Male Sterile2 Encodes a Plastid-Localized Fatty Acyl Carrier Protein Reductase Required for Pollen Exine Development in Arabidopsis

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Male Sterile2 (MS2) is predicted to encode a fatty acid reductase required for pollen wall development in Arabidopsis (Arabidopsis thaliana). Transient expression of MS2 in tobacco (Nicotiana benthamiana) leaves resulted in the accumulation of significant levels of C16 and C18 fatty alcohols. Expression of MS2 fused with green fluorescent protein revealed that an amino-terminal transit peptide targets the MS2 to plastids. The plastidial localization of MS2 is biologically important because genetic complementation of MS2 in ms2 homozygous plants was dependent on the presence of its amino-terminal transit peptide or that of the Rubisco small subunit protein amino-terminal transit peptide. In addition, two domains, NAD(P)H-binding domain and sterile domain, conserved in MS2 and its homologs were also shown to be essential for MS2 function in pollen exine development by genetic complementation testing. Direct biochemical analysis revealed that purified recombinant MS2 enzyme is able to convert palmitoyl-Acyt Carrier Protein to the corresponding C16:0 alcohol with NAD(P)H as the preferred electron donor. Using optimized reaction conditions (i.e. at pH 6.0 and 30°C), MS2 exhibits a Kₘ for 16:0-Acyl Carrier Protein of 23.3 ± 4.0 μM, a Vₘₚₐₜ of 38.3 ± 4.5 nmol mg⁻¹ min⁻¹, and a catalytic efficiency/Kₘ of 1,873 μmol-s⁻¹. Based on the high homology of MS2 to other characterized fatty acid reductases, it was surprising that MS2 showed no activity against palmitoyl- or other acyl-coenzyme A; however, this is consistent with its plastidial localization. In summary, genetic and biochemical evidence demonstrate an MS2-mediated conserved plastidial pathway for the production of fatty alcohols that are essential for pollen wall biosynthesis in Arabidopsis.

In flowering plants, the life cycle alternates between diploid sporophyte and haploid gametophyte generations. Pollen grains play a biologically protective role for the haploid male sperm cells surrounded by the outer cell wall lipidic biopolymers called the exine (Blackmore et al., 2007; Li and Zhang, 2010; Ariizumi and Toriyama, 2011). Pollen exine protects the gameto-phyte against pathogen attack, dehydration, and UV irradiation as well as facilitates the pollination process, including pollen recognition and adhesion to the stigma. The highly durable exine that occurs throughout flowering plants is thought to play an essential role in land colonization by plants (Chaloner, 1976).

The exine is mainly composed of the biopolymer sporopollenin and contains two sublayers, the sexine and nexine (Zinkl et al., 1999). The biochemical nature of pollen exine remains largely unknown because of the technical difficulties in purifying and obtaining large quantities of materials for analysis. In addition, sporopollenin is highly insoluble, resistant to degradation, and exceptionally stable (Brooks and Shaw, 1968; Bubert et al., 2002). Current evidence suggests that the major components of sporopollenin are derivatives of aliphatics, such as fatty acids and phenolic compounds (Bubert et al., 2002; Blackmore et al., 2007).

Tapetum, the innermost sporophytic anther wall layer, is thought to play a major role in actively synthesizing and secreting sporopollenin precursors onto the microspore surface for pollen exine polymerization and patterning (Bedinger, 1992; Li and Zhang, 2010; Ariizumi and Toriyama, 2011). The model dicot plant Arabidopsis (Arabidopsis thaliana) and many other plants have a secretory-type tapetum with spe-
cialized structures such as tapetosomes in the cells, which accumulate lipidic components (Huysmans et al., 1998). The outer surface of Arabidopsis pollen grains displays elegant reticulate cavities with abundant pollen coat (tryphine) deposited inside the pollen exine.

Pollen exine patterning appears to include at least three major developmental events: callose wall formation, primexine formation, and sporopollenin synthesis (Ariizumi and Toriyama, 2011). Exine formation commences after meiosis, with the accumulation of lipidic precursors onto the primexine surrounding newly formed microspores between the callose wall and the microspore plasma membrane (Paxson-Sowders et al., 2001; Blackmore et al., 2007). After the first pollen mitosis, the synthesis of the exine is almost complete; and during later stages of pollen exine formation, the pectocellulosic intine and the tryphine, called the pollen coat, are deposited onto the pollen wall (Piffanelli et al., 1998). Recent genetic and biochemical investigations showed that some genes, including \textit{MALE STERILITY1 (MS1)}, \textit{MS2}, \textit{CER1}, \textit{NO EXINE FORMATION1}, \textit{FACE-LESS POLLEN1}, \textit{CYP703A2}, \textit{ACYL-COA SYNTHETASE5 (ACOS5)}, \textit{CYP704B1}, \textit{TETRAKETIDE α-PYRONE REDUCTASE1} and -2 (TKPR1/2), \textit{LAP6/POLYKETIDE SYNTHASE A (PKSA)}, and \textit{LAP5/POLYKETIDE SYN-}

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\caption{Pollen phenotype of the \textit{ms2} mutant. Pollen grains isolated from a wild-type (WT) plant and a \textit{ms2} plant were detected by DAPI staining (A and B), \textit{I2-KI} staining (C and D), acetylasis treatment (E and F), and SEM (G and H). Arrows in D show the rough outer surface of \textit{ms2} pollen grains. Bars = 10 μm (A–F) and 5 μm (G and H).}
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\caption{Subcellular localization analysis of \textit{MS2} in protoplasts. A, Diagrams of the constructs of the full-length \textit{MS2} cDNA with and without the sequence encoding the putative transit peptide (TP) fused with the N terminus of GFP under the control of the \textit{CaMV 35S} promoter (\textit{pro35S:MS2-GFP} and \textit{pro35S:MS2A-N-GFP}, respectively) and the fragment encoding the putative transit peptide of \textit{MS2} fused with YFP under the control of the \textit{CaMV 35S} promoter (\textit{pro35S:TP-YFP}). B to E, An Arabidopsis protoplast expressing \textit{MS2-GFP} showing green fluorescent signals (B), the chlorophyll autofluorescence signals in the chloroplasts (C), blue fluorescent signals by coexpressing mitochondrion-localizing F1ATPase-γ-RFP (D), and the merged signals (E). \textit{F} to \text{I}, An Arabidopsis protoplast expressing \textit{MS2A-N-GFP} showing green fluorescent signals in the cytoplasm (F), the chlorophyll autofluorescence signals (G), blue fluorescent signals by coexpressing mitochondrion-localizing F1ATPase-γ-RFP (H), and the merged signals (I). \textit{J} to \text{L}, An Arabidopsis protoplast expressing \textit{TP-YFP} showing yellow fluorescent signals (J), the chlorophyll autofluorescence signals in the plastids (K), and the merged signals (L). Bars = 10 μm.}
\end{figure}
recombinant MS2 enzyme from Acyl Carrier Protein (ACP) reductase, and the purified involvement of the plastid in primary fatty alcohol mutants. This work, therefore, demonstrates the significance of MS2 subcellular localization, and the necessary for localization to the plastid. The biological MS2 possesses an N-terminal transit peptide that is C16:0 alcohol in the presence of NAD(P)H. In addition, to convert the preferred substrate palmitoyl-ACP to From endogenous bacterial fatty acids. Bacteria expressed in the tapetum shortly after the microspore contained a putative plastid-targeting (or mitochon-
derived fatty acids 

RESULTS

ms2 Pollen Grains Have Defective Pollen Exine

Defective pollen grains of ms2 were incubated with 4',6-diamidino-2-phenylindole (DAPI) and iodine-potassium iodide (I2-KI). DAPI staining showed that both the wild type and ms2 display normal mature pollen grains with a single vegetative nucleus and two sperm nuclei at the mature pollen developmental stage (Fig. 1, A and B). I2-KI staining revealed that ms2 pollen grains had a rough outer surface compared with the wild type (Fig. 1, C and D), consistent with the defective pollen wall development of ms2. In agreement with previous reports (Aarts et al., 1997; Dobritsa et al., 2009), ms2 pollen grains were sensitive to acetoxylation treatment (Fig. 1, E and F), and scanning electron microscopy (SEM) analysis revealed that the ms2 pollen grains collapsed and attained a severely shrunk appearance (Fig. 1, G and H).

The MS2 Protein Is Localized to Plastids

Previous investigation predicted that MS2 (At3g11980) and a MS2 homolog (At3g56700) were localized in chloroplasts based on the presence of an N-terminal transit peptide (Doan et al., 2009). Our TargetP 1.1, ChloroP 1.1, and MitoProt II version 1.101 sequence analyses consistently suggested that the MS2 protein contained a putative plastid-targeting (or mitochon-

Figure 3. Localization of MS2 expression in transgenic plants. A to C, A transgenic plant line expressing full-length MS2 cDNA fused with GFP under the control of the MS2 promoter (proMS2:MS2-GFP) in the wild type, showing green fluorescent signals in the tapetal layer (A), autoluminescence signals of chlorophyll (B), and the merged signals (C). D to F, A transgenic plant line expressing MS2 cDNA without the sequence encoding the putative transit peptide fused with GFP under the control of the MS2 promoter (proMS2:MS2-N-GFP) showing green fluorescent signals (D), autoluminescence signals of chlorophyll (E), and the merged signals (F). Bars = 100 μm.

MS2 Encodes a Fatty Acyl-ACP Reductase

The expression of MS2 was shown only in tapetal cells at or very shortly after young microspores were released from the tetrad by in situ RNA hybridization and promoter fused with the GUS reporter gene analyses (Aarts et al., 1997). In this study, we observed the GFP signal of proMS2-GFP in the tapetal cells at the free microspore stage in the stable transgenic lines (Fig. 3, A–C). Furthermore, the fluorescent signal in the anthers of transgenic lines harboring proMS2ΔN-GFP showed higher expression signals in tapetal cells and anther locules compared with those of transgenic lines containing proM2-GFP (Fig. 3, D–F), suggesting that the N-terminal transit peptide of MS2 is responsible for correct MS2 localization, the absence of which possibly affects its turnover.

The biological importance of the MS2 localization to plastids was confirmed from the observation that the construct proM2:MS2ΔN without the N-terminal transit peptide-encoding sequence of MS2 could not complement the defective pollen wall development in ms2

Figure 4. Functional complementation of the ms2 mutant using truncated MS2 cDNA fragments. A, Diagrams of the constructs of the full-length MS2 cDNA under the control of the MS2 promoter (proMS2:MS2), MS2 cDNA without the sequence encoding the putative transit peptide (proMS2:MS2ΔN), MS2 cDNA without the sequence encoding the SD (proMS2:MS2ΔSD), MS2 cDNA without the sequence encoding the NAD-binding 4 domain (proMS2:MS2ΔNBD), MS2 cDNA without the sequence encoding the NAD(P)H-binding motif (proMS2:MS2ΔNBM), and MS2 cDNA without the sequence encoding the active site motif YX(3)K of NADP-dependent proteins (proMS2:MS2ΔASM). B to M, These constructs were transformed to the ms2 mutants individually, and pollen grains were detected by acetolysis treatment (B–G) and SEM analysis (H–M). Bars = 20 μm (B–G) and 5 μm (H–M). [See online article for color version of this figure.]
homozygous mutants, whereas proM2:MS2 rescued the defect of ms2 pollen wall development (Fig. 4, A, B, C, H, and I). The Arabidopsis Rubisco small subunit protein contains an N-terminal transit peptide (RTP) that has been shown to effectively target this protein to chloroplasts (Kim et al., 2010a). To test whether RTP can functionally replace the N-terminal transit peptide of MS2 protein, we made the construct proMS2:RTP-MS2ΔN', in which the fragment of RTP translationally replaced the N-terminal transit peptide of MS2 (Fig. 5A) and transformed it to ms2 mutants. Twenty-one independent transgenic lines containing proMS2:RTP-MS2ΔN' in the ms2 homozygous background were obtained. Acetolysis treatment and SEM analysis showed that 10 transgenic lines displayed normal pollen exine development compared with the wild type (Fig. 5, B and E), suggesting the rescued pollen wall phenotype of ms2 mutants by this construct. The pollen grains of four transgenic lines showed partially resistant to acetolysis treatment and less defective outer surface analyzed by SEM, suggesting a partially rescued phenotype of pollen exine formation (Fig. 5, C and F). Seven transgenic lines showed defects of pollen exine development similar to ms2 (Fig. 5, D and G), which may have resulted from the difference between the RTP and the N-terminal transit peptide of MS2. These results confirmed that the localization of MS2 to plastids is critical for its function in anther development.

NAD(P)H-Binding Domain and Sterile Domain Are Essential for MS2 Function

Consistent with a previous investigation (Aarts et al., 1997), analysis of the MS2 protein using the SMART protein structure prediction tool (http://smart.embl-heidelberg.de/) revealed that the MS2 protein contains two conserved domains: the NAD(P)H-binding domain (NBD) between residues 135 and 438 and the sterile domain (SD) between residues 534 and 614.

MS2 and other FARs were observed to contain the conserved NAD(P)H-binding motif between residues 133 and 143 ([I/V/F][I/L/V]TGXTGFL(G/A)] and the active site motif YX(3)K of NADP-dependent proteins between residues 357 and 368 (Fig. 4A; Aarts et al., 1997; Doan et al., 2009), implying a functional role of these motifs.

To examine whether these domains/motifs are essential for MS2 function, we made several constructs containing the mutated MS2 fragments driven by the MS2 promoter (Fig. 4A). The construct containing the deleted SD sequence (proMS2:MS2ΔSD) or deletion of the NBD domain (proMS2:MS2ΔNBD) could not rescue the defective pollen exine development in the ms2 homozygous background (Fig. 4, A, D, E, J, and K), suggesting an essential role of both SD and NBD domains in MS2 function. Furthermore, the constructs deleted for the NAD(P)H-binding motif or the active site motif YX(3)K of NADP-dependent proteins failed to complement the defective pollen exine phenotype of ms2 in the transgenic plants (Fig. 4, A, E, G, L, and M), suggesting the essential role of these two conserved motifs in the NBD of MS2.

Purified Recombinant MS2 Enzyme Has Fatty Acyl-ACP Reductase Activity

Although MS2 is predicted as a fatty acid reductase involved in fatty alcohol formation and recombinant bacteria expressing MS2 can produce C14:0, C16:0, and C18:1 alcohols (Aarts et al., 1997; Doan et al., 2009), a comprehensive biochemical characterization of this enzyme has not been performed. To this end, we cloned the full-length MS2 cDNA into the expression vector pET30a (Novagen) and introduced it into E. coli BL21 (DE3). The recombinant MS2 protein was purified by affinity chromatography and subjected to SDS-PAGE. Compared with the predicted size of about 73 kD, the molecular mass of the affinity-purified protein ran somewhat faster on SDS-PAGE (Fig. 6).
Because the data presented herein show that MS2 is localized in plastids in which long-chain fatty acids are de novo synthesized and while esterified to ACP (Li-Beisson et al., 2010), we analyzed MS2 activity with acyl-ACPs. Using 14C-labeled fatty acyl-ACPs, we detected strong activity only with [1-14C]16:0-ACP, trace activity with [1-14C]18:0-ACP, and no activity with [1-14C]14:0-ACP, [1-14C]16:1-ACP, and [1-14C]18:1-ACP (Supplemental Table S1), indicating that MS2 prefers palmitoyl-ACP as a substrate. Furthermore, no activity was detected with the equivalent [1-14C]acyl-CoA substrates (data not shown). To further investigate the substrate specificity of recombinant MS2, unlabeled myristoyl-ACP, palmitoyl-ACP, palmitoleoyl-ACP, and stearoyl-ACP were tested with the purified MS2 enzyme in the presence of NAD(P)H, and only palmitoyl-ACP was reduced to C16:0 alcohol (Fig. 7). The specificity of MS2 against palmitoyl-ACP was confirmed by using deuterium-labeled 7,7,8,8-d4-palmitoyl-ACP as substrate, which yielded a product at the same retention time as hexadecanol but with a mass ion +4 relative to the non-mass-labeled standard (Supplemental Fig. S2).

To more comprehensively characterize the MS2 enzyme, we compared the palmitoyl-ACP-reducing activity in the presence of NAD(P)H versus NADH. Under the same experimental conditions, chromatographic analysis revealed that the recombinant MS2 has higher activity in the presence of NAD(P)H relative to that using NADH, and no C16:0 alcohol was detectable if NAD(P)H or NADH was omitted (Fig. 8), suggesting that MS2 activity is dependent on the presence of either NAD(P)H or NADH. Furthermore, we show that the optimal pH value for the MS2 activity is 6.0 (Fig. 9). Additionally, assays at various temperatures showed that the optimal working temperature for the MS2 converting palmitoyl-ACP to the C16:0 alcohol is 30°C (Fig. 9). Under the condition of optimum pH and temperature, the recombinant MS2 has a K_m of 23.3 ± 4.0 μM for palmitoyl-ACP and a V_max of 38.3 ± 4.5 nmol mg⁻¹ min⁻¹ (Table I).

Fatty Alcohol Production in Tobacco Leaves Transiently Expressing MS2

To demonstrate the physiological role of MS2 activity in producing fatty acyl alcohols, we transiently expressed the MS2 protein in tobacco leaves using a cowpea mosaic virus RNA-2-based expression system (Sainsbury and Lomonossoff, 2008). The constructed

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**Figure 6.** Analysis of Ni²⁺-NTA column affinity-purified MS2. Lane 1, prestained protein markers; lane 2, 10 μL of eluted solution from the Ni²⁺-NTA affinity column incubated with the protein from control bacteria containing the empty vector pET30a; lane 3, 10 μL of purified His tag fused-MS2 protein from the Ni²⁺-NTA affinity column incubated with protein from pET30a-MS2.

**Figure 7.** Substrate specificity analysis of recombinant MS2. Gas chromatograms of metabolites generated from the incubation of myristoyl-ACP (A), palmitoyl-ACP (B), palmitoleoyl-ACP (C), and stearoyl-ACP (D) with affinity-purified MS2 in the presence of NAD(P)H are shown.
vector pEAQ:MS2 was transformed into tobacco leaves, and overexpression of MS2 protein was confirmed by western-blot analysis using an MS2-specific antibody (see "Materials and Methods") of the tobacco leaf extracts 3 d after Agrobacterium tumefaciens infiltration (Supplemental Figs. S3 and S4). The lipidic compositions of chloroform-extractable cuticular waxes, cutin monomers, and total soluble lipids were analyzed in the transgenic lines using gas chromatography-mass spectrometry (GC-MS) and gas chromatography-flame ionization detection (GC-FID; Bonaventure et al., 2004; Franke et al., 2005) and compared with lines carrying the empty vector pEAQ as the control.

From GC-FID analysis, we observed that the amounts of total wax and total soluble lipids per cm² of tobacco leaves in the pEAQ:MS2 lines were very close to those of the control lines. In contrast, the total cutin amount per cm² of tobacco leaves was significantly higher in the pEAQ:MS2 lines compared with the control lines (Fig. 10A). Interestingly, compared with the control lines, significant increases of the contents of hexacosanol, octadecanol, and C29 alkane were observed in the wax of tobacco leaves harboring pEAQ:MS2 (Fig. 10B; Supplemental Table S2) as well as increased levels of hexacosanol and C20 fatty acid in internal lipids (Fig. 10C; Supplemental Table S3). In addition, significant accumulations of hexacosanol, octadeccanol, octadecanoic acid, oleic acid, eicosanoic acid, docosanoic acid, and unknown cutin monomer were observed in the cutin monomers of the transgenic tobacco leaves (Fig. 10D; Supplemental Table S4). These results suggested that MS2 can synthesize fatty alcohols in plants.

DISCUSSION

MS2 Encodes a Fatty Acyl-ACP Reductase Essential for Normal Pollen Exine Development

Male reproductive development in higher plants is indispensable for the synthesis of fatty acid derivatives (Li and Zhang, 2010). Our results show that purified recombinant MS2 is able to produce C16:0 alcohol, which is required for pollen exine development. Although the biochemical nature of pollen exine remains exclusive, we previously showed that pollen exine may share a common lipidic biosynthetic pathway with that of cutin, which is an insoluble polymer matrix containing the major constituents of hydroxylated and epoxy hydroxylated C16 and C18 fatty acids as well as minor components such as fatty alcohols (Li et al., 2010; Zhang et al., 2010). Fatty alcohols with C16 and C18 occupy 0% to 8% of cutin composition (Pollard et al., 2008). CYP704B2 belongs to a conserved and ancient subfamily of fatty acid hydroxylases and is specifically expressed in the tapetum and the microspore. Mutation of CYP704B2 causes aborted pollen.
Table 1. Kinetic properties of MS2 catalyzing palmitoyl-ACP
Enzyme activity was measured by the assay with affinity-purified MS2 protein and various concentrations of palmitoyl-ACP. Catalytic efficiency ($k_{cat}/k_{m}$) means and ± values are based on four replicates.

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<th>$k_{m}$ (µM)</th>
<th>$V_{max}$ (nmol mg$^{-1}$ min$^{-1}$)</th>
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<td>23.3 ± 4.0</td>
<td>38.3 ± 4.5</td>
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grains without detectable exine and undeveloped another cuticle with reduced levels of cutin monomers. Recombinant CYP704B2 catalyzes the production of ω-hydroxylated fatty acids with 16 and 18 carbon chains, suggesting that CYP704B2 is involved in the biosynthesis of the two biopolymers sporopollenin and cutin (Li et al., 2010). Accordingly, we propose that the MS2-derived fatty alcohol may account for pollen exine development. Consistently, we recently genetically characterized a close homolog of MS2 in rice called Defective Pollen Wall (DPW). $dpw$ displays defective anther development and aborted pollen grains with an abnormal exine, causing complete male sterility. Additionally, $dpw$ anthers have significantly reduced levels of cutin monomers and cuticular wax composition as well as soluble fatty acids and alcohols (Shi et al., 2011). $dpw$, like MS2, is able to produce C16 primary alcohols.

MS2 has a spatiotemporal expression pattern in tapetal cells (Aarts et al., 1997; Fig. 3) that is consistent with the tapetal role in synthesizing and secreting lipidic sporopollenin precursors (Li and Zhang, 2010). This expression pattern is similar to other tapetum-expressed genes, such as the fatty acid-metabolizing genes CYP703s (Morant et al., 2007; Aya et al., 2009), ACOS5 (de Azevedo Souza et al., 2009), CYP704Bs (Dobritsa et al., 2009; Li et al., 2010; Yi et al., 2010), TKPR1/2 (Grienenberger et al., 2010), and LAP6/PKSA and LAP5/PKSB (Tang et al., 2009; Dobritsa et al., 2010; Kim et al., 2010b) as well as WBC27/ABCG26 (Quilichini et al., 2010; Xu et al., 2010; Choi et al., 2011; Dou et al., 2011), OsC6 (Zhang et al., 2010), and DPW (Shi et al., 2011).

In this study, we show that the purified recombinant MS2 has a strong preference for converting palmitoyl-ACP to hexadecanol that is dependent on the electron donor NAD(P)H, but it can also use NADH with lower efficiency. Compared with previous investigations, this study provides biochemical insight into plastidially localized enzymes with sequence homology to the FARs from the green alga Euglena gracilis (Kolattukudy, 1970), jojoba (Metz et al., 2000), oilseed rape (Brassica napus; Hu et al., 2006), and Arabidopsis (Rowland et al., 2006; Doan et al., 2009; Domergue et al., 2010). In the Arabidopsis genome, eight FAR-like sequences were identified, and five FARs have been biochemically investigated (Doan et al., 2009). The Arabidopsis FARs share sequence similarity with the jojoba FAR (30% to 54% identity), and CER4 has the highest sequence identity to that of jojoba (54%; Doan et al., 2009). Yeast cells expressing Arabidopsis CER4 generated C24:0 and C26:0 primary alcohols, and CER4 is required for the development of epidermal cells of aerial organs and roots (Rowland et al., 2006). Bacteria expressing MS2 are capable of producing C14:0, C16:0, and C18:1 alcohols (Doan et al., 2009). We are currently unable to reconcile this observation with our biochemical assays, in which we failed to detect activity with [1-14C]C14:0 substrates. It is possible that bacteria expressing MS2 have more complex metabolic activities mediated by MS2 and bacterial enzymes that are not revealed in purified enzyme assays. Unlike MS2, the FAR enzyme from jojoba is able to convert acyl-CoAs to C16:0 and C18:0 fatty alcohols, which are required for wax production in jojoba seeds (Metz et al., 2000). Silk moth FAR-expressing yeast cells could convert C14-20 fatty acids to alcohols and preferentially to C15 and C16 fatty acids (Moto et al., 2003). Two mouse enzymes, FAR1 and FAR2, were shown to reduce fatty acyl-CoA ester substrates into fatty alcohols (Cheng and Russell, 2004).

Unlike the recombinant MS2, the recombinant DPW can convert both palmitoyl-ACP and palmitoyl-CoA into hexadecanol despite its higher activity to palmitoyl-ACP (Shi et al., 2011). In addition, the $K_m$ value of the recombinant MS2 toward palmitoyl-ACP was determined to be about 6.5-fold higher than that of DPW, suggesting that DPW has higher affinity for palmitoyl-ACP than MS2. Moreover, the recombinant MS2 has trace activity against [1-14C]18:0-ACP that is not detectable for DPW. Furthermore, the DPW activity depends exclusively on NAD(P)H (Shi et al., 2011), whereas MS2 can use either NAD(P)H or NADH. These results suggest that MS2 homologs have undergone substantial diversification during the course of the evolution of monocots and dicots.

Alignment analysis revealed that MS2, DPW, and FARs from Arabidopsis (CER4), jojoba, wheat (TAA1a), silk moth, and mouse share conserved NAD(P)H-binding and active site motifs (Doan et al., 2009; Shi et al., 2011). In this study, we demonstrate the biological importance of these motifs in anther development by genetic complementation (Fig. 4), implying that these motifs are essential for enzyme function. In addition, the critical role of the SD conserved in MS2 and other homologs (Aarts et al., 1997) was also empirically demonstrated in this investigation (Fig. 4).

**MS2 Represents a Conserved Plastid-Localized Lipid Metabolism Pathway That Operates during Anther Development**

Plant anther cells contain various types of diversified plastids, and plastids within tapetal cells are thought to be associated with lipid synthesis and secretion for pollen wall synthesis (Clement and Pacini, 2001). Plant de novo biosynthesis of fatty acids with short carbon chain lengths of up to C18 is mainly in plastids (Öhberg et al., 1979; Li-Beisson et al., 2010). We revealed that the MS2/DPW protein is localized to chloroplasts, and a genetic complementation assay shows that the
N-terminal transit peptide is required for the MS2/DPW function in anther development (Figs. 4 and 5; Shi et al., 2011). A similar N-terminal transit peptide is also observed in other MS2 homologs (Doan et al., 2009; Shi et al., 2011). Therefore, our data show that a plastid-localized and conserved pathway of converting fatty acids to fatty alcohols is required for male reproductive development in monocots and dicots (Shi et al., 2011).

In conclusion, we have characterized an Arabidopsis plastid-localized fatty acyl-ACP reductase, MS2, that is able to reduce palmitoyl-ACP to C16:0 alcohol. The plastidial MS2 fatty alcohol production pathway is essential for pollen exine synthesis. Moreover, the subcellular localization of MS2 in plastids, mediated by its N-terminal transit peptide, NBD, and SD and the active site motif YX(3)K, is required for MS2 function in anther development. This work reveals a key and conserved step of primary fatty alcohol synthesis for pollen wall biosynthesis in Arabidopsis.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana Columbia ecotype) plants were grown in a greenhouse at 22°C and 70% relative humidity under continuous illumination. The ms2 mutant, a T-DNA insertion line (SAIL_92_C07), was obtained from the Arabidopsis Biological Resource Center. Tobacco (Nicotiana benthamiana) plants were grown in a greenhouse at 25°C with 16 h of supplemental light per day.

Staining, SEM, and Acetolysis Treatment

For DAPI staining, pollen grains were fixed in formaldehyde acetic acid, immersed in 0.2% DAPI, and viewed by fluorescence microscopy under UV light (Leica DM2500). For I2-KI staining, pollen grains were immersed in I2-KI solution and photographed with a microscope (Leica DM2500). For SEM, pollen grains were air dried in silica, coated with palladium-gold in a sputter coater (Hummer), and then observed in an autoscan scanning electron microscope (JEOL) with an acceleration voltage of 30 kV. Acetolysis treatment was performed as described previously (Aarts et al., 1997).

Figure 10. Analysis of wax, internal lipids, and cutin in transient tobacco leaves. A, Total wax, internal lipids, and cutin amounts per unit surface area (mg cm−2) in tobacco leaves by transient expression of MS2 (white bars) or empty vector (black bars). B, Wax constituent amounts per unit surface area (mg cm−2) in tobacco leaves by transient expression of MS2 (white bars) or empty vector (black bars). C, Internal lipid amounts per unit surface area (mg cm−2) in tobacco leaves by transient expression of MS2 (white bars) or empty vector (black bars). D, Cutin monomer amounts per unit surface area (mg cm−2) in tobacco leaves by transient expression of MS2 (white bars) or empty vector (black bars). The values indicate means of five biological replicates ± so. **P < 0.01. Compound names are as follows: C16-1-OL, hexacosanol; C18-1-OL, octadecanol; C16-FA, hexadecanoic acid; C18-FA, octadecanoic acid; C18(2/3)-FA, linoleic and linolenic acid; C18(1)-FA, oleic acid; C20-FA, eicosenoic acid; C22-FA, docosanoic acid; C24-FA, etracosanoic acid; C16-2HFA, 2-hydroxy-hexadecanoic acid; C23-2HFA, 2-hydroxy-tricosanoic acid; C24-2HFA, 2-hydroxy-tetracosanoic acid; C25-2HFA, 2-hydroxy-pentacosanoic acid; C26-2HFA, 2-hydroxy-hexacosanoic acid; C16-ω-HFA, 16-hydroxyhexadecanoic acid; C18-1-ω-HFA, 18-hydroxy-octadecanoic acid; C18(1)-DFA, octadecene-1,18-dioic acid; UI, unknown cutin monomer; ALK, alkane; BR, branched aliphates.
**Expression, Purification, and Activity Assay of Recombinant MS2**

BL21 (DE3) E. coli cells containing pET30a-MS2 were grown at 30°C to an optical density at 600 nm of 0.6, and MS2 expression was induced by the addition of 0.5 mM isopropyl β-D-thiogalactoside and further incubation at 20°C for 48 h. The cell pellet was collected by centrifugation at 4°C and resuspended in phosphate-buffered saline buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, and 1.76 mM KH2PO4, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride and 5 mM dithiothreitol and then lysed by French press. The lysis solution was clarified by centrifugation (45,000g) and loaded onto a nickel-nitritolactic acid agarose (Ni2+-NTA) affinity column (Merck) following the manufacturer’s instructions. The bound proteins were eluted with phosphate-buffered saline containing 70 mM imidazole and detected by 10% SDS-PAGE. Nonspecific proteins purified from pET30a-transformed bacteria were used as a negative control.

C16-ACP, 7,7,8,8-d4-C16-ACP, C14-ACP, C18:0-ACP, C16:1-ACP, [1-14C]C14:0-ACP, [1-14C]C16:0-ACP, [3-14C]C16:1-ACP, [1-14C]C18:0-ACP, and [1-14C]18:1-ACP were synthesized enzymatically (Rock and Garvin, 1999) using recombinant spinach (Spinacia oleracea)ACP-I (Broadwater and Fox, 1999). For the substrate preference analysis using unlabeled fatty acyl-ACP, the reductase activity of MS2 was analyzed as described by Metz et al. (2000). The reaction mixture of 200 μL for the MS2 assay contained 15 mM NADP(H), 25 mM HEPES-NaOH, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 10% (w/v) glycerol, and 0.3% (w/v) CHAPS plus the desired substrate. NADP(H) was replaced by NADH or water for the catalytic activity analysis of MS2. The reaction was initiated by the addition of the enzyme, and for negative control experiments, an equivalent volume of eluted buffer from the Ni2+-NTA column that had been incubated with protein from cells expressing the empty pET30a vector was added. Incubations were performed at 30°C for 60 min and terminated by the addition of an equal volume of chloroform:methanol (2:1). The organic phase was extracted with 1.2 volumes of hexane, and 2 μL of this extract was subjected to GC-MS analysis.

The Agilent GC apparatus was coupled with an Agilent 5975C inert XL MSD with triple axis detector. The detector and injector temperature were maintained at 300°C. The oven temperature of 120°C was increased to 240°C at a rate of 15°C min⁻¹ and held at 240°C for 2 min.

For substrate preference with [1-14C]labeled fatty acyl-ACPs, the 150-μL reaction was carried out as described above and incubated at 30°C for 30 min. For pH optimization, 50 μL MES/50 μL Bis-Tris propane buffer with pH range from 5.0 to 9.0 was used, and the reaction was incubated for 15 min. For temperature optimization, the optimum MES buffer (pH 6.0) was used and incubated at 10°C, 16°C, 25°C, 30°C, 35°C, and 42°C. For kinetics analysis of MS2 using [1-14C]16:0-ACP, the reaction was carried out at optimum pH (50 mM MES, pH 6.0) and temperature (30°C) for 10 min. The reactions were extracted twice with 2 volumes of hexane. The radioactivity of the hexane fraction was measured by liquid scintillation counting. As described above, extracts from bacteria expressing pET30a were used as the negative control.

**Analysis of Wax, Internal Lipids, and Cutin in Tobacco Leaves**

Tobacco leaf discs used for the analysis of wax, internal lipids, and cutin were prepared as described (Cameron et al., 2006) with modifications. Leaf discs (1.5 cm diameter) were excised using a cork borer from the leaves 3 days after argoinfiltration of pET30a-MS2, frozen immediately in liquid N2, freeze dried, and then stored in silica gel at room temperature. Each sample contained six leaf discs derived from three individual plants.

Cuticular waxes were extracted from the leaf discs described above by immersing tissues for 30 s in 1 mL of chloroform containing 10 μg of l-1 kanamycin to an optical density at 600 nm of 0.8 and induced using 1 mM isopropyl β-D-thiogalactoside.

After 48 h of induction at 20°C, the cell pellet was collected by centrifugation at 4°C. The fusion protein purification and antibody preparation were performed as described by Huang et al. (2003). The antiserum was collected after four injections. The antibody was purified according to the method described by Ritter (1991).

Total soluble proteins were extracted from the wild-type buds and pEAQ-MS2-transformed tobacco leaves using the protocol described by Huang et al. (2003) and separated by 10% SDS-PAGE. After electrophoresis, the proteins were electronically transferred onto nitrocellulose membranes and incubated with the purified antibodies (1:100 dilution) as described by Huang et al. (2003).
tetracosane (Fluka) as an internal standard. The extracts were transferred to reaction vials and a gentle stream of nitrogen gas. The dried wax residues were derivatized by adding 20 μL of N,N-bis-trimethylsilyl trifluoroacetamide (Macherey-Nagel) and 20 μL of pyridine and incubated for 40 min at 70°C. These derivatized samples were then analyzed by GC-FID (Agilent Technologies) and GC-MS (Agilent gas chromatograph coupled to an Agilent 5973N quadrupole mass selective detector).

For total internal lipid extraction, leaf discs that had been used in the wax extraction were reextracted with 1 mL of chloroform:methanol (1:1, v/v). This extraction was repeated three times to ensure that no internal lipids were left in the samples. The combined lipid extracts were then dried under a gentle stream of nitrogen gas to about 100 μL. The remaining delipidated leaf discs were dried over silica gels and used to analyze the monomer composition of cutin polyester as described by Franke et al. (2005). All internal lipid samples and cutin samples were trans-esterified in 1 mL of 1 M methanolic HCl for 2 h at 80°C. After the addition of 2 mL of saturated NaCl and 20 μg of dotriacontane (Fluka) as an internal standard, the hydrophobic monomers were subsequently extracted three times with 1 mL of hexane. The organic phases were combined and dried under a stream of nitrogen gas, and the remaining samples were derivatized as described above. GC-MS and GC-FID analyses were performed as the warm analysis. Results of leaf wax, internal lipids, and cutin analyses were expressed relative to unit surface area of tobacco leaves.

Supplemental Data

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

Supplemental Figure S1. Subcellular localization of MS2 in tobacco leaves.

Supplemental Figure S2. 7,7,8,8-d4-Palmitoyl-ACP specificity of recombinant MS2 and the mass spectra of fatty alcohols.

Supplemental Figure S3. Specificity detection of MS2 antibody.

Supplemental Figure S4. Western-blot analysis of MS2 expressed in tobacco leaves.

Supplemental Table S1. Substrate preference of MS2.

Supplemental Table S2. Detailed wax compositions in transient tobacco leaves.

Supplemental Table S3. Detailed internal lipid compositions in transient tobacco leaves.

Supplemental Table S4. Detailed cutin compositions in transient tobacco leaves.

Supplemental Table S5. List of the primers used for vector construction.

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LITERATURE CITED


M52 Encodes a Fatty Acyl-ACP Reductase


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