MOLECULAR CLONING, CHARACTERIZATION AND PROMOTER ANALYSIS OF A NOVEL MDR-TYPE ABC TRANSPORTER GENE (GBMDR1) FROM GINKGO BILOBA

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Abstract - A novel MDR-type ABC transporter cDNA (designated as Gbmdr1) was cloned and characterized for the first time from the gymnosperm plant species, Ginkgo biloba, using RACE technique. The full-length cDNA of Gbmdr1 was 4275 bp, and it contained a 3840 bp open reading frame (ORF) encoding a polypeptide of 1279 amino acid with a predicted isoelectric point of 8.22 and molecular mass of 139.6 kDa. The deduced GbMDR1 protein consisted of four domains including two TMDs (74-351, 716-993) and two NBDs (428-607, 1067-1251). The promoter of the Gbmdr1 was also cloned by the Genome Walking method. Sequence analysis demonstrated that there were many regulatory elements in the Gbmdr1 promoter and the TATA box was located at -52--56 bp upstream of the transcriptional start site. Sequence alignment and molecular evolution analysis revealed that GbMDR1 was a plant MDR-like ABC transporter protein, and that it has a further relationship with many other MDRs of plant species. Southern blot analysis indicated that Gbmdr1 belonged to a small multi-gene family. Tissue expression analysis indicated that Gbmdr1 expression was high in stems and leaves but low in roots. These results show that GbMDR1 is a MDR-like ABC transporter protein that may be involved in the transport and accumulation of secondary metabolites.

Key words: ABC transporter-Ginkgo biloba-MDR-Gbmdr1-molecular cloning

Abbreviations: MDR, multidrug resistance; ABC, ATP-binding cassette; PAF, platelet-activating factor; GBE, Ginkgo biloba leaf extract;

INTRODUCTION

Ginkgo biloba L. is one of the oldest living tree species, usually referred to as a “living fossil”. The extract from Ginkgo biloba leaves (GBE) contains many active ingredients, including flavonol and flavone glycosides, diterpene lactones, ginkgolides, sesquiterpenes, iron-based superoxide dismutase, p-hydroxybenzoic acid, ascorbic acid and catechin (Jacobs and Browner, 2000). The ginkgolides are platelet-activating factor (PAF) antagonists, capable of reducing platelet activation and aggregation, thereby having the potential to improve blood circulation. Bilobalide, a sesquiterpene trilactone constituent of GBE, can reduce cerebral edema produced by triethyltin, decrease cortical infarct volume in certain stroke models, and reduce damage from cerebral ischemia (Defeudis et al., 2002). While these secondary metabolites are potentially toxic to the plant cells, the plants are able to grow without serious disruption of their basic metabolism (Sato et al., 1994). Recently, it has been found that biosyntheses and accumulation of secondary metabolites are highly regulated in a temporal and
spatial manner in plant organs; these metabolites are often transported from source cells to neighboring cells, or even further to other tissues or remote organs (Yazaki, 2005). ATP-binding cassette (ABC) transporters are involved in some plant systems and responsible for the mechanism in transport across biological membranes (Yazaki, 2006).

The ABC-transporter superfamily is a large, ubiquitous and diverse group of proteins, most of which mediate transport across biological membranes. Typically, the ABC transporters are composed of two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). The ABC genes have been divided into several clusters or subfamilies corresponding to phylogenetic pathways and structural features. The three best-characterized subfamilies are the pleiotropic drug resistance (PDR), multidrug resistance (MDR), and multidrug resistance-associated protein (MRP) subfamilies (Theodoulou, 2000; Jasinski et al., 2003). The MDRs represent the second largest ABC protein subfamily and the largest full-molecule ABC transporter subfamily in plants (Rea, 2007). Until now, many MDRs, with a membership of 22 in Arabidopsis (Sánchez-Fernández et al., 2001) and 24 in rice (García et al., 2004), have been identified in plant species, however, there are very few reports on the cloning of ABC transporter genes in medicinal plants, with exception for Cjmdr1 from Coptis japonica (Shitan et al., 2003) and Crmdr1 from Catharanthus roseus (Jin et al., 2007).

In the present study, we report for the first time that a MDR-type ABC transporter gene (designated as Gbmdr1, GenBank Accession Number DQ779968) and the promoter sequence have been isolated and characterized from G. biloba by the RACE (rapid amplification of cDNA ends) technique. The promoter sequence analysis and expression pattern of Gbmdr1, the properties of the characteristic ABC transporter domain, and phylogenetic analysis of GbMDR1 protein were examined. This study may facilitate our further understanding of the molecular mechanism in the transport of the secondary metabolites in medicinal plants.

MATERIALS AND METHODS

Plant materials

Fresh four-leaf-stage seedlings of G. biloba growing at 25°C in a controlled-growth greenhouse (16 h light/8 h dark) were used in the experiment. The leaves, stems and roots were collected separately, frozen immediately in liquid nitrogen and stored at an ultra low temperature (-80°C) for preparing RNA and DNA extraction. Total RNA was extracted with a Plant (Leaves) RNA Mini Kit (Watson Biotechnologies, Inc., Shanghai, China). Genomic DNA was isolated from the leaves of G. biloba by using a cetyltrimethylammonium bromide (CTAB) method (Sambrook et al, 1989). The quality and concentration of RNA and genomic DNA were measured by agarose gel electrophoresis and spectrophotometry before later steps.

Cloning of Gbmdr1 core cDNA fragment

The core cDNA fragment of the Gbmdr1 gene was cloned by using SuperscriptTM II Reverse Transcriptase according to the manufacturer’s instructions (Takara, Dalian, China). The forward primer FMDR and the reverse primer RMDR (Table 1) used for the cloning of Gbmdr1 core cDNA were designed and synthesized according to the two conserved regions obtained by aligning the NBD domains of mdr sequences in Arabidopsis thaliana (GenBank accession no. Atmdr1 AC006922, Atmdr2 AL161564, Atmdr4 AC004411, Atmdr7 AB019223, Atmdr8 AC022521), Coptis japonica (AB043999), Oryza sativa (AJ535070), Solanum tuberosum (U52079), Sorghum bicolor (AY372819) and Triticum aestivum (AB055077) deposited in Genbank. PCR was carried out in a total volume of 50 μl containing 2 μl 25 ng/μl cDNA under the following condition: the template was denatured at 94°C for 3 min followed by 40 cycles of amplification (1 min at 94°C, 1 min at 52°C and 3.5 min at 72°C) and finally extended for 10 min at 72°C.

3’ RACE of the Gbmdr1 cDNA

According to the protocol of the BD SMARTTM
MOLECULAR CLONING, CHARACTERIZATION AND PROMOTER ANALYSIS OF GBMDR1

According to the protocol of the BD SMARTTM RACE cDNA Amplification Kit (Clontech, CA, USA), 1 μg of total RNA was reversely transcribed with primer 3’-CDS primer A, which annealed to the tail of the RNA and served as an extended template for PowerScript RT. The forward primers used for the cloning of the partial coding sequence of G. biloba were designed and synthesized according to the core cDNA fragment of the G. biloba gene obtained earlier. The first round of PCR was performed with Sgbmdr3-1 as the forward primer and Universal Primer A Mix (UPM) (Table 1) as the reverse primer. PCR was carried out in a total volume of 50 μl containing 2.5 μl 5 ng/μl 3’-RACE-Ready cDNA under the following conditions: 25 cycles of amplification (94°C for 30 s, 68°C for 30 s, 72°C for 4 min). Subsequently, nested PCR was performed using Sgbmdr3-2 as the forward primer and the Nested Universal Primer A (NUP) (Table 1) as the reverse primer in 20 cycles of amplification (94°C for 30 s, 68°C for 30 s, 72°C for 4 min).

5’ RACE of the Gbmdr1 cDNA

According to the protocol of the BD SMARTTM RACE cDNA Amplification Kit (Clontech, CA, USA), 1 μg of total RNA was reversely transcribed with primer 5’-CDS primer coupled with (dC) tailing and BD SMART II A Oligo, which annealed to the tail of the RNA and served as an extended template for Power Script RT. The reverse primers used for the cloning of the partial coding sequence of G. biloba were designed and synthesized according to the core cDNA fragment of G. biloba gene obtained earlier. The first round of PCR was performed with Sgbmdr5-1 as the reverse primer and Universal Primer A Mix (UPM) as the forward primer (Table 1). PCR was carried out in a total volume of 50 μl containing 2 μl 5 ng/μl 5’-RACE-Ready cDNA under the following conditions: the template was denatured at 94°C for 5 min and then subjected to 40 cycles of amplification (94°C for 1 min, 58°C for 1 min, 72°C for 4 min) followed by 10 min at 72°C. Subsequently, the nested PCR was performed using Sgbmdr5-2 as the reverse primer and the Nested Universal Primer A (NUP) (Table 1) as the forward primer at 94°C for 5 min, and then for 40 cycles of amplification (94°C for 1 min, 60°C for 1 min, 72°C for 1.5 min), followed by 10 min at 72°C.

The 3’ and 5’ PCR products were purified and subcloned into pMD 18-T vector (Takara, Dalian, China) and sequenced.

Generation of the full-length cDNA of Gbmdr1

By comparing and aligning the sequences of the core fragment, the 5’ RACE and 3’ RACE products, the full-length cDNA sequence of Gbmdr1 was deduced; this was confirmed by sequencing the full-length fragment amplified with two gene-specific primers, GbmdrF35 and GbmdrR35 (Table 1).

Cloning of Gbmdr1 promoter sequence

The cloning manipulation was performed according to the protocol of the Universal Genome Walker Kit (Clontech, CA, USA) with slight modification. An aliquot of 1 μg total DNA was digested with DraI, EcoRV, PvuII and StuI (Takara, Dalian, China), respectively, and four DNA libraries were constructed. After purification of the digested DNA fragments, the four DNA libraries were used as templates separately for PCR. The first round of PCR was performed with GbWalker5-1 (Table 1) as the forward primer and the Clontech AP1 primer (Table 1) as the reverse primer. PCR was carried out in a total volume of 50 μl containing 3 μl 5 ng/μl purified DNA fragments under the following conditions: the template was performed with 7 cycles of amplification (denatured at 94°C for 25 s, 72°C for 3 min). Subsequently, the nested PCR was performed using GbWalker5-2 (Table 1) as the reverse primer and Clontech AP2 primer (Table 1) as the forward primer under the following conditions: 5 cycles of amplification (94°C for 25 s, 72°C for 3 min) and then 32 cycles of amplification (94°C for 25 s, 67°C for 3 min) followed by 7 min at 67°C.

Bioinformatic analyses and phylogenetic construction

The obtained sequences were analyzed by using web bioinformatic tools (http://www.ncbi.nlm.nih.gov and http://cn.expasy.org). The nucleotide sequence,
deduced amino acid sequence and Open Reading Frame (ORF) encoded by Gbmdr1 were analyzed and sequence comparison was conducted through database search using the BLAST program (NCBI, National Center for Biotechnology Services, http://www.ncbi.nlm.nih.gov). The structural domains of GbMDR1 were analyzed online (http://plantsp.sdsc.edu). The secondary structure of GbMDR1 was analyzed by SOPMA (Geourjon and Deleage, 1995). The promoter sequence of Gbmdr1 was analyzed online with PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The phylogenetic analysis of GbMDR1 and MDRs from other plant species retrieved from GenBank was aligned with CLUSTAL W (1.81) using default parameters, and subsequently a phylogenetic tree was constructed by the neighbor-joining (NJ) method (Saitou and Nei, 1987) with MEGA version 3.0 (Kumar et al., 2001). The reliability of the tree was measured by bootstrap analysis with 1000 replicates (Felsenstein, 1985).

### Southern blot analysis

The genomic DNA (20 μg per lane) were digested overnight at 37°C with EcoRI, KpnI and NotI (Takara, Dalian, China), respectively, separated by 1% agarose gel electrophoresis in TBE buffer and then transferred onto a positively charged Hybond-H+ nylon membrane (Amersham Pharmacia, England). PCR was performed with GbSouthF and GbSouthR (Table 1) as primers, and with the G. biloba cDNA as a template. An aliquot of 70 ng purified PCR product containing the 383 bp sequence flanking poly-(A) tail was used as the template in a total volume of 50 μl for probe-labeling. Probe labeling (biotin), hybridization and signal detection were performed using

### TABLE 1. Primers used in the cloning and analysis of Gbmdr1.

<table>
<thead>
<tr>
<th>Name of Primer</th>
<th>Primer Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMDR</td>
<td>5'-CAAGA(T/C)GC(T/C)(A/C)T(T/G)GG(A/T/G)GA(G/A)AAGG-3'</td>
</tr>
<tr>
<td>RMDR</td>
<td>5'-GCAC(T/G)GT(T/G)GC(T/C)TCAT(A/C/T)AG(T/C)AG-3'</td>
</tr>
<tr>
<td>Sgbmdr3-1</td>
<td>5'-GAGGGGCCAACATTGGTTGGAAT-3'</td>
</tr>
<tr>
<td>Sgbmdr3-2</td>
<td>5'-ACTCAGGTAGGAGAAGGAGGTGC-3'</td>
</tr>
<tr>
<td>Sgbmdr5-1</td>
<td>5'-ACGAACGGCTTTCTCCTGCAAACG-3'</td>
</tr>
<tr>
<td>Sgbmdr5-2</td>
<td>5'-GGGAACATATTGCCAGCGTCACCAGG-3'</td>
</tr>
<tr>
<td>GbmdrF3S</td>
<td>5'-GAAGCAATCTGTTTTCCGTGGAGGTTGAGT-3'</td>
</tr>
<tr>
<td>GbmdrR35</td>
<td>5'-GGCAACAGATCAAAATGTGTTGCTTGTCACTATA-3'</td>
</tr>
<tr>
<td>GbWalker5-1</td>
<td>5'-GGCATAATGATCTGTCAGCAAACGTGAGT-3'</td>
</tr>
<tr>
<td>GbWalker5-2</td>
<td>5'-CAACCCATCCACGGAAAACAGATTGCCCT-3'</td>
</tr>
<tr>
<td>GbmdrRT-F</td>
<td>5'-CAGAAAAATAGTAGCGGAGGAGTTCATTG-3'</td>
</tr>
<tr>
<td>GbmdrRT-R</td>
<td>5'-GGCACACAGATCAAAATGTGTTGCTT-3'</td>
</tr>
<tr>
<td>GbSouth F</td>
<td>5'-CATCAGCTCTCCAAACAAAATCATGTA-3'</td>
</tr>
<tr>
<td>GbSouth R</td>
<td>5'-GGCACACAGATCAAAATGTGTTGCTT-3'</td>
</tr>
<tr>
<td>UPM</td>
<td>5'-CTAATACGACTCACTATAGGGCAAGCAGTATTCAACGCAGATG-3'</td>
</tr>
<tr>
<td>NUP</td>
<td>5'-CTAATACGACTCACTATAGGGCAAGCAGTATTCAACGCAGATG-3'</td>
</tr>
<tr>
<td>18SF</td>
<td>5'-ATGATAATCGACGGATTCGCC-3'</td>
</tr>
<tr>
<td>18SR</td>
<td>5'-CCTGGATGTGATAGGCGAGTGC-3'</td>
</tr>
<tr>
<td>AP1</td>
<td>5'-GGCACACAGATCAAAATGTGTTGCTT-3'</td>
</tr>
<tr>
<td>AP2</td>
<td>5'-ACTCAGATCGCTGAGCAGG-3'</td>
</tr>
</tbody>
</table>
Gene Images Random Prime Labeling Module and CDP-Star Detection Module following the manufacturer's instructions (Amersham Pharmacia, UK). The hybridized signals were visualized by exposure to Fuji X-ray film at room temperature for 30 min.

**Transcription pattern analysis**

To investigate the Gbmdr1 expression pattern in different tissues of *G. biloba*, semi-quantitative RT-PCR analysis was carried out as follows: total RNA was extracted from roots, stems and leaves, followed by incubation with RNase-free DNase I at 37°C for 30 min according to the manufacturer's instructions (Takara, Dalian, China). Subsequently, an aliquot of 1 µg total RNA was used as the template in PCR reaction with the forward primer GbmdrRT-F and reverse primer GbmdrRT-R (Table 1) by using a One Step RNA PCR Kit (TaKaRa, Dalian, China). Meanwhile, two primers, 18SF and 18SR (Table 1), were also used to amplify the 18S rRNA gene in the quantitative one-step RT-PCR as control. The template was reversely transcribed at 50°C for 30 min and denatured at 94°C for 2 min, followed by 35 cycles of amplification (94°C for 30 s, 55°C for 50 s, 72°C for 1 min) and by extension at 72°C for 10 min. The PCR products were separated on 1% agarose gels stained with ethidium bromide (10 µg/ml). Densities of the target bands were measured using Furi FR-200A ultraviolet analyzer (Furi Tech., Shanghai, China).

**RESULTS AND DISCUSSION**

*Cloning of the full-length cDNA of Gbmdr1*

By comparing and aligning the sequences of the core fragment, the 5' RACE and 3' RACE products, the full-length cDNA sequence of Gbmdr1 was deduced, which was subsequently confirmed by sequencing. The full-length cDNA of Gbmdr1 (GenBank accession no.: DQ779968) was 4275 bp in length and contained an ORF of 3840 bp, flanked by stretches of 106 bp and 329 bp at the 5'- and 3'-untranslated regions, respectively (Fig. 1). The start codon (ATG) and stop codon (TGA) including a short polyA tail were recognized in the sequence. The ORF encoded a predicted polypeptide of 1279 amino acid residues with a predicted molecular weight of 139.6 kDa and an isoelectric point of 8.22. Because all the mdr gene sequences found in plant species are about 4000 bp in length, we considered the sequence of Gbmdr1 cDNA as a MDR-type ABC transporter gene.

*Analysis of the Gbmdr1 promoter sequence*

On analysis of the 5' RACE and the upstream sequences in the coding region, it was found that the putative transcription start is located at the G (106 bp) in the 5' untranslated region upstream of the start codon ATG. The analysis of upstream sequence in the Gbmdr1 gene was carried out online with PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). It was found that there were ten TATA box-like elements, eleven CAAT box-like elements and one GC box (-936--941 bp) element. After comparing the locations of these elements with the transcriptional start site, the TATAAA element located at -52--56 bp upstream of the transcriptional start site seems to be the TATA box of the Gbmdr1 promoter. The CAAT box (-124--121 bp) is found at the site upstream of the TATA box (Fig. 2). Interestingly, the results also demonstrated that there are many light-responsive elements and cis-acting regulatory elements involved in light responsiveness in the promoter, such as AE-box, ATCT-motif, Box I, GT1-motif, I-box, Sp1, GATA-motif, CATT-motif, G-Box, L-Box and TCT-motif. This fact suggested there should be some relationships between the Gbmdr1 gene expression and light stimulation in *G. biloba* cells. Sidler et al (1998) had reported that AtPGP1 (alias AtMDR1) mediates hypocotyl length in the light. They found that overexpression of the AtPGP1 gene within a certain light fluence rate window causes *Arabidopsis* plants to develop longer hypocotyls compared with those of the wild type, whereas hypocotyls of transgenic and wild-type plants are indistinguishable in the dark. In addition, the Gbmdr1 promoter contains an MRE element (MYB binding site involved in light responsiveness) and CGTCA-motif (cis-acting regulatory element involved in the MeJA-responsiveness). Therefore, we postulated that
Fig. 1. The full-length cDNA sequence and the deduced amino acid sequence of Crmdr1 gene. The start codon (ATG) is underlined and the stop codon (TAA) is underlined in italics. The deduced amino acid sequence is shown beneath the nucleotide sequence. A short polyA tail is recognized in the sequence and shadowed.

1                    GAAGCAATCTGTGTTTCCGTGGATGGGTTGCAGTTGAGAGGAGGCT
47      TATTAAACTCTCAGTGTTCGCAGCACAGATCATATGCTCTGAGTGCAGACAGGCGTGAAACA
107      ATGTATTCTGGAAAAAATGTTGCAGAAAATGGTTTCCAAAAGAGTGCATATGAAGGGAAT
M  Y  S  G  K  N  V  A  E  N  G  F  Q  K  S  A  Y  E  G  N        20
167      CACAAGGAAGAGCCTGGTGAAACTGTGAAGGTGAAGTTGAAGGCCGGATGCGAGAG
H  K  E  E  S  L  G  D  N  C  E  G  E  V  E  G  R  M  R  E        40
227      AAGAAAACATTGGGAGATGGAAGGGCCGGTCGTCGACGCCGACAGAACTAGCGTTCTAT
K  T  L  E  D  G  E  A  A  S  Q  P  F  Q  K  V  A  F  60
287      AACCTTTTTTTGTAAGTACAGTGGTTGGAATTCTTCTCAGGGCCTGGTTCTATCGGA
K  L  F  S  Y  A  D  G  W  D  Y  L  L  M  A  V  G  S  I  G        80
347      GCCTGGCAGCAGATGAGCAGCGCTGAGGGTTTTTCTCTATTTTCTCGGAAAGCTATTAAT
A  C  A  H  G  A  S  V  P  V  F  F  I  F  F  G  K  L  I  N       100
407      TGTATTGGACTTGCTTATTTGGATCCCCCTGCTGTAACGCACACGGTCGCTATGTTCT
C  I  G  L  A  Y  L  D  P  P  A  V  T  H  T  V  A  M  Y  S        120
467      TTGGACCTCGTATATTGGAAGGTTGGTCTGGATGGGGACTAGGTTGGCATGC
L  D  F  V  Y  L  G  V  V  V  L  F  S  S  W  T  E  V  A C       140
527      TGGATGTACACTTGAGAAAGGCAGGCTACTCGCATGCGATTGACGTACTTAAGAGCTATG
W  M  Y  T  G  E  R  Q  A  T  R  M  R  L  T  Y  L  R  A  M        160
587      CTTAACGAGGATGATCTTCTTCTCAGCGTCGGACCGGTTGGAAGGTAGTGGCTGCC
L  N  Q  D  V  S  F  D  T  D  A  T  G  G  E  V  V  A A       180
647      ATTACTAGTGACACTATTGTAGTCCAAGATGCCATCGGGGAGAAGGTGGGGAATTTCCTG
I  T  S  D  T  I  V  V  Q  D  A  I  G  E  K  V  G  N  F  L       200
707      CATTACATGGGTGCTGGTTTGTTGGAGTGGTTTTCGCTGGTGGCAATTTG
H  Y  M  G  R  F  V  A  G  F  A  V  G  S  A  V  W  Q  L        220
767      AGCCCTGGTGACGCTGGCAATAGTTCCCTTAGTGCAGATCGCTATTTCT
S  L  V  T  L  A  L  A  G  G  Y  A F       240
827      GTTGATAGCTGGGCTCACCAGGTCGAGGCTGAGAACGCTTATATCAGAACGGGGGAATGCA
V  V  T  G  L  T  S  R  S  N  I  K  A  G  G  A       260
887      GAAGAGGTGATATTGGAAGTGGGACTGTGTTGTATGCTGGGAGAAAGAGCGGTT
E  E  V  I  G  N  T  V  Y  A  F  V  G  E  R  A  V       280
947      CGTTCATACAAACTGCATGATGGAAGATATAGGGAAGAGTGCATAGC
R  N  V  Y  F  S  Y  P  S  R  P  D  V  V  F  Q  N  L S       320
1007     AAAGGTTTGGTTTGGACATATGCTTCTTCTCTTTGATGCTGGCGTGGATTT
K  G  L  G  L  H  M  C  L  L  F  W  A  L  L  L       340
1067     TGGATACATGCGTATGAGATGACATGACTGACTGGTGGTCTGCTGGCTT
I  S  S  G  S  R  T  G  N  K  A  K  V  E  G  N  I  E  L       360
1127     ATGCTTAATGTAGTTATGCTGACTATGGACAGGCTTCCTGGATTTGAGTCGCG
M  L  N  V  V  I  S  G  L  G  Q  A  A  P  D  L  T A       380
1187     TTCGGAGGCAGACGGAATGTGCAATTCCCCATTTTCAGATGATAATATCCTTATCTTCT
F  G  R  S  A  A  Y  S  I  F  Q  M  I  N  R  N  S A       400
1247     ATAAATTTGCGTTTCTCAGTACGTGTAATAAAATATGCGAAGGTTGAGGAAATATGCACTA
I  S  S  G  S  R  T  G  N  K  L  A  V  G  N  I E       420
1307     CGGAGATGTAATCATCTCAGCTATCTTCACTGCGCTAGTGGAGTATTTCCAGATCTGTAGT
R  N  V  Y  F  S  Y  P  S  R  P  D  V  V  F  Q  N  L S       440

Fig. 1. Continued

1347  TTTAGAATTCCGCGGGGAAGTTGTTGCCATTGTTGCGGGGAGCGGTTCTGGGAAAAGC  
F R I P A G K V V A I V G G S G S G K S  440
1427  ACCGTATTTCCTCCTACGAGCGGTTTTATGATCCTTTCAGCGGAAGTGATGTTGAT  
T V I S L I E R Y D P V G M L D  460
1547  CAAGAGCCATTATTTGCCACAAATGATAGTGAGAAGATACATGGGAAATGAC  
Q E P A L F A S I T R E N I L Y G K N D  500
1607  GCATCCAGGAGGAAATTTGTGACGGCTGCTAAGCTATCAGTGCTATTCTGCTATT  
A S T E I V Q A A K L S D A Y L F I N  520
1667  AACCTCCTATGCTATGAACTCAGGTAGGAGAACGAGGGGTGCAGTTATCTGGGGGC  
N L P D Y E T Q V G Q V L S G  540
1727  CAGAAACAGCGAATTGCAATATCTAGAGCCATACTGAAGAATCCTTCAATCCTCCTTT  
G H N I R S L E L K W L R G Q I G L V N  560
1787  GACGAAGCAGATTGCTTTGGAATGCGAATCTGGAAGAAAGTGAAGGAAAGCTGGAC  
N L P D R Y E T Q V G E R G V Q L S G  580
1847  CGGGTGATGGTTGGTAGAACCACGGTTGTAGTTGCCCATGTGGCTCTCTCGACCGTGAAAAAC  
R V M V G R T T V V A H R L S T V K N  600
1907  GCGGACATTATCGCAGTCGTTCAAAACGGTAAAATTGTAGAATGTGGCGATCATGAAGAT  
A D I A V Q N G K I V E C G D H E D  620
1967  CTGATAAGAAGAGAGGGAGGAGCATATGCTGCCCTTGTCAAACTGCAAGAGACACGTCAA  
L I R R E G Y A A L V Q K E A L D  640
2027  GCTGATCGAGGTTGCTCAGTCTGCTATCCTGCACCGTGAAAAC  
R V M G R T T V V A H R L S T V K N  600
2087  ATTTCTAGGAGAACATTCAGCTTTGGAGCGAGTGTAAGCTCGGATAAAGATTCGGTGGGA  
I S R R T F S G A V S P D D K D S V G  680
2147  GCCGTTTTCCAAGCGTTTTGGTTTCGGATCAGATGAACGGGGGCTCTCTGGTCGAAAAGGTT  
A F S K R F G S D Q M N G G S L V E K  700
2207  TCACTGAAAAAGGTGTGGTTAAAAATGCTGCTCCAGACTGGAATGTGATGCTCTTTCTGGGCA  
S L K R L F M K A A P D W M Y G L F G A  720
2267  GCCGGAGGACATTATGCGAGGACTCAGTGCTCCTTTGGACTGGATGACTCAGGCT  
A G A I F A G A Q M P L A N V T Q A  740
2327  TTGGTGGCTCATTAGTCCAGCAGATATACGCTATACCAAAACGTGAAGTCCCGCAAAATCTCG  
L V A F Y S P D G Y V T K R E V R K I S  760
2387  CTAAGGTTTTGGTATGCTTGGCAATAATGCAGATTGTGGTGGTATGCAATCTAATCTCAAC  
L W F C S G A I L T V V A H V R I H L N  780
2447  TTCCGAATAGTGAGGAGCAAGATATGCTGTGGTGGAGATGATGTTTGAGCGATCG  
F G M G M G E R L R L V R E M F G A I  800
2507  CTAGAAATAGTGAAGTGGTTGAGCGATAACGACAACCAACAGCGGTTGGTTCTTCTCT  
L R N E V W F D D N D N S G L V S S  820
2567  CGCCCTCGGCGTGGAATGACCTCTTGTGGAACCTCCTGGTGATCTGGTGGACATCTCT  
R L S A D T A L V R T L V V D R V T I L  840
2627  ATACAAAAACTACCTCAGTGACCCTCCTTCTACTTACATTGCTCATTGAACATGGCCT  
I Q N I A L I V T S F T A I F E Q W R  860
2687  ATACACTGTGACATATGGCGCACTCCTCCTACTTATAGCATCTCTATGAGGAGAGG  
I T L V I L A T Y P L I A S H M S E R  880
Fig. 1.  Continued

2747      TTTTTATGCACGGTTATGGAGGAAACCTGAGCAAAGCATACCTCAAAGCTAACATGCTG
2807      GCCACTGAGCTGTGACAAACATAAGAAGCGGAGGCTTCTGTGAGGAAAGAAAGTG
2867      ATCGATCCTGTACGAGAGATTTGAGAGCTCCTTTGGATGATCTTCAGGCGGCACAA
2927      TTGGTGGTACTCTTCGACCCTCATTAAACACTACCAGGCGTCTTTCGGATCTGTAATGAAA
2987      ATAGCAGGGATTTGCTACGGGGTTGCACAGTGCTGCATGTTTAGCTCTTACGGTTTGCC
3047      ATCGATCTGTTCAGCAGAGAGTTGGAGGAGCCGAGAAGGAGATCATTTATGCGCGGTCAA
3107      GCCACTGAAGCTGTGAGCAACATAAGAACGGTGGCGGCCTTCTGTGCGGAAGAGAAAGTG
3167      AAAATCCCTCCGGAGCTCCTCCAGGTTGAAAATTTGGAAGGTTGAG
3227      CTCAAGCAGTTGGTTTTGAGTTCTCAGACGGCCTGAGTAATTTTCAGAAATTTCTTC
3287      AACCTCAGAGTGCGAGCAGGTCGAAGCGTGGCACTGGTCGGTTCCAGCGGGTCGGAG
3347      CTCAAGCATGTGGATTTCAGCTATCCTTCGAGGCCCGATGTGATAATTTTCAAAGATTTC
3407      GACGGCAAGGATATCAGAAAAGTGAAGGCGAGGTCATTGCGGAAACACATAGGTAAGGTACGGTA
3467      CAGCAGGAGCCTGCTCTGTTCGCCACAACCATATACGAGAATATCATGTATGGAAGAGAA
3527      GGAGCGACAGAGGCGGAGGTGATTGAAGCTGCAAAGCTGGCCAATGCGCACAGCTTTATC
3587      AGTAGTCTGCCGGATGGATATCAGACGGAAGTGGGTGAGAGGGGAGTTCAGTTATCGGT
3647      GGGCAGAAGCAAAGAGTCGCCATTGCCAGAGCAGTGCTGAAGGATCCTGCCATTCTTCTG
3707      TTAGACGAGGCTCAGCAGCTCTGTGCAGCCGCAGTCAAAGAAGAATGTGCAACAGCCATTA
3767      GACCGTTTGATGAAGAACCGAACTACTGTTATGATTGCCCATCGGTTATGTCTACCATTCAG
3827      AATGCTGATGTCATCTCTGTCTTGCAAGAGGGTAAAGTAGCGGAGCAAGGTACTCATTCC
3887      AGTCTTCTCAGGCAAGGATGTGCTCCTATCAGGCGTACGGCTGCAAAAATACGTTGGAAGT
3947      TGACTTTTCCTTCTGATATGCATTTCTCACAAGCAGTACCTCGTACGTGGTGTTCT
4007      CTGCGAAGGCTTACCAACGAGTTAGTGATGACCCGCTCCCATGCTTGACCTACCATCATC
4067      TTTTGTACCTAAATTTTATGTTATTAGTACAGGCAAGCAACATTTGATCTGTGACAA
4127      TTTTTTATGCACGGTTATGGAGGAAACCTGAGCAAAGCATACCTCAAAGCTAACATGCTG
4187      AAAAAAAAAAAAAAAAAAAAAA
the expression of the Gbmdr1 gene is involved in a development process regulated by light and regulatory factors in *G. biloba* plants.

**Bioinformatic analyses of Gbmdr1**

Using gene and protein prediction programs on different websites (www.ncbi.nlm.nih.gov, http://plantsp.sdsc.edu), the putative translation product of the Gbmdr1 gene exhibited the typical features of “full-molecule” ABC transporter proteins. According to the results of the prediction and alignment, the deduced protein of Gbmdr1 consisted of four domains including two TMDs (74-351, 716-993) and two NBDs (428-607, 1067-1251) with the arrangement of forward orientation “TMD1-NBD1-TMD2-NBD2”, which was identical to the typical functional MDR-type ABC transporters. Three highly-conserved motifs common to all ABC proteins, “Walker A”, “Walker B” and C motifs, were also found in the two conserved NBDs of GbMDR1 (Fig. 3), implying that GbMDR1 might combine and hydrolyze ATP to provide energy for the transport of certain substrates. C motif is also called ABC signature motif, which distinguishes ABC transporters from other NTP binding proteins with Walker sequences (Theodoulou, 2000).
In addition, the result of BlastP also showed that the predicted GbMDR1 from *G. biloba* belonged to the MDR subfamily of ABC transporters. The secondary structure of GbMDR1 by SOPMA analysis showed that the deduced GbMDR1 protein contained 50.59% alpha helices, 17.90% extended...
strands, 5.63% beta turns and 25.88% random coils. The alpha helix constituted domination of more than half of the secondary structure of GbMDR1, which was consistent with the transmembrane domains of other MDR proteins and suggested that GbMDR1 might be a transmembrane protein. Using the transmembrane helix prediction program online (http://www.cbs.dtu.dk/services/TMHMM/), nine transmembrane helices were identified in the GbMDR1, five of which reside at the amino-terminal and four at the carboxyl-terminal halves. This is basically in agreement with the human P-gp model (human P-gp is an MDR-type ABC transporter in human beings), predicting that human P-gp contains a tandem re-

![Phylogenetic tree analysis of MDRs from Ginkgo biloba and other plant species by MEGA version 3.0 from CLUSTAL W alignments. The neighbor-joining method is used to construct the tree. The MDRs used in phylogenetic tree analysis are from plants A. thaliana (from AtMDR1 to AtMDR22, GenBank accession no.: AAD31576, CAB79451, CAB80675, AAC34225, CAB80676, AAC27839, BAB10822, AAG10628, CAB78807, AAF17668, BAB02129, AAG51476, BAB02627, CAB75766, AAG51482, AAG10627, CAB71875, BAB02852, BAB02854, BAB02855, BAB02858, BAB02613), C. japonica (GenBank accession no.: BAB62040) and G. biloba (GenBank accession no.: ABG75919).]
peat of six transmembrane helices, each set followed by an ATP binding domain – the “two-times-six paradigm” (Loo and Clarke, 2005).

The result of sequence alignment by using the Vector NTI Suite 8.0 and BLAST showed that the predicted GbMDR1 from G. biloba exhibited identity to MDR proteins from Arabidopsis thaliana (GenBank accession no.AAD31576), Coptis japonica (BAB62040), Gossypium hirsutum (AAF23176) and Triticum aestivum (BAB85651), with 48.4%, 43.7%, 66.7% and 42.8% identities, respectively. The CrMDR1 from Catharanthus roseus showed 59.8%, 62.5%, 60.0% and 58.2% identity to the MDRs from the above plant species, respectively (Jin et al., 2007). This result indicates that the identity of the Gbmdr1 sequence with the other mdr sequences is relatively low, which is in accord with the fact that G. biloba is one of the most ancient gymnosperm plant species and it has further relation with higher plants.

After comparing full-length Gbmdr1 cDNA with the two other full-length ABC transporter cDNAs from other two medicinal plants, Catharanthus roseus (Jin et al., 2007) and Taxus cuspidata (unpublished), both of which belong to the MDR subfamily in structure, it was found that the identity of GbMDR1 with CrMDR1 and TcMDR1 is 42.20% and 44.30%, respectively, whereas that of CrMDR1 with TcMDR1 is 62.9%. This fact also reflects that GbMDR1 is in a lower evolution position in the plant kingdom.

Molecular evolution analysis

To investigate the evolutionary relationship among GbMDR1 and other MDRs in plants, a phylogenetic tree was constructed using the deduced amino acid sequences of GbMDR, all the putative twenty-two MDRs in the genome of A. thaliana and the only MDR protein from the medicinal plant C. japonica (GjMDR1) (Fig 4). The result showed that GbMDR1
was distinctly separated from most other MDRs and it was grouped into a cluster with only AtMDR2 and AtMDR10, indicating that GbMDR1 had a further relationship with most other MDRs of plant species. On the other hand, it was noticed that AtMDR4 and CjMDR, which have been characterized to be importers of certain substrates (Shitan et al., 2003; Terasaka et al., 2005), and AtMDR4 and AtMDR19, characterized to be exporters (Geisler et al., 2005; Geisler et al., 2003), were classified separately into the other clusters different than GbMDR1, suggesting that GbMDR1 may play a role in different manner in *G. biloba* cells.

**Southern blot analysis**

To investigate whether the Gbmdr1 gene belongs to a multi-gene family, *G. biloba* genomic DNA (20μg per lane) was digested with EcoRI, KpnI or NotI. Except for EcoRI that cuts once within the coding region of Gbmdr1, the other two enzymes do not cut within the coding sequence. For the hybridization processing, the 383 bp fragment flanking the poly-A tail of Gbmdr1 cDNA was used as the probe. The hybridization was carried out with a high stringency wash at 65°C. The result revealed that there were several specific hybridization bands, ranging from 11kb to 0.5kb (Fig. 5), indicating that Gbmdr1 belonged to a multi-gene family. Similar results were reported for mdr genes from *Coptis japonica* (Shitan et al., 2003) and Crmdr1 from *Catharanthus roseus* (Jin et al., 2007), which consisted of more than two members for each with different expression profiles.

**Tissue expression analysis of the Gbmdr1 gene**

Expression analysis showed that Gbmdr1 constitutively expressed in all tested tissues. However, the transcripts were higher in the stem and leaf and lower in the root (Fig. 6), indicating that GbMDR1 functions to transport substrates more actively in stems and leaves than in roots. It can be concluded that GbMDR1 might transport certain substrates synthesized in all parts of *G. biloba* but with more activity in the stems and leaves.

From the analyses above, GbMDR1 is more ancient than other known MDR proteins in plant species but is homologous with them. All the analyses results strongly suggest that GbMDR1 is a plant MDR-like ABC transporter protein involved in the transmembrane transport of certain substrates regulated by light and regulatory factors. Further research will be focused on the purification and functional analysis of GbMDR1, so as to gain more clues on the reactive mechanism of MDR proteins in medicinal plants and finally to obtain some high-yielding plants of valuable secondary metabolites by transferring mdr genes into their source plants.

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