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RESEARCH ARTICLE

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Cis-acting elements analysis and function detection of xylem specific promoter from *Populus*

Abstract The cis-acting elements of JCesAP, YCesAP, and MDCesAP were analyzed by the PLACE database, the results showed that three sequences all had a core element and upstream promoter element containing TATA-box and CAAT-box. The sequences related xylem-specific and wound-defense functions were found in YCesAP and MDCesAP; and the "core xylem gene set" in JCesAP, indicating that three sequences were xylem-specific and provided with wound-defense functions. Meanwhile, some other elements were also detected, including some tissue-specific elements and some regulative ones that were induced by light, temperature, and water. For investigating their functions, three sequences of JCesAP, YCesAP, and MDCesAP were respectively combined with the report gene GFP, and those reconstructed GFP genes were respectively transferred into the genome of *Populus tomentose* by *Agrobacterium*-mediated transformation method. The detected results of GFP expressions in hygromycin B resistant calli of *P. tomentosa* showed that three sequences all possessed the promoter functions with tissue-specific characteristics.

Keywords *Populus*, promoter, cis-elements analysis, function detection

1 Introduction

Poplar plantations occupy a great geographic range in China, and the genus makes important contributions to meeting the global need for paper, timber, and other wood-based products. However, according to recent statistics, a great loss to the forestry production is brought by serious pests, especially wood boring and leaf eating larvae. Hu (2006) estimates that in China alone, an average annual occurrence area of the forest pests and diseases reaches 8 million hectare and causes economic losses of more than 50 billion RMB. Coleopteran pests are a constant threat to poplar trees, and definitely, they are the most important pests in China, causing a decrease in wood quality or defoliations that result in slower growth rates. It is urgent to prevent and control the poplar insect pests especially Cerambycidae.

Genetic engineering technology is often used in protection of plant diseases and insect pests. Specific promoters are used to regulate and control the foreign gene expressing time-specially, tissue-specially, and quantitatively; it is an important strategy often used in recent years to study transgenic plants (Potenza et al., 2004).

Xylem cellulose synthase (CesA) gene will be expressed specifically in the xylem during the normal growth of plants, but when the trees are subjected to some external pressure, the gene will be expressed in both xylem and phloem (Wu et al., 2000). According to the 5′ untranslated region of xylem cellulose synthase of *Populus tremuloides* (Wu et al., 2000), three sequences of JCesAP, YCesAP, and MDCesAP were cloned from Canada poplar (*Populus canadensis* Moench. - "1"), *Populus canadensis* cv. 'I-214', and triploid Chinese white poplar (*Populus tomentosa* Carr.-93) respectively in earlier studies (Zhang et al., 2006, 2009). The main purpose of this paper is to find cis-acting elements in JCesAP, YCesAP, and MDCesAP and to screen the xylem-specific promoters of three sequences. If the xylem-specific promoters can be used to incorporate Coleoptera-resistant genes into poplar genomic DNA, these genes will be expressed in the xylem specially. It will not only promote the development of poplar beetle-resistance breeding but also provide a new effective approach to regulate and control the stem-boring pests especially Cerambycidae.

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2 Materials and methods

2.1 Experimental materials

2.1.1 Plant materials

Tissue culture plantlets of *P. tomentosa*, conserved by the College of Forestry, Agricultural University of Hebei, Baoding, China, were used as the material for detecting the function of the candidate promoters.

2.1.2 Bacterial strain and plasmids

Agrobacterium tumefaciens strain AGL-1 and three plasmids, pJCesAPGFP, pYCesAPGFP, and pMDCesAPGFP reconstructed from pCambia1302 plasmisds, in which the 35S promoters of report gene GFP were replaced by JCesAP, YCesAP, and MDCesAP, respectively (Zhang et al., 2006, 2009), were preserved in the College of Forestry, Agricultural University of Hebei.

2.2 Methods

2.2.1 Analysis of promoter sequences

The potential cis-acting elements of the three sequences were analyzed by the PLACE program (Plant cis-acting regulatory DNA elements) online (Higo et al., 1999; Yu et al., 2007; Zong et al., 2007).

2.2.2 *Agrobacterium tumefaciens*-mediated genetic transformation system

The plant expression vectors, pJCesAPGFP, pYCesAPGFP, and pMDCesAPGFP (Zhang et al., 2006, 2009), were transformed to *A. tumefaciens* strain AGL-I by electroporation (Horsch, 1985). Leaves of tissue culture plantlets of *P. tomentosa* were used as explants and transformed by *Agrobacterium*-mediated transformation method (Dence, 1992). The leaves transformed by the pCambia1302 containing *CaMV35S*-GFP were used as a positive control, and nontransformed leaves were used as a negative one.

The coculture medium was MS_0, the differentiation medium was MS + TDZ ($1.0\ mg \cdot L^{-1}$) + NAA ($0.1\ mg \cdot L^{-1}$); and the selective medium was differentiation medium + hygromycin B ($10\ mg \cdot L^{-1}$) + cefotaxime sodium ($400\ mg \cdot L^{-1}$).

2.2.3 GFP activity assay

Lecia DM 4000 B fluorescence microscope was used for detecting the activity of GFP gene expression of the slices of hygromycin resistant calli.

3 Results

3.1 Analysis of candidate promoter sequences

3.1.1 Analysis of transcriptional regulatory modules

The cis-acting elements of the three sequences were detected by the PLACE database. Results showed that JCesAP, YCesAP, and MDCesAP all had the core elements and upstream promoter element, including TATA-box and CAAT-box, and therefore, the three sequences were presumed to be the promoter sequences.

3.1.2 Analysis of xylem-specific elements

Plant MYB transcription factors contain an MYB-conserving region concerned with plant development and metabolic regulation broadly. These factors are especially important in the regulation of plant phenylpropanes secondary metabolism and the regulation of the lignification progress of the plant vascular tissues (Tian et al., 2007). Lignin is a major representation in the xylem vessel elements and phloem fibers and secondary wall-thickening formed by the induction of development, stress, and pathogen infection. It plays an important role in plant vascular tissue differentiation and defense system (Lu and Song, 1999). Lignin synthesis can be influenced by phenylalanine ammonialyase (PAL) activity. PAL is the key enzyme during the process of phytoalexin biosynthesis and lignification. An *MYB* binding site was predicted in YCesAP and MDCesAP by the PLACE, which was related to the vascular tissue lignification progress, and another binding site for OsTBP2 was also found in YCesAP. The binding site for OsTBP2 was found in the promoter of rice PAL gene encoding phenylalanine ammonialyase; OsTFIIB stimulated the DNA binding and bending activities of OsTBP2 and synergistically enhanced OsTBP2-mediated transcription from the pal promoter, as shown in Table 1.

3.1.3 Analysis of wound-induced cis-acting elements

When plants are wounded by outside forces or damaged by the pests, they produce some small molecular substances and polysaccharides that induce a series of defense genes expressed as signal substances. These defensive genes not only promote wound healing but also inhibit the invasion of pathogens. Some of these defensive genes, as related cis-acting elements, contain the sequence of P-box, A-box, L-box (Logemann et al., 1995; Zhang et al., 2007), and so on. In addition, according to concerning references (Gao et al., 2005; Su et al., 2007), the cis-acting element W-box [(T)(T)TGAC(C/T) sequence] could be combined with the WRKY proteins specifically, whose expression was induced mainly by pathogens, injury, and signal molecule SA (Eulgem et al., 2000). W-box with a higher frequency

Table 1 Distribution of xylem-specific elements

elements and sequences	function	site in fragment		
		JCesAP	YCesAP	MDCesAP
MYBPLANT MACCWAMC	plant MYB binding site	–	753(+)	20(+)
XYLAT ACAAAGAA	cis-element identified among the promoters of the core xylem gene set	320(−)	–	–
TATABOXOSPAL TATTTAA	binding site for OsTBP2	–	298(+)	–

in the promoter genes was related to disease resistance, wound, and plant defense responses (Ulker and Somssich, 2004). The similar elements were predicted by the PLACE database in the three sequences (Table 2).

In addition, the target site for trans-acting StDof1 protein controlling guard cell-specific gene expression was also found in the three sequences.

In Tables 1 and 2, it can be seen that the cis-acting elements related to xylem-specific and wound-defensive functions were detected in YCesAP and MDCesAP. Although there were no such elements in JCesAP, some other ones were found and identified among the promoters of the "core xylem gene set". The three sequences were preliminary proven to be xylem-specific and provided with wound-defense functions. It was also reported in related references that (G/C) (G/C) TATG sequence (Yuan et al., 2002) was probably related to the phloem tissue-specific promoter and appeared in JCesAP and MDCesAP.

3.1.4 Plant hormone-induced cis-acting elements

Plant hormones regulate plant cell elongation, division, and differentiation, and they also control the progress of plant growth, development, dormancy, germination, rooting, flowering and fruiting, fruit-ripening, and so on. The plant hormones can not act on DNA directly; each must be bound to its receptor and activate its receptor protein first. Activated receptor proteins then initiate the expression of the specific genes, thus leading to a series of physiological responses. The hormone-induced cis-acting elements were also found in the three sequences, which are shown in Table 3.

Auxin and cytokinin are the two main kinds of hormones in the regulation of vascular tissue differentiation. The former is necessary for the cambium formation and tracheid differentiation and has an influence on xylem and phloem differentiation, while the later can increase the tissue sensitivity to auxin. Cytokinin regulates the vascular tissues differentiation with auxin mainly in the early stages of development (Aloni, 1995). The elements were found in MDCesAP including auxin response factor (ARF) binding site and the ones similar to ARF, the auxin-induced element found in YCesAP. "CACGCAAT" sequence showed a constitutive activity with TGTCTC element, conferring inducibility to the auxin, and TGACG motifs were found in many promoters and involved in transcriptional activation of several genes by auxin and/or salicylic acid. Many ARR1-related binding sites were predicted in the three sequences, indicating that auxin and cytokinin synergistically induced xylem and phloem differentiation in MDCesAP and YCesAP. Additionally, a number of ABA, ethylene, and GA responsive element were also predicted in the sequences, as shown in Table 3.

3.1.5 Other cis-acting elements

Some regulative elements were also detected in the three sequences induced by light, temperature, and water through the PLACE database. These regulative elements, such as I-box, LTRE, and CCAAT box play a crucial role in sensing a variety of stress signals.

3.2 Function detecting of candidate promoter sequences

3.2.1 Calli induction

After coculture with *A. tumefaciens* for 48 h, the explants were grown on the selective medium for another 20 days.

Table 2 Distribution of wound-induced cis-acting elements

elements and sequence	function	site in fragment		
		JCesAP	YCesAP	MDCesAP
BOXLCOREDCPAL ACCWWCC	box-L-like sequences in carrot (D.c.) PAL1	–	615(+)	–
−300CORE TGTAAAG	−300 element core; P-box	–	–	704(+)
ACGTABOX TACGTA	A-box	–	477(+) 477(−)	–
WBOXNTERF3 TGACY	W-box, maybe involved in activation of *ERF3* gene by wounding	–	156(+) 291(+) 226(−)	156(+)
WBOXNTCHN48 CTGACY	W-box, NtWRKY1,2,4 bound to W-box	–	155(+)	155(+)

Table 3 Distribution of hormone-induced cis-acting elements

elements and sequence	function	site in fragment		
		JCesAP	YCesAP	MDCesAP
ARFAT TGTCTC	auxin response factor binding site	–	–	439(−)
SEBFCONSSTPR10A YTGTCWC	similar to the auxin response element	–	–	439(−) 466(−)
CACGCAATGMGH3 CACGCAAT	auxin-inducibility	–	70(+)	–
ASF1MOTIFCAMV TGACG	*ASF-1* binding site	–	370(+)	–
ARR1AT NGATT	cytokinin regulator gene *ARR1* binding site; *ARR1*-binding element	41(+) 335(+) 352(+) 476(+) 617(+) 740(+) 434(+) 592(+) 597(+) 602(+) 727(+) 62(−) 181(−) 195(−) 533(−) 581(−) 630(−) 735(−)	163(+) 771(+) 488(+) 581(+) 716(+) 100(−) 116(−) 403(−) 428(−) 656(−)	88(+) 201(+) 520(+) 750(+) 360(+) 503(+) 774(+) 665(+) 172(−) 195(−) 274(−) 283(−) 348(−) 457(−) 472(−) 578(−) 717(−)
DPBFCOREDCDC3 ACACNNG	*ABA*-responsive element	–	572(−) 678(−)	622 (+)
MYB1AT WAACCA	*MYB* binding site; *ABA*-inducibility	–	752(+) 757(+) 128(+) 252(−)	550(−)
ERELEE4 AWTTCAAA	ethylene responsive element	–	358(+)	–
PYRIMIDINEBOXHVEPB1 TTTTTTCC	*GA*-responsive element	–	693(−)	–
ARFAT TGTCTC	auxin response factor binding site	–	–	439(−)
SEBFCONSSTPR10A YTGTCWC	similar to the auxin response element	–	–	439(−) 466(−)
CACGCAATGMGH3 CACGCAAT	auxin-inducibility	–	70(+)	–
ASF1MOTIFCAMV TGACG	ASF-1 binding site	–	370(+)	–
ARR1AT NGATT	cytokinin regulator geneARR1 binding site; ARR1-binding element	41(+) 335(+) 352(+) 476(+) 617(+) 740(+) 434(+) 592(+) 597(+) 602(+) 727(+) 62(−) 181(−) 195(−) 533(−) 581(−) 630(−) 735(−)	163(+) 771(+) 488(+) 581(+) 716(+) 100(−) 116(−) 403(−) 428(−) 656(−)	88(+) 201(+) 520(+) 750(+) 360(+) 503(+) 774(+) 665(+) 172(−) 195(−) 274(−) 283(−) 348(−) 457(−) 472(−) 578(−) 717(−)
DPBFCOREDCDC3 ACACNNG	ABA-responsive element	–	572(−) 678(−)	622(+)
ERELEE4 AWTTCAAA	ethylene responsive element	–	358(+)	–
PYRIMIDINEBOXHVEPB1 TTTTTTCC	GA-responsive element	–	693(−)	–

A few hygromycin B resistant calli could grow up and differentiation on the selective medium. Nontransformed plants would be albino and die (data not shown).

3.2.2 GFP expression in transgenic calli

Green fluorescent protein (GFP) is a special protein derived from jellyfish (*Aequorea victoria*) and sea pen (*Renilla reniformis*), which absorbs blue light and emits green fluorescence without exogenous substrates or cofactors. Such fluorescence can be easily detected with a fluorescent microscope.

Nontransformed calli emitted red fluorescence (Fig. 1a), and high levels of GFP fluorescence could be observed in the positive control with pCambia1302 (Fig. 1b). Also, the green fluorescence was observed in local tissues of the calli transformed by *A. tumefaciens* containing pJCesAPGFP, pYCesAPGFP, and pMDCesAPGFP respectively (Fig. 1c, d and e). Therefore, it seemed that the three fragments had promoter function with tissue-specific characteristics.

4 Discussion

Promoters act like a “switch” to regulate and control the expression of gene in plants accurately. With the wide application of plant transgenic technology, it has become necessary that specific promoters of plants are cloned and

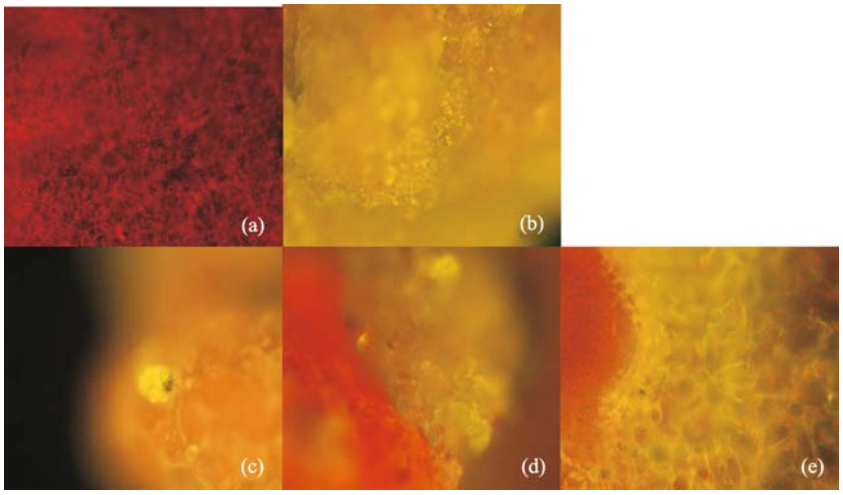

Fig. 1 GFP detection in calli of transgenic plants by fluorescence microscope (400×)
Note: (a), (b), (c), (d), and (e) represent nontransformed calli, calli transformed by *A. tumefaciens* containing pCAMBIA1302, calli transformed by *A. tumefaciens* containing pJCesAPGFP, calli transformed by *A. tumefaciens* containing pYCesAPGFP, and calli transformed by *A. tumefaciens* containing pMDCesAPGFP, respectively.

analyzed in the research of plant genetic engineering (Lu et al., 2004). The cis-elements of JCesAP, YCesAP, and MDCesAP were analyzed by PLACE program online, and the GFP activity of the resistant calli was detected and analyzed, indicating that the three sequences were tissue-specific and offered wound-defensive functions.

It has been reported (Zhang et al., 2007) that when P-box, A-box, and L-box respond to the wound, they need additional cooperating elements. Although P-box, A-box, and L-box were found in the three sequences, their co-operative elements were not found, and therefore, their wound-inductive response needs further research.

Besides the above specific elements, other elements with specific expression regulation ones were also detected in the three fragments induced by light, temperature, and water. Integrated analyses showed that the minimum active units and the specific elements can be used to do a deletion analysis of the three fragments, which still needs further study.

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