

# The *Arabidopsis* stearoyl-acyl carrier protein-desaturase family and the contribution of leaf isoforms to oleic acid synthesis

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**Abstract** In plants, changes in the levels of oleic acid (18:1), a major monounsaturated fatty acid (FA), results in the alteration of salicylic acid (SA)- and jasmonic acid (JA)-mediated defense responses. This is evident in the *Arabidopsis ssi2/fab2* mutant, which encodes a defective stearoyl-acyl carrier protein-desaturase (S-ACP-DES) and consequently accumulates high levels of stearic acid (18:0) and low levels of 18:1. In addition to SSI2, the *Arabidopsis* genome encodes six S-ACP-DES-like enzymes, the native expression levels of which are unable to compensate for a loss-of-function mutation in *ssi2*. The presence of low levels of 18:1 in the *fab2* null mutant indicates that one or more S-ACP-DES isozymes contribute to the 18:1 pool. Biochemical assays show that in addition to SSI2, four other isozymes are capable of desaturating 18:0-ACP but with greatly reduced specific activities, which likely explains the inability of these SSI2

isozymes to substitute for a defective *ssi2*. Lines containing T-DNA insertions in *S-ACP-DES1* and *S-ACP-DES4* show that they are altered in their lipid profile but contain normal 18:1 levels. However, overexpression of the *S-ACP-DES1* isoform in *ssi2* plants results in restoration of 18:1 levels and thereby rescues all *ssi2*-associated phenotypes. Thus, high expression of a low specific activity S-ACP-DES is required to compensate for a mutation in *ssi2*. Transcript level of *S-ACP-DES* isoforms is reduced in high 18:1-containing plants. Enzyme activities of the desaturase isoforms in a 5-fold excess of 18:1-ACP show product inhibition of up to 73%. Together these data indicate that 18:1 levels are regulated at both transcriptional and post-translational levels.

**Keywords** SSI2/FAB2 · Stearoyl-ACP-Desaturase · Oleic acid · Salicylic acid · Jasmonic acid

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## Abbreviations

S-ACP-DES	Stearoyl-ACP-desaturase
FA	Fatty acid
18:1	Oleic acid
18:0	Stearic acid
SCD	Stearoyl-CoA-desaturase

## Introduction

Plants respond to stress treatments by modulating various phytohormone triggered pathways (reviewed in Kachroo and Kachroo 2006). Upon recognition of pathogens, the host plants initiate one or more signal

transduction pathways that eventually avert pathogen colonization. Resistance is often associated with increased expression of defense genes, including the pathogenesis-related (*PR*) and plant defensin (*PDF1.2*) genes, which coincides with accumulation of the phytohormones salicylic acid (SA) and jasmonic acid (JA), respectively. Although SA and JA activate distinct signaling pathways, there is a growing body of evidence that shows that these pathways do not function entirely independently and are involved in a complex signaling network. The mechanism of cross talk between SA and JA signaling pathways in plant defense response remains to be elucidated.

In addition to the SA- and JA-mediated defense pathways, fatty acids (FA)-derived signaling has also started to emerge as one of the important defense pathways (Vijayan et al. 1998; Kachroo et al. 2001, 2003b, 2004, 2005; Weber 2002; Li et al. 2003; Yaeno et al. 2004). In *Arabidopsis*, oleic acid (18:1) has been implicated to participate in SA- and JA-mediated defense pathways (Kachroo et al. 2001, 2003a, b, 2004, 2005). Oleic acid is one of the major monounsaturated FA of membrane glycerolipids in both plants and animals. In plants, de novo FA biosynthesis occurs exclusively in the plastids and, upon its synthesis, 18:1 can either enter glycerolipid synthesis via the prokaryotic pathway in the chloroplasts or is exported out of plastids as CoA thioesters to enter the eukaryotic glycerolipid synthesis pathway. The first step of the plastidial glycerolipid synthesis is mediated by the *ACT1* encoded glycerol-3-phosphate (G3P) acyltransferase, which catalyzes the acylation of 18:1 to G3P and initiates formation of phosphatidic acid and other chloroplastic lipids.

In mammalian systems, the formation of 18:1 is catalyzed by stearoyl-CoA-desaturase (SCD) (see review by Dobrzyn and Ntambi 2005). The levels of SCD play an important role in metabolic processes and altered expression of the SCD gene appears to be associated with cancer (Lu et al. 1997; Thai et al. 2001), obesity (Enser 1975; Sivaramakrishnan and Pynadath 1982; Smith et al. 1999; Bassilian et al. 2002), diabetes (Worcester et al. 1979; Shimomura et al. 1999; Waters and Ntambi 1994), and neurological disease (Breuer et al. 2004). Recent studies using mice carrying a naturally occurring mutation or a targeted disruption in *SCD1* have revealed the central role of this protein in metabolic control and in addition has identified *SCD1* as a potential target for the treatment of obesity, diabetes and other metabolic disorders (Zheng et al. 1999; Miyazaki et al. 2001). SCDs have been studied in several systems and several mammalian genomes including rat and mouse have several isoforms of this

gene (Mihara 1990; Ntambi et al. 1988). Although the specific roles of each isoform is not clear, there is some evidence suggesting that multiple isoforms of SCD may be related to the tissue-specific expression and/or substrate specificities of the different isozymes (Zheng et al. 2001).

In plants, 18:1 formation is catalyzed by the soluble stearoyl-acyl-carrier-protein-desaturase (*S-ACP-DES*). The members of *S-ACP-DES* are specific for particular substrate chain length and introduce double bond between specific carbon atoms. Since *S-ACP-DES* are the only plant enzymes which catalyze conversion of 18:0 to 18:1 in plants, their activity primarily regulates the ratios of saturated to monounsaturated FAs. The *S-ACP-DES* has been purified from several plants and the encoding genes characterized from several different species (Shanklin and Somerville 1991; Thompson et al. 1991; Cahoon et al. 1996, 1998; Whittle et al. 2005). The archetypal *S-ACP-DES* from castor has been the focus of extensive structure–function studies and is the only member of the acyl-ACP desaturase family for which a three-dimensional crystal structure has been reported (Lindqvist et al. 1996). Antisense suppression of *S-ACP-DES* has also been used as a strategy to increase the saturated FA content in transgenic *Brassica napus* (Knutzon et al. 1992).

The *Arabidopsis* genome carries seven *S-ACP-DES*-like genes, including the *SSI2/FAB2*. The *ssi2* allele encodes a *S-ACP-DES* of reduced function and, as a result the mutant plants accumulate increased levels of 18:0 and reduced levels of 18:1 (Lightner et al. 1994; Kachroo et al. 2001). The mutant plants are dwarfed, constitutively activated in SA-mediated signaling but repressed in certain JA-mediated responses (Shah et al. 2001; Kachroo et al. 2001). We have previously shown that a reduction in 18:1 levels is the likely cause of altered defense signaling in *ssi2* plants (Kachroo et al. 2001, 2003a, b, 2004, 2005). The altered morphology, as well as defense-related phenotypes in *ssi2*, can be restored by elevating the endogenous 18:1 levels via second-site mutations in the *act1* (Kachroo et al. 2003b) or the *gly1* genes (Kachroo et al. 2004; Nandi et al. 2004). The *GLY1* gene encodes G3P dehydrogenase, which catalyzes the formation of G3P from dihydroxyacetone phosphate (DHAP).

In this study, we have attempted to define the physiological roles of the different *S-ACP-DES* isoforms of *Arabidopsis*, particularly with respect to defense signaling. We have analyzed the expression of these genes in different tissues and also examined the effects of overexpression of *S-ACP-DES1* and *SSI2* in both wild-type as well as the *ssi2* backgrounds. Characterization of lines carrying T-DNA insertions in *S-ACP-DES1*

and 4 isoforms showed that these influence the lipid profiles but not the 18:1 levels. However, unlike the *ssi2* mutation, a defect in *S-ACP-DES1* and 4 isoforms does not lead to defective SA- or JA-mediated signaling. Biochemical analyses of five *S-ACP-DES* isozymes shows that *SSI2* plays a dominant role in determining the 18:1 pool in *Arabidopsis* leaves.

## Materials and methods

### Plant growth conditions, transgenic analyses and generation of knock-out lines

Plants were grown in the MTPS 144 Conviron (Winnipeg, MB, Canada) walk-in-chambers at 22°C, 65% relative humidity and 14 h photoperiod. T-DNA lines generated by the Salk Institute genomic analysis laboratory (Alonso et al. 2003) were obtained from ABRC.

The full length cDNA corresponding to *S-ACP-DES1* and *SSI2* genes were PCR amplified using linked primers and cloned downstream of a double 35S promoter in pRTL2.GUS. For *Arabidopsis* transformation, the fragment containing the promoter, cDNA and terminator was removed from pRTL2-*SSI2* and pRTL2-*S-ACP-DES1* vectors and cloned into binary vectors pBIN19 and pBAR1, respectively. These clones in binary vectors were moved into *Agrobacterium tumefaciens* strain MP90 by electroporation and were used to transform *Arabidopsis* via the floral dip method (Clough and Bent 1998). Selection of transformants was carried out on media containing kanamycin (pBIN19), or BASTA (pBAR1).

The presence of T-DNA in the *S-ACP-DES1* and 4 was confirmed by amplifying an insertion site using left border and gene specific primer and sequence analysis of the amplified product. Knock-outs (KO's) homozygous for the insertion were confirmed by genomic and RT-PCR analysis, which showed an absence of product in these lines. KO's were further analyzed in T4 and T5 generations.

### RNA extraction and Northern analyses

Small-scale extraction of RNA from one or two leaves was performed with the TRIzol reagent (Invitrogen), following the manufacturer's instructions. Northern blot analysis and synthesis of random-primed probes for *PR-1* and *PDF1.2* were carried out as described (Kachroo et al. 2001).

### Reverse transcription-PCR

RNA quality and concentration were determined by gel electrophoresis and determination of A<sub>260</sub>. Reverse transcription (RT) and first strand cDNA synthesis was carried out using Superscript II (Invitrogen). Two to three independent RNA preparations were used for RT-PCR and each of these were analyzed at least twice by RT-PCR. The RT-PCR was carried out for 35 cycles in order to determine absolute levels of transcripts. The number of amplification cycles was reduced to 23–25 in order to evaluate and quantify difference among transcript levels before they reached saturation. Gene-specific primers used for RT-PCR analysis were, ACT1 (At1g32200) atgactctcagcttttctcc and ctaattccaaggttgaca, MOD1 (At2g05990) gatcgaaatcatgtctgaatccagtga and gtacgacgtcctaattctgtctgtaagg, FATA1 (At4g13050) ggacgaaatcatgtgaagcttctgtgta and agtcggatccttaacttgaa-ggcttctt, FATA2 (At3g25110) ggacctcgagatcctgatggtg-gccacctctgcta and agtctctagatcgcacttacgggtgcagttcccaa, ACX1 (At4g16760) atggaaggaattgatcacctcg and ttaac-catatcgagtatcaagc, ACS9 (At1g77590) ggacgtcgaccaat-gattccttatgctgg and agtctctcagttaggcatataacttggtga, PED1 (At2g33150) atggagaagcgcgatcgagagac and ctagc-gagcgtccttggaaca, MFP2 (At3g06860) atggattcacgaacc-aagggg and ttacaaccgtgagctggattg.

### FA, lipid and SA analyses

FA analyses of isolated chloroplasts and leaf tissue were carried out as described (Dahmer et al. 1989; He et al. 2002). Lipid profiles and acyl group identification were carried out using the automated electrospray ionization-tandem mass spectrometry facility at the Kansas Lipidomics Center. SA and SAG were extracted and measured from 0.25 g of fresh weight leaf tissue as described (Bowling et al. 1997).

### S-ACP-DES expression, purification, assay, regiospecificity and product inhibition

The putative signal peptide regions of all *S-ACP-DES* proteins were predicted based on TargetP and ChloroP analyses. cDNAs for the truncated proteins were amplified such that they lacked the N-terminal sequence corresponding to the putative signal peptide (Table 1), and the subsequent amino acid was converted to a methionine. The cDNAs were cloned into the NcoI site of pET-28a vector and expressed without a tag. Purification and determination of desaturase activity were carried out as described (Cahoon and Shanklin 1997). Regiospecificity was determined by mass spectral analysis of the pyrrolidine adducts as

Table 1 The *Arabidopsis* S-ACP-DES gene family

Designation	Locus	Length <sup>a</sup> (a.a)	MW <sup>b</sup> (kDa)	TP <sup>c</sup> (a.a)	Predicted localization <sup>d</sup>
SSI2/FAB2	At2g43710	401	45.69	35	C (stroma)
S-ACP-DES1	At5g16240	391	45.02	36	C (stroma)
S-ACP-DES2	At3g02610	411	46.95	44	C (stroma)
S-ACP-DES3	At5g16230	401	45.86	35	C (stroma)
S-ACP-DES4	At3g02620	396	45.5	68	C (stroma)
S-ACP-DES5	At3g02630	396	45.3	29	C (stroma)
S-ACP-DES6	At1g43800	391	44.15	44	M/C (stroma)

<sup>a</sup> Length of the pre-processed protein is indicated as number of amino acids (a.a)

<sup>b</sup> Molecular weight of mature polypeptide

<sup>c</sup> Length of the N-terminal transit peptide (TP)

<sup>d</sup> Predictions based on Target P; C, Chloroplast; M, Mitochondria

described before (Cahoon and Shanklin 1997). Product inhibition was carried out by addition of a 5-fold excess of unlabelled product to a standard <sup>14</sup>C-labeled acyl-ACP assay (Whittle et al. 2005).

#### Cell death staining and pathogen infection

Trypan blue staining for cell death detection was performed as described (Chandra-Shekara et al. 2006). Infections with *Hyaloperonospora parasitica* Emco5 were performed by spraying asexual inoculum suspension as described (Kachroo et al. 2001). Landsberg (Ler) and Col-0 ecotypes were used as resistant and susceptible controls, respectively. The *ssi2* plants were derived from the Nössen ecotype, which show slightly more susceptibility to Emco5 as compared to Col-0 (Kachroo et al. 2003).

#### Results

The *Arabidopsis* S-ACP-DES family consists of seven highly conserved members

A database search of the *Arabidopsis* genome identified six genes predicted to encode proteins with high sequence similarity to SSI2 (Fig. 1). Of the seven S-ACP-DES genes (Table 1), three are located in tandem on chromosome 3, two in tandem on chromosome 5 and one each on chromosomes 1 and 2. The predicted peptides encoded by the seven *Arabidopsis* isoforms range from 391 to 411 amino acids (aa) and all except S-ACP-DES6 are predicted to localize to the plastids (Table 1). Although TargetP (Emanuelsson et al. 2000), predicts localization of S-ACP-DES6 to the mitochondria, the difference between the scores for a mitochondrial target peptide versus a plastidal transit

peptide is very low (0.004) and the program places this prediction in the lowest reliability class. Sequence comparisons and phylogenetic analysis of the different isozymes conducted using DNASTAR program revealed that the S-ACP-DES2 and 4 isozymes are the most similar, showing ~90% similarity at the aa level, followed by S-ACP-DES1 and 5, which are ~86% similar (supplemental Fig. 1A, B). Strikingly, the SSI2 protein showed highest relatedness to a heterologous desaturase from castor plant (S-ACP-DES<sub>Rc</sub>; Shanklin and Somerville, 1991), followed by S-ACP-DES1 and 5 (72–74% similarity) and S-ACP-DES2, 3, 4, 5, 6 (64–66% similarity). The S-ACP-DES6 protein shows maximum divergence from other desaturases, arising from a completely separate branch point (Fig. 1). Sequence comparisons of regions between the predicted translational start site and ~500 bp upstream showed 65% identity between S-ACP-DES2 and 4 and 35% identity among the remaining isoforms (data not shown).

The *S-ACP-DES* genes exhibit tissue-specific differences in their expression

The low level of sequence similarity in the 5' untranslated regions of the various *S-ACP-DES* genes suggests a possible divergent tissue-specific expression. To provide clues as to possible physiological roles of each of the seven isozymes, we examined their expression patterns in various tissues of wild-type plants. Reverse transcriptase (RT)-PCR analysis of RNA from leaf, stem, root, flower and silique using gene-specific primers for the seven isoforms revealed that all except *S-ACP-DES2*, 4 and 6 showed detectable transcript in all these tissues (Fig. 2). Furthermore, except *S-ACP-DES6*, all of the other isoforms showed tissue specificity with increased or reduced levels of transcripts in

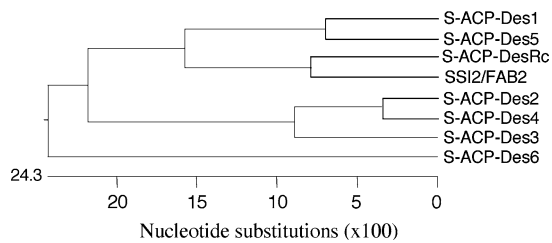


Fig. 1 Sequence conservation in the *Arabidopsis* S-ACP-DES family. Phylogenetic analysis of predicted sequences of *Arabidopsis* S-ACP-DES proteins including SSI2, S-ACP-DES1, 2, 3, 4, 5 and 6 and a  $\Delta^9$  desaturase from castor (S-ACP-DES<sub>Rc</sub>). Phylogenetic analysis was carried out using the Megalign program in the DNASTAR package

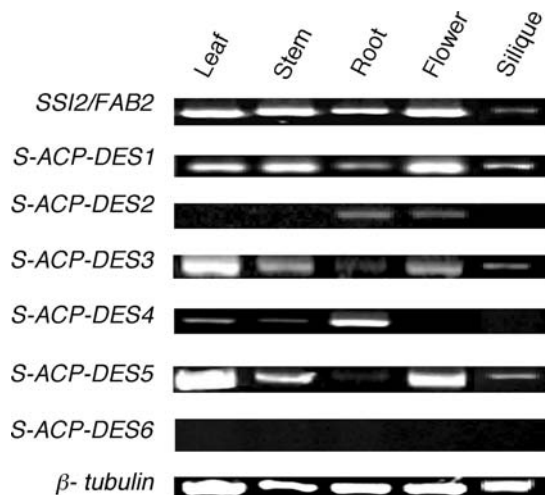


Fig. 2 Tissue-specific expression of the *Arabidopsis* S-ACP-DES family. RT-PCR analysis of RNA extracted from leaf, stem, root, flower and silique tissues of Col-0 plants using gene-specific primers for SSI2, S-ACP-DES1, 2, 3, 4, 5 and 6. The level of  $\beta$ -tubulin was used as an internal control to normalize the amount of cDNA template

one or more tissues. For example, S-ACP-DES<sub>4</sub> showed relatively high expression in roots compared to its levels in leaf, stem, flower and silique, while S-ACP-DES<sub>3</sub> showed high expression in leaves, but not in roots. The S-ACP-DES<sub>5</sub> isoform was expressed at high levels in leaf, stem and flower tissues, but at low levels in roots and siliques. The S-ACP-DES<sub>2</sub> isoform was only expressed at low levels in roots and flowers, while S-ACP-DES<sub>6</sub> was not detected in any of the tissues analyzed. The expression patterns observed in our study are largely consistent with the data available from the Massively Parallel Signature Sequencing project (<http://mpss.udel.edu/at/>).

We next determined the FA profile of leaf, stem, root, flower and silique of wild-type plants to evaluate how they differed in their 18:1 content (Table 2). Since S-ACP-DES might use 16:0 or 18:0 as substrates,

emphasis was placed on analysis of 16:1 and 18:1 levels. In plants 18:1 can also be synthesized upon extension of 16:1 $\Delta^9$  and these differ from 18:0 desaturation products in double bond position; 18:1 derived from 18:0 has a  $\Delta^9$  double bond while 18:1 derived from 16:1 $\Delta^9$  has a  $\Delta^{11}$  double bond. The 18:1 levels in roots were ~2.5-times higher than levels in leaves and although 18:1 $\Delta^9$  levels were comparable, the majority of the 18:1 in root was present as the  $\Delta^{11}$  isomer. The 18:1 $\Delta^9$  levels in flowers were comparable to those of leaves but the flowers consistently showed a ~2-fold higher levels of 18:1 $\Delta^{11}$ . By comparison, the 16:1 levels in leaves were ~9-fold higher as compared to those in flower, root or stem tissues. Strikingly, even though silique tissue showed low expression of SSI2 and other isoforms, they contained highest levels of 18:1 $\Delta^9$ . Similarly, leaf and flower tissues showed high expression of several isoforms but did not contain a proportional increase in their 18:1 levels (Fig. 2, Table 2).

Expression of the S-ACP-DES isoforms is not affected by a mutation in *ssi2*

Although the various S-ACP-DES isozymes are similar in sequence to SSI2, these enzymes are unable to complement the reduced 18:1 levels in the *ssi2* mutant plants (Kachroo et al. 2001). This raises the possibility that either a mutation in *ssi2* affects the expression of the various isoforms or that these isozymes have different substrate specificities. In mammalian systems, it was reported that alterations in the expression of a specific desaturase affected the expression of other isoforms. For example, *SCD1*-deficient mice lack *SCD3* expression in their preputial gland, although the expression of *SCD2* remains unaltered (Miyazaki et al. 2002). We therefore examined the expression patterns of the S-ACP-DES isoforms in the *ssi2* mutant. RT-PCR analysis using gene-specific primers revealed that the expression patterns for SSI2, S-ACP-DES<sub>1</sub>, 4, and 5 were similar to those in leaves of wild-type plants (Fig. 3). However, the levels of the S-ACP-DES<sub>3</sub> transcript were reduced in *ssi2* leaves, as compared to those in wild-type plants, indicating that the *ssi2* mutation results in slight repression of the S-ACP-DES<sub>3</sub> isoform. The S-ACP-DES<sub>2</sub> and 6 transcripts were undetectable in *ssi2* leaf tissue (data not shown).

We next determined transcript levels of the various S-ACP-DES isoforms in *ssi2 act1* and *ssi2 gly1* plants. Our previous work has shown that second-site mutations in the *act1* or the *gly1* genes, resulting in increased 18:1 levels, are able to restore wild-type-like phenotypes in the *ssi2* mutant (Kachroo et al. 2003a,

Table 2 Tissue-specific FA composition of Col-0, *SD1* KO and *SD4* KO plants

Tissue <sup>a</sup>	Genotype	Fatty acid <sup>b</sup>								
		16:0	16:1	16:2	16:3	18:0	18:1 $\Delta^9$	18:1 $\Delta^{11}$	18:2	18:3
Leaf	Col-0	16.5 ± 1.1	3.5 ± 0.1	0.7 ± 0.2	13.5 ± 0.8	1.0 ± 0.2	2.3 ± 0.3	0.3 ± 0	13.7 ± 1.0	48.5 ± 2.1
	<i>sd1</i> ko	16.5 ± 0.4	3.9 ± 0.2	0.4 ± 0	13.4 ± 0.3	1.0 ± 0.3	2.4 ± 0.6	0.4 ± 0	11.4 ± 1.4	50.6 ± 1.8
	<i>sd4</i> ko	18.5 ± 0.8	3.8 ± 0.4	0.3 ± 0.1	12.2 ± 0.5	1.2 ± 0.3	2.0 ± 0.4	0.4 ± 0	13.3 ± 1.7	48.3 ± 1.6
Flower	Col-0	25.5 ± 1.2	0.4 ± 0	0.4 ± 0	4.8 ± 0.4	1.4 ± 0.3	2.2 ± 0.2	0.8 ± 0.1	27.4 ± 1.1	37.1 ± 2.1
	<i>sd1</i> ko	25.4 ± 0.5	1.0 ± 0.1	nd <sup>c</sup>	3.9 ± 0.1	1.5 ± 0	2.2 ± 0.3	1.9 ± 0.3	25.5 ± 1.0	38.6 ± 1.2
	<i>sd4</i> ko	25.2 ± 0.2	0.7 ± 0.1	nd	4.1 ± 0.2	1.2 ± 0.1	1.9 ± 0.2	0.9 ± 0	29.1 ± 1.1	36.9 ± 1.2
Root	Col-0	27.3 ± 0.5	0.4 ± 0	nd	nd	2.4 ± 0.4	1.9 ± 0.5	4.6 ± 0.5	46.0 ± 1.5	17.0 ± 1.0
	<i>sd1</i> ko	26.0 ± 2.3	0.1 ± 0	0.2 ± 0.1	nd	2.0 ± 0.2	2.0 ± 0.2	5.5 ± 0.9	43.7 ± 4.0	20.5 ± 2.0
	<i>sd4</i> ko	27.7 ± 0.9	nd	nd	nd	1.8 ± 0.1	1.6 ± 0.1	4.5 ± 0.8	45.3 ± 2.0	19.1 ± 1.5
Stem	Col-0	20.3 ± 1.1	0.4 ± 0	0.5 ± 0	8.5 ± 0.5	1.1 ± 0.1	1.4 ± 0.2	0.4 ± 0	24.3 ± 2.7	43.1 ± 2.4
	<i>sd1</i> ko	21.7 ± 1.0	2.1 ± 0.4	0.2 ± 0	6.8 ± 0.8	1.0 ± 0	2.1 ± 0.7	0.9 ± 0.2	23.9 ± 3.1	41.3 ± 3.4
	<i>sd4</i> ko	21.2 ± 0.4	2.5 ± 0	0.3 ± 0	7.9 ± 0.4	1.1 ± 0.2	1.7 ± 0.4	0.3 ± 0.1	24.6 ± 1.8	40.4 ± 2.6
Silique	Col-0	22.0 ± 0.9	1.0 ± 0.1	0.3 ± 0	4.7 ± 0.5	2.7 ± 0.2	5.3 ± 0.4	1.3 ± 0.4	32.3 ± 1.2	30.4 ± 2.1
	<i>sd1</i> ko	22.3 ± 1.0	1.5 ± 0.2	0.1 ± 0.1	4.9 ± 0.3	2.0 ± 0	4.4 ± 1.0	1.0 ± 0.3	30.3 ± 2.2	33.5 ± 1.9
	<i>sd4</i> ko	22.1 ± 0.5	1.4 ± 0.1	0.3 ± 0.1	4.1 ± 0.2	2.1 ± 0.2	4.1 ± 0.7	1.1 ± 0.3	33.8 ± 1.9	31.0 ± 2.7

<sup>a</sup> All measurements were made on plants grown at 22°C

<sup>b</sup> Data are described as mol% ± SE ( $n = 6$ )

<sup>c</sup> nd, not detected

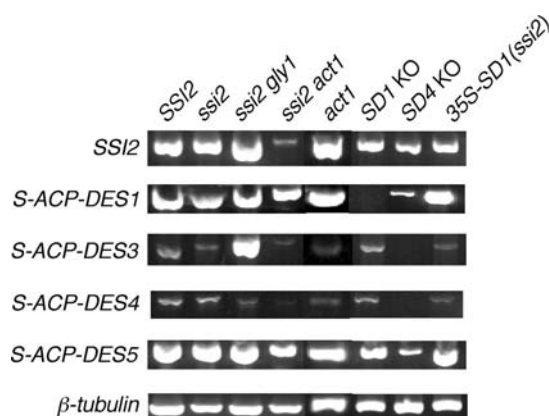


Fig. 3 Expression of the *S-ACP-DES* isoforms in various genetic backgrounds. RT-PCR analysis of RNA extracted from *SSI2* (Nö), *ssi2*, *ssi2 gly1*, *ssi2 act1*, *act1*, *SD1* KO, *SD4* KO and *35S-SD1(ssi2)* leaves, using gene-specific primers for *SSI2*, *S-ACP-DES1*, 3, 4 and 5. The level of  $\beta$ -tubulin was used as an internal control to normalize the amount of cDNA template

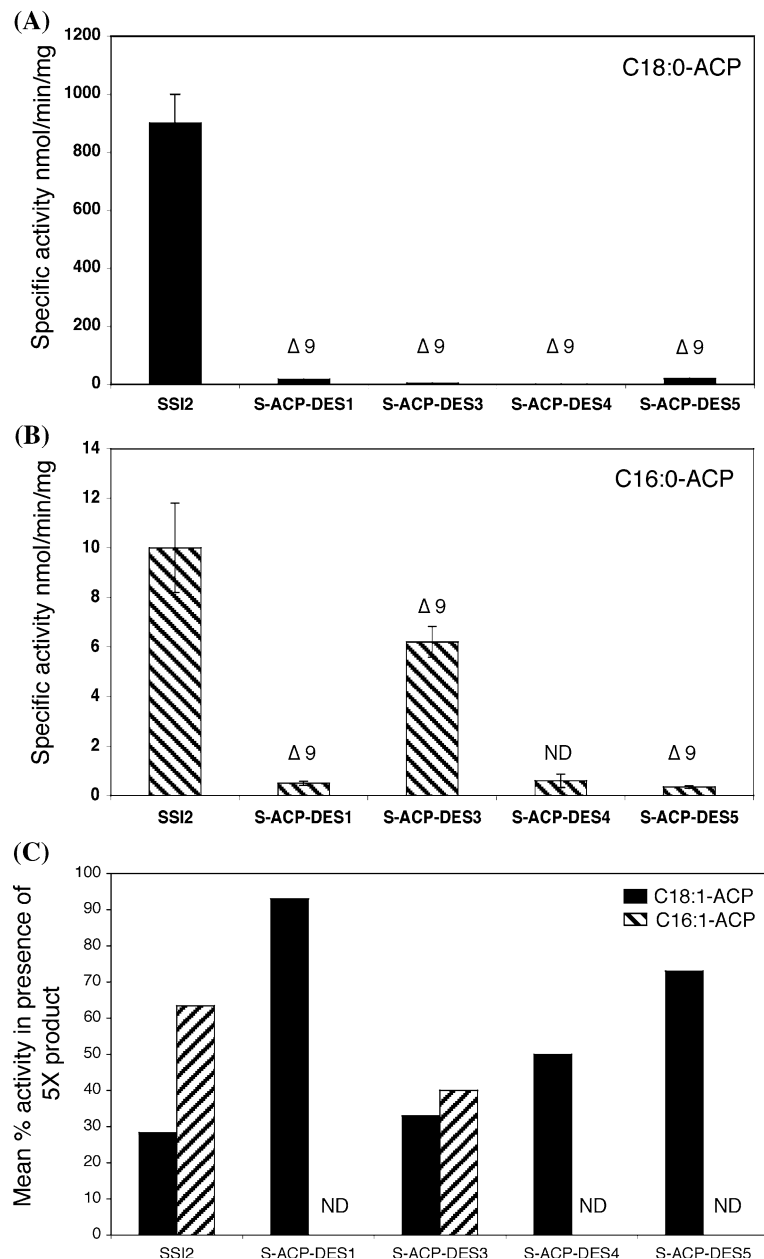
2004). In the *ssi2 gly1* plants, levels of the *S-ACP-DES3* transcript were increased, while all the other isoforms were expressed at their wild-type levels (Fig. 3). By contrast, *ssi2 act1* plants showed a substantial reduction in the transcript levels of *SSI2*, *S-ACP-DES3* and 4 transcripts, and a slight reduction in the transcript levels of *S-ACP-DES1* and 5, as compared to wild-type. Both *S-ACP-DES2* and 6 transcripts were undetectable in *ssi2 act1* and *ssi2 gly1* plants (data not shown). Repression of *S-ACP-DES* isoforms in *ssi2 act1* plants, which contain ~3-fold higher 18:1 as compared to wild-type, suggests that the 18:1 levels may be under feedback control and that

higher than threshold levels of 18:1 result in downregulation of various *S-ACP-DES* isoforms. However, we discounted this possibility because the *act1* single mutant, which like *ssi2 act1*, accumulated high levels of 18:1, but did not show repression of *S-ACP-DES* isoforms. These observations suggest that presence of *ssi2* mutation in *act1* plants was responsible for repression of *S-ACP-DES* isoforms.

The *S-ACP-DES* isozymes have greatly reduced specific activities as compared to *SSI2*

To determine how various *S-ACP-DES* isozymes contribute to the FA pool in the leaf, we next examined the enzymatic activities of *S-ACP-DES* 1, 3, 4, and 5 isozymes, which showed detectable mRNA in leaf tissue. The substrate specificities, specific activities and regiospecificity of these were determined for C14:0-, C16:0-, and C18:0-ACP substrates. The *SSI2*, *S-ACP-DES1*, 3, 4 and 5 enzymes were expressed in *Escherichia coli* and the purified proteins were assayed for their desaturase activity (Cahoon and Shanklin 1997). Biochemical analyses showed that the *SSI2*, *S-ACP-DES1* and 5 proteins utilize C18:0-ACP as a preferred substrate, while *S-ACP-DES3* preferentially utilizes C16:0-ACP as a substrate (Fig. 4A, B). The *S-ACP-DES4* isozyme showed very low activity on both C16:0- and C18:0-ACP substrates. In comparison to *SSI2*, the specific activities of *S-ACP-DES1*, 3, and 5 were ~53-, ~219- and ~45-fold less on C18:0-ACP substrate and ~20-, ~1.6- and ~29-fold less on C16:0-ACP substrate, respectively (Fig. 4A, B).

**Fig. 4** Enzymatic activities, substrate specificities, regiospecificity and product inhibition of S-ACP-DES isozymes. Specific activities analysis was carried out with ~90% pure preparations of bacterially expressed S-ACP-DES proteins. Activities are reported in nmol/min/mg and the error bars represent SE ( $n = 4$ ). Enzymatic studies were carried out using C18:0-ACP (A) or C16:0-ACP (B) as substrates and castor desaturase was used as a positive control (Cahoon et al. 1998). The specific activity of the SSI2 proteins is as previously reported (Kachroo et al. 2001). Regiospecificity of S-ACP-DES 1, 3, 4 and 5 enzymes was determined by mass spectral analysis of the pyrrolidine adducts of their reaction products and is indicated at the top of each bar. The S-ACP-DES4 catalyzed reaction on C16:0-ACP substrate did not accumulate sufficient product for double bond analysis (ND = not determined). (C) Mean percent activity of SSI2, S-ACP-DES1, 3, 4 and 5 in presence of 5-fold excess of product (C16:1-ACP or C18:1-ACP). The rates of desaturation of C16:0-ACP were insufficient to perform the product inhibition analysis of S-ACP-DES1, 4 and 5 (ND = not determined)



We further determined the regiospecificities of S-ACP-DES 1, 3, 4 and 5 enzymes by mass spectral analysis of the pyrrolidine adducts of their reaction products. S-ACP-DES1, 3, 4 and 5 preferentially desaturated C18:0-ACP substrate at C9 position (Fig. 4A). With the C16:0-ACP substrate, S-ACP-DES1, 3 and 5 also exhibited a preference for the C9 position (Fig. 4B). Technical problems precluded the determination of regiospecificity for S-ACP-DES4.

The possibility of product inhibition of their activities was investigated by the addition of a 5-fold excess of unlabeled product (either C16:1- or C18:1-ACP) to a standard  $^{14}\text{C}$ -labeled acyl-ACP assay. In contrast to the

castor  $\Delta 9$ -18:0-ACP desaturase, which was reported to be insensitive to a 30-fold excess of unlabelled 18:1-ACP product (Whittle et al. 2005), the desaturases investigated in this report showed 7–73% inhibition in the presence of 5-fold excess of unlabeled product (Fig. 4C). The highest level of inhibition was shown by SSI2 (73%), followed by S-ACP-DES3 (67%), S-ACP-DES4 (50%), S-ACP-DES 5 (27%) and S-ACP-DES1 (7%). The rates of desaturation of C16:0-ACP were insufficient to perform the product inhibition analysis of all but SSI2 and S-ACP-DES3, which showed 37 and 40% product inhibition, respectively. Because S-ACP-DES1, 3, 4, 5 are poorly active on C16:0 and

C18:0-ACP substrates, we investigated the possibility that they may recognize C14:0-ACP, similar to a desaturase isolated from geranium trichomes (Schultz et al. 1996). However, no activity was detected on C14:0-ACP, even in the presence of a large excess of the purified proteins (data not shown). The observation of product inhibition in the presence of monounsaturated acyl-ACPs, along with recent reports that a desaturase from ivy recognizes monounsaturated substrates (Whittle et al. 2005), prompted us to test whether S-ACP-DES1, 3, 4, 5 enzymes exhibit activity towards C16:1- or C18:1-ACP substrates. None of the four desaturases showed any detectable activity towards C16:1- or C18:1-ACP, even in the presence of a large excess of the enzyme (data not shown).

Taken together, these data show that S-ACP-DES1, 3, 4, and 5 can desaturate C16:0- and C18:0-ACP substrates at C9 position. Furthermore, these data also indicate that SSI2 is regulated at post-translational level and is perhaps the major activity responsible for the synthesis of 18:1 $\Delta^9$ .

Knock-out mutations in *S-ACP-DES1* and *4* alter lipid profiles but do not alter defense phenotypes

Next, we investigated the function of the S-ACP-DES isoforms by analyzing the effect of loss-of-function mutations in the genes encoding these isozymes. T-DNA knock-out (KO) lines were identified in *S-ACP-DES1*, 2, 3, 4 and 5 isoforms but in spite of analyzing three generations of selfed heterozygous plants we were unable to obtain homozygous line for *S-ACP-DES2*, 3 and 5. At present it is not clear if homozygosity in these desaturase KO genes is associated with reduced viability or lethality. Homozygous KO lines were obtained for *S-ACP-DES1* (*SD1* KO) and *S-ACP-DES4* (*SD4* KO). The *SD1* KO line carried a T-DNA insertion in the first intron, while the *SD4* KO line carried an insertion in the third exon. Plants carrying homozygous insertions were identified using gene-specific primers flanking the T-DNA insertion site and a T-DNA left border primer. The KO phenotype was confirmed by RT-PCR analysis, which showed absence of any detectable transcript for *S-ACP-DES1* or *4* genes, respectively (Fig. 3). RT-PCR analysis of *SSI2*, *S-ACP-DES3* and *5* transcripts in the KO lines revealed that disruption of *SD4* downregulated expression of *S-ACP-DES1*, 3 and 5 genes but a disruption of *SD1* only resulted in a slight reduction of *S-ACP-DES3*. No transcripts for *S-ACP-DES2* and *6* genes were detected in *SD1* KO and *SD4* KO lines (data not shown). Unlike *ssi2* plants, the *SD1* KO and *SD4* KO lines showed a wild-type-like

morphological phenotype, absence of any visible or microscopic cell death (data not shown) and basal level expression of *PR-1* (Fig. 5A). Their JA responsiveness was also unaltered, and both *SD1* KO and *SD4* KO lines induced high levels of the *PDF1.2* (Plant Defensin 1.2) transcript upon exposure to JA (Fig. 5B). Analysis of response to inoculation with a virulent isolate of *Hyaloperonospora parasitica* revealed that both *SD1* and *4* KO lines behaved similar to wild-type plants, and were susceptible to the pathogen (Fig. 5C).

To examine if a loss-of-function mutation in *S-ACP-DES1* and *4* genes had any effects on various steps of FA synthesis and/or  $\beta$ -oxidation, we analyzed the expression of some representative genes involved in these processes in wild-type and KO lines. RT-PCR analysis of genes involved in FA synthesis showed that the leaf tissue of *SD1* KO line expressed wild-type-like levels of *MOD1* (enoyl-ACP-reductase) and *FATA1* (acyl-ACP thioesterase), but showed reduced expression of *FATA2* (acyl-ACP thioesterase) and failed to amplify any transcript for *ACS9* (acyl-CoA synthetase) and *ACT1* (G3P acyltransferase). The leaf tissue of *SD4* KO line expressed wild-type levels of *MOD1* but showed reduced levels of *ACT1*, *FATA1* and *FATA2*, and did not amplify any transcript for *ACS9* (Fig. 6). By comparison, the *ssi2* leaves showed wild-type-like levels of *MOD1*, *ACT1* and *FATA2* but increased amounts of the *ACS9* and *FATA1* transcripts. Analysis of genes involved in FA  $\beta$ -oxidation revealed a marginal reduction in expression of *PED1* (acetyl CoA acyl transferase) gene and no detectable expression levels of *ACX1* (acyl-CoA oxidase) and *MFP2* (multifunctional protein) genes in *SD1* KO plants. By comparison, *SD4* KO lines also showed marginal reduction in *PED1* transcript and no detectable amplification of *ACX1* transcript but these plants contained normal levels of *MFP2* transcript. In contrast to *SD1* and *SD4* KO lines, *ssi2* plants were up-regulated in expression of *ACX1* gene but similar to *SD1* KO did not amplify *MFP2* transcript (Fig. 6).

To determine if these alterations in the expression of FA metabolism genes result in an altered FA/lipid profile in these KO lines, we carried out FA as well as lipid analysis of the *SD1* KO and *SD4* KO lines. Unlike *ssi2* plants, the *SD1* and *4* KO plants showed no significant differences in 18:0 accumulations compared to wild-type plants in any of the tissues analyzed (Table 2). Furthermore, the FA profile of leaf tissue did not show any change in their total or chloroplastic FA content (Table 2 and data not shown). FA profiling revealed a ~5–6-fold increase in 16:1 levels in stems of *SD1* KO and *SD4* KO plants. In addition, the *SD1* KO plants also showed a ~2.3-fold increase in the 18:1 $\Delta^{11}$  content in flowers.

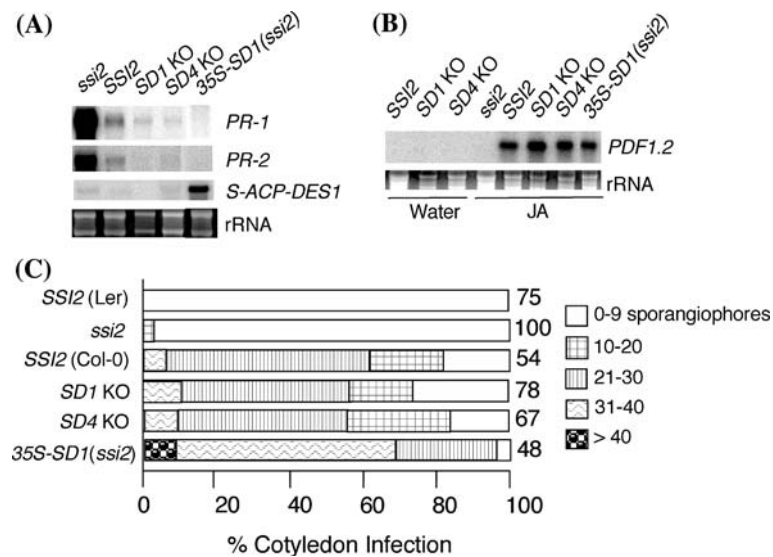


Fig. 5 *PR*-levels, JA-responsiveness and pathogen response of *SD1 KO*, *SD4 KO* and *35S-SD(ssi2)* plants. (A) Northern blot analysis of *PR-1*, *PR-2* and *S-ACP-DES1* gene expression in *ssi2*, *SSI2* (Col-0), *SD1 KO*, *SD4 KO* and *35S-SD1(ssi2)* plants. Each lane shows RNA from a single plant. Ethidium bromide staining of rRNA was used as a loading control. (B) Northern blot analysis of *PDF1.2* as a measure of JA-responsiveness of *ssi2*, *SSI2*, *SD1 KO*, *SD4 KO* and *35S-SD1(ssi2)* plants. The 4-week-old plants were treated with water or 50  $\mu$ M JA and the samples were collected 48 h post-treatment. Ethidium bromide staining

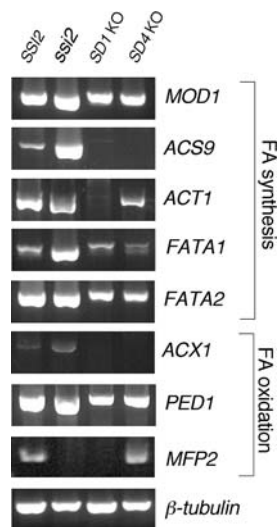
of rRNA was used as a loading control. (C) Comparison of *Hyaloperonospora parasitica* ecotype Emco5 growth on cotyledons of 7-day-old seedlings. Various genotypes, listed on the left were inoculated by spraying conidiospores at a concentration of  $10^6$  spores/ml. Pathogen growth was assessed by counting the number of sporangiophores per cotyledon at 8 days after inoculation. The shading of each box indicates the severity of infection, based on the number of sporangiophores per cotyledon (see key at right). Numbers at right of the sample boxes indicate the number of cotyledons assayed. Ler, *Landsberg erecta*

Analysis of total lipid content in leaves from *SD1 KO* and *SD4 KO* plants showed a ~22% and 16% reduction, as compared to wild-type, respectively (Fig. 7A). However, the total lipid content in *SD1 KO* and *SD4 KO* leaves was higher than the total lipid content in *ssi2*. Similarly, the levels of monogalactosyldiacylglycerol (MGDG) in leaves from *SD1 KO* and *SD4 KO* was lower than wild-type plants but higher than that of *ssi2* (Fig. 7B). There were no significant changes in the profiles of other leaf lipids between wild-type and KO lines. Analysis of acyl carbon species showed that the *SD1 KO* and *SD4 KO* plants contained reduced levels of 34:6 (no. of carbons:no. of double bonds) species of acyl carbon on MGDG, 34:4 on phosphatidylglycerol (PG) and 34:3 on phosphatidic acid but accumulated higher levels of 34:3, 36:3, 40:3, 42:2 and 43:3 acyl species on phosphatidylserine (PS) (Fig. 7C).

#### Overexpression of S-ACP-DES1 complements the *ssi2* mutation

In order to determine whether various isoforms are active in vivo and can contribute to the total 18:1 pool if present at sufficiently high levels, we overexpressed *S-ACP-DES1* in *ssi2* plants (*35S-SD1-ssi2*), because it

has high structural similarity to *SSI2* and similar substrate specificity for 18:0. Twenty-five T1 transgenic plants were obtained, and all of these showed wild-type-like morphology (Fig. 8A). Three lines were analyzed in the T<sub>2</sub> generation and these segregated ~3 wild-type-like: 1 *ssi2*-like (56 wild-type-like, 22 *ssi2*-like;  $\chi^2 = 0.42$ ,  $P = 0.51$ ). PCR analysis of genomic DNA amplified *S-ACP-DES1* cDNA transgene only in the wild-type-like plants and not in the *ssi2*-like plants, suggesting that the wild-type-like phenotype co-segregated with the presence of transgene. This was further confirmed by analyzing the *S-ACP-DES1* transcript levels in the transgenic plants; 14 plants were tested and all showed increased levels of the *S-ACP-DES1* transcript (Fig. 5A). The *35S-SD1-ssi2* lines lacked spontaneous cell death lesions and increased expression of the *PR-1* gene; both of these phenotypes are triggered by the *ssi2* mutation (Figs. 8B, 5A). In addition, the JA-related phenotypes of *ssi2* plants were also restored in the *35S-SD1-ssi2* lines and, unlike in *ssi2* plants, exposure to JA induced high levels of expression of the *PDF1.2* gene in *35S-SD1-ssi2* plants (Fig. 5B). Overexpression of *S-ACP-DES1* also restored the wild-type-like responses to pathogen inoculations and these transgenic *ssi2* plants were as susceptible as the wild-type parent (Fig. 5C). This



**Fig. 6** Expression analysis of the genes involved in FA metabolism in *ssi2*, *SD1* KO and *SD4* KO plants. RT-PCR analysis of RNA extracted from *SSI2* (Col-0), *ssi2*, *SD1* KO and *SD4* KO plants. Gene specific primers for enoyl-ACP-reductase (*MOD1*), FA-CoA synthetase (*ACS9*), G3P acyltransferase (*ACT1*), acyl-ACP-thioesterase (*FATA1* and *A2*), acyl-CoA oxidase (*ACX1*), acetyl CoA acyl transferase (*PED1*) and multifunctional protein (*MFP2*) were used to detect transcript levels for the corresponding genes in various genotypes. The level of  $\beta$ -tubulin was used as an internal control to normalize the amount of cDNA template

result correlated well with the reduction in SA levels in the *S-ACP-DES1* overexpressing lines; in comparison to *ssi2*, the *35S-SD-ssi2* plants showed basal levels of SA and SA glucoside (SAG), similar to the levels seen in wild-type plants (Fig. 8C). Taken together, these results show that overexpression of *S-ACP-DES1* was sufficient to replace *SSI2* functionally in the *ssi2* mutant line.

Since a reduction in 18:1 levels in the *ssi2* mutant plants is implicated in the altered phenotypes, the *35S-SD1-ssi2* transgenic lines were analyzed for their leaf FA content. The 18:1 levels in these plants were restored to wild-type-like levels (Table 3). In addition, 16:3 levels, which are lower in *ssi2* plants, were also restored to wild-type-like levels. Analysis of the chloroplastic FAs from *ssi2* and *35S-SD1-ssi2* plants showed wild-type-like 18:1 levels in *35S-SD1-ssi2* plants (Fig. 8D). To determine if *S-ACP-DES1* overexpression impacted the lipid profile, we measured the levels of individual lipids and the levels of total acyl carbons on each of these lipids. The overexpression of *S-ACP-DES1* in *ssi2* plants increased the total lipid, MGDG, digalactosyldiacylglycerol (DGDG) and phosphatidylglycerol (PG) content to an intermediate level between that seen for wild-type and *ssi2* plants (Fig. 7A, B). Particularly, overexpression of

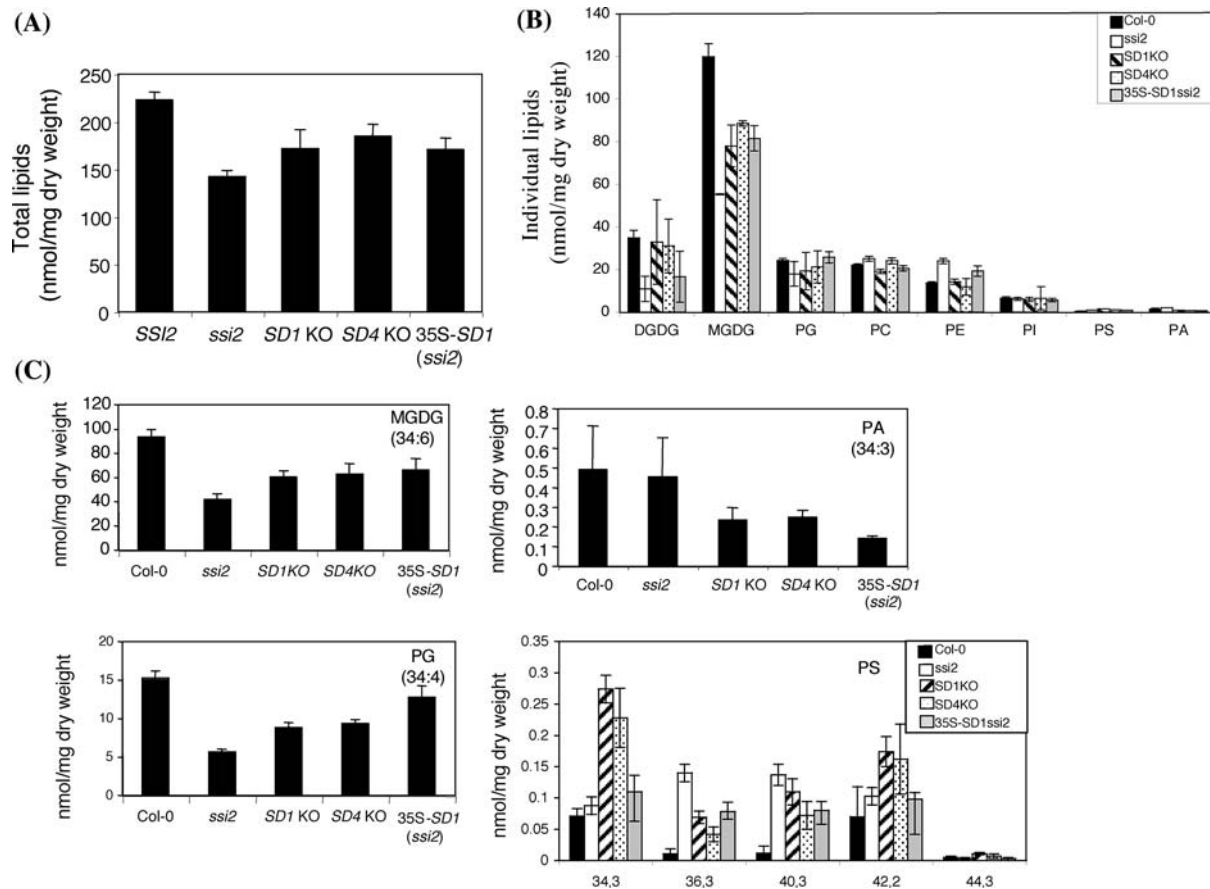
*S-ACP-DES1* increased the levels of 34:6 species of acyl carbon on MGDG, 34:6 and 34:3 on DGDG and 34:3 and 34:4 on PG; the levels of these acyl carbons were reduced in *ssi2* leaves as compared to wild-type (Fig. 7C). There were no significant changes in the profiles of other leaf lipid, lyso-lipids or the total acyl carbons on each lipid (Fig. 7B, C). These data suggest that the increased levels of *S-ACP-DES1* enzyme are sufficient to restore normal FA levels in *ssi2* plants.

**Overexpression of *SSI2* and *S-ACP-DES1* in wild-type plants does not alter morphological or defense phenotypes**

Our data thus far suggests that it is crucial for plants to maintain certain threshold levels of 18:1 for proper defense signaling. To determine if increased levels of 18:1-ACP sensitizes the defense-signaling pathway, we overexpressed *SSI2* and *S-ACP-DES1*, that differ in specific activity by ~50-fold, in wild-type plants under the same promoter. Transgenic plants (ecotype Col-0) expressing *SSI2* (*35S-SSI2* Col-0) and *S-ACP-DES1* (*35S-SD1* Col-0) under control of the CaMV 35S promoter were generated, and these showed high levels of the *SSI2* or the *S-ACP-DES1* transcripts, respectively (data not shown). Both *35S-SSI2* Col-0 and *35S-SD1* Col-0 plants were morphologically indistinguishable from wild-type plants and showed normal SA- and JA-responsiveness; exogenous application of SA and JA induced expression of *PR-1* and *PDF1.2* genes, respectively, in the overexpressing plants (data not shown). To assess if increased expression of *SSI2* and *S-ACP-DES1* affected 18:1 content, we determined the FA levels from the overexpressing plants. Interestingly, 18:1 levels in both *35S-SSI2* Col-0 and *35S-SD1* Col-0 plants were similar to that seen in wild-type plants (Fig. 8D), suggesting that desaturase activity is not limiting for accumulation of 18:1 in Col-0 plants.

## Discussion

Plants are dependent on the stearyl-ACP desaturase for the synthesis of the monounsaturated FA 18:1 and its polyunsaturated derivatives. Our previous work has demonstrated the importance of 18:1 in normal defense signaling in *Arabidopsis* (Kachroo et al. 2001). A mutation in the *ssi2* gene, resulting in reduced 18:1 levels, constitutively upregulates the SA-mediated pathway and represses the JA-mediated pathway. Besides *SSI2*, the *Arabidopsis* genome encodes six other *S-ACP-DES*s, which show high levels of



**Fig. 7** Total lipid content, lipid profile and and total acyl species present on each lipid. (A) Total lipid content from leaves of *SSI2*, *ssi2*, *SD1 KO*, *SD4 KO* and *35S-SD1 (ssi2)* plants. (B) Comparison of levels of individual lipids in genotypes shown in A. Symbols for various components are DGDG, digalactosyldiacylglycerol;

MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid. (C) Levels of acyl carbon species present in individual lipids. For A, B and C, error bar represents SD ( $n = 5$ )

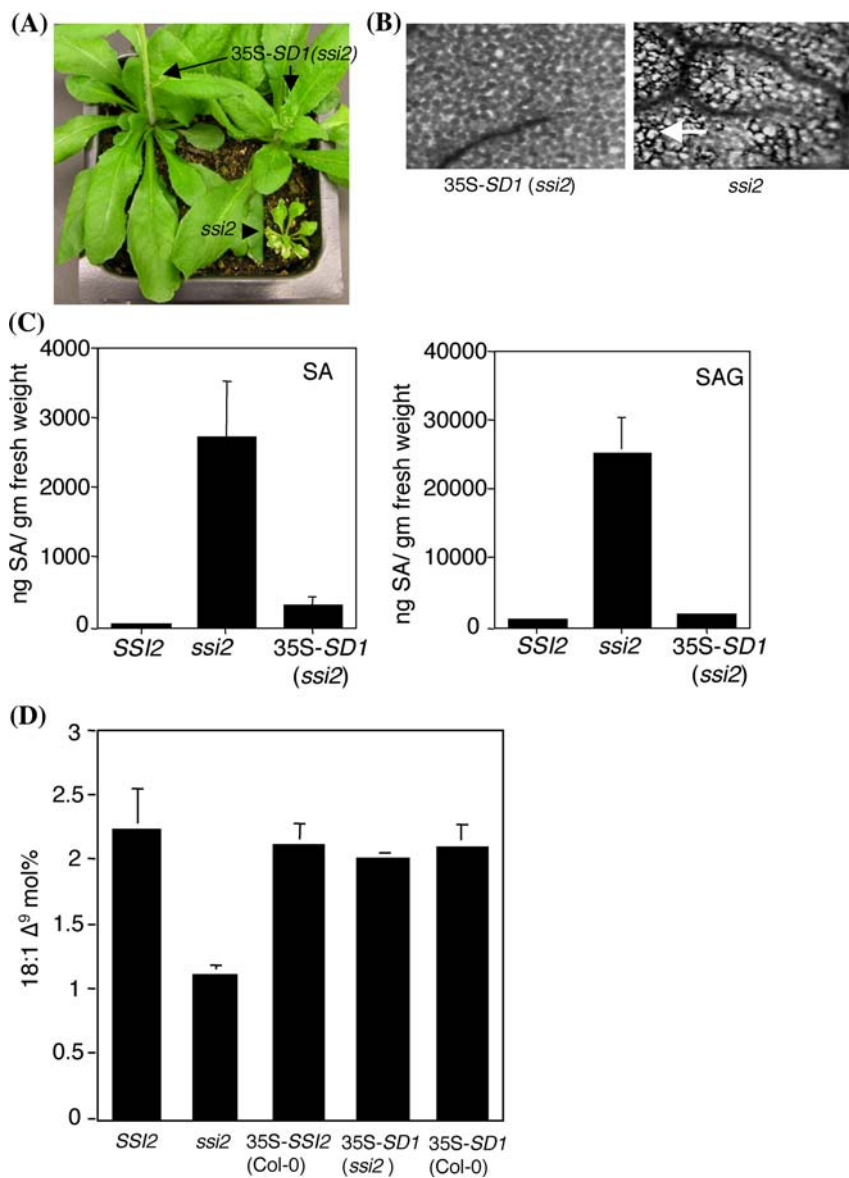
sequence conservation (supplemental Fig. 1A, B). In this study, we have examined the enzymatic activities and the possible physiological roles of the various *Arabidopsis* S-ACP-DES isozymes. Our data suggests that S-ACP-DES isozymes are likely to participate in various biological processes and are capable of making minor contributions to the total 18:1 pool in the plant.

Even though the SSI2 protein shares up to 74% amino acid sequence similarities with the other isozymes, these are unable to substitute for a defective *ssi2* protein in the mutant plants. This raises the question as to the role of these isozymes in plants and whether they contribute to the total 18:1 pool. Although both the *ssi2* and *fab2* mutations reduce the desaturase activity by 90 or 100%, respectively (Kachroo et al. 2001), they do not completely abolish the 18:1 content in the mutant plants; both *ssi2* and *fab2* plants continue to accumulate low levels of 18:1. This suggests that other S-ACP-DES isozymes make

minor contributions to the total 18:1 pool. This is further supported by the observation that, except for SSI2, all other S-ACP-DES enzymes tested showed very low specific activity towards C16:0- and C18:0-ACP substrates in vitro. The observed low specific activity of S-ACP-DES1 also explains why its overexpression was required to complement the various *ssi2*-associated phenotypes. Taken together, these observations suggest that the majority of the 18:1 pool in the plant results from SSI2 activity, while the other isozymes make minor contributions.

Various S-ACP-DES isoforms are likely to participate in normal physiological processes. This is suggested by the observation that a loss-of-function mutation in *S-ACP-DES1* or *4* resulted in a reduction in the total lipid content by ~23% and ~17%, respectively. Similarly, a mutation in *ssi2* also causes ~36% reduction in their lipid content as compared to wild-type plants. However, unlike the *ssi2* mutation, KO

**Fig. 8** Morphological and molecular phenotypes of *ssi2* and *35S-SD1(ssi2)* plants. **(A)** Comparison of the morphological phenotypes displayed by 4-week-old soil grown, *ssi2* and *35S-SD1(ssi2)* plants. **(B)** Microscopy of trypan-blue stained leaves from *ssi2* and *35S-SD1(ssi2)* plants. Leaves from *ssi2* plants contain intensely stained areas of dead cells (marked by a white arrow), while the *35S-SD1(ssi2)* leaves exhibit no cell death. **(C)** Endogenous SA and SA glucoside (SAG) levels in leaves of 4-week-old, soil-grown, wild-type, *ssi2* and *35S-SD1(ssi2)* plants. The values are presented as a mean of three replicates. The error bars represent SD. **(D)** The levels of chloroplastic 18:1 in *SSI2*, *ssi2*, *35S-SSI2* (Col-0) and *35S-SD1* (in *ssi2* and Col-0 backgrounds) plants. The values are a mean of six independent replicates. The error bars represent SD



**Table 3** Fatty acid composition of total leaf lipids from *SSI2*, *ssi2*, *35S-SSI2*, *35S-SD1(ssi2)*, *SD1KO* and *SD4KO* plants

Genotype <sup>a</sup>	Fatty acid <sup>a</sup>							
	16:0	16:1	16:2	16:3	18:0	18:1 <sup>b</sup>	18:2	18:3
<i>SSI2</i> (Col-0)	14.7 ± 0.3	3.4 ± 0.1	0.5 ± 0.2	15.1 ± 1.2	1.1 ± 0.3	2.2 ± 0.5	12.9 ± 0.7	49.7 ± 1.3
<i>ssi2</i> (Nö) <sup>c</sup>	15.9 ± 1.1	2.8 ± 0.3	0.2 ± 0.2	11.7 ± 0.4	16.9 ± 1.7	0.8 ± 0.1	12.1 ± 0.8	39.2 ± 1.3
<i>35S-SSI2</i> (Col-0)	15.6 ± 0.5	3.9 ± 0.4	0.9 ± 0.2	14.4 ± 1.6	1.1 ± 0.3	2.0 ± 0.7	15.0 ± 1.8	46.8 ± 1.6
<i>35S-SD1</i> ( <i>ssi2</i> )	12.6 ± 0.4	3.6 ± 0.1	1.0 ± 0.1	14.2 ± 0.6	5.1 ± 1.3	2.1 ± 0.4	12.8 ± 1.0	48.4 ± 0.9
<i>SD1KO</i> (Col-0)	14.4 ± 0.4	4.6 ± 0.1	1.2 ± 0.1	17.1 ± 0.2	0.4 ± 0	2.4 ± 0.3	14.2 ± 0.1	45.5 ± 1.0
<i>SD4KO</i> (Col-0)	15.3 ± 0.3	4.3 ± 0.8	1.1 ± 0.1	16.1 ± 0.7	0.5 ± 0	2.5 ± 0.1	15.2 ± 0.3	44.8 ± 1.2

<sup>a</sup> All measurements were made on plants grown at 22°C. Data are described as mol% ± SE (*n* = 6)

<sup>b</sup> 18:1<sup>9</sup>

<sup>c</sup> FA profile of Nö ecotype is similar to that of Col-0 (Kachroo et al. 2003b)

lines of *S-ACP-DES1* or 4 did not reduce total leaf or chloroplastic 18:1 content and did not show any morphological or defense phenotypes. It is possible that the effects of mutations in *SSI2* and *S-ACP-DES1* and 4 may trigger a reduction in lipid content by impacting different physiological processes. For instance, a mutation in *SSI2* may influence plant phenotypes due to lack of 18:1 whereas a mutation in *S-ACP-DES1* or 4 could influence various physiological processes via downregulation of the FA biosynthetic enzymes. These results are similar to those reported for *SCD1* deficient mice carrying a targeted disruption in the *SCD1* gene that have reduced body mass and exhibit increased oxidation of FAs (Ntambi et al. 2002). Unlike the *scd1* mutation, a mutation in *ssi2* also upregulated genes involved in FA synthesis, which suggests that plants possibly react to deficiencies in 18:1 by adjusting their FA metabolism.

Interestingly, wild-type-like levels of various *S-ACP-DES* isoforms was insufficient to complement a defect in *ssi2* plants. This could be because *ssi2* plants continue to accumulate wild-type levels of *ssi2* protein, which competes with other desaturases for substrate and/or cofactors. Thus, increased levels of a low specific activity desaturase would be required to out compete defective *ssi2* protein. This would also explain why increased expression of *S-ACP-DES1* was required to complement *ssi2* phenotypes. However, this possibility can be discounted because *fab2* plants, which contain a null mutation in *SSI2*, shows severe stunted morphological phenotype and like *ssi2* plants are defective in SA- and JA-mediated defense pathways.

Because overexpression of *SSI2* or *S-ACP-DES1* in wild-type plants did not result in increased accumulation of 18:1, it suggests that synthesis of 18:1-ACP is either tightly regulated or that it is rapidly metabolized. It is also possible that overexpression is unable to increase 18:1-ACP content beyond the wild-type levels because of substrate or cofactor limitations. Downregulation of *SSI2* transcript in *ssi2 act1* plants, which carry high levels of 18:1, suggests that 18:0-ACP desaturation is regulated via feedback control. This is further supported by the observation that a 5-fold excess of 18:1-ACP substrate resulted in 73% reduction in activity of *SSI2*, which is perhaps the major activity responsible for the synthesis of 18:1 $\Delta^9$ . It is also conceivable that increased 18:1 in the *ssi2 act1* plants inhibits the further formation of 18:1 by downregulating *SSI2* transcript levels. However, unlike *ssi2 act1* plants, *act1* single mutants carry wild-type levels of the *SSI2* transcript even though the 18:1 levels in *act1* plants are similar to those in *ssi2 act1* plants. An important difference between *act1* and *ssi2 act1* plants is that the latter contain high levels of 18:0 in addition

to high levels of 18:1. It is possible that, due to the increased abundance of the 18:0-ACP substrate, the *ssi2 act1* plants have the potential to generate even more 18:1-ACP, which could be potentially detrimental to the plant. Interestingly, the levels of the other *S-ACP-DES* isoforms in *ssi2 act1* plants were also downregulated, which further indicates that these isozymes may also be contributing to the 18:1 pool. These observations suggest that the expression of *S-ACP-DES*s is tightly regulated in order to exercise control over 18:1 levels, further emphasizing the importance of this monounsaturated FA in the normal, as well as defense-related physiology of the plant.

In comparison to *ssi2 act1*, the *ssi2 gly1* plants did not show any repression of *SSI2* transcript even though these plants accumulated wild-type-like levels of 18:1 (Kachroo et al. 2004). One possible explanation for this difference could be that the levels of 18:1 in *ssi2 gly1* plants are 2–3-fold lower than those in *ssi2 act1* plants. Furthermore, *ssi2* phenotypes reappear in 4-week-old *ssi2 gly1* plants but not in *ssi2 act1* plants (Kachroo et al. 2004). The reappearance of phenotypes in *ssi2 gly1* plants correlates with an age-dependent decline in 18:1, which suggests that the increased levels of 18:1 present during the early stages of development are sufficient to complement *ssi2* phenotypes. We have previously attributed the increased levels of 18:1 in *ssi2 gly1* plants to the reduced availability of G3P for acylation to 18:1, resulting from a mutation in the G3P dehydrogenase gene in these plants. Our present results have revealed that in comparison to *ssi2 act1* plants, the *ssi2 gly1* plants show marked increase in levels of the *S-ACP-DES3* transcript. Whether the increased levels of *S-ACP-DES3* in these plants contribute to the increase in 18:1 levels and thereby the complementation of *ssi2* phenotypes remains to be investigated.

In conclusion, we have shown that *S-ACP-DES* isoforms contribute to the 18:1 pool, although a major portion of this pool reflects contributions from *SSI2* activity. We have also shown that, unlike *SSI2*, the other isoforms have much lower specific activities. Furthermore, when *SSI2* and *S-ACP-DES1* isozymes, differing in specific activity by ~50-fold were overexpressed, they restored wild-type levels of 18:1 in *ssi2* plants along with wild-type responses to pathogen challenge. The data presented here supports our hypothesis that the *ssi2*-associated, altered defense signaling results from reduced levels of 18:1.

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## References

- Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, Gadri-nab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N, Hom E, Karnes M, Mulholland C, Ndubaku R, Schmidt I, Guzman P, Aguilar-Henonin L, Schmid M, Weigel D, Carter DE, Marchand T, Risseeuw E, Brogden D, Zeko A, Crosby WL, Berry CC, Ecker JR (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* 301:653–657
- Bassilian S, Ahmed S, Lim SK, Boros LG, Mao CS, Lee WN (2002) Loss of regulation of lipogenesis in the Zucker diabetic rat, II. Changes in stearate and oleate synthesis. *Am J Physiol Endocrinol Metab* 282:E507–E513
- Bowling SA, Clarke JD, Liu Y, Klessig DF, Dong X (1997) The *cpr5* mutant of *Arabidopsis* expresses both *NPRI*-dependent and *NPRI*-Independent resistance. *Plant Cell* 9:1573–1584
- Breuer S, Pech K, Buss A, Spitzer C, Ozols J, Hol EM, Heussen N, Noth J, Schwaiger FW, Schmitt AB (2004) Regulation of stearoyl-CoA desaturase-1 after central and peripheral nerve lesions. *BMC Neurosci* 20:15–23
- Cahoon EB, Mills LA, Shanklin J (1996) Modification of the fatty acid composition of *Escherichia coli* by coexpression of a plant acyl-acyl carrier protein desaturase and ferredoxin. *J Bacteriol* 178:936–939
- Cahoon EB, Shanklin J (1997) Approaches to the design of acyl-ACP desaturases with altered fatty acid chain-length and double bond positional specificities. In: Williams JP, Khan MU, Lem NW (eds) *Physiology, biochemistry and molecular biology of plant lipids*. Kluwer Academic Publishers, The Netherlands, pp 374–376
- Cahoon EB, Shah S, Shanklin J, Browse J (1998) A determinant of substrate specificity predicted from the acyl-acyl carrier protein desaturase of developing cat's claw seed. *Plant Physiol* 117:593–598
- Chandra-Shekara AC, Gupte M, Navarre DA, Raina S, Raina R, Klessig D, Kachroo P (2006) Light-dependent hypersensitive response and resistance signaling to Turnip Crinkle Virus in *Arabidopsis*. *Plant J* 45:320–334
- Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16:735–743
- Dahmer ML, Fleming PD, Collins GB, Hildebrand DF (1989) A rapid screening for determining the lipid composition of soybean seeds. *J Am Oil Chem Soc* 66:534–538
- Dobrzyn A, Ntambi JM (2005) The role of stearoyl-CoA desaturase in the control of metabolism. *Prostaglandins Leukot Essent Fatty Acids* 73:35–41
- Emanuelsson O, Nielsen H, Brunak S, Heijne GV (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J Mol Biol* 300:1005–1016
- Enser M (1975) Desaturation of stearic acid by liver and adipose tissue from obese-hyperglycaemic mice (*ob/ob*). *Biochem J* 148:551–555
- He Y, Fukushige H, Hildebrand DF, Gan S (2002) Evidence supporting a role of jasmonic acid in *Arabidopsis* leaf senescence. *Plant Physiol* 128:876–884
- Kachroo P, Shanklin J, Shah J, Whittle EJ, Klessig DF (2001) A fatty acid desaturase modulates the activation of defense signaling pathways in plants. *Proc Natl Acad Sci USA* 98:9448–9453
- Kachroo P, Kachroo A, Lapchik L, Hildebrand D, Klessig D (2003a) Restoration of defective cross talk in *ssi2* mutants; role of salicylic acid, jasmonic acid and fatty acids in *SSI2*-mediated signaling. *Mol Plant-Microbe Interact* 11:1022–1029
- Kachroo A, Lapchik L, Fukushige H, Hildebrand D, Klessig D, Kachroo P (2003b) Plastidial fatty acid signaling modulates plants epigenetically suppressed in phenylalanine ammonia-lyase expression do not develop systemic acquired resistance in response to infection by tobacco mosaic virus. *Plant Cell* 10:281–293
- Kachroo A, Venugopal SC, Lapchik L, Falcone D, Hildebrand D, Kachroo P (2004) Oleic acid levels regulated by glycerolipid metabolism modulate defense gene expression in *Arabidopsis*. *Proc Natl Acad Sci USA* 101:5152–5157
- Kachroo P, Venugopal SC, Navarre DA, Lapchik L, Kachroo A (2005) Role of salicylic acid and fatty acid desaturation pathways in *ssi2*-mediated signaling. *Plant Physiol* 139:1717–1735
- Kachroo A, Kachroo P (2006) Salicylic acid-, jasmonic acid- and ethylene-mediated regulation of plant defense signaling. In: Jane Setlow (ed) *Genetic engineering, principles and methods*. Springer pubs., vol. 28, In press
- Knutzon DS, Thompson GA, Radke SE, Johnson WB, Knauf VC, Kridl JC (1992) Modification of *Brassica* seed oil by antisense expression of a stearoyl-acyl carrier protein desaturase gene. *Proc Natl Acad Sci USA* 89:2624–2628
- Li C, Liu G, Xu C, Lee GI, Bauer P, Ling HQ, Ganai MW, Howe GA (2003) The tomato suppressor of prosystemin-mediated responses2 gene encodes a fatty acid desaturase required for the biosynthesis of jasmonic acid and the production of a systemic wound signal for defense gene expression. *Plant Cell* 15:1646–1661
- Lightner J, Wu J, Browse J (1994) A mutant of *Arabidopsis* with increased levels of stearic acid. *Plant Physiol* 106:1443–1451
- Lindqvist Y, Huang W, Schneider G, Shanklin J (1996) Crystal structure of delta9 stearoyl-acyl carrier protein desaturase from castor seed and its relationship to other di-iron proteins. *EMBO J* 15:4081–4092
- Lu J, Pei H, Kaeck M, Thompson HJ (1997) Gene expression changes associated with chemically induced rat mammary carcinogenesis. *Mol Carcinog* 20:204–215
- Mihara K (1990) Structure and regulation of rat liver microsomal stearoyl-CoA desaturase gene. *J Biochem* 108:1022–1029
- Miyazaki M, Gomez FE, Ntambi JM (2002) Lack of stearoyl-CoA desaturase-1 function induces a palmitoyl-CoA Delta6 desaturase and represses the stearoyl-CoA desaturase-3 gene in the preputial glands of the mouse. *J Lipid Res* 43:2146–2154

- Miyazaki M, Man WC, Ntambi JM (2001) Targeted disruption of stearoyl-CoA desaturase1 gene in mice causes atrophy of sebaceous and meibomian glands and depletion of wax esters in the eyelid. *J Nutr* 131:2260–2268
- Nandi A, Welte R, Shah J (2004) The *Arabidopsis thaliana* dihydroxyacetone phosphate reductase gene suppressor of fatty acid desaturase deficiency 1 is required for glycerolipid metabolism and for the activation of systemic acquired resistance. *Plant Cell* 16:465–477
- Ntambi JM, Buhrow SA, Kaestner KH, Christy RJ, Sibley E, Kelly TJ Jr, Lane MD (1988) Differentiation-induced gene expression in 3T3-L1 preadipocytes. Characterization of a differentially expressed gene encoding stearoyl-CoA desaturase. *J Biol Chem* 263:17291–17300
- Ntambi JM, Miyazaki M, Stoehr JP, Lan H, Kendzierski CM, Yandell BS, Song Y, Cohen P, Friedman JM, Attie AD (2002) Loss of stearoyl-CoA desaturase-1 function protects mice against adiposity. *Proc Natl Acad Sci USA* 99:11482–11486
- Schultz DJ, Cahoon EB, Shanklin J, Craig R, Cox-Foster DL, Mumma RO, Medford JI (1996) Expression of a delta 9 14:0-acyl carrier protein fatty acid desaturase gene is necessary for the production of omega 5 anacardic acids found in pest-resistant geranium (*Pelargonium hortorum*). *Proc Natl Acad Sci USA* 93:8771–8775
- Shah J, Kachroo P, Nandi A, Klessig DF (2001) A recessive mutation in the *Arabidopsis SSI2* gene confers SA- and NPR1-independent expression of *PR* genes and resistance against bacterial and oomycete pathogens. *Plant J* 25:563–574
- Shanklin J, Somerville C (1991) Stearoyl-acyl-carrier-protein desaturase from higher plants is structurally unrelated to the animal and fungal homologs. *Proc Natl Acad Sci USA* 88:2510–2514
- Shimomura I, Bashmakov Y, Horton JD (1999) Increased levels of nuclear SREBP-1c associated with fatty livers in two mouse models of diabetes mellitus. *J Biol Chem* 274:30028–30032
- Sivaramakrishnan MR, Pynadath TI (1982) Increased liver oleic acid synthesis in cholesterol-fed rabbits. *Atherosclerosis* 41:21–25
- Smith SB, Mersmann HJ, Smith EO, Britain KG (1999) Stearoyl-coenzyme A desaturase gene expression during growth in adipose tissue from obese and crossbred pigs. *J Anim Sci* 77:1710–1716
- Thai SF, Allen JW, DeAngelo AB, George MH, Fuscoe JC (2001) Detection of early gene expression changes by differential display in the livers of mice exposed to dichloroacetic acid. *Carcinogenesis* 22:1317–1322
- Thompson GA, Scherer DE, Foxall-Van, Aken S, Kenny JW, Young HL, Shintani DK, Kridl JC, Knauf VC (1991) Primary structures of the precursor and mature forms of stearoyl-acyl carrier protein desaturase from safflower embryos and requirement of ferredoxin for enzyme activity. *Proc Natl Acad Sci USA* 88:2578–2582
- Vijayan P, Shockey J, Levesque CA, Cook RJ, Browse J (1998) A role for jasmonate in pathogen defence of *Arabidopsis*. *Proc Natl Acad Sci USA* 95:7209–7214
- Waters KM, Ntambi JM (1994) Insulin and dietary fructose induce stearoyl-CoA desaturase 1 gene expression of diabetic mice. *J Biol Chem* 269:27773–27777
- Weber H (2002) Fatty acid derived signals in plants. *Trends Plant Sci* 7:217–224
- Whittle E, Cahoon EB, Subrahmanyam S, Shanklin J (2005) A multifunctional acyl-acyl carrier protein desaturase from *Hedera helix* L. (English Ivy) can synthesize 16- and 18-carbon monoene and diene products. *J Biol Chem* 280:28169–28176
- Worcester NA, Bruckdorfer KR, Hallinan T, Wilkins AJ, Mann JA, Yudkins J (1979) The influence of diet and diabetes on stearoyl coenzyme A desaturase (EC 1.14.99.5) activity and fatty acid composition in rat tissues. *Br J Nutr* 41:239–252
- Yaeno T, Matsuda O, Iba K (2004) Role of chloroplast trienoic fatty acids in plant disease defense responses. *Plant J* 40:931–941
- Zheng Y, Eilertsen KJ, Ge L, Zhang L, Sundberg JP, Prouty SM (1999) *Scd1* is expressed in sebaceous glands and is disrupted in the asebia mouse. *Nat Genet* 23:268–270
- Zheng Y, Prouty SM, Harmon A, Sundberg JP, Stenn KS, Parimoo S (2001) *Scd3-a* novel gene of the stearoyl-CoA desaturase family with restricted expression in skin. *Genomics* 71:182–191