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Isolation and characterization of *BrMDR1* a novel MDR-type ATP-binding cassette (ABC) transporter in *Brassica rapa* L.

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Abstract - A cDNA clone encoding a MDR-like ABC transporter protein was isolated from *Brassica rapa* seedlings, through rapid amplification of cDNA ends (RACE). This gene (named as *Brmdr 1*; GenBank accession no.: DQ296184) had a total length of 4222 bp with an open reading frame of 3900 bp, and encoded a predicted polypeptide of 1300 amino acids with a molecular weight of 143.1 kDa. The BrMDR1 protein shared 71.0, 62.5, 60.0 and 58.2% identity with other MDR proteins isolated from *Arabidopsis* thaliana (AAN28720), *Coptis japonica* (CjMDR), *Gossypium hirsutum* (GhMDR) and Triticum aestivum (TaMDR) at amino acid level, respectively. Southern blot analysis showed that *Brmdr1* was a low-copy gene. Expression pattern analysis revealed that *Brmdr1* constitutively expressed in the root, stem petals and stamens, but with lower expression in leaves and open flowers. The domains analysis showed that BrMDR1 protein possessed two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs) arranging in “TMD1-NBD1-TMD2-NBD2” direction, which is consistent with other MDR transporters. Within NBDs three characteristic motifs common to all ABC transporters, “Walker A”, “Walker B” and C motif, were found. These results indicate that BrMDR1 is a MDR-like ABC transporter protein that may be involved in the transport and accumulation of secondary metabolites.

Key words - ABC transpoter, *Brassica rapa*, gene expression, MDR, secondary metabolism

Introduction

Plant cells synthesize both primary and secondary metabolites. Primary metabolites are necessary for basal growth and maintenance of the cell and include certain nucleic acids, amino acids, proteins, fats and carbohydrates. In contrast, secondary metabolites are a chemically diverse group of compounds that includes alkaloid compounds (e.g., terpenoid indole alkaloids and indole alkaloids), phenolic compounds (e.g., quinones, lignans and flavonoids), and terpenoid compounds (e.g. monoterpenoids, iridoids, sesquiterpenoids, diterpenoids and triterpenoids). Up to now, more than 100,000 kinds of secondary metabolites have been identified (Schulz and Kolukisaoglu, 2006). Many of them show strong biological activities and have been used as medicines. This vast variety of secondary metabolites may play important roles in the life cycle of sessile plants. Some reports indicated that secondary metabolites protect plants from the attacks of herbivores and pathogens, and the injuries of different physical and chemical factors such as ultraviolet irradiation (Harborne, 1990; Bouwmeester *et al*., 2003). Although the secondary metabolites themselves may be poisonous to the plant cells, the plants can develop without apparent influence on their basic metabolism (Sato *et al*., 1990). In this process, it has been found that ATP-binding cassette (ABC) transporters play an important role (Yazaki, 2005; 2006). ABC transporters are the largest protein family known, typically composed of two transmembrane domains (TMDs) and two nucleotide domains (NBDs). NBDs are relatively conservative and are able to combine and hydrolyze ATP to release energy while TMDs are able to utilize the energy to choose and transport substrates across membranes (Theodoulou, 2000). The completion of the *Arabidopsis* and rice genome sequencing revealed 129 and 128 ABC transporters, respectively, which are much more than those of other organisms. They may transport certain secondary metabolites across membranes and lead to their accumulation in specific parts of plants or cells (Yazaki, 2005; 2006). Multidrug-resistance proteins (MDR) are one subfamily of ABC transporters. Including the first isolated

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ABC transporters in plants, MDR proteins constitute the largest subfamily in full-size ABC transporters and the second largest subfamily in all ABC transporters in *Arabidopsis thaliana* (Jasinski *et al*., 2003).

In this paper, we describe for the first time, the isolation and characterization of a MDR-type ABC transporter gene from *B. rapa*. This is also the first cloned ABC transporter gene from *B. rapa*. The expression pattern of *Brmdr1*, the phylogenetic analysis and properties of the characteristic ABC transporter domains of BrMDR1 proteins were also studied. All of these may help us further understand the molecular basis for the regulation of the production and the accumulation of the secondary metabolites in *B. rapa*.

Materials and Methods

Plant materials

Chinese cabbage (*B. rapa* var. 'Sosongche') plants were grown in the greenhouse at Hankyong National University, Anseong, Korea. Different parts of the plant, i.e. young (first to third from the shoot apex), mature (fourth to sixth from shoot apex) and old (eighth and ninth from shoot apex) leaves, internodal segments, flower buds, open flowers, pods and roots (branched side roots) from 2-month-old nursery grown plants were used as plant materials.

Stress treatments

Two-month-old potted mature plants of *B. rapa* var. Sosongche were subjected to different stress conditions in the following manner. MJ treatment was applied on leaves detached from plants and kept on paper soaked in 1/10 Murashige-Skoog (MS) basal medium by painting on the ataxia surface of the leaves, and the tray containing the leaves was sealed with saran wrap. In control experiments, similar leaves were painted with double-distilled water containing the same amount of ethanol required for dissolving MJ. For each treatment, young leaves, the first to the third from the shoot apex, were used. The leaves were harvested at different time points by snap freezing in liquid nitrogen, and stored at - 80℃ for further analyses.

Cloning of *Brmdr1* cDNA and gene

Cloning of *Brmdr1* core cDNA fragment: Total RNA was isolated from vegetative tissues (roots, stem and leaves) as well as reproductive tissues (flower buds, open flowers, stamens and petals) of *B. rapa* “Sosonche” using the Trizol method. First-strand cDNA synthesis was carried out with 5 μg of total RNA using oligo-dT15 primer (Promega, Seoul, Korea) and Superscript TM II Reverse Transcriptase (TaKaRa, Japan) following the manufacturer’s instruction, and used as the template for PCRs. Two consensus sequences, obtained by aligning the NBD domains in *A. thaliana, Oryza sativa, Solanum tuberosum, Sorghum bicolor, Triticum aestivum mdr* sequences deposited in Genbank, BF-1 (5'-CAAGA(T/C)GC(T/C)(A/C)T(T/G)GG(A/T/G) GA(G/A)AAGG-3') and BR-1 (5'-GCACT(T/G)GT(T/G)GC(T/C)TCATC(A/C/T)AG (T/C)AG-3'), were used as the pair of primers in the PCR reaction in a total volume of 50 μl containing 5 μl 10 × *Ex Taq* buffer, 6 μl 2.5 mM dNTP Mix, 2 μl 10 μM primer FMDR, 2 μl 10 μM primer RMDR, 2 μl 25 ng/l cDNA and 0.5 μl 5 U/μl *ExTaq* polymerase. The PCR reaction was carried out under the following condition: the template was denatured at 94℃ for 3 min followed by 40 cycles of amplification (1 min at 94℃, 1 min at 52℃ and 3.5 min at 72℃) and by 10 min at 72℃.

3' RACE of the *Brmdr1* cDNA: According to the protocol of the BD SMART^{TM} RACE cDNA Amplification Kit (Clontech, CA, USA), about 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 *B. rapa* were designed and synthesized according to the core cDNA fragment of *B. rapa* gene obtained earlier. The first round PCR was performed with *Brmdr3-1* (5'-AAACTGCCACAGGGTCTGGACACAATG-3') as the forward primer and Universal Primer A Mix (UPM 5'-CTAATACGACTCACTATAGGGCAA GCAGTGGTATCAACGCAGAGT-3' and 5'-CTAATACGACTCACTATAGGGC-3') 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, 1 μl 10 μM primer *Brmdr3-1*, 5 μl 10 × primer UPM, 5 l 10 × BD Advantage 2 PCR buffer, 1 μl 10 mM dNTP Mix and 1 μl 50 × BD Advantage 2 Polymerase Mix under the following condition: 25 cycles of amplification (94℃ for 30 s, 68℃ for 30 s, 72℃ for 4 min). Subsequently, the nested PCR was performed using *Brmdr3-2* (5'-TCTGGACACAA

TGGTCGGTGAGCA-3') as the forward primer and the Nested Universal Primer A (NUP 5'-AAGCAGTGGTATCA ACGCAGAGT-3') as the reverse primer under the condition of 20 cycles of amplification (94℃ for 30 s, 68℃ for 30 s, 7 2℃ for 4 min).

5' RACE of the *Brmdr1* cDNA: According to the protocol of the BD SMART^{TM} RACE cDNA Amplification Kit (Clontech, CA, USA), about 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 *B. rapa* were designed and synthesized according to the core cDNA fragment of *B. rapa* gene obtained earlier. The first round PCR was performed with *Brmdr5-1* (5'-TCGGTAGCATTTGATTTCCCGTAGAG-3') as the reverse primer and Universal Primer A Mix (UPM 5'-CTAATACGACTCACTATAGGGCAAG CAGTGGTA TCAACGCAGAGT-3' and 5'-CTAATACGACTCACTAT AGGGC-3') as the forward primer. PCR was carried out in a total volume of 50 μl containing 2 μl 5 ng/μl 5'-RACE-Ready cDNA, 2 μl 10 μM primer *Brmdr5-1*, 2 μl 10 × primer UPM, 5 μl 10 × *ExTaq* PCR buffer, 6 μl 2.5 mM dNTP Mix and 0.5 μl 5 U/μl *ExTaq* polymerase under the following condition: the template was firstly denatured at 94℃ for 5 min and then subjected to 40 cycles of amplification (94℃ for 1 min, 58℃ for 1 min, 72℃ for 4 min) followed by 10 min at 72℃. Subsequently, the nested PCR was performed using *Brmdr5-2* (5'-ATCTGAACTTCTGGTCTGGCTGGA-3') as the reverse primer and the Nested Universal Primer A (NUP 5'-AAGCA GTGGTATCAACGCAGAGT-3') as the forward primer under the condition of 94℃ for 5 min and then 40 cycles of amplification (94℃ for 1 min, 60℃ for 1 min, 72℃ for 1.5 min) followed by 10 min at 72℃. The 3' and 5' PCR products were purified and subcloned into pGEM-T vector (Promega, Seoul, Korea) followed by sequencing.

Generation of the full-length cDNA of Brmdr1: By comparing and aligning the sequences of the core fragment, the 5' RACE and 3' RACE products, the full-length cDNA sequence of *Brmdr1* was deduced, which was confirmed by sequencing the full-length fragment amplified with two gene-specific primers, BrmdrFL (5'-GATACTCGGTTGCGGCAAACAA GGAAAGCACATCT-3') and BrmdrRL (5'-GGTGAAAAA GTGAAGGCTTGCAATTGAATTCAACA-3').

Comparative and bioinformatic analyses

Comparative and bioinformatic analyses of *Brmdr1* were carried out online at the websites (http://www.ncbi.nlm.nih.gov; http://cn.expasy.org). The nucleotide sequence, deduced amino acid sequence and open reading frame (ORF) encoded by *Brmdr1* were analyzed and the sequence comparison was conducted through database search using BLAST program (NCBI, National Center for Biotechnology Services, http://www.ncbi.nlm.nih.gov). The structural domains of BrMDR1 were analyzed online at the website (http://plantsp.sdsc.edu). The phylogenetic analysis of BrMDR1 and MDRs from other plant species was aligned with CLUSTAL W (1.81) using default parameters. A phylogenetic tree was constructed using MEGA version 3.0 (Kumar *et al*. 2001) from CLUSTAL W alignments. The neighbor-joining method (Saitou and Nei 1987) was used to construct the tree.

Southern blot analysis

Genomic DNA (20 μg/sample) was isolated from leaves of *B. rapa* using a cetyltrimethylammonium bromide (CTAB) method (Sambrook *et al*. 1989), digested overnight at 37℃ with *Bam*HI, *Eco*RI and *Hind*III (Takara, Seoul, Korea), separated by 1% agarose gel electrophoresis and blotted onto Hybond-N^{+} nylon membrane (Amersham, England, UK). The Gene Images random priming labeling module and Gene Images CDP-Star detection module were used for probe labeling, hybridization and detection procedures (GIBCO-BRL, CA, USA). Biotin labeled sequence of 370 bp flanking poly-(A)$_n$ tail was used as the probe in hybridization, which was amplified with two gene-specific primers, Brmdr-SouthernF (5'-AAGATCCTCGAATTCTCTTGCTTGA-3') and Brmdr-SouthernR (5'-GGTGAAAAAGTGAAGGCTTGCA ATT-3').

Expression analysis of *Brmdr1* gene

Realtime PCR analysis of *Brmdr1* : To investigate the *Brmdr1* expression pattern in different tissues of *B. rapa*, total RNA was extracted from vegetative tissues (roots, stem and leaves) as well as reproductive tissues (flower buds, open

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   1 ggtcccaagaatctgttcagatagaagaagaaataaagtcattagaacaatggcgttggtccgaa
  66 atgcaaggacttgagcttctccctgaaccttcctcaaatagtaacagcaactcaagaaacccagaaacagagctccaagaacatccgccg
      M  Q  G  L  E  L  L  P  E  P  S  S  N  S  N  S  N  S  R  N  P  E  T  E  L  Q  E  H  P  P
 156 gagatgggaaacggcggtggtactcctcctcctcctcctccggcaacagttgaggagcctaaaaaagcagagattcgtggagttgcgttc
      E  M  G  N  G  G  G  T  P  P  P  P  P  P  A  T  V  E  E  P  K  K  A  E  I  R  G  V  A  F
 246 aaagagcttttcagattcgcagatgggttagattatgtactgatgacaattggctctgttggtgctttcgtccacggctgctctttgcct
      K  E  L  F  R  F  A  D  G  L  D  Y  V  L  M  T  I  G  S  V  G  A  F  V  H  G  C  S  L  P
 336 ctgttcctcagattcttcgccgatctcgtgaactccttcggttctaacgctaataacgtcgacaagatgatgcaagaagttctcaagtat
      L  F  L  R  F  F  A  D  L  V  N  S  F  G  S  N  A  N  N  V  D  K  M  M  Q  E  V  L  K  Y
 426 gcgctttactttcttgtcgttggtgctgcaatctgggcttcctcctgggcagagatttcgtgttggatgtggactggagagagacaaaca
      A  L  Y  F  L  V  V  G  A  A  I  W  A  S  S  W  A  E  I  S  C  W  M  W  T  G  E  R  Q  T
 516 acgaagatgaggataaagtacttagaagctgctttaaaccaagacattcagttcttcgacacagaggttcgaacttcagatgttgtctcc
      T  K  M  R  I  K  Y  L  E  A  A  L  N  Q  D  I  Q  F  F  D  T  E  V  R  T  S  D  V  V  S
 606 gccattaacaccgacgctgttatggttcaagacgccatcagcgagaaattgggtaacttcatccattacatggcgttggtgactatcgcg
      A  I  N  T  D  A  V  M  V  Q  D  A  I  S  E  K  L  G  N  F  I  H  Y  M  A  L  V  T  I  A
 696 gtggttccgttgatagctgtgatcggaggaatccacaccacaactctctctaagctctctaacaagagtcaggagtctctttcacaagct
      V  V  P  L  I  A  V  I  G  G  I  H  T  T  T  L  S  K  L  S  N  K  S  Q  E  S  L  S  Q  A
 786 ggtaatatcgtcgaacagacagtggtacagatcagggtagtaatggcttttgtcggagagtcaagagcctctcaagcttactcatcagct
      G  N  I  V  E  Q  T  V  V  Q  I  R  V  V  M  A  F  V  G  E  S  R  A  S  Q  A  Y  S  S  A
 876 ttgaagacagctcagaaactcggttacaaaacaggtttcgctaaaggaatgggacttggcgcgacttacttcgtcgtcttctgttgctac
      L  K  T  A  Q  K  L  G  Y  K  T  G  F  A  K  G  M  G  L  G  A  T  Y  F  V  V  F  C  C  Y
 966 gctctcttgctctggtacggtggctatctcgtccgccaccatttgaccaacggtggtctcgctatagccaccatgttcgccgtcatgatc
      A  L  L  L  W  Y  G  G  Y  L  V  R  H  H  L  T  N  G  G  L  A  I  A  T  M  F  A  V  M  I
1056 ggtggcttggggttgggacaatcagtaccgagcatggctgcgtttgcgaaagctaaagttgctgctgcaaagatctttagaatcattgat
      G  G  L  G  L  G  Q  S  V  P  S  M  A  A  F  A  K  A  K  V  A  A  A  K  I  F  R  I  I  D
1146 cacaagccaacgatagagcgtaacagcgagtccggcgtggagctagagtctgtcacgggtctcgtcgagcttaaaaacgttgacttttcg
      H  K  P  T  I  E  R  N  S  E  S  G  V  E  L  E  S  V  T  G  L  V  E  L  K  N  V  D  F  S
1236 tacccgtcaagaccagatgttaagatccttaacgacttcacactctctgtacccgccgggaagactatagctttggtcggaagcagtggt
      Y  P  S  R  P  D  V  K  I  L  N  D  F  T  L  S  V  P  A  G  K  T  I  A  L  V  G  S  S  G
1326 tcgggcaaaagcactgtcgtttcgcttatcgagcggttttacgacccgacctcaggacaagttttactagatgggcacgacctgaagaca
      S  G  K  S  T  V  V  S  L  I  E  R  F  Y  D  P  T  S  G  Q  V  L  L  D  G  H  D  L  K  T
1416 ctgaagctcaaatggttaagacaacagatcggtcttgtgagccaagaaccagccttgttcgccacttccatcaaagagaatatactctta
      L  K  L  K  W  L  R  Q  Q  I  G  L  V  S  Q  E  P  A  L  F  A  T  S  I  K  E  N  I  L  L
1506 ggccgtccagacgcagatcaagtcgaggtagaagaagcagctcgagtcgcaaatgctcattccttcatcatcaaactacctgatggcttc
      G  R  P  D  A  D  Q  V  E  V  E  E  A  A  R  V  A  N  A  H  S  F  I  I  K  L  P  D  G  F
1596 gacacacaggttggggagagaggactgcagctttcaggtggacagaagcaaagaatagcgatagcaagagccatgttaaagaacccggcg
      D  T  Q  V  G  E  R  G  L  Q  L  S  G  G  Q  K  Q  R  I  A  I  A  R  A  M  L  K  N  P  A
1686 atactttactagatgaagccacaagcgctttggattctgaatcagagaagctagtgcaagaagctttggaccgtttcatgataggaagg
      I  L  L  L  D  E  A  T  S  A  L  D  S  E  S  E  K  L  V  Q  E  A  L  D  R  F  M  I  G  R
1776 acgactctaatcatcgctcaccgtctctccaccatccgcaaagctgaccttgtagctgtgcttcagcaaggaagtgtctccgagattggt
      T  T  L  I  I  A  H  R  L  S  T  I  R  K  A  D  L  V  A  V  L  Q  Q  G  S  V  S  E  I  G
1866 actcacgacgagcttttcgccaagggagagaatggtatctactctaagcttattaaaatgcaagaggcagctcatgaaaccgctatgaac
      T  H  D  E  L  F  A  K  G  E  N  G  I  Y  S  K  L  I  K  M  Q  E  A  A  H  E  T  A  M  N
1956 aacgcaagaaagagtagtgctagaccgtctagtgctagaaactcggttagctcaccgataatagctcggaactcttcttatggaaggtca
      N  A  R  K  S  S  A  R  P  S  S  A  R  N  S  V  S  S  P  I  I  A  R  N  S  S  Y  G  R  S
2046 ccgtactcacggagactctccgacttctcaactactgacttcagcctctccgtcgaagcttcttcatacccaaactaccgacacgataag
      P  Y  S  R  R  L  S  D  F  S  T  T  D  F  S  L  S  V  E  A  S  S  Y  P  N  Y  R  H  D  K
2136 ctacccttcaaggaccaagccaactccttctggcggctagcgaaaatgaactctccggagtggaagtacgctctcgtcggttctgtaggc
      L  P  F  K  D  Q  A  N  S  F  W  R  L  A  K  M  N  S  P  E  W  K  Y  A  L  V  G  S  V  G
2226 tcagtcatctgcggctcactcagcgccttctttgcatacgtcctcagcgcggtcctaagcatctactacaacccggaccacaattacatg
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2316 atcaaacagattgataaatactgttacctcttgatcggtctctcttctgcggctctcatcttcaacacgctccagcattctttctgggac
      I  K  Q  I  D  K  Y  C  Y  L  L  I  G  L  S  S  A  A  L  I  F  N  T  L  Q  H  S  F  W  D
2406 attgtgggggagaatctcaccaagagggtccgtgagaagatgcttaccgctgtgctcaagaacgagatggcttggtttgatcaagaggag
      I  V  G  E  N  L  T  K  R  V  R  E  K  M  L  T  A  V  L  K  N  E  M  A  W  F  D  Q  E  E
2496 aatgagagcgcgagaatctcggcgaggttggctcttgatgctaataacgtgagatcagctataggagataggatctcggtcattgtgcag
      N  E  S  A  R  I  S  A  R  L  A  L  D  A  N  N  V  R  S  A  I  G  D  R  I  S  V  I  V  Q
2586 aacacagcgttgatgcttgttgcttgcactgctgggtttgttctgcaatggagactcgctctcgtcttggtcgcagtcttccctgtagtt
      N  T  A  L  M  L  V  A  C  T  A  G  F  V  L  Q  W  R  L  A  L  V  L  V  A  V  F  P  V  V
2676 gttgctgcaaccgtcttacagaaaatgttcatgactggattctctggagaccttgaagcagcgcatgccaagggaacacagctcgctggt
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2766 gaagccatagctaatgtcagaacagtcgcagcctttaactcagaagcaaagattgttcgtctctacactgcaaacctcgaaccaccgttg
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2856 aaacgctgcttctggaaaggacagatagctggaagtggctacggtgtagctcagttctgtctctacgcatcttacgctctagggctatgg
      K  R  C  F  W  K  G  Q  I  A  G  S  G  Y  G  V  A  Q  F  C  L  Y  A  S  Y  A  L  G  L  W
2946 tacgcgtcgtggcttgtgaaacacggaatctccgacttctccaaaaccataagagtcttcatggttctgatggtctcagctaatggcgca
      Y  A  S  W  L  V  K  H  G  I  S  D  F  S  K  T  I  R  V  F  M  V  L  M  V  S  A  N  G  A
3036 gcagagacgctcacattagctcctgatttcatcaaaggtggtcaggcaatgaggtcggttttcgaacttcttgatcgaaaaaccgagatc
      A  E  T  L  T  L  A  P  D  F  I  K  G  G  Q  A  M  R  S  V  F  E  L  L  D  R  K  T  E  I
3126 gaaccggatgatcttgacaccacgccggttccagaccggttacgcggcgaagtggagctgaaacacattgatttctcttacccttctaga
      E  P  D  D  L  D  T  T  P  V  P  D  R  L  R  G  E  V  E  L  K  H  I  D  F  S  Y  P  S  R
3216 ccagacattcaagttttccgtgaccttagccttcgcgctagagctggcaagactttggcccttgtcggtccgagcgggtgcggtaaaagc
      P  D  I  Q  V  F  R  D  L  S  L  R  A  R  A  G  K  T  L  A  L  V  G  P  S  G  C  G  K  S
3306 tcagtcatctccctgatccagagattctacgaaccgtcctcaggccgagtcttgatagacggtaaagacataaggaagtacaatttgaag
      S  V  I  S  L  I  Q  R  F  Y  E  P  S  S  G  R  V  L  I  D  G  K  D  I  R  K  Y  N  L  K
3396 gccataaggaaacacatagccatagtccctcaagagccttgcttgttcggcacgacaatatacgaaaacatcgcatacggacacgaatgc
      A  I  R  K  H  I  A  I  V  P  Q  E  P  C  L  F  G  T  T  I  Y  E  N  I  A  Y  G  H  E  C
3486 gcgacagaagcagagatcatacaagccgcaacgctagccagcgcgcacaagttcatatccgcgttgcctgatggctacaagacttatgtc
      A  T  E  A  E  I  I  Q  A  A  T  L  A  S  A  H  K  F  I  S  A  L  P  D  G  Y  K  T  Y  V
3576 ggagagagaggcgttcagctgtcgggaggacagaaacagaggatagcgatagcacgtgccttggtgaggaaggcagagatcatgctgctc
      G  E  R  G  V  Q  L  S  G  G  Q  K  Q  R  I  A  I  A  R  A  L  V  R  K  A  E  I  M  L  L
3666 gatgaggccacaagtgctcttgatgcagagtcagagaggtcggtgcaagaggcgttggaccaggcttgttctggtagaacatctattgtc
      D  E  A  T  S  A  L  D  A  E  S  E  R  S  V  Q  E  A  L  D  Q  A  C  S  G  R  T  S  I  V
3756 gtggctcataggttgtctacaatcaggaacgcacacgtgatcgcggtgattgatgatggtaaagttgtggaacaaggatcgcattcgcat
      V  A  H  R  L  S  T  I  R  N  A  H  V  I  A  V  I  D  D  G  K  V  V  E  Q  G  S  H  S  H
3846 cttctcaagaactatcccgatggaatctacgcgaggatgatacagttgcagaggtttacgcatactcaggtgattggtatgacgtcaggg
      L  L  K  N  Y  P  D  G  I  Y  A  R  M  I  Q  L  Q  R  F  T  H  T  Q  V  I  G  M  T  S  G
3936 tcgagttctagggttaaggaagatgatgcttagtacttgctactttggtaatatagaagaagagagaacagagttgaaaaaaaaagaca
      S  S  S  R  V  K  E  D  D  A  *
4026 acacgcactactagaacatgttctgtataaactctgcgagctcatttttcttctttttatgttgtaattttgaggttactttatttttgg
4116 aactacgtttcagaattgatggtatagtattattattattgtgtattattatgagtaatctaattgttcatctgtgtaatatcagtttat
4206 cccaaaaaaaaaaaaaa
```

Fig. 1. The full-length cDNA sequence and the deduced amino acid sequence of *Brmdr1* gene. The start codon ATG is underlined and the stop codon TAA is underlined italically. The deduced amino acid sequence is shown beneath the nucleotide sequence. The polyadenylation signal AATAA is boxed and the polyA tail is underlined and painted with shadow.

flowers, stamens and petals), respectively, followed by incubation with RNase-free DNase I at 37℃ for 30 min according to the manufacturer's instruction (Takara, Seoul, Korea). Subsequently, an aliquot of 1 μg total RNA was used as the template in PCR reaction with the forward primer BrmdrRT-F (5'-TTCTCAAGCAATGGCATTGGCACCT-3') and reverse primer BrmdrRT-R (5'-GGTGAAAAAGTGAAGGCTTGCAATT-3') by using One Step RNA PCR Kit (TaKaRa, Seoul, Korea). Meanwhile, two primers, 18SF (5'-ATGATAACTCGACGGATCGC-3') and 18SR (5'-CTTGGATGTGGTAGCCGTTT-3'), were also used to amplify 18S rRNA gene in the quantitative one-step RT-PCR as control. The template was reversely transcribed at 50℃ for 30 min and denatured at 94℃ for 2 min, followed by 35 cycles of amplification (94℃ for 30 s, 55℃ for 50 s, 72℃ for 1 min) and by extension at 72℃ for 10 min. The PCR products were separated on 1% agarose gels stained with ethidium bromide (10 μg/ml).

Results and Discussion

Cloning of the full-length cDNA of *Brmdr1*

By comparing and aligning the sequences of the core fragment, the 5' RACE and 3' RACE products, the full-length cDNA sequence of *Brmdr1* was deduced, which was subsequently confirmed by sequencing. The full-length cDNA of *Brmdr1* (GenBank accession no.: DQ296184) had 4226 bp in length and contained an ORF of 3903 bp, flanked by stretches of 66 and 257 bp at the 5'- and 3'-untranslated regions, respectively. The translation stop codon and one putative polyadenylation signal site were recognized in the 3'-untranslated region, which were followed by a short polyA tail. The ORF encoded a predicted polypeptide of 1301 amino acid residues with a predicted molecular weight of 137.1 kDa and an isoelectric point of 8.44 (Fig. 1).

Comparative and bioinformatic analyses of *Brmdr1*

Sequence alignment using Vector NTI Suite 8.0 and BLAST

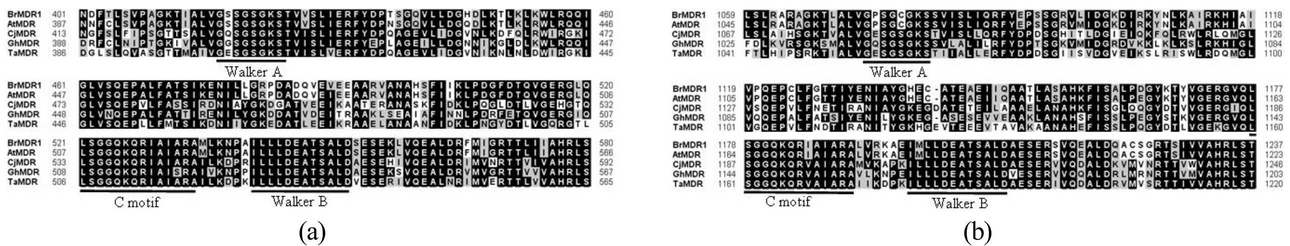

Fig. 2. Amino acid identity of the NBD domains of plant MDR-type ABC transporter proteins. (A) N-Terminal NBD domains. (B) C-Terminal NBD domains. Five NBD domains are aligned. The identical amino acids are showed in white with black background and the conserved amino acids are showed in black with gray background. The three conserved motifs common to all ABC transporters, "Walker A", "Walker B" and C motifs, are underlined. The aligned MDRs are from *A. thaliana* (AtMDR, GenBank accession no.: AAD31576), *C. japonica* (CjMDR, GenBank accession no.: BAB62040), B. rapa (BrMDR1, GenBank accession no. DQ296184), *G. hirsutum* (GhMDR, GenBank accession no.: AAF23176) and *T. aestivum* (TaMDR, GenBank accession no.: BAB85651).

showed that the predicted BrMDR1 from *B. rapa* had high identity with MDRs from other plant species, such as *A. thaliana* (GenBank accession no.: AAD31576), *C. japonica* (GenBank accession no.: BAB62040), *G. hirsutum* (GenBank accession no.: AAF23176) and *T. aestivum* (GenBank accession no.: BAB85651), with the identity of 59.8, 62.5, 60.0 and 58.2%, respectively. Using gene and protein prediction programs on different websites (www.ncbi.nlm.nih.gov; http://plantsp.sdsc.edu), the putative translation product of *Brmdr1* gene exhibited typical features of "full-molecule" ABC transporter proteins. According to the result of the prediction and alignment, the deduced protein of *Brmdr1* is consisted of four domains including two TMDs and two NBDs with the arrangement of forward orientation "TMD1-NBD1-TMD2-NBD2", which is identical with the typical functional MDR-type ABC transporters. Three highly-conserved motifs common to all ABC proteins, "Walker A", "Walker B" and C motif were also found in the two conservative NBDs of Br MDR1 (Fig. 2), which implicated BrMDR1 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 the Walker sequences (Theodoulou 2000). In addition, the results of BlastP also showed that the predicted BrMDR1 from *B. rapa* belongs to the MDR subfamily of ABC transporters.

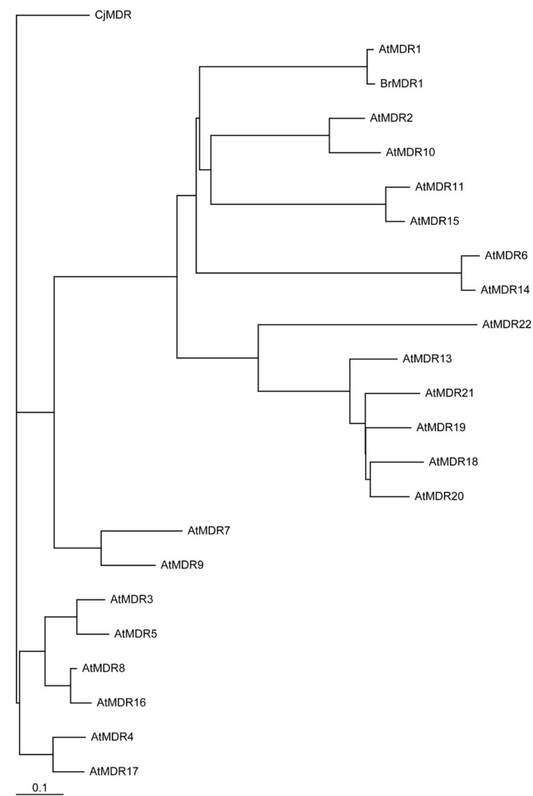

Fig. 3. Phylogenetic tree analysis of MDRs from *Brassica rapa* and other species by MEGA version 3.0 from CLUSTAL W alignments. The neighbour-joining method is used to construct the tree. The MDRs used in phylogenetic tree analysis are from plants including *A. thaliana* (from AtMDR1 to AtMDR22, GenBank accession no.:AAD31576, CAB79451, CAB80675, AAC34225, CAB80676, AAC27839, BAB10822, AAG10628, CAB78807, AAF17668, BAB02129, AAG51476, BAB02627, CAB75766, AAG51482, AAG10627, CAB71875, BAB02852, BAB02854, BAB02855, BAB02858, BAB02613), *B. rapa* (BrMDR1, GenBank accession no.: DQ296184) and C. japonica (CjMDR, GenBank accession no.: BAB62040).

Molecular evolution analysis

Brmdr1 was the first mdr gene cloned from Brassica species. Therefore it would be interesting to investigate its evolutionary

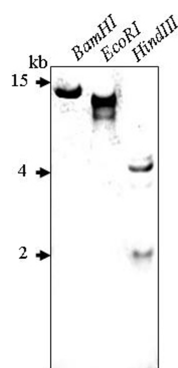

Fig. 4. Southern blot analysis of *Brmdr1*. Genomic DNA isolated from *B. rapa* leaves is digested overnight, respectively, with *BamHI*, EcoRI and HindIII, followed by hybridization with the 370 bp fragment as the probe.

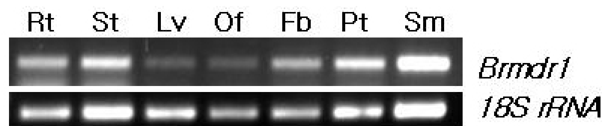

Fig. 5. Expression profile of *Brmdr1* in different *B. rapa* tissues. Total RNA is isolated from vegetative tissues (roots (Rt), stem (St), leaves (Lv)) as well as reproductive tissues (flower buds (Fb), open flowers (Of), stamens (Sm) and petals (Pt)) respectively, and subjected to semi-quantitative one-step RT-PCR analysis. The 18S rRNA gene is used as the control to show the normalization of the amount of templates used in PCR reaction.

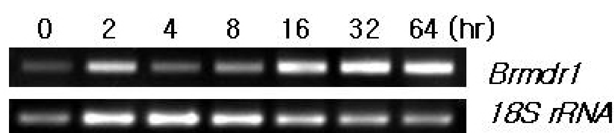

Fig. 6. Transcript's levels of *Brmdr1* at the different time points after exposure to MJ treatment. Total RNA is isolated at the 2hr, 4hr, 8hr, 16hr, 32hr and 64hr after exposure to MJ treatment, respectively, and subjected to semi-quantitative one-step RT-PCR analysis. The 18S rRNA gene is used as the control to show the normalization of the amount of templates used in PCR reaction.

position on the phylogenetic tree composed of a variety of MDRs. Using MEGA version 3.0 from CLUSTAL W alignments, a phylogenetic tree of MDRs was built up from *A. thaliana*, C. japonica and C. roseus (Fig. 3). In order to see the phylogenetic position of BrMDR1 in the whole MDR subfamily and even further to infer its possible function in *B. rapa*, we included all the putative 22 MDRs in the genome of *A. thaliana* and the only MDR protein from medicinal plants, CjMDR1. According to the phylogenetic tree, all MDRs contained are classified into three clusters. BrMDR1 was classified into the same cluster with AtMDR4 and CjMDR, which had been characterized to be importer of certain subtrates (Shitan *et al*. 2003; Santelia *et al*. 2005; Terasaka *et al*. 2005), while AtMDR1 and AtMDR19, characterized as exporters (Geisler *et al*. 2003; Windsor *et al*. 2003; Geisler *et al*. 2005), were classified into the other two clusters, respectively. It was supposed that BrMDR1 might act as an importer of certain substrates. From the sequence analyses above, BrMDR1 was found to have many characteristics common in the MDR subfamily. All the analytical results strongly suggest that BrMDR1 is a plant MDR-like ABC transporter protein involved in the transmembrane transport of certain substrates, probably in an inward direction. The purification and relevant analysis of BrMDR1 protein from *B. rapa* will further illustrate the structure and function of BrMDR1 in the future.

Southern blot analysis

In order to determine the genomic organization of *Brmdr1* in *B. rapa*, Southern blot analysis was carried out by digesting the genomic DNA of *B. rapa* with the restriction enzymes *Bam*HI, *Eco*RI and *Hind*III, respectively, followed by hybridization with the 370 bp fragment including 3'-untranslated sequence of *Brmdr1* cDNA generated by PCR with primers Brmdr-SouthernF and Brmdr-SouthernR as the probe. In the probe region and the *Brmdr1* cDNA, there were no cleavage sites for these endonucleases. The result revealed that only one to two clear hybridization bands were present in each lane, indicating that *Brmdr1* was a low-copy gene (Fig. 4).

Tissue expression analysis of *Brmdr1* gene

To investigate the expression profile of *Brmdr1* in different tissues including vegetative tissues (roots, stem and leaves) as well as reproductive tissues (flower buds, open flowers, stamens and petals), respectively of *B. rapa*, total RNA was isolated from these tissues and subjected to semi-quantitative one-step RT-PCR analysis using primers BrmdrRT-F and BrmdrRT-R. The result showed *Brmdr1* expressed in all the tested tissues, but with lower expression in open flowers and leaves (Fig. 5), indicating that BrMDR1 proteins might function to transport substrates more actively in roots and stems than in leaves. Furthermore, to investigate the mechanism of *Brmdr1* trans-

cription under stressed conditions, total RNA (1 μg) extracted from leaves of *B. rapa* was used as template to detect *Brmdr1* transcript's levels at the different time points after exposure to MJ treatment (Fig. 6). Accumulation of secondary metabolites often occurs in plants subjected to stresses including various elicitors or signal molecules, among which MJ is a commonly-used elicitor as an artificial stress for the plants to test the expression changes of the biosynthetic enzyme genes for certain secondary metabolites (Zhao *et al*. 2005). In our study, the results showed no apparent differences in the expression of *Brmdr1* at different time points, indicating that the expression of *Brmdr1* was not induced by MJ treatment. It is supposed that *Brmdr1*, as a transporter gene, may obey a law different from the biosynthetic enzymes of secondary metabolites.

Up to now, a number of enzymes involved in the biosynthetic pathway of alkaloids and transcription factors regulating the expression of the enzyme genes have been identified and some of them have been characterized in *B. rapa* (Majse *et al*. 2006). But up to now, there has not been any report on the cloning of ABC transporter genes from Brassica species. In this paper, we report, for the first time, the cloning and characterization of ABC transporters in *B. rapa.* The results of this study will help us to understand more about the regulation mechanism of production and accumulation of secondary metabolites both in *B. rapa* and in other vegetable plants.

Acknowledgements

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