Identification of the Wax Ester Synthase/Acyl-Coenzyme A:Diacylglycerol Acyltransferase WSD1 Required for Stem Wax Ester Biosynthesis in Arabidopsis1,2[W][OA]

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Wax esters are neutral lipids composed of aliphatic alcohols and acids, with both moieties usually long-chain (C16 and C18) or very-long-chain (C20 and longer) carbon structures. They have diverse biological functions in bacteria, insects, mammals, and terrestrial plants and are also important substrates for a variety of industrial applications. In plants, wax esters are mostly found in the cuticles coating the primary shoot surfaces, but they also accumulate to high concentrations in the seed oils of a few plant species, including jojoba (Simmondsia chinensis), a desert shrub that is the major commercial source of these compounds. Here, we report the identification and characterization of WSD1, a member of the bifunctional wax ester synthase/diacylglycerol acyltransferase gene family, which plays a key role in wax ester synthesis in Arabidopsis (Arabidopsis thaliana) stems, as first evidenced by severely reduced wax ester levels of in the stem wax of wsd1 mutants. In vitro assays using protein extracts from Escherichia coli expressing WSD1 showed that this enzyme has a high level of wax synthase activity and approximately 10-fold lower level of diacylglycerol acyltransferase activity. Expression of the WSD1 gene in Saccharomyces cerevisiae resulted in the accumulation of wax esters, but not triacylglycerol, indicating that WSD1 predominantly functions as a wax synthase. Analyses of WSD1 expression revealed that this gene is transcribed in flowers, top parts of stems, and leaves. Fully functional yellow fluorescent protein-tagged WSD1 protein was localized to the endoplasmic reticulum, demonstrating that biosynthesis of wax esters, the final products of the alcohol-forming pathway, occurs in this subcellular compartment.

The cuticle is a thin hydrophobic layer that covers the outermost surface of the primary aerial tissues of terrestrial plants. It controls nonstomatal water loss (Riederer and Schreiber, 2001), protects plants from UV radiation (Krauss et al. 1997; Solcovchenko and Merzlyak, 2003; Pfündel et al., 2006), minimizes deposition of dust, pollen, and pathogen spores (Barthlott and Neinhuis, 1997), and mediates a variety of plant interactions with insects (Eigenbrode and Espelie, 1995; Müller, 2006), as well as bacterial and fungal pathogens (Carver and Gurr, 2006; Leveau, 2006). In addition, the cuticle prevents fusions between plant organs during development (Lolle et al., 1998; Sieber et al., 2000).

The cuticle framework is provided by cutin, an insoluble plant-specific polyester composed of C16 and C18 hydroxy and epoxy fatty acids and glycerol (Heredia, 2003). The cutin matrix is embedded in and overlaid with cuticular waxes, complex mixtures of mostly very-long-chain fatty acid (VLCPA) derivatives easily extractable by organic solvents (Jetter et al., 2006).

Cuticular wax biosynthesis involves elongation of saturated C16 and C18 fatty acyl-CoAs to VLCPA precursors between 24 and 34 carbons in length and their subsequent modification by either the alkane-forming (decarbonylation) or the alcohol-forming (acyl reduction) pathway. The alkane pathway results in the synthesis of aldehydes, alkanes, secondary alcohols, and ketones, whereas the alcohol pathway yields primary alcohols and wax esters (Kunst et al., 2006). Because they are derived from VLCPAs, chain lengths of cuticular wax constituents on Arabidopsis (Arabidopsis thaliana) fluorescence stems range from C24 to C34, with the exception of wax esters, dimeric compounds consisting of fatty acids and fatty alcohols with chain lengths between C38 and C52 (Lai et al., 2007).

The ester content in plant cuticular waxes varies greatly among species and, to some extent, even within

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species. For example, Arabidopsis leaf and stem cuticular waxes contain only 0.1% to 0.2% and 0.7% to 2.9% of wax esters, respectively (Jenks et al., 1995), whereas esters accumulate to 85% in leaf wax of the carnauba palm (*Copernicia cerifera*; Kolattukudy, 1976). Wax esters also serve as seed storage reserves in jojoba (*Simmondsia chinensis*), where they constitute approximately 97% of the seed oil (Benzioni and Forti, 1989). Carnauba and jojoba are the main commercial sources of plant wax esters, which have diverse pharmaceutical, cosmetic, and food applications, in addition to their traditional use as lubricants. Wax esters are not only found in cuticles of many plant species, but are also widely distributed in insects (ubiquitously in the exoskeleton, but also in beeswax) and mammals (among others in humans and whales), as well as in bacteria (storage lipids in *Acinetobacter* spp.; Kolattukudy, 1976; Ishige et al., 2003; Wältermann et al., 2006).

Even though major steps in plant cuticular wax biosynthesis have been proposed, our knowledge of the enzymes involved and the type of biochemical reactions that they catalyze is incomplete. Forward genetic approaches using a collection of wax-deficient Arabidopsis *eceriferum* (cer) mutants and maize (*Zea mays*) glossy mutants resulted in the identification of several genes encoding wax biosynthetic enzymes, including *CER6*, *CER10*, *GL8A*, and *GL8B* (Millar et al., 1999; Fiebig et al., 2000; Hooker et al., 2002; Xu et al., 1997; Dietrich et al., 2005; Zheng et al., 2005) required for fatty acid elongation. Several other mutants, specifically *cer1*, *cer2*, *wax2/cer3*, *gl1*, and *gl2*, were also useful for the identification of proteins required for the synthesis of wax components by the alkane pathway (for review, see Kunst et al., 2006), but the biochemical activities of these proteins are presently unknown. Only the enzyme catalyzing the last two enzymatic steps of this pathway leading from alkanes to secondary alcohols and ketones have been identified through a reverse genetic approach and characterized as a

**Figure 1.** Structure of *WSD1* gene and transcript levels in mutant alleles of *WSD1*. A, Exon-intron structure of the *WSD1* gene, with white boxes representing exons. Arrows indicate T-DNA insertion sites. B, Semiquantitative RT-PCR analysis of steady-state *WSD1* transcript leaves in stems of *wsd1-1* and *wsd1-2* mutants and wild-type plants. The bottom image shows expression of *GAPC* as a loading control.

**Figure 2.** Cuticular wax composition on the stems of Arabidopsis. A, Compound classes in wild-type and *wsd1* mutant stem wax. B, Chain length distributions within compound classes generated by the alcohol (acyl reduction) pathway. Each value is the mean of the wax load on five individual plants. Error bars indicate SDs. C, GC analysis of ester fractions within wax mixtures from stems of wild-type and mutant *wsd1-2* plants. Wax esters were first concentrated and purified via TLC and then characterized by GC-MS.
P450-dependent midchain alkane hydroxylase (MAH1; Greer et al., 2007).

For the alcohol-forming pathway, only the first of the two steps, the conversion of VLCFAs to primary alcohols by the fatty acyl-CoA reductase CER4, has been investigated at the molecular level (Rowland et al., 2006). Furthermore, compositional analysis of wax esters and free primary alcohols from Arabidopsis wild type and cer mutants demonstrated that the primary alcohols formed by the CER4 enzyme serve as substrates for biosynthesis of the alkyl esters found in the stem wax mixture (Lai et al., 2007). It was also reported that the composition of stem wax esters in Arabidopsis is dominated by C16 acyl groups and C26 to C30 alkyl groups, suggesting that the wax synthase (WS) enzyme may have a preference for these substrate chain lengths. However, the WS enzyme involved in wax ester production has not been identified.

In an attempt to clone and characterize the gene/enzyme responsible for wax ester biosynthesis in Arabidopsis stem epidermis, we focused our attention on gene families exhibiting high sequence homology with the three types of WS enzymes known to catalyze wax ester formation in other organisms. The first type, the mammalian WS enzymes, have the highest activity with acyl-CoAs ranging from C14 to C24, with C20:1 as the preferred substrate, and exhibits the highest activity with C14 to C18 alcohols (Kalscheuer and Steinbüchel, 2003; Stöveken et al., 2005). In Arabidopsis, there are 11 annotated WS/DGAT genes whose functions are currently unknown. However, recently a WS/DGAT bifunctional enzyme has been shown to be involved in the synthesis of wax esters composed of a VLCFA esterified to a short- to medium-chain alcohol (≧C12) in the petunia (Petunia hybrida) petal (King et al., 2007).

To narrow down the number of candidate genes, we decided to concentrate on the jojoba-type WS and WS/DGAT genes up-regulated in the stem epidermis during active wax synthesis (Suh et al., 2005). We identified a WS/DGAT gene (At5g37300) with the highest levels of expression in the upper, rapidly expanding part of the stem, and a high ratio of epidermis-to-total stem expression as the prime candidate for functional analysis using reverse genetics. In this study, we report on the characterization of this candidate gene, named WSD1. We show that the protein it encodes is indeed involved in stem wax ester formation and is the major enzyme catalyzing this last step of the alcohol-forming pathway of wax biosynthesis.

### RESULTS

Among the Arabidopsis genes annotated as WS/DGAT, WSD1 showed the highest expression level in the stem epidermis and a high expression ratio of epidermis to total stem in a microarray experiment (Suh et al., 2005). To determine whether this gene is involved in wax ester formation in Arabidopsis stems, we obtained mutant lines wsd1-1 (SALK_067714) and wsd1-2 (SALK_118165; Alonso et al., 2003) from the Arabidopsis Biological Resource Center (ABRC) and analyzed them for disruption of the WSD1 gene using PCR. The wsd1-1 allele had a T-DNA insertion in the second exon of the WSD1 gene, 643 bp downstream from the predicted translation start site, and wsd1-2 carried a T-DNA insertion in the 5’-untranslated region of the WSD1 gene, 180 bp upstream of the predicted translation start site (Fig. 1A). Semiquantitative reverse transcription (RT)-PCR analyses conducted to assess steady-state levels of WSD1 gene transcript in mutant alleles revealed no detectable WSD1 transcript in wsd1-1 and reduced transcript accumulation in wsd1-2 (Fig. 1B).

### Stem Cuticular Wax Phenotypes of wsd1 Mutants

Insertions in the WSD1 gene did not result in a glossy stem phenotype or altered appearance and density of epicuticular wax crystals on the stem surface (scanning electron micrographs not shown), characteristic of a number of wax-deficient mutants studied to date. To further investigate the effect of WSD1 dis-

### Table 1. Chain length distribution of wax esters

Relative compositions (%) are given.

<table>
<thead>
<tr>
<th>Ester Chain Length</th>
<th>Wild Type</th>
<th>wsd1-1</th>
<th>wsd1-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>C38</td>
<td>1.7</td>
<td>12.9</td>
<td>16.1</td>
</tr>
<tr>
<td>C39</td>
<td>0.2</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C40</td>
<td>6.6</td>
<td>28.2</td>
<td>23.2</td>
</tr>
<tr>
<td>C41</td>
<td>0.4</td>
<td>nd</td>
<td>tr</td>
</tr>
<tr>
<td>C42</td>
<td>20.4</td>
<td>27.4</td>
<td>18.2</td>
</tr>
<tr>
<td>C43</td>
<td>0.7</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C44</td>
<td>29.5</td>
<td>13.6</td>
<td>19.0</td>
</tr>
<tr>
<td>C45</td>
<td>1.4</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C46</td>
<td>21.4</td>
<td>7.4</td>
<td>12.3</td>
</tr>
<tr>
<td>C47</td>
<td>0.5</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C48</td>
<td>10.6</td>
<td>10.4</td>
<td>11.1</td>
</tr>
<tr>
<td>C49</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C50</td>
<td>3.8</td>
<td>nd</td>
<td>tr</td>
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<tr>
<td>C51</td>
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<td>nd</td>
<td>nd</td>
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<td>C52</td>
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<td>C53</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C54</td>
<td>1.2</td>
<td>tr</td>
<td>tr</td>
</tr>
</tbody>
</table>

*nd, Not detected. tr, Trace amount (i.e. <0.1%).
ruption on wax accumulation and specifically wax ester formation, we therefore extracted total stem waxes from the \textit{wsd1-1} and \textit{wsd1-2} mutants and wild-type plants and analyzed them by gas chromatography (GC). No differences in the absolute amounts and the relative proportions of most wax components, including aldehydes, alkanes, secondary alcohols, ketones, fatty acids, primary alcohols, phenyl-ethyl esters, and triterpenoids, were found in mutant lines in comparison to the wild type (Fig. 2A). The only compound class affected by \textit{wsd1} mutations was the alkyl esters, which were severely reduced in both mutants (Fig. 2). The alkyl ester profile detected in the wild-type wax included chain lengths ranging from C\textsubscript{38} to C\textsubscript{48} (Fig. 2B). None of these wax esters was detectable in the total wax extracts from the mutant stems. Therefore, a more detailed analysis of wax esters from wild-type and mutant stem cuticular wax mixtures was carried out after separation of wax compound classes by thin-layer chromatography (TLC). In the wild-type wax, ester chain lengths varied between C\textsubscript{38} and C\textsubscript{48}, with a predominance of even-numbered homologs and a maximum at C\textsubscript{44} (Fig. 1; Table I). Only trace amounts of even-numbered wax esters and no odd-numbered wax esters were detected in either \textit{wsd1-1} or \textit{wsd1-2} mutant. The chain length distribution of mutant esters was shifted relative to the wild type, resulting in a predominance of the C\textsubscript{40} homolog.

In Vitro Characterization of WSD1 Using Heterologous Expression in \textit{Escherichia coli}

Because WSD1 has significant sequence similarity to the WS/DGAT from \textit{A. calcoaceticus} ADP1, the question arises as to whether the WSD1 protein has both WS and DGAT activity. To test this, we heterologously expressed the WSD1 cDNA in \textit{E. coli} BL21 (DE3). WS assays were carried out with crude protein extract using radiolabeled [1-\textsuperscript{14}C]palmitoyl-CoA as the acyl donor and 1-octadecanol (C\textsubscript{18} alcohol) as acyl acceptor, whereas [1-\textsuperscript{14}C]palmitoyl-CoA and 1,2-dipalmitoylglycerol were used as substrates in DGAT activity assays. The reaction products were detected by autoradiography following TLC separation and the specific activities of WSD1 were determined by scintillation counting. Our results showed that WSD1 is a bifunctional enzyme, which has high WS activity (84.4 ± 5.5 pmol/mg min) and substantially lower DGAT activity (7.7 ± 1.0 pmol/mg min; Fig. 3). The ratio of 10.9 between WS and DGAT activity of WSD1 was similar to the ratio of these activities (11.1) determined for the \textit{A. calcoaceticus} ADP1 bifunctional enzyme (Kalscheuer and Steinbüchel, 2003).

In Vivo Characterization of WSD1 Using Heterologous Expression in Yeast

To verify that WSD1 is indeed a bifunctional enzyme with WS and DGAT activity, we introduced the WSD1 cDNA into the yeast (\textit{Saccharomyces cerevisiae}) mutant \textit{H1246} deficient in storage lipid biosynthesis (Sandager et al., 2002). TLC analysis of the lipids extracted from the recombinant yeast cells expressing the WSD1...
revealed that these cells did not accumulate wax esters or triacylglycerols (TAGs) when grown in inducible medium alone (Fig. 4, lane 2) or when supplemented with palmitic acid (C\textsubscript{16:0}; Fig. 4, lane 3). In contrast, cultivation in inducible medium containing palmitic acid together with octadecanol (C\textsubscript{18} alcohol) led to the formation of wax esters (Fig. 4, lane 4). Similarly, when yeast cells were fed with palmitic acid together with tetracosanol (C\textsubscript{24} alcohol) or octacosanol (C\textsubscript{28} alcohol), both very-long-chain fatty alcohols were incorporated into wax esters (Fig. 4, lanes 5 and 6). The wax esters generated in these three experiments were purified by preparative TLC and their exact structures determined by GC-mass spectrometry (MS) analysis as octadecyl (C\textsubscript{18}), tetracosyl (C\textsubscript{24}), and octacosyl (C\textsubscript{28}) esters of palmitic (C\textsubscript{16}) acid, respectively (Fig. 5). Unlike wax ester biosynthesis, heterologous expression of WSD1 in yeast did not yield detectable amounts of TAG under any condition tested (Fig. 4).

Organ- and Tissue-Specific Expression of WSD1

To determine the organ-specific expression of the WSD1 gene, quantitative PCR (qPCR) and RT-PCR were performed. In both experiments, the highest WSD1 mRNA accumulation was detected in flowers, followed by stem tops and leaves. Very low levels of transcript were found in roots and seeds only by qPCR (Fig. 6, A and B). To confirm these results and more precisely define the tissue specificity of WSD1 expression, the 1,978-kb genomic fragment upstream of the WSD1 coding region was fused to the GUS reporter gene (\textit{PWSD1}:GUS). Consistent with qPCR and RT-PCR results, histochemical analysis of GUS activity in transgenic plants harboring \textit{PWSD1}:GUS showed a high level of expression in the top 3 cm of stems (Fig. 7A). Cross sections indicated WSD1 expression in all cell types (Fig. 7B), and this result was verified by in situ hybridization (Fig. 7C; Supplemental Fig. S1). Furthermore, GUS activity was apparent in the hydathodes and veins of 20-d-old cauline leaves (Fig. 7D), filaments of stamens (Fig. 7E), and siliques (Fig. 7F), but not in seeds, roots, and young seedlings (data not shown).

To investigate the subcellular localization of the WSD1 enzyme in Arabidopsis, the \textit{WSD1} cDNA was fused with the yellow fluorescent protein (YFP) coding sequence at its 5’ end and expressed in the \textit{wsd1-1} mutant under the control of the 35S promoter. Expression of the \textit{P\textsubscript{35S}:YFP:WSD1} construct complemented the wax ester deficiency of the \textit{wsd1-1} mutant (Fig. 8) by restoring stem wax ester levels to 30% to 50% of the wild type, thus confirming that the mutant phenotype was caused by the T-DNA insertion in the \textit{WSD1} gene. At the same time, the complementation of the \textit{wsd1} phenotype by the YFP-tagged WSD1 demonstrated that the addition of YFP did not interfere with WSD1 folding or function and allowed us to localize the functional protein within epidermal cells. Protein sequence analysis using the DAS transmembrane prediction server (http://www.sbc.su.se/~miklos/DAS) classified WSD1 as an integral membrane protein containing at least four transmembrane domains (Supplemental Fig. S2). This predicted membrane association was experimentally verified by examination of the \textit{P\textsubscript{35S}:YFP:WSD1} transgenic plants in which YFP fluorescence uniformly labeled a reticulate network typi-
cal of the endoplasmic reticulum (ER; Fig. 9A). The YFP fluorescence signal colocalized with the signal from hexyl rhodamine B, a dye that labels the ER in plants (Boevink et al., 1996, Fig. 9, B–D).

DISCUSSION

Alkyl esters are the final products of the alcohol pathway of cuticular wax biosynthesis and account for up to 3% of total wax on Arabidopsis inflorescence stem. These wax esters consist mainly of C\textsubscript{16} acyl and C\textsubscript{26} to C\textsubscript{30} alkyl moieties (Lai et al., 2007), but the identity of the WS that catalyzes their formation was previously unknown. Based on sequence similarities to enzymes that mediate wax ester biosynthesis in other organisms and high levels of expression at the time of maximal wax deposition (Suh et al., 2005), we predicted that WSD1, encoded by a WS/DGAT-type gene At5g37300, is the WS involved in stem wax ester production. Several lines of evidence discussed below demonstrate that this is indeed the case.

Phenotypic analysis of two independent wsd1 mutants with T-DNA insertions in the WSD1 gene revealed deficiencies in the accumulation of wax esters in both lines. These compounds were not detectable in the total wax extracts from either the loss-of-function allele wsd1-1 or the partial-loss-of-function line wsd1-2 (Fig. 2). Cuticular wax ester deficiencies in wsd1 mutants were successfully complemented by the introduction of the wild-type WSD1 (Fig. 8), indicating that inactivation of this gene was responsible for the observed mutant phenotypes. Collectively, these results support the conclusion that the WSD1 enzyme is required for wax ester biosynthesis in Arabidopsis stem.

In contrast to the jojoba WS, which could not be functionally expressed in microorganisms (Lardizabal et al., 2000), we were successful in expressing active WSD1 in E. coli and yeast. Introduction of WSD1 into E. coli conferred upon these recombinant cells the ability to synthesize both wax esters and TAGs, indicating that WSD1 is a bifunctional WS/DGAT enzyme (Fig. 3). Both WS and DGAT activities measured in vitro for WSD1 were comparable to those of the A. calcoaceticus ADP1 enzyme (Kalscheuer and Steinbüchel, 2003), with the WS activity approximately 10-fold greater than the DGAT activity. When WSD1 was expressed in yeast, wax ester synthesis could only be accomplished when the growth medium was supplemented with both the acyl and the alkyl substrates required for their production. In agreement with the established composition of
Arabidopsis stem wax esters (Lai et al., 2007), WSD1 readily utilized C16 acyl and C24 or C28 alkyl substrates. Feeding of palmitic acid (C16 acid) with C18 alcohol also resulted in wax ester formation (Fig. 4), indicating that WSD1 uses a relatively broad spectrum of acyl acceptors. However, esters containing short-chain alcohols could not be detected in yeast H1246 expressing WSD1, even though potential acyl acceptors, such as ethanol and isoamyl alcohol, were likely present in our yeast strain and were accessible to the heterologous enzyme. This assumption is based on previous findings that yeast expressing the A. calcoaceticus WS/DGAT enzyme or petunia wax synthase PhWS1 can utilize endogenous short-chain alcohols like ethanol and/or isoamyl alcohol and incorporate them into esters (Kalscheuer et al., 2004; King et al., 2007).

WSD1 expression was detected in Arabidopsis shoots, most prominently in flowers, in the top 3 cm of inflorescence stems and in leaves, consistent with a role for this gene in cuticular wax production (Figs. 6 and 7). Surprisingly, WSD1 expression was not restricted to the epidermal cells like most other genes involved in wax deposition (Xia et al., 1997; Millar et al., 1999; Kurata et al., 2003; Pighin and Zheng et al., 2004; Rowland et al., 2006; Greer et al., 2007), but detected in all cell types by GUS activity assays and by in situ RNA hybridization. The significance of WSD1 expression in tissues other than the epidermis is unclear at present.

The subcellular localization of the active YFP-WSD1 by confocal microscopy demonstrated that this enzyme resides in the ER membranes (Fig. 9). Because the biosynthesis of fatty acyl and alkyl substrates for wax ester production also takes place at this site (Lessire et al., 1985; Kunst and Samuels, 2003; Zheng et al., 2005; Rowland et al., 2006; Samuels et al., 2008), this result implies that the whole alcohol pathway of wax biosynthesis is associated with the ER. Recently, the midchain hydroxylase that catalyzes the final steps of the alkane pathway, the formation of secondary alcohols and ketones, was also found to be present in the ER (Greer et al., 2007). Thus, the ER is the cellular compartment where most, if not all, of the cuticular wax components are generated. These wax components then need to be delivered from the ER to the ATP-binding cassette transporters in the plasma membrane (Pighin and Zheng et al., 2004; Bird et al., 2007) for export to the cuticle.

Based on all the evidence discussed above, we conclude that WSD1 plays a major role in the formation of wax esters in the stem of Arabidopsis. This enzyme is involved in cuticle formation, and in this physiological function acts as WS by using primary alcohols as acyl acceptors. This raises the question of whether wax ester formation is the only function of WSD1 or whether the enzyme can also use diacylglycerols as acyl acceptors and thus function as a DGAT. Results from three of our experiments address this question.

First, unlike the ADP1 A. calcoaceticus enzyme, which restored TAG biosynthesis when expressed in the quadruple mutant of yeast H1246 deficient in storage lipid production (Kalscheuer et al., 2004), introduction of WSD1 failed to restore TAG biosynthesis in this yeast mutant, suggesting that WSD1 does not have DGAT activity in vivo (Fig. 4). Because this result is in contrast with our cell-free assays using WSD1 heterologously expressed in E. coli, it further suggests that WSD1 may have DGAT activity only in vitro, but not in vivo, or that the DAG substrate is not accessible to the enzyme. Alternatively, the WSD1 enzyme may show different substrate specificities depending on whether E. coli or yeast was used as host for heterologous expression, as reported for the A. calcoaceticus WS/DGAT (Kalscheuer et al., 2004). The lack of in vivo DGAT activity for the members of the WS/DGAT family has also been reported previously for the PhWS1 from petunia when expressed in yeast (King et al., 2007). Similarly, the majority of the 15 WS/DGAT homologs from Mycobacterium tuberculosis H37Rv showed no DGAT or WS activity (Wältermann et al., 2006) in yeast. Second, WSD1 transcript was virtually undetectable in seeds of Arabi-
dopsis wild type (Fig. 6). Third, wsd1-1 and wsd1-2 mutants did not exhibit reduced seed oil content in comparison to wild-type seeds (Supplemental Fig. S3). Based on these results, it seems unlikely that WSD1 is involved in storage TAG formation in a physiological context.

A large family of 11 sequences, including WSD1, has been tentatively annotated as WS/DGATs in the Arabidopsis genome (Fig. 10A; Supplemental Fig. S4), so it is conceivable that at least some of these predicted WSD enzymes can work as DGATs. They have similar sequence (16.0%-20.5% amino acid sequence identity over their entire length) and size, ranging from 476 to 523 residues in length with short N-terminal or C-terminal extensions in a few cases. In addition, like the A. calcoaceticus WS/DGAT, the WSD1-WSD11 sequences contain a highly conserved condensing domain with a proposed active-site motif (146HHXXXDG152) in their N-terminal region (Fig. 10B; Supplemental Fig. S4) suggested to be essential for catalytic activity in the acyl-CoA acyltransferase reactions involved in wax ester and TAG formation (Kalscheuer and Steinbüchel, 2003). Whether any of these proteins actually have WS or DGAT activity remains to be determined. An interesting

Figure 9. Subcellular localization of YFP::WSD1 expression in transgenic 35S:YFP::WSD1 Arabidopsis lines. A, YFP::WSD1 is localized in the ER in the wsd1-1 leaf epidermal cells. B to D, Localization of the ER network stained by hexyl rhodamine B (B) and colocalization with YFP::WSD1 labeled network (C); D shows merging images of B and C. One centimeter = 10 μm.

Figure 10. Phylogenetic tree of the 11 Arabidopsis proteins related to the bifunctional WS/DGAT ADP1 of A. calcoaceticus. A, Phylogenetic analysis by MEGA2.0 was performed using the neighbor-joining tree with 1,000 replicates; the handling gap option was pairwise deletion. Sequence alignments were assembled by the ClustalW algorithm version 1.83. B, Protein sequence alignment of a region of the Arabidopsis WSD1 and WS/DGAT from A. calcoaceticus strain ADP1, which includes a putative active site motif (boxed).
candidate is WSD9 (At5g12420), which shows high expression levels in seeds (Schmid et al., 2005) and may, therefore, be involved in seed TAG synthesis. Other WSDs may act as acyltransferases in the formation of other lipid esters, possibly even in the oligomerization or polymerization reactions leading to cutin or suberin production.

In summary, we have shown that WSD1, a member of the Arabidopsis WS/DGAT enzyme family, is the WS that catalyzes the biosynthesis of wax esters, dimeric cuticular wax components found on Arabidopsis shoots. This enzyme resides in the ER and uses long-chain and very-long-chain primary alcohols with C<sub>16</sub> fatty acid for wax ester production.

MATERIALS AND METHODS

Plant Material and Growth Conditions

T-DNA insertion mutant lines SALK_067714 and SALK_118165 were obtained from the ABRC (www.arabidopsis.org). Seeds were stratified for 3 to 4 d at 4°C and plants were grown on Sunshine Mix 5 (Sun Gro Horticulture) under continuous white fluorescent light (80–100 μE m<sup>−2</sup> s<sup>−1</sup>). The temperature was set at 20°C to 22°C.

Real-Time PCR and RT-PCR

Total RNA was extracted from each sample by using the RNaseasy mini kit (Qiagen) and treated with RNase-free DNase (Promega). Total RNA (2.5 μg) was used for reverse transcriptase reactions. First-strand cDNA synthesis was performed using the SuperScript III platinum two-step qRT-PCR kit with SYBR green (Invitrogen). Glyceraldehyde-3-phosphate dehydrogenase (GAPC) was used as a reference. Primers for WSD1 (forward 5′-CTGATGTGTTGACCAAGTATTG-3′ and reverse 5′-TCTGCCACACCTTATATAACACC-3′) and for GAPC (forward 5′-AATGGAGAGAGGTGGGACAAGTTTGTACAAAAAGCAGGCTTCATGAAAGCGGAAAAAGTTATG-3′ and reverse 5′-GGGGACCACTTTGTACAAAAAGCAGGCTTCATGAAAGCGGAAAAAGTTATG-3′) were used for real-time PCR. The real-time PCR reaction included an initial 5-min denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 2 min, and a final 5-min extension at 72°C. The quantitative real-time PCR was performed using a Bio-Rad miniOpticon real-time PCR detection system (Bio-Rad) according to the manufacturer’s directions.

WT PCR was carried out in an MJ Research PTC 200 thermocycler with WSD1 primers: forward 5′-ATGAAAGCGGAAAAAGTTATGG-3′ and reverse 5′-GGGGACCACTTTGTACAAAAAGCAGGCTTCATGAAAGCGGAAAAAGTTATG-3′) and reverse 5′-GGGGACCACTTTGTACAAAAAGCAGGCTTCATGAAAGCGGAAAAAGTTATG-3′) were used for real-time PCR. The real-time PCR reaction included an initial 5-min denaturation at 95°C for 2 min, followed by 20 cycles of 94°C for 30 s, 56°C for 30 s, and a final 5-min extension at 72°C.

Wax Analysis

Cauline leaves, flowers, and silique were removed from the inflorescence stems and cuticular waxes extracted by immersing the stems twice for 30 s into chloroform at room temperature. Both chloroform extracts were combined and C<sub>44</sub> alkane was added as internal standard. The solvent was removed under a gentle stream of nitrogen and the wax mixtures were treated into chloroform at room temperature. Both chloroform extracts were combined and C<sub>44</sub> alkane was added as internal standard. The solvent was removed under a gentle stream of nitrogen and the wax mixtures were treated with bis-3,5-dinitrobenzoyl chloride (Sigma) in pyridine for 1 h at 70°C to transform all hydroxy-containing compounds into the corresponding trimethylsilyl derivatives. The wax composition was determined with capillary GC (5890N; Agilent) and a mass spectrometer detector (5975N; Agilent). GC was carried out with temperature-programmed on-column injection at 53°C, oven 2 min at 50°C, raised by 40°C min<sup>−1</sup> to 200°C, held for 2 min at 200°C, raised by 3°C min<sup>−1</sup> to 320°C, and held for 30 min at 320°C, and He carrier gas inlet pressure was programmed for constant flow of 1.4 mL min<sup>−1</sup>. The quantitative analyses of wax mixtures were carried out by capillary GC with flame ionization detector (FID) under the same GC conditions as above, but with H<sub>2</sub> carrier gas at a constant flow of 2 mL min<sup>−1</sup>. Wax loads were determined by comparing GC-FID peak areas against internal standard and dividing by the surface area determined for the corresponding sample. Stem surface areas were calculated by measuring the projected stem areas in photographs and multiplying by π.

In TLC analysis, wax mixtures from stems of wild-type, mutant, and transgenic plants were separated on silica gel 60 (EMD Chemicals) using hexane-diethylcarbonate-acetic acid (90:5:1 [v/v/v]) and visualized under UV light after spraying with primuline (Sigma).

Analysis of Seed Oil Fatty Acid Composition

To determine the fatty acid composition of the seed oil, fatty acid methyl esters were prepared by refluxing the seed samples in 2 mL of 1 n methanolic HCl for 90 min at 80°C. After cooling, 2 mL of 0.9% NaCl solution and 150 μL of hexane were added and the mixture was vortexed vigorously. The fatty acid methyl esters in the hexane phase were analyzed by GC-liquid chromatography as described previously (Kunst et al., 1992). The identity of fatty acids in the samples was determined by comparing retention times with those of standards (Sigma).

Constructs and Plant Transformation

For the GUS activity assays, the 1,978-bp fragment immediately upstream of the WSD1 coding region was amplified by PCR from genomic Arabidopsis DNA using forward 5′-GGGACAACAGTTTCAAAAGGACGTCGAA-GATCAGGGTTATG-3′ and reverse 5′-GGGACAACATTGTTCAAAACGGAAGTTATG-3′). The amplified fragment was introduced into a donor vector pDONR221 (Invitrogen), resulting in pDONR221-WSD1 promoter constructs, and then the WSD1 promoter was cloned into a binary vector pGWB3 (Nakagawa et al., 2007) to create pGWB3-WSD1 promoter:GUS construct.

To generate an N-terminally YFP fusion to WSD1, the WSD1 coding region was amplified from wild-type cDNA using gene-specific primers (forward 5′-GGGACAACAGTTTCAAAAGGACGTCGAA-GATCAGGGTTATG-3′ and reverse 5′-GGGACAACATTGTTCAAAACGGAAGTTATG-3′). The PCR product was cloned into the pEARLEYGATE104 vector (Earley et al., 2006) behind the constitutive cauliflower mosaic virus 35S promoter using Gateway cloning kits (Invitrogen) to create the pPEARLEYGATE104-YFP:WSD1 construct. The resulting construct was transformed into wild type Col-0 and mutant wsd1-1 using the Agrobacterium-mediated floral-dip plant transformation method (Clough and Bent, 1998).

GUS Activity Analysis

The tissues collected from transgenic plants harboring the WSD1 promoter:GUS construct were incubated in GUS assay buffer as described previously (Jefferson, 1987). After staining, samples were cleared by incubation in 95% (v/v) ethanol overnight. For tissue sectioning, GUS-stained stems were fixed and embedded in Spurr’s resin (Rowland et al., 2006). Tissue sections were prepared on a Leica Ultracut microtome (Reichert-Jung) and mounted on glass slides. The commonly observed staining patterns were recorded using a dissecting microscope fitted with a digital camera (SPOT Diagnostics).

Laser-Scanning Confocal Microscopy

Tissues from 10-d-old transgenic seedlings harboring the 35S:YFP:WSD1 were immersed in 1.6 M hexyl rhodamine B solution (Molecular Probes) for 10 to 30 min, then mounted in distilled water and observed in a Zeiss LSM 5 Pascal confocal laser-scanning microscope (Carl Zeiss; http://www.zeiss.com). YFP fluorescence was detected using excitation of 514 nm with a 535- to 580-nm band-pass emission filter. Hexyl rhodamine B was excited with a 543-nm argon ion laser line with a 600- to 650-nm band-pass emission filter. All confocal images obtained were processed with LSM 5 Image Browser (Carl Zeiss) and Adobe Photoshop 5.0 software.

In Situ Hybridization

Tissue fixation, sectioning, hybridization, signal detection, and probe synthesis were performed as previously described (Hooker et al., 2002; Hepworth et al., 2005). The probes used for hybridization were obtained by PCR amplification from WSD1 cDNA using primers incorporating the 17 RNA polymerase-binding site. For the antisense probe, the primers used were (forward, 5′-CACGATTCGATGAGAGAG-3′) and reverse, 5′-TAAATTTCCCTCACCATCC-3′). The PCR product was cloned into the pPEARLEYGATE104 vector (Earley et al., 2006) behind the constitutive cauliflower mosaic virus 35S promoter using Gateway cloning kits (Invitrogen) to create the pPEARLEYGATE104-YFP:WSD1 construct. The resulting construct was transformed into wild type Col-0 and mutant wsd1-1 using the Agrobacterium-mediated floral-dip plant transformation method (Clough and Bent, 1998).
Heterologous Expression of WSD1 in *Escherichia coli* and Yeast

For expression in *E. coli*, full-length WSD1 cDNA was cloned into the expression vector pGEX4T2 (GE Healthcare) using BamHI and Xhol restriction sites. The resulting plasmid pGEX4T2::WSD1 was transformed into *E. coli* BL21 (DE3). Recombinant WSD1 was grown in Luria-Bertani medium for 6 h at 37°C in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside. Cells were then harvested, washed, resuspended in 125 mM sodium phosphate buffer (pH 7.4), and disrupted by glass beads.

For expression in yeast (*Saccharomyces cerevisiae*), full-length WSD1 cDNA was amplified by PCR using forward and reverse primers 5'-AGAGCAGTCATGAAAGGAAAAGATTAGG-3' and 5'-AGACCTGAGCTCAAACTCTTCCGTTTGTTGAAA-3', respectively. The PCR product was cloned into the BamHI-Xhol-restricted vector pESC-URA (Stratagene) with the Gal1 inducible GAL1 promoter. The generated construct was transformed into yeast strain HI246 defective in storage lipid accumulation (Samlager et al., 2002). Recombinant yeast cells were cultivated in synthetic minimal dropout medium lacking uracil and containing 2% (w/v) Gal for 48 h at 30°C. Primary alcohols (C18, C24, and C28) were dissolved in hot ethanol and added to the culture medium to a final concentration of 0.1% (w/v), where indicated.

Determination of Enzyme Activities in Vitro

Enzyme activities were determined according to the method reported by Kalscheuer et al. (2004). Briefly, 3.75 mM octadecanol (C18 alcohol) and 4.72 mM [1-14C]palmitoyl-CoA and 3.75 mM 1,2-dipalmitoylglycerol were used as substrates for WS activity, and 4.72 mM [1-14C]palmitoyl-CoA and 3.75 mM 1,2-dipalmitoylglycerol were used as substrates for DGAT activity. One hundred micrograms of crude extract protein from recombinant *E. coli* was used in each assay. The assay mixtures were incubated for 30 min at 35°C and the reactions terminated by adding 500 μL of chloroform phase. Lipids were extracted from the chloroform phase and separated by TLC as described above. Radioactivity of reaction products was measured by scintillation counting.

Yeast Lipid Analysis

For extraction of total lipids, yeast cells were harvested by centrifugation and 2 mL of methanol were added to each sample. After 5 min, 4 mL of chloroform and 1 mL of 0.9% NaCl were added, the two-phase system was mixed by vortexing, and the chloroform phase transferred to a new tube and concentrated under a stream of nitrogen. Wax esters were purified by preparative TLC and analyzed by GC-MS and GC-FID as described above.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. In situ hybridization analysis of WSD1 expression in Arabidopsis stem.

Supplemental Figure S2. Transmembrane domain prediction for WSD1.

Supplemental Figure S3. GC analyses of fatty acid methyl esters prepared from mature Arabidopsis seeds.

Supplemental Figure S4. Sequence alignment of the WS/DGAT (WSD) family from Arabidopsis.

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