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RESEARCH ARTICLE

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Construction of a bidirectional promoter and its transient expression in *Populus tomentosa*

Abstract Simultaneous introduction of multiple genes into plants is a critical step in plant genetic engineering to manipulate multiple functional genes in metabolic engineering and trait stacking. It is important to construct a bidirectional promoter for transforming two or more genes into plants simultaneously. The widely used unidirectional CaMV35S promoter has been modified to a bidirectional promoter in this work by fusing a CaMV35S minimal promoter (Pmini) at its end in opposite orientation to the original promoter. To test its bi-directional transcriptional activities, two widely used histochemically visible reporter genes, *gus*A (*β*-glucuronidase) from *Escherichia coli* and *gfp* (Green Fluorescent Protein) from *Aequorea victoria*, were fused to the terminus of the bidirectional promoter in different orientations ending with NOS terminator sequences. The transient expression of the *gus*A and *gfp* genes were detected by histochemical staining for GUS and by fluorescence microscopy for GFP. The direction of transient expression of GUS and GFP in Agrobacterium mediated 3–4 days transformed leaf discs of *Populus tomentosa*, indicating that the promoter did have bidirectional transcriptional activities simultaneously in cells and tissues. It was discussed that this bidirectional promoter could possibly be applied in woody plant engineering.

Keywords *Populus tomentosa*, bidirectional promoter, green fluorescent protein gene (*gfp*), *β*-glucuronidase gene (*gus*A), transient expression

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1 Introduction

Plant growth is the result of gene expression specifically. Gene expression is influenced by many internal and external factors. Transcription is an important step of gene expression that is regulated by many cis-acting elements and trans-acting factors. Transcription starts when RNA polymerase binds to a promoter, a particular sequence of DNA located at the upstream of a gene. A promoter is a crucial cis-acting element recognized by the RNA polymerase, which determines the direction and efficiency of transcription (Zhu, 2004).

Simultaneous introduction of multiple genes into plants is a critical step in plant genetic engineering to manipulate multiple functional genes in metabolic engineering and trait stacking. There are different strategies for this kind of genetic engineering, including 1) fusion of genes to produce fusion proteins with bi-functionality or multiple-functionality, 2) multiple and stacking genes to generate the different enzymes, 3) co-incubation with two or more agrobacteria which contain different vectors harboring different genes to co-transform plants and 4) bidirectional promoter strategy. Homologue sequence, more than 90 bp within two promoters, often leads to gene silencing in transgenic plants (Flavell, 1994). It is necessary to construct a bi-directional promoter to avoid gene silencing and promote genetic engineering. In this research, a bidirectional promoter is constructed based on the CaMV35S promoter. Bidirectional promoter activity is detected in transgenic *Populus tomentosa* by two reporter genes (*gus*A and *gfp*).

2 Materials and methods

Plasmids, bacteria and plant. The plasmids pBI121 and modified p131G are reserved in our lab. *Escheria coli* JM109 and *Agrobacterium tumefaciens* LBA4404 are also supplied by our lab. Leaves are taken from *Populus tomentosa* tissue cultured about 20 days in our lab.

Enzymes and chemicals. Restriction enzymes (*Hin*d III and *Eco*R I), T4 ligase, ExTaq enzymes and pMD 18-T Vector were purchased from TaKaRa Biotechnology (Dalian) Co, Ltd.. Primers were synthesized by SBS Genetech (Beijing). DNA sequencing was performed by Shenyou Biotechnology Co., Ltd (Shanghai). All other chemicals were of the highest available quality from various suppliers in China.

Instruments. The main instruments used in this work are an OLYMPUS BH2 microscope, Nikon E4500 digital camera, PE-9600 (Perkin Elmer) PCR equipment, Beckman Compact High Performance Centrifuge and temperature-controlling shaker.

2.1 Construction of binary expression vector with bidirectional promoter and dual reporter genes

A bidirectional promoter was obtained by fusing a 51 bp CaMV35S Pmini promoter to the CaMV35S promoter on the opposite direction (Xie et al., 2000). The construction of bidirectional and binary expression vector pBDGG was described in Fig. 1. The sequence of 5′ primer D1 was 5′-AAGCTTGGTACCCC-TTCGCAAGACCCTTC-3′, providing a *Hin*dIII restriction site (AAGCTT), a *Eco*R I restriction site (GGTACC) and 17 bp partial Pmini sequence. The sequence of 3′ primer D2 was 5′-GAAGCTTGTCACT-GGATTTTGGTTTTAGG-3′, providing a *Hin*dIII restriction site (AAGCTT) and 22 bp NOS terminator sequence. The polymerase chain reaction (PCR) was performed with p131G as template DNA and D1 and D2 as primers to amplify the Pmini-gfp-NOS sequence (1.1 kb). The PCR followed the standard procedure of 94°C 5 min for predenaturation, 25 cycles of 94°C for 30 s, 67°C for 30 s, 72°C for 1 min, 72°C for 10 min for elongation and 4°C for reservation. The 1.1 kb PCR product was reclaimed and subcloned into the pMD18 T-vector to construct cloning vector pTMGN.

The pTMGN was digested with *Hin*d III and the digested 1.1 kb fragment was ligated to *Hin*d III digested pBI121 to construct the bidirectional and binary expression vector pBDGG. The ligation products were transformed into *E. coli* strain JM109 and selected on the LB culture media with 50 mg/L kanamycine. The transformants were cultivated to obtain the plasmids. The plasmids were identified by *Hin*d III digestion for inserting fragments and *Eco*R I digestion for inserted fragment direction.

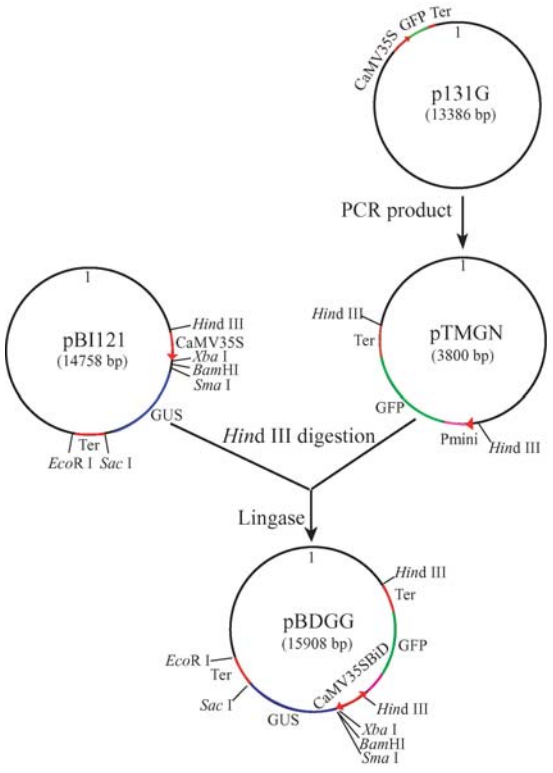

Fig. 1 The construction of bidirectional and binary expression vector pBDGG

2.2 *Agrobacterium* cultivation and transformation

The binary vector pBDGG was transferred into *A. tumefaciens* (strain LBA4404) using the freeze/thaw method (An, 1987). The transformants were selected on the solid YEB culture media with 50 mg/L rifamycin and 50 mg/L kanamycine. The transformants were identified with PCR. *A. tumefaciens* with pBDGG were cultured in liquid YEB culture media with 20 mg/L rifamycin and 50 mg/L kanamycine at 28°C, 200 r/min to OD_{600} 0.6–0.8. *A. tumefaciens* were then further cultured for transforming with 98%–99% liquid YEB without antibiotics at the same condition for 5–6 h until OD_{600} 0.4–0.6.

2.3 Plant tissue culture and transformation

The tissue culture media of *P. tomentosa* for differentiation is MS + 0.5 mg/L 6-BA + 0.2 mg/L NAA. The leaves were cut into 0.5–1.0 cm^2 pieces and soaked in the above cultured *A. tumefaciens* with pBDGG for 15 min. The soaked pieces were cultured on the differentiation media 3–4 d at 26°C in darkness. The unsoaked pieces were cultured at the same condition as control.

2.4 GUS staining assay and GFP visualized detection

The verification of instant expression of *gus*A and *gfp* genes was performed with transformed poplars of 3–4 d. Both *β*-glucuronidase and green fluorescent protein activity detection were using the same samples, except specific indication. GUS activity was histochemically assayed with 1 mmol/L indigogenic substrate (X-Gluc) in 50 mmol/L NaH_2PO_4, pH 7.0 at 37°C for 4 h (Jefferson et al. 1987). The stained pieces were fixed with FAA and bleached until the control whitened. The specific GUS

blue images were histochemically observed under an OLYMPUS BH2 microscope and pictures were taken with a Nikon 4500 digital camera mounted to the fluorescence microscope. The same materials were observed and subjected to ultraviolet light in a lamphouse to initiate GFP activity.

3 Results

3.1 Identification of binary expression vector pBDGG

The pTMGN was digested with *Hin*d III and the digested 1.1 kb fragment was ligated to *Hin*d III digested pBI121 to construct pBDGG, the bidirectional and binary expression vector. The plasmid pBDGG was identified first by *Hin*d III digestion to confirm the existence of the inserted fragment. There was a 1.1 kb fragment (Fig. 2, lane 2) after pBDGG digestion with *Hin*d III and there were no fragments (Fig. 2, lane 3) in pBI121 with the same digestion. The result showed that the 1.1 kb fragment was inserted into pBI121 at *Hin*d III site.

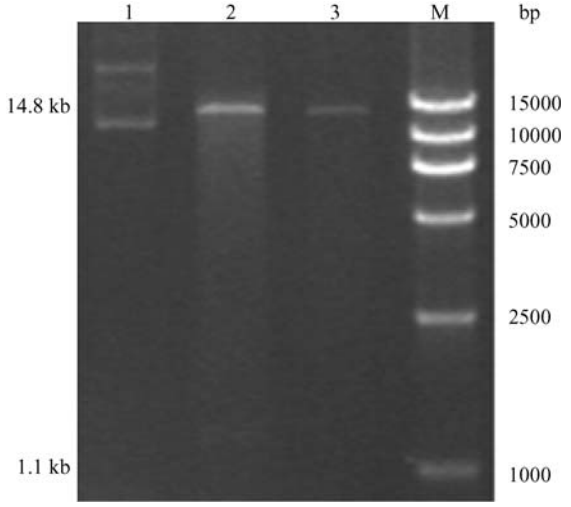

Fig. 2 Identification of binary expression vector pBDGG by *Hin*d III digestion.
Lane 1: plasmid pBDGG. Lanes 2 and 3: pBDGG and pBI121 digested by *Hin*d III

In constructing pBDGG, the single enzyme Hind III was used to digest pBI121 to make the cohesive ends uniform. Although there was an inserted fragment in the ligation product, the inserted fragment direction was not clear. There were two *Eco*R I sites in pBI121 and the inserted DNA by analyzing their sequence. The direction of inserted DNA was identified by *Eco*R I of pBDGG. If the direction of inserted fragment was correct, there were two 12 kb and 3 kb bands after the digestion (Fig. 3, lanes 3–4). If the direction of inserted fragment was wrong, there were two 11 kb and 4 kb bands after the digestion (Fig. 3, lanes 1–2). The results of plasmid pBDGG digested with *Hin*d III and *Eco*R I showed that a binary expression vector was obtained.

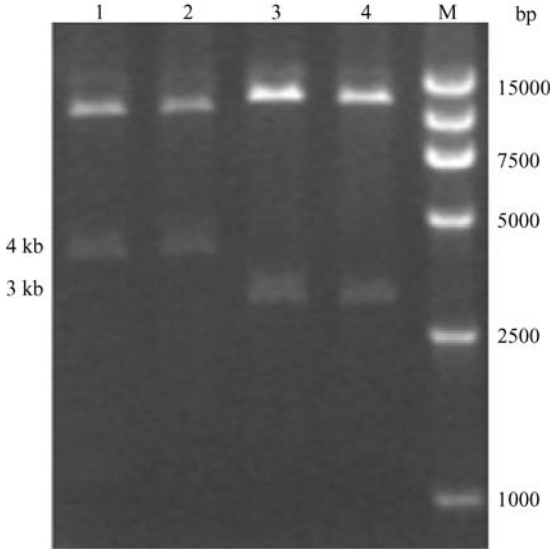

Fig. 3 Identification of the insertion DNA orientation of pBDGG.
Lanes 1–2: the recombination plasmid with wrongly orientated DNA insert; Lane 3–4: the recombination plasmid with correctly orientated DNA insert.

3.2 The detection of *gus*A instant expression

The vector pBDGG is a plant transformation binary vector facilitating a bidirectional promoter fusing dual histochemically visible and detectable reporter genes *gus*A and *gfp*. The activity of the constructed bidirectional promoter was confirmed by instant expression in transgenic *P. tomentosa of gus*A and *gfp*. GUS activity (as shown in blue at Fig. 4(b)) could be easily observed around the wound of leaf pieces after 3–4 d transformation. There was no GUS activity at the control (Fig. 4(a)). The result showed that the constructed bidirectional promoter had one directional activity in the *gus*A gene direction.

3.3 The detection of *gfp* instant expression

The *gfp* instant expression was detected at the excitation of ultraviolet light using the same material. The green fluorescence excited by GFP (Fig. 4(d)) can be easily distinguished from the red fluorescence excited by chloroplast between transgenic plants and wild type (non-transgenic plants, Fig. 4(c)). The *gfp* gene expression showed that the constructed bidirectional promoter had another directional activity in the *gfp* gene direction.

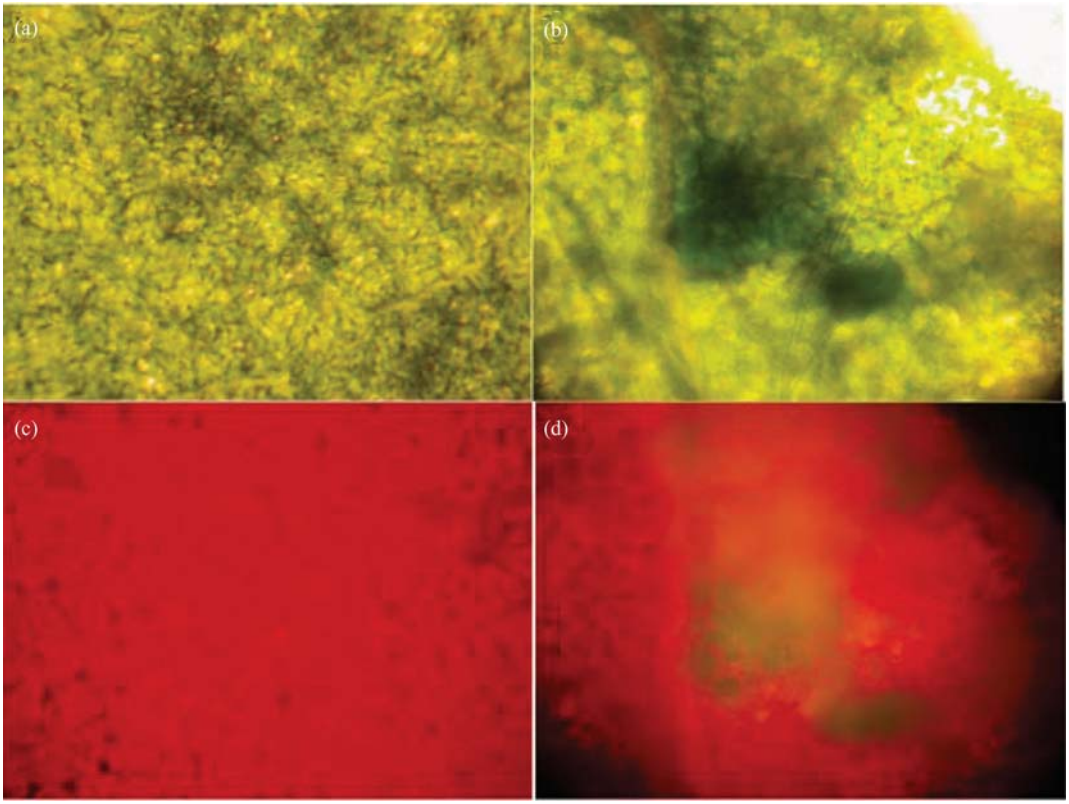

Fig. 4 The instant expression of *gfp* and *gus*A gene fused with bidirectional promoter in *P. tomentosa*
The control leaf staining with X-Gluc (a) and red fluorescence under UV light (c). GUS staining (b) and GFP fluorescence (d) in transgenic poplar leaf.

4 Discussion

The green fluorescene and GUS activity detected in transgenic *P. tomentosa* show that the constructed bidirectional promoter has bidirectional activity in woody plants. The result is consistent with the transgenic *Nicotiana tabacum* and *Arabidopsis thaliana* (unpublished). The bidirectional promoter can be used in tree genetic engineering to improve the transgenic efficiency by one bidirectional promoter with two or more functional genes (one missile with two satellites).

The bidirectional promoter fusing with different functional genes cannot only be used to transform more genes simultaneously, but also to express the genes more efficiently. The transformed genes may express individually or in coordination to improve transgenic plant properties. The transformed plant properties sometimes have no obvious improvement because the transformed genes may be silent or may not be the key genes. Sorbitol content in transformed tobacco of D-glucito-6-phosphate dehydrogenase gene (*gut*D) and mannitol-1-phosphate dehydrogenase gene (*mtl*D) was increased and the plants exhibited the ability to accumulate mannitol. The salt tolerance of tobacco was also improved (Tarczynski et al., 1992; Mitchell, 1993; Liu et al., 1995a, 1995b, 1996). The plant properties of transformed *mtl*D/*gut*D genes were better than the transformed single gene (Liu et al., 1995a). The salt tolerance of transformed plants of America black poplar × cathay poplar hybrid (Fan et al., 2002a) and 84K poplar (Fan et al., 2002b) with *mtl*D/*gut*D genes was increased differently because of the different loci and different copy number in transgenic plants. The results were also consistent with transgenic rice (Wang et al., 2000). Transgenic rice with *mtl*D/*gut*D genes grew in 0.75% NaCl and produced seeds, but the control rice died only after 10 d at the same condition (Wang et al., 2000). Thus, transforming two genes in osmosis molecular synthesis may have a better effect than transforming only a single gene because the two genes may behave coordinately at metabolism. Transgenic plants have better improvement using the bidirectional promoter fusing with different functional genes than a single gene.

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