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Gene Note

Unusual expression of an *Arabidopsis* ATP-binding cassette transporter *ABCC11*

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Abstract Plant genomes contain more than 120 genes encoding ATP-binding cassette (ABC) proteins, which can be classified into 8 groups. Among them, ABCC/MRP (Multidrug resistance-associated protein) family members are proposed to function in general as MgATP-energized pumps involved in detoxification mechanisms, mainly by transporting glutathione-xenobiotic (GS-X) conjugates and other bulky amphipathic anions across membranes. Recent reports demonstrated that 15 ABCC subfamily members in *Arabidopsis thaliana* are widely expressed in the plant body and suggested that these proteins play a general function for the survival of plant cells. In this study, it was found that a member of this family, *AtABCC11*, which was thought to encode a full-sized ABCC-type transporter, gives an unusual transcript of a much smaller size than the full-size mRNA covering the entire open reading frame, and therefore may not be functional as a full-sized ABC transporter in plants. This study also assessed the promoter activity of *AtABCC11* compared with its closest paralogue, *AtABCC12*.

Key words: ABC protein, *Arabidopsis thaliana*, GUS expression, northern analysis.

The ATP-binding cassette (ABC) superfamily is the largest transporter family in living organisms, ranging from bacteria to humans (Higgins 1992; Garcia et al. 2004). In humans, ABC transporters have received considerable attention as deficiencies or mutations in their genes are associated with severe diseases, whereas in plants only a limited number of mutants of ABC transporters show defects in growth and morphology, probably due to their high redundancy. These transporters are involved in the movement of various substrates, including ions, carbohydrates, lipids, xenobiotics, drugs, and heavy metals across membranes. In the *Arabidopsis* genome, more than 120 genes encoding ABC proteins have been identified (Garcia et al. 2004), but their functions and substrates remain to be clarified in most cases. Some ABC transporters have been characterized as showing involvement in the transport of auxin, chlorophyll catabolites (Lu et al. 1998; Terasaka et al. 2005), pathogen-related compounds (Kobae et al. 2006), and conjugates of heavy metals (Lee et al. 2005). A plant ABC transporter controlling water stress via anion and calcium channel regulation has also been reported as well (Suh et al. 2007).

ABC transporters possess two hydrophobic domains (TMD) containing six membrane spans, and two hydrophilic nucleotide-binding domains (NBD) facing the cytosol, which are organized in tandem in full-sized ABC transporters. We have been working on the transporters localized to the vacuolar membrane (Otani et al. 2005), and there are several tonoplast localized ABC transporters, most of which belong to the ABCC/MRP (multidrug resistance-associated protein) subfamily (Martinoia et al. 2002). Typically ABCC transporters have an additional hydrophobic domain (TMD0, including 3 to 5 transmembrane spans) in the N-terminal part of the polypeptide.

Phylogenetic analysis of the 15 AtABCCs divided them into two subclades; clade I consisted of AtABCC1 (At1g30400), 2 (At2g34660), 11 (At1g30420), and 12 (At1g30410) and clade II contained the 10 other members (Figure 1A). The nomenclature in this paper is in accordance with the recent unification of plant ABC protein nomenclature (Verrier et al. 2008). Current evidence suggests that AtABCC1 and AtABCC2 localize to the tonoplast and exhibit glutathione *S*-conjugate transport activity in an ATP-dependent manner (Frelet-Barrand et al. 2008). However, two other AtABCC members 11 and 12 in clade I, which are also presumed to localize to the vacuoles, have not been characterized in detail. This study characterized the expression of the

Abbreviation: ABC, ATP-binding cassette; GUS, beta-glucuronidase; MRP, Multidrug resistance-associated protein; NBD, nucleotide-binding domain; ORF, open reading frame; TMD, transmembrane domain; UTR, untranslated region.
This article can be found at http://www.jspcmb.jp/

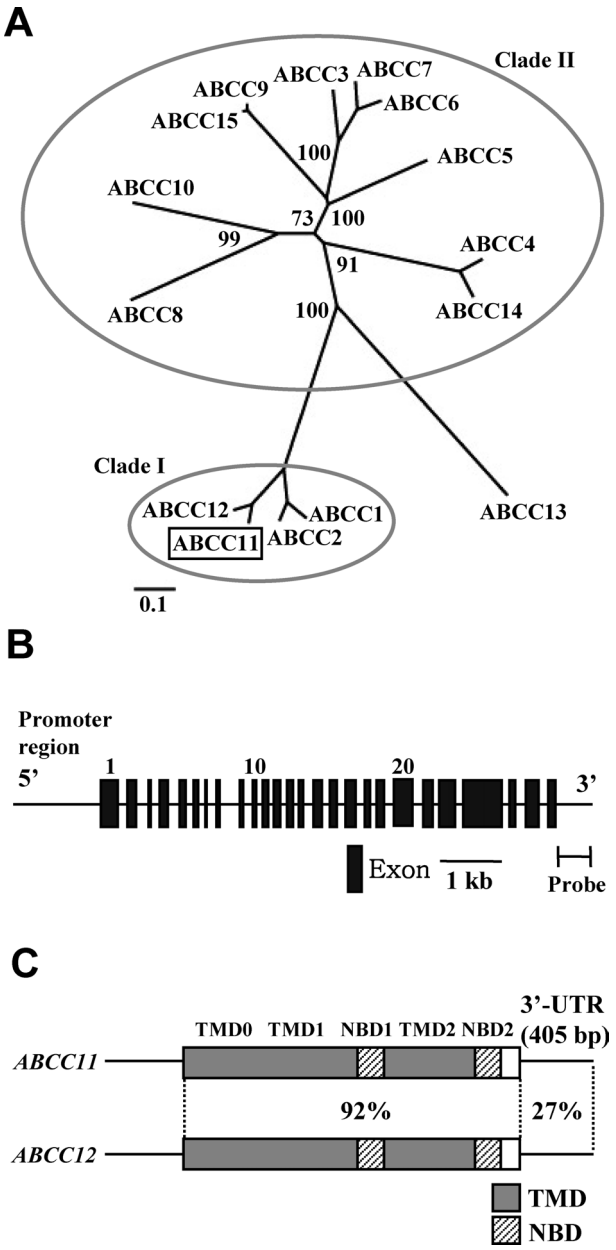

Figure 1. Features of the *AtABCC11* gene and its gene products. (A) Phylogenetic tree of the *Arabidopsis* ABCC subfamily. Numbers indicate the bootstrap values. (B) Intron-exon structure of *AtABCC11*. This gene consists of 26 exons and is located on chromosome 1. The 398 bp fragment at the 3′-end was used as a probe. The upstream 1.8 kb region was used in promoter GUS analyses. (C) Protein features of AtABCC11 to AtABCC12. Transmembrane domains (TMD) and nucleotide-binding domains (NBD) are depicted. The sequence identity of the mRNAs is also shown; the open reading frame was 92%, whereas that of the 3′-UTR was 27%.

AtABCC11 gene to find an unusual sized transcript, suggesting that this ABCC member may not be translated as a full-size ABC transporter in *Arabidopsis thaliana*.

To analyze the expression pattern of *AtABCC11*, total RNA was isolated using a RNeasy Plant Mini-Kit (Qiagen, Valencia, CA, USA) from fourteen-day-old seedlings, which had been treated with various phytohormones and abiotic stresses. Due to the high sequence similarity between *AtABCC11* and 12 in their open reading frames (ORFs) (92% nucleotide identity), the 3′-untranslated region (UTR) of *AtABCC11* (27% nucleotide identity) was selected for the gene-specific probe (Figure 1B, C). To prepare the *AtABCC11* probe, its 3′-UTR was amplified using the primers ABCC11-f3 (5′-TTGATGACGGAATTTGCATTGTTAC-3′) and ABCC11-r3 (5′-AGCCGAGTATCCTGACCAGCTAGA-3′) using GoTaq polymerase (Promega). Agarose gel electrophoresis of RNA, transfer onto Hybond-N^+ membranes (GE healthcare UK Ltd.), and hybridization with the 398 bp *AtABCC11* fragment (positions +4509 to +4907) were performed using standard procedures. The last stringent wash was done with 0.2×SSC (1×SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS at 60°C. As shown in Figure 2A, the *AtABCC11* transcript was not detectable at the expected size (*ca*. 4.5 kb) in the northern analysis of plants under various treatments, but a band at *ca*. 400 bp was found. In addition, the organ-specific expression analysis of *AtABCC11* gene was also performed by northern blot using the RNA prepared from various *Arabidopsis* organs (Figure 2B). There was no accumulation of *AtABCC11* transcript detectable at 4.5 kb in all organs tested, whereas a transcript of small size at *ca*. 400 bp was detected in the northern analyses. To confirm the unusual size of the *AtABCC11* transcript, the same RNA samples from seedlings as applied to northern analyses was reverse transcribed using SuperScript III (Invitrogen, CA, USA), followed by treatment with RNase H (Invitrogen, CA, USA), and then both ORF regions of *AtABCC11* and *AtABCC12*, and their 3′-UTRs, were amplified using PCR with the primers shown in Figure 2C. Although all *AtABCC12* fragments corresponding to the two ORF regions and a 3′-UTR were apparently amplified, *AtABCC11* expression was only detected using its 3′-UTR. These results strongly supported the finding that the *AtABCC11* gene is not expressed as a full-size ABC transporter but gives an unusual transcript of *ca*. 400 bp corresponding to the 3′-region. Only one short EST 1361098 corresponding to the 3′-region of the *AtABCC11* gene is recorded in the *Arabidopsis* database, which is also consistent with the present experimental observations.

The expression level of this short transcript of *AtABCC11* was altered by various treatments, *e.g*. up-regulated by 2,4-D and down-regulated by darkness, and the highest expression level was observed in flowers (Figure 2A, B). Among plant ABC proteins characterized thus far, the *AtABCC1* gene is also strongly expressed in flowers, while the up-regulation of gene expression by dark treatment is known in *AtABCC2* (Frelet-Barrand et al. 2008). This small RNA molecule

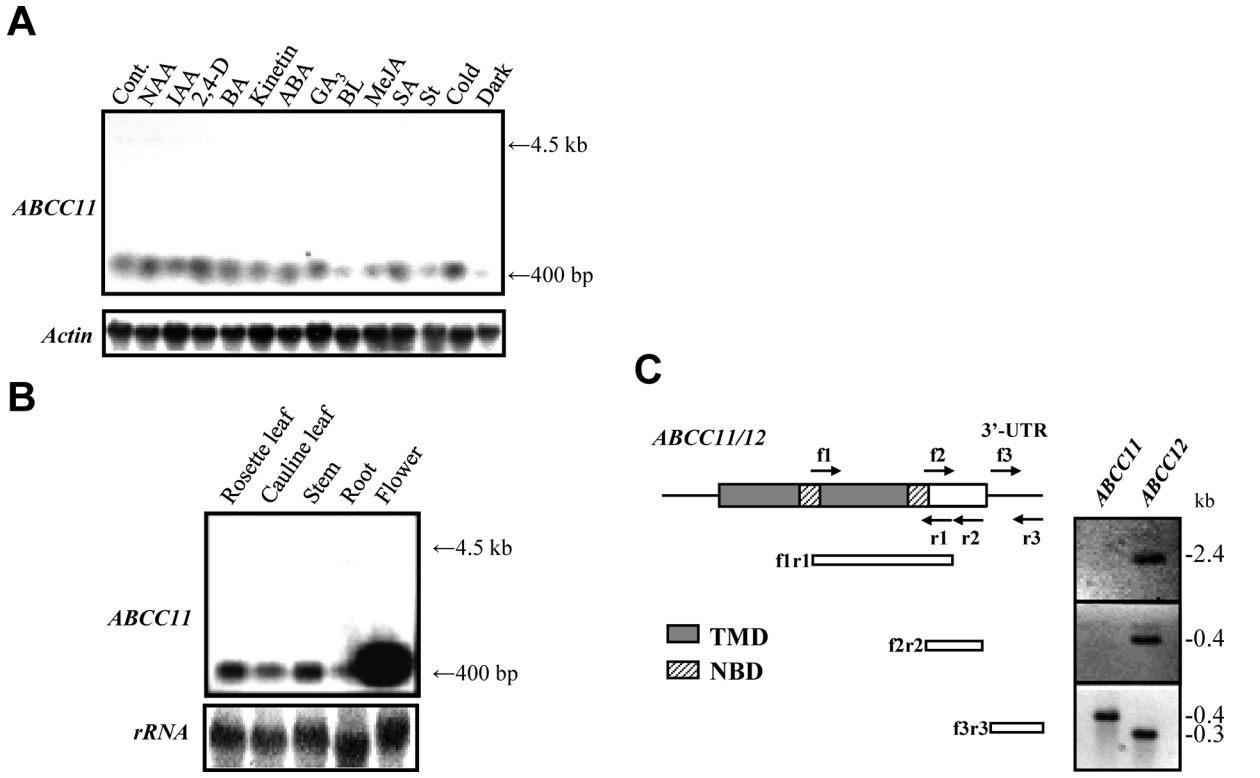

Figure 2. mRNA accumulation pattern of *AtABCC11* under various treatments in *Arabidopsis*. (A) Northern blot analysis of *AtABCC11* under various treatments in *Arabidopsis* seedlings. Cont., untreated control. Fourteen-day-old seedlings were treated for 24 h with 10 μM 1-naphthaleneacetic acid (NAA), 10 μM 3-indoleacetic acid (IAA), 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 10 μM 6-benzyladenine (BA), 10 μM kinetin, 10 μM abscisic acid (ABA), 10 μM gibberellic acid (GA_3), 10 μM brassinolide (BL), 100 μM methyl jasmonate (MeJA), 100 μM salicylic acid (SA), or 10 μM stigmasterol (St). Cold (4°C/low light) and dark treatments were also performed for 24 h. Total RNA (8 μg) prepared from whole seedlings was probed with a ^{32}P-labeled *AtABCC11* fragment (top panel). Loading controls are shown using β-actin (bottom panel). The experiment was repeated twice with similar results. (B) Organ-specific expression of *AtABCC11* in *Arabidopsis*. Loading controls are shown using *rRNA* (bottom panel). The experiment was repeated three times with similar results. (C) RT-PCR analysis of *AtABCC11* gene. The primers used were ABCC11-f1 (5′-GGTTTCATTTGGGGTTTAT-3′), ABCC11-r1 (5′-CTAACAAGAAGAACTGTTTCC-3′), ABCC12-f1 (5′-GTAAAGCA-CAGTTACTATCAGCTTTT-3′), ABCC12-r1 (5′-ACTGGAGATTGTGGTATGATACTTAG-3′), ABCC11-f2 (5′-GTAGGAAACAGTTCTTCTTGT-3′), ABCC12-f2 (5′-CTAAGTATCATACCACAATCTCCAGT-3′), ABCC11/12-r2 (5′-CAGTCGATGATGGTATTCAA-3′), ABCC12-f3 (5′-TTTGTGACAACAAGAAAGATAATG-3′) and ABCC12-r3 (5′-CATTTGAAGCTCTTGTATGTTATGA-3′) for amplifications of *AtABCC11* and *AtABCC12*. The amplicon by the primer pair f1 and r1 is shown as f1r1, and the other two fragments are also shown in the same manner. The experiment was repeated four times with similar results.

might be functional as an endogenous non-coding RNA that regulates the expression pattern of other genes involved, for example, in stress responses or developmental processes (Lu and Huang 2008).

To characterize the promoter activity of *AtABCC11*, *ProABCC11*: beta-glucuronidase (GUS) and *ProABCC12*: GUS transformants were produced for comparison. To create these promoter regions, PCR primers annealing at −1799 to +18 bp and −1658 to +18 were made; ABCC11pro-Fw (5′-GTCGACAAA-TGGTCACTAGCCTTCCAAAATG-3′), ABCC11pro-Rv (5′-GGATCCACCCATCCTTTTTCAATCAAAC-CTC-3′) and ABCC12pro-Fw (5′-GTCGACACGATGA-AAGGCTTACACTAATAGA-3′), ABCC12pro-Rv (5′-GGATCCCAAGGCTTCAAAACCCATCTC-3′). The underlined positions are non-native sequences representing *Sal*I and *Bam*HI restriction sites, respectively. PCR was performed using genomic DNA as a template and KOD-Plus polymerase (TOYOBO). The PCR product was cloned into the cloning vector, pCR-Blunt II-TOPO (Invitrogen, CA, USA) for sequencing and then re-cloned into the binary vector, pBI101 (CLONTECH). *A. tumefaciens* GV3101 (pMP90) was transformed with the binary vector and introduced into wild-type *Arabidopsis* by the floral dip method (Clough and Bent 1998). Kanamycin-resistant plants of the T_2 generation were stained histochemically to detect GUS activity. GUS staining was done according to the method reported previously (Terasaka et al. 2005). Two-week-old plantlets and adult plants were used, but no GUS activity was observed in both materials of different developmental stages (Figure 3A, C), whereas GUS gene expression driven by the *AtABCC12* promoter was apparently expressed throughout the whole plant (Figure 3B). Prolonged GUS staining did not give any detectable signal in *ProABCC11*:GUS transformants (data not

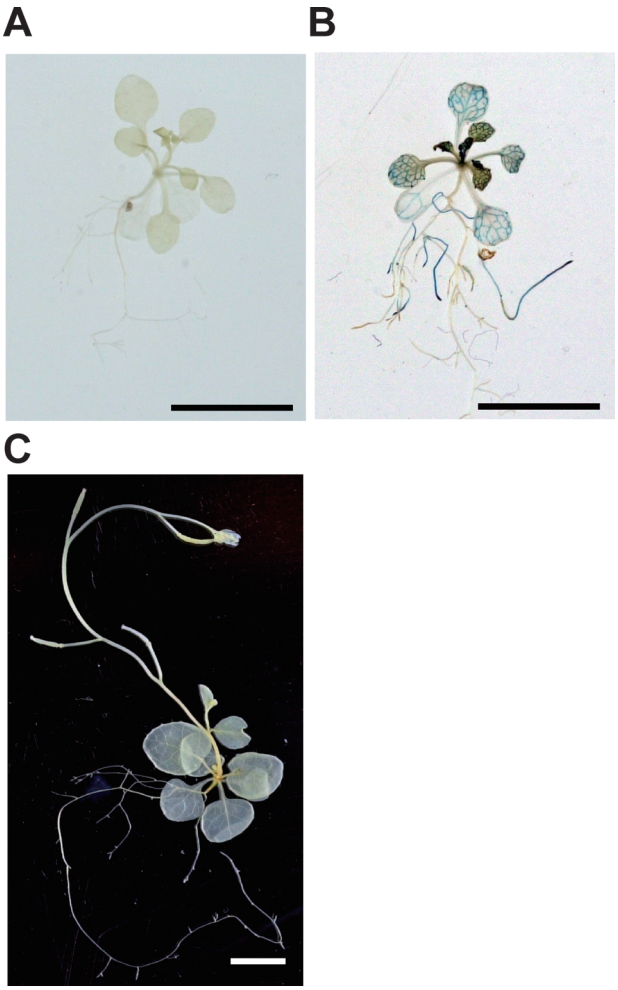

Figure 3. Promoter GUS analysis of *AtABCC11* and *AtABCC12*. The promoter regions of *AtABCC11* (1.8 kb) and *AtABCC12* (1.7 kb) were fused to the *GUS* gene and introduced into *Arabidopsis*. GUS staining was done using T_2 generation plants. Bars=5 mm. (A) GUS staining analysis performed using 2-week-old sterile grown *ProABCC11 : GUS* seedlings. (B) GUS staining analysis performed using 2-week-old sterile grown *ProABCC12 : GUS* seedlings. (C) GUS staining analysis of *ProABCC11 : GUS* was performed with 5-week-old sterile grown adult plants.

shown). These results supported the hypothesis that the *AtABCC11* gene is not expressed to yield a full-size mRNA coding for a full-size ABCC-type transporter in *A. thaliana*.

To gain further insights into the promoter of *AtABCC11*, the promoter region of *AtABCC11* was compared with that of *AtABCC12*. Multiple sequence alignment indicated that the promoter regions between *AtABCC11* and *AtABCC12* shared low sequence similarity (19% identity at the nucleotide level). Computer analysis demonstrated that the *AtABCC11* promoter region has less *cis*-regulatory elements than *AtABCC12*, i.e., the *AtABCC11* promoter had three *W*-Boxes, while the *AtABCC12* promoter had an additional four *W*-Boxes, an as1-like element, and a *G*-Box as reported previously (Kolukisaoglu et al. 2002). In addition, a promoter prediction program (http://www.fruitfly.org/seq_tools/promoter.html) indicated that the TATA box in the *AtABCC11* promoter sequence is located more distant (300 bp) from the putative initiation codon, than that in the *AtABCC12* promoter.

Terrestrial plants are incapable of escaping unfavorable environmental conditions, such as xenobiotic stresses, by moving. Instead, they rely heavily on xenobiotic transporters to survive these conditions. Thus, how plants transport and detoxify xenobiotics is a fundamental question. It has been shown that *AtABCC1* and 2 are involved in xenobiotic transport into vacuoles. This study analyzed *AtABCC11* in *Arabidopsis* a new member of ABCC clade I, which was proposed to consist of vacuolar transporters. It was concluded that *AtABCC11* does not give RNA species corresponding to the full-size ABC transporter on the basis of the following evidence: (i) full-sized *AtABCC11* mRNAs were not detected in gel blot analysis; (ii) RT-PCR and northern analysis showed that only the 3′-UTR is expressed in *AtABCC11*; (iii) promoter GUS analysis showed that *ProABCC11* activity was not detected in either seedling or adult *ProABCC11* transformants, while *ProABCC12*, the promoter of the closest paralogue, was active in whole plants in the same experiment.

The short transcript corresponding to the 3′-region (400 bp) of *AtABCC11* suggests that there might be a region showing promoter activity in the *AtABCC11* ORF. In fact, potential sequences that may have promoter activity containing a putative TATA box (at *ca*. 4.0 kb from start codon) and some putative *cis*-elements such as GCN2, GCR4, and NIT2 were found in the *AtABCC11* ORF, which may be the reason of the unusual short transcript (data not shown). Future studies will determine whether or not the small RNA species of *AtABCC11* play a physiological role in plants.

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