Characterization of Arabidopsis ABCG11/WBC11, an ATP binding cassette (ABC) transporter that is required for cuticular lipid secretion†

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†This paper is dedicated to the memory of Vincent R. Franceschi (1953–2005).

Summary
ABCG11/WBC11, an ATP binding cassette (ABC) transporter from Arabidopsis thaliana, is a key component of the export pathway for cuticular lipids. Arabidopsis wbc11 T-DNA insertional knock-out mutants exhibited lipidic inclusions inside epidermal cells similar to the previously characterized wax transporter mutant cer5, with a similar strong reduction in the alkanes of surface waxes. Moreover, the wbc11 knock-out mutants also showed defects not present in cer5, including post-genital organ fusions, stunted growth and a reduction in cutin load on the plant surface. A mutant line previously isolated in a forward genetics screen, called permeable leaves 1 (pel1), was identified as an allele of ABCG11/WBC11. The double knock-out wbc11 cer5 exhibited the same morphological and biochemical phenotypes as the wbc11 knock-out. A YFP-WBC11 fusion protein rescued a T-DNA knock-out mutant and was localized to the plasma membrane. These results show that WBC11 functions in secretion of surface waxes, possibly by interacting with CER5. However, unlike ABCG12/CER5, ABCG11/WBC11 is important to the normal process of cutin formation.

Keywords: cuticle, wax, cutin, ABC transporter, cell wall, Arabidopsis thaliana.

Introduction
Aerial surfaces of the primary plant body are coated by a waxy cuticle that serves physiological, ecological and developmental roles. Its primary function is to act as a hydrophobic barrier limiting non-stomatal water loss, but it also provides an interface between the plant and the environment. For example, the cuticle surface can, to various degrees, repel water, and has therefore been implied in defense against pathogenic spores that require high humidity for germination (Neinhuis and Barthlott, 1997). In addition, the cuticle plays an important role in preventing post-genital organ fusion: plant lines overexpressing a fungal cutinase or carrying mutations that result in an alteration in cutin organization often have a ‘fiddlehead’ phenotype, where fusions amongst floral organs deform the growing inflorescence (Nawrath, 2006). Plant cuticles consist of cutin, a lipid polyester, as well as intracuticular wax, which is embedded within the cutin matrix, and epicuticular wax, which is deposited on the surface of the cuticle (Jetter et al., 2000).

Cutin is a polymer of fatty acid derivatives that are ester-linked and, possibly through cross-linking with glycerol, form a complex three-dimensional matrix (Graça et al., 2002; Nawrath, 2002; Stark and Tian, 2006). In Arabidopsis this polymer is primarily made up of dicarboxy-, hydroxy- and dihydroxy- C16 and C18 fatty acids (Bonaventure et al., 2004; Franke et al., 2005; Xiao et al., 2004). Plant cuticular wax typically consists of homologous series of very long chain fatty acid derivatives, as well as diverse terpenoid and phenolic components, depending upon the species, organ and developmental state (Jetter et al., 2006). Cuticular lipid biosynthesis is believed to occur exclusively within epidermal cells using plastid-derived C16 and
C18 fatty acids (Browse and Somerville, 1991; Nawrath, 2002). It is likely that cutin monomer biosynthesis is, at least in part, localized to the endoplasmic reticulum (ER) as the C16 and C18 fatty acids subsequently undergo a number of microsomal hydroxylation steps (Kolattukudy, 1984), one of which is performed by the cytochrome P450 CYP86A8 (LACERATA), an ω-hydroxylase required for normal cuticle formation (Wellesen et al., 2001). Similar to cutin biosynthesis, wax biosynthesis has been localized to the ER, as demonstrated by GFP fusions of enzymes required for cuticular wax production such as CER6 and CER10 (Kunst and Samuels, 2003; Zheng et al., 2005). Similarly, the fatty acyl-CoA reductase required for wax primary alcohols and esters, CER4, is also localized to the ER when expressed in yeast (Rowland et al., 2006).

How the cutin and wax components are exported from the epidermal cell and assembled into the complex, three-dimensional, hydrophobic structure on the plant surface is not well understood. Although nothing is known about cutin precursor export, a single ATP binding cassette (ABC) transporter, CER5, has been shown to be involved in wax secretion to the surface (Pighin et al., 2004). The stems of cer5-1, and stems of a knock-out mutant cer5-2, still had 41% of the wild-type wax load, suggesting that additional transporters are involved in wax secretion to the surface (Pighin et al., 2004). No other elements of the wax export machinery have been identified to date.

CER5 is a member of the WBC subfamily of Arabidopsis ABC transporters (Sanchez-Fernandez et al., 2001), named after the canonical WHITE-BROWN complex of Drosophila melanogaster (Ewart et al., 1994). It is also designated as ABCG12 for its similarity to the human lipidic transporters of the ABCG subfamily. The human ABCG subfamily members are emerging as important lipid transporters, including the sterol-transporting heterodimer ABCG5/ABCG8 and ABCG2, which forms a homodimer that functions in xenobiotic export (Wang et al., 2006; Xu et al., 2004). ABCG/WBC-type proteins are believed to be ‘half’-transporters, requiring a second ABCG/WBC polypeptide to create a fully functional ABC transporter with two ABC and two transmembrane domains (Dreesen et al., 1988). This subfamily provides interesting candidates for the additional wax exporters that must exist to account for the presence of cuticular wax in the cer5 loss-of-function mutant.

Many ABC transporter genes show a high level of expression in the stem epidermis (Suh et al., 2005). To pinpoint ABC transporters involved in cuticular lipid export within the WBC subfamily, we evaluated candidates with high expression in the stem epidermis for similar patterns of gene expression with CER5 by applying a Genevestigator (Zimmermann et al., 2004) gene expression correlation approach. Using 673 microarray data sets from aerial organs, we identified an ABC transporter WBC11 (At1g17840) that showed a high degree of correlation with CER5 (R² = 0.83) as a prime candidate for further analysis.

The objective of this study was to assess the function of WBC11 in cuticular lipid export using a reverse genetics approach. Our results show that mutations in WBC11 cause reduced wax load and accumulation of intracellular lipidic inclusions. Surprisingly, mutations in WBC11 also result in dwarfism, sterility, post-genital organ fusions and reduced cutin load.

Results

Genetic characterization of mutant alleles of WBC11

Three T-DNA insertional mutants lines, wbc11-1, -2 and -3, were obtained from the Salk Institute and characterized in this study (Alonso et al., 2003; Figure 1a). The two alleles wbc11-1 and wbc11-2 had T-DNA insertions 404 and 346 base pairs upstream of the predicted translation start site, respectively, and both showed a reduction, but not elimination, of gene transcript. The wbc11-3 allele is an insertion at the 141st base pair of the 6th exon, which resulted in no detectable WBC11 transcript (Figure 1b).

![Figure 1. Mutant alleles of WBC11.](image-url)

- **(a)** The locations of T-DNA insertions and pel1-1 WBC11 are mapped: wbc11-1, -2 and -3 are Salk T-DNA insertions in the 5’ untranslated region (UTR) (wbc11-1 and -2) and 6th exon (wbc11-3). 761C10 and 590C03 are GABI-KAT T-DNA lines in the 2nd and 6th exons, respectively. The mutant pel1-1 is a point mutant in the 1st intron that creates a premature stop codon at amino acid 97. Exons are shown as black boxes.
- **(b)** Semi-quantitative RT-PCR of steady-state WBC11 and CER5 mRNA in wbc11 mutants compared with wild-type Columbia-0. Expression of ACTIN1 was used as a loading control for the corresponding lanes above. RNA was isolated from stem tissue.

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Using a toluidine blue test for detection of cuticular defects, Tanaka et al. (2004) identified seven permeable leaves (pel) mutants. The pel1 genetic locus was mapped to the upper arm of chromosome 1 to a contig comprising 12 overlapping bacterial artificial chromosome (BAC) clones. In the positional candidate gene approach, we examined phenotypes of more than 20 loss-of-function T-DNA mutants from the corresponding region, and found that only two insertion mutants in At1g17840 (ecotype Columbia, Col-0, lines GK-590C03 and GK-761C10; Rosso et al., 2003) showed organ fusion defects typical for some cuticular mutants, a reduced cuticular wax load and produced non-fertile flowers. For the allelism test, pel1-1 heterozygotes (ecotype Ler) were pollinated by GK-590C03 plants heterozygous at the At1g17840 locus. Progeny resembling the pel1-1 line segregated in a 46:15 (wild type : pel1-1 mutant) ratio. This is consistent with the expected 3:1 ratio ($\chi^2 = 0.0055, P = 0.941$), indicating that pel1-1 is another allele of the At1g17840 gene. Sequencing of genomic DNA confirmed that the pel1 mutant allele has a C-to-T transition resulting in a premature UGA stop codon at amino acid position 97.

Mutations in WBC11 cause reduced growth and post-genital organ fusions

Plants homozygous for wbc11-3 were dramatically smaller than wild-type plants, with rosette leaves rarely exceeding 0.5 cm in length. In addition to their reduced size, rosette leaves were misshapen and occasional leaf–leaf fusions were observed (Figure 2a, inset). At maturity, wbc11-3 stems were very slender and highly branching, ending in terminal sterile flowers (Figure 2a). The two alleles with decreased, but not eliminated, transcript levels, wbc11-1 and wbc11-2, were normal in size, leaf shape and fertility (Figure 2b). When embryos were isolated from heterozygous wbc11-3/ WBC11 siliques, two populations of embryos were observed: of 109 embryos, 20 were smaller and less pigmented, consistent with a 3:1 phenotypic segregation of a single recessive allele ($\chi^2 = 2.57, P = 0.109$; Figure 2c).

The knock-down mutants, wbc11-1 and -2 had extensive organ fusions, many of which showed a fiddlehead-like phenotype resulting from stem–stem and stem–leaf fusions (Figure 2b). At such fusion sites, the outer periclinal wall of the epidermal cells adhered to the corresponding cells of adjacent organs. In some cases, the strength of the adhesion led to the epidermis being stretched between the adjacent stems (Figure 3a,b). Tears in the stretched epidermis could be observed using scanning electron microscopy (Figure 3c, arrow). In wbc11-3, leaf–leaf fusions were commonly observed (Figure 3d). In addition, aberrations in leaf trichome morphology could be also observed: trichomes were commonly two-pronged instead of the normal tripartite shape and were also often stunted, misshapen or even collapsed (Figure 3d,e).

Mutations in WBC11 cause reduced cuticular wax and cutin loads

To determine if disruptions in WBC11 cause alterations in epicuticular wax, we examined wild-type (Col-0) and mutant...
Consistent with the overall growth abnormalities, stem epidermal cells of *wbc11-3* were noticeably more irregular than those of wild type. Also, whereas plates, rods and columnar-shaped crystals were observed in both wild-type and mutant genotypes, only the *wbc11-3* plants additionally...
Table 1 Composition of cuticular waxes on inflorescence stems of Arabidopsis wild type (Col-0) and wbc11 mutant lines. Mean values (μg cm⁻²) of total wax loads and coverage of individual compound classes are given with SEs (n = 5)

<table>
<thead>
<tr>
<th></th>
<th>Total wax</th>
<th>Fatty acids</th>
<th>Aldehydes</th>
<th>Alkanes</th>
<th>Sec. alcohols</th>
<th>Ketone</th>
<th>Primary alcohols</th>
<th>Esters</th>
<th>Not identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0 (wild type)</td>
<td>19.5 ± 1.1</td>
<td>0.10 ± 0.04</td>
<td>0.48 ± 0.23</td>
<td>7.7 ± 0.4</td>
<td>2.1 ± 0.1</td>
<td>4.5 ± 0.2</td>
<td>1.7 ± 0.1</td>
<td>0.93 ± 0.13</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>wbc11-1</td>
<td>7.9 ± 0.3</td>
<td>0.02 ± 0.01</td>
<td>0.10 ± 0.03</td>
<td>1.6 ± 0.2</td>
<td>12* ± 0.1</td>
<td>2.9* ± 0.1</td>
<td>10* ± 0.1</td>
<td>0.20* ± 0.03</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>wbc11-2</td>
<td>9.1 ± 0.6</td>
<td>0.01 ± 0.007</td>
<td>0.16 ± 0.02</td>
<td>2.2 ± 0.4</td>
<td>15 ± 0.1</td>
<td>32* ± 0.2</td>
<td>10* ± 0.1</td>
<td>0.23* ± 0.02</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>wbc11-3</td>
<td>9.3 ± 1.3</td>
<td>0.03 ± 0.007</td>
<td>0.10 ± 0.03</td>
<td>1.0 ± 0.2</td>
<td>25 ± 0.4</td>
<td>2.7 ± 0.7</td>
<td>04* ± 0.1</td>
<td>0.25 ± 0.13</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>wbc11-3 cer5-2</td>
<td>9.9 ± 0.7</td>
<td>0.05 ± 0.01</td>
<td>0.20 ± 0.08</td>
<td>1.0 ± 0.1</td>
<td>2.3 ± 0.1</td>
<td>3.7 ± 0.1</td>
<td>0.6* ± 0.1</td>
<td>0.55 ± 0.05</td>
<td>1.4 ± 0.3</td>
</tr>
</tbody>
</table>

*aMeans are significantly different from each other (ANOVA, F(4,20) = 28.860, P < 0.001). Post hoc comparisons using Tukey’s HSD test (P < 0.05) revealed that all mutants were significantly lower than wild type, but not different from each other.

*bMeans are significantly different from each other (ANOVA, F(4,20) = 101.324, P < 0.001). Post hoc comparisons using Tukey’s HSD test (P < 0.05) revealed that all mutants were significantly lower than wild type; wbc11-1 and -2 were not significantly different from each other but were significantly larger than wbc11-3 and wbc11-3 cer5-2.

*Values are significantly different from wild type (parametric means comparison using Games–Howell test, P < 0.05).

Figure 5. Epidermal wax load is reduced in wbc11 stems. Sections of whole stems were chloroform extracted and analyzed for wax using GC-FID/MS. Wax loads are expressed in μg of wax per cm² of stem surface area. The genotype of each sample is shown in the upper right of the charts. Each wax constituent is designated by carbon chain length and is labeled by chemical class along the x-axis. Each value is the mean of five independent measurements of individual plants. *Means significantly different (P < 0.05) from wild type. Error bars = SE.
had thickened plates that appeared to be aggregated columnar-shaped crystals that were not observed in wild type (Figure 4d, arrow).

The reduced number of epicuticular wax crystals on the surface of wbc11-3 suggested to us that WBC11 may be involved in transport of wax components. To test this hypothesis, we examined the cuticular wax load and composition of stems from the wbc11 alleles using gas chromatography with flame ionization detection (GC-FID). The total wax load (total chloroform-extractable lipids) in wbc11-1, -2 and -3 was reduced by 41–56% compared with wild type (Col-0). Most of the change in wax load could be attributed to a 75–90% reduction of alkanes, the most abundant class of wax components on Arabidopsis stems (Table 1). In all three mutant plant lines, the largest reduction was always observed in a single wax compound, nonacosane (C29 alkane; Figure 5). Furthermore, alkane levels in different wbc11 alleles appeared to be correlated with the levels of detectable WBC11 transcript in each line, that is, the knock-out allele wbc11-3 had the strongest reduction in alkanes, which were significantly lower than those found in wbc11-1 and -2 (Table 1). Significant reductions of C26 and C28 primary alcohols were also observed in all mutant lines. Also, significant reductions were observed in the C44 and C46 esters in the wbc11-1 and -2 knock-down alleles (Figure 5). In contrast, total loss of WBC11 transcript in wbc11-3 resulted in no significant change in the levels of C29 secondary alcohols and C29 ketone. In the wbc11-1 line, these compounds were reduced, but relatively less so compared with the reduction of nonacosane (Figure 5).

Because CER5 had also been implicated in wax secretion, and the knock-out cer5-2 mutant had a similar reduction in wax load, predominantly caused by the reduction in the C29 alkane (Pighin et al., 2004), we wanted to know if wax loads would be even further reduced in plants lacking both ABC transporters. wbc11-3 cer5-2 double mutants were obtained by crossing wbc11-3/WBC11 heterozygotes with the cer5-2 knock-out mutant. Surprisingly, the total wax load of the double mutants, the genotypes of which were confirmed by PCR, did not show any significant difference in total wax load compared with wbc11-3 (Table 1). Moreover, the contribution of individual compounds to the surface wax of wbc11-3 cer5-2 was comparable with that of wbc11-3 (Figure 5).

The presence of fusions in all wbc11 mutants, which have been correlated with defects in cutin, led us to examine if wbc11 lines had a reduction of cutin monomers in the stem compared with wild type. Strikingly, a strong 65% decrease in total cutin monomers was found in the knock-out allele wbc11-3 (1.14 μg cm\(^{-1}\) ± 0.41 SE) compared with wild type (3.29 μg cm\(^{-1}\) ± 0.07 SE), which was not detected in the cer5-2 mutant (3.74 μg cm\(^{-1}\) ± 0.14 SE). This reduction was reflected in a 35–70% reduction of almost all individual cutin monomers extracted, including the most predominant monomer, C18:2 dicarboxylic acid (Figure 6). Some of the unsubstituted C16-C18 fatty acid monomers, which did not exhibit a strong decrease, are likely to be mostly arising from residual soluble lipids. Interestingly, almost all cutin monomer levels in the knock-down alleles wbc11-1 and wbc11-2, which both displayed extensive stem–leaf fusions but no stunted growth phenotype, were not significantly lower than in wild type; the only exceptions being a small reduction of C22 and C24 fatty acid monomers in wbc11-1 (Figure 6).

**Alterations in epidermal cell ultrastructure in wbc11 mutants**

Because of the homology of WBC11 and CER5 genes (76% similar, 54% identical at the amino acid level), we examined the epidermal cell ultrastructure of wbc11-3 to determine if it, like cer5-1, had lipidic inclusions in the cytoplasm (Pighin et al., 2004). In wild-type epidermal cells, the large central vacuole, surrounded by a thin layer of cortical cytoplasm and plasma membrane, dominated the epidermal cells (Figure 7a). Inside the stem epidermal cells of wbc11 mutants, inclusions were observed in the cytoplasm that...
created large cytoplasmic protrusions into the central vacuole (Figure 7b). Examination of the inclusions at higher magnification revealed that they tended to aggregate into dense coils of trilamellar (dark-light-dark) ribbons (Figure 7c and inset). When \textit{wbc11-3} stems were frozen, then fractured transversely and observed in cryo-SEM, inclusions appeared as concentric aggregated sheets within the cytoplasm, which correlated with the structures observed in thin sections (Figure 7f cf. Figure 7c).

In addition to the accumulation of cytoplasmic inclusions, \textit{wbc11-3} plants showed dramatically altered cuticle morphology. Transverse sections of wild-type stems showed the thin (<100 nm), continuous electron-dense cuticle (Figure 7d). However, \textit{wbc11-3} stems had numerous regions where the cuticle appeared to be more diffuse, disorganized, and only lightly stained (Figure 7e).

**Yellow fluorescent protein-WBC11 fusion protein complements \textit{wbc11-3} defects and is localized to the plasma membrane**

To confirm that the defects in growth, cuticle structure, and cuticular lipid levels in the \textit{wbc11} mutants were all caused by disruptions in the \textit{WBC11} gene, we expressed the cDNA of the gene as a YFP fusion in the knock-out mutant \textit{wbc11-3} plants, under the control of the 35S promoter. Twelve independent transgenic lines were recovered, eleven of which had wild-type growth habit and fertility suggesting that the defects in the mutant were restored. When \textit{wbc11-3} plants expressing YFP-WBC11 were analyzed for wax load, the levels observed were not significantly different from wild-type levels, but were significantly higher than in \textit{wbc11-3} plants (Figure 8a). In all isolated transgenic lines, YFP fluorescence was observed in the periphery of the cells and was coincident with the plasma membrane (PM) stain, FM4-64 (Bolte et al., 2004; Figure 8b–d). When the signal from the YFP-WBC was merged with the PM label from FM4-64, the signals co-localize, as seen in the inset in Figure 8d. When the YFP-WBC expressing stems were observed with a propidium iodide counter-stain, a membrane-impermeable dye which stains the cell wall (Figure 8d,e–g), the YFP fluorescence appeared directly appressed to the stained cell wall (Figure 8g, inset). To further confirm the YFP-WBC11 localization to the plasma membrane, we compared seedlings expressing YFP-WBC11 with seedlings expressing a tonoplast-localized GFP-\(\delta\)-TIP (At3g16240; Cutler et al., 2000). GFP-\(\delta\)-TIP was found localized to small vacuoles in addition to the periphery of the cells, but was not co-localized with the FM4-64-labeled PM (Figure S1a–c).

**Expression of WBC11 in Arabidopsis**

To characterize the expression patterns of \textit{WBC11}, a 2.6-kb fragment, upstream of the predicted translation start site of
WBC11, was used as a promoter to drive the expression of GUS in transgenic Arabidopsis plants. GUS activity was detected throughout the shoot system, particularly in stems (Figure 9a). Transverse sections of stems demonstrated GUS activity not only in the epidermis, but also in the cortical cells (Figure 9b). Trichomes on both stems and leaves also showed significant levels of GUS staining (Figure 9c). In contrast to the stems, GUS activity in leaves was restricted to the epidermal layers (Figure 9e). Flower buds, prior to anthesis, showed staining in all whorls, but GUS activity was strongest in anthers (Figure 9f). Upon anthesis, expression in the floral whorls was markedly reduced, except in the carpel (Figure 9g). Expression in the carpel was retained after pollination and during expansion of the silique; in addition, strong staining was observed in developing seeds (Figure 9h). Dissection of these seeds revealed GUS activity predominantly in the endosperm, with additional GUS activity detected in the embryo protoderm (data not shown).

GUS expression was also detected in root tips and emerging secondary root tips, even as early as the initial cell divisions of the pericycle (Figure 9i–k). Longitudinal sections of root tips revealed that, as in the stem, the WBC11 5'-promoter conferred expression primarily in the epidermis (or protoderm) and outermost cortical cells, but also in the root cap (Figure 9j). To confirm the GUS expression patterns of WBC11 in wild-type plants, we used real-time PCR. WBC11 transcript was detected in all organs, but was strongest in aerial organs and seeds, consistent with the promoter-GUS findings (Figure 9o).

Discussion

This study identifies WBC11 as a key component of the cuticular lipid export pathway. We have characterized three Arabidopsis wbc11 T-DNA insertion mutants, which either reduce or abolish WBC11 transcript accumulation. The wbc11 mutants had inflorescence stem cuticles deficient in both cutin and wax, disorganized cuticle structure, fiddlehead-type post-genital organ fusions, changes in the numbers and shapes of epicuticular wax crystals, as well as accumulations of lipidic inclusions within the cytoplasm. We have also confirmed the permeable cuticle mutation pel1 to be an allele of the WBC11, providing further evidence to show that WBC11 is required for cuticle formation. Thus WBC11 is the second ABC transporter, besides CER5, involved in the export of cuticular lipids from the epidermal cells.

WBC11 expression was detected in the epidermal cells of stems and leaves, consistent with the role of this gene in the formation of the cuticle. Although WBC11 transcript was detected in roots using real time PCR, they had the lowest level of expression of all organs tested. The expression in roots was restricted to root tips, specifically to the protoderm, root cap cells and lateral root primordia; expression of WBC11 was not observed in mature, differentiated cells. The function of WBC11 in developing root tips is not clear; however, other genes shown to be involved in cuticle formation are also expressed in roots, namely FDH (Lolle et al., 1992; Pruitt et al., 2000; Yephremov et al., 1999), LCR (Wellesen et al., 2001), WAX2/YRE (Chen et al., 2003; Kurata et al., 2003), LACS2 (Schnurr et al., 2004), ACE/HTH (Krollowski et al., 2003; Kurdyukov et al., 2006a), CER5 (Pighin

Figure 8. YFP-WBC11 is localized in the epidermal cell plasma membrane and complements the wbc11-3 phenotype.
(a) wbc11-3 plants expressing YFP-WBC11 show a normal stem wax load compared with wild type. Each value is the mean of five independent measurements of individual plants. *Means significantly different from wild type (ANOVA \( F_{12,15} = 52.045, \ p < 0.001 \); Tukey's HSD, \( p < 0.05 \). Error bars = SE.
(b–g) Confocal micrographs of wbc11-3 stems expressing YFP-WBC11 fusion protein under the constitutive 35S promoter. (b, e) YFP-WBC11 is localized to the periphery of stem epidermal cells (green). (c) FM4-64 staining of plasma membranes (red). (d) Overlay of (b) and (c). (f) Propidium iodide (PI) stains cell walls (red). (g) Overlay of (e) and (f). The YFP-WBC11 signal is coincident with FM4-64 but not with PI. Bar = 10 \( \mu \)m.
et al., 2004), BDG (Kurdyukov et al., 2006b) and CER4 (Rowland et al., 2006).

There are four possible hypotheses for the combined cutin and wax deficiency in wbc11: (i) WBC11 is only indirectly involved in cuticle formation, and the cutin and wax alterations are effects of an unknown transport function of WBC11; (ii) WBC11 transports only wax, and the cutin phenotype is an indirect effect; (iii) WBC11 transports only cutin precursors, and the wax phenotype is an indirect effect; (iv) WBC11 transports both wax and cutin precursors. We find (i) unlikely, given the high degree of similarity of WBC11 to known lipid transporters such as CER5 (76% similarity) and human ABCG5 (47%) and ABCG8 (49%; Pighin et al., 2004; Wang et al., 2006). Therefore, the transport of lipids by WBC11 is consistent with conservation of function in this gene family. Assuming the simplest scenario, that cuticular lipids are substrates, this raises the question of whether the function of WBC11 is to transport cutin, wax or both.

**WBC11 is required for normal cutin deposition**

The wbc11-3 plants exhibited a 65% reduction of cutin monomers. In contrast, the levels of cutin monomers in the knock-down alleles, wbc11-1 and -2, were not significantly different from wild type, with the exception of only C22 and C24 unsubstituted fatty acids. This suggests that complete elimination of WBC11 transcript was necessary to cause a significant drop in cutin deposition.

In addition to the reduction in extractable cutin monomers, wbc11-3 plants had regions where the cuticle was more disorganized, more diffuse and electron-translucent. Similar disorganized regions of the cuticle have also been found in mutants with cuticle defects caused by alterations in cutin composition, including wax2, bodyguard, lacs2 and lacerata (Chen et al., 2003; Kurdyukov et al., 2006b; Lolle et al., 1992; Schnurr et al., 2004; Wellesen et al., 2001), and Arabidopsis plants overexpressing fungal cutinase (Sieber et al., 2000).

The absence of WBC11 transporter also resulted in extensive post-genital stem–stem, stem–leaf and leaf–leaf fusions, which are often found in mutants with cutin-specific defects (Tanaka and Machida, 2006). Reduction in cutin content alone is not likely to be sufficient to cause organ fusions, because att1 plants, which have 30% lower cutin monomer levels, were not reported to have organ fusions (Xiao et al., 2004). On the other hand, some mutants with disorganized cuticles, but normal absolute levels of cutin,
such as bodyguard (Kurdyukov et al., 2006b), exhibit post-genital fusions, suggesting that disorganization of cutin may be the key factor resulting in fusion of organs.

In addition to organ fusions found in all of the wbc11 alleles, the knock-out wbc11-3 mutant was dwarf and sterile. These latter defects are likely to be pleiotropic effects of the impaired cuticle. Reduced growth and fertility have been commonly observed in plants with cuticle defects including ale1, lacerata, wax2, lacs2 and bodyguard (Chen et al., 2003; Kurdyukov et al., 2006b; Pighin et al., 2004; Schnurr et al., 2004; Tanaka et al., 2001; Wellesen et al., 2001). It is possible that wax transport defects of wbc11-3 lead to infertility and dwarfism. However, mutants with wax loads comparable with wbc11-3, such as cer5 (Pighin et al., 2004), or more severely reduced wax loads, such as cer6, did not have changes in growth (Hooker et al., 2002; Millar et al., 1999).

Taken together, the disorganized cuticular layer, reduced levels of cutin monomers and organ fusions of wbc11 mutants provide compelling evidence of the importance of this ABC transporter to the process of cutin formation.

**WBC11 is required for wax transport**

Similar to cer5 mutants, wbc11-3 had fewer epicuticular wax crystals than the wild type, suggesting a lower wax load. All T-DNA mutants of WBC11 had a wax load that was 40–50% that of wild type. wbc11-3 was further found to accumulate trilamellar inclusions in the stem epidermal cells, as observed in TEM, and lipidic sheets, as observed in cryo-SEM. Lipidic inclusions were also observed in cer5-1, which accumulated wax in the epidermal cells; thus, it seems likely that the inclusions in wbc11-3 are the same in nature (Pighin et al., 2004). Unfortunately, because of the strongly hydrophobic nature of the wax substrates, we cannot currently assay WBC11 transport activity biochemically. Nevertheless, these data strongly support the hypothesis that WBC11, like CER5, is directly involved in wax transport.

Interestingly, the wax load deficiency in wbc11 alleles was largely the result of a 75–90% reduction in C29 alkane (nonacosane), the most abundant component of wild-type stem wax. Furthermore, nonacosane levels appeared to correlate with levels of wbc11 transcript, which, if the transporter is moving wax molecules directly, indicates WBC11 shows a substrate preference for alkanes, and specifically nonacosane. In contrast to the alkanes, some wax components, such as the C29 secondary alcohols and C29 ketone, were not as much reduced in the wbc11-3 knock-out as in with the wbc11-1 and -2 knock-down mutants. This result could represent a compensatory effect induced by the total loss of WBC11 gene transcript, for example an increase in the biosynthesis and/or the transport of wax components other than nonacosane. This compensation does not appear to involve CER5 at the transcriptional level, as CER5 expression was not changed in wbc11 mutants compared with wild type. Furthermore, the wbc11-3 cer5-2 double mutant had similar wax loads. It follows that other transporters, besides WBC11 and CER5, are involved in wax export on Arabidopsis stems. In support of this latter hypothesis, we note that other ABC transporters were also found up-regulated in the transcriptome of epidermal cells, as compared to neighbouring tissues (Suh et al., 2005).

**WBC11 may physically interact with CER5**

Analysis of the cer5 and wbc11 mutants demonstrated identical strong reductions in the predominant wax component for Arabidopsis stems, nonacosane, and accumulation of intracellular lipidic inclusions. Therefore, these two ABC transporters may have similar, but separate, roles in wax export. If this is the case, then mutations in both genes in the wbc11-3 cer5-2 double mutant should show an additive effect, and result in a lower wax load than that found in either single mutant. In contrast to this prediction, the cuticular lipid analysis of the double mutant revealed that there was no difference in wax load between the single wbc11-3 and cer5-2 mutants and the corresponding wbc11-3 cer5-2 double mutant. These results indicate that both WBC11 and CER5 gene products act in the same pathway or complex. As the ABCG/WBC subfamily of ABC transporters are ‘half-transporters’, and require dimerization to be functional, it is conceivable that CER5 and WBC11 form heterodimers that function in the export of waxes, particularly nonacosane. Protein–protein interaction studies are currently underway to test this hypothesis directly.

**Can WBC11 transport both cutin precursors and wax?**

Our results provide evidence that WBC11 is involved in export of both cutin precursors and wax. At this point, however, it cannot be ruled out that WBC11 is specific for only one of these cuticle lipid classes, and the loss of function has a secondary, indirect effect on the accumulation of the other component. Such an indirect effect could be mediated by altered physicochemical properties of the mutant wax, which in turn disrupt cutin assembly, or by altered transport of a wax component playing a signaling role for cutin biogenesis. However, it appears unlikely that WBC11 is directly involved in wax transport only, because mutations in CER5, which result in a similar reduction of wax load as wbc11 mutations, show neither a decrease in cutin monomers nor any cutin defects. An extreme hypothesis would be that cutin deposition is influenced mostly by very low abundance wax compounds, which thus may play a kind of signaling role in cuticle biogenesis (Lolle and Pruitt, 1999). However,
there is no experimental evidence that such a signaling pathway exists in the extracellular matrix.

It also seems unlikely that WBC11 is directly involved in cutin transport only. There is little evidence in the literature to suggest that cutin defects are coupled with loss of wax. On the contrary, mutants with altered cutin (including bodyguard and lacs2) as well as cutinase overexpressing plants do not show any detrimental effect on wax load, but instead have higher wax loads (Kurdyukov et al., 2006b; Schnurr et al., 2004; Sieber et al., 2000). Although other mechanisms cannot be ruled out, the simplest hypothesis is that WBC11 is directly involved in both cutin precursor and wax transport. If WBC11 is involved in transporting cutin precursors, then we must assume that assembly of the cutin polyester is extracellular. Thus the exported cutin precursors could be, for example, in the form of oligomers, acylglycerols or free fatty acids.

This raises the question of how this protein could transport substrates as diverse as these two classes of lipids. Arabidopsis cutin is rich in 18:2 and 16:0 dicarboxylates and a variety of C16 and C18 hydroxy-fatty acids (Bonaventure et al., 2004; Franke et al., 2005). In contrast, the wax components have much longer aliphatic chains, fully saturated carbon backbones and typically only one functional group. Many ABC transporters are capable of accepting a wide range in substrates (Chang, 2003). For example, a human orthologue of Arabidopsis WBC11, ABCG2/BRCP is capable of transporting a diverse array of hydrophobic xenobiotics (Xu et al., 2004). Still, despite the substrate promiscuity of ABC transporters, it is difficult to explain how WBC11 would discriminate between fairly similar wax molecules, such as nonacosane and the oxygenated C29 molecules, and at the same time be capable of transporting the structurally different cutin precursors.

However, we must keep in mind that WBC11 is a half-transporter, and thus may represent only one-half of one or more full-transporters composed of itself and another WBC family member. Such is the case with the Drosophila WHITE, BROWN and SCARLET gene products, where WHITE pairs with BROWN or SCARLET to form two different transporters to transport tryptophan- or guanine-derivatives, respectively (Dreesen et al., 1988). Thus, we propose a model where WBC11 forms different dimers to export wax or cutin precursors. Specifically, WBC11 and CER5 may form a heterodimer that functions in the transport of wax, particularly C29 alkane, whereas WBC11 may partner with itself to form a homodimer, or with an unidentified ABCG/WBC transporter, for the transport of cutin precursors. If WBC11 does have function in multiple interactions as described, then it is likely to have a higher affinity to form the cutin-transporting dimer, as the cutin monomer levels were reduced in the knock-out wbc11-3, but not in the knock-downs, wbc11-1 and -2.

Conclusions

In summary, we have characterized the Arabidopsis WBC11 transporter using a reverse genetics approach, and have demonstrated that it is required for the accumulation of both cutin and wax. We have further shown that WBC11 is localized to the plasma membrane where it may interact with CER5. These findings lead to interesting testable hypotheses, including: (i) heterodimers between CER5 and WBC11 transport wax; and (ii) WBC11 homodimers, or heterodimers with an unknown partner, are required for cutin accumulation.

Experimental procedures

Plant material and growth conditions

Arabidopsis thaliana seeds were stratified for 3–4 days at 4°C, and plants were grown on a mixture of sunshine mix 5 (Sun Gro Horticulture Inc., http://www.sungro.com) under white fluorescent light (80–100 μE m−2 sec−1) in a constant photoperiod at 21°C. Salk T-DNA insertional mutant lines were obtained from the Arabidopsis Biological Resource Centre (http://www.arabidopsis.org). Seeds of the pel1-1 mutant were a gift to AY received from Prof. Y. Machida (Nagoya University).

Stem harvest and surface area measurements

Primary stems were harvested from 5-6-week-old plants. After removal of the cauline leaves and siliques, stems were photographed with a digital camera. Surface areas were calculated by measuring the area of the two-dimensional projection of the stem, assuming a cylindrical geometry.

Cryo-scanning electron microscopy

Segments from the apical 4–6 cm of stem were mounted onto cryo-SEM stubs using graphite paste and plunged into liquid nitrogen. Frozen stems were transferred into an Emitech K1250 cryo-system, where stems were fractured and water sublimed for 10 min at −110°C. Samples were viewed with a Hitachi S4700 field emission SEM (Nissei Sangyo America Ltd.; http://www.hitachi.us) using an accelerating voltage of 1.5 kV and a working distance of 12 mm.

High-pressure freezing and transmission electron microscopy

Segments (1–2 mm thick) from the apical 0.5–1 cm of the bolting stem or basal leaves were high-pressure frozen, freeze-substituted and embedded in Spurr’s resin as previously described by Rensing et al. (2002). Briefly, stem and leaf segments were cut in 0.2 mm and subsequently high-pressure frozen in a Balzers HPM 010 (Bal-tec AG; http://www.bal-tec.com). Frozen samples were freeze-substituted for 5 days in 2% osmium tetroxide and 8% dimethoxy-propane in acetone using a dry ice-acetone bath. Tissues were then warmed to −20°C for 4 h to 4°C for 4 h and to room temperature for a final 4 h. Tissues were rinsed in fresh acetone and gradually infiltrated and embedded with Spurr’s resin. Embedded tissues were

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sectioned at 70 nm and mounted onto uncoated 300-mesh hexagonal grids, stained with 2% uranyl acetate in 70% methanol for 20 min and Reynold’s lead citrate for 10 min. Digital images were captured with a Hitachi 7600 (Nissai Sangyo America Ltd.) at 80 kV.

**Laser-scanning confocal microscopy**

Stem sections from 5-week-old plants or hypocotyls from 5-day-old seedlings were mounted in distilled water and observed on a Zeiss LSM 510 Meta laser scanning microscope (Carl Zeiss Inc., http://www.zeiss.com). GFP fluorescence was detected using excitation of 488 nm with a 505–530-nm band-pass filter. YFP fluorescence was detected using excitation of 514 nm with a 535–580-nm band-pass filter. For cell wall staining, tissue was immersed in FM4-64 solution (10 µM) for 10 min. Propidium iodide and FM4-64 staining was detected using an excitation of 514 nm with a 635–680-nm band-pass filter. Images were processed with LSM 5 Image Browser (Carl Zeiss Inc.) and Corel Photopaint 8.0 software (Corel, http://www.corel.com).

**Polyester analysis**

Surface waxes of whole stems from 6-8-week-old plants were first extracted as described above. Stems were then immediately processed to extract and quantify lipid polyesters according to the method described by Suh et al. (2005). Briefly, after extensive dilution of the ground stems, the resulting dry residue was depolymerized using the sodium methoxide method. Polyester monomers were separated, identified and quantified by GC-MS. Splitless injection was used and the mass spectrometer run in scan mode over 40–500 amu (electron impact ionization), with peaks quantified on the basis of their total ion current. Polyester monomer quantities were expressed per unit of surface area, which was determined as indicated for waxes. The initial wax extractions were used to verify the wax loads.

**Transgene constructs and plant transformation**

The native WBC11 promoter was amplified from genomic DNA using gene specific primers (forward primer 5'-GGGGACACCTCTTGTAGTTGAGTTAGAGAGTACG-3' and reverse primer 5'-ATGGAGTTAGAGAGTACG-3'). The PCR product was cloned into pDONR 221 using BP Clonase (Invitrogen) to create pD-WBC11. Following this, the WBC11 promoter cassette was transferred to pGBW3 (T. Nakagawa, unpublished data) using LR Clonase (Invitrogen) to create pWBCT11::GUS and was confirmed by sequencing. The WBC11 open reading frame (ORF) was amplified from wild-type cDNA using gene-specific primers (forward primer 5'-GGGGACACCTCTTGTAGTTGAGTTAGAGAGTACG-3' and reverse primer 5'-GGGGACACCTCTTGTAGTTGAGTTAGAGAGTACG-3') and the PCR product was cloned into pDONR 221 using BP Clonase (Invitrogen) to create pD-WBC11. Following this, the WBC11 ORF was transferred to pEARLEYGATE 104 (Earley et al., 2006) to create an N-terminal YFP-fusion to WBC11 using the CaMV 3SS promoter for constitutive expression in plants. Arabidopsis plants (wild-type Col-0, and heterozygotes wbc11-3/ WBC1) were transformed using the floral dip method (Clough and Bent, 1998).

**Gene expression analysis**

Total RNA was extracted from whole-stem segments using TRIzol reagent (Invitrogen). Double-stranded cDNA was synthesized from approximately 15 mg of total RNA by using a double-stranded cDNA synthesis kit (Fermentas, http://www.fermentas.com) with oligo-d(T) primer. A 2-µl sample of a 20-µl reaction (approximately 3 µg of cDNA) was used for semi-quantitative RT-PCR reactions with gene-specific primers for At1g17840 (forward primer 5'-ATGGGATATAGAAGACAGAC-3' and reverse primer 5'-CCATCTGGGCTCCATC-3'), At1g51500 (forward primer 5'-ATGGAGTCTAGAAGATGACG-3' and reverse primer 5'-ATTAAATGGTGGTAAGAAGC-3'), At5g33000 (forward primer 5'-GATCTTTGCAGAAAACATTGGGAGGATGTT-3' and reverse primer 5'-GGACTTGTGATTAGAAAGAGATGAAACA-3'). The PCR reaction was performed in an MJ Research PTC 200 thermocycler (Waltham, MA, USA) using Hot Start Taq (Fermentas) with the following conditions: initial denaturation 95°C for 4 min, followed by twenty cycles of 95°C for 30 sec, 55°C for 45 sec, 72°C for 120 sec and a final 10-min extension at 72°C. Quantitative Real-time PCR was performed using SYBR Green to monitor dsDNA synthesis using a Bio-Rad miniOpticon real-time PCR detection system (Bio-Rad, http://www.bio-rad.com) according to the manufacturer’s directions. RNA levels were normalized using ACTIN2 (At3g18780) as a housekeeping gene. Efficiencies were calculated at 496 David Bird et al.

Statistical analysis

Data analysis was conducted with SYSTAT 12 software (Systat Software, http://www.systat.com). When assumptions of normalcy and homoscedasticity were met, significance was analyzed using ANOVA and Tukey’s honestly significant differences (HSD) post-hoc tests. In cases of heterogeneous variance, the Games–Howell multiple comparisons test was used to analyze significance (Sokal and Rohlf, 1995).
using a 10-fold serial dilution series of DNA. Primers for WBC11 (forward primer 5'-GCCGCAADAAATGCTTTTTG-3'; reverse primer 5'-CTTTTCCACACTATTTCCACGC-3') and ACT2 (forward primer 5'-CCAGAGGATGCTATGTTGGTGA-3'; reverse primer 5'-GAGGAGGCTGCTGAAGAA-3') were used. Reaction conditions were as follows: initial denaturation 95°C for 2 min, followed by forty cycles of 95°C for 20 sec, 55°C for 10 sec, 72°C for 10 sec and a final 5-min extension at 72°C. A dissociation analysis was conducted in order to check for the specificity of primers to the genes of interest. Results were analyzed using the Bio-Rad OPTICON MONITOR 3 software (Bio-Rad).

GUS expression analysis

Expression of GUS in transgenic WBC11promoter::GUS plants (wild-type Col-0 background) was assayed as described by Koizumi et al., 2000 with the modification of a 4-h incubation at 37°C. Stained seedlings were mounted directly in water. Alternatively, tissue specimens were processed using microwave embedding into Spurr’s resin. Sections (2-μm thick) were cut on a Leica Ultracut microtome (http://www.leica.com), mounted on glass slides and imaged using a QCAM digital camera (QiMaging, http://www.qimaging.com) and OPENLAB 4.01 (Improvision, http://www.improvision.com).

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Stem epidermal wax load is restored in wbc11 plants expressing 35S::YFP-WBC11.

Figure S2. Comparison of YFP-WBC11 and tonoplast-localized δ-TIP-GFP.

This material is available as part of the online article from http://www.blackwell-synergy.com

References


