE in (a) represents the doubled enhancer sequence of CaMV 35S promoter. K326 is non-transformed tobacco tsp – total leaf soluble protein as described in Figure 1. The vertical bars in (b) represent the standard errors calculated by the program SSPS12.0. The number of plants analyzed for each group is indicated in parentheses.

As shown in Figure 2b, the average GUS activity in pBdENP-transgenic tobacco plants was the highest, being 1.2-,1.5-, 3.2- and 6-fold of that of pBI121-, pBENII-, pBCod1- and pBNO-transgenic tobacco plants, respectively. Though the difference of GUS activities between pBdENP- and pBI121-transgenic plants was not significant, the activity of the former was a little higher that that of the latter, implicating that in phloem tissues the reconstituted NP promoter could be stronger than the CaMV 35S promoter. Statistical analysis of these data indicates that the differences in average GUS activities between transgenic plants of pBdENP with that of the pBENII, pBCod1 and pBNO were all significant at a 95% confidence level. Since the activity of NPII is nearly the same as that of NPO (Figure 1b) the GUS activity of pBdENP-transgenic plants should be about 5-fold higher than that of plants expressing NPII-gus (pBNII). Thus, doubling the −465/−85 region of the NP promoter significantly increased the promoter activity of NPII. This enhancing function is even stronger than that of the CaMV 35S doubled enhancer exerted on NPII (pBENII). Surprisingly, the CaMV 35S doubled enhancer sequence did not change the phloem specificity of the NP promoter as revealed by histochemical staining (Figure 3).

Histochemical staining analyses (Figure 3) showed that unlike the plants expressing 35SP-Gus (pBI121), the GUS activity was strictly localized in phloem of the pBdENP- and pBENII-transformed tobacco plants, similar to that observed in pBCod1-transgenic tobacco plants. Consistent with the results shown in Figure 2b, the results shown in Figure 3 confirmed that the enhanced NP promoter (pBdENP) directs the highest GUS activity in phloem tissues.

Discussion

Though many phloem-specific promoters have been characterized from vascular plants, plant viruses and phytopathogenic bacteria, the unique advantages of using phloem-specific promoters derived from plant genes, are attractive in plant biotechnology. In this manuscript we report the isolation of the NP promoter fragment from genomic DNA pools of *C. maschata* and its characterization in transgenic tobacco plants by deletion analysis and construction of an enhanced NP promoter.

The phloem-specificity of GUS expression could be judged roughly by the ratio of mv/leaf; the higher the ratio, the higher the phloem specificity. The ratio of mv/leaf (Table 1) together with the results of histochemical GUS staining (data not shown) confirmed that the NP promoter drove GUS expression predominantly in phloem tissues, though the specificity was lower than that of the CoYMV promoter.

The purpose of deletion analysis and construction of an enhanced NP promoter was to identity the possible cis-element for improving the promoter activity without changing its tissue

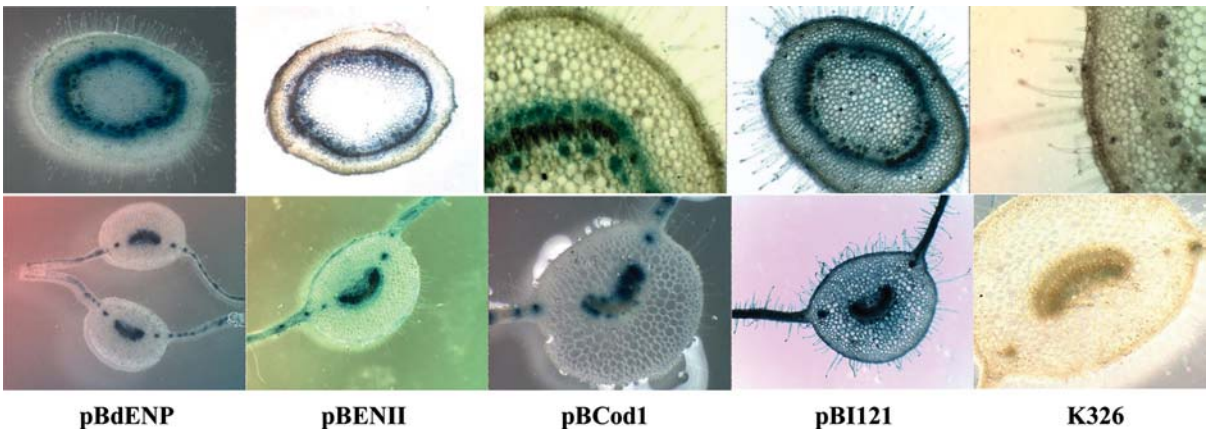

Figure 3. Histochemical staining of cross-sections of stems (upper-part) and petioles (lower-part) in transgenic tobacco plants. The expression vectors used to transform plants are indicated below each picture. K326 refers to non-transformed tobacco plant.

specificity and to evaluate the usefulness of the enhanced NP promoter. In the case of pBLNG, the presence of a strong CaMV 35S promoter directly next to NP promoter-GUS would possibly affect the real expression activity of NP promoter thus making the analysis complicated, therefore a different set of expression vectors, pBNO–pBNIV and pBdENP without an internal reference expression cassette 35SP-luc were used to evaluate the promoter activity in transgenic tobacco plants. In these cases, to obtain a statistically valuable data, a great number of individual transgenic plants were used in the analyses. In Figures 1b and 2b, more than 40 and 32 transgenic plants for each group were analyzed for GUS activity respectively, and the results were statistically analyzed using the program SPSS 12.0. These results indicate the −465/−171 region of NP promoter may contain enhancing elements and is necessary for the promoter activity (Figure 1b); doubling the region of −465/−85 could significantly increase the promoter activity when compared with that resulted from pBNO (−833/+8) (Figure 2b). The strength of the enhanced NP promoter is slightly higher than that of the CaMV 35S promoter (Figure 2b). Considering the 35S promoter is a strong constitutive promoter, the actual activity of the enhanced NP promoter in phloem tissue should be significantly higher than 35SP. It is also interesting to see that the enhanced NP promoter could drive GUS expression at a level of 2.2-fold higher than that obtained with the strong phloem-specific CoYMV promoter (Figure 2b), although the activity of NPII alone is only 77.8% of that of the CoYMV promoter (Table 1). Taken together, the above data showed that by doubling the positive regulatory region we were able to create a modified version of the NP promoter with stronger activity and without altering the phloem specificity. The enhanced NP promoter could be of practical value in biotechnological applications, especially for dicotyledonous plants.

Hehn and Rohde (1998) identified a highly conserved motif 'ATAAGAACGAATC' involved in the phloem strength and specificity when they compared the Coconut Foliar Decay Virus (CFDV) promoter sequence with other phloem-specific promoters from Rice Tungro Bacilliform Virus (RTBV), CoYMV, rolC of *Agrobacterium rhizogenes* and the pea gultamine synthase gene GS3A. Another vascular tissue-specific promoter from a rice GRP gene (Osgrp-2) was also found to contain this conserved sequence (Liu et al., 2003). In all these promoters, this motif is localized upstream of the TATA box. Similar motifs with high homology to the above-mentioned 13-bp conserved sequence were also found 262 bp (−298/−310) upstream of the TATA box in the NP promoter (GenBank AY312402) and 37 bp (−68/−80) upstream of TATA box in the *C. maxima PP2* promoter (Thompson & Larkins,1996). However, the sequence of the *C. maschata PP2* promoter is quite different from that of *C. maxima PP2* promoter (Jiang et al., 1999), even though their *PP2* protein and the cDNA coding sequence share over 90% identity and the sequences downstream of the TATA box in these two promoters share over 80% identity (Bostwick et al., 1994; AY312402). Deletion of this 13-bp conserved motif from NPII resulted in dramatic decrease in GUS activity (Figure 1b) and duplication of this motif and its surrounding sequences significantly increased the GUS activity in pBdENP transgenic plants (Figure 2b). These results imply that the 13-bp motif might be responsible for the enhanced activity of the reconstituted NP promoter, though function of other sequences in −465/−85 in enhancement of the promoter activity could not be ruled out.

Acknowledgement

This work was supported by the National High Technology Program of China (2001AA12071 and 2002AA227031).

References

Balachandran S, Xiang Y, Schobert C, Thompson GA and Lucas WJ (1997) Phloem sap proteins from *Cucurbita maxima* and *Ricinus communis* have the capacity to traffic cell-to-cell through plasmadesmata. *Proc Natl Acad Sci USA* **94**: 14150–14155.

Bostwick DE, Skaggs MI and Thompson GA (1994) Organization and characterization of *Cucurbita* phloem lectin genes. *Plant Mol Biol* **26**: 887–897.

Bostwick DE and Thompson GA (1993) Nucleotide sequence of a pumpkin phloem lectin cDNA. *Plant Physiol* **102**: 693–694.

Bradford MM (1976) A rapid and sensitive mothod for quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**: 248–254.

Dinant S, Clark AM, Zhu Y, Vilaine F, Palauqui J-C, Ku siak C et al. (2003) Diversity of the superfamily of phloem lectins (phloem protein 2) in angiosperms. *Plant Physiol* **131**: 114–128.

Gómez G and Pallás V (2001) Identification of an *in vitro* ribonucleoprotein complex between a viroid RNA and a phloem protein from cucumber plants. *MPMI* **14**: 910–913.

Hehn A and Rohde W (1998) Characterization of cis-acting elements affecting strength and phloem specificity of the coconut decay virus promoter. *J Gen Virol* **79**: 1495–1499.

Horsch RB, Fry JE, Hoffmann NF, Eichholtz D, Rogers SG and Fraley RT (1985) A simple and general methods for transferring genes into plants. *Science* **227**: 1229–1231.

Jefferson RA, Kavanagh TA and Beven MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion maker in higher plants. *EMBO* J **6**: 3901–3907.

Jiang H, Qin HM, Yu HM and Tian YC (1999) Cloning and function of the phloem protein gene promoter from *Cucurbita maxima*. *J Agricultural Biotechnol* **7**: 63–68 (in Chinese with an English abstract).

Kleinig H, Thones J, Dorr I and Kollmann R (1975) Filament formation in vitro of sieve tube protein from *Cucurbita maxima* and Cucurbita pepo. *Planta* **127**: 163–170.

Li TY, Tian YC, Qin XF, Mang KQ, Li WG, He YG et al. (1994) Transgenic tobacco plants with efficient insect resistance. Science in China (Series B), **37**: 1479–1488.

Liu ZZ, Wang JL, Huang X, Xu WH, Liu ZM and Fang RX (2003) The promoter of a rice glycine-rich protein gene, Osgrp-2, confers vascular-specific expression in transgenic plants. *Planta* **216**: 824–833.

Medberry SL, Lockhart BEL and Olszewski NE (1992) The Commelina yellow mottle virus promoter is a strong promoter in vascular and reproductive tissue. *Plant Cell* **4**: 185–192.

Owens RA, Blackburn M and Ding B (2001) Possible involvement of phloem lectin in long-distance viroid movement. *MPMI* **14**: 905–909.

Paterson AH, Brubaker CL and Wendel JF (1993) A rapid method for extraction of cotton genomic DNA suitable for RFLP or PCR analysis. *Plant Mol Biol Rep* **11**: 122–127.

Read SM and Northcote DH (1983) Chemical and immunological similarities between the phloem proteins of three genera of the Cucurbitaceae. *Planta* **158**: 119–127.

Shelton AM, Zhao JZ and Roush RT (2002) Economic, ecological, food safety and social consequences of the development of Bt transgenic plants. *Annu Rev Entomol* **47**: 845–881.

Siebert PD, Chenchik A, Kellogg DE, Lukyanov KA and Lukyanov SA (1995) An improved PCR method for walking in uncloned genomic DNA. *Nucleic Acids Res* **23**: 1087–1088.

Thompson GA and Larkins BA (1996) Phloem-specific promoter. US patent US005495007A.

Van Damme EJM, Barre A, Rouge P and Peumans WJ (2004) Potato lectin: an updated model of a unique chimeric plant protein. *Plant* J **37**: 34–45.

Wang MB, Boultor D and Gatehouse JA (1994) Characterization and sequencing of a cDNA clone encoding the phloem protein *PP2* of *Cucurbita pepo*. *Plant Mol Biol* **24**: 159–170.

Wu B, Pan RQ, Lu R and Tian YC (1999) Deletion analysis and functional studies of the promoter from Commelina yellow mottle virus. *Acta Microbiol Sinica* **39**: 15–22 (in Chinese with an English abstract).

Yuan ZQ, Jia YT, Wu JH and Tian YC (2002) Comparison of three phloem-specific promoters in transgenic tobacco plants. *J Agric Biotechnol* **10**: 6–9 (in Chinese with an English abstract).