Running head: CHS-like proteins in pollen exine development

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LAP5 and LAP6 encode anther-specific proteins with similarity to chalcone synthase essential for pollen exine development in Arabidopsis thaliana

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ABSTRACT

Pollen grains of land plants have evolved remarkably strong outer walls referred to as exine that protect pollen and interact with female stigma cells. Exine is composed of sporopollenin, and while the composition and synthesis of this biopolymer are not well understood, both fatty acids and phenolics are likely components. Here we describe mutations in the Arabidopsis thaliana LAP5 and LAP6 that affect exine development. Mutation of either gene results in abnormal exine patterning, whereas pollen of double mutants lacked exine deposition and subsequently collapsed, causing male sterility. LAP5 and LAP6 encode anther-specific proteins with homology to chalcone synthase (CHS), a key flavonoid biosynthesis enzyme. lap5 and lap6 mutations reduced the accumulation of flavonoid precursors and flavonoids in developing anthers, suggesting a role in the synthesis of phenolic constituents of sporopollenin. Our in vitro functional analysis of LAP5 and LAP6 using 4-coumaroyl-CoA yielded bis-noryangonin (a commonly reported derailment product of CHS), while similar in vitro analyses using fatty acyl-CoA as the substrate yielded medium-chain alkyl pyrones. Thus, in vitro assays indicate that LAP5 and LAP6 are multi-functional enzymes and may play a role in both the synthesis of pollen fatty acids and phenolics found in exine. Finally, the genetic interaction between LAP5 and an anther gene involved in fatty acid hydroxylation (CYP703A2) demonstrated that they act synergistically in exine production.
Pollen grains of land plants are surrounded by complex cell walls that are divided into three layers: (i) an outer exine, itself a multilayered structure, primarily made of sporopollenin, (ii) an inner intine, made primarily of cellulose, and (iii) a lipid- and protein-rich pollen coat in the crevices of exine. The exine is morphologically diverse, provides protection against environmental stresses and bacterial and fungal attacks, and plays a role in species-specific adhesion (Zinkl et al., 1999; Edlund et al., 2004).

Several studies indicate that sporopollenin is a complex polymer composed of fatty acids and phenolic compounds (Guilford et al., 1988; Ahlers et al., 1999; Wiermann et al., 2001). Sporopollenin is remarkably strong and chemically resistant, making it difficult to determine its precise composition by direct chemical analysis. Ozonolysis has yielded simple straight- and branched-chain monocarboxylic acids, typical of fatty acid breakdown (Shaw, 1971), as well as phenolic acids, such as \( p \)-hydroxybenzoic, \( m \)-hydroxybenzoic, and protocatechuic acids. Additional evidence for phenolic compounds came from degradation experiments or studies showing incorporation of radiolabelled phenylalanine and \( p \)-coumaric acid into sporopollenin (Shulze Osthoff and Wiermann, 1987; Rittscher and Wiermann, 1988; Gubatz et al., 1993), while immunolocalization studies with anti-\( p \)-coumaric acid antibodies demonstrated the occurrence of phenols in exines of different plant species (Niester-Nyveld et al., 1997).

While a growing number of genes have been identified that are important for exine development, still relatively little is known about the genetic network that governs the formation of this structure and the pathways that lead to its biosynthesis are far from being understood. In recent years, the importance of fatty acid-derived components in sporopollenin composition has been revealed through the identification of several Arabidopsis genes (\( MS2 \) (Aarts et al., 1997), \( CYP703A2 \) (Morant et al., 2007), \( CYP704B1 \) (Dobritsa et al., 2009) and \( ACOS5 \) (de Azevedo Souza et al., 2009)), which are important for exine production and involved in fatty acid metabolism. Less is known concerning the role of phenolics in sporopollenin biosynthesis, and the key synthetic and regulatory genes specifically associated with this aspect of sporopollenin biosynthesis are absent from the literature.

Phenolic compounds are a large class of secondary metabolites that play a variety of biological roles (Hahlbrock and Scheel, 1989). Most plant phenolics are products of phenylpropanoid metabolism, including lignins, coumarins, stilbenes, and flavonoids. A well-characterized biosynthetic pathway leads to the biosynthesis of flavonoids (Supplemental Fig.
S1). Chalcone synthase (CHS) catalyses the first committed step in this pathway using 4-coumaroyl-CoA provided by 4-coumaroyl:CoA ligase (4CL) as a substrate. Flavonoids are important for pollen germination and plant fertility in several plant species (Coe et al., 1981; van der Meer et al., 1992; Fischer et al., 1997; Napoli et al., 1999), while a null mutation in the Arabidopsis CHS gene, TT4, results in plants with normal fertility and an absence of flavonoids in the mature stamens (Burbulis et al., 1996; Ylstra et al., 1996). This suggests that flavonoids are either not required for Arabidopsis male fertility or that TT4-independent flavonoid synthesis occurs in anthers, perhaps transiently and at an earlier developmental stage, through a mechanism that has not been detected in previous experiments.

Recently, an anther-specific gene ACOS5 was described that is essential for exine production and sporopollenin biosynthesis (de Azevedo Souza et al., 2009). ACOS5 is related to a phenylpropanoid enzyme 4-coumaroyl:CoA ligase (4CL), but encodes a novel medium- to long-chain fatty acyl-CoA synthetase. In this study we describe the identification and characterization of two highly conserved anther-specific genes that are involved in pollen exine development, likely participate in sporopollenin biosynthesis, and similar to ACOS5, are related to, yet distinct from, an enzyme of the phenylpropanoid pathway. Our results provide further insight into the mechanism that leads to the formation of sporopollenin.

**RESULTS**

*lap5* and *lap6* Mutants Have Defective Exine Patterning

We recovered less adhesive pollen mutants *lap5* and *lap6* in a screen for genes that play a role in Arabidopsis pollen-stigma adhesion (Nishikawa et al., 2005). Both mutants had morphological defects in exine structure that were apparent under light or scanning electron microscopy (SEM) (Fig. 1). The mutant anthers and pollen appeared glossy under a dissection stereomicroscope (Fig. 1, A-C). The pollen adhered tightly to the anthers and was not easily released. Under SEM, *lap5* and *lap6* pollen grains exhibited abnormal exine patterning (Fig. 1, D-I): *lap5* pollen lacked the characteristic reticulate structure (Fig. 1H), and *lap6* pollen grains had a more extensively covered surface with broader muri and smaller lacunae (Fig. 1I).
grains of both mutants collapsed more easily under vacuum than the wild type, although the thickness of the exine layer was unaltered (data not shown).

Defects in some Arabidopsis exine mutants (ms2 (Aarts et al., 1997), flp1 (Ariizumi et al., 2003), and cyp704b1 (Dobritsa et al., 2009)) render pollen sensitive to acetolysis, an oxidative process often used by palynologists to prepare exine samples (Erdtman, 1960). We incubated lap5 and lap6 pollen in a mixture of acetic anhydrate and sulfuric acid but did not observe sensitivity. Interestingly, lap6, but not lap5 exine consistently showed reduced staining, suggesting that the two mutants are different in the quality or amounts of the exine constituents that react with the acetolysis mixture (Fig. 1, J-L).

Mapping LAP5 and LAP6

Both lap5 and lap6 mutations segregated as single-locus, homozygous recessive mutations with phenotypic ratios (wild-type:mutant) of 1008:326 (lap5) and 740:219 (lap6), consistent with 3:1 segregation (chi squared, $P>0.5$ and $0.5>P>0.1$, respectively). Using bulked segregant analysis (Michelmore et al., 1991), we mapped LAP5 to chromosome 4 and LAP6 to chromosome 1 (see Materials and Methods). Additional PCR-based markers were used to refine the locations to within 59 kb for LAP5 and 140 kb for LAP6. One gene in each interval, At4g34850 and At1g02050, respectively, contained mutations within open reading frames that co-segregated with the lap5 and lap6 mutant phenotypes. Both candidate genes encoded predicted chalcone synthase (CHS) family proteins. The mutations in the genes resulted in missense codons, causing a G227E conversion in lap5, and an I132T conversion in lap6. In addition to the lap6-1 allele that we isolated, Arabidopsis stock centers contained two lines with T-DNA or transposon insertions in At1g02050 (SALK_134643, lap6-2 and N172991, lap6-3) (Fig. 2). We confirmed the positions of these insertions in the first exon (lap6-2) and in the second exon close to the end of the ORF (lap6-3). Both insertion alleles had pollen and anther phenotypes identical to those of lap6-1 (data not shown).

To confirm the identity of the LAP5 and LAP6 genes, complementation constructs were created containing the entire At4g34850 or At1g02050 genes, including the native promoters, and transformed, respectively, into lap5-1 and lap6-1. Wild-type anther and pollen phenotypes were restored in 38/40 BASTA-resistant T1 plants for lap5. Of the remaining 2 plants, one had a
lap5 phenotype, and the other lacked pollen and was sterile. For lap6, 35/40 BASTA-resistant T1 plants had restored the wild-type phenotype. The remaining 5 plants had the lap6 phenotypes. We confirmed the presence of the lap5-1 and lap6-1 mutations in the rescued lines using CAPS markers. These results indicate that wild-type copies of At4g34850 and At1g02050 can complement the lap5-1 and lap6-1 mutants, respectively, and that the constructs used contained sufficient cis-regulatory elements to drive expression in a spatially and temporally relevant manner.

LAP5 and LAP6 Encode Chalcone Synthase Superfamily Proteins

The predicted LAP5 and LAP6 proteins are similar to enzymes in the chalcone synthase superfamily (Fig. 3). Members of this superfamily are plant-specific type III polyketide synthases (PKS) and catalyze condensing reactions that generate the backbone required for the synthesis of a large number of phenylpropanoid compounds, such as flavonoids and stilbenes (Schröder, 1997; Austin and Noel, 2003). BLAST results revealed that, in addition to these two genes and a well-characterized chalcone synthase TT4 (Shirley et al., 1995), Arabidopsis contains a third CHS-related gene, At4g00040.

CHSs (EC 2.3.1.74) from different plants often share 80-90% sequence identity (Niesbach-Klösgen et al., 1987). In contrast, LAP5, LAP6 and At4g00040 share only 40% sequence identity with the Arabidopsis CHS TT4 or with chalcone synthases from other organisms. However, they share 64% (LAP5-LAP6), 76% (At4g00040-LAP5) and 62% (At4g00040-LAP6) identity with each other and are members of a male-organ-specific CHS-like protein clade, which includes proteins from dicots, monocots, and gymnosperms (BA42 from B. napus (Shen and Hsu, 1992), YY2 from O. sativa (Hihara et al., 1996), SICHS from S. latifolia (Ageez et al., 2005), CHSLK from N. sylvestris (Atanassov et al., 1998) (Fig. 3). The evolutionarily distant gymnosperm Pinus radiata also has a CHS-like protein PrChS1 expressed in male cones (Walden et al., 1999) with 68%, 68%, and 64% sequence identity with LAP5, LAP6, and At4g00040, respectively. An ortholog of these male-organ-specific proteins exists in the genome of the moss Physcomitrella patens (NCBI XP_001781520), in addition to orthologs of a canonical CHS.
LAP5, LAP6 and At4g00040 have a predicted gene structure similar to that of CHS, stilbene synthase (STS) and related type III PKS genes, including a conserved cysteine codon (Cys60 in the numbering scheme of the well-characterized enzyme CHS2 from alfalfa *Medicago sativa*) that is split by a short intron between the first and the second nucleotides (Fig 2 and 3). In addition to this intron, LAP5, unlike the majority of this family’s members, has a second intron. Four absolutely conserved residues (Cys164, Phe215, His303, Asn336) that define the catalytic machinery in the active centers of CHS-related enzymes (Lanz et al., 1991; Ferrer et al., 1999; Jez et al., 2000b; Austin et al., 2004) are present in LAP5, LAP6, At4g00040, as well as in male-specific CHS-like proteins from other species (Fig. 3). However, known male-specific CHS-like proteins, as well as LAP5, LAP6, and At4g00040, have a large number of amino acid changes specific to this clade (Fig. 3A).

The lap5-1 and lap6-1 point mutations affect highly conserved amino acids: Ile132 in LAP6, replaced with threonine in lap6-1, is present in all 42 CHS, STS, and CHS-like proteins examined (Fig. 3A and data not shown). Gly227 in LAP5, replaced with glutamic acid in lap5-1, is conserved in all LAP5/LAP6-like proteins (Fig. 3A).

**LAP5 and LAP6 Have Anther-specific Expression Patterns**

We searched publicly available expression databases, including Genevestigator (Zimmermann et al., 2004) (https://www.genevestigator.ethz.ch/) and MPSS (Meyers et al., 2004) (http://mpss.udel.edu/at/) to identify LAP5 and LAP6 tissue expression patterns. According to microarray and MPSS data, LAP5 and LAP6 were specifically expressed in young anthers and in flower buds (stages 9 and 10), and transcripts were absent in flower buds from stamen-lacking *ap3* and *ag* mutants. In addition, a search of ATTED-II and BAR Expression Angler databases that analyze microarray expression data and predicted cis-regulatory elements to build networks of genes that are likely to be co-expressed (Obayashi et al., 2007; Toufighi et al., 2005) (http://www.atted.bio.titech.ac.jp/), (http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools_expression_angler.cgi) suggested that LAP5 and LAP6 were expressed together and highly co-expressed with several other genes involved in exine production including ACOS5 (de Azevedo Souza et al., 2009), MS2 (Aarts et al., 1997), CYP703A2 (Morant et al., 2007), CYP704B1 (Dobritsa et al., 2009), and DRL1 (Tang et al., 2008). To confirm LAP5 and LAP6
expression patterns, we transformed wild-type plants with β-glucuronidase (GUS) under the control of the LAP5 and LAP6 promoters. In the resulting T1 plants, GUS staining was observed only in anthers of stage 9 and 10 buds (Fig. 4). Taken together, these data indicate that LAP5 and LAP6 are male-organ-specific members of the chalcone synthase family and are expressed in anthers coincident with the timing of exine formation.

lap5 lap6 Double Mutants Have Enhanced Exine Defects

A lap5-1 lap6-1 double mutant was created to explore the relationships between the LAP5 and LAP6 genes in pollen development. While both single mutations resulted in fertile pollen with abnormally patterned exine, the double mutant was sterile, produced brown shriveled anthers with almost no pollen and failed to produce elongated seed pods or siliques (Fig. 5). Sections of developing lap5-1 lap6-1 anthers showed normal pollen development until stage 9 (Fig. 6), but lacked the exine typically found in wild type at stage 9 (green staining around the pollen grains, Fig. 6C) (Sanders et al., 1999). Pollen in the double mutant subsequently collapses, with almost no pollen remaining by the stage 11 (Fig. 6H).

Neither At4g00040 nor TT4 can Substitute for LAP5 or LAP6

Given the high similarity of the predicted At4g00040 protein to LAP5, LAP6, and male-specific CHS-like enzymes from several organisms, we tested whether this gene also plays a role in pollen development. Three lines with DNA insertions in At4g00040, including one with an insertion in the middle of the predicted ORF (Fig. 2), were available from Arabidopsis stock centers. We confirmed the insertion positions in each line, and found that each contained anthers and pollen indistinguishable from wild type.

Because gene expression databases indicated that At4g00040 was not male-specific, but instead was expressed in various organs and stages of development, we next tested whether driving expression of this gene in stage 9-10 anthers would rescue lap5 or lap6 defects. We placed a wild-type copy of At4g00040 under the control of a LAP5 or LAP6 promoter and introduced these constructs into lap5-1 and lap6-1 mutants. 10 BASTA-resistant T1 plants were analyzed for each of the four resulting combinations, but none had reversed the mutant
phenotype. Thus, despite its high similarity to male-specific CHS-like proteins, At4g00040 apparently is unable to substitute for LAP5 or LAP6 in pollen development.

In addition, we tested if expression of Arabidopsis CHS TT4 in stage 9-10 anthers would rescue lap5 lap6 defects by expressing it under LAP5 promoter and introducing into the lap5-1/lap6-1 plants. None of the 36 BASTA-resistant T1 plants analyzed (11 lap5-1/lap5-1 lap6-1/lap6-1; 18 lap5-1/lap6-1/lap6-1; 7 lap6-1/lap6-1) had mutant phenotypes reversed. Thus, canonical CHS is also unable to substitute for LAP5 and LAP6 in pollen development.

**lap5-1 and lap6-1 Affect Production of Anther Chalcone, Naringenin, Other Flavonoids, and Additional Metabolites**

Because LAP5 and LAP6 resemble CHS proteins, we hypothesized they might catalyze the production of phenolic components needed to form sporopollenin. We collected metabolic profiles from stage 9/10 anther extracts from wild-type, lap5-1, lap6-1, and the lap5-1 lap6-1 double mutant (see Materials and Methods). Using ultra high performance liquid chromatography and gas chromatography coupled to mass spectrometry (UPLC-MS and GC-MS), a broad spectrum of primary and secondary metabolites were separated, detected and characterized. 83 metabolites were characterized with UPLC-TofMS (Supplemental Tables SI-IV), and 45 of these were annotated using available authentic standards or comparison to previously published data. The annotated metabolites predominantly belonged to three chemical groups: phenolics (mainly flavonoids), glucosinolates and long-chain fatty acids.

The levels of the phenolics, chalcone and naringenin, formed in the first committed step of the flavonoid pathway, were significantly reduced in the lap mutants (11.8-fold, lap5 and 5.6-fold, lap6) and were undetectable in the double mutant (Fig. 7; Supplemental Table SI). In addition, several other flavonoids were reduced in both mutants, including dihydrokaempferol andisorhamnetin 3-sophoroside. Kaempferol-3-O-gentiobioside-7-O-rhamnoside was not detected in lap5, and kaempferol 3-glucoside-7-p-coumarylglucoside was not detected in lap6. Levels of kaempferol-3-galactoside-7-rhamnoside, luteolin 7-O-rhamnoside and 6-methoxytaxifolin were significantly reduced in lap6 and 6-methoxytaxifolin not detected in the double mutant. Robinin was reduced in lap5 and almost absent in the double mutant samples.
Surprisingly, increases (almost 6-fold) of quercetin 3-glucoside-7-rhamnoside were observed in the lap6 mutant and another flavone, apigenin-6-C-glucoside, was detected only in lap6.

In addition to changes in the levels of phenolics, we also detected changes in the levels of other compounds in mutant anther extracts. For instance, several glucosinolate compounds were observed only in the mutant plants, as were some fatty acids, such as α-eleosteric acid and 10,16-dihydroxypalmitic acid (Supplemental Tables SII-III). Given the possible role of 16-hydroxypalmitic acid in the biosynthesis of sporopollenin (Dobritsa et al., 2009), accumulation of this fatty acid in the lap6 and lap5 lap6 mutants could be a consequence of the overall changes in sporopollenin composition in the mutant anthers, which could result in changes in fatty acid polymerization. Significant changes in several unidentified compounds were also found in the mutant extracts (Supplemental Table SIV).

The relative abundances of 96 molecules of known chemical structures, including amino acids, Krebs cycle intermediates, carbohydrates, fatty acids derivatives and several other primary metabolites were determined by GC/MS (Supplemental Table SV). Several primary metabolites were altered in the mutant lines, the most noticeable being a general increase in the levels of amino acids and their catabolic products. Levels of L-asparagine, L-valine and 4-aminobutiric acid were increased in all mutant lines, with the most pronounced changes observed in the double mutant samples. Additionally, levels of pipecolic acid, a degradation product of L-lysine were increased 13.6-fold in lap5 mutant and 6.1-fold in the double mutant. Several changes in the levels of carbohydrates were observed. The most evident change was a dramatic reduction of maltose levels in all three mutant samples. Because maltose is a starch hydrolysis product, this suggests a reduction in starch pools.

**Enzymatic Activity of LAP5 and LAP6**

The metabolic profiling data of the lap5 and lap6 mutants revealed decreased flavonoid levels, suggesting that LAP5 and LAP6 may play a role in anther flavonoid biosynthesis. Heterologous gene expression and *in vitro* enzymatic assays were performed to investigate the function of LAP5 and LAP6 proteins. Enzymatic reactions were performed in the presence of 4-coumaroyl-CoA and malonyl-CoA, CHS substrates. The reaction products were analyzed by HPLC-UV/Vis-ITMS (Fig. 8). The empty-vector extract and the Arabidopsis CHS TT4 were
used as the negative and the positive controls, respectively (Fig. 8, A and B). The product of TT4/CHS (Fig. 8B) was identified as naringenin by comparing its retention time, UV and mass spectrum with those obtained with the authentic naringenin standard (Figure 8C). Naringenin, the expected CHS product, was not present in the LAP5 or LAP6 assays. However, two other products, peaks 1 and 2, were found (Fig. 8, D and E), which were absent in both the positive and negative controls. The mass spectra of the two peaks observed in the LAP5 and LAP6 product profiles were almost identical and dominated by three major ions $m/z = 229, 185, 143$ with slightly different abundance ratios (Supplemental Fig. S2). These two peaks were unambiguously identified as trans- and cis-bisnoryangonin (i.e. 4-coumaroylacetic acid lactone) based upon the observed ions at $m/z$ 229 [M-H$^-$], 185 [M-H-CO$_2$]$^-$ and 143 [retro Diels-Alder reaction: M-H-C$_3$H$_2$O$_3$]$^-$ as previously described (Jindaprasert et al., 2008) and characteristic UV absorption maxima at 365 nm (Shiokawa et al., 2000). The MS assignments were further confirmed by MS/MS of the molecular ion $m/z$ 229 which gave two fragment ions: $m/z$ 185 and 143 (Akiyama et al., 1999). The in vitro activities of LAP5 and LAP6 were also investigated at different pH levels (pH 5.0, 8.0, and 9.0). Bisnoryangonin was found to be the only product in both LAP5 and LAP6 assays at all pH levels. We also tested whether combining both LAP5 and LAP6 would result in a different activity because chalcone and stilbene synthases are known to function as homodimers (Ferrer et al., 1999; Shomura et al., 2005) and are also capable of forming in vitro heterodimers with partial catalytic activities (Tropf et al., 1995). However, bisnoryangonin was again the only detected product.

In vitro formation of trans- and cis-bisnoryangonin has been observed with other recombinant CHS (Akiyama et al., 1999; Shiokawa et al., 2000). It is formed through the condensation of one molecule of 4-coumaroyl-CoA and two molecules of malonyl-CoA (Supplemental Fig. S3). Like coumaroyltriacetic acid lactone (CTAL, a condensation product of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA), bisnoryangonin has been reported as an enzymatic ‘derailment’ product (i.e., an incomplete biosynthetic product that detaches from the enzyme prior to completion of all the enzymatic steps) of CHS or CHS-like proteins (Kreuzaler and Hahlbrock, 1975; Shiokawa et al., 2000). The formation of the derailment products probably reflects the differences between the in vitro and the in vivo environments, and was attributed to the lack of subsequent reactions in the in vitro environments (Eckermann et al., 2003). Targeted analyses of the UPLC-QTOF-MS data did not show the
presence of bisnoryangonin or its glycosides in the wild-type plants, indicating that bisnoryangonin is unlikely the in vivo physiological product of LAP5 and LAP6.

LAP5 and LAP6 belong to the type III polyketide synthase (PKS) superfamily, which produces a wide variety of secondary metabolites using different starter CoA esters, different elongation cycles, and different cyclization mechanisms (Austin and Noel, 2003; Flores-Sanchez and Verpoorte, 2009). Acetyl-CoA is one of the most common starters in polyketide synthesis and leads to the formation of both aromatic and pyrone polyketides. To determine whether LAP5 and LAP6 proteins have PKS activity in the presence of acetyl-CoA, 4-coumaroyl-CoA was replaced with acetyl-CoA in a separate experiment. However, no product was observed, indicating that acetyl-CoA is unlikely a physiological substrate.

While these experiments were in progress, another group reported on the biochemical characterization of the LAP5 and LAP6 proteins (Mizuuchi et al., 2008). The proteins were shown in vitro to catalyze condensation reactions using medium- and long-chain fatty acyl-CoA and malonyl-CoA as starter molecules, resulting in the formation of triketide and tetraketide α-pyrone lipids. We repeated these experiments using a variety of acyl-CoA (chain length C4-C18) as substrates. The empty-vector extract was used as a negative control. Similar to Mizuuchi et al. (2008), we found that LAP5 and LAP6 are also capable of accepting fatty acyl-CoA as starters and performing condensation reactions with malonyl-CoA to form triketide (major product) and tetraketide (minor product) α-pyrone lipids (Fig. 9 and Supplemental Figs. S4-S6; data for one protein are shown; the other demonstrated identical activity). However, unlike Mizuuchi et al. (2008), who observed conversion of C4-C20 fatty acyl-CoA, only C6-C12 were converted into the corresponding tri- or tetraketide pyrones under our experimental conditions. In addition to LAP5 and LAP6, TT4/CHS was also assayed with fatty acyl-CoA as a substrate. We found that it also possessed an in vitro activity comparable to that of LAP5 and LAP6 towards fatty acyl-CoAs, accepting C6-C10 substrates and catalyzing condensation reactions to form tri- and tetraketide pyrones (Fig. 9 and Supplemental Figs. S5-S6). In reaction with hexanoyl-CoA catalyzed by TT4/CHS, a minor product was detected, which was identified as tetraketide resorcylic acid (Supplemental Fig. S5) based on its mass spectrum and its retention time relative to triketide pyrone (Zhou et al., 2008). In contrast, tetraketides found in octanoyl-CoA (Fig. 9) and decanoyl-CoA (Supplemental Fig. S6) experiments were tetraketide pyrones rather than
tetraketide resorcylic acid, suggesting that tetraketides can undergo different cyclization in solutions (Supplemental Fig. S4).

To determine if LAP5/LAP6 serve as multifunctional proteins that could generate cross-linked phenolics and fatty acids in exine, we have performed a multi-substrate assay for LAP5 and TT4/CHS using hexanoyl-CoA, 4-coumaroyl-CoA and malonyl-CoA at a 1:1:3 molar ratio as substrates. 4-hydroxy-6-pentyl-2-pyrone and bisnoryangonin were obtained with LAP5 (at a molar ratio of 32:1), and 4-hydroxy-6-pentyl-2-pyrone and naringenin were obtained with TT4/CHS (at a molar ratio 11:1) (Supplemental Fig. S7). No novel products different from the compounds obtained in separate experiments were detected, with the exception of bisnoryangonin found in the reaction catalyzed by TT4/CHS.

Mutations Affecting LAP5 and a Gene from the Fatty Acid Hydroxylation Pathway Have Synergistic Effects

A recently described gene CYP703A2 is necessary for normal exine development, and encodes a protein that hydroxylates lauric acid, potentially providing fatty acid building blocks required for sporopollenin biosynthesis (Morant et al., 2007). We created a double mutant between lap5-1 and a hypomorphic lap4-1 allele of CYP703A2 (Dobritsa et al., 2009). The lap4-1 mutant causes plants to produce functional pollen that, like lap5 and lap6, appears glossy and strongly adheres to anthers. When this pollen was stained with the exine-specific fluorescent dye auramine O and examined under a laser scanning confocal microscope (LSCM), we observed a strong reduction in the thickness of exine, which nonetheless exhibited reticulate pattern (Fig. 10, C and G). Patches of exine were often missing on the surface of the lap4-1 pollen (Fig. 10C). The lap4-1 lap5-1 double mutant was fertile, but produced glossy pollen that did not easily separate from the anthers. When we examined the lap4-1 lap5-1 pollen grains under LSCM, we found that they exhibited a more severe defect in their surface structure than either lap5-1 or lap4-1 single mutants. The pollen surface showed no reticulate pattern and almost no auramine O fluorescence (Fig. 10, D and H). When optical sections through the middle of the grains were obtained to determine exine thickness, we found that in the lap4-1 lap5-1 double mutant exine was virtually missing (Fig. 10H). Interestingly, in many of those pollen grains areas of somewhat increased fluorescence were located longitudinally (Fig. 10D).
DISCUSSION

Defects in LAP5 and LAP6 Proteins Have Broad Metabolic Consequences

This study underscores that a disruption of a single gene can have wide-ranging consequences on the metabolic composition, revealing the intricacy of interactions among metabolic pathways. Collected metabolomics data indicate that alterations in the expression of LAP5 and LAP6 genes result in the differential accumulation of several flavonoids and of two lipids (10,16-dihydropalmitic acid and α-eleostearic acid), and profoundly affect the biosynthesis of a variety of metabolites outside the fatty acid or phenylpropanoid pathway. It has been previously suggested (Rohde et al., 2004) that primary and secondary metabolism are interlocked through the phenylalanine pool. Thus, it is possible that a reduced carbon flux into the phenylpropanoid pathway affects the homeostasis of amino acid and carbohydrate metabolism. Possibly, part of the surplus carbon resulting from the reduced phenolic flux in the lap5 and lap6 mutants is redirected towards increased amino acids. Under normal conditions, 20% of all fixed carbon is directed into the shikimate pathway that generates aromatic amino acids (Herrmann, 1995). One of the major sinks of this pathway is the conversion of phenylalanine to cinnamic acid via phenylalanine lyase (PAL), which is the first enzyme in the phenylpropanoid pathway. Interestingly, the reduction in LAP5 and LAP6 activity has dramatic consequences for the accumulation of several amino acids, not only the aromatic ones. Similarly, increases in the abundance of multiple amino acids were previously observed in mutants with defective PAL activity (Rohde et al., 2004). Also, depletion of tyrosine and tryptophan pools via over-expression of Trp and Tyr decarboxylases alters levels of both aromatic and non-aromatic amino acids (Guillet et al., 2000). These results led to a proposed mechanism that controls amino acids homeostasis across individual biosynthetic pathways (Guillet et al., 2000; Rohde et al., 2004).

The accumulation of amino acids in lap5 and lap6 mutants may have additional effects. Beta-alanine and the L-valine biosynthetic intermediate, 2-oxoisovalerate, are used in the formation of pantothenic acid (White et al., 2001), which is subsequently converted to coenzyme A (Kupke et al., 2003). We interpret the accumulation of branched-chain amino acids (such as valine and leucine), putrescine, GABA and beta-alanine in the lap5 and lap6 anthers as a result
of altered CoA biosynthesis. A similar metabolic model was previously suggested by (Broeckling et al., 2005) to describe experimentally observed changes of several metabolites. CoA serves as a carrier for organic acids, including acetic acid (utilized in fatty acid biosynthesis, glycolysis, Krebs cycle, amino acids biosynthesis), 4-coumaric acid and malonic acid that are used in flavonoid biosynthesis. Thus, levels of CoA might, in turn, provide feedback control for the production of flavonoids and fatty acids.

Amino acids also provide a link between phenylpropanoids and glucosinolates, another class of secondary metabolites that was increased in lap5 and lap6 mutants. Glucosinolates are derived from various amino acids (Halkier and Gershenzon, 2006), and their close metabolic connection (Knill et al., 2008) might account for the observed increases in glucosinolate levels in our mutants.

**LAP5 and LAP6 Are Paralogs Required for Exine Biosynthesis**

Here, we have shown that LAP5 and LAP6 are essential for the production of pollen exine. Both genes are specifically expressed during the period of exine synthesis in developing anthers and belong to a male-specific clade of the chalcone synthase superfamily. Point mutations lap5-1 and lap6-1 result in the formation of fertile pollen with aberrant exine patterning; available insertions (likely null alleles) in lap6 show an identical phenotype. Double mutants (lap5-1 lap6-1) in both genes abolish exine deposition, followed by pollen collapse and male sterility. Given the sequence similarity of these genes and the results of in vitro enzymatic assays, a possible interpretation of the double mutant phenotype is that the genes are functionally redundant, with the single mutants reducing the levels of the same compound(s). On the other hand, since the lap5 and lap6 mutants have different exine morphologies, responses to acetolysis and metabolite profiles, it is conceivable that their functions are somewhat distinct.

In addition to LAP5 and LAP6, Arabidopsis has a third paralog, At4g00040, which, despite sequence similarity with known male-specific CHS family proteins, does not exhibit anther-specific expression nor appear to participate in pollen development. Several other plant species also have multiple orthologs of LAP5 and LAP6. Poplar *Populus trichocarpa* has 3 orthologous proteins ([http://genome.jgi-psf.org/Poptr1/Poptr1.home.html](http://genome.jgi-psf.org/Poptr1/Poptr1.home.html)), and at least two orthologs are present in the *Brassica rapa*, tomato, maize, and rice genomes. Classical chalcone
and stilbene synthases are known to function as homodimers (Ferrer et al., 1999; Shomura et al., 2005). However, an in vitro study with mutated proteins that were inactive as homodimers demonstrated that CHS and STS are capable of forming heterodimers with partial catalytic activities (Tropf et al., 1995). It would be interesting to determine if LAP5 and LAP6 can function as heterodimers, thus potentially expanding the repertoire of reaction substrates and products. Although our experiments in combining LAP5 and LAP6 in CHS reaction have not resulted in the change of activity in a heterodimer, it is probable that the substrates used were not physiological.

**LAP5 and LAP6: novel type III polyketide synthases**

Canonical chalcone synthases, such as Arabidopsis TT4 or alfalfa CHS2, are the most ubiquitous and best characterized members of the plant type III PKS family, which is often referred to as the CHS family. They condense a CoA-ester from the phenylpropanoid pathway (usually, 4-coumaroyl-CoA) with three molecules of malonyl-CoA to form a tetraketide intermediate and a new aromatic ring of chalcone. Stilbene synthases, which are found in a relatively small number of plants, share approximately 70% sequence identity with CHS and use the same substrates (4-coumaroyl-CoA and malonyl-CoA) to perform three condensation reactions. The final steps of STS tetraketide ring closure are mechanistically different than those of CHS, enabling the formation of stilbenes, such as resveratrol. Other members of the type III PKS superfamily, such as acridone synthase, benzophenone synthase, valerophenone synthase, also perform three condensation reactions, but use different substrates, while still other type III PKS enzymes utilize a different number of condensation reactions (Austin and Noel, 2003; Karppinen et al., 2008).

Sequence comparisons among different members of CHS superfamily provide few clues to their enzymatic functions, and amino acid residues that cause differences between catalytic activities remain unknown. For instance, replacement of only 8 amino acids is sufficient to convert alfalfa CHS to STS (Austin et al., 2004). Yet, there is no apparent STS consensus sequence, as eight STS-derived mutations in pine that can convert CHS to STS are not conserved among STS enzymes from different species. Of the two CHS-like proteins from *Pinus strobus* that share 87.6% identity only one has CHS activity, whereas the other one is completely
inactive with any starter CoA-esters from the phenylpropanoid pathway or with linear CoA starters. Instead, it performs a single condensation reaction with a diketide derivative and methylmalonyl-CoA (Schröder et al., 1998). Out of the five CHS-like genes in hop *Humulus lupulus* only one encodes CHS activity, whereas the others prefer substrates unrelated to flavonoid metabolism (Novak et al., 2006). Additionally, a 2-pyrone synthase (2PS) from *Gerbera hybrida* shares 74% sequence identity with two CHSs from the same species, yet exhibits a very different activity: it uses acetyl-CoA as a substrate, performs only two condensation reactions, and folds the triketide intermediate into a pyrone ring structure (Eckermann et al., 1998). Replacement of only three amino acids in CHS is sufficient to convert it to 2PS (Jez et al., 2000a). To complicate matters further, members of the CHS family are promiscuous *in vitro*. CHS is capable of accepting non-physiological substrates such as benzoyl-CoA or aliphatic acyl-CoA (Schüz et al., 1983); use of isovaleryl-CoA as a substrate for CHS from pine *P. sylvestris* was sufficient to convert it to pyrone synthase that produces a pyrone derivative (Zuurbier et al., 1998), and *Gerbera hybrida* 2PS is able to produce pyrone using isovaleryl-CoA or benzoyl-CoA as substrates (Eckermann et al., 1998).

LAP5, LAP6, and other members of their clade in the CHS superfamily share approximately 40% sequence identity with CHS. At the amino acid level, they are much more dissimilar to the canonical CHS than 2PS and other members of this superfamily with demonstrated non-CHS activity. While there are limitations to primary structure analysis for members of this family, several notable sequence differences could be important for LAP5 and LAP6 enzymatic activity: 1) The conserved residue, Thr197, is important for determining the volume of the coumaroyl-binding pocket in the active center of CHS and possibly for the preference for 4-coumaroyl-CoA as a substrate (Jez et al., 2000a). In addition, the hydroxyl of Thr197 interacts with the carbonyl of the chalcone product (Ferrer et al., 1999). LAP5, LAP6, and other members of the male-specific CHS-like clade contain an invariant glycine at the corresponding position, potentially affecting a substrate preference. *lap5-1* mutation highlights the importance of this residue, replacing it with the larger and negatively charged amino acid, glutamate. 2) Lys62, an amino acid that sits at the entrance to the CoA-binding tunnel of CHS2 and forms a hydrogen bond with a phosphate of CoA (Ferrer et al., 1999), is replaced with Thr or Ser in LAP5, LAP6 and related proteins. 3) Homodimers of CHS have two independent active sites, each of which is composed almost entirely of residues from a single monomer. The only
residue that comes from a different monomer is Met137 that protrudes into a hole on the surface of the adjoining monomer to form a part of the cyclization pocket (Ferrer et al., 1999). This Met residue is conserved in all CHS, STS, and most other members of the superfamily with identified biochemical activity. Met137 is replaced by Leu or Phe in the LAP5/LAP6-related proteins.

The physiological role of LAP5 and LAP6, as that of many other members of type III PKS superfamily, remains unclear. Given that very few amino acid changes are sufficient to convert CHS into a protein with a different activity and the level of LAP5/LAP6 divergence from CHS, we did not readily expect LAP5 and LAP6 to catalyze CHS-type reactions. This assumption is consistent with the results of the in vitro protein assays and with the apparent inability of the TT4/CHS to rescue the lap5 and lap6 pollen defects. Interestingly, despite the fact that LAP5 and LAP6 do not exhibit in vitro CHS activity, the metabolic profiles of the lap5 and lap6 mutants revealed clear reduction in anther chalcones and flavonoids. It is presently unknown whether this reduction is directly caused by the absence of LAP5 and/or LAP6 activities or whether this is an indirect consequence of the general changes stemming from altered sporopollenin synthesis and exine development. Thus, a strong possibility remains that LAP5 and LAP6 function in the production of phenylpropanoid precursors of sporopollenin.

The biochemistry results presented here and by Mizuuchi et al. (Mizuuchi et al., 2008) demonstrated that in vitro LAP5 and LAP6 are also able to accept a variety of fatty acyl-CoA and convert them to corresponding pyrone lipids in the condensation reactions with malonyl-CoA. Homology modeling of LAP5 and LAP6 predicted that, while they share the three-dimensional overall fold with other plant type III PKS proteins, they have an unusually long substrate-binding cavity (Mizuuchi et al., 2008), consistent with their ability to accept long substrates. This feature resembles the long substrate-binding tunnel of the bacterial PKS-18 from M. tuberculosis, whose crystal structure was resolved (Sankaranarayanan et al., 2004) and which also produces long-chain pyrones from fatty acyl-CoA starters (Saxena et al., 2003). Interestingly, however, we also observed similar activity towards fatty acid derivatives with the bona fide CHS, TT4, which is known to accept other aliphatic acyl-CoA substrates (Schüz et al., 1983). This further highlights the in vitro multi-functionality of the type III PKS superfamily enzymes and demonstrates that conclusions on the physiological roles of these proteins from in vitro studies should be approached with caution.
If long-chain pyrones were produced in the developing anthers by LAP5 and LAP6, one would expect to see a differential accumulation of these compounds in the wild type and the lap5 and lap6 mutants. However, the metabolite analyses did not identify long-chain pyrones in the developing anthers. One possibility is that these compounds are among the metabolites, which were not positively identified (Supplemental Table SIV) and some of which were significantly reduced in mutants. Another possibility is that they undergo rapid polymerization and incorporation into sporopollenin and, therefore, escape detection during a metabolomics analysis. It remains to be demonstrated that fatty acyl-CoA molecules serve as in vivo substrates for LAP5 and LAP6.

Despite the overlapping in vitro biochemical activity, TT4/CHS does not rescue the lap5 and lap6 pollen phenotypes when expressed under the control of the LAP5 promoter. It is possible that the intracellular environment may affect the in vivo activity of this enzyme or that TT4/CHS does not get access to the same substrates as LAP5 and LAP6 because it is sequestered in a metabolon dedicated to flavonoid metabolism (Winkel, 2004).

Phenylpropanoid Pathway and Sporopollenin Biosynthesis Pathway: Possible Parallels

While the precise structure of sporopollenin remains undetermined, chemical studies have suggested that it is composed of fatty acid and phenolic compounds (Guilford et al., 1988; Wiermann et al., 2001). Several genes and corresponding mutants have been described that confirm the important role of fatty acid metabolism in sporopollenin production. CYP703A2 and CYP704B1 are cytochromes P450 that have fatty acid hydroxylase activities and use lauric acid and long-chain fatty acids, respectively, as preferred substrates (Morant et al., 2007; Dobritsa et al., 2009), whereas MS2 is an anther-specific gene encoding a fatty acyl reductase (Aarts et al., 1997; Doan et al., 2009). Mutations in each of these genes severely impair pollen wall production. A recently described gene ACOS5 encodes a fatty acyl-CoA synthetase, which has an in vitro preference for medium-to long-chain fatty acids (de Azevedo Souza et al., 2009). ACOS5 protein is related to 4-coumaroyl-CoA ligase, an enzyme of the general phenylpropanoid pathway that acts directly upstream of and provides the substrate (4-coumaroyl-CoA) for chalcone synthase. Thus, it is very tempting to speculate that LAP5 and LAP6, which are related to CHS and are able to utilize medium-chain fatty acyl-CoA in vitro, function immediately
downstream of ACOS5. This is in agreement with mutant analysis of these three genes. An insertion in ACOS5 results in the formation of brown shrunken anthers that lack pollen, causing male sterility ((de Azevedo Souza et al., 2009) and A.A.D., unpublished data). Sections of developing anthers from this mutant revealed that pollen develops normally until stage 9 when an exine deficiency was observed, followed by subsequent collapse of the pollen grains (de Azevedo Souza et al., 2009; Supplemental Fig. S8). This phenotype is identical to that of the lap5 lap6 double mutant and is very distinct from the phenotype of the cyp703a2 cyp704b1 ms2 triple mutant, which has viable pollen despite its lack of the normal exine layer and a characteristic zebra exine phenotype (Dobritsa et al., 2009). The more severe phenotypes point to a more central role that the ACOS5/LAP5/LAP6 pathway plays in the sporopollenin biosynthesis compared with the fatty acid-modifying CYP703A2, CYP704B1, and MS2.

Similarly to CYP703A2, CYP704B1, and MS2 (Morant et al., 2007; Dobritsa et al., 2009), ACOS5 (de Azevedo Souza et al., 2009) and LAP5/LAP6 are highly conserved in plant lineages, with homologs present in the genomes of angiosperms, gymnosperms and bryophytes but absent in green algae. The ability to synthesize sporopollenin is believed to be a critical evolutionary innovation at a time when plants colonized land, which allowed plants to protect spores from harmful effects of UV irradiation and desiccation (Bowman et al., 2007; Morant et al., 2007; de Azevedo Souza et al., 2009). Existence of ACOS5 and LAP5/LAP6 orthologs in the genome of moss P. patens indicates that this possible variation of the phenylpropanoid pathway was an ancient adaptation.

DRL1, another anther-specific protein in Arabidopsis related to a protein from the phenylpropanoid pathway (dihydroflavonol 4-reductase (DFR, Supplemental Fig. S1), whose function is presently unknown, has also been recently found to be important for exine development (Tang et al., 2008). DRL1 is similarly conserved in land plants. Expression analysis of microarray data from the wild type and several transcription factor mutants with abnormal anther and pollen development (Wijeratne et al., 2007, Yang et al., 2007; Xing and Zachgo, 2008, Xu et al., 2010) demonstrates that DRL1 is co-expressed with ACOS5, LAP5, LAP6, CYP703A2, CYP704B1, and MS2. This makes DRL1 another strong candidate for an enzyme that is involved in the biochemical pathway leading to sporopollenin biosynthesis.
MATERIALS AND METHODS

Plant Material

lap5-1, lap6-1, and the lap4-1 allele of CYP703A2 were isolated as described (Nishikawa et al., 2005) from CS6242, a cer6-2 Landsberg erecta (Ler) variety, mutagenized with 1.25 mM ethyl nitrosourea. lap6-2 (SALK_134643) and lap6-3 (N172991) were from the Arabidopsis Biological Resource Center (ABRC) and the European Arabidopsis Stock Center (NASC), respectively. T-DNA insertions in At4g00040 were from NASC (N180467, N162784; both in the 3’ end of the ORF) and from ABRC (SAIL_829_D04; in the middle of the ORF). A transposon insertion in At1g62940 (N123936) was from NASC. Plants were grown in a greenhouse at 22°C with a 16-h-light/8-h-dark photoperiod.

Genetic Mapping

Positional cloning was used to map lap5-1 and lap6-1 (backcrossed three times to CER6 Ler) in populations generated by crossing to wild-type Columbia. Bulked segregant analysis demonstrated linkage between lap5-1 and CIW7 and NGA1107 on the bottom of chromosome 4 and between lap6-1 and ZFPG on the top of chromosome 1 (markers are available at the Arabidopsis Information Resource http://www.arabidopsis.org/). Fine mapping of lap5-1 in 657 F2 plants localized it to a 59 kb region between markers T4L20E2 and F11111P1; similarly, mapping analysis of 378 F2 plants localized lap6-1 to 140 kb between T1N6A and T7I23G1. Candidate genes were sequenced and polymorphisms relative to wild-type Ler were found in At4g34850 for lap5-1 and At1g02050 for lap6-1. The lap5-1 mutation removes a BamHI site. A corresponding CAPS marker was designed (lap5-850-CF, GAGCCTGCATCAAGAACTGG and lap5-850-CR, CGGTTCAAGATGGCTGGTC) to score the lap5-1 alleles in the segregating populations. To score the lap6-1 alleles, a dCAPS marker was designed (lap6-2050-CF, GCAGCTCCAACCTAGGTCG, lap6-2050-BR1, GGTTGCATCAAGGAATGGGGGAAGGCCAGTGGAAAGATA, EcoRV cleaves the wild-type allele).

Complementation Constructs
LAP5 (from 631 nt upstream of the start codon to 264 nt downstream of the stop codon) was amplified with Platinum Pfx DNA polymerase (Invitrogen) using lap5-850-EF2 (GTGTACCGGTCATCAGTGGGAAATGTCTCTCAG) and lap5-850-ER (CCATGGGATAGAGATCCAACTCCTGGAC). LAP6 (from 479 nt upstream of the start codon to 148 nt downstream of the stop codon) was amplified using lap6-2050-ER2 (GTGTACCGGTTAGAGATGGGAAATGTCTCTCAG) and lap6-2050-EF (CCATGGGCACTTGGGTAAACGACACGATGGTG). PCR products were digested with AgeI/NcoI, and incorporated into a modified binary vector pGreenII02229 (Hellens et al., 2000; von Besser et al., 2006). lap5-1 and lap6-1 plants were transformed by the floral dip method (Clough and Bent, 1998) and transgenic plants were selected with BASTA.

GUS, At4g00040, and TT4 Expression Constructs

LAP5 and LAP6 promoters were amplified (lap5-850-EF3, GGATCCCATCAGTGGGAAATGTCTCTCAG and lap5-850-FR, ACCGGTTTGTATCTGTTTGTGGAAGACTC; lap6-2050-DF2, ACCGGTTTCTTTTTCAAGCGCAGAG and lap6-2050-ER2), digested with BamHI/AgeI, and incorporated into a modified vector pGreenII02229, creating LAP5pr-pGR111 or LAP6pr-pGR111 constructs. The β-glucuronidase gene (GUS) was amplified with GUS-AF (CACCATGGGATGTTACCTGCAGTCTCTCAAG) and GUS-CR (CCACTAGTTACGTGTCCAGTCTCAGTCT), digested with NcoI/SpeI, and cloned into NcoI/SpeI-cut LAP5pr-pGR111 or LAP6pr-pGR111. TT4 was amplified with TT4-AF (TCTCCCATGGATGGTGCTGGGTCTTCTTCTTCT), and TT4-CR (AGGGACTATTAGAGATGGGAAACCGCTTG), and similarly cloned in LAP5pr-pGR111. At4g00040 was amplified with 040-AF (ACCAGTATGTTGCTCCGCAAGGATGAG) and 040-AR2 (CCATGGGTCAATACAGGGAAGGCTCTCAG), digested with AgeI/NcoI, and cloned into AgeI/NcoI-digested LAP5pr-pGR111 or LAP6pr-pGR111.

Phylogenetic Analysis
CHS family proteins analyzed and their accession numbers from the Entrez Protein database are:

- *Brassica napus* CHS A2 (AAC31912), *Zea mays* CHS C2 (P24825) and CHS Whp (P24824),
- *Bromheadia finlaysonia* CHS (O23729), *Hypericum androsaemum* CHS (Q9FUB7), *Ipomoea purpurea* CHS-D (AAK39111), *Lycopersicon esculentum* CHS (P23419), *Pinus sylvestris* CHS (P30079), *Medicago sativa* CHS2 (P30074), *Daucus carota* CHS9 (Q9SB26), *Allium cepa* CHS (AAO63020), *Petunia x hybrida* CHSA (P08894) and CHSJ (P22928), *Ipomoea nil* CHS (O22046), *Gerbera hybrida* 2-PS (P48391), *Ruta graveolens* ACS (Q9FSC0), *Rheum palmatum* ALS (AAS87170), *Pinus radiata* PrCHS1(AAB80804), *Silene latifolia* SICH (BAE80096), *Vitis vinifera* STS (CAA54221), *Nicotiana sylvestris* CHSLK (CAA74847), *Oryza sativa* YY2 (BAA23618) and CHSS6 (BAC21541), *Physcomitrella patens* CHS-like (XP_001781520).

Additional sequences were obtained from The Institute for Genomic Research (http://www.tigr.org) *Zea mays* CHSS1 and CHSS2, *Brassica rapa* CHSS1 and CHSS2, and *Lycopersicon esculentum* CHS1 and CHS2. Predicted proteins were aligned using ClustalW (MegAlign, DNAStar, Lasergene, Madison, WI, USA). A phylogenetic tree was created using the neighbor-joining method; bootstrap values were obtained (trials=1000, seed=111). Polyketide synthase PKS18 from *Mycobacterium tuberculosis* (A70958) was used as an outgroup.

**Microscopy**

Dissection stereomicroscopy (50X-90X magnification) was used to assess anther/pollen phenotypes. Anther images were captured using Zeiss SteReo Lumar V12 (80X magnification) fitted with a Zeiss AxioCam MRc5 digital camera. SEM was performed as described (Nishikawa et al., 2005). For developmental analysis, inflorescences were fixed in 4% paraformaldehyde, 0.5% glutaraldehyde, 0.05 M phosphate buffer, pH 7.2 for 2 h (RT), washed in phosphate buffer (4 times, 15 min), dehydrated in ethanol (15%, 30%, 45%, 60%, 70%), ethanol:n-butanol (50%:35%, 40%:55%, 25%:75%, v/v), and 100% n-butanol, infiltrated with paraffin, and cut into 5 μm sections. Slides were processed in two changes of xylene, gradually rehydrated in an ethanol series followed by water, and stained with 0.1% toluidine blue. Auramine O staining, GUS staining and acetolysis were performed as described (Dobritsa et al., 2009).
Metabolomics

Anthers from stage 9 and 10 flower buds (staged according to (Smyth et al., 1990)) were collected on dry ice; three replicates composed of 60 anthers each were analyzed per genotype. Samples were lyophilized, homogenized and extracted in 50 μl of 80% methanol with the internal standard 2 μg umbelliferone for 2 h. Half the sample was used for ultra performance liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer (UPLC-QTOFMS), using an ACQUITY UPLC system (Waters Corp.) equipped with a binary solvent delivery system and an autosampler. The mobile phases were 0.1% acetic acid in water (eluent A) and 100% acetonitrile (eluent B). UPLC separations used a Waters 2.1 x 100 mm, BEH C18 column with 1.7 μm particles, a linear gradient of 95%:5% to 30%:70% eluents A:B, a flow rate of 0.56 ml/min, column temperature of 60°C, and sample temperature of 4°C (autosampler). Each wash cycle used 0.8 ml of 100% methanol and 2 ml of 0.1% acetic acid in water. A blank (80% methanol) was injected between every five samples. Mass spectrometry was performed using a Waters QTOFMS Premier operating in the negative electrospray ionization mode, with the nebulization gas at 850 l/h (350°C), the cone gas at 50 l/h (120°C). QTOFMS data were collected between 50 and 2000 m/z and were acquired using an independent reference lock-mass ion via the LockSpray interface, with the LockSpray frequency set at 10 s in the centroid mode. Raffinose (m/z 503.1612) was used as the reference compound and delivered at a concentration of 50 fmol/mL and flow rate of 0.2 mL/h.

GC/MS was performed on the remaining portion of the anther extracts (three replicates, ~25 μl each). 500 μl of 80% MeOH containing an internal standard of 2.5 μg ribitol was added and samples were incubated for 1 h (50°C), equilibrated to room temperature, and incubated for 1 h at 50°C following the addition of 500 μl of chloroform containing 1 μg of docosanol (an internal standard). 400 μl of H2O was added and phases were separated by centrifugation at 2900 g (30 min, 4°C). Equal amounts of each phase were collected, transferred to 2 ml vials, and dried at room temperature under nitrogen (organic phase) or in air (aqueous phase). Polar extracts were methoximated in pyridine with 20 μl of 1.5% methoxyamine–HCl, briefly sonicated, suspended at 50°C, and incubated with 20 μl commercial derivatization solution containing MSTFA+1% TMCS (1 h, 50°C). Non-polar extracts were suspended in 0.5 ml chloroform, hydrolyzed with
0.5 ml 1.25M HCl in MeOH (4 h, 50°C), and then evaporated under nitrogen. Polar and non-polar extracts were analyzed with an Agilent 6890 GC coupled to a 5973 MSD. Samples were injected at a 1:1 split ratio, and the inlet and transfer lines were maintained at 280°C. Separation was achieved with a temperature program of 80°C for 2 min, ramped at 5°C /min to 315°C and held for 12 min on a 60 m DB-5MS column (J&W Scientific, 0.25 mm ID, 0.25 lm film thickness) and a constant flow of 1 ml/min helium. Mass spectra were collected from 50 to 800 m/z. For each compound, a Student’s t-test was performed to assess statistical differences.

**Protein Expression and Purification**

LAP5, LAP6 and TT4 ORFs were PCR amplified from cDNA that was prepared from RNA isolated from stage 9-10 buds (Dobritsa et al., 2009). The following primers were used: lap5-start1 (CACCATGGGTAGCATCGACGCAGCAGTGGGT) and lap5-stop1 (TCAGACATCAAGGTTTCGAGC), lap6-start1 (CACCATGTCTTAATTCTCGATAATGGGTGGT), and lap6-stop1 (TTAGGAAGAGGTGAGGCTGGATG), TT4-start1 (CACCATGGTGATGGCTGGTGCTTTCTTC) and TT4-stop1 (TTAGGAGGAACGCTGTGCAAGAC). Start codons (ATG) are underlined in the start primers; stop codons are underlined in the stop primers. Bold corresponds to the mutagenized nucleotides that introduce silent mutations for improved expression in *E. coli*. The PCR products were cloned into pET100-D/Topo vector from Invitrogen (Carlsbad, CA, USA), and resulting constructs were transformed into *E. coli* BL21 Star (DE3). 500 mL cultures of transformed *E. coli* were grown at 37°C in LB media containing 50 μg/mL kanamycin until OD₆₀₀=0.8. After induction with 0.3 mM isopropyl 1-thio-β-D-galactopyranoside, the cultures were grown for 16 h at 16°C. Cells were pelleted, harvested and resuspended in extraction buffer (50 mM NaH₂PO₄, pH 8.0, 500 mM NaCl, 10% glycerol, 10 mM β-mercaptoethanol, 10 mM imidazole) supplemented with protease inhibitors. After brief sonication, cells were homogenized in an EmulsiFlex-C3 high-pressure homogenizer (Avestin, Ottawa, Canada). The homogenate was clarified at 30,000 and 50,000 x g for 20 min each, and incubated with Talon Metal Affinity Resin (Clontech) for 1 h at 4°C and then loaded onto a disposable column. After a 50-mL extraction buffer wash, proteins were eluted with Talon elution buffer (extraction buffer supplemented with 250 mM imidazole) and 1 mL fractions were collected. Protein purity was
analyzed on 12.5% SDS-PAGE. Protein-rich elution fractions (1-6) were pooled (fractions 1-6 were similarly pooled for vector-only control; fractions 1-4 from vector-only control contained proteins detectable by Coomassie blue staining), dialyzed in 2 L storage buffer (20 mM HEPES, 1 mM EDTA, 200 mM KCl, 0.01% NaN₃, 10% (v/v) glycerol, pH 7.4) overnight, concentrated using Centriprep filters (Millipore) to approximately 1 mg/mL, and stored on ice. Similarly processed extracts from vector-only transformed *E. coli* were used as negative controls.

**Synthesis of 4-coumaroyl-CoA**

4-coumaroyl-CoA was synthesized as previously described (Stoeckigt and Zenk, 1975). The *N*-hydroxysuccinimide ester of 4-coumaric acid was first synthesized from 4-coumaric acid (C-9008, Sigma-Aldrich) and *N*-hydroxysuccinimide (130672, Sigma-Aldrich) in the presence of *N*,*N*'-dicyclohexylcarbodiimide (36650, Sigma-Aldrich). After purification, the *N*-hydroxysuccinimide ester of 4-coumaric acid was then converted into 4-coumaroyl-CoA through thioester exchange between the *N*-hydroxysuccinimide ester and coenzyme A. The resulting 4-coumaroyl CoA was purified on a Diethylaminoethyl (DEAE)-Sephacel (GE Healthcare) column (1.6 x 20 cm) and lyophilized.

**Protein Activity Assays**

The activities of LAP5 and LAP6 were determined by monitoring the enzymatic reaction products with HPLC-UV/Vis-ITMS (ion trap mass spectrometry). The *TT4* gene product, TT4/CHS, was used as a positive control and the empty-vector extract was used as a negative control. The enzymatic reactions were performed in a 100-µL reaction buffer (100 mM of potassium phosphate buffer, pH 7.0) containing 15 µM of 4-coumaroyl-CoA, 45 µM of malonyl-CoA (M4263, Sigma-Aldrich), and 50 µg of the purified proteins. In the separate assays, 4-coumaroyl-CoA was replaced with acetyl-CoA (A2181, Sigma-Aldrich) with other conditions remaining unchanged. Reactions were allowed to continue for 4 hours at 37°C and quenched with the addition of 20 µl of formic acid and extracted with 200 µL of ethyl acetate twice. The mixtures were vortexed and briefly centrifuged to assist in phase partitioning. The ethyl acetate phases were collected, combined and evaporated to dryness under nitrogen stream, redissolved in methanol (35 µL), and subjected to HPLC-UV/Vis-ITMS analyses as previously described (Farag et al., 2007). Briefly, 30 µL aliquots of the reaction products were injected onto an
Agilent 1100 series II HPLC system (Hewlett-Packard, Palo Alto, CA) which was coupled to a Bruker Esquire ion-trap mass spectrometer. Separations were achieved using a reverse-phase C18 column (5 µm, 4.6 mm I.D. x 250 mm, J.T.Baker, Phillipsburg, NJ) and a linear gradient of 5–70% B (v/v) over 60 min at a flow rate of 0.8 mL/min. The mobile phases consisted of solvent A (0.1% acetic acid (v/v) in water) and solvent B (acetonitrile). UV spectra were obtained by scanning from 200 to 600 nm and the mass spectra were acquired in negative electrospray ionization mode with a mass scan range of 50-2200 m/z using an electrospray ionization voltage of 4.0 kV. Tandem mass spectrometry (MS/MS) was performed with manual selection of precursor ion and the fragmentation energy set at 1.5. To determine the activities of LAP5, LAP6 and TT4/CHS towards fatty acyl-CoA, 4-coumaroyl-CoA was replaced with 8 n-alkyl fatty acyl-CoA (butyryl-, hexanoyl-, octanoyl-, decanoyl-, lauroyl-, myristoyl-, palmitoyl-, stearoyl-CoA (Sigma-Aldrich), with the reaction conditions, product processing and analyses remaining unchanged.

The Arabidosis Genome Initiative locus numbers for the genes analyzed in this article are as follows: LAP5 (At4g34850), LAP6 (At1g02050), At4g00040, CYP703A2 (At1g01280), TT4 (At5g13930).

Supplemental material

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

Supplemental Figure S1. Biosynthesis of plant phenolic compounds.

Supplemental Figure S2. Mass spectra of the two products from the LAP6 enzymatic assay.

Supplemental Figure S3. In vitro formation of trans- and cis-bisnoryangoninc.

Supplemental Figure S4. Condensation of fatty acyl-CoA with malonyl-CoA leads to the formation of triketide pyrones, tetraketide pyrones and tetraketide resorcylic acid.

Supplemental Figure S5. HPLC-ITMS UV analyses of the products of the enzymatic reactions with hexanoyl-CoA and malonyl-CoA as substrates.

Supplemental Figure S6. HPLC-ITMS UV analyses of the products of the enzymatic reactions with decanoyl-CoA and malonyl-CoA as substrates.

Supplemental Figure S7. HPLC chromatograms of the enzymatic assays with multiple substrates (coumaroyl-CoA, hexanoyl-CoA and malonyl-CoA).
Supplemental Figure S8. Defects of pollen and anther development in acos5 mutant strongly resemble those in lap5-1 lap6-1 mutant.

Supplemental Table SI.

Supplemental Table SII.

Supplemental Table SIII.

Supplemental Table SIV.

Supplemental Table SV.

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LITERATURE CITED


FIGURE LEGENDS

Figure 1. *lap5* and *lap6* mutants have defects in anther and pollen exine morphology. (A-C) Compared to the wild-type anthers (A), anthers of *lap5-1* (B) and *lap6-1* (C) mutants appear glossy and do not easily shed pollen (arrowhead in A). (D-I) SEM of pollen grains and exine. Wild-type grains (D,G) have a regular reticulate exine pattern, while *lap5-1* (E,H) and *lap6-1* (F,I) mutations cause pollen to collapse more easily and disrupt exine, changing the pattern or resulting in a more extensively covered surface. (J-L) Similar to wild type (J), *lap5-1* (K) and *lap6-1* (L) pollen does not demonstrate sensitivity to acetolysis. The *lap6-1* grains, however, exhibit decreased reactivity to acetolysis-dependent staining. Scale bars: 100 μm (A-C), 10 μm (D-F and J-L), 5 μm (G-I).

Figure 2. The *lap5* and *lap6* defects map to At4g34850 and At1g02050, respectively. *LAP5*, *LAP6*, and At4g00040 have gene structures similar to CHS/STS family members, including the conserved position of an intron separating the first and second nucleotides in a cysteine codon (TGC). Exons are shown as black rectangles. Positions of the *lap5-1* and *lap6-1* point mutations and the *lap6-2*, *lap6-3*, and At1g00040 insertions are indicated with black triangles.

Figure 3. *LAP5*, *LAP6* and At1g00040 belong to a male-specific clade of the chalcone/stilbene synthase family. (A) Amino acid alignment of protein sequences from *LAP5*, *LAP6*, At1g00040, CHS, STS and CHS/STS-like proteins from several plant species (two-thirds of the protein length is shown, the remainder shows a similar trend). Residues identical to *LAP5* are shaded blue; those identical to the *A. thaliana* CHS TT4 are shaded yellow; those that match the majority consensus from 17 sequences are orange. Amino acid numbers correspond to CHS2 from *M. sativa*. Proteins with demonstrated male-specific expression patterns are marked with asterisks; the *lap5-1* and *lap6-1* lesions are indicated, and two of the four amino acids critical for active center formation (C164 and F215) are boxed. M137, important for dimer formation, is conserved in the CHS-like enzymes (boxed) but replaced with L/F in enzymes more similar to *LAP5*. (B) A phylogenetic tree demonstrates a clear segregation of the *LAP5*/LAP6-like proteins in a clade separate from the other members of the CHS superfamily. Red bar, chalcone synthases; blue bar, sequences similar to *LAP5*; green bar, four CHS-like proteins with demonstrated non-CHS activity (2-pyrone synthase (2PS), aloesone synthase (ALS), acridone
synthase (ACS), stilbene syntase (STS)). Numerical values indicate bootstrap support for each node. Polyketide synthase PKS18 from *M. tuberculosis* was used as an outgroup.

**Figure 4.** LAP5 and LAP6 are expressed specifically in developing anthers. *LAP5pr::GUS* (A-D) and *LAP6pr::GUS* (E-H) promoter fusion constructs are expressed in anthers of stage 9 and 10 buds (B,F), but not at earlier stages (A,E), or later stages (stage 11 (C,G), mature anthers (D,H)). Scale bar, 100 μm. All images are to the same magnification.

**Figure 5.** A *lap5-1 lap6-1* double mutant is sterile and lacks pollen. (A) Left to right: siliques from wild-type, *lap5-1*, *lap6-1*, and a *lap5-1 lap6-1* double mutant. (B) Mature anther from *lap5-1 lap6-1* demonstrates complete absence of pollen grains (compare with Fig. 1A for a wild-type anther).

**Figure 6.** Pollen produced by a *lap5-1 lap6-1* double mutant lacks exine and degenerates. Anther development in the wild-type *Arabidopsis* (A-D) and a *lap5-1 lap6-1* double mutant (E-H). Anther locules at stages 6 (A,E), 7 (B,F), 9 (C,G), and 11 (D,H) are shown. Exine is deposited around developing pollen grains at stage 9 in the wild type (green halo around the pollen grains, C), but not in *lap5 lap6* (G). Insets in (C,G) are higher magnification views of boxed areas. Little pollen is visible in *lap5 lap6* by stage 11 (H). Scale bars: 20 μm (A-H); 10 μm, insets.

**Figure 7.** Levels of chalcone and naringenin are affected in the *lap5* and *lap6* mutants. Example of UPLC-QTOFMS chromatograms obtained from *lap5-1*, *lap6-1*, the *lap5-1 lap6-1* double mutant and wild-type (WT) anther extracts.

**Figure 8.** HPLC-UV-MS chromatograms of the enzymatic assays with 4-coumaroyl-CoA and malonyl-CoA as substrates. (A) Empty-vector control; (B) TT4/CHS. Inserts are UV and MS spectra of the enzymatic reaction product (naringenin); (C) Naringenin standard. The inserts are the UV and MS spectra; (D) LAP5. The inserts are the close-up view of peaks 1 and 2, and their UV spectra; (E) LAP6. The inserts are the close-up view of peaks 1 and 2, and their UV spectra.

**Figure 9.** HPLC-ITMS UV analyses of the products of the enzymatic reactions with octanoyl-CoA and malonyl-CoA as substrates. (A-C) UV chromatograms (287 nm) of the products of
enzymatic reactions in the presence of (A) empty-vector control; (B) LAP5; (C) TT4/CHS; (D) Mass spectrum of the major enzymatic product, 4-hydroxy-6-heptyl-2-pyrone, of the reaction catalyzed by LAP5; (E) Mass spectrum of the major enzymatic product, 4-hydroxy-6-heptyl-2-pyrone, of the reaction catalyzed by TT4.

**Figure 10.** Exine from the lap5-1 and cyp703a2 (lap4-1) double mutant has defects stronger than either single mutant. (A-D) Confocal images of the exine surface from wild-type (A,E), lap5-1 (B,F), lap4-1 (C,G), and a lap4-1 lap5-1 double mutant (D,H) pollen grains stained with auramine O. Arrow in (A) indicates an aperture. Arrow in (D) indicates a longitudinal area of increased fluorescence. (E-F) Optical sections through the middle of pollen grains allow visualization of exine thickness. Scale bar, 10 μm. All images are to the same magnification.

**Supplemental Figure S1.** Biosynthesis of plant phenolic compounds. Shown are reactions catalyzed by phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumaroyl ligase (4CL), chalcone synthase (CHS), stilbene synthase (STS), chalcone isomerase (CHI), flavonoid 3-hydroxylase (F3H), flavanone 3’-hydroxylase (F3’H), flavonol synthase (FS), and dihydroflavonol reductase (DFR).

**Supplemental Figure S2.** Mass spectra of the two products observed in the LAP6 enzymatic assay. Upper panel (A) is the spectrum of product 1 and the lower panel (B) is product 2.

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**Supplemental Figure S6.** HPLC-ITMS UV analyses of the products of the enzymatic reactions with decanoyl-CoA and malonyl-CoA as substrates. (A-C) UV chromatograms (287 nm) of the products of enzymatic reactions in the presence of (A) empty-vector control; (B) LAP6; (C) TT4/CHS; (D) Mass spectrum of the major enzymatic product, 4-hydroxy-6-nonyl-2-pyrone, of the reaction catalyzed by LAP6; (E) Mass spectrum of the major enzymatic product, 4-hydroxy-6-nonyl-2-pyrone, of the reaction catalyzed by TT4.

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**Supplemental Figure S8.** Defects of pollen and anther development in the acons5 mutant strongly resemble those in the lap5-1 lap6-1 double mutant (compare with Fig. 6). Mutation in ACOS5 impairs exine deposition and leads to pollen degeneration and male sterility. Anther development in wild-type (A-D) and acons5 mutant (E-H). Anther locules at stages 6 (A,E), 7 (B,F), 9 (C,G), and 11 (D,H) are shown. Exine (visible as green halo in C and D) is not deposited around developing pollen grains in the acons5 mutant (G). Pollen undergoes degeneration in the mutant, with almost none visible by stage 11 (H). Scale bar, 40 μm. All images are to the same magnification.
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