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**RESEARCH PAPER**

# Jasmonates meet fatty acids: functional analysis of a new acyl-coenzyme A synthetase family from *Arabidopsis thaliana*

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## Abstract

***Arabidopsis thaliana*** **contains a large number of genes encoding carboxylic acid-activating enzymes, including long-chain fatty acyl-CoA synthetase (LACS), 4-coumarate:CoA ligases (4CL), and proteins closely related to 4CLs with unknown activities. The function of these 4CL-like proteins was systematically explored by applying an extensive substrate screen, and it was uncovered that activation of fatty acids is the common feature of all active members of this protein family, thereby defining a new group of fatty acyl-CoA synthetase, which is distinct from the known LACS family. Significantly, four family members also displayed activity towards different biosynthetic precursors of jasmonic acid (JA), including 12-oxo-phytodienoic acid (OPDA), dinor-OPDA, 3-oxo-2(2′-[*Z*]-pentenyl)cyclopentane-1-octanoic acid (OPC-8), and OPC-6. Detailed analysis of *in vitro* properties uncovered significant differences in substrate specificity for individual enzymes, but only one protein (At1g20510) showed OPC-8:CoA ligase activity. Its *in vivo* function was analysed by transcript and jasmonate profiling of *Arabidopsis* insertion mutants for the gene. OPC-8:CoA ligase expression was activated in response to wounding or infection in the wild type but was undetectable in the mutants, which also exhibited OPC-8 accumulation and reduced levels of JA. In addition, the developmental, tissue- and cell-type specific expression pattern of the gene, and regulatory properties of its promoter were monitored by analysing promoter::GUS reporter lines. Collectively, the results demonstrate that OPC-8:CoA ligase catalyses an essential step in JA biosynthesis by initiating the β-oxidative chain shortening of the carboxylic acid side chain of its precursors, and, in accordance with this function, the protein is localized in peroxisomes.**

Key words: *Arabidopsis thaliana*, fatty acyl-CoA synthetase, jasmonate biosynthesis, oxylipins, plant defence, wound response.

## Introduction

Jasmonic acid (JA) and its cyclic precursors, collectively referred to as jasmonates, constitute a family of bioactive oxylipins, which are derived from oxygen-containing fatty acids and regulate a variety of defence responses and developmental processes in plants. The pathway of JA biosynthesis has been unravelled, and most, but not all, of the participating enzymes have been identified (Turner *et al*., 2002; Schaller *et al*., 2005; Wasternack, 2006). Phospholipases, such as DAD1, releasing linolenic acid (C18:3) or hexadecatrienoic acid (C16:3) from membrane lipids in the chloroplast are considered to initiate the pathway (Ishiguro *et al*., 2001; Schaller *et al*., 2005). These polyunsaturated fatty acids are converted to the cyclopentenones 12-oxo-phytodienoic acid (OPDA) or dinor-OPDA (dnOPDA), respectively, by the plastidic

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enzymes 13-lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC) (Stenzel *et al*., 2003*b*; Schaller *et al*., 2005). OPDA and dnOPDA may then be re-esterified to plastid-specific galacto- and phospholipids, which accumulate massively upon wounding (Stelmach *et al*., 2001; Andersson *et al*., 2006; Buseman *et al*., 2006). Alternatively, OPDA and dnOPDA can be released from the chloroplast and function directly as signal molecules in plant defence responses (Stintzi *et al*., 2001; Taki *et al*., 2005). How OPDA is exported from the plastid and exerts its function is currently unknown.

For conversion to JA, OPDA and dnOPDA are translocated into peroxisomes. Current evidence indicates that import is at least partially mediated by the peroxisomal ATP-binding cassette (ABC) transporter COMATOSE (CTS1, also known as PXA1/PED3) (Zolman *et al*., 2001; Theodoulou *et al*., 2005). Both compounds are reduced by OPDA reductase 3 (OPR3) to 3-oxo-2-(2′-[*Z*]-pentenyl)-cyclopentane-1-octanoic acid (OPC-8) and 3-oxo-2-(2′-[*Z*]-pentenyl)-cyclopentane-1-hexanoic acid (OPC-6), respectively (Schaller *et al*., 2000; Stintzi and Browse, 2000; Strassner *et al*., 2002). The final steps of JA biosynthesis comprise several cycles of β-oxidation, by which an even number of carbons is removed from the carboxyl side chains of OPC-8 and OPC-6, giving rise to JA (Miersch and Wasternack, 2000; Schaller *et al*., 2005). It has long been assumed that these steps are catalysed by the three core enzymes of peroxisomal fatty acid β-oxidation, acyl-CoA oxidase (ACX), multifunctional protein (MFP), and 3-ketoacyl-CoA thiolase (KAT). More recently it has been demonstrated that specific isoforms of these enzymes participate in wound-induced JA biosynthesis, i.e. ACX1, ACX5, and KAT2 in *Arabidopsis* and ACX1A in tomato (Castillo *et al*., 2004; Afitlhile *et al*., 2005; Li *et al*., 2005; Schilmiller *et al*., 2007). For initiation and completion of β-oxidation, two additional reactions are required, which have largely been neglected in connection with JA biosynthesis: (i) activation of a suitable precursor to the CoA ester by a CoA ligase; and (ii) release of the final product (JA) from the penultimate intermediate jasmonoyl-CoA by a thioesterase. While an enzyme catalysing the latter reaction has not yet been identified, an OPC-8:CoA ligase (named OPCL1) was recently described that accounts for a fraction of the JA synthesized in response to wounding (Koo *et al*., 2006), whereas redundant or unknown enzymatic functions are responsible for ~60% of the accumulating JA. Other CoA ligases with the *in vitro* capacity to convert OPDA and OPC-6 to the corresponding CoA esters also exist (Schneider *et al*., 2005), but their physiological roles remain enigmatic.

Focusing on CoA ester formation, it is important to note that the superfamily of 63 predicted acyl-activating (adenylate-forming) enzymes in *Arabidopsis thaliana*, which was originally defined by the presence of a diagnostic AMP-binding domain, comprises several distinct subgroups of proteins (Shockey *et al*., 2003). All of these enzymes utilize ATP to convert an organic acid to an adenylate intermediate, from which the acyl group is transferred in a second step to an ultimate acceptor, which in most cases is CoA. Well-known enzymes operating by this two-step mechanism include acetyl-CoA synthetase, long-chain fatty acyl-CoA synthetase (LACS), and 4-coumarate:CoA ligase (4CL). One subgroup of the superfamily was found to catalyse the ATP-dependent *in vitro* adenylation of the plant hormones JA, salicylic acid (SA), or indole-3-acetic acid (IAA), suggesting participation in hormone signalling without CoA ester formation (Staswick *et al*., 2002, 2005; Shockey *et al*., 2003). In fact, one enzyme of this group, JAR1, was shown to conjugate activated JA with isoleucine, and genetic evidence supports the notion that JA-Ile is an essential component of jasmonate signalling in *Arabidopsis* (Staswick and Tiryaki, 2004). The recent identification of several COI1-interacting JAZ proteins, some of which apparently mediate their regulatory function in a JA-Ile-dependent manner, further adds to our understanding of the molecular mechanism of jasmonate action (Chini *et al*., 2007; Thines *et al*., 2007).

Members of the LACS family have been extensively studied and found to fulfil important functions in various organelles and tissues by providing the acyl-CoA pool for phospholipids and triacylglycerol biosynthesis or for lipid degradation via β-oxidation (Graham and Eastmond, 2002; Baker *et al*., 2006). However, a relationship of this family to JA biosynthesis has not been observed.

The four 4CL isoforms in *Arabidopsis* constitute another well-studied group of the adenylate-forming superfamily but they share only low similarity with the fatty acid-activating LACS enzymes at the amino acid sequence and gene structure level (Ehlting *et al*., 1999; Shockey *et al*., 2003; Hamberger and Hahlbrock, 2004). Typically, 4CLs catalyse the activation of 4-coumarate and other cinnamic acid derivatives to the corresponding CoA esters, thereby providing the phenylalanine-derived building block for the different branches of phenylpropanoid metabolism.

In this context, a clade of nine proteins has been identified in the superfamily of adenylate-forming enzymes that displays high sequence similarity to 4CLs (Schneider *et al*., 2003, 2005). The current study focuses on the systematic, functional analysis of the whole clade of these proteins and it revealed a new class of fatty acyl-CoA synthetases comprising four enzymes with the *in vitro* capacity to activate different JA precursors. By detailed biochemical characterization using a broad range of substrates, one of these enzymes was identified as OPC-8:CoA ligase (OPCL1) that participates in wound-induced JA biosynthesis.

## Materials and methods

### Plant material and growth conditions

*Arabidopsis thaliana* wild-type (Col-0, Ws-0) and mutant plants were soil grown in controlled environment chambers under a regime of a 10 h light period at 150–200 μE $m^{-2}$, at 20–22 °C, and 60% relative humidity. Insertion mutants of genes encoding acyl-CoA synthetases were identified using the SIGnAL T-DNA Express *Arabidopsis* gene mapping tool (http://signal.salk.edu/). The T-DNA insertion lines SALK_140659 (At1g20510-KO-1), SALK_050214 (At4g05160), SALK_003233 (At5g63380), and SALK_067285 (At1g20500) were distributed by NASC (http://arabidopsis.info/); the GABI-Kat line GK-550H05-020728 (At1g20510-KO-2) was obtained from the Max Planck Institute for Plant Breeding Research (http://www.gabi-kat.de/). Homozygous insertion mutants were identified by PCR using T-DNA- and gene-specific primer sets as described on the T-DNA Express home page. To evaluate whether or not the isolated mutants were mRNA nulls, semi-quantitative RT-PCR was performed on RNA isolated from control and wounded leaves using gene-specific primer pairs for *OPCL1* (At1g20510), all other acyl-CoA synthetase genes, and numerous additional genes. Primer sequences are available on request.

For effector screening, *Arabidopsis* seedlings were grown in hydroponic culture in 48-well microtitre plates (NUNC, Roskilde, Denmark). Each well contained 0.5 ml of Murashige and Skoog (MS) liquid medium and 0.5% sucrose into which 2–4 surface-sterilized seeds were placed. The microtitre plates were incubated in a growth cabinet (Percival AR-75L) at 24 °C and a light/dark cycle of 16/8 h.

### Cloning and expression of acyl-CoA synthetase cDNAs

Total RNA was isolated from different tissues of *A. thaliana* (Col-0) and transcribed into cDNA using Superscript Reverse Transcriptase II (Invitrogen, Carlsbad, CA, USA). The gene of interest was then amplified by RT-PCR as previously described (Schneider *et al*., 2005) using primer pairs listed in Supplementary Table S2 available at *JXB* online. PCR fragments were cloned into the pQE-32 expression vector (Qiagen, Hilden, Germany), thereby adding an N-terminal $His_6$ tag to the encoded protein. All clones were sequenced by the ADIS service unit (Max Planck Institute for Plant Breeding Research, Köln, Germany) and any deviation from the sequences available from the MIPS database (Munich Information Center for Protein Sequences, Neuherberg, Germany) was corrected by site-directed mutagenesis using the Quikchange® II Site-Directed Mutagenesis kit (Stratagene, Amsterdam-Zuidoost, The Netherlands). Cloning of At5g63380 and At4g05160 has been described previously (Schneider *et al*., 2005).

Heterologous expression in *Escherichia coli* strains DH5α or M15 and purification of recombinant acyl-CoA synthetases was carried out as previously described (Stuible *et al*., 2000; Schneider *et al*., 2005). Enzyme purity was inspected by SDS gel electrophoresis, and protein concentrations determined according to Bradford (1976) with bovine serum albumin as standard.

### Determination of enzymatic activity

To screen a large number of carboxylic acids for activation to CoA esters by acyl-CoA synthetases, the consumption of ATP was determined by a luciferase-coupled assay as previously described (Schneider *et al*., 2005). For detailed kinetic analyses with selected substrates, acyl-CoA synthetase activity was determined by following AMP formation in a coupled spectrophotometric assay with myokinase, pyruvate kinase, and lactate dehydrogenase (Schneider *et al*., 2005). $K_m$ and $V_{max}$ values were obtained by linear regression of v/s against s (Hanes plots) from at least three independent experiments. Activity towards cinnamic acids was determined by the spectrophotometric 4CL assay previously described (Ehlting *et al*., 1999) with standard concentrations of the cinnamic acid derivative (0.2 mM), ATP (5.5 mM), and CoA (0.3 mM).

### Complementation of bacterial acyl-CoA synthetase (FadD)

Two *E. coli* strains lacking fatty acyl-CoA synthetase activity, LS1907 (*fadD::Kan*$^r$) and LS1908 (*ΔfadR fadD::Kan*$^r$), kindly provided by Dr Paul N Black (Albany Medical College, New York, NY, USA), were used for complementation assays. All of the *Arabidopsis* genes encoding putative acyl-CoA synthetases were cloned into the bacterial expression vector pQE-32 (Qiagen, Hilden, Germany) under the control of the T5 promoter and the resulting plasmids transferred into both *E. coli* mutants. The resulting transformants were analysed for their ability to grow on solid medium (1.5% agar) and in liquid minimal medium M9 (Miller, 1972) containing 0.5% Brij58 and 0.1% fatty acids of various chain lengths (decanoic acid, dodecanoic acid, oleic acid) as the only energy and carbon source.

### Transient expression and subcellular localization of *OPC-8:CoA ligase* in planta

The cDNA of OPCL1 (At1g20510) was PCR-amplified using the primer pair attb1-20510 (5′-TCATGGCTTCAGTGAATTCTCG-3′) and attb2-20510 (5′-CTCAAAGCTTGGAGTTGGAAG-3′), and inserted into the expression vector pENSGYFP, leading to N-terminal fusion with the yellow fluorescent protein (YFP), using the Gateway® cloning system (Invitrogen, Karlsruhe, Germany) as previously described (Schneider *et al*., 2005). The dsRED fluorescent protein with the C-terminal extension -SRL served as a peroxisomal marker protein (Schneider *et al*., 2005). Both vectors allow the *in planta* expression of proteins under the control of the constitutive cauliflower mosaic virus (CaMV) double 35S promoter. For transient *in planta* expression, detached leaves of 4-week-old *A. thaliana* (Col-0) plants were bombarded with 1 μm gold particles coated with vector DNA using the Biolistic® PDS-1000/He Particle Delivery System (Bio-Rad Laboratories, München, Germany) and subsequently placed in a growth chamber for 36 h (Schneider *et al*., 2005). Fluorescence microscopy was carried out using an LSM 510 Meta confocal laser microscope (Carl Zeiss, Jena, Germany). An argon laser was used as excitation source (514 nm) and light emission was detected in the range of 570–634 nm for red fluorescent protein (RFP) constructs and 535–545 nm for YFP constructs. Images were recorded and processed using LSM 510 3.2 software (Carl Zeiss, Jena, Germany).

### Generation and analysis of GUS reporter lines

β-Glucuronidase (GUS) reporter lines of gene *OPCL1* (At1g20510) were created using Gateway® Technology (Invitrogen, Carlsbad, CA, USA). A 1066 bp portion of the At1g20510 promoter was amplified by PCR of *A. thaliana* (Col-0) genomic DNA using primers P1-20510 (5′-attb1-ACGGATACCACTTCTGGAAG-3′) and P2-20510 (5′-attb2-CATTTGGCGGGAGTGGGATTGG-3′), and cloned into vector pJawohl11 (kindly provided by Dr B. Ülker, Max Planck Institute for Plant Breeding Research, Cologne, Germany) upstream of *uidA*. The resulting plasmid was transferred into *Arabidopsis* (Col-0) plants by *Agrobacterium*-mediated transformation (Bechtold *et al*., 1993). Transformants were selected on kanamycin-containing medium and lines homozygous for a single insertion identified in the $T_2$ generation. These lines were grown in soil and liquid MS medium and inspected for GUS expression at different developmental stages and after various treatments. For histochemical staining, seedlings or detached organs were placed in

the GUS substrate solution (50 mM sodium phosphate buffer, pH 7.0, 0.1% Triton X-100, 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide, and, after 5 min vacuum infiltration, samples were incubated overnight at 37 °C and subsequently destained by a series of washes in 80% ethanol (Jefferson, 1987).

For the analysis of the promoter response to different effectors, plants grown in hydroponic culture for 2 weeks were treated with various compounds dissolved in dimethylsulphoxide (DMSO; or only DMSO as control) and incubated for 24 h (or the time indicated). Subsequently, seedlings were either immediately used for histochemical GUS staining or, after removal of the medium, frozen in liquid nitrogen for subsequent quantitative determination of GUS activity in a microtitre plate-based assay as previously described (Sprenger-Haussels and Weisshaar, 2000). The quantitative analysis is based on at least three biological replicates.

### *RNA isolation and collection of gene expression data*

Total RNA was extracted from fully developed leaves of 4-week-old plants using RNAwiz (Ambion, Austin, TX, USA) according to the manufacturer's instructions. cRNA labelling, hybridization to the GeneChip ATH-1 (Affymetrix, Santa Clara, CA, USA), and collection of fluorescence data were performed as recommended by the manufacturer. The expression value of each gene chip was scaled globally to the value of 500 arbitrary fluorescence units, and the background was <59 arbitrary fluorescence units for all chips. The microarray data set has been deposited in the Array Express data library (http://www.ebi.ac.uk/arrayexpress/) with the accession number E-MEXP-546.

### *Synthesis of jasmonic acid precursors*

OPC derivatives were obtained as described by Miersch and Wasternack (2000). OPDA and dnOPDA were prepared from α-linolenic acid and (7*Z*,10*Z*,13*Z*)-hexadeca-7,10,13-trienoic acid, respectively, by a flax seed extract according to Vick *et al*. (1980), purified by reversed-phase (RP) HPLC, and checked for purity by GC-MS. (7*Z*,10*Z*,13*Z*)-hexadeca-7,10,13-trienoic acid could be obtained by alkaline saponification of a lipid extract from tomato leaves and separation of the 16:3 fatty acids by RP-HPLC. All compounds represent mixtures of stereoisomers, which were used without further separation.

### *Quantification of jasmonates*

Rosette leaves of 7- to 8-week-old *Arabidopsis* plants were wounded by squeezing the leaf blade six times with forceps, avoiding damage of the mid-vein. Plant material was collected at various times after treatment and frozen in liquid nitrogen. Jasmonates were extracted and quantified as previously described in at least three independent experiments (Stenzel *et al*., 2003*b*).

## Results

### *Large-scale* in vitro *substrate analysis of adenylate-forming enzymes*

To characterize functionally the group of nine putative adenylate-forming proteins from *Arabidopsis* (Group B of the phylogeny presented in Supplementary Fig. S1 at *JXB* online) that is closely related to 4CLs (Group A), a recently developed screen for substrates of CoA ester-forming enzymes was applied (Schneider *et al*., 2005). All of the cDNAs were cloned and the proteins were affinity purified via their attached N-terminal $His_6$ tags after heterologous expression in *E. coli*. As an exception, no correctly spliced cDNA variant of At1g20490 could be isolated from several *Arabidopsis* tissues or found in public expressed sequence tag (EST) databases. Thus, this protein was excluded from further analysis. The remaining eight proteins were subjected to the highly sensitive luciferase-based activity assay that determines ATP consumption by CoA-ligase reaction using cinnamic acids, fatty acids, all of the potential jasmonic acid precursors, and a broad range of additional organic acids. A decrease in the ATP concentration to <10% of its initial value after 2 h of incubation has previously been proven to represent a significant activity (Schneider *et al*., 2005). Only two proteins, At4g19010 and At1g20500, were capable of activating cinnamic acid derivatives, ferulic acid and sinapic acid, respectively (Table 1), but otherwise their substrate utilization pattern was distinct from that of established 4CLs. Four of the proteins showed a high and differential activity when tested with fatty acids of variable chain length (Table 1). At4g19010 showed the highest activity with short-chain fatty acids (C6, C7) but also converted C14 efficiently. At4g05160 displayed a preference for C6, C7, and long-chain fatty acids, whereas fatty acids of intermediate chain length were less efficiently converted. In contrast, At5g63380 accepted a broad range of medium- to long-chain fatty acids, whereas At1g20510 activated a narrower range of fatty acids excluding polyunsaturated fatty acids.

In view of previous findings (Schneider *et al*., 2005), it was of particular interest to analyse all of the proteins for their activity towards intermediates of JA biosynthesis. Indeed, four out of eight proteins displayed distinct and differential *in vitro* conversion of JA precursors to the corresponding CoA esters. Three proteins, At4g05160, At5g63380, and At1g20500, showed high activity towards only a single compound, namely OPC-6 or OPDA, whereas the protein At1g20510 accepted several substrates, OPDA, dnOPDA, OPC-8, and OPC-6 (Table 1), confirming and extending previous observations (Koo *et al*., 2006). Importantly, At1g20510 was the only enzyme with the capacity to activate OPC-8 efficiently. JA itself and its direct precursor OPC-4 were not converted by any of the proteins tested (Table 1).

Numerous additional substrate classes were tested in the activity screen, including methyl-, phenyl-, hydroxy-, and bifunctional fatty acids, as well as a broad range of benzoic acid derivatives and amino acids, but with few exceptions none was converted to the CoA ester to any significant extent (Supplementary Table S1 at *JXB* online). The proteins At1g62940, At1g20480, and At5g38120 were the only family members for which no appreciable or only marginal *in vitro* activity toward any of the tested substrates could be detected. It is concluded

**Table 1.** *Substrate utilization spectrum of* Arabidopsis *acyl-CoA synthetases*

Purified recombinant proteins were incubated for 2 h with the indicated substrates and residual ATP was determined by a luciferase-based assay as previously described (Schneider *et al*., 2005). Values represent relative luciferase activity, normalized to the reaction containing At4CL2 and sinapic acid in which no ATP consumption occurs (100%). Activities of residual ATP amounts <10% are shown in bold italics, 10–25% in bold, and 25–50% in italics. Note: some of the values for At4g05160 and At5g63380 have been reported previously (Schneider *et al*., 2005); they are included here for better comparison.

| Substrate | At4CL2 WT (%) | At1g62940 (%) | At4g19010 (%) | At4g05160 (%) | At5g63380 (%) | At1g20510 (%) | At1g20480 (%) | At5g38120 (%) | At1g20500 (%) |
|---|---|---|---|---|---|---|---|---|---|
| **Cinnamic acids** | | | | | | | | | |
| Cinnamic acid | 50 | 102 | **20** | 118 | 65 | 85 | 84 | 95 | 68 |
| 2-Coumaric acid | *33* | 72 | 85 | 69 | | 97 | 70 | 88 | 67 |
| 3-Coumaric acid | **5** | 76 | 81 | 88 | | 122 | 90 | 82 | 80 |
| 4-Coumaric acid | **4** | 102 | **18** | 106 | 50 | 81 | 69 | 72 | *47* |
| Caffeic acid | **4** | 97 | *37* | 96 | 69 | 92 | 68 | 81 | 61 |
| Ferulic acid | 50 | 107 | **5** | 148 | 94 | 123 | 98 | *47* | *27* |
| Sinapic acid | 102 | 105 | 74 | 104 | 120 | 84 | 82 | 91 | **8** |
| **Fatty acids** | | | | | | | | | |
| Acetic acid | 87 | 87 | 106 | 99 | 85 | 80 | 89 | 89 | 108 |
| Propanoic acid | 91 | 81 | 108 | 107 | 98 | 86 | 81 | 109 | 97 |
| Butanoic acid | 97 | 90 | 91 | 81 | 86 | 88 | 81 | 97 | 106 |
| Pentanoic acid | 110 | 85 | **16** | **23** | 67 | 80 | 104 | 77 | 91 |
| Hexanoic acid | *46* | 83 | ***6*** | ***1*** | *47* | 78 | 100 | 79 | 93 |
| Heptanoic acid | *26* | 76 | ***8*** | ***6*** | **20** | 63 | 107 | 78 | 87 |
| Octanoic acid | 70 | 85 | **18** | **18** | ***8*** | 78 | 101 | 102 | 118 |
| Nonanoic acid | 54 | 107 | **18** | *43* | ***0*** | 56 | 112 | 108 | 82 |
| Decanoic acid | 93 | 78 | *27* | **11** | ***1*** | ***9*** | 104 | 90 | 90 |
| Dodecanoic acid | 109 | 83 | **17** | **25** | ***3*** | ***0*** | 104 | 98 | *42* |
| Tetradecanoic acid | 85 | 59 | ***9*** | ***0*** | ***1*** | ***0*** | 94 | 88 | *45* |
| Hexadecanoic acid | 94 | 52 | **25** | ***0*** | ***2*** | ***3*** | 62 | 92 | 63 |
| Octadecanoic acid | 98 | *29* | 54 | ***0*** | ***0*** | ***10*** | 86 | 96 | 75 |
| Oleic acid (C18:1) | 84 | *47* | 63 | ***0*** | ***3*** | ***1*** | *46* | 61 | *45* |
| Linoleic acid (C18:2) | 109 | *43* | *25* | ***0*** | ***0*** | 78 | 83 | 77 | 49 |
| Linolenic acid (C18:3) | 111 | *46* | **17** | ***0*** | ***1*** | 94 | 97 | 99 | *48* |
| **Plant hormones and precursors** | | | | | | | | | |
| JA | 107 | 97 | 99 | 110 | 118 | 68 | 80 | 92 | 102 |
| OPC-4:0 | 95 | 80 | 88 | *47* | 89 | *48* | 83 | 95 | 93 |
| OPC-6:0 | 96 | 80 | 83 | ***5*** | 82 | ***1*** | 97 | 101 | ***4*** |
| OPC-8:0 | 111 | 92 | 88 | *41* | **22** | ***0*** | 96 | 94 | 62 |
| dnOPDA | 93 | 81 | 77 | *34* | 103 | ***7*** | 71 | 87 | 56 |
| OPDA | 101 | 92 | 92 | **21** | ***0*** | ***1*** | 74 | 105 | *26* |
| Indole-acetic acid (IAA) | 105 | 75 | 67 | 99 | 114 | 76 | 96 | 90 | 93 |
| Indole-propanoic acid (IPA) | 80 | 72 | 52 | 92 | | | 91 | 116 | 103 |
| Indole-butanoic acid (IBA) | 110 | 68 | 72 | 59 | 89 | 70 | 89 | 93 | 85 |

that these proteins may require modification, cofactors, or other particular conditions for activity, or, alternatively, utilize other substrates.

### *Complementation of bacterial growth by* Arabidopsis *acyl-CoA synthetases*

To confirm the observed fatty acyl-CoA synthetase activity by an *in vivo* method, a growth complementation test was applied to two *E. coli* strains, which are defective in fatty acid utilization. *Escherichia coli* wild type contains a single fatty acyl-CoA synthetase (FadD) which, together with the fatty acid transport protein, the enzymes of β-oxidation, and their transcriptional regulator (FadR), is an essential component of the fatty acid uptake and utilization system (DiRusso *et al*., 1999). To determine whether the *Arabidopsis* fatty acyl-CoA synthetases could complement *fadD* and support bacterial growth on fatty acids as sole energy and carbon source, the *E. coli fadD* mutant was transformed with expression vectors harbouring each of the proteins, and growth on agar plates and in liquid medium supplemented with oleic acid (C18:1) was examined. Growth on medium-length fatty acids (C12–C8) requires the absence of the transcriptional repressor FadR, which dissociates from *fad* genes upon binding of long-chain fatty acyl-CoA esters but not medium-chain acyl-CoA esters (DiRusso *et al*., 1999; DiRusso and Black, 2004). Correspondingly, to test for growth complementation on medium-chain fatty acids, the *E. coli fadD fadR* double mutant was transformed and tested for growth on media containing decanoic or dodecanoic acid.

The results presented in Fig. 1 clearly demonstrate that the enzymes showing high *in vitro* activity toward various fatty acids also supported growth of *E. coli* on various fatty acids. Beyond that, the enzymes showing weak *in vitro* activity towards fatty acids, i.e. At1g62940 and

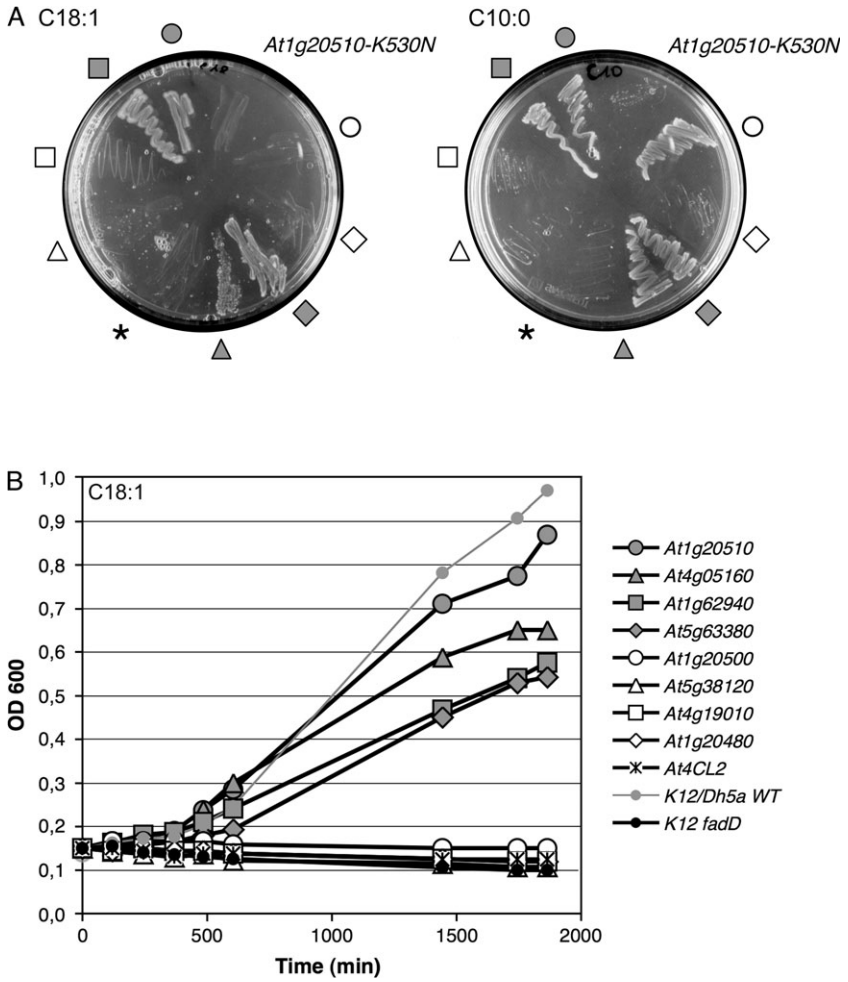


**Fig. 1.** Complementation of bacterial fatty acyl-CoA synthetase (*fadD*) by *Arabidopsis* acyl-CoA synthetases. *Arabidopsis* cDNAs were cloned into the pQE-32 expression vector under the control of the T5 promoter and the resulting plasmids transferred into two *E. coli* strains, LS1907 (*fadD::Kan*$^r$) and LS1908 (*ΔfadR fadD::Kan*$^r$), that are defective in the endogenous acyl-CoA synthetase (*fadD*) and hence cannot utilize fatty acids. The resulting transformed strains were monitored for growth (37 °C) in minimal medium (M9) containing 0.1% fatty acid as sole carbon source. (A) Plate assays showing growth of the indicated mutant strains (*fadD*) on oleic acid (C18:1) or double mutant strains (*fadD fadR*) on decanoic acid (C10:0). (B) Growth rates of bacterial strains in liquid medium containing oleic acid (C18:1).

At1g20500, also supported bacterial growth in liquid culture at rates indistinguishable from the more active enzymes (Fig. 1). Apparent discrepancies that cannot be explained yet are the lack of growth complementation by the enzyme At4g19010 and the differential growth support on medium containing oleic acid or decanoic acid by the enzyme At1g20500. In contrast, enzymes showing no appreciable activity towards fatty acids, e.g. At1g20480, At5g38120 as well as At4CL2 and At1g20510-K530N, which is defective in the active site, also did not support bacterial growth on fatty acids. In general, the growth complementation assays confirmed the results of the *in vitro* activity assay and, based on these properties, the investigated group of enzymes are defined as a new class of acyl-CoA synthetase accepting various fatty acids and fatty acid derivatives.

### *Catalytic efficiency of* Arabidopsis *acyl-CoA synthetases*

The critical assessment of substrate specificity and possible *in vivo* function of enzymes converting multiple substrates requires knowledge of their detailed kinetic properties. Therefore, all enzymes were subjected to a quantitative spectrophotometric assay, in which the rate of AMP formation by acyl-CoA synthetase is enzymatically coupled to NADH oxidation (Schneider *et al.*, 2005). Obviously, At1g20510 attracted particular attention since it activated a range of JA precursors.

All enzymes displayed Michaelis–Menten kinetics, and the derived kinetic parameters ($K_m$, $k_{cat}$, and $k_{cat}/K_m$) for their preferred substrates are summarized in Table 2. It is evident that protein At1g20510 readily converted OPDA, dnOPDA, OPC-8, OPC-6, and myristic acid (C14:0) with

**Table 2.** *Kinetic properties of* Arabidopsis thaliana *acyl-CoA synthetases that are active towards JA precursors*

Enzyme activity was determined by the lactate dehydrogenase-coupled assay with affinity-purified proteins and varying concentrations of the substrates listed. All values are the mean ±SD of at least three independent determinations.

| Substrate | At4g05160 | | | At5g63380 | | | At1g20510 | | | At1g20500 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) |
| Hexanoic acid | 75±18 | 1.26±0.09 | 16.7 | 6302±1548 | 2.79±0.25 | 0.4 | ND[a] | ND | – | ND | ND | – |
| Nonanoic acid | 6±1 | 1.38±0.12 | **230.7** | 2125±1700 | 19.87±9.68 | 9.4 | ND | ND | – | ND | ND | – |
| Tetradecanoic acid | 54±7 | 2.31±0.47 | 42.8 | 35±10 | 2.00±0.16 | 57.2 | 36±22 | 0.96±0.2 | 26.4 | ND | ND | – |
| OPDA | 93±36 | 0.30±0.08 | 3.2 | 11±5 | 2.62±0.93 | **237.9** | 27±13 | 1.56±0.4 | 56.2 | ND | ND | – |
| dnOPDA | ND | ND | – | ND | ND | – | 76±17 | 1.78±0.6 | 23.4 | ND | ND | – |
| OPC-8:0 | ND | ND | – | ND | ND | – | 19±4 | 1.27±0.1 | **65.4** | ND | ND | – |
| OPC-6:0 | 187±33 | 0.41±0.06 | 2.2 | ND | ND | – | 94±38 | 1.49±0.3 | 15.2 | 169±29 | 1.94±0.23 | **11.5** |

[a] ND, not determinable, activity too low, or no saturation with substrate. Highlighted in bold for each enzyme are the substrates converted with the highest catalytic efficiency ($k_{cat}/K_m$).

similar $k_{cat}$ but different $K_m$ values. With a $k_{cat}/K_m$ value of 65.4 $s^{-1}$ $mM^{-1}$, OPC-8 represented the most efficiently converted substrate of protein At1g20510, but OPDA ($k_{cat}/K_m$=56 $s^{-1}$ $mM^{-1}$) was converted with similar efficiency, whereas dnOPDA, OPC-6, and tetradecanoic acid showed significantly lower $k_{cat}/K_m$ values (Table 2). Substitution of the catalytic Lys530 in At1g20510 yielded a protein variant (At1g20510-K530N) that was inactive towards all substrates, demonstrating a specific enzymatic reaction (result not shown).

In the case of protein At1g20500, OPC-6 was the only substrate listed in Table 2 that was efficiently converted and therefore allowed the determination of kinetic constants, yielding a $k_{cat}/K_m$ value of 11.5 $s^{-1}$ $mM^{-1}$. The additional activity of At1g20500 with sinapic acid was verified by the standard 4CL assay, yielding a $K_m$ of 182±29 μM, $k_{cat}$ of 0.12±0.04 $s^{-1}$, and $k_{cat}/K_m$ of 0.66 $s^{-1}$ $mM^{-1}$, which is low activity in comparison with bona fide 4CLs (Schneider *et al*., 2003; Costa *et al*., 2005). For At4g05160 and At5g63380, it has previously been established that in addition to fatty acids, the JA precursors OPC-6 and OPDA were the preferred substrates (table 2 in Schneider *et al*., 2005).

Finally, At4g19010 allowed the determination of $K_m$ (47.5 μM), $k_{cat}$ (0.43 $s^{-1}$), and $k_{cat}/K_m$ values (9.1 $s^{-1}$ $mM^{-1}$) only for tetradecanoic acid. With decanoic, nonanoic, and shorter fatty acids (C6–C4), decreasing activities were observed, which could not be saturated at higher substrate concentrations and hence did not allow determination of accurate kinetic constants. The apparent activity of the enzyme with ferulic acid was likewise too low for activity determination in the standard 4CL assay.

Based on the highest catalytic efficiency ($k_{cat}/K_m$), the protein At1g20510 is classified as OPC-8:CoA ligase, previously designated OPCL1 (Koo *et al*., 2006), whereas the functions assigned to At1g20500 and At5g63380 are OPC-6:CoA ligase and OPDA:CoA ligase, respectively. In contrast, At4g05160 has to be considered a fatty acyl-CoA synthetase, with marginal albeit not negligible activity towards OPDA and OPC-6 (Table 2).

### *Expression patterns of acyl-CoA synthetases*

JA biosynthesis in plants is stimulated by various conditions, such as infection, wounding, and treatment with jasmonates (Stenzel *et al*., 2003*a*, *b*; Schaller *et al*., 2005), and the enzymes participating in this pathway are known or expected to be co-regulated. Thus, to analyse the correlation of the new group of acyl-CoA synthetases with JA biosynthesis, transcript profiles of infected *Arabidopsis* leaves were analysed using Affymetrix whole genome chips.

In unchallenged *Arabidopsis* (Ws-0) leaves, the highest steady-state mRNA levels were detected for At4g05160, OPDA:CoA ligase (At5g63380), OPCL1 (At1g20510), and At1g20490, whereas transcripts of the remaining

family members were barely detectable (Fig. 2). Upon infection of the leaves with *Pseudomonas syringae* pv. *tomato* (*Pst*) carrying *avrRpm1*, a strong increase in the amount of mRNA for OPCL1 (At1g20510) was observed (Fig. 2). In fact, this was the only transcript of any of the acyl-CoA synthetases that increased in abundance. Inoculation of leaves with *Pst* carrying *avrRps4* yielded similar results (not shown). Control treatment of leaves with $MgCl_2$ did not significantly affect expression of any of the gene family members. When analysing the expression of other genes encoding JA biosynthetic enzymes, LOX3 (At1g17420), AOC3 (At3g25780), OPR3 (At2g06050), and ACX1 (At1g16760) were found to be co-regulated with OPC-8:CoA ligase (At1g20510), whereas LOX2 (At3g45140) and AOS (At5g42650) mRNAs were highly abundant in both $MgCl_2$-treated and infected leaves, which indicates stress-induced expression resulting from the infiltration process (Fig. 2). LOX1, AOC1, and AOC4 mRNAs were barely detectable (Fig. 2), as were ACX2-5 mRNAs (not shown).

In an independent experiment, it was verified that MeJA treatment also caused a strong and transient accumulation of OPCL1 (At1g20510) mRNA with a time course identical to that of AOS (result not shown). Collectively, these results demonstrate a tight co-regulation of OPCL1 (At1g20510) with other enzymes involved in JA biosynthesis.

### *OPC-8:CoA ligase participates in JA biosynthesis*

Two independent T-DNA insertion lines of OPCL1 (At1g20510) of *Arabidopsis* (Col-0) were obtained from the SALK (KO-1) and GABI-Kat (KO-2) collections and compared with wild-type and knockout (KO) lines defective for other single acyl-CoA synthetases and combinations thereof, in particular At4g05160 and At5g63380, as these enzymes were also active towards JA precursors. Homozygous plants were selected by PCR analysis (Supplementary Fig. S2 at *JXB* online) and subjected to phenotypic and biochemical characterization. None of these plants showed any obvious differences in growth, root and flower development, fertility, reproduction, or morphology compared with wild-type plants (results not shown), despite the fact that semi-quantitative RT-PCR analysis with gene-specific primers failed to detect mRNA of the *OPCL1* (At1g20510) gene, which in Col-0 wildtype accumulated upon wounding (Fig. 3). A similar wound-induced transcript accumulation was observed for other JA biosynthetic genes, e.g. *AOS* and *OPR3*, in both the At1g20510-KO lines and Col-0 wildtype (Fig. 3). In contrast, the transcript levels of acyl-CoA synthetases with potentially redundant functions in JA biosynthesis, i.e. At4g05160, At5g63380, and At1g20500, remained unaltered in wounded mutant and wild-type plants or were undetectable (Fig. 3). From these results it is apparent that the At1g20510-KO lines represent mRNA null

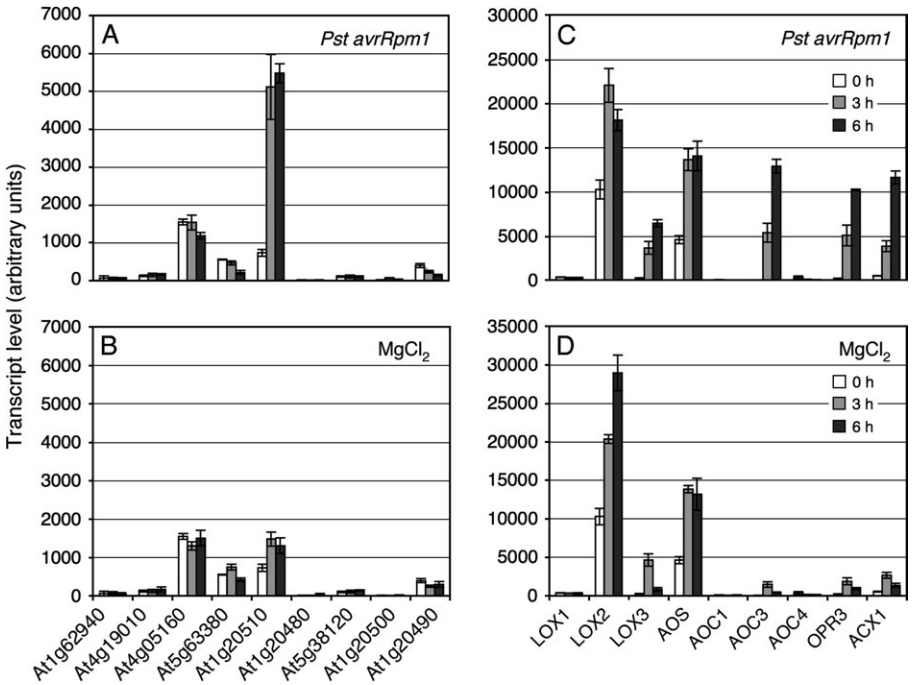


**Fig. 2.** Expression levels of acyl-CoA synthetases and JA biosynthetic genes in *Arabidopsis* leaves in response to infection with *Pseudomonas syringae* pv. *tomato* (*Pst*). Total RNA was extracted from leaves of 4-week-old *Arabidopsis* plants that were infiltrated with *Pst* (DC3000) expressing the avirulence gene *avrRpm1* (A, C) or mock-treated with 10 mM $MgCl_2$ (B, D) for the times indicated. Relative transcript levels were determined as described in Materials and methods using *Arabidopsis* genome microarrays (Affymetrix ATH1 genome array).

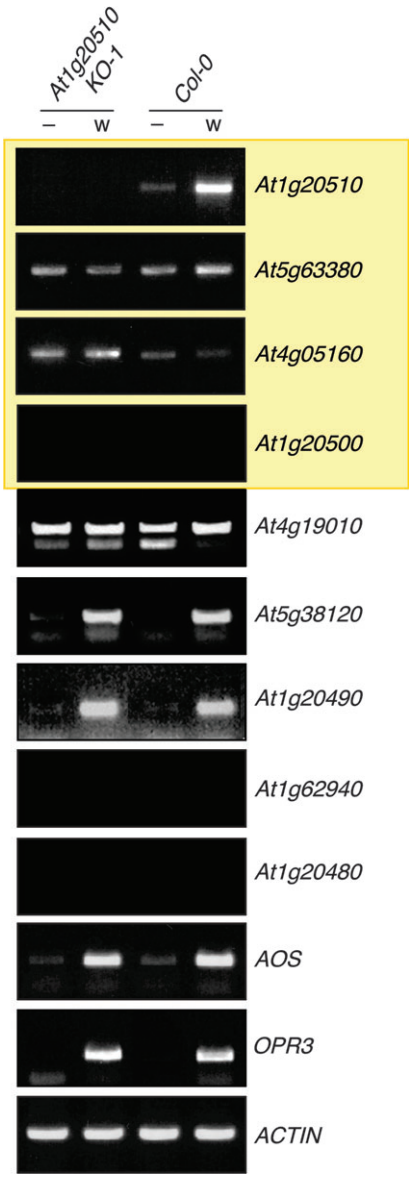


**Fig. 3.** Expression of acyl-CoA synthetase genes in response to wounding of leaves of *Arabidopsis* wild-type plants (Col-0) and lines defective for OPC-8:CoA ligase (At1g20510-KO). RT-PCR of RNA isolated from leaves of the *Arabidopsis OPCL1* knockout line (At1g20510-KO-1) and wild-type plants (Col-0) before and 1.5 h after wounding. The acyl-CoA synthetases active towards JA precursors are marked by the yellow box. *Actin* mRNA was used to normalize transcript levels in each sample, and all primer pairs were designed such that the product spanned the first intron (see Materials and methods). For RNAs that could not be detected in leaves (e.g. At1g20500, At1g62940, and At1g20480), the functionality of the primer pairs was proven with RNA isolated from different organs (e.g. roots, flowers, and siliques); in all cases products of the predicted sizes were obtained. Similar results were obtained with the second, independent KO-line (At1g20510-KO-2) derived from the GABI-Kat collection (not shown).

phenotypes and further established that *OPCL1* is co-expressed with other JA biosynthetic genes. Based on their induced expression in response to wounding, At5g38120 and At1g20490 may represent alternative candidates for participating in JA biosynthesis although functional evidence for this is lacking. The genes encoding the remaining acyl-CoA synthetases showed a differential mode of expression in wounded *Arabidopsis* leaves, which was either constitutive (*At1g19010*) or undetectable (*At1g62940* and *At1g20480*) (Fig. 3).

Next jasmonate profiles were determined in leaves of *Arabidopsis* wild-type (Col-0) and *opcl1* mutant (At1g20510-KO-1) plants. Wounding caused a transient, >20-fold increase in the amount of JA in wild-type plants, but in the mutants JA levels were consistently found to be ~40% lower at all time points analysed (Fig. 4A). Corresponding profiles were also established for other jasmonates (Supplementary Fig. S3 at *JXB* online), but for clarity only their relative amounts at 1.5 h after wounding are presented in Fig. 4B. From these results it is apparent that OPC-8 accumulated to ~2-fold higher levels in wounded leaves of the *opcl1* mutant (At1g20510-KO-1 line) in comparison with wild-type plants. Interestingly, OPC-6 and OPC-4 also hyper-accumulated significantly in the mutant, whereas the levels of OPDA and dnOPDA, although also strongly increased upon wounding, were not significantly different in leaves of mutant and wild-type plants (Fig. 4B). Similar alterations of JA levels were determined for the second *opcl1* mutant (At1g20510-KO-2 line), which is homozygous for an independent T-DNA insertion (derived from the GABI-Kat collection), and the triple mutant At1g20510-KO-1 At4g05160-KO At5g63380-KO, whereas in the double mutant At4g05160-KO At5g63380-KO, and the corresponding single mutants, the JA contents remained unchanged in comparison with wild-type plants (Supplementary Fig. S4 at *JXB* online). From these results, it is concluded that only mutation of the *OPCL1* gene caused a partial metabolic block in the JA biosynthetic pathway downstream of OPC-8, whereas an impact of the other jasmonate-activating acyl-CoA synthetases was undetectable under the conditions tested. This provides strong evidence that the protein At1g20510 functions as OPC-8:CoA ligase *in vivo* and that it contributes to ~40% of wound-induced JA biosynthesis.

### *Cellular localization and expression pattern of OPC-8:CoA ligase*

In order to fulfil a role in JA biosynthesis *in vivo*, OPCL1 (At1g20510) is expected to reside in the peroxisome, where its predicted substrate, OPC-8, is generated from OPDA by the action of OPR3. To confirm that the C-terminal tripeptide (-SKL) that is present at the C-terminus of the protein indeed functions as a peroxisomal targeting signal, a YFP-tagged version of OPCL1 (At1g20510) was transiently expressed together with a RFP-tagged peroxisomal marker protein in *Arabidopsis* leaves (Schneider *et al.*, 2005). Confocal imaging revealed a punctate labelling that is characteristic for peroxisomes, and

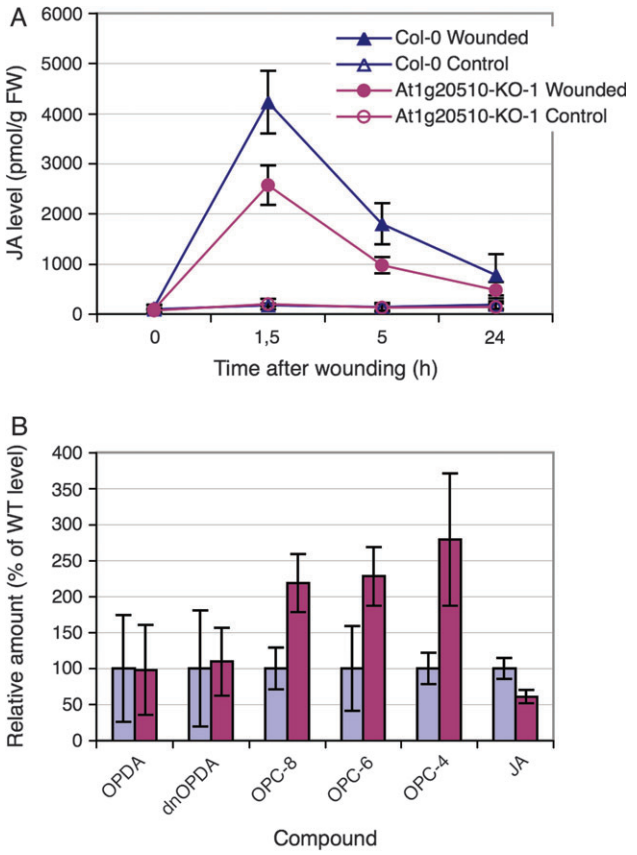


**Fig. 4.** Wound-induced accumulation of JA and its biosynthetic precursors in *Arabidopsis* leaves. Leaves of 5-week-old *Arabidopsis* wild-type (Col-0) and *opcl1* mutant plants (At1g20510-KO-1 line) were wounded six times with tweezers, collected 0, 1.5, 5, and 24 h after the onset of treatment, immediately frozen, and stored in liquid nitrogen for subsequent extraction and analysis of oxylipins by HPLC/GC-MS (see Materials and methods). The data show the mean and SD of at least three independent determinations. (A) Time course of wound-induced JA accumulation in leaves of wild-type (Col-0) and *opcl1* mutant plants (At1g20510-KO-1). (B) Relative levels of JA and different precursors at 1.5 h after wounding of *opcl1* mutant (At1g20510-KO-1) plants (red bars), with 100% representing the level of the corresponding compound in wild-type (Col-0) plants (blue bars).

a complete three-dimensional overlap of yellow and red fluorescent particles (Fig. 5), demonstrating that OPC-8:CoA ligase is targeted to peroxisomes.

To study the organ- and tissue-specific expression of the *OPCL1* gene, a 1 kb promoter sequence was fused to the GUS gene and integrated into the *Arabidopsis* genome. Several independent transgenic lines homozygous for a single copy of the reporter gene were analysed for their GUS activity. Seedlings grown for 2 weeks in hydroponic culture showed weak GUS expression in the vascular system of cotyledons, leaves, hypocotyls, and roots, as well as in hydathodes (Fig. 6A). Upon MeJA application to the medium, expression of *OPCL1p::GUS* was strongly up-regulated, leading to intensified GUS staining of the vascular system throughout the whole plant (cotyledons, leaves, hypocotyl, and roots) and lower expression in the rest of the leaf lamina (Fig. 6B, C). These results indicate that the transgene comprises a functional promoter mediating expression similar to the endogenous gene. Closer inspection of *OPCL1p::GUS* expression in the roots of MeJA-treated plants revealed high GUS activity in the zone of cell division proximal to the root tip, which itself was devoid of staining (Fig. 6D). In the elongation zone, only weak staining of a few superficial cells was observed. The vascular system also showed strong staining (Fig. 6D), as did primordial cells of lateral roots (Fig. 6F, G). At the tip, lateral roots showed a staining pattern similar to the primary root (Fig. 6H).

In mature plants, expression of *OPCL1p::GUS* was also primarily observed in the vasculature of leaves, stems, and roots, although increasing expression in mesophyll cells of the leaf was observed as the plants aged (Fig. 6I). Conditions that led to increased levels of jasmonates, such as wounding, also led to increased GUS staining in plants expressing *OPCL1p::GUS* (Fig. 6I). In flowers and siliques of different developmental stages, likewise no appreciable expression of *OPCL1* could be detected, although weak GUS staining was observed in the young parts of the bolting stem (Fig. 6J, K), whereas in old cauline leaves expression was clearly detectable similar to that in old rosette leaves (not shown).

Analysis of different *OPCL1p::GUS* plant lines revealed that treatment for 24 h with 100 μM MeJA resulted in maximum GUS activity, which was >20-fold higher than in untreated plants (Fig. 7A). Using the same experimental conditions, the gene-activating capacity of various additional jasmonates was explored, including biosynthetic intermediates and synthetic derivatives. The results demonstrate that OPC-4, OPC-6, and the synthetic structural JA analogue indanoyl-isoleucine activated the *OPCL1p::GUS* reporter gene to the same extent as JA or MeJA, whereas OPC-8, OPDA, and coronalon were even superior activators (Fig. 7A). In contrast, dnOPDA, γ-linolenic acid (LinA), and the derivatives cucurbic acid and 12-hydroxy-jasmonic acid (12-OH-JA) activated the reporter gene only weakly, whereas jasmonic acid-12-sulphate (12-$SO_4$-JA) was completely inactive, similar to its lack of activation of numerous other JA-related genes (Miersch *et al.*, 2008). Efficient activation of *OPCL1p::GUS* by OPC-8 may indicate the existence of new regulatory circuits in the JA biosynthesis and signalling network, such as positive feed-forward regulation of the biosynthetic gene, *OPCL1*, by the substrate, OPC-8, of the encoded protein, OPC-8:CoA ligase. Alternatively, OPC-8 may mimic the activity of OPDA. In contrast, the promoter of the *LOX2* gene, which also strongly responded to JA and MeJA, was not activated by OPC-8 and only weakly stimulated by OPDA (Fig. 7B).

When 2-week-old plants containing the *OPCL1p::GUS* gene were inoculated with *Pst* carrying *avrRpm1* or *avrRps4* by vacuum infiltration, increased expression of

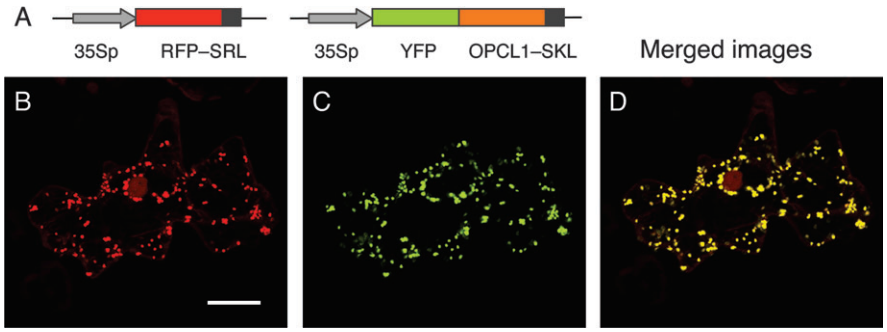


**Fig. 5.** Subcellular localization of OPC-8:CoA ligase in *Arabidopsis* leaves. (A) Structural features of the reporter constructs 35Sp::RFP-SRL, serving as peroxisomal marker, and 35Sp::YFP-OPCL1-SKL, comprising the natural C-terminus -SKL, both under the control of the cauliflower mosaic virus 35S promoter (35Sp). Both expression plasmids were co-transformed into *Arabidopsis* leaves by particle bombardment. Single epidermal cells were inspected by confocal laser scanning microscopy using appropriate filter sets for simultaneous and selective recording of RFP (B) and YFP (C) fluorescence. Optical sections were used for the reconstruction of three-dimensional images of the cellular labelling patterns. The YFP signals coincide with the RFP fluorescence of the peroxisomal marker as evident from the merged images (D), clearly demonstrating that OPC-8:CoA ligase (At1g20510) is targeted to peroxisomes. Scale bar=25 μm.

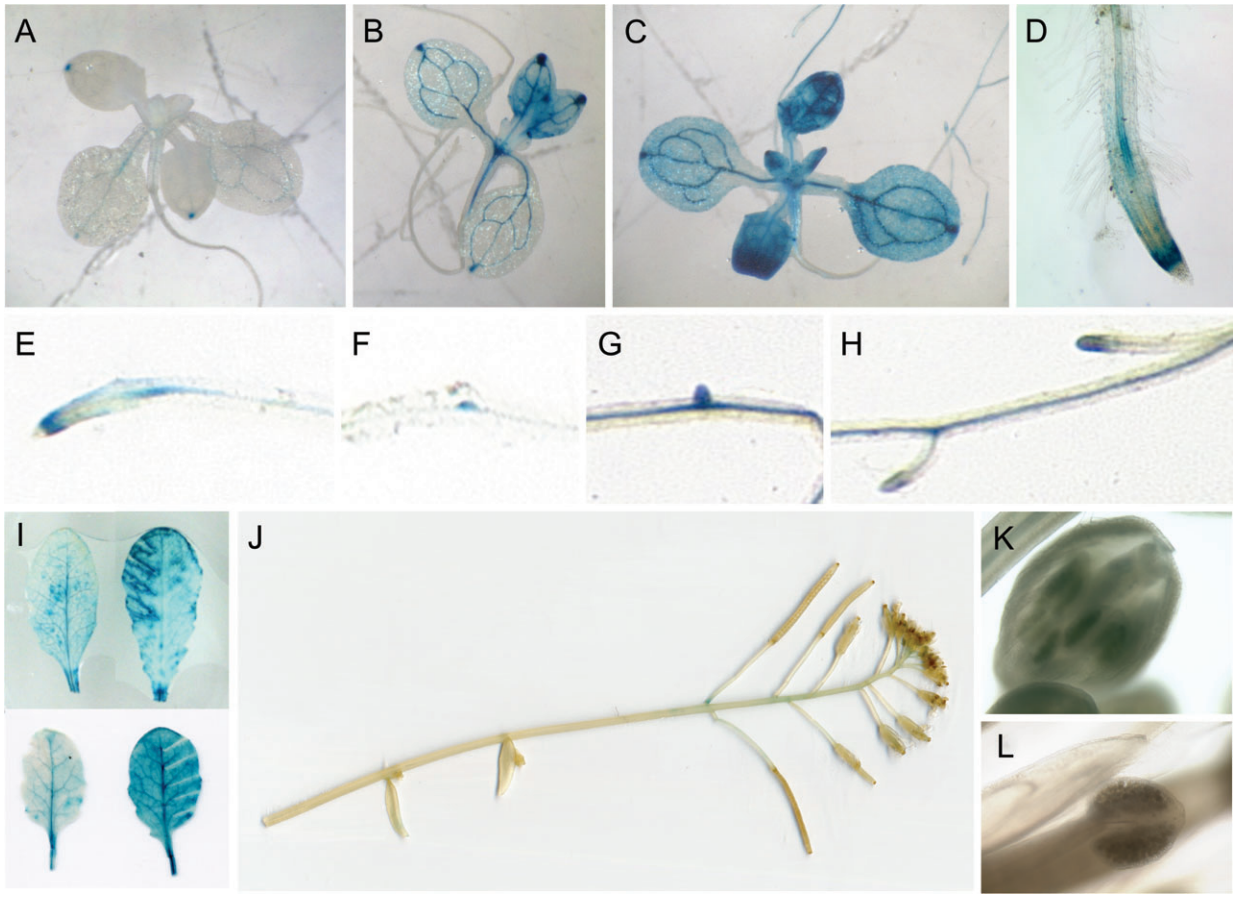


**Fig. 6.** Developmental and tissue-specific expression of the *OPCL1* (At1g20510) gene. *Arabidopsis* lines homozygous for a single insertion of the reporter gene, comprising a 1 kb promoter of the *OPCL1* gene and the GUS reporter, were generated and selected as outlined in Materials and methods. Reporter activity was revealed by X-gluc staining. (A) A seedling germinated and grown for 2 weeks in liquid medium. (B and C) Two-week-old seedlings (line 1-2) after treatment for 24 h with 10 μM and 100 μM MeJA, respectively. (D) Root tip of a 2-week-old seedling after treatment with MeJA (100 μM for 24 h). (E–H) Consecutive root portions along the main axis of 2-week-old plants grown in liquid culture following treatment with MeJA (100 μM for 24 h). (I) Leaves of a 6-week-old plant before and after wounding for 1.5 h (top) or 24 h (bottom). (J) Bolting stem and flowers of an 8-week-old plant. (K) Flower bud. (L) Anther.

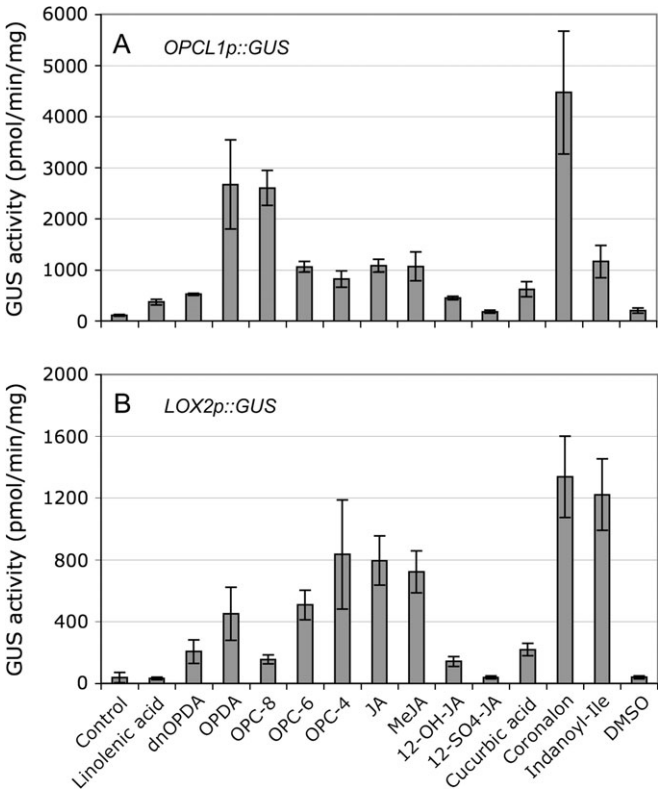


**Fig. 7.** Activation of the *OPCL1* promoter by different jasmonates. *Arabidopsis* plants homozygous for a single insertion of the reporter gene, comprising a 1 kb promoter of the *OPCL1* gene (line 1-2), were grown in liquid medium for 2 weeks and were treated with the indicated compounds (20 ppm) for 24 h (A). Similarly, transgenic *Arabidopsis* plants containing the *LOX2p::GUS* reporter gene were treated under the same conditions (B). GUS activity was quantified in plant extracts using 4-methylumbelliferyl-β-D-glucuronide as substrate in a fluorimetric assay and related to total extractable protein. The data represent the average of at least three biological replicates (±SD).

the reporter gene was observed (Supplementary Fig. S5 at *JXB* online), which is in accordance with the transcript accumulation pattern (Fig. 2).

In conclusion, the gene encoding OPC-8:CoA ligase (At1g20510) shares many features with other JA biosynthetic genes and, together with the *in vitro* and *in vivo* properties of the encoded protein, this strongly indicates an essential function in jasmonate biosynthesis.

## Discussion

In the present study, the function of a group of proteins with high sequence similarity to 4CLs but exhibiting the catalytic capacity to activate long-, medium-, and short-chain fatty acids to the corresponding CoA esters was systematically explored. Two interesting features are connected with this new type of acyl-CoA synthetase: (i) individual enzymes displayed a broad but distinct spectrum of substrate (fatty acid) utilization; and (ii) four of them also showed high activity towards different JA precursors.

The first finding was rather unexpected because of the abundance of known fatty acid-activating proteins in *Arabidopsis*, which include nine LACSs and two additional proteins, AAE7 (At3g16910) and AAE11 (At1g66120), that preferentially activate butanoic and hexanoic acid, respectively, thus representing short-chain fatty acyl-CoA synthetases (Shockey *et al*., 2002, 2003). In comparison with other organisms, *Arabidopsis* contains an unusually large number of fatty acid-activating enzymes. In this context, it is noteworthy that the 4CL-like proteins At4g19010 and At4g05160 are unique amongst their family members by likewise showing high activity towards short-chain fatty acids, in particular hexanoic and heptanoic acid. The biological significance of such enzymatic activity is currently unknown, but in several plants short- to medium-chain fatty acids, such as nonanoic acid, have been found to inhibit seed germination and to impair membrane functions such as ion transport (Berrie *et al*., 1979; Metzger and Sebesta, 1982; Babiano *et al*., 1984). Whether or not this implies a biological role for nonanoic acid in plants is not known but, given the inhibitory effect on plant growth and development, it seems essential to prevent accumulation of medium-chain fatty acids, which could potentially arise from premature hydrolysis of acyl-CoA intermediates during fatty acid β-oxidation. Peroxisomes harbour the complete β-oxidation machinery, including at least one acyl-CoA thioesterase, ACH2, which displays high levels of activity towards a wide range of acyl-CoA substrates (Tilton *et al*., 2004). It is proposed that acyl-CoA synthetases acting on short- and medium-chain fatty acids may serve an important salvage function for substrates that are not or only inefficiently activated by the conventional LACS system. This may also apply to short- and medium-chain fatty acids generated in other cellular compartments, and hence the function of peroxisomal acyl-CoA synthetases may include the vectorial translocation of fatty acids similar to the established roles of FadD in *E. coli* or of Faa1p and Faa4p in *Saccharomyces cerevisiae* (Færgeman *et al*., 2001; Weimar *et al*., 2002; Zou *et al*., 2003; DiRusso and Black, 2004). In accordance with such function, most, but not all, of the new acyl-CoA synthetases complemented the *fadD* mutation in *E. coli*. The lack of growth complementation by some of the active enzymes is not a unique finding, because, similarly, only one of the five LACS isoforms from rat that are active towards fatty acids *in vitro* was able functionally to substitute *E. coli* FadD regarding growth (Caviglia *et al*., 2004).

The second result of the present investigation was the identification of enzymes capable of converting JA precursors to the corresponding CoA esters. It has generally been assumed that JA biosynthesis involves three cycles of β-oxidation, but until recently it was not known whether these steps are catalysed by specific

enzymes or the general fatty acid degradation machinery localized in the peroxisomes (Miersch and Wasternack, 2000; Schaller *et al*., 2005; Baker *et al*., 2006). In any event, conversion of the carboxylic acid substrate to the corresponding CoA ester is required to initiate β-oxidation (Fig. 8). There has been a previous report on two adenylate-forming enzymes, At4g05160 and At5g63380, having the capacity to activate the JA precursors OPC-6 and OPDA *in vitro* (Schneider *et al*., 2005). Here, two additional enzymes with the potential to contribute to JA biosynthesis were characterized, the protein At1g20500 selectively activating only OPC-6, and OPC-8:CoA ligase (OPCL1), which showed a relatively broad substrate specificity by also accepting other JA pathway intermediates (Table 1), a property that was not previously recognized. It should be noted that OPCL1 (At1g20510) has recently been identified independently as OPC-8:CoA ligase that participates in JA biosynthesis using detailed

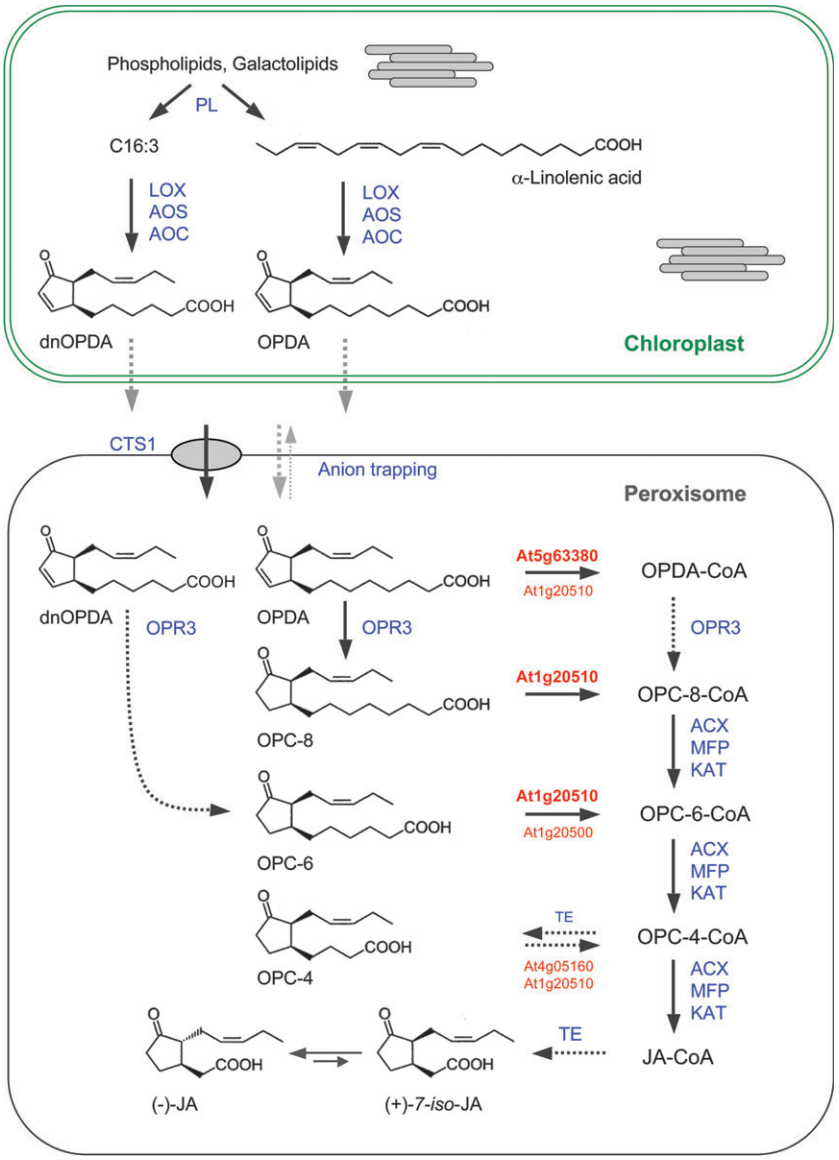


**Fig. 8.** Pathways of JA biosynthesis. JA biosynthesis is initiated in the chloroplast starting from α-linolenic acid (C18:3) or hexadecatrienoic acid (C16:3), which are released from membrane lipids by phospholipase (PL) and converted to OPDA and dinor-OPDA (dnOPDA), respectively, by consecutive action of lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC). Proteins facilitating export of OPDA and dnOPDA from the chloroplast are unknown; active uptake of both compounds by peroxisomes is mediated by the ABC transporter COMATOSE (CTS1) but may also include passive mechanisms (anion trapping). Within the peroxisome, OPDA and dnOPDA are converted by oxophytodienoic acid reductase 3 (OPR3) to OPC-8 and OPC-6, respectively. Conversion of all of these acids to the corresponding CoA esters by the indicated acyl-CoA synthetases (labelled in red) is a prerequisite for β-oxidative shortening of the side chain catalysed by consecutive action of acyl-CoA oxidase (ACX), multifunctional protein (MFP), and ketoacyl-CoA thiolase (KAT), yielding jasmonoyl-CoA from which (+)-7-*iso*-JA is released by thioesterase (TE) and spontaneously epimerized to (–)-JA. Dotted arrows indicate putative reactions for which the participation of specific enzymes has not yet been demonstrated.

co-expression analysis as an alternative approach (Koo *et al*., 2006), instead of a biochemical screening strategy that was applied here, but its biochemical properties have not been described.

Knowledge of their biochemical properties allows the acyl-CoA synthetases activating JA precursors to be precisely positioned in a revised scheme of JA biosynthesis (Fig. 8), which underscores the functional redundancy at the level of CoA ester formation and the essential role of this step for β-oxidation to occur.

The four recombinant proteins capable of converting JA precursors to CoA esters showed significant differences in substrate specificity, which may point to different biological functions. The protein At1g20500 selectively activated OPC-6 and sinapic acid, but was inactive towards fatty acids *in vitro*. At4g05160 activated OPC-6 and OPDA, although with considerably lower efficiency than fatty acids, whereas OPC-8 and dnOPDA were not converted. The protein At5g63380 selectively activated OPDA, in addition to fatty acids. In contrast, OPCL1 (At1g20510), the only enzyme identified so far that catalysed efficient CoA ester formation of OPC-8, also activated OPDA, dnOPDA, OPC-6, and long-chain fatty acids (e.g. C14) (Table 2). Thus, the presence or absence of the double bond in the cyclopentenone ring of the substrate (OPDA versus OPC-8) did not restrict its utilization by OPC-8:CoA ligase, nor did the length of the carboxylic acid side chain, at least within the limit of C8–C6. It should be noted that OPC-4 was not efficiently activated, neither by OPCL1 nor by any other protein analysed to date.

In spite of these *in vitro* activities, OPCL1 (At1g20510) is the only acyl-CoA synthetase for which an essential *in vivo* contribution to JA biosynthesis has now been firmly established (this work and data published by Koo *et al*., 2006). Two independent mutants defective for the gene consistently showed reduced JA accumulation upon wounding, with a concomitant accumulation of OPC-8, OPC-6, and OPC-4, which is in accordance with a partial metabolic block of the predicted conversion of OPC-8 to the CoA ester. The persistence of ~50–60% of the JA level found in wild-type plants indicates that OPCL1 (At1g20510) is responsible for only a part of the wound-induced JA production. However, the identity of alternative acyl-CoA synthetases contributing to JA biosynthesis remains unknown since mutants defective for the other enzymes that activate JA precursors, in particular At4g05160, At5g63380, At1g20500, and various combinations of double and triple mutants, did not display any significant alteration in would-induced JA accumulation (Supplementary Fig. S4 at *JXB* online; and similar results published by Koo *et al*., 2006). Collectively, the present data point to the existence of alternative biosynthetic routes for JA production in *Arabidopsis*, a conclusion also derived from the recent analysis of the acyl-CoA oxidase (ACX) protein family (Schilmiller *et al*., 2007).

The specific and selective activation of OPDA (and no other jasmonate) by the protein At5g63380 further indicates that alternative routes of JA production may operate, at least under certain restrictive conditions, in addition to the generally accepted pathway outlined in Fig. 8, comprising: (i) OPDA import into peroxisomes; (ii) reduction of OPDA by OPR3; (iii) conversion of OPC-8 to the CoA ester; (iv) β-oxidative shortening of the side chain; and (v) release of JA from jasmonoyl-CoA (Li *et al*., 2005; Baker *et al*., 2006; Delker *et al*., 2006; Schilmiller *et al*., 2007). Such an alternative scenario may include facilitated import of OPDA into peroxisomes mediated by OPDA-CoA ligase (At5g63380), similar to the vectorial translocation of fatty acids in *E. coli* and yeast mediated by FadD and Faa1p/Faa4p, respectively. However, for this step to contribute to JA production, the imported OPDA-CoA must be reduced to OPC-8-CoA by OPR3 (Fig. 8).

At present, little is known about the mechanisms of OPDA translocation from the plastid, where it is synthesized and stored in galactolipids and phospholipids (Stelmach *et al*., 2001; Andersson *et al*., 2006; Buseman *et al*., 2006), to the peroxisome. Recent evidence indicates that the ABC transporter CTS1 contributes to OPDA import into peroxisomes (Theodoulou *et al*., 2005). However, the *cts* mutant retained significant levels of JA, indicating alternative routes of OPDA translocation into peroxisomes, either by a passive mechanism, such as ion trapping (Theodoulou *et al*., 2005), or by an unidentified transporter system, which may include or operate in combination with the OPDA-CoA ligase (At5g63380).

The mutants defective for OPCL1 (At1g20510) accumulated not only OPC-8, as predicted from its main activity, but also OPC-6, which is not surprising in view of the activity profile of the protein (Table 2). OPC-6 originates from dnOPDA (Fig. 8), and in the mutant a considerable fraction of the total OPC-6-activating activity has been lost. The remaining CoA ligases apparently activate OPC-6 less efficiently (e.g. At4g05160) or are expressed at only a very low level in leaves (e.g. At1g20500). In contrast, the origin and fate of OPC-4, which also accumulated in the mutant, are intriguing. No pathway specifically generating OPC-4 is known, and as an intermediate of β-oxidation the unesterified compound is not expected to appear. This may suggest its release from the CoA ester intermediate by the unspecific action of thioesterase, which is predicted to hydrolyse the end-product of β-oxidation, jasmonoyl-CoA (Fig. 8). Lack of a specific enzyme catalysing CoA ester formation of OPC-4 may account for its 10-fold higher levels of accumulation compared with OPC-8 and OPC-6. However, the transient nature of OPC-4 accumulation also suggests that metabolizing enzymes must exist, e.g. the low activities displayed by At4g05160 or At1g20510 may be sufficient (Table 1).

Expression analyses of the *OPCL1* gene applying RNA blot analysis, RT-PCR, and an *OPCL1* promoter–reporter construct revealed that OPCL1 was the only acyl-CoA synthetase that was up-regulated by infection of *Arabidopsis* with *P. syringae* concomitant with specific isoforms of other JA biosynthetic genes such as *LOX3*, *AOC3*, *OPR3*, and *ACX1* (Fig. 2). Other members of the latter gene families were either constitutively expressed in *Arabidopsis* leaves and not induced by infection (*LOX2* and *AOS*) or expressed at a very low level (*LOX1*, *AOC1*, and *AOC4*), suggesting that they encode non-redundant functions (Delker *et al*., 2006). The recent finding that activated expression of JA biosynthetic genes, including *OPCL1* (At1g20510), in response to *P. syringae* occurred not only in directly inoculated *Arabidopsis* leaves but also systemically, lends further support to the hypothesis that jasmonates (JA or one of its derivatives) may act as a long-distance systemic signal mediating systemic acquired resistance (Truman *et al*., 2007). This concept is also supported by grafting experiments using JA-deficient and JA signalling mutants of tomato (Schilmiller and Howe, 2005). The fact, that *OPCL1* expression is particularly pronounced in the vasculature of *Arabidopsis* roots and leaves is in accordance with such a role. In tomato, expression of *AOC* and accumulation of JA have also been demonstrated to occur preferentially in the vascular system (Hause *et al*., 2003; Stenzel *et al*., 2003*a*).

JA biosynthesis is subject to control by a positive feedback loop, i.e. the expression of all JA biosynthetic genes, including *OPCL1* (At1g20510), is activated by JA or MeJA (Stenzel *et al*., 2003*b*; Castillo *et al*., 2004; Taki *et al*., 2005; Delker *et al*., 2006). When analysing the activating potential of different jasmonates on reporter gene expression mediated by the *OPCL1* promoter in transgenic plants, it was observed that, in comparison with JA or MeJA, treatment of transgenic seedlings with OPDA, OPC-8, and coronalon resulted in much higher activities (Fig. 7A). A signalling function of OPDA that is distinct from that of JA or MeJA has previously been established (Weiler *et al*., 1994; Stintzi *et al*., 2001; Taki *et al*., 2005) and has been related to the electrophilic properties of the reactive α,β-unsaturated carbonyl moiety that is present in the molecule (Stintzi *et al*., 2001; Almeras *et al*., 2003). However, the comparable activities of OPC-8 and OPDA and the almost complete lack of activity of dnOPDA, which also contains an unsaturated α,β-unsaturated carbonyl group, suggest that the double bond in the cyclopentenone ring is not important for the differential signalling function of OPDA versus JA (Fig. 7A). Similar conclusions were drawn from recent expression profiling experiments using different jasmonates (Taki *et al*., 2005). Whether or not OPC-8 is a signalling molecule in its own right, rather than a structural and functional mimic of OPDA, remains to be established. Its activity may point to the existence of a previously undescribed feed-forward regulatory loop in the jasmonate signalling network, by which the gene encoding a JA biosynthetic protein is activated by the substrate of the respective protein. Such a mechanism seems biologically plausible, but its function *in vivo* still needs to be confirmed, although the lack of activation of *LOX2* gene expression by OPC-8 provides additional evidence in support of such a mechanism. The much lower or even missing activation of *OPCL1* gene expression by 12-OH-JA or 12-$HSO_4$-JA, respectively, corresponds to recent results obtained with tomato, which also demonstrate that both compounds failed to activate JA biosynthetic genes and wound-responsive genes (Miersch *et al*., 2008). This led to the conclusion that the abundant JA derivatives 12-OH-JA and 12-$HSO_4$-JA are products of the mechanism involved in switching off the JA signalling pathway, at least for a subset of genes.

The detailed biochemical and functional analysis of a previously uncharacterized group of acyl-CoA synthetases provides new insight into lipid metabolism as well as the JA biosynthesis and signalling networks. The results reported here build the foundation for additional work that is required to understand fully the role of different jasmonates in plant defence and development. It is these kinds of analyses that are needed in the post-genomic era, if we are to define the biological functions of all of the *Arabidopsis* genes.

## Supplementary data

The following supplementary data are available at *JXB* online:

**Figure S1.** Phylogeny of *Arabidopsis* 4CLs and related 4CL-like proteins of the superfamily of adenylate-forming enzymes.

**Figure S2.** Identification of two *Arabidopsis* lines homozygous for defective OPC-8:CoA ligase.

**Figure S3.** Time courses of accumulation of different JA precursors in wounded leaves of *Arabidopsis* wild-type and mutant defective for OPC-8:CoA ligase (At1g20510-KO-1).

**Figure S4.** Time course of JA accumulation in different mutants defective for single and multiple acyl-CoA synthetases.

**Figure S5.** Activation of the OPC-8:CoA ligase promoter in response to infection by *Pseudomonas syringae* pv *tomato* (Pst).

**Table S1.** Activity profile of *Arabidopsis* acyl-CoA synthetases with additional substrates.

**Table S2.** Primers used for cloning of *Arabidopsis* acyl-CoA synthetases.

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