

A Serine Carboxypeptidase-Like Acyltransferase Is Required for Synthesis of Antimicrobial Compounds and Disease Resistance in Oats

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Serine carboxypeptidase-like (SCPL) proteins have recently emerged as a new group of plant acyltransferases. These enzymes share homology with peptidases but lack protease activity and instead are able to acylate natural products. Several SCPL acyltransferases have been characterized to date from dicots, including an enzyme required for the synthesis of glucose polyesters that may contribute to insect resistance in wild tomato (*Solanum pennellii*) and enzymes required for the synthesis of sinapate esters associated with UV protection in *Arabidopsis thaliana*. In our earlier genetic analysis, we identified the *Saponin-deficient 7* (*Sad7*) locus as being required for the synthesis of antimicrobial triterpene glycosides (avenacins) and for broad-spectrum disease resistance in diploid oat (*Avena strigosa*). Here, we report on the cloning of *Sad7* and show that this gene encodes a functional SCPL acyltransferase, SCPL1, that is able to catalyze the synthesis of both *N*-methyl anthraniloyl- and benzoyl-derivatized forms of avenacin. *Sad7* forms part of an operon-like gene cluster for avenacin synthesis. Oat SCPL1 (*SAD7*) is the founder member of a subfamily of monocot-specific SCPL proteins that includes predicted proteins from rice (*Oryza sativa*) and other grasses with potential roles in secondary metabolism and plant defense.

INTRODUCTION

Plants produce a diverse array of natural products. These compounds serve important ecological functions, for example by providing protection against pathogens and herbivores (Dixon, 2001). Many plant-derived natural products are modified by the addition of acyl groups (D'Auria, 2006). Acylation of natural products contributes to structural and functional diversity and has in addition been linked to the chemical stability of a number of compounds. The best-studied family of plant natural product acyltransferases is the BAHD family, the members of which are collectively responsible for acylation of a wide range of compounds (Yang et al., 1997; Dudareva et al., 1998; Fujiwara et al., 1998; St Pierre et al., 1998; Walker et al., 2002; Luo et al., 2007,

2009; Grienemberger et al., 2009). Members of the BAHD family are involved in the synthesis of important natural products, including the anticancer drugs vinblastine (St Pierre et al., 1998) and taxol (Kingston 1994; Walker et al., 2002). More recently, serine carboxypeptidase-like (SCPL) proteins have emerged as a new group of acyltransferase enzymes that are able to modify plant natural products (Lehfeldt et al., 2000; Li and Steffens, 2000; Shirley et al., 2001; Fraser et al., 2007; Weier et al., 2008). These two different classes of acyltransferases operate by distinct mechanisms. While BAHD acyltransferases use CoA-thioesters as the acyl donors, SCPL acyltransferase enzymes use *O*-glucose esters (Shirley and Chapple, 2003; Milkowski and Strack, 2004; Milkowski et al., 2004; Baumert et al., 2005; Stehle et al., 2008).

The SCPL acyltransferases that have been cloned and characterized to date are all of dicot origin (Lehfeldt et al., 2000; Li and Steffens, 2000; Shirley et al., 2001; Fraser et al., 2007; Weier et al., 2008). Oats (monocots belonging to the genus *Avena*) produce acylated defense compounds known as avenacins. Avenacins are antimicrobial triterpene glycosides (saponins) that are synthesized in the roots and confer broad-spectrum disease resistance to soil-borne pathogens (Hostettmann and Marston, 1995; Papadopoulou et al., 1999). The ability to synthesize avenacins is restricted to the genus *Avena* and has evolved since the divergence of oats from other cereals and grasses (Haralampidis et al., 2001; Qi et al., 2004, 2006; Mylona et al., 2008). There are four structurally related avenacins, A-1, B-1,


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
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A-2, and B-2 (Hostettmann and Marston, 1995) (Figure 1A). Avenacins A-1 and B-1 are acylated with *N*-methyl anthranilate at the C-21 carbon position, while avenacins A-2 and B-2 are acylated with benzoate at this position. Avenacin A-1 is the most abundant avenacin found in young oat roots. This compound has strong fluorescence under ultraviolet illumination due to the presence of the *N*-methyl anthraniloyl group. Previously, we have exploited this property to screen for reduced root fluorescence mutants of diploid oat (*Avena strigosa*) that are unable to produce avenacins (Papadopolou et al., 1999). Avenacin-deficient mutants of *A. strigosa* are compromised in disease resistance, indicating that avenacins provide protection against microbial attack (Papadopolou et al., 1999). We have cloned the gene for the first committed step in the pathway, *Saponin deficient 1* (*Sad1*), which encodes the oxidosqualene cyclase enzyme β -amyrin synthase (Haralampidis et al., 2001) and the gene for a second early pathway step, *Sad2*, which encodes a novel cytochrome P450 (Qi et al., 2006). *Sad1* and *Sad2* are adjacent genes that lie ~ 70 kb apart in the *A. strigosa* genome (Qi et al., 2006). Remarkably, five of the six other loci that we have defined by mutation as being required for avenacin synthesis also cosegregate with *Sad1* and *Sad2*, indicating that most of the genes for the pathway are likely to be clustered (Qi et al., 2004). Genes for the majority of the plant secondary metabolic pathways that have been so far characterized (such as those for anthocyanin biosynthesis) are generally unlinked. However operon-like gene clusters for secondary metabolic pathways are emerging as a new theme in plant biology (Frey et al., 1997; Qi

et al., 2004; Wilderman et al., 2004; Shimura et al., 2007; Field and Osbourn, 2008; Osbourn and Field, 2009).

Mutants affected at *Sad7*, which is required for avenacin synthesis, accumulate avenacin biosynthetic intermediates lacking the *N*-methyl anthraniloyl or benzoyl groups (des-acyl avenacins A and B) (Figure 1B) (Qi et al., 2004). Here, we report the cloning of *Sad7*, and we show that this gene, which forms part of the avenacin gene cluster, encodes an SCPL acyltransferase that is required for avenacin acylation and plant defense. SAD7 (SCPL1) is the founder member of a subfamily of SCPL proteins that also includes uncharacterized SCPL proteins from other cereals and grasses. Our demonstration that SCPL1 is a functional acyltransferase with a role distinct from those of other characterized enzymes from this family therefore opens up opportunities for identifying new enzymes, metabolites, and pathways with possible protective functions in biotic and/or abiotic stress tolerance in other cereals.

RESULTS

SCPL1 Is Synonymous with *Sad7*

Previously, we reported the identification of *A. strigosa* mutants #376 and #616, both of which are deficient in avenacin acylation (Papadopolou et al., 1999; Qi et al., 2004). We have subsequently shown that the mutations in #376 and #616 are allelic and have assigned these two mutants as independent mutant alleles

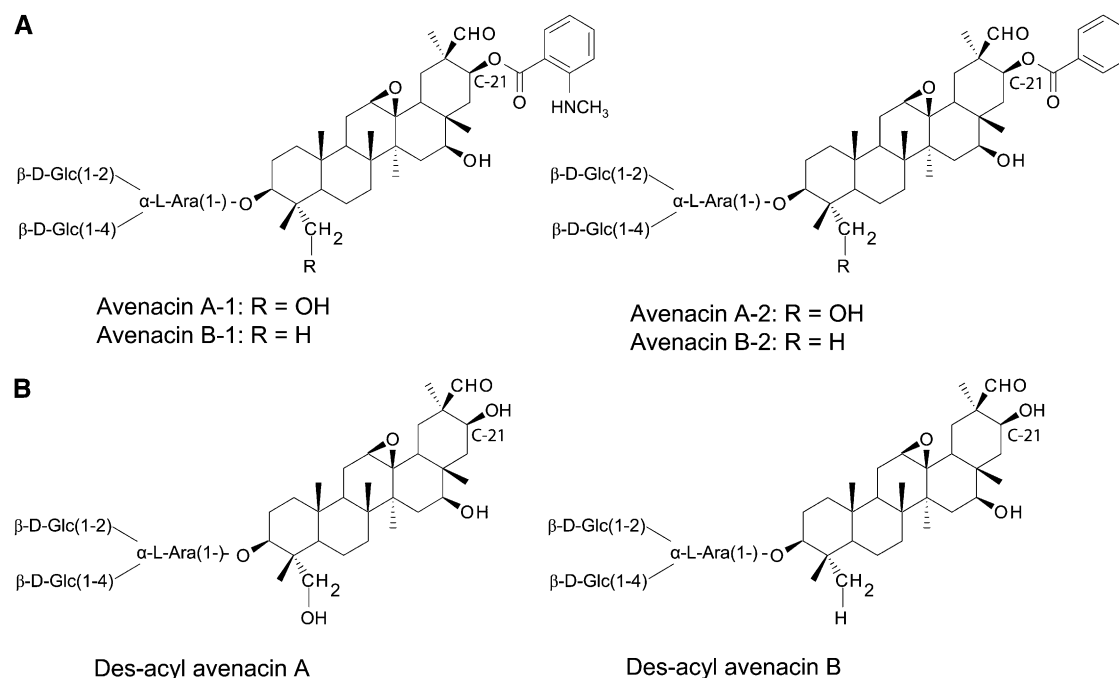


Figure 1. Structures of Avenacins and Des-acyl Avenacins.

(A) Avenacins A-1 and B-1 (left) are esterified at the C-21 position with *N*-methyl anthranilate, and avenacins A-2 and B-2 (right) with benzoate.
(B) Des-acyl avenacins A and B.

of *Sad7*. Genetic analysis indicates that *Sad7* cosegregates with the two previously cloned avenacin biosynthesis genes, *Sad1* and *Sad2* (Papadopoulou et al., 1999; Qi et al., 2004). We extended the BAC contig spanning the *Sad1* and *Sad2* genes (Qi et al., 2006) and discovered a third gene, *SCPL1* (Figure 2A). *SCPL1* is predicted to encode an SCPL protein.

The synthesis of avenacins is tightly regulated and is restricted to the epidermal cells of the root tip (Haralampidis et al., 2001). Previously, we have shown that *Sad1* and *Sad2* are expressed specifically in the root tip cells (Haralampidis et al., 2001; Qi et al., 2006). RNA gel blot analysis indicated that expression of *SCPL1* was also root specific (Figure 2B). Furthermore, mRNA in situ hybridization revealed that expression of *SCPL1* within the root tip is restricted to the epidermal cells (Figure 2C), as is the case for *Sad1* and *Sad2* (Haralampidis et al., 2001; Qi et al., 2006). These cells are the site of avenacin accumulation (Osbourne et al., 1994; Haralampidis et al., 2001). The expression pattern of *SCPL1* is therefore consistent with a role for *SCPL1* in avenacin biosynthesis.

Since some dicot SCPL proteins are known to have acyltransferase functions (Lehfeldt et al., 2000; Li and Steffens, 2000; Shirley et al., 2001; Fraser et al., 2007; Weier et al., 2008), and *Sad7* is known to be genetically linked to *Sad1* and *Sad2*, *SCPL1* became a candidate avenacin acyltransferase gene. We sequenced the *SCPL1* gene in mutants #376 and #616 and identified a single nucleotide polymorphism (SNP) in each. These mutations are both predicted to give rise to amino acid changes (in mutant #376, the mutation was C236T corresponding to an amino acid change of P79L, and in mutant #616, from C410T corresponding to S137F) (see Supplemental Figure 1 online). We identified a third candidate *sad7* mutant (#19) by surveying for

SNPs in *SCPL1* in a wider collection of ~90 uncharacterized avenacin-deficient mutants (Qi et al., 2006). DNA sequence analysis revealed that mutant #19 had also undergone a non-synonymous point mutation in the *SCPL1* coding sequence (C1388T, corresponding to T463I) (see Supplemental Figure 1 online). Liquid chromatography–mass spectrometry (LC-MS) analysis of root extracts confirmed that mutant #19, like mutants #376 and #616, is defective in avenacin acylation. All three mutants failed to synthesize avenacins A-1, B-1, A-2, and B-2 and instead accumulated des-acyl avenacins A and B (Figure 3; see Supplemental Figure 2 online). Collectively, these data indicate that *SCPL1* is synonymous with *Sad7* and is required for avenacin acylation.

Oat *SCPL1* Is a Member of a Monocot-Specific Subfamily of SCPLs

The plant SCPL acyltransferases that have been characterized to date and functionally confirmed as acyltransferases are marked with blue circles in Figure 4. Most of these are either from *Arabidopsis thaliana* or *Brassica napus* and fall within a Brassicaceae-specific subgroup of the serine carboxypeptidase/SCPL protein family within Clade 1A (Fraser et al., 2005). These enzymes include the sinapoylglucose:malate sinapoyltransferase from *Arabidopsis* (SNG1), which is required for the synthesis of the UV protectant sinapoylmalate (Lehfeldt et al., 2000), and sinapoylglucose:choline sinapoyltransferases (SCTs) from *Arabidopsis* (SNG2) and *B. napus* (Bn SCT1 and Bn SCT2), which are required for the formation of sinapoylcholine in seeds (Shirley et al., 2001; Weier et al., 2008). The wild tomato (*Solanum pennellii*) SCPL acyltransferase GAC, which catalyzes

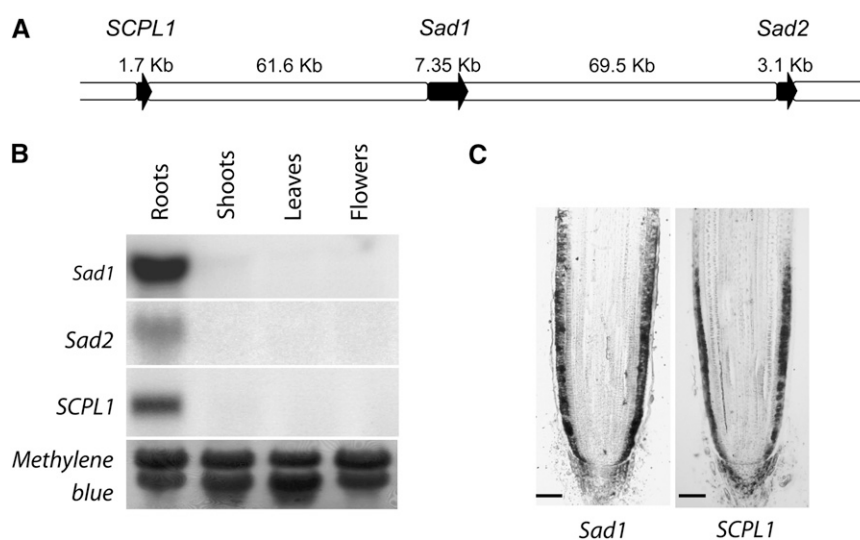


Figure 2. *SCPL1*, a Gene That Is Predicted to Encode an SCPL Protein, Is Linked to and Coexpressed with *Sad1* and *Sad2*.

(A) BAC contig showing the positions of the *Sad1*, *Sad2*, and *SCPL1* genes.

(B) RNA gel blot analysis of transcripts of *SCPL1* and the two cloned avenacin pathway genes *Sad1* and *Sad2*; RNA loading was monitored with methylene blue.

(C) mRNA in situ localization of *Sad1* and *SCPL1* transcripts in *A. strigosa* root tips. No signal was observed for sections probed with sense control probes. Bars = 100 μ m.

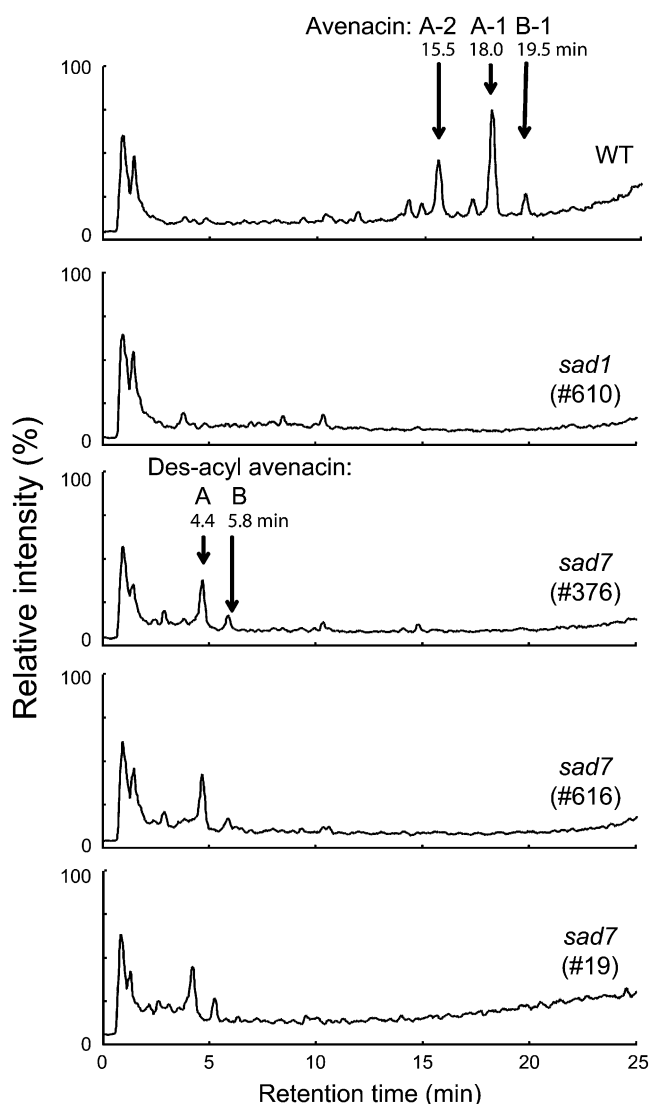


Figure 3. *sad7* Mutants Accumulate Des-Acyl Avenacins.

LC-MS analysis of root extracts of wild-type and mutant oat lines. Total ion chromatograms for wild-type (WT), *sad1*, and *sad7* mutants reveal accumulation of unacylated intermediates (des-acyl avenacins A and B) in the *sad7* mutants. The peak at 4.4 min is comprised of a major ion at 984 amu, corresponding to avenacin A-1/A-2 minus the acyl group [$M + Na^+$] (des-acyl avenacin A) (Figure 1B). Similarly, the peak at 5.8 min is comprised of a major ion at 968 amu, which corresponds to avenacins B-1/B-2 minus the acyl group [$M + Na^+$] (des-acyl avenacin B) (Figure 1B). The identity of these compounds is supported by MS2 fragmentation data (see Supplemental Figures 2A to 2C online). The minor avenacin B-2 is present only in trace amounts in root extracts from wild-type seedlings but can be detected by MS.

the formation of glucose polyesters associated with insect resistance in wild tomato (Li and Steffens 2000), does not lie within the Brassicaceae-specific subgroup but does group within Clade 1A (Fraser et al., 2005).

Oat SCPL1 (indicated by a red circle in Figure 4) defines a previously uncharacterized monocot-specific group of SCPL

proteins within Clade 1A. This group of SCPL proteins also contains uncharacterized sequences from rice (*Oryza sativa*) and from the model grass species *Brachypodium distachyon*. As mentioned earlier, avenacins are only produced by members of the genus *Avena* and not by other cereals and grasses (Hostettmann and Marston, 1995). *Sad1* and *Sad2* have both arisen relatively recently by gene duplication, divergence, and acquisition of new functions and have undergone accelerated evolution (Haralampidis et al., 2001; Qi et al., 2006). Oats are more closely related to *B. distachyon* than to rice. However, our phylogenetic analysis shows that the closest homologs of SCPL1 from rice and *B. distachyon* (encoded by *Os10g01134* and *Bd SCPL1*, respectively) are more similar to each other than to oat SCPL1, suggesting functional diversification of oat SCPL1. Consistent with this, single nucleotide differences between oat SCPL1 and *Bd SCPL1* and between oat SCPL1 and *Os10g01134* are biased toward nonsynonymous changes (codon-based test of neutrality: $d_n - d_s = 3.78$ versus *Bd SCPL1* and 3.36 versus *Os10g01134*, $P < 0.01$ for both comparisons; Nei and Gojobori, 1986).

***sad7* Mutants Accumulate *N*-Methyl Anthraniloyl-O-Glucose**

Avenacin A-1 causes the root tips of wild-type *A. strigosa* seedlings to fluoresce strongly under UV illumination, while roots of *sad1* mutants have little or no fluorescence (Papadopolou et al., 1999). By contrast, the root tips of *sad7* mutants still have some UV fluorescence (although less than those of the wild type) despite lacking avenacins (Figure 5). We used LC-MS analysis in combination with a fluorescence detector to investigate the nature of the fluorescent material present in the root tips of *sad7* mutants. Extracts from the root tips of *sad7* mutants contained elevated levels of a fluorescent compound with a mass spectrum consistent with that predicted for *N*-methyl anthraniloyl-O-glucose (Figure 6A; see Supplemental Figures 2D to 2F online). The levels of this compound were clearly elevated in *sad7* root extracts compared with extracts from roots of the wild type and *sad1* mutants (Figure 6B). These results suggest that SCPL1, like the other characterized dicot SCPL acyltransferases (Shirley and Chapple, 2003; Milkowski and Strack, 2004; Milkowski et al., 2004; Baumert et al., 2005; Stehle et al., 2008), uses an O-glucose ester as the acyl donor substrate.

The SCPL1 Protein Is Posttranslationally Processed and Is Likely to Function as a Heterodimer

The oat SCPL1 gene encodes a predicted protein of 493 amino acids. This includes a predicted 21 amino acid hydrophobic N-terminal signal peptide, removal of which will result in a 51.4-kD protein. The presence of the predicted signal peptide suggests targeting of the protein to the secretory pathway or, more likely, to the vacuole. Avenacins are known to accumulate in the vacuoles of oat root epidermal cells (Mylona et al., 2008), and the only SCPL acyltransferase that has been localized to date (the *Arabidopsis* 1-O-sinapoylgucose:malate sinapoyltransferase) is found in the vacuole (Hause et al., 2002).

Several dicot SCPL proteins have also been shown to undergo further processing and to be cleaved into two subunits (a small

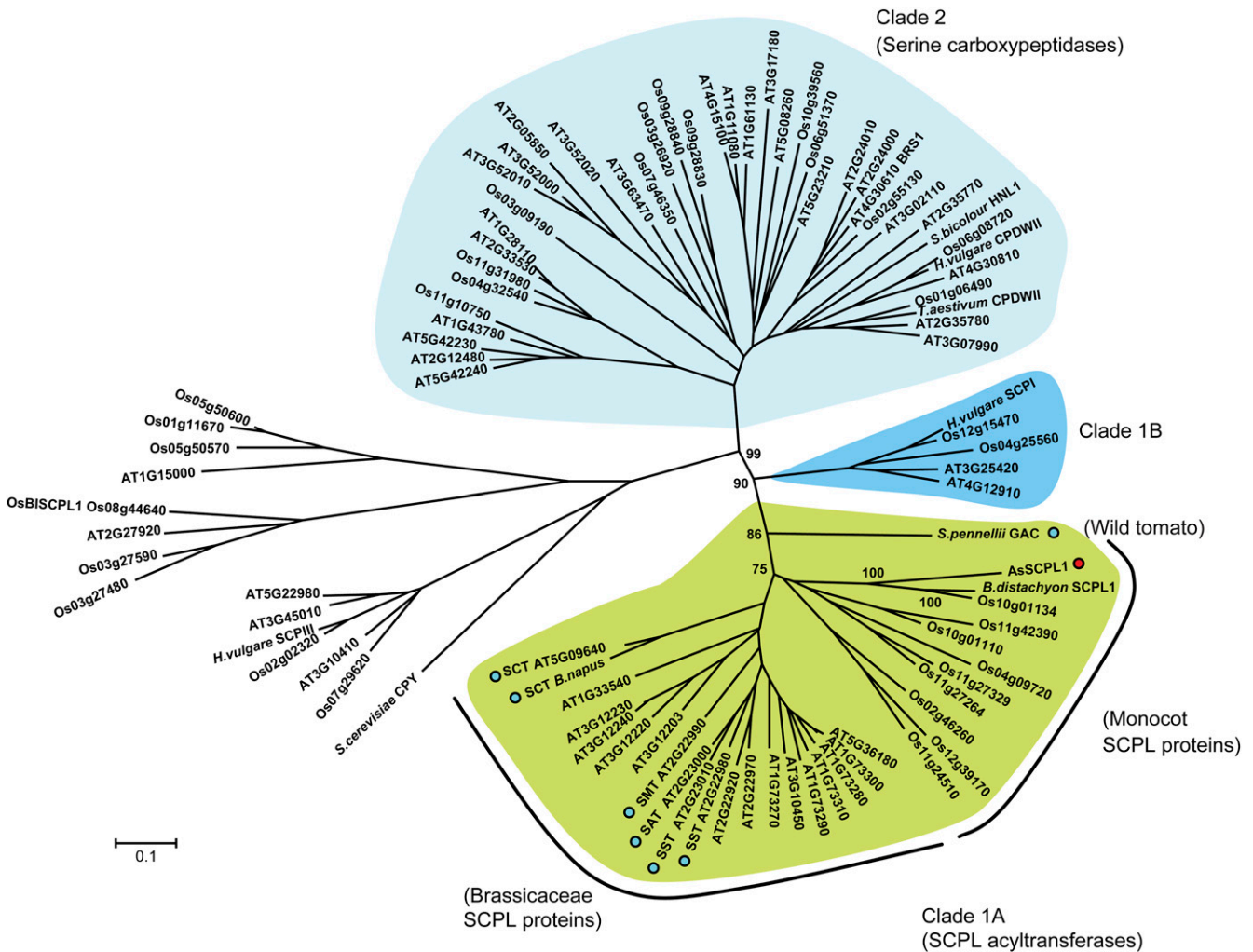


Figure 4. Oat SCPL1 Defines a Clade of Monocot-Specific SCPL Proteins.

A neighbor-joining phylogenetic analysis of SCP and SCPL proteins is shown. Bootstrap values (percentage values from 1000 replicates) are presented for key branches. The scale bar indicates 0.1 substitutions per site. The highlighted clades are based on the classification of Fraser et al. (2005). Blue circles indicate functionally characterized SCPL acyltransferase enzymes that have previously been reported: SCT, sinapoylglucose:choline sinapoyltransferases from *Arabidopsis* (Shirley et al., 2001) and *B. napus* (Weier et al., 2008); SMT, *Arabidopsis* sinapoylglucose:malate sinapoyltransferase (Lehfeldt et al., 2000); SAT, *Arabidopsis* sinapoylglucose:anthocyanin sinapoyltransferase (Fraser et al., 2007); SST, *Arabidopsis* sinapoylglucose:sinapoylglucose sinapoyltransferase (Fraser et al., 2007); GAC, glucose acyltransferase from wild tomato (Li and Steffens, 2000). Oat SCPL1 (SAD7) is indicated by the red circle. Protein sequence accession numbers, sequence alignment, and further details of the sequences used for this phylogenetic analysis are provided in Supplemental Table 1, Supplemental Data Set 1, and Supplemental Figure 1 online.

subunit and a large subunit) that together function as a heterodimer (Liao and Remington, 1990; Li and Steffens, 2000; Shirley et al., 2001; Zhou and Li, 2005). However, not all SCPL proteins undergo such processing (Hause et al., 2002; Stehle et al., 2006). Comparison of the amino acid sequences of oat SCPL1 with those of characterized SCP and SCPL proteins (some of which undergo cleavage and some of which do not) reveals that SCPL1 has an extended linker region typical of SCPL proteins that are cleaved into two subunits (Figure 7A). Based on this comparison, we predict that SCPL1 is cleaved in this region and is likely to function as a heterodimer consisting of a large subunit of ~ 29 kD and a small subunit of ~ 19 kD.

Oat SCPL1 was expressed in *Escherichia coli* and purified from inclusion bodies and the purified protein used to raise specific antisera in rabbits (see Supplemental Materials and Methods online). Immunoblot analysis identified bands corresponding to the small (19 kD) and large (29 kD) subunits of SCPL1 in soluble protein extracts from wild-type oat roots as predicted (Figure 7B). A third band of ~33 kD was also detected. This may correspond to the large subunit plus the internal cleaved linker peptide, suggesting that SCPL1 cleavage is a two-step process with the detachment of the small subunit followed by the removal of the linker peptide from the large subunit (Figure 7C). In some experiments, a band at 52 kD was also detected at low levels in

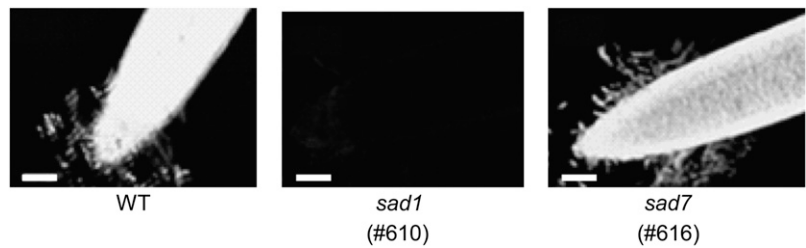


Figure 5. Roots of *sad7* Mutants Contain UV Fluorescent Material.

Unlike other avenacin-deficient mutants, such as *sad1*, *sad7* mutants exhibit some blue fluorescence under UV illumination (although this is weaker than that observed in wild-type roots). Images of 3-d-old roots under UV illumination (wavelength 290 to 320 nm). Bars = 100 μ m.

wild-type oat roots (data not shown). This presumably corresponds to the full-length unprocessed protein. The levels of the SCPL1 protein were either reduced (#376) or abolished (#616 and #19) in all three *sad7* mutants (Figure 7B), although the levels of SCPL1 transcript were not substantially altered in these mutants (see Supplemental Figure 3 online). Sequence alignment revealed that the sites of all three mutations are highly conserved in other plant SCPL proteins (see Supplemental Figure 1 online). Based on alignment with the wheat (*Triticum aestivum*) CPDWII protein (Liao and Remington, 1990), the mutation in #376 (P79A) corresponds to a Pro residue in the large subunit that forms a loop between two β -sheets within the active site, which might be expected to impair the activity of the protein. The mutated amino acid in mutant #616 (S137F) lies within a β -sheet in the large subunit, while the mutated residue in mutant #19 (T463L) is located in a β -sheet in the small subunit. Since the mutations in #616 and #19 affect conserved residues within the SCPL1 secondary structure, they may affect protein folding, resulting in targeting of the misfolded protein for degradation by the proteasome.

SCPL1 Restores Avenacin Acylation

We then performed functional analysis of SCPL1 using a plant virus-based system to express the oat protein in *Nicotiana benthamiana* leaves (Cañizares et al., 2006; Sainsbury et al., 2008). The SCPL1 open reading frame was cloned into the coding sequence of the RNA-2 component of cowpea mosaic virus (CPMV) under the control of the CaMV35S promoter. *N. benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* cells carrying the CPMV-SCPL1-1 construct. SCPL1 protein was readily detectable in protein extracts from infiltrated leaves by immunoblot analysis and appeared to be correctly processed (Figure 8). In addition to the expected 19-, 29-, and 33-kD bands, a 52-kD band was also detected that presumably corresponds to the full-length protein (Figure 7C). Attachment of a 6xHis epitope tag to the C terminus of the SCPL1 protein resulted in lower levels of protein accumulation and a reduction in correctly processed SCPL1 protein (Figure 8). SCPL1 protein expression was barely detectable when the His-tag was attached to the N terminus in place of the signal peptide (see Supplemental Figure 4 online).

Des-acyl avenacins and *N*-methyl anthraniloyl-O-glucose are not commercially available, and purification of these compounds

is problematic because they are present in the root tips of *sad7* mutants at only very low levels (Figures 3 and 6). We therefore assayed for acyltransferase activity by incubation of desalted protein preparations from SCPL1-expressing *N. benthamiana* leaves with methanolic extracts from roots of *sad7* mutants,

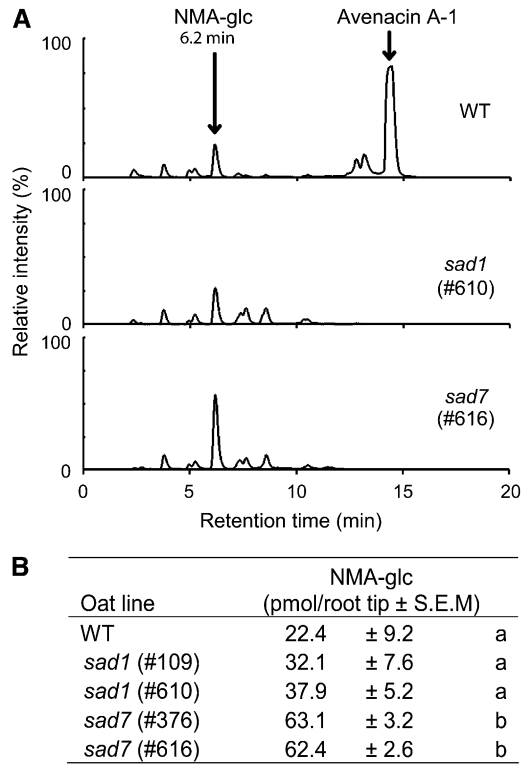


Figure 6. *sad7* Mutants Accumulate *N*-Methyl Anthraniloyl-O-Glucose.

(A) LC-MS analysis of fluorescent components of root extracts from wild-type (WT) and mutant *A. strigosa* lines. The peak eluting at 6.2 min consists of a major ion of 336 amu corresponding to *N*-methyl anthraniloyl-O-glucose (NMA-glc) [M+Na⁺]. The identity of this compound is supported by MS2 fragmentation data (see Supplemental Figures 2D to 2F online).

(B) Quantification of *N*-methyl anthraniloyl-O-glucose from LC separations based on fluorescence ($n = 3$) \pm SE. Different letters (a or b) indicate significant differences in *N*-methyl anthraniloyl-O-glucose levels ($P < 0.01$, t test with Bonferroni correction). The concentration of avenacin A-1 in wild-type root extracts was 103.2 pmol/root tip \pm 18.

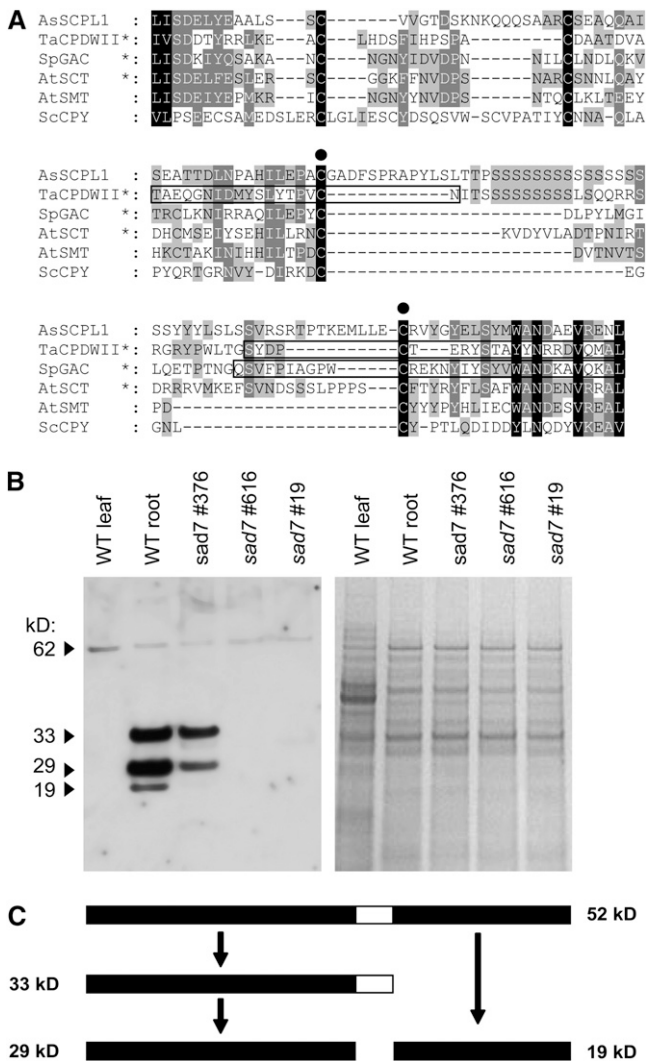


Figure 7. SCPL1 Is Posttranslationally Cleaved.

(A) Alignment of SCP and SCPL protein sequences covering the site of posttranslational cleavage. Sequences known to be posttranslationally cleaved are indicated with an asterisk. Sequences shown are SCPL1 (SAD7) (amino acids 210 to 411); CPDWII, *T. aestivum* Ser carboxypeptidase II (P08819) (amino acids 185 to 356); GAC *S. pennellii* glucose acyltransferase (AAF64227) (amino acids 205 to 380); SCT, *Arabidopsis* sinapoylglucose:choline sinapoyltransferase (AY033947) (amino acids 216 to 391); SMT, *Arabidopsis* sinapoylglucose:malate sinapoyltransferase (AF275313) (amino acids 201 to 350); and CPY, *Saccharomyces cerevisiae* carboxypeptidase Y (1YSC) (amino acids 174 to 334 of the mature peptide sequence). The regions of the *T. aestivum* CPDWII and *S. pennellii* GAC enzymes that have been shown to be present in the mature proteins are boxed, indicating the likely sites of cleavage (Liao and Remington, 1990; Li and Steffens, 2000). Similar amino acids are highlighted in black, dark, or light gray (100, >60, and >40% similarity, respectively). Conserved Cys residues that are likely to form intersubunit disulphide bond are indicated by black circles.

(B) Immunoblot analysis of protein extracts from roots of seedlings of wild-type (WT) and *sad7* mutant *A. strigosa* lines probed with anti-SCPL1 antisera (left) and a replicate gel stained with Coomassie blue (right). A weaker background band of ~62 kD was detected on the immunoblot

followed by LC-MS analysis of the reaction products to determine whether SCPL1 could restore avenacin synthesis. Assays with protein preparations from SCPL1-expressing *N. benthamiana* leaves yielded all four avenacins (Table 1). Avenacins were not formed in assays with protein preparations from *N. benthamiana* leaves that had been infiltrated with *A. tumefaciens* carrying the empty vector, with boiled protein preparation from *N. benthamiana* leaves expressing the SCPL1 protein, or when active SCPL1 protein was incubated with methanolic extracts from roots of *sad1* mutants (which do not contain des acyl avenacins). Thus, SCPL1 is able to catalyze the transfer of both *N*-methyl anthraniloyl- and benzoyl-groups to des-acyl avenacins. While the native protein was effectively processed and active, attachment of a 6XHis epitope tag at either the C- or the N-terminal end of the protein resulted in severely reduced or abolished levels of the processed SCPL1 protein and acyltransferase activity (Figure 8; see Supplemental Figure 4 online). Stehle et al. (2008) have also found that epitope tagging of the *Arabidopsis* sinapoylglucose:malate sinapoyltransferase enzyme abolished acyltransferase activity. Subsequent experiments involving incubation of SCPL1 protein preparations with HPLC-purified fractions that were enriched for des-acyl avenacin A and *N*-methyl anthraniloyl-O-glucose, respectively (see Supplemental Figure 5 online), resulted in the formation of avenacin A-1 (34.6 pmoles/ μ g protein/h \pm 5.95), while avenacin was not detected in control reactions performed with protein preparations from *N. benthamiana* leaves infiltrated with *A. tumefaciens* carrying the empty vector. Full biochemical analysis of the kinetic properties of SCPL1 will depend on the development of methods for the synthesis/purification of the desacyl avenacin and *N*-methyl anthraniloyl-O-glucose substrates in quantity and for purification of the functional protein and will form the basis of future work.

DISCUSSION

Here, we report on the characterization of a new SCPL acyltransferase, SCPL1, which is required for the synthesis of defense compounds in oats. The SCPL1 protein forms part of a previously uncharacterized monocot-specific subfamily of SCPL proteins within Clade 1A (Fraser et al., 2005). We show by genetic analysis that SCPL1 is synonymous with *Sad7*, a gene that we had previously defined by mutation as being required for avenacin acylation and plant defense (Qi et al., 2006). *sad7* mutants with defined SNPs in the SCPL1 (*Sad7*) gene accumulate des-acyl avenacins and the glucose ester *N*-methyl anthraniloyl-O-glucose. The accumulation of *N*-methyl anthraniloyl-O-glucose is consistent with the use of a glucose ester acyl donor

in samples from both leaves and roots. Since this is larger than the predicted full-length SCPL1 protein and SCPL1 is expressed only in roots, this is unlikely to be an SCPL1 gene product. Replicate blots probed with pre-immune sera did not result in the detection of any bands.

(C) Proposed processing of the SCPL1 protein from the 52-kD full-length protein, via a 33-kD intermediate, to the mature 29- and 19-kD subunits.

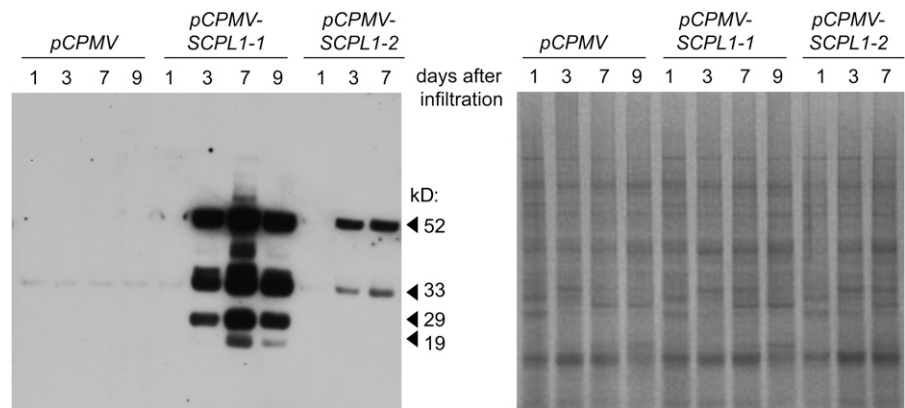


Figure 8. Heterologous Expression of SCPL1 in *N. benthamiana* Leaves Using the CPMV Expression System. Left, immunoblot using polyclonal antisera raised against SCPL1. Total soluble protein extracts were prepared from *N. benthamiana* leaves that had been infiltrated with *A. tumefaciens* carrying either the CPMV empty vector construct (*pCPMV*), CPMV vector modified to express the SCPL1 protein (*pCPMV-SCPL1-1*), or a C-terminal His-tagged version of this (*pCPMV-SCPL1-2*). Samples were collected 1, 3, 7, and 9 d after leaf infiltration. Right, replicate gel stained with Coomassie blue showing protein loading.

substrate, as has been shown for other plant SCPL acyltransferase enzymes (Shirley and Chapple, 2003; Milkowski and Strack, 2004; Milkowski et al., 2004; Baumert et al., 2005; Stehle et al., 2008). We further show by functional analysis that SCPL1 is able to catalyze the formation of avenacin A-1 from des-acyl avenacin A and *N*-methyl anthraniloyl-*O*-glucose. Together, these data demonstrate that *SCPL1* encodes a new SCPL acyltransferase that is required for the synthesis of acylated plant defense compounds (avenacins) in oats.

BAHD acyltransferases that catalyze the transfer of benzoate to various natural products have previously been reported (Yang et al., 1997; Walker et al., 2002). The absence of all four avenacins from root extracts of *sad7* mutants together with the observation that SCPL1 can catalyze the formation of all four avenacins indicates that this SCPL acyltransferase can catalyze the transfer of both *N*-methyl anthraniloyl and benzoyl groups onto des-acyl avenacins. Since *sad7* mutants accumulate the fully glycosylated structures shown in Figure 1B and avenacins are sequestered in the cell vacuoles (Mylona et al., 2008), it is likely that the addition of the acyl group occurs late in the pathway, most probably in the vacuole. Further experiments will determine whether this is the case and will also elucidate the mechanisms by which avenacin precursors are transported to the vacuole.

sad7 mutants have enhanced susceptibility to fungal pathogens, indicating that avenacin acylation is important for disease resistance (Papadopoulou et al., 1999). The acyl groups may contribute directly to the biological activity of the compounds, as has been observed for acylated saponins from tea (Yoshikawa et al., 2005) and for the anticancer drug taxol (Kingston, 1994). Alternatively, acylation may confer increased chemical stability, as has been suggested for anthocyanins and flavanols (Nakajima et al., 2003; Luo et al., 2007). Des-acyl avenacins A and B are present at very low levels in root extracts of *sad7* mutants, and purification of these compounds for use in assays of chemical and biological activity therefore represents a challenge. Future

work will involve the development of chemical and enzymatic methods that will enable the generation of the des-acyl avenacins in sufficient quantity to allow full biochemical analysis of the enzymatic properties of SCPL1 and will also allow the biological and physicochemical properties of these des-acyl compounds to be compared with those of the fully acylated avenacins. Acylated triterpenes have been reported from other plant species, and some of these, like the avenacins, are conjugated with *N*-methyl anthranilate or benzoate. For example, Yoshikawa et al. (1994, 1997) identified a number of acylated triterpenoid saponins, sitakissosides, in *Stephanotis lutchuensis*, including some that are acylated with *N*-methyl anthranilate. Triterpenoid saponins that are acylated with benzoate and that exhibit anti-leishmanial activity have also been reported from *Maesa balansae* (Germonprez et al., 2004). Triterpenes have a diverse range of biological activities and are an important source of pharmaceuticals and other commercially valuable compounds (Francis et al., 2002; Sparg et al., 2004; Liby et al., 2007).

Table 1. Avenacin Acyltransferase Activity Assay				
Protein Preparation	Avenacin Acyltransferase Activity (pmoles/μg/h)			
	A-1	A-2	B-1	B-2
<i>pCPMV</i>	n.d.	n.d.	n.d.	n.d.
<i>pCPMV-SCPL1-1</i>	8.10 (±1.34)	9.13 (±1.27)	1.09 (±0.14)	1.66 (±0.26)

Formation of avenacins following incubation of *sad7* mutant root extract with desalted protein extract from *N. benthamiana* expressing the SCPL1 protein 7 d after infiltration. Avenacins were analyzed by LC-MS with selective ion monitoring. Values are means (*n* = 3) ± SE, expressed as pmoles of avenacin per μg total protein per h (quantification based on a standard curve of avenacin A-1). *pCPMV*, empty vector control; *pCPMV-SCPL1-1*, recombinant cowpea mosaic virus containing the *SCPL1* cDNA. n.d., not detected.

The genes for avenacin synthesis are genetically linked (Papadopoulos et al., 1999; Qi et al., 2004). Previously, we have cloned and characterized two genes from this cluster: *Sad1*, which encodes the oxidosqualene cyclase that catalyzes the first committed step in avenacin synthesis (Haralampidis et al., 2001; Qi et al., 2004), and *Sad2*, which encodes a second early pathway enzyme (a cytochrome 450 required for modification of the triterpene skeleton) (Qi et al., 2006). *Sad1* and *Sad2* are adjacent genes within the *A. strigosa* genome and are ~70 kb apart (Qi et al., 2006). *Sad7* lies within ~60 kb of *Sad1* and is the third component of this operon-like gene cluster to be functionally characterized. *Sad1* and *Sad2* both appear to have been recruited from sterol metabolism by gene duplication, rapid sequence divergence, and acquisition of new function (Haralampidis et al., 2001; Qi et al., 2004, 2006). The *Sad7* gene also appears to have undergone accelerated sequence divergence, although the function of the ancestral gene is unknown. There is no evidence for a link between *Sad7* and sterol metabolism, although we note that acylated sterols have been identified in the grains of several cereals (Norton, 1994).

The reason why genes for some plant metabolic pathways are clustered and others are not is not yet understood. Genetic linkage of functionally related genes will facilitate coinheritance, which is expected to confer a selective advantage since gene clusters of this kind are generally associated with production of defense-related compounds (Frey et al., 1997; Papadopoulos et al., 1999; Shimura et al., 2007; Field and Osbourn, 2008; Osbourn and Field, 2009). Interference with the integrity of the avenacin gene cluster can on occasion lead not only to failure to produce protective pathway end products but also to the accumulation of toxic intermediates. This is the case for mutations at *Sad3*, a locus that is required for avenacin glucosylation (Mylona et al., 2008). However, mutants affected at the other linked loci that we have defined so far, including *Sad7*, do not have any obvious growth defects. Clustering may also be important for coordinate regulation of expression of pathway genes at the levels of chromatin and nuclear organization (Field and Osbourn, 2008; Osbourn and Field, 2009).

The SCPL acyltransferases that have been cloned and characterized previously are from dicots (Lehfeldt et al., 2000; Li and Steffens, 2000; Shirley et al., 2001; Fraser et al., 2007; Weier et al., 2008). These include an enzyme required for the synthesis of glucose polyesters that may contribute to insect resistance in wild tomato (GAC) (Li and Steffens, 2000) and SNG1, an enzyme required for the synthesis of sinapate esters associated with UV protection in *Arabidopsis* (Lehfeldt et al., 2000). SCPL1 is required for broad-spectrum disease resistance in cereals (Papadopoulos et al., 1999; Qi et al., 2004) and defines a subfamily of SCPL proteins that appears to be restricted to the Gramineae (Figure 4). The other members of this group include predicted SCPL proteins of unknown function from rice and from *B. distachyon*. The ability to synthesize avenacins is restricted to the genus *Avena*. Rice, *B. distachyon*, and other cereals and grasses do not appear to synthesize these compounds (Hostettmann and Marston, 1995). The functions of the predicted SCPL proteins from these other species are not known. However, it is possible that they may also be required for the synthesis of acylated compounds that provide protection against biotic

and/or abiotic stresses. The characterization of the oat SCPL acyltransferase SCPL1 as the founder member of a monocot-specific subfamily of SCPL acyltransferases opens up new opportunities for combining reverse genetics-based approaches with metabolomics to evaluate the function of SCPL proteins in other cereals and grasses.

METHODS

Plant Material

Wild-type and mutant *Avena strigosa* lines are described by Papadopoulos et al. (1999) and Qi et al. (2006).

BAC Contig Development, Gene Expression Analysis, and Identification of New *sad7* Alleles

The *A. strigosa* BAC library and development of the original ~100-kb BAC contig spanning *Sad1* and *Sad2* have been described previously (Qi et al., 2006). This BAC contig was extended by screening the BAC library with single/low-copy number probes designed from the ends of BAC clones. Overlapping BAC clones were verified by PCR and genetic fingerprinting. Sequencing of BAC clones was performed by standard BAC shotgun sequencing. *SCPL1*, *Sad1*, and *Sad2* are the only predicted genes within this sequenced region. RNA gel blots were performed as described previously (Qi et al., 2004, 2006). For mRNA in situ hybridization analysis, full-length antisense probes labeled with digoxigenin-11-UTP (Roche) were prepared from the cDNAs for *SCPL1* and *Sad1*. Tissue section preparation and hybridization were performed as by Qi et al. (2006). Screens for SNPs in the *SCPL1* gene in uncharacterized avenacin-deficient *A. strigosa* mutants were performed using the Surveyor mutation detection kit (Transgenomic) according to the manufacturer's protocol.

Sequence Comparisons and Phylogenetic Analysis

Alignment of 92 plant SCPL proteins (see Supplemental Table 1 and Supplemental Figure 1 online) was performed using Muscle 3.6 (Edgar, 2004) with default parameters. The closest relative of oat SCPL1 in the *Brachypodium distachyon* genome was identified using a BLAST search. Predicted mature protein sequences were used in phylogenetic analysis (see Supplemental Figure 1 online) excluding signal peptides and linker regions. Mega 4.0 software (Tamura et al., 2007) was used to construct a neighbor-joining phylogeny and for assessment of sequence diversity. Evolutionary distances were computed using the Poisson correction method. All positions containing gaps and missing data were eliminated from the data set (complete deletion option).

Immunoblot Analysis

Expression of SCPL1 in *Escherichia coli* and generation of polyclonal antisera are described in the Supplemental Materials and Methods online. For immunoblot analysis, soluble protein was extracted from the terminal 0.5 cm of roots of 5-d-old seedlings in 25 mM Tris-HCl buffer, pH 7.0, containing 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1% (w/v) polyvinyl polypyrrolidone, and 0.5 mM DTT. Proteins were denatured in the presence of Nupage reducing agent (Invitrogen) and separated and blotted onto nitrocellulose using Nupage gels (4 to 12% acrylamide gradient) according to the manufacturer's protocol. Membranes were probed with anti-SCPL1 antisera (1/1000 dilution), followed by detection with peroxidase-labeled secondary antibody according to the manufacturer's protocol (Sigma-Aldrich).

Expression of SCPL1 in *Nicotiana benthamiana*

Recombinant CPMV plasmids bearing the AsSCPL1 coding sequence (see Supplemental Materials and Methods online for details) were transformed into *Agrobacterium tumefaciens* strain LBA 4404 and *N. benthamiana* leaf infiltration performed as previously described (Cañizares et al., 2006). Cultures were coinfiltrated with *A. tumefaciens* carrying a plasmid encoding the P19 silencing suppressor protein from tomato bushy stunt virus (Voinnet et al., 2003). Leaf tissue was harvested and frozen in liquid nitrogen. Protein extraction and immunoblot analysis was performed as described above.

Metabolite Analysis and Acyltransferase Assays

All metabolite extractions were prepared as follows: root tips from 5-d-old seedlings were harvested, ground in liquid nitrogen, and extracted with 75% methanol at 4°C for 1 h. Root extracts were centrifuged for 10 min at 12,000g, and the supernatant dried under a vacuum, resuspended in 100% methanol, and centrifuged again. The supernatant was analyzed using a Surveyor HPLC system connected to a DecaXPplus ion trap (Thermo Fisher). A Surveyor FL plus fluorescence detector was used for detection of fluorescent compounds. Data were analyzed using Xcalibur (Thermo Fisher). For quantification of *N*-methyl anthranilate glucose, samples were run on a Waters 2695 HPLC fitted with a 474 Fluorescence detector with excitation at 353 nm and emission at 441 nm. A standard curve was established using *N*-methyl anthranilic acid. The *N*-methyl anthraniloyl-*O*-glucose peak was collected and its identity confirmed by MS. Details of the conditions used are in the Supplemental Materials and Methods online.

Acyltransferase assays were performed using using methanolic extract from 1 g of *sad7* mutant roots as the source of substrates. The extract was dried under vacuum and resuspended in 0.6 mL 1% DMSO in water. Protein preparations for use in activity assays were generated by extraction of 1 g of *N. benthamiana* leaf tissue 6 d after infiltration with *A. tumefaciens* cultures in 50 mM MES, pH 6.5, 150 mM NaCl, and 1% polyvinyl pyrrolidone. Extracts were desalted using PD-10 columns (GE Healthcare). Assays were performed in 100- μ L reactions containing 80 μ g desalted protein, 25 mM MES, pH 6.5, and 15 μ L oat root metabolite extract at 30°C for 2 h. Reactions were stopped by snap freezing in liquid nitrogen. The samples were run on an Agilent 1100 LC-MS system with a single quadrupole detector. Details of the conditions used are in Supplemental Materials and Methods online.

Details of the partial purification of des-acyl avenacin A and *N*-methyl anthraniloyl-*O*-glucose are provided in the Supplemental Materials and Methods online. HPLC-enriched des-acyl avenacin A and *N*-methyl anthraniloyl-*O*-glucose fractions were used in acyltransferase assays at concentrations equivalent to those used in the assays that were performed with the crude root extract, as assessed by LC-MS. Assays were performed in reactions containing 23 μ g of desalted protein extract from SCPL1-expressing *N. benthamiana* leaves or empty vector control in a total volume of 15 μ L of 25 mM MES, pH 6.5. Reactions were incubated at 30°C for 24 h. Avenacin A-1 formation was monitored by LC-MS using a Sciex 4000 QTrap triple quadrupole mass spectrometer (Applied Biosystems) in selective reaction monitoring mode to achieve maximal sensitivity. Details of the conditions used can be found in the Supplemental Materials and Methods online.

Accession Numbers

The *A. strigosa* SCPL1 cDNA sequence can be found in the GenBank/EMBL data libraries under accession number FJ475130. Other plant SCPL protein sequence accession numbers can be found in Supplemental Table 1 online.

Supplemental Data

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

Supplemental Figure 1. Sequence Alignment Used to Generate the Phylogenetic Tree in Figure 4.

Supplemental Figure 2. Mass Spectra and Fragmentation Pattern of Compounds That Accumulate in Roots of *sad7* Mutants.

Supplemental Figure 3. RNA Gel Blot Analysis of the SCPL1 Transcript in Wild-Type and *sad7* Mutant Roots.

Supplemental Figure 4. Effects of Epitope Tagging of the SCPL1 Protein on Protein Levels, Processing, and Enzyme Activity.

Supplemental Figure 5. Mass Spectra of HPLC Fractions Enriched for Des-Acyl Avenacin A and *N*-Methyl Anthraniloyl-*O*-Glucose.

Supplemental Table 1. Protein Sequences Used in the Phylogenetic Tree Shown in Figure 4.

Supplemental Table 2. Oligonucleotide Primer Sequences

Supplemental Materials and Methods.

Supplemental Data Set 1. Text File of the Alignment in Supplemental Figure 1.

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A Serine Carboxypeptidase-Like Acyltransferase Is Required for Synthesis of Antimicrobial Compounds and Disease Resistance in Oats

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