An ABC Transporter Gene of *Arabidopsis thaliana*, *AtWBC11*, is Involved in Cuticle Development and Prevention of Organ Fusion

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Cuticle, including wax and cutin, is the barrier covering plant aerial organs and protecting the inner tissues. The *Arabidopsis thaliana* ATP-binding cassette (ABC) transporter CER5 (*AtWBC12*) has been identified as a wax exporter. In agreement with the latest report of another wax exporter, *AtWBC11*, here we show that *atwbc11* mutants displayed organ fusions and stunted growth, and became vulnerable to chlorophyll leaching and toluidine blue staining. Chemical analysis showed that wax and cutin monomers were both reduced in the *atwbc11* mutant. *AtWBC11* was widely expressed in aerial organs. Interestingly, we found that the expression was light dependent, and the phytohormone ABA up-regulated *AtWBC11* expression. We also found that while the *AtWBC11* promoter had a broad pattern of activity, the expression was converted to epidermis specific when the reporter gene was fused to *AtWBC11* cDNA. Furthermore, RNA blot analysis supported epidermis-specific expression of *AtWBC11*. Our results support that *AtWBC11* is involved in cuticle development.

**Keywords:** ABC transporter — *Arabidopsis thaliana* — Cuticle — Cutin — Wax.

Abbreviations: ABC, ATP-binding cassette; FAE, fatty acid elongase; GC-MS, gas chromatography–mass spectrometry; GFP, green fluorescent protein; GUS, β-glucuronidase; NBD, nucleotide-binding domain; RT–PCR, reverse transcription-PCR; SEM, scanning electron microscopy; TB, toluidine blue; T-DNA, transfer DNA; TEM, transmission electron microscopy; TMD, transmembrane domain; VLCFA, very long chain fatty acid; WBC, white–brown complex.

**Introduction**

Plant primary aerial organs, such as young stems and leaves, are covered by cuticle which is the first site where plants interact with the environment. Unlike the cell wall underneath, which consists of both hydrophilic and hydrophobic components, cuticle is highly hydrophobic and mainly composed of fatty acids and their derivatives. Structurally, cuticle can be divided into the inner cutin layer and outer wax layer. The cutin layer is a polymer matrix composed of hydroxy and epoxy fatty acids connected via intermolecular esterification (Nawrath 2002, Heredia 2003), whereas wax is an amorphous mixture of very long chain fatty acids (VLCFAs; carbon chain length >18) and their derivatives (Post-Beittenmiller 1996, Jenks et al. 2002, Kunst and Samuels 2003). The plant cuticle protects inner tissues from biotic and abiotic stresses (e.g. non-stomatal water loss, bacterial and fungal pathogens, herbivore attacks, temperature extremes and UV light), and prevents organ fusion during plant development (Lolle et al. 1998, Lolle and Pruitt 1999, Sieber et al. 2000).

Many genes involved in cuticle lipid synthesis have been identified (Post-Beittenmiller 1996, Jenks et al. 2002, Heredia 2003, Yephremov and Schreiber 2005). Generally, at least in *Arabidopsis*, the plastid-produced C16/C18 fatty acids serve as common intermediates for both wax and cutin synthesis. For wax precursors, C16/C18 fatty acids are elongated to produce VLCFAs, which is catalyzed by an enzyme complex, fatty acid elongase (FAE). VLCFAs are further modified to make different kinds of wax precursors (e.g. ketones, alkanes and aldehydes). On the other hand, C16/C18 fatty acids are also modified to produce major monomers of cutin (e.g. C16 and C18 dicarboxylic acids).

The cuticle monomers are synthesized inside the epidermal cells and deposited outside the epidermis. This raises an interesting issue in cuticle research: how cuticle (wax and cutin) monomers are exported to the cell surface (Kunst and Samuels 2003). In this process, these aliphatic materials have to pass through the plasma membrane of epidermal cells. The involvement of ATP-binding cassette (ABC) transporters in wax transport across the cell plasma membrane has been proposed (Kunst and Samuels 2003). It was found that cer5 mutant plants had a reduction of epicuticular wax load (wax deposited on the plant surface), but the amount of the total wax (epicuticular plus intracellular) did not differ much between cer5 and the wild-type plants, suggesting a role for CER5 as a wax transporter. CER5 encodes an ABC transporter, namely *AtWBC12* (Sánchez-Fernández et al. 2001, Pighin et al. 2004). This is the first evidence of an ABC transporter involved in movement of cuticle lipids. Considering the

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common features of cuticle monomers and the large ABC transporter gene family in *Arabidopsis*, there should be other ABC transporters involved in cuticle monomer secretion. Moreover, the transport of cutin monomer is still an enigma.

In this article, we present evidence that another *Arabidopsis* ABC transporter, AtWBC11, is required for plant cuticle development, which agrees with the latest report (Bird et al. 2007). Our detailed expression analysis revealed that light signaling and the phytohormone ABA were involved in regulation of *AtWBC11* expression. Interestingly, although the *AtWBC11* promoter displayed a broad pattern of activity, the fusion of *AtWBC11* cDNA to reporter gene converted the expression pattern to epidermis specific, in agreement with the epidermal distribution of *AtWBC11* transcripts in aerial organs.

**Results**

Isolation and genetic analysis of *atwbc11* mutants

In previous work, we have isolated a cotton ABC transporter, GhWBC1. We found that overexpression of GhWBC1 in *Arabidopsis* led to the phenotype of short siliques and reduced fertility (Zhu et al. 2003). We assumed that it was the heterodimer formed between GhWBC1 and *Arabidopsis* ABC transporters that interfered with the function of endogenous ABC transporters. The closest homolog of GhWBC1 is AtWBC11, and a reverse genetics approach was then employed to investigate the function of *AtWBC11*.

Transfer-DNA (T-DNA) insertion lines were collected from the Arabidopsis Biological Resource Center (ABRC, www.arabidopsis.org) (Alonso et al. 2003). Two mutants (Salk_043637 and Salk_072079) were identified for the *AtWBC11* (At1g17840) locus. Filled and open boxes represent exons and introns, respectively. The T-DNA insertion sites of *AtWBC11* locus contributed to the *atwbc11* mutant phenotype of *atwbc11-4* and *atwbc11-5* mutants. We then used *ProAtWBC11::AtWBC11* cDNA to complement the *atwbc11-5* mutant plants (Fig. 1B). We found that light signaling and the phytohormone ABA were involved in regulation of *AtWBC11* expression. A low level of *AtWBC11* expression could be detected in *atwbc11-4*, but not in *atwbc11-5* (Fig. 1C), suggesting that the mutations were caused by T-DNA insertion in the *AtWBC11* locus. Because abnormal plants of either *atwbc11-4* or *atwbc11-5* produced no seeds, the mutations were kept in a heterozygous state.

To assess the effects of T-DNA insertions on *AtWBC11* gene expression, RNA blot analysis was performed. The *AtWBC11* transcripts were undetectable in either *atwbc11-4* or *atwbc11-5* mutant plants (Fig. 1B). We then used the more sensitive reverse transcription-PCR (RT-PCR) to examine the expression of *AtWBC11*. A low level of *AtWBC11* expression could be detected in *atwbc11-4*, but not in *atwbc11-5* (Fig. 1C), suggesting that *atwbc11-5* was a knockout and *atwbc11-4* a knockdown mutant. We then used *atwbc11-5* for most of the analyses.

To exclude the possibility that mutations in other sites closely linked to the *AtWBC11* locus contributed to the phenotype of *atwbc11* mutants, we examined the ability of *AtWBC11* to complement the *atwbc11* mutant. An ~2kb genomic region upstream of the *AtWBC11* translation start codon was used as the promoter (*ProAtWBC11*) to drive the *AtWBC11* cDNA (*ProAtWBC11::AtWBC11*) (Supplementary Fig. S1). Because homozygous *atwbc11* mutants were sterile, complementation was performed on the heterozygous *atwbc11* plants and analyzed by examining segregating progeny of the transformants. The T1 generation plants were screened by PCR to identify the lines carrying the expected T-DNA insertion. The T2 plants also displayed phenotypic segregation, and the presence of the transgene showed that the abnormal plants all contained homozygous T-DNA insertions, whereas the plants with a heterozygous T-DNA insertion were indistinguishable from the wild-type (data not shown), suggesting that the mutations were recessive and the phenotype was caused by T-DNA insertion in the *AtWBC11* locus. Because abnormal plants of either *atwbc11-4* or *atwbc11-5* produced no seeds, the mutations were kept in a heterozygous state.
that had a ProAtWBC11::AtWBC11 transgene were selected for subsequent analysis. They all grew normally and stably transmitted the wild-type phenotype to the successive generations, corroborating that ProAtWBC11::AtWBC11 can fully complement the atwbc11-5 mutation (Supplementary Fig. S1). Therefore, it is the AtWBC11 gene mutation that caused organ fusion and other developmental abnormalities.

Analysis of the atwbc11 mutant phenotype

The atwbc11-4 and atwbc11-5 mutants exhibited morphological and developmental abnormalities similar to wbc11-1, -2 and -3 plants (Bird et al. 2007), though subtle differences were found with regard to trichome morphology.

The atwbc11 mutants were indistinguishable from the wild-type plants until the appearance of true leaves. For the wild-type seedlings, trichomes developed well on the first pair of true leaves (Fig. 2A). However, there were only a few distorted trichomes of smaller size on the first pair of atwbc11-5 leaves, which were hardly observable by the naked eyes (Fig. 2B). Another feature of the atwbc11-5 mutant was that the seedlings grew a little faster than the wild-type: when the wild-type seedlings expanded the first pair of true leaves and the third and fourth leaves were just emerging (Fig. 2A), the third and fourth leaves of the atwbc11-5 plant had already fully expanded and the fifth leaf began to emerge (Fig. 2B). Roots of the atwbc11-5 seedlings appeared normal in our laboratory conditions (Fig. 2C).

In the later growth stage, organ fusions among aerial tissues were frequently observed (Fig. 2D). In extreme cases, the aboveground tissues all fused together and the plant did not expand and died. The atwbc11-5 mutant also became much smaller than the wild-type (Fig. 2D, E). Organ fusions between stems and leaves, siliques and leaves, and among other aerial organs were observed (Fig. 2E). Unlike Arabidopsis fiddlehead (jdl) (Yephremov et al. 1999, Pruitt et al. 2000), another organ fusion mutant, the atwbc11-5 flowers did not fuse together (Fig. 2E). The atwbc11-5 siliques did not elongate and failed to produce seeds (Fig. 2E), as was also the case for atwbc11-4 plants. Bird et al. (2007) reported that the fertility of wbc11-1 and wbc11-2 plants, which were also knockdown mutants, was normal. This discrepancy might be explained by the stronger repression of AtWBC11 in our atwbc11-4 plants, or by different growth conditions. The atwbc11-5 leaf trichomes were much smaller than those of the wild-type, and some even ceased growing at very early stages (Fig. 2F). The two-pronged trichomes described by Bird et al. (2007) were rarely observed in our experiment and, generally, the trichomes on atwbc11-4 or atwbc11-5 leaves had three branches. Similarly, epidermal cells of atwbc11-5 leaves were much smaller than those of the wild-type (Fig. 2G). The mutant stem was thinner than that of the wild-type and displayed a light-green color (Fig. 2H), which was a trait of Arabidopsis eceriferum (cer) mutants (Koornneef et al. 1989). Scanning electron microscopy (SEM) observation showed that the wax crystals on the stem surface were reduced in atwbc11-4 and atwbc11-5 plants in comparison with the wild-type (Fig. 2I). Wax crystal reduction was less significant on the atwbc11-4 stem surface, which can be explained by the leaky expression of AtWBC11 in atwbc11-4 plants (Fig. 1C).

TB staining can be used to visualize plant cuticle defects (Tanaka et al. 2004). Like the Arabidopsis bodyguard (bdg) mutant, which also had the organ fusion phenotype (Kurdyukov et al. 2006a), atwbc11-5 plants could be stained by TB solution (Fig. 2J). The atwbc11 plant tissues released chlorophyll faster than the wild-type plants when immersed in 80% ethanol (Fig. 2K), just as other organ fusion mutants did (Lolle et al. 1998). These results indicate that the mutations of AtWBC11 affected the cuticle impermeability.

In the fusion sites, the organs were connected by adhering epidermal cell layers (Fig. 3A). The fusion could also occur between just a few cells (Fig. 3B). The epidermal cell identity was not changed according to the cell shape and TB staining pattern (Fig. 3A, B). In wild-type plants, the cuticle appeared as an electron-opaque layer under transmission electron microscopy (TEM) (Fig. 3C). For atwbc11-5 plants, the cuticle of the epidermal cells outside fusion sites seemed normal except that it was less electron opaque (Fig. 3D). In organ fusion sites, the epidermal cell walls of both sites came into direct contact, and the electron-opaque structure corresponding to the cuticle layer lost the uniform structure and became discontinuous or even absent (Fig. 3E, F).

Reduced cuticle lipids in atwbc11 mutant

SEM observation revealed that the amount of the wax crystals was significantly reduced on the stem surface of atwbc11 mutants (Fig. 2I). We quantified the total wax coverage on inflorescence stems. The wax load in atwbc11-4 and atwbc11-5 inflorescence stems was reduced by ~39% and ~70%, respectively, compared with the wild-type (Fig. 4A). Bird et al. (2007) reported that another knockout mutant, wbc11-3, had a reduction of ~52% in wax load, which was lower than that of atwbc11-5. The amount of most wax components, including alkanes, aldehydes, fatty acids, ketones, and primary and secondary alcohols, on the atwbc11-5 stem surface was reduced (Fig. 4A). The most striking change was that C29 alkane, a major wax constituent of Arabidopsis, was reduced to ~7.7% of the
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The amount of C29 ketone was also clearly decreased; C29 secondary alcohol showed a slight decrease. In addition to the overall reduction of the amount of wax, the relative contents of the atwbc11-5 epicuticular wax were changed and the decrease of each component was disproportionate (Fig. 4A). C29 alkane made up only ~13% of the atwbc11-5 stem wax, with ~54% in the wild-type load.

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Fig. 2 Phenotype of atwbc11 mutants. (A) and (B) Seven-day-old wild-type (A) and atwbc11-5 (B) seedlings can be easily distinguished by their trichome development on the first pair of true leaves. Inset: the enlargement of the first pair of true leaves, co, cotyledon. The numbers refer to the order of the leaves produced from the plant. (C) Seven-day-old wild-type (left) and atwbc11-5 (right) seedlings grown on the vertical plates. (D) Three-week-old wild-type (left) and atwbc11-5 plants grown in soil with moderate (middle) and severe (right) phenotypes. Bars = 1 cm. (E) Five-week-old wild-type (left) and atwbc11-5 (right) plants grown in soil. The organ fusions among aerial organs can be observed (as shown by the arrowhead). (F) Scanning electron micrographs of trichomes on the leaf of the wild-type (left) and atwbc11-5 (right). Bars = 100 µm. (G) Scanning electron micrographs of adaxial epidermal cells of the rosette leaves of 3-week-old wild-type (left) and atwbc11-5 (right) plants. Bars = 20 µm. (H) Inflorescence stems of wild-type (left) and atwbc11-5 (right) plants. (I) Scanning electron micrographs of epicuticular wax on stems of wild-type and atwbc11 mutant plants. Bar = 10 µm. (J) Ten-day-old wild-type (left) and atwbc11-5 (right) plants were stained with toluidine blue. (K) Chlorophyll release from aerial parts of 4-week-old wild-type and atwbc11-4 mutant plants.
35% and 43% of the atwbc11-5 wax, respectively, whereas in the wild-type the figures were 19% and 11%, respectively. It seems that the reduction of C29 alkane played a major role in the changes of wax composition.

It was reported that wbc11-3 plants had an ~65% decrease of cutin content (Bird et al. 2007). We analyzed the cutin contents of atwbc11-5 stems and found that the atwbc11-5 mutant had a reduction of ~43% in total cutin monomers (Fig. 4B), consistent with its less electron-opaque cuticle (Fig. 3D). The decrease was reflected by most cutin monomers. The most dramatic change was the decrease of the predominant monomer, C18:2 dicarboxylic acid (Fig. 4B), although other dicarboxylic acid monomers also decreased. Again, relative compositions of the atwbc11-5 cutin were different from those of the wild-type. Except for dicarboxylic acids, the relative content of other classes of cutin monomers was increased in atwbc11-5 (Fig. 4B), largely due to the reduced amount of C18:2 dicarboxylic acid.

Characterization of AtWBC11 protein

AtWBC11 has been annotated as a half-size ABC transporter belonging to the white–brown complex (WBC) subfamily, in an analysis of all putative ABC transporter genes in the Arabidopsis genome (Sánchez-Fernández et al. 2001). Among these WBCs, only CER5 was functionally defined as a wax exporter (Pighin et al. 2004).

AtWBC11 contains 703 amino acid residues with a predicted molecular mass of 78 kDa. Protein sequence analysis revealed the location of characteristic domains of ABC transporters near the N-terminus, including the Walker A and B motif and ABC signature constituting the nucleotide-binding domain (NBD), and a transmembrane domain (TMD) containing six transmembrane α-helices near the C-terminus (H1–H6) (Fig. 5A). Alignment of sequences of AtWBC11, GhWBC1 and CER5 showed that these three proteins have high similarities in the N-terminal NBD domain, but only GhWBC1 and AtWBC11 share an overall similarity in the whole protein sequence, including the C-terminal TMD (Fig. 5A).

To determine the exact subcellular location of AtWBC11, we introduced a Pro35S::GFP-AtWBC11 construct into Arabidopsis. Examination of root cells revealed that green fluorescent protein (GFP) fluorescence was located exclusively in the periphery of the cells (Fig. 5C). In contrast, Pro35S::GFP plants showed fluorescence signal throughout the cell (Fig. 5B). When the roots were plasmolyzed, GFP–AtWBC11 fluorescence was clearly separated from the cell wall (Fig. 5D). Comparing this with the reported fluorescence distribution pattern of different GFP localizations (Cutler et al. 2000), these results demonstrate that GFP–AtWBC11 was localized on the plasma membrane, supporting the notion of Bird et al. (2007) that AtWBC11 is a plasma membrane protein.

Expression patterns of AtWBC11

RT–PCR analysis revealed that AtWBC11 is expressed in various organs, with the highest level of transcripts in inflorescences and the lowest in roots (Fig. 6A), a pattern also reported by Bird et al. (2007). The AtWBC11 expression was extremely weak in roots and the transcripts became detectable only when PCR was performed with a greater number of cycles (Fig. 6A). The distribution of AtWBC11 transcripts agreed with the phenotype because the developmental abnormality was observed only in the aboveground organs. This spatial expression pattern...
was shared by several *Arabidopsis* genes related to organ fusion and epidermal defects, such as *FDH* (Yephremov et al. 1999, Pruitt et al. 2000), *BDG* (Kurdyukov et al. 2006a) and *HOTHEAD* (*HTH*) (Kurdyukov et al. 2006b).

Further analysis showed that light was required for *AtWBC11* transcription (Fig. 6B). Absence of light for 24 h resulted in a dramatic reduction of the *AtWBC11* mRNA level, and continuous darkness for 72 h nearly abolished *AtWBC11* expression. When light was restored, the *AtWBC11* transcription was also restored. The expression of *CER6*, a key condensing enzyme gene in *Arabidopsis* wax production (Millar et al. 1999, Fiebig et al. 2000), also showed a dependency on light (Hooker et al. 2002). We then
**Fig. 5** Analysis of AtWBC11 protein. (A) Alignment of the amino acid sequence of AtWBC11 (AY080792), GhWBC1 (AY255521) and CER5 (AF412079). The sequences were aligned with ClustalW (Thompson et al. 1994) using default parameters through EMBnet (http://www.ch.embnet.org/software/ClustalW.html), and the shading was created by BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html). The transmembrane domain is predicted by TMHMM (Krogh et al. 2001) through CBS Prediction Servers (http://www.cbs.dtu.dk/services/TMHMM/). (B–D) AtWBC11 is a plasma membrane protein. Bars = 50 µm. (B) Roots of Pro35S::GFP transgenic plants with ubiquitous fluorescence distribution throughout the cell. (C) and (D) Roots of Pro35S::GFP-AtWBC11 transgenic plants. (D) The roots were treated with 10% sucrose and plasmolyzed.
checked the effects of phytohormones on \textit{AtWBC11} expression. The 7-day-old seedlings suspended in half-strength MS medium were treated with different phytohormones for 5 h. Northern blot analysis showed that ABA induced \textit{AtWBC11} expression, whereas cytokinin repressed the expression. Treatment by other hormones did not cause a drastic change of the \textit{AtWBC11} expression level (Fig. 6C).

To investigate the tissue specificity of \textit{AtWBC11} expression, we fused its promoter (Pro\textit{AtWBC11}) to the \(b\)-glucuronidase (GUS) and the GFP reporter gene, respectively. GUS activity was detected in various tissues of Pro\textit{AtWBC11}::GUS plants (Fig. 7A–E). In roots, GUS activity was present only in the lateral root primordia and the root tip (Fig. 7A–C). This limited expression explains the low level of \textit{AtWBC11} transcripts in roots. In the aerial organs, the \textit{AtWBC11} promoter drove GUS expression in young leaves, trichomes and the stems (Fig. 7D, E). The Pro\textit{AtWBC11}::GFP showed a similar expression pattern (data not shown).

A striking finding is that the reporter gene expression was not confined to the epidermis: GUS activity, or GFP fluorescence, was present in the subepidermal cells of the stem (Fig. 7E, F). To corroborate that Pro\textit{AtWBC11} was active beyond epidermal cells, we examined the expression of GUS in subepidermal cells by peeling off the epidermal cells from the stem. RNA blot analysis showed that the GUS transcript level in the subepidermal cells was a little lower but comparable with that in the whole stem (Fig. 7G), suggesting that, unlike the \textit{CER6} and \textit{CER5} promoters that are epidermis specific (Hooker et al. 2002, Pighin et al. 2004), the \textit{AtWBC11} promoter activity is not confined to the epidermis.

The activity of the \textit{AtWBC11} promoter in shoot subepidermal cells was also reported in the recent article by Bird et al. (2007). However, we found that there was only
a trace amount of AtWBC11 transcripts in the subepidermal cells, suggesting an epidermal distribution of the AtWBC11 mRNA (Fig. 7G). To investigate this discrepancy, we chose ProAtWBC11::GFP-AtWBC11 (atwbc11-5) plants for further analysis. ProAtWBC11::GFP-AtWBC11 was able to complement the atwbc11-5 mutant fully (data not shown), thus the N-terminal GFP tagging did not affect AtWBC11 function. In ProAtWBC11::GFP-AtWBC11 transgenic plants, GFP fluorescence was observed in the lateral root primordia and root tip (Fig. 8A, B), as observed in ProAtWBC11::GUS plants. In aerial organs, the GFP fluorescence was present in epidermis only. This epidermis-specific distribution was consistently observed in leaves (Fig. 8C), stems (Fig. 8D–F) and siliques (Fig. 8G, H). Arabidopsis siliques have three epidermis-like structures: the exocarp constituting the outer epidermis of the siliques, and endocarp and septum constituting the chamber in which the ovules develop into seeds. The GFP signal was detected in all three layers. The ProAtWBC11::GFP-AtWBC11 transgene in the wild-type plants showed the same expression pattern (data not shown). These results indicate that, in aerial parts, AtWBC11 transcripts are concentrated in the epidermis. We also examined the distribution of GFP-AtWBC11 transcripts by peeling off the stem epidermis. RNA blot analysis showed that GFP-AtWBC11 transcripts were mainly present in epidermal cells (Fig. 8I).
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Discussion

We have shown that AtWBC11 is involved in cuticle development. Two T-DNA insertion mutants were characterized. The atwbc11 mutants exhibited organ fusions and became vulnerable to chlorophyll extraction and TB staining, suggesting that the plants were defective in the maintenance of the epidermis barrier. Chemical analysis of cuticle lipids demonstrated that both wax and cutin monomer were reduced in the AtWBC11 knockout mutant. Thus, besides CER5, AtWBC11 is the second ABC transporter involved in the transportation of cuticle lipids across the plasma membrane. These findings are compatible with the latest report (Bird et al. 2007).

Many genes identified to date as affecting cuticle formation share a similar epidermis-specific expression pattern (Suh et al. 2005). Bird et al. (2007) reported that the AtWBC11 promoter drove GUS expression beyond the shoot epidermis, which is consistent with our observations obtained from the ProAtWBC11::GUS or the ProAtWBC11::GFP plants. However, we found that endogenous AtWBC11 mRNAs were distributed in shoot epidermis. The discrepancy between the AtWBC11 promoter activity and the transcript distribution suggests that there is an additional regulatory step. Furthermore, ProAtWBC11::GFP-AtWBC11 displayed an epidermis-specific fluorescence distribution pattern, which resembled the epidermis-specific expression of AtWBC11 in shoots. Because the only difference between ProAtWBC11::GFP and ProAtWBC11::GFP-AtWBC11 is the presence of the AtWBC11 coding region, this additional regulation must rely on this region. In ProAtWBC11::GFP-AtWBC11 plants, not only the GFP-AtWBC11 fusion transcripts but also the fusion protein accumulated specifically in shoot epidermis, suggesting that the additional regulation did not occur at the protein level. This regulation may involve degradation of the transcripts or suppression of the promoter activity in subepidermal cells. Further studies are required to understand the nature of this regulation and its significance in plant cuticle development.

The cuticle protects plants from environmental stresses. On the other hand, environmental signals also affect cuticle development. CER6 is an enzyme involved in wax production (Fiebig et al. 2000), and its expression was shown to be dependent on light (Hooker et al. 2002). Our results presented here demonstrate that light is essential for AtWBC11 gene transcription (Fig. 6B). Light is not only an environmental signal but is also the energy source for plant growth and metabolism, including biosynthesis of cuticle lipids. CER6 is involved in wax synthesis and AtWBC11 in wax transport, hence correlation of their expression by light signaling might be a tool used by plants to respond to environmental changes. The phytohormone ABA induces the expression of a number of genes that respond to drought stress, including CER6 (Hooker et al. 2002). We found that ABA upregulates AtWBC11 expression. Wax accumulation can be induced by drought stress (Jenks et al. 2002), which also triggers the ABA production and signaling. Thus, the response of CER6 and AtWBC11 expression to ABA treatment might explain how plants regulate cuticle development upon drought stress. The mechanism and biological significance of AtWBC11 gene regulation are worthy of further investigation.

ABC transporters have long been hypothesized to be involved in cuticle transport (Kunst and Samuels 2003). A recent report suggested that ABC transporters may also be involved in the biosynthesis of suberin, which is structurally similar to cutin, by exporting aliphatic compounds (Soler et al. 2007). The Arabidopsis genome has a large ABC transporter gene family with ~120 members, and AtWBC11 is a member of the WBC subfamily comprising 29 members (Sánchez-Fernández et al. 2001, García et al. 2004, Sugiyama et al. 2006). Plant WBC transporters show similarity to human ABC transporters from the ABCG subfamily (e.g. ABCG2, 5 and 8), which were believed to function in lipid and drug export (Doyle et al. 1998, Berge et al. 2000). CER5 (AtWBC12) is the first plant WBC protein identified with a biological function (Pighin et al. 2004). The cer5 mutant grew normally except for the reduced wax load, suggesting that the role of CER5 is limited in wax transport. Repression of CER6 caused a more severe wax reduction of >90%; however, the plant displayed no organ fusion phenotype (Millar et al. 1999). On the other hand, knockout mutation of AtWBC11 led to pleiotropic phenotypes including organ fusions and altered cuticle permeability. These phenotypic changes cannot be explained solely by AtWBC11’s export of wax. The deformed cuticle and reduction in the amount of cutin monomers strongly suggest that the loss of function of AtWBC11 prevents the flipping of cutin monomers. AtWBC11 is therefore the first transporter shown to be involved in cutin transport (Bird et al. 2007, and this report).

The half-size ABC transporters, including WBC members, need to form a dimer in order to function. Dimerization can effectively broaden the substrate spectrum of ABC transporters, as shown by the research on three fruitfly ABC transporters, White, Brown and Scarlet (Mackenzie et al. 1999). In Arabidopsis, CER5 and AtWBC11 have high sequence similarities and share a similar epidermis-specific expression pattern. Heterodimerization of CER5 and AtWBC11 is possibly involved in wax transport because their mutants displayed a similar wax-less phenotype. However, the possible involvement of other dimers (e.g. CER5 homodimer and AtWBC11 homodimer) in wax transport cannot be excluded.
Whether AtWBC11 forms a homodimer or heterodimer in cutin transport is unknown. Besides CER5 and AtWBC11, AtWBC1, 18 and 19 were reported to be up-regulated in shoot epidermis (Suh et al. 2005), which makes them good candidates for cuticle exporters. Another possibility is that AtWBC11 forms different dimers with several WBC transporters to transport cutin monomers. Because of gene redundancy, mutation of one of these WBCs may not lead to a visual phenotype. In this scenario, AtWBC11 could act like the fruitfly White transporter which can dimerize with Brown to transport guanine and with Scarlet to transport tryptophan (Mackenzie et al. 1999). Further work on mutant screening and protein–protein interaction studies will give more clues to understanding the mechanisms of cuticle lipid transportation by ABC transporters.

The cuticle changes in atwbc11 affected the development of epidermal cells, as demonstrated by smaller pavement cells and deformed trichomes. In other cases, the specialized epidermal cells (e.g. trichomes and guard cells) might also be affected by cuticle changes. The trichomes were reported to be defective in the Arabidopsis yore-yore (yre) cer1 double mutant (Kurata et al. 2003), while YRE (WAX2) was involved in both wax and cutin production (Chen et al. 2003, Kurata et al. 2003) and CER1 (Aarts et al. 1995) was involved in wax production. The Arabidopsis HIC (for high carbon dioxide) gene encodes a putative 3-ketoacyl-coenzyme A synthase in the biosynthetic pathway of VLCFAs. HIC was specifically expressed in guard cells and its loss of function significantly increased the stomatal index and stomatal opening under an elevated CO₂ environment (Gray et al. 2000). Cotton fiber is also a class of specialized epidermal cells. Lipid metabolism is highly active in developing fiber cells, and the expression of related enzymes, such as the acyl-CoA-binding protein, 3-ketoacyl-CoA synthase and 3-ketoacyl-CoA reductase, and lipid transfer proteins was up-regulated during the fiber elongation period (Li et al. 2002, Ji et al. 2003, Gou et al. 2007), suggesting that VLCFAs play important roles in cotton fiber development. Previously, we isolated a cotton ABC transporter gene, GhWBC1, which is highly expressed in developing fiber cells (Zhu et al. 2003). Since two of the Arabidopsis homologs of GhWBC1, CER5 (AtWBC12) (Pighin et al. 2004) and AtWBC11 (Bird et al. 2007, and this report), are both involved in cuticle lipid movement, it is possible that GhWBC1 has a similar function in cotton fiber.

Materials and Methods

Plant material and growth conditions

Plants of A. thaliana (Columbia-0, Col-0 ecotype) were grown indoors (22°C) under a 16 h photoperiod at 50–60% relative humidity. For growth on the medium, the seeds were surface sterilized in 20% (v/v) bleach containing 0.1% Triton X-100 (v/v) for 15 min, washed four times with sterile water, plated on half-strength MS medium (0.8% sucrose and 0.8% agar) and kept for synchronization at 4°C for 2 d. Finally, plants were transferred to soil about 7 d after germination.

Isolation of T-DNA insertion mutants

PCRs with a T-DNA left border primer (LBb1, 5′-AACCACTTGGACCGCTTGCTG-3′) and gene-specific primers (Salk_043637, 5′-ACGAAAGAATGCCATCAAGGGTC-3′ and 5′-CACCATCTCCATAGCTACCATAC-3′; Salk_072079, 5′-CCCACTTTGAAGCGATG3′ and 5′-TGGTCGGGAAATCAAGCTTGA-3′) were used to identify T-DNA insertion mutants of atwbc11-4 and -5, respectively. The insertion sites were verified by sequencing of T-DNA flanking sequences.

Vector construction and plant transformation

The pCAMBIA1300 vector (CAMBIA, Canberra, Australia) was used as a backbone for transgenic constructs. The HindIII–EcoRI fragment of the pBI101 vector (Clontech, Palo Alto, CA, USA) was inserted into the corresponding sites of pCAMBIA1300 to produce p1300/GUS vector. The 2 kb promoter and coding region of AtWBC11 was amplified by PCR and verified by sequencing. The AtWBC11 promoter was inserted into p1300/GUS upstream of GUS to generate the ProAtWBC11::GUS construct. Then the EGFP coding region (Zhu et al. 2003) was used to replace GUS of ProAtWBC11::GUS to form ProAtWBC11::GFP. The AtWBC11 coding region was used to replace GUS in ProAtWBC11::GUS to form ProAtWBC11::AtWBC11 for complementation analysis. The ProAtWBC11::GFP-AtWBC11 construct was obtained by in-frame fusion of the AtWBC11 coding region with GFP in ProAtWBC11::GFP. To generate the ProAtWBC11::GFP-AtWBC11 construct, the 35S promoter from pBI121 (Clontech) was used to replace the AtWBC11 promoter in ProAtWBC11::GFP-AtWBC11. Transgenic plants were generated by a floral dip method (Clough and Bent 1998) and screened on half-strength MS agar medium containing 50 μg ml⁻¹ hygromycin. Analysis was performed on T₂ or T₃ plants.

Microscopic observation

For TEM, samples were fixed with glutaraldehyde (2%) in sodium cacodylate (0.05 M, pH 7.2) for 2 h at room temperature and post-fixed with OsO₄ (1%) in sodium cacodylate at 4°C overnight. After dehydration with a graded ethanol series, the material was infiltrated in Spurr’s standard epoxy resin. Ultrathin sections (60–70 nm) were prepared and stained with uranyl acetate and alkaline lead citrate, and then observed with an H-7650 transmission electron microscope (Hitachi High-Technologies, Tokyo, Japan). For semi-thin sections (2μm), material was fixed as for TEM, sections were stained with TB and microscopy pictures were taken with a BX51 microscope (Olympus, Tokyo, Japan). Confocal images were obtained with an LSM510 laser scanning confocal microscope (Zeiss, Jena, Germany). For SEM, plant materials were fixed in a solution of 5% (v/v) acetic acid, 45% (v/v) ethanol and 5% (v/v) formaldehyde at 4°C overnight, dehydrated in a graded ethanol series, then critical point dried in liquid carbon dioxide and coated with gold. Samples for wax crystal observation were directly coated with gold. The samples were visualized with a JSM-6360LV scanning electron microscope (JEOL, Tokyo, Japan).
**RNA analysis**

Total RNAs were isolated from plant materials using Trizol reagent (Invitrogen, Carlsbad, CA, USA). For RNA blot analysis, total RNA was separated on a 1.2% formaldehyde denaturing gel and transferred to a Hybond-N filter membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). The membrane was hybridized with PerfectHyb solution (Toyobo, Osaka, Japan). The probe was randomly labeled with [32P]dCTP using the Random Primer DNA Labeling Kit (TAKARA, Dalian, PR China). The blots were exposed to a phosphor screen that was scanned with a BAS 1800II phosphor imager (FUJI photofilm, Tokyo, Japan). RT–PCR was performed as described by Zhu et al. (2003). The primers were AT1 (5'-TTGTTTGACTATTA CCACACTTCTG-3') and AT2 (5'-AGAACAAAGAAGAGGT AGTAGGTG-3') for AtWBC11, and Actin5 (5'-TCTTCTCAA TCTATCTTCTCCC-3') and Actin3 (5'-GACCTGCTCTCAT CATACTCG-3') for Arabidopsis Actin2. The PCR product obtained with the AT1 and AT2 primers was used as the AtWBC11 probe in RNA blot analysis.

**GUS assay, TB staining and chlorophyll leaching experiment**

Histochemical GUS assays were performed according to a published protocol (Jefferson et al. 1987). For TB staining, 10-day-old plants were stained at room temperature for 2 min in freshly prepared 0.05% TB solution (Tanaka et al. 2004). The samples were rinsed with water before being photographed. The chlorophyll-leaching method (Lolle et al. 1997) was used with minor modifications. The aboveground parts (~0.2 g) of 4-week-old plants were prepared and pooled. The samples were weighed before addition of 80% ethanol. The extraction was conducted at room temperature with gentle shaking. Aliquots were taken after 10, 20, 30, 40, 50, 60, 90 and 120 min, and the chlorophyll content was measured and calculated as described (Lolle et al. 1997).

**Chemical analysis**

Surface wax extraction and analysis were performed as described (Pighin et al. 2004) with minor modifications. Bolting stems were extracted by hexane and the inner standard was 19:0 FAME (methyl nonadecanoate). For surface wax extraction, bolting stems were extracted in hexane for about 30 s. Gas chromatography–mass spectrometry (GC-MS) analysis was carried out using a 6890N GC system equipped with a quadrupole 5973N mass selective detector (Agilent Technologies Inc., Santa Clara, CA, USA). GC analysis was carried out using a 6890N GC system equipped with an FID detector. Peaks were identified by their electron-impact MS spectra. Quantification of compounds was based on peak areas, which were converted to mass units by comparison with the internal standard. Wax monomer quantities were expressed per unit of surface area, which is calculated by assuming the stem is a cylinder. The diameter of the stems was measured under a microscope. Cutin lipids were analyzed as described (Kurdyukov et al. 2006b). Briefly, the free lipids were removed and the resulting dry residue was depolymerized using the methanolic HCl method. The monomers were separated, identified and quantified by GC-MS.

**Supplementary material**

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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**References**


