CLONING AND EXPRESSION ANALYSIS OF BPLSPL2, A NOVEL SBP-BOX GENE FROM BETULA PLATYPHYLLA

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ABSTRACT
A cDNA representing the squamosa promoter binding protein-box genes (SBP-box gene), BplSPL2, containing the full coding sequence was obtained from Betula platyphylla by 5′ rapid amplification of cDNA (5′RACE). Sequence analysis showed that BplSPL2 was 925 bp in length with a 525-bp open-reading frame encoding 175 amino acids. By comparing the expression patterns of BplSPL2 and BplSPL1 using quantitative real-time PCR, we determined that the expression of the former is inflorescence-specific and that it is temporally expressed during inflorescence development. Overall, the 2 SBP-box genes exhibit different expression patterns.


Keywords: Betula platyphylla, BplSPL2, cloning; expression

Introduction
Genetic and molecular studies have uncovered a large number of genes that control different steps in flower development, including flowering time, flower meristem, and organ identity. In particular, the ABC model has been proposed for the specification of floral organ identity (5), and the discovery of the D and E function genes has enhanced the understanding of the regulatory network for floral development (6, 16). Presently, considerable advances have been made to elucidate the genetic mechanism of floral development in many species.

Squamosa-promoter binding protein (SBP) was first identified in Antirrhinum majus and was assumed to be involved in the regulation of early flower development because the SBP-box can bind with the promoter of floral meristem identity gene Squa/AP1 (11). SBP-box genes encode proteins sharing a conserved DNA-binding domain of 79 amino acid residues sufficient to bind to a palindromic GTAC core motif (2, 11). Thus far, SBP-box genes have been identified in many plants. They play critical roles in regulating flower and fruit development as well as other physiological processes (1, 9, 12, 15). In Arabidopsis, constitutive expression of SPL3 can cause early flowering (4); SPL8 affects pollen sac development and acts as a local regulator in a subset of GA-dependent developmental processes (20, 24); SPL14 functions as a transcriptional regulator that is involved in not only sensitivity to FB1 but also the development of normal plant architecture (19); SPL9 and SPL15 control shoot maturation in Arabidopsis under microRNA regulation (17, 18, 23).

BpSPL1 is the first SBP-box gene cloned from Betula pendula. It is expressed in inflorescences, shoots, and leaves. Over-expression of BpSPL1 has no effect on the phenotype in Arabidopsis, and Southern blot analysis has revealed that there are at least 2 SBP-box genes in B. pendula (14), indicating the existence of other SBP-box gene(s) associated with flower and fruit development. In this paper, we report the cloning of a novel SBP-box gene, BplSPL2, expressed by Betula platyphylla based on an expressed tag sequence (EST; DQ012539, containing the entire 3′ coding sequence) that was derived from a temporal subtracted cDNA library (22), along with a primary study on its expression.

Materials and Methods

Plant materials
At an interval of 2 days, female inflorescence was sampled from birch forest in the yard of Northeast Forestry University from April 30th to June 2nd in 2004, and male inflorescence was sampled from June 14th to August 6th in 2004. The samples were separated into 7 groups of female inflorescence (F1 to F7) and 5 groups of male inflorescence (M1 to M5) according to the developmental stage (Table 1). The samples were quickly frozen with liquid nitrogen after sampling and stored at -70°C before extraction of total RNA.

RNA isolation and cDNA synthesis
The total RNA of B. platyphylla inflorescence was isolated using a modified hexadecyltrimethylammonium bromide (CTAB)/phenol method from different tissues at various developmental stages (21). The integrity of total RNA was verified by electrophoresis on a 0.8% agarose gel, and its quality and amount was measured using a UV-spectrometer (Eppendorf).

First-strand cDNA synthesis was conducted using the SMART™ RACE cDNA Amplification Kit.

Cloning of the full-length coding sequence of BplSPL2
The following gene-specific primer (GSP) and nested GSP were designed from the sequence of the EST (DQ012539) to perform 5′ rapid amplification of cDNA ends (5′ RACE) using SMART™ RACE cDNA Amplification Kit.

Inflorescence sampling periods

<table>
<thead>
<tr>
<th>Stage</th>
<th>Sampling periods</th>
<th>Developing status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1 of female inflorescence (F1)</td>
<td>April 30th-May 2nd</td>
<td>Pollination completion</td>
</tr>
<tr>
<td>Stage 1 of female inflorescence (F2)</td>
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<td>Pollen tube elongation</td>
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<tr>
<td>Stage 1 of female inflorescence (F3)</td>
<td>May 9th-May 11th</td>
<td>Pollen tube elongation</td>
</tr>
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<td>Stage 1 of female inflorescence (F4)</td>
<td>May 13th-May 15th</td>
<td>Ovule development initiation</td>
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<tr>
<td>Stage 1 of female inflorescence (F5)</td>
<td>May 17th-May 19th</td>
<td>Ovule developing</td>
</tr>
<tr>
<td>Stage 1 of female inflorescence (F6)</td>
<td>May 21st-May 23rd</td>
<td>Meiosis initiation</td>
</tr>
<tr>
<td>Stage 1 of female inflorescence (F7)</td>
<td>May 25th-May 27th</td>
<td>Sac formation</td>
</tr>
<tr>
<td>Stage 1 of male inflorescence (M1)</td>
<td>June 14th-June 16th</td>
<td>Stamens anlage formation</td>
</tr>
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<td>Stage 1 of male inflorescence (M2)</td>
<td>June 21st-June 26th</td>
<td>Filament developing</td>
</tr>
<tr>
<td>Stage 1 of male inflorescence (M3)</td>
<td>July 3rd-July 10th</td>
<td>Archesporium formation</td>
</tr>
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<td>Stage 1 of male inflorescence (M4)</td>
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<td>Sporogenous cell formation</td>
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the SMART RACE cDNA kit (BD Biosciences) with the primers GSP2 (5′-AGTAAATAGGTGATACAGAACAAGG-3′) and NGSP-2 (5′-CTGATGAGTGCCTGTGCCCTTG-3′), according to the manufacturer’s instructions. The 5′ RACE products were ligated into the pGEM-T vector and sequenced on the ABI PRISM 3730 DNA sequencer, using which a pair of primers was designed for end to end PCR, and a final PCR reaction was performed.

To test the spatial specificity of the target gene expression, we prepared cDNA from root, stem, and leaf samples after total RNA isolation and reverse transcription. Real-time PCR (RT-PCR) was performed with the positive control of cDNA obtained from various inflorescence materials at different developmental stages and DNA sample.

Temporal expression analysis of BplSPL2 in inflorescence tissue

To prepare the outer reference for quantitative real time PCR (qRT-PCR), we amplified a 375-bp fragment of BplSPL2 using the primer GSP2 and another gene-specific primer, GSP1 (5′-GCTCAAGGTGTCTGCTGTGGTGGG-3′).

After purification with a PCR purification kit (Promega), the concentration of the product was determined by using a UV-spectrometer. Then, the PCR product was diluted by 5 grds (function as standard), i.e. 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹, and 10⁻¹⁰ to obtain the postulated copies of approximately 10⁵, 10⁴, 10³, 10², and 10, respectively. Using cDNA (50 ng) templates for every developmental stage, we performed qRT-PCR at 95°C for 1 min, followed by 94°C for 30 sec, 68°C for 30 sec, 72°C for 1 min, and 82°C for 1 sec, for a total of 40 cycles.

Results and Discussion

Sequence analysis of BPLSPL2

The isolated target cDNA designated as BplSPL2 (Genbank accession no. AY921636) is 925 bp in length, with an open reading frame of 525 bp encoding 175 amino acids. The deduced amino acid sequence of BplSPL2 shares a 62% identity with that of Gossypium hirsutum SBP transcription factor (Genbank accession no. AA V51939), 56% with Arabidopsis thaliana SPL4 (Genbank accession no. CAB56583), and 68% with Betula pendula BpSPL1 (Genbank accession no. CAD90157).

Moreover, sequence analysis in the DNA level revealed the existence of 1 intron in length of 1713 bp within BplSPL2.

To determine the phylogenetic situation of BplSPL2, we constructed a phylip tree (Fig. 1) with TreeTop based on the SBP domain sequences of the majority of SBP-box genes from 4 kinds of angiosperms in the public database. The results indicate that the phylogenetic tree is markedly similar to that constructed by Cardon (3).

It is clear that BplSPL2 and BpSPL1 belong to the same clade. Moreover, the N-terminal of BplSPL2 is acidic in character, as determined by isoelectric point calculation and, therefore, could be involved in transcriptional activation.

BpSPL1 is the first SBP-box gene cloned from B. pendula; its nucleotide sequence is similar to Antirrhinum SBP2 and Arabidopsis SPL3, but it does not contain an intron typical to all other known SBP-box genes (14). In the phylogenetic tree constructed by Guo, SBPs were classified into 2 groups on the basis of the sequence of the SBP domain for Arabidopsis and rice, and group II consisted of 7 clades, namely, Ia to Iig (10). BpSPL1 and BplSPL2 belong to clade IId, as determined using the phylogenetic tree. In general, SBP-box genes in the same clade are likely to exhibit the same exon-intron structure and, usually, the members in clade IId have 1 intron with the exception of BpSPL1, which has no intron.

Expression pattern of BplSPL2

No PCR product could be derived from the cDNA template for root, stem, and leaf in the special expression test, indicating that BplSPL2 expression is inflorescence-specific (Fig. 2).

The results of RT-PCR demonstrated that the expression of BplSPL2 is developmentally regulated (Fig. 3b and Fig. 3c). They also showed in male inflorescence, BplSPL2 was expressed stably, except in the stage M3 (corresponding to the development period of anther differentiation), while in female inflorescence, BplSPL2 was expressed at a low level before...
The sequence of the squamosa-promoter binding protein-box genes were obtained from a public database and involve Arabidopsis (AtSPL1-16), snapdragon (AmSBP1-5), maize (ZmLig1, ZmSBP1-5), and rice (OsSPL1-18).

Fig. 1. Reconstruction of the phylogenetic relationships between the SBP-box genes.

Stage F4 (corresponding to the period of ovule development), and reached its maximum in stage F7 (corresponding to the development period of megaspore formation); these findings indicated that BplSPL2 may play a role in ovule and fruit development.

In order to study the difference in the expression patterns between the 2 SBP-box genes in birch, we cloned BplSPL1 from B. platyphylla by RT-PCR (sequence analysis showed no difference from BpSPL1 extracted from B. pendula). Compared to BplSPL2, BplSPL1 was expressed stably in female inflorescence, except in stage F6 (corresponding to the meiosis stage), while in male inflorescence, BplSPL1 appeared to peak in stage M3 with a gradual decrease during the flank periods.
Conclusions
Based on the findings of this study, it is clear that BplSPL1 and BplSPL2 share little similarity in expression patterns during inflorescence development; it is likely that they have different target gene. Three AP1-like genes with different expression patterns have been identified in B. pendula, i.e., BpMADS3, BpMADS4, and BpMADS5 (8). BpMADS3 is almost similar in sequence to AP1 and SQUA, but it seems to have the highest expression in late developmental stages. BpMADS4 is almost similar in sequence to the Arabidopsis gene, FRUITFULL, but is expressed, in addition to developing inflorescences, in shoots and roots. BpMADS4 has been proved to have a central role in inflorescence initiation (7). Constitutive expression of BpMADS4 in poplar can induce broad changes in senescence and winter dormancy, but no early flowering (Hoenicka). BpMADS5 is also similar to FRUITFULL; its expression seems to be inflorescence-specific. The BpFULL1::BARNASE construct has potential biotechnological applications in the prevention of flower formation (13). Interestingly, the expression of BpMADS5 expression continues during fruit development like that of BplSPL2 and may be a potential target of BplSPL2.

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