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A Novel Bi-directional Promoter Cloned from Melon and Its Activity in Cucumber and Tobacco

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A bi-directional promoter, DP, was cloned by PCR amplification using the genomic DNA of melon as template. Analysis of its *cis*-acting elements in both directions revealed a series of inducible regulatory elements and some enhancer elements. To evaluate its transcriptional activity, DP in both directions was then cloned into vector pBI121 to replace the CaMV 35S promoter. DP in both directions also was inserted downstream of CaMV 35S to investigate whether the double promoter might affect expression of the *uidA* reporter gene at higher levels. Transient expression in cucumber leaves, stems, and fruits as well as in tobacco leaves and stems showed that DP in both directions drove transcription to much higher levels than did the single promoter CaMV 35S. However, activity of the double promoter was lower than the corresponding activity of the single promoter DP in both directions. These results demonstrate that DP is a natural bi-directional promoter, with much more activity than is found with the CaMV 35S promoter. Furthermore, in cucumber and tobacco, it is not suitable to insert DP in either direction downstream of the CaMV 35S promoter to form a double promoter.

Keywords: bi-directional promoter, double promoter, transient transformation, *uidA* reporter gene

Genetic transformation of plants is a popular tool for improving certain characteristics or establishing new traits. Molecular farming is defined as the creation of pharmaceutically important and commercially valuable proteins, with the goal of providing a safe and inexpensive means for the mass production of recombinant proteins. Compared with animal systems, eukaryotic expression in plants is less expensive, easier to scale-up, and safer (Fischer and Emans, 2000). Such a system also can be utilized to produce abundant proteins, e.g., mammalian antibodies (Hiatt et al., 1989). Many strategies have been followed to improve the expressional level of a gene of interest, such as targeting particular proteins into the intercellular space or endoplasmic reticulum (ER) (Conrad and Fiedler, 1998). However, one of the most convenient methods is to utilize promoters with either a high level of transcriptional activity or an enhancer domain for downstream expression. For example, activity of a double CaMV 35S promoter that drives transcription of the GUS gene can be two-fold greater than that of a single CaMV 35S promoter (Suo et al., 2006).

Despite the prevalence of transgenic plants, the most widely used constitutive promoters are still obtained from the Cauliflower Mosaic Virus 35S transcript (Odell et al., 1985) or from the nopaline and octopine synthase genes (An et al., 1988; Ellis et al., 1987). This is because very few endogenous, constitutive plant promoters have been characterized that drive the expression of actin (Zhang et al., 1991), ubiquitin (Callis et al., 1990), or a cryptic promoter (Foster et al., 1999). Although the CaMV 35S promoter is widely utilized in transgenic research, expression varies among plants with the same transgene construct (Mirkovitch et al., 1984). For instance, in stably transformed plants, overall expression of full-size IgG under the control of the 35S promoter can range from 0.35% (Van Engelen et al., 1994) to 1.3% of the total soluble protein (TSP) in tobacco leaves (Hiatt et al., 1989). To overcome this problem, scientists have employed promoters from plants to achieve greater expression of the gene of interest and to lower the variability of inter-transformant expression in the T0, T1, and T2 generations (Chen et al., 2006). However, the limited number of candidates means that screening for stronger promoters is now a crucial issue in transgenic research. Even with the use of highly active promoters, one may find homology-based gene silencing when the same kind of promoter drives expression of multiple genes within a single transgenic plant (Bhullar et al., 2003). Thus, applying bi-directional promoters to drive expression is a desirable approach for transferring multiple genes to generate a transgenic plant with several desirable traits.

Bi-directional promoters have been reported in the human genome (Takai and Jones, 2004) and in other organisms, such as Marek's disease virus (Ding et al., 2004). Artificial bi-directional promoters also have been generated, including one that is multifactorial for high-level expression of transgenes in plants, and which is predictably, stably, and simultaneously expressed in both directions (Chaturvedi et al., 2006). Although not yet fully examined, natural bi-directional promoters are also present in plants, e.g., the oleosin promoter in *Brassica* napus (Sadanandom et al., 1996) and the CaTin1–CaTin1-2 promoter in Capsicum annuum (Shin et al., 2003). Here, we focused on a natural, potentially bi-directional promoter cloned from the melon genome, and investigated its activity in both directions using the *uidA* reporter gene in cucumber and tobacco.

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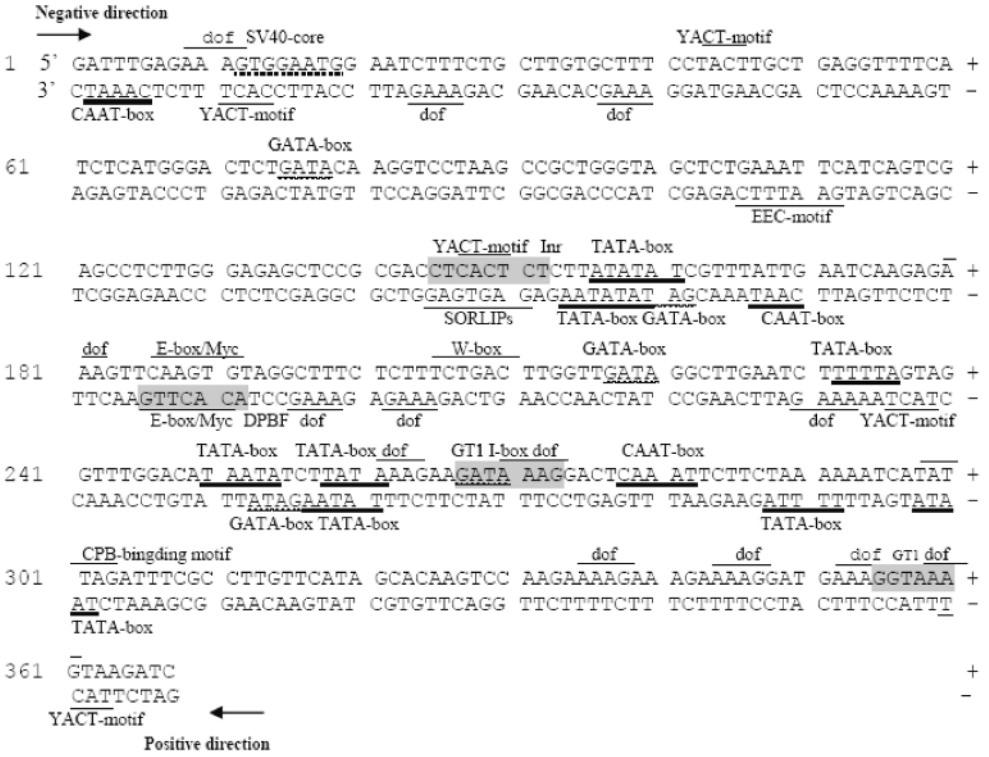

Figure 1. Nucleotide sequence of DP promoter (368 bp). Various *cis*-acting elements are presented on two strands.

MATERIALS AND METHODS

Plant Materials, Strains, and Reagents

Escherichia coli DH5α, the pMD18-T vector, and restriction enzymes were obtained from TaKaRa (Dalian), while *Agrobacterium tumefaciens* EHA105 was kindly provided by Prof. Rongxiang Fang.

Seeds of melon (cv. Ribentianbao) were bought from a local market and germinated in a growth chamber under a 12-h photoperiod at 25/19°C (day/night). When the seedlings had developed two true leaves, genomic DNA was prepared. In addition, the leaves and stems of *Cucumis* inbreeding line P2 were harvested from seedlings with four to six true leaves. Their fruits were obtained at 10 d after pollination. All cucumber tissues were kindly provided by Dr. Shengli Du. Finally, the leaves and stems of tobacco (*Nicotiana tabacum* 'Samsun') were harvested when plants from Professor Defu Chen had acquired six true leaves.

Cloning of the Potential Promoter DP

Melon genomic DNA was prepared from 1 g of young leaves, using cetyltrimethyl ammonium bromide according to the method described by Yamagata et al. (1994). To obtain the DNA fragment for DP, PCR amplification was performed with 1 μg of melon genomic DNA as template plus a forward primer (CM1, TCGCATGCGATCTTACTTTACC) and a reverse primer (CM2, TCGGATCCGATTTGAGAAAG) that were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China) based on the promoter of cucumisin cDNA (Yamagata et al., 2002). PCR conditions were: 94°C for 10 min; then 30 cycles of 1 min at 94°C, 70 s at 55°C, and 100 s at 72°C; followed by a final 10 min at 72°C. The amplified fragment (368 bp) was cloned into the pMD18-T vector (subclone named pMD-DP), then sequenced by Sangon Biotechnology Co., Ltd. (Figure 1).

Vector Constructs and Transformation into *Agrobacterium tumefaciens*

To investigate the promoter activity of DP, we produced four constructs and a binary vector, pBI121, for use as the backbone to generate transgenic constructs. In all cases, the positive direction of DP was defined as going from CM1 to CM2. For constructs pBI-35S-DPF containing DP in the positive direction and pBI-35S-DPR with DP in the negative direction, the DNA fragment DP from vector pMD-DP (digested with *Bam*HI) was inserted into the binary vector pBI121 (Figure 2) at the *Bam*HI site downstream of the CaMV 35S promoter in both directions. Vector pBI-35S-DPF was then digested with *Hind*III and *Xba*I, after which the large recovered fragment was filled in with *Pfu* DNA polymerase according to the method of Yang et al. (2005). Afterward, this filled-in fragment was ligated, using T4 DNA ligase, at 16°C for 12 h to generate the vector pBI-DPF, which contained only the DP promoter in the positive direction but lacked the CaMV 35S promoter. For construct pBI-DPR, containing only the DP promoter in the negative direction, the CaMV 35S promoter in pBI121 was replaced by DP cut from vector pMD-DP at the *Hind*III and *Xba*I sites. These four complete constructs and vector pBI121 were transformed into *A. tumefaciens* EHA105 according to

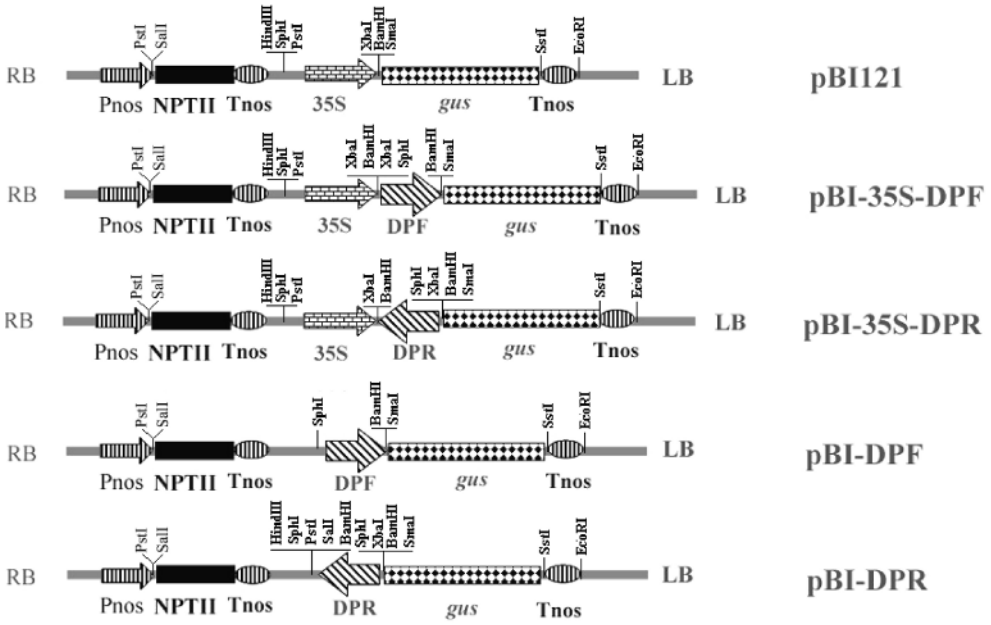

Figure 2. Maps of five constructs. Pnos, promoter from nos gene of *A. tumefaciens*, NPTII; Tnos, terminator from *nos* gene of *A. tumefaciens*; *gus*, β-glucuronidase gene; DPF, DP in positive direction; DPR, DP in negative direction. RB, right border of T-DNA; LB, left border of T-DNA.

the method described by Höfgen and Willmitzer (1988).

Explant Preparation and Gene Transformation

Fruits, leaves, and stems were harvested from cucumber and tobacco plants, then sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 5 min and rinsed five times with sterile distilled water. They were also filter-dried to remove any remaining surface water. Afterward, the cucumber fruits were cut into pieces (0.5 cm × 0.5 cm × 0.2 cm), while the stems of cucumber and tobacco were excised transversely (0.2 cm thickness) and their leaves cut into pieces of 0.5 cm × 0.5 cm. All of these tissues served as our explants.

Agrobacterium tumefaciens EHA105 that either lacked any construct or harbored one of five constructs -- pBI121, pBI-35S-DPF, pBI-35S-DPR, pBI-DPF, or pBI-DPR (Figure 2) -- was used for transformations. A single bacterial colony was first transferred to a liquid LB medium containing 50 mg L^{-1} kanamycin and 50 mg L^{-1} streptomycin, and incubated at 28°C for 2 d. These bacteria were then centrifuged and re-suspended in a liquid MS medium. For gene transformation, explants were infected with this *Agrobacterium* (OD600 ≈ 0.4) for 30 min, then filter-dried to remove any excess. All explants and bacteria were co-cultured for 3 d under darkness in an MS medium containing 0.7% agar.

GUS Histochemical and Fluorometric Assays

After co-cultivation, the explants were washed with sterile distilled water. GUS histochemical assays were performed by immersing samples for 8 h in a GUS staining solution at 37°C, according to the method of Jefferson et al. (1987). Afterward, the samples were de-stained in a 9:1 solution (v: v) of ethanol and acetic acid. Explants that had been infected with a plasmid-free *Agrobacterium* suspension were used as the blanks. Each treatment comprised 25 to 30 explants, and three repeats were conducted.

Up to 30 explants per treatment were frozen and ground in liquefacient N_2 with a sterilized mortar and pestle until homogenized, then transferred into a 10-mL centrifuge tube to which was added 6 mL of icy GUS extraction buffer [50 mM Na_2HPO_4 (pH 7.0), 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% (w/v) sodium lauryl sarcosine (w/v), and 0.1% (w/v) Triton X-100]. This compound was then centrifuged for 10 min at 4000 rpm. The supernatant was used for measuring protein concentration according to the method of Bradford (1976), with bovine serum albumin (BSA) as the standard. GUS activity was determined with emission and excitation filters set at 455 nm and 365 nm, as directed by Jefferson (1987). This experiment was repeated three times.

Data Analysis

We used the following computation formula for GUS activity:

$$\text{GUS activity (pmol MU min}^{-1}\text{ mg}^{-1}) = \frac{''1''}{''2''} \times \frac{1}{''3''} \times \frac{1}{''4''}$$

Where, *FI* was the fluorescence intensity, "1" represented FI min^{-1}, "2" was = FI pmol-1 MU, "3" = sample volume (μL), and "4" was the extract concentration of the total protein (mg μL^{-1}).

Because the value of FI $pmol^{-1}$ MU was invariable in one fluorescence spectrophotometer, GUS activity was calculated as FI * mg^{-1} * min^{-1} (fluorescence intensity per min per mg soluble protein) to represent the relative level of each sample in this study.

Statistical comparisons of data among treatments were made by analysis of variance (ANOVA), and averages were evaluated with a t-test, using Microsoft Excel 2003 software.

RESULTS

Characterization of DP

A 368-bp DNA fragment was obtained by PCR amplification with the genomic DNA of melon as template. The PCR product was sequenced and aligned with sequences in GenBank via the Blastn program (NCBI). No highly similar sequences were found in that database. However, when the promoters were compared with the plant *cis*-element (PLACE) and plant *cis*-acting regulatory element (PLANTCARE) databases, several matches to known regulatory elements were found in both directions of the cloned fragment (named DP). This suggested that DP might drive the expression of genes downstream in two directions (Fig. 1).

The bi-directional DP contained several possible TATA and CAAT boxes (Fig. 1). Additional elements were identified that could generally be divided into four types. These included cis-acting elements similar to those previously described in defense-related and elicitor-responsive genes, e.g., DOF-box, E-box, DPBF-core (Lopez-Molina and Chua, 2000), GT1-core, MYC-core (Abe et al., 2003), and W-box; four cis-acting elements involved in the enhancement of gene expression: CPB-binding motif (Fusada et al., 2005), EEC-motif (Kucho et al., 2003), GATA-box, and SV40-core (Weiher et al., 1983); two elements involved in light-responsive genes: I-box (Terzaghi and Cashmore, 1995) and SORLIPs (Jiao et al., 2005); and elements of Mem1 (mesophyll expression module 1), i.e., the CACT-motif, a key component of Mem1 (mesophyll expression module 1) found in the *cis*-regulatory element in the distal region of the phosphoenolpyruvate carboxylase (ppcA1) of the C4 dicot *Flaveria trinervia* (Gowik et al., 2004). Interestingly, in the negative strand of DP, an Inr element was identified that is especially dependent on the light-responsive transcription of psaDb, but not a TATA box (Nakamura et al., 2002). Therefore, we speculated that this promoter might drive the expression of genes downstream in both directions. Nevertheless, it should be noted that the presence of a particular motif does not alone dictate the activity of a promoter. There is evidence that interaction between elements is necessary for differential activity in some cases, and spacing between elements may also be a factor.

Qualitative Analysis of the Promoter Function of DP in Both Directions

To evaluate whether DP works as a promoter to drive expression of genes downstream, we transferred five constructs (Fig. 2) into different tissues of cucumber and tobacco. After co-cultivation, the explants were GUS-assayed. All tissues – cucumber leaves, stems, and fruits, and tobacco leaves and stems – that had been infected with *A. tumefaciens* harboring pBI-DPF or pBI-DPR showed blue staining whereas no such coloring emerged in the corresponding blank. This indicated that DP could drive the expression of *uidA* reporter gene in both directions at a high level (Fig. 3). Nevertheless, it was difficult to visually distinguish color differences among explants transferred with construct pBI-DPF, pBI-DPR, or pBI121, which made it hard to confirm which had higher driving activity -- DP in either direction or the CaMV 35S promoter.

We also inserted DP in both directions downstream of the CaMV 35S promoter to investigate the relationship between those promoters as well as to monitor activity of the double promoter. Again, no visible differences in coloration were obvious among explants transformed with constructs pBI-35S-DPF or pBI-35S-DPR versus those transformed with constructs pBI-DPF, pBI-DPR, or pBI121 (Figure 3). Therefore, it was difficult to determine the effect of the double

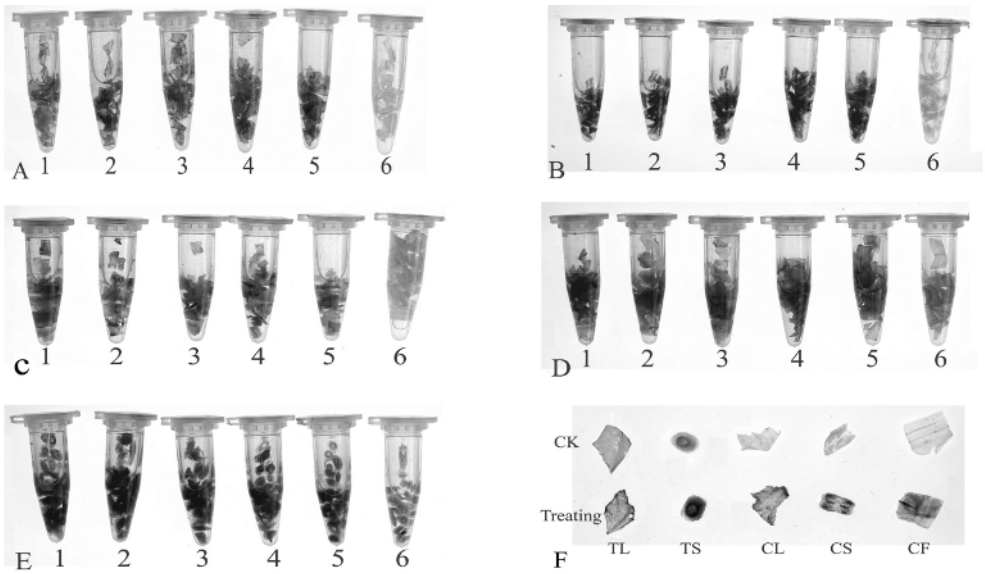

Figure 3. Results of GUS histochemical assays in cucumber leaves, stems, and fruits, and tobacco leaves and stems. Explants 1, 2, 3, 4, 5, and 6 were transformed with construct pBI-35S-DPF, pBI-DPF, pBI-35S-DPR, pBI-DPR, pBI121, or nothing as blank, respectively. Tissues are from cucumber leaves (**A**), stems (**B**), and fruits (**C**); and tobacco leaves (**D**) and stems (**E**). **F**, Explants Letter from all five tissue types were transferred with pBI-DPR or nothing as CK. TL, tobacco leaves; TS, tobacco stems; CL, cucumber leaves; CS, cucumber stems; CF, cucumber fruits.

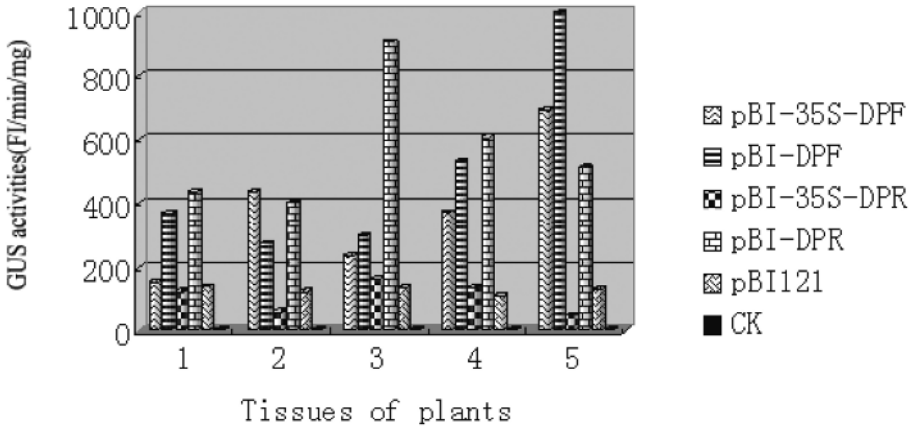

Figure 4. Transient expression of *uidA* reporter gene in cucumber leaves (1), stems (2), and fruits (3), or tobacco leaves (4) and stems (5).

promoter on the expression level of GUS when the CaMV 35S promoter and DP in either direction were combined to drive one gene downstream in any of our experimental tissues.

Quantitative Analysis of DP Activity in Cucumber and Tobacco Tissues

When the sensitive and quantitative fluorometric assay was employed, no remarkable difference was found among GUS activities in different tissues transformed with pBI121 (Fig. 4). This demonstrated that the CaMV 35S promoter acted as a constitutive promoter rather than as a tissue-specific promoter in either cucumber or tobacco. In fact, GUS activity in pBI-DPF and pBI-DPR transformants was 2.23- to 7.86-fold greater than that of pBI121. This indicated that DP in both directions could drive the high-level expression of genes downstream in all five explant types. Moreover, promoter activity was obviously different among tissues, but also much higher than that of the CaMV 35S. GUS activity in pBI-35S-DPF-transformed explants from the leaves and fruits of cucumber and the leaves and stems of tobacco was higher than that measured in the pBI-121-transformed explants, but was lower than that of the corresponding pBI-DPF transformants. Therefore, we conclude that the presence of the CaMV 35S promoter restrained the promoter activity of DP in the positive direction in those particular tissues from cucumber and tobacco. However, for cucumber stems, GUS activity was much higher in pBI-35S-DPF- or pBI-DPF-transformed explants than in those transformed with pBI121, thereby suggesting that the relationship between the CaMV 35S promoter and DP in the positive direction was interdependent. Furthermore, GUS activity in the pBI-35S-DPR-transformed explants was much lower than the corresponding activity of pBI-DPR transformants, regardless of tissue type. Finally, for cucumber leaves and stems and for tobacco stems, GUS activity was lower in pBI-35S-DPR-transformed explants than in those containing pBI121.

Based on these results, we conclude that the relationship between CaMV 35S and DPR is contradictory when the latter is placed downstream of the former to drive one gene in all five tissues of cucumber and tobacco. Therefore, it is not appropriate to utilize the CaMV 35S promoter and DP in both directions to form a double promoter when transferring the expression cassette into cucumber or tobacco to generate transgenic plants.

DISCUSSION

Bi-directional promoters have the potential advantage of simultaneously expressing two genes in an organism, thus halving the number of promoters needed for producing transgenes (Li et al., 2004). Artificial promoter modules that express transgenes at a high level in plants can be generated by combining the motifs that contribute to gene expression (Sawant et al., 2001). Likewise, artificial promoters can be designed to regulate expression in response to a number of elicitors by aborative analysis. Suitable sequence combinations that lack long sequence domains with homology to a particular plant genome can circumvent homology-based gene silencing (Bhullar et al., 2003). Therefore, when these concepts are combined to form bi-directional promoters, one can obtain genetic expression that remains stable and predictable from generation to generation and in response to a variety of external factors (Chaturvedi et al., 2006). Here, we cloned a promoter from the genomic DNA of melon and used it for high-level expression of the *uidA* reporter gene downstream in both the positive and negative direction. This demonstrated that we could develop a bi-directional promoter for both cucumber and tobacco.

The strategy commonly followed for generating artificial bi-directional promoters places a 35S core promoter in a divergent orientation that is positioned upstream of a native 35S promoter; the resultant promoter simultaneously expresses two transgenes. This approach has been further applied for bi-directionalization of other naturally occurring promoters (Xie et al., 2001). Theoretically designed DNA sequences can consist of several putative regulatory sequence motifs (Chaturvedi et al., 2006). One characteristic of these bi-directional promoters is their smaller sequence, with the shortest being just over 400 bp long. However, the bi-directional promoter reported here differs from those in earlier studies in that it originates from the melon genome, has a sequence length of only 368 bp, and does not contain two

exactly replicated sequences, unlike another bi-directional promoter that possesses two native 35S promoters and two minimal promoters (*Pmec*) (Chaturvedi et al., 2006). In contrast, our DP comprises several commonly found small core sequence motifs (Figure 1) that can function as a bi-directional promoter to drive the transcription of genes downstream in both directions at a much higher level than does the CaMV 35S promoter. The lack of any identical long-sequence domains in DP also makes it useful for circumventing homology-based gene silencing in transgenic plants.

When DP in both directions replaced the CaMV 35S promoter to drive transient expression of the *uidA* reporter gene downstream (Figure 4), expression was much higher than that associated with the CaMV 35S promoter (Positive direction: 2.67-, 2.28-, 2.23-, 4.82-, and 7.86-fold greater in cucumber leaves, stems, and fruits, and tobacco leaves and stems, respectively; Negative: 3.17-, 3.32-, 6.80-, 5.51-, and 4.05-fold higher, respectively, in the leaves, stems, and fruits of cucumber, and the leaves and stems of tobacco). These increases may have been related to the arrangement and presence of multiple copies of various *cis*-acting motifs in both directions of DP (Figure 1). For example, four TATA-boxes occurred in the positive direction and six in the negative direction. Likewise, this promoter had two (positive) and one (negative) CAAT-box, a common *cis*-acting element in the promoter and enhancer regions (Shirsat et al., 1989). A CPB-binding motif is the sequence critical for cytokinin-enhanced protein binding *in vitro* (Fusada et al., 2005). Other enhancer elements exist, e.g., an EEC-motif with the sequence GANTTNC and an SV40-core with the sequence GTGGWWHG. The most noteworthy is element GATA, with DP having two in the positive direction and three in the negative direction. Commonly called a GATA-box, it is required for high levels of light-regulated and tissue-specific expression in plants (Lam and Chua, 1989).

Although DP activities in both directions were much higher than those measured for CaMV 35S in our five tissues, they also varied among explant types. This may have contributed to the different numbers of *cis*-elements in the two directions for DP -- such as the GATA-box, with two in the positive direction and three in the negative direction -- as well as the different kinds of *cis*-elements found in DP, such as only one CPB-binding motif in the negative direction. Chaturvedi et al. (2006) have observed the phenomenon of enhanced expression by two reporter genes in response to treatment with SA, NaCl, or IAA in both transiently and stably transformed tobacco leaves, which may determine the presence and arrangement of multiple copies of various *cis*-acting motifs, e.g., a W-box, *as-1*, *OCS cis*-acting elements, and GT-1 box. We also found a series of such inducing elements, including a DOF-box, E-box, DPBF-core, GT1-core, MYC-core, and W-box, which may indicate that the expression level of genes driven by DP in both directions can be somewhat boosted in response to wounding, light, or cold.

Currently, one of the most convenient methods for enhancing the expression level for genes of interest in transgenic plants is to utilize either promoters with high-level transcriptional activity or an enhancer domain. Following this strategy, we placed DP in both directions downstream of the CaMV 35S promoter to generate constructs pBI-35S-DPF and pBI-35S-DPR (Figure 2). Our objective was to elevate the expression level of GUS to some extent under the double promoters. Surprisingly, however, all GUS activity in the pBI-35S-DPF or pBI-35S-DPR-transformed explants always was considerably lower than the corresponding activities of pBI-DPF or pBI-DPR transformants, with one exception -- the pBI-35S-DPF-transformed explants from cucumber stems (Figure 4). This may have been an example of transgene silencing, a universal phenomenon now associated with the extensive application of transgenic biotechnology. Therefore, further experiments are required.

β-Glucuronidase (*gus*), first reported by Jefferson et al. (1987), has been widely used as a reporter gene for studying transcriptional regulation in transgenic plants. Its application is based on the assumption that the level of GUS activity is indicative of the rate of *gus* transcription driven by the particular promoter being investigated (Jeon et al., 1994). Because its histochemical assay is convenient, the *uidA* gene was also used in our study to examine the promoter activity of DP in both directions. Blue staining in all wounded tissues, especially the leaves of cucumber and tobacco (Figure 3f), further demonstrated the value of using *Agrobacterium*-mediated transformation to insert genes of interest into plants. Although GUS is a relatively stable protein, under certain conditions, an increase in the rates of its degradation or inactivation may mask a rise in its transcription rate. This is particularly true during rapid changes in a developmental or metabolic state (Shaul et al., 1999), and it explains why we performed our histochemical and fluorometric assays after the explants and *A. tumefaciens* colonies were co-cultivated for 3 d. An increase in the rates of GUS degradation or inactivation may also have contributed to the generally much lower GUS activity in pBI-35S-DPF- or pBI-35S-DPR-transformed explants compared with the pBI-DPF or pBI-DPR transformants; the exception being the pBI-35S-DPF transformed explants from cucumber stems (Figure 4).

In summary, we believe we are the first to report the cloning of a novel and potential bi-directional promoter from the melon genome, with a goal of driving high-level expression of *uidA* downstream. Because previous methods that utilize an enhancer upstream of a promoter with high activity may not always be effective strategies, we propose that this bi-directional promoter, DP, will be helpful in generating transgenic plants that contain specific genes of interest.

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STATEMENT FOR REQUEST

If any scientist wants to utilize the DP described in this paper, we can provide any of these vectors without hesitation.

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