A new class of oxidosqualene cyclases directs synthesis of antimicrobial phytoprotectants in monocots

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Many plants synthesize antimicrobial secondary metabolites as part of their normal program of growth and development, often sequestering them in tissues where they may protect against microbial attack. These include glycosylated triterpenoids (saponins), natural products that are exploited by man for a variety of purposes including use as drugs [Hostettmann, K. & Marston, A. (1995) *Saponins* (Cambridge Univ. Press, Cambridge, U.K.)]. Very little is known about the genes required for the synthesis of this important family of secondary metabolites in plants. Here we show the novel oxidosqualene cyclase AsbAS1 catalyzes the first committed step in the synthesis of antifungal triterpenoid saponins that accumulate in oat roots. We also demonstrate that two sodium azide-generated saponin-deficient mutants of oat, which define the *Sad1* genetic complementation group, are defective in the gene encoding this enzyme and provide molecular genetic evidence indicating a direct link between AsbAS1, triterpenoid saponin biosynthesis, and disease resistance. Orthologs of *AsbAS1* are absent from modern cereals and may have been lost during selection, raising the possibility that this gene could be exploited to enhance disease resistance in crop plants.

Triterpenoid saponins are secondary metabolites that are produced by many different plant species, primarily dicots (1). The natural role of saponins in plants is likely to be in defense against attack by pathogens and pests (1–3). These molecules also have considerable commercial value and are exploited as drugs and medicines, adjuvants, foaming agents, sweeteners, taste modifiers, and cosmetics (1). The enzymes, genes, and biochemical pathways involved in saponin biosynthesis are largely uncharacterized, despite the considerable interest in this important group of natural products. A more detailed understanding of these secondary metabolite pathways and of the genes that are involved would facilitate the development of plants with altered or novel saponin content, either by classical plant breeding or by transformation-mediated genetic modification.

Triterpenoid saponins, like sterols, are synthesized from mevalonic acid via the isoprenoid pathway, the two pathways diverging after 2,3-oxidosqualene (1). Synthesis of sterols in plants involves cyclization of 2,3-oxidosqualene to cycloartenol mediated by the oxidosqualene cyclase enzyme, cycloartenol synthase (4). For triterpenoid saponin synthesis, 2,3-oxidosqualene is cyclized to one of a number of different potential products, the most common being β-amyrin (1). Until recently, a key question in understanding triterpenoid biosynthesis has centered around whether the generation of different cyclization products from 2,3-oxidosqualene involves distinct oxidosqualene cyclase enzymes or whether these reactions may be mediated by a single enzyme (5–7). This question has now been resolved by the cloning and characterization of triterpene synthases that cyclize 2,3-oxidosqualene to β-amyrin (β-amyrin synthases) or to other triterpenoid products (lupeol synthases and multifunctional triterpene synthases) from a diverse collection of dicots, which includes ginseng (*Panax ginseng*; refs. 8 and 9), pea (*Pisum sativum*; ref. 10), olive (*Olea europaea*; ref. 11), dandelion (*Taraxacum officinale*; ref. 11), and thalecress (*Arabidopsis thaliana*; refs. 12–14). These triterpene synthase enzymes share overall structural relatedness with oxidosqualene cyclases that are required for synthesis of sterols (cycloartenol synthases in plants and lanosterol synthases in animals and fungi), but are distinct from them and form separate subgroups within the oxidosqualene cyclase superfamily (reviewed in ref. 15).

Work in our laboratory has focused on avenacins, antifungal triterpenoid saponins that accumulate in the roots of oat (*Avena* spp.; refs. 16 and 17). These secondary metabolites have been implicated as chemical defenses against attack by soil fungi (17, 18). Interestingly, other cereals and grasses do not synthesize avenacins and are generally deficient in antifungal saponins of any kind. The first committed step in avenacin biosynthesis is the cyclization of 2,3-oxidosqualene to β-amyrin (1, 19, 20). The subsequent conversion of β-amyrin into antifungal avenacins has not been biochemically characterized but is predicted to be a multistep process involving cytochrome P450-dependent monooxygenases, glycosyltransferases, and other enzymes. Previously, we isolated a collection of sodium azide-generated saponin-deficient (*sad*) mutants of oat that are defective in avenacin biosynthesis and demonstrated that these mutants are impaired in their resistance to fungal pathogens (21). The mutants define at least seven genetic loci (*Sad1*–*Sad7*; ref. 21 and K.P. and A.O., unpublished data) that are as yet uncharacterized at the molecular genetic level. The isolation of genes for avenacin biosynthesis from oat will provide tools for the analysis of the evolution and regulation of saponin biosynthesis in monocots and may also have potential for the development of improved disease resistance in cultivated cereals. Therefore, our recent efforts have focused on molecular genetic and biochemical dissection of this secondary metabolite pathway.

Here we show that the synthesis of β-amyrin in oat is catalyzed by the novel β-amyrin synthase enzyme AsbAS1. This enzyme, which to our knowledge is the first triterpene synthase to be characterized from monocots, is clearly distinct from dicot triterpene synthases and also from all other oxidosqualene cyclases that have so far been described from plants, animals, and fungi. Thus, AsbAS1 defines a new class of oxidosqualene cyclases. We also demonstrate that *A. strigosa* mutants belonging to the *Sad1* complementation group (21) are mutated in the gene

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AJ311790 (*A. strigosa* cycloartenol synthase cDNA; *AsCS1*) and AJ311789 (*A. strigosa* β-amyrin synthase gene; *AsbAS1*)].

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encoding this enzyme, thereby providing a direct link between AsbAS1, avenacin biosynthesis, and disease resistance. Finally, we provide evidence to indicate that other cultivated cereals do not contain sequences that are closely related to *AsbAS1*.

Methods

Plant Material. The wild-type diploid oat accession used in these experiments was *Avena strigosa* accession S75 (21). The mutant lines of *A. strigosa* were all homozygous F_3 progeny derived from crosses between S75 and the original *sad* mutant lines described by Papadopoulou *et al.* (21).

Construction and Sequence Analysis of Oat Root cDNA Libraries. Total RNA was extracted from the terminal 3 mm of roots of 3-day-old seedlings by using the RNeasy Plant Mini kit (Qiagen, Chatsworth, CA), and mRNA isolation was carried out by using the Dynabeads mRNA Purification kit (Dynal, Great Neck, NY). cDNA was synthesized with the ZAP-cDNA Synthesis kit and cloned into the bacteriophage vector lambda ZAP Express (both from Stratagene). Plasmid clones were generated from the phagemid library by *in vivo* excision, using ExAssist helper phage and *Escherichia coli* strain SOLR, according to the ZAP-cDNA Gigapack III Gold Cloning kit manual (Stratagene). A second library that was enriched for sequences expressed in the root tip was constructed by subtraction. cDNA was synthesized from the terminal 3 mm of the root (''tester'') and from the remainder of the root (''driver'') with the SMART PCR cDNA Synthesis kit (CLONTECH), and subtraction was carried out by using the PCR-Select cDNA Subtraction kit (CLONTECH) following the supplier's instructions. The amplified subtracted cDNA fragments were purified by using the Qiagen PCR Purification kit and ligated into the pGEM-T vector (Promega) before electroporation into ELECTROMAX DH10B competent cells (GIBCO/BRL).

DNA templates for sequencing were isolated by using the 96-Well Alkaline Lysis Miniprep kit (Advanced Genetic Technologies, Gaithersburg, MD) or the QIAprep Spin Miniprep kit (Qiagen). Sequencing reactions were performed by using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with fluorescent sequencing (FS) AmpliTaq DNA polymerase (Perkin–Elmer) and analyzed on ABI 377 sequencing gels. Similarity searches were carried out by using the BLASTX program (22). Where necessary, the isolation of longer cDNA clones from the lambda ZAP library was carried out by using random-primed ^{32}P-labeled probes and following standard procedures (23).

Yeast Expression Experiments. Yeast expression experiments were carried out essentially as described by Kushiro *et al.* (8). Full-length cDNAs corresponding to *AsbAS1* and *AsCS1* were cloned into the yeast expression vector pYES2 (Invitrogen) and transformed into the yeast strain GIL77 (24). After galactose induction, cells were extracted with hexane, and the extracts were analyzed by TLC (solvent mix 1:1 hexane/ethylacetate). Reaction products were detected as described (21). HPLC analysis was carried out by using a Nucleosil 5 C18 column (4.6 × 250 mm; Hichrom, Reading, U.K.) with 95% acetonitrile (flow rate 1 ml/min) at 40°C with detection at 202 nm. α- and β-Amyrin, lupeol, and cycloartenol (Apin Chemical, Abingdon, U.K.) were used as standards for TLC and HPLC analysis.

Isolation of the *AsbAS1* Gene. Genomic DNA was isolated from 3–5-day-old seedlings of *A. strigosa* S75 by using the DNeasy Plant Mini kit (Qiagen). Primers based on the *AsbAS1* cDNA were used to isolate the corresponding gene by PCR, using Expand Long Template polymerase (Roche Diagnostics, Lewes, U.K.). A single band of ≈7.4 kb was obtained. This band was purified by using the Qiagen Gel Extraction kit and cloned into the pGEM-T vector (Promega). Two independent clones were subjected to automated sequencing as described previously. Approximately 2 kb of the 5′ region of the *ASbAS1* gene was also isolated by using the GenomeWalker kit (CLONTECH) and sequenced. For analysis of *sad1* mutants, the *AsbAS1* genes (including the 2-kb 5′ region) were PCR-amplified with high-fidelity *Taq* polymerase (Herculase), and 3 independent clones for each were sequenced to 6-fold coverage.

Southern and Northern Blot Analyses. Seeds of *A. strigosa* and other cereals were germinated as described previously. High molecular weight genomic DNA was isolated from 7-day-old seedlings by using the DNeasy Plant Mini kit (Qiagen). Total RNA was isolated as for cDNA library construction. Southern and Northern blot analyses were carried out by using standard methods (23). Hybridizations were performed at different stringencies (55, 60, and 65°C) with washes as described by Church and Gilbert (25), using ^{32}P-labeled cDNA probes.

***In Situ* Hybridization.** A 1-kb *Eco*RI fragment from the 5′ end of the *AsbAS1* cDNA was cloned into the pBluescript II SK(+) vector. This fragment was PCR-amplified by using the M13–20 and the M13 reverse primers. Digoxigenin-labeled sense and antisense RNA probes were synthesized by using T3 and T7 RNA polymerases, respectively (Roche Diagnostics). Tissue preparation and *in situ* hybridization were carried out as described (26).

Single Nucleotide Polymorphism (SNP) Analysis. F_2 seedlings from crosses between the S75 wild-type and each *sad1* mutant (nos. 109 and 610) were scored for the saponin-deficient phenotype as described (21). SNP analysis was carried out with 83 progeny from the S75 × no. 109 cross and 96 progeny from the S75 × no. 610 cross. DNA fragments encompassing the point mutations in the *AsbAS1* sequences (274 bp for mutant no. 109 and 329 bp for mutant no. 610) were amplified by PCR with primer pairs 5′-CATATCCATTATGACGACGAATCAACC-3′ (Amy109F)/5′-TCTATACCAACCTGTGCCTTCATTCC-3′ (Amy109R) and 5′-GTCGCTACATTTACAATCAACAGGCA-3′ (Amy610F)/5′-ATACCGACAACCATATTTTTCCCCA-3′ (Amy610R), respectively. PCR products were purified by using QIAquick Spin columns (Qiagen) and sequenced, using a minisequencing method based on single primer chain extension (27). Reactions were carried out in 20 μl of 0.05 mM each of dATP, dCTP, dGTP, and ddTTP/20 mM Tris·HCl, pH 8.8/10 mM KCl/10 mM $(NH)_4SO_4$/2 mM $MgSO_4$/0.1 mg/ml BSA/0.1% Triton X-100/0.6 units of *Pfu* DNA polymerase (Promega)/0.5 μM primer/3 μl of purified PCR product. Primer extension was carried out at 94°C for 1 min, 28 cycles for 10 seconds at 94°C, 10 seconds at 52°C, and 10 seconds at 72°C by using primers 5′-AATTCAGGCGAATTTGGGTTT-TCT-3′ (Amy109sp) and GTACTTGGGCCAAGAAC-CATTTTT (Amy610sp), which anneal to the target DNAs two nucleotides upstream of the site of SNPs in mutant nos. 109 and 610, respectively. Primer extension products were separated on 15% denaturing polyacrylamide gels and silver-stained.

Results and Discussion

Characterization of Oxidosqualene Cyclases Expressed in Oat Roots. Expressed sequence tag (EST) analysis of cDNAs from specific plant tissues has proven to be a valuable tool for the identification of genes for secondary metabolite biosynthesis (28). Several lines of evidence indicate that the tips of young oat roots are the sites of synthesis of avenacins. Incorporation of radioactivity from *R*[2-^{14}C]MVA into β-amyrin and avenacins occurs primarily in this part of the root (19), and enzyme assays indicate that β-amyrin synthase activity is also restricted to the root tips (20). We therefore constructed two cDNA libraries from the root tips

of diploid oat (*A. strigosa*) with the objective of isolating sequences encoding β-amyrin synthase and other enzymes involved in the synthesis of avenacins. One of these was a full-length lambda ZAP library, whereas the other was a subtracted library that had been enriched for sequences expressed in the root tip.

Sequence analysis of a total of 2,200 cDNA clones (2,000 from the lambda ZAP library and 200 from the subtracted library) revealed two distinct sequences that were predicted to encode oxidosqualene cyclases. One was represented twice in the lambda ZAP library clones. This sequence was highly homologous to cycloartenol synthase sequences from other plants [e.g., amino acid sequence identities of 87% with *Oryza sativa* cycloartenol synthase (GenBank accession no. AF169966) and 75% with *A. thaliana* cycloartenol synthase (GenBank accession no. U02555)] and represents the presumed *A. strigosa* cycloartenol synthase gene (designated *AsCS1*). The second predicted oxidosqualene cyclase sequence was identified in the subtracted cDNA library. This partial sequence was used as a probe to screen the lambda ZAP library to isolate the full-length cDNA. DNA sequence analysis of ≈350 bp of the 5′ and 3′ ends of 8 independent positive clones indicated that these clones all corresponded to the same sequence. Determination of the full-length cDNA sequence of the predicted oxidosqualene cyclase product revealed that this was clearly distinct from *AsCS1* (55% amino acid identity), and thus we reasoned that this may encode the *A. strigosa* β-amyrin synthase.

Two independent cDNA clones containing the complete predicted ORF of the candidate β-amyrin synthase were cloned into the expression vector pYES2 and transformed into the yeast strain GIL77 (24). GIL77 lacks lanosterol synthase and so accumulates 2,3-oxidosqualene, thus favoring the synthesis of novel 2,3-oxidosqualene cyclization products by heterologous expression of oxidosqualene cyclases. The *P. ginseng* β-amyrin synthase clone $pOSC_{PNY}$ was used as a positive control (8). Yeast cell extracts were prepared after induction of expression with galactose. TLC analysis revealed the presence of a compound with the same R_f value as β-amyrin in extracts from the cells expressing the *P. ginseng* β-amyrin synthase (Fig. 1*A*, lane 4) and also in extracts from cells containing the *A. strigosa* constructs (Fig. 1*A*, lanes 2 and 3). The compound was detectable only after galactose induction (data not shown) and was not present in extracts of cells that had been transformed with the empty pYES expression vector (Fig. 1*A*, lane 1). HPLC analysis confirmed that the yeast cells expressing the *A. strigosa* candidate β-amyrin synthase cDNA accumulated a product with a retention time that was identical to that of β-amyrin (Fig. 1*B*). We therefore concluded that this cDNA sequence did indeed encode *A. strigosa* β-amyrin synthase. β-Amyrin or other triterpenoid products were not observed when the full-length *AsCS1* cDNA was expressed in yeast (data not shown). Cycloartenol does not normally accumulate in sufficient amounts to be detected by TLC or HPLC in yeast extracts, probably as a result of metabolic conversion by the yeast strain (8, 30).

The *A. strigosa* β-amyrin synthase gene (*AsbAS1*) was cloned and sequenced after PCR amplification. Southern blot analysis, using seven different restriction enzymes, indicated that *AsbAS1* was present as a single copy in the *A. strigosa* genome (data not shown). The deduced amino acid sequence showed similarity to oxidosqualene cyclases and contained a conserved DCTAE motif implicated in substrate binding (31) and four conserved QW motifs that are characteristic for this family of enzymes (ref. 32; Fig. 1*C*). Amino acid sequence comparisons show that AsbAS1 is clearly distinct from the other β-amyrin synthases that have been cloned from plants to date (Fig. 1*C*). Remarkably, phylogenetic analysis indicates that AsbAS1 shares greater amino acid sequence similarity with lanosterol synthases from animals and fungi than it does with triterpenoid synthases or cycloartenol synthases from plants, and this grouping received near complete bootstrap support (498/500 replications; Fig. 1*D*). AsbAS1 is therefore a novel enzyme that defines a new class of plant β-amyrin synthases. Little is known about the amino acid residues that are required for sterol and triterpenoid determination in oxidosqualene cyclases (15). Given that there are substantial mechanistic differences between these cyclization processes, the closer relatedness of AsbAS1 to sterol synthases rather than to triterpenoid synthases raises intriguing questions about the enzymology and evolution of the oxidosqualene cyclase superfamily.

Expression of *AsbAS1* in Oats. *AsbAS1* is expressed strongly in the roots with little or no detectable transcript in other plant tissues (Fig. 2*A Left* and *Center*), consistent with the organ-specific accumulation of the saponins (18) and with the biochemical information indicating that the root tips are the site of synthesis (19, 20). The *AsbAS1* transcript was barely detectable in the shoot meristem (Fig. 2*A Right*), indicating that strong expression is not a general meristem-related phenomenon. In contrast, transcripts for the *A. strigosa AsCS1* gene were detectable in all tissues examined by Northern blot analysis (Fig. 2*A*).

The major avenacin (A-1) is autofluorescent under UV light and is localized in the root epidermis (ref. 33; see also Fig. 2*B*). *In situ* hybridization with digoxigenin-labeled antisense *AsbAS1* RNA indicates that the gene is expressed primarily in the root tip epidermis and the root cap, coincident with the localization of avenacin A-1 (Fig. 2 *C* and *D*). In contrast, *AsCS1* is expressed throughout the cells of the root tip (data not shown). *AsbAS1* expression also occurs in the elongation zone (Fig. 2*D*). In this region of the root, expression is not restricted to the epidermis. The fate of β-amyrin in these other cells is not known. Triterpenoids may act as structural components of membranes under certain conditions in some plant tissues (5, 34). Alternatively other minor nonfluorescent avenacins (16, 17) may be produced in the inner part of the root.

Characterization of Saponin-Deficient Oat Mutants Belonging to the *Sad1* Genetic Complementation Group. Two of the 10 *sad* mutants isolated in our original mutant screen (nos. 109 and 610) represent different mutant alleles at an uncharacterized locus known as *Sad1* (21). Roots of *sad1* mutants accumulate radiolabeled 2,3-oxidosqualene when fed with the ^{14}C-labeled precursor mevalonic acid, suggesting that the triterpenoid pathway is blocked between 2,3-oxidosqualene and β-amyrin (20). *sad1* mutants also lack detectable βAS activity but are unimpaired in CS activity and sterol biosynthesis (20). The transcript levels for *AsbAS1* are substantially reduced in roots of *sad1* mutants, whereas *AsCS1* transcript levels are unaffected (Fig. 3*A*). The transcript levels of both genes are not obviously affected in other *sad* mutants such as *sad2* mutant no. 791 (Fig. 3*A*). These results suggest that the *sad1* mutants are mutated either in the *AsbAS1* gene itself or in a gene involved in the regulation of expression of *AsbAS1*.

To resolve these two possibilities, we determined the DNA sequences of the complete *AsbAS1* genes of both *sad1* mutants including the ≈2 kb of 5′ untranslated sequence. Single point mutations were identified in the *AsbAS1* genes of each mutant that are predicted to result in premature termination of translation within the *AsbAS1* coding sequence (Fig. 3*B*). The reason for the reduction in *AsbAS1* transcript levels in *sad1* mutants is unknown but may involve nonsense-mediated mRNA decay, a phenomenon that has been studied most extensively in yeast and animals (35) and has also been reported in plants (36–39). Single nucleotide polymorphism analysis of F_2 populations derived from crosses between the wild-type S75 and mutant no. 109 (83 progeny) and between S75 and mutant no. 610 (96 progeny) indicated that there was no recombination between *AsbAS1* and

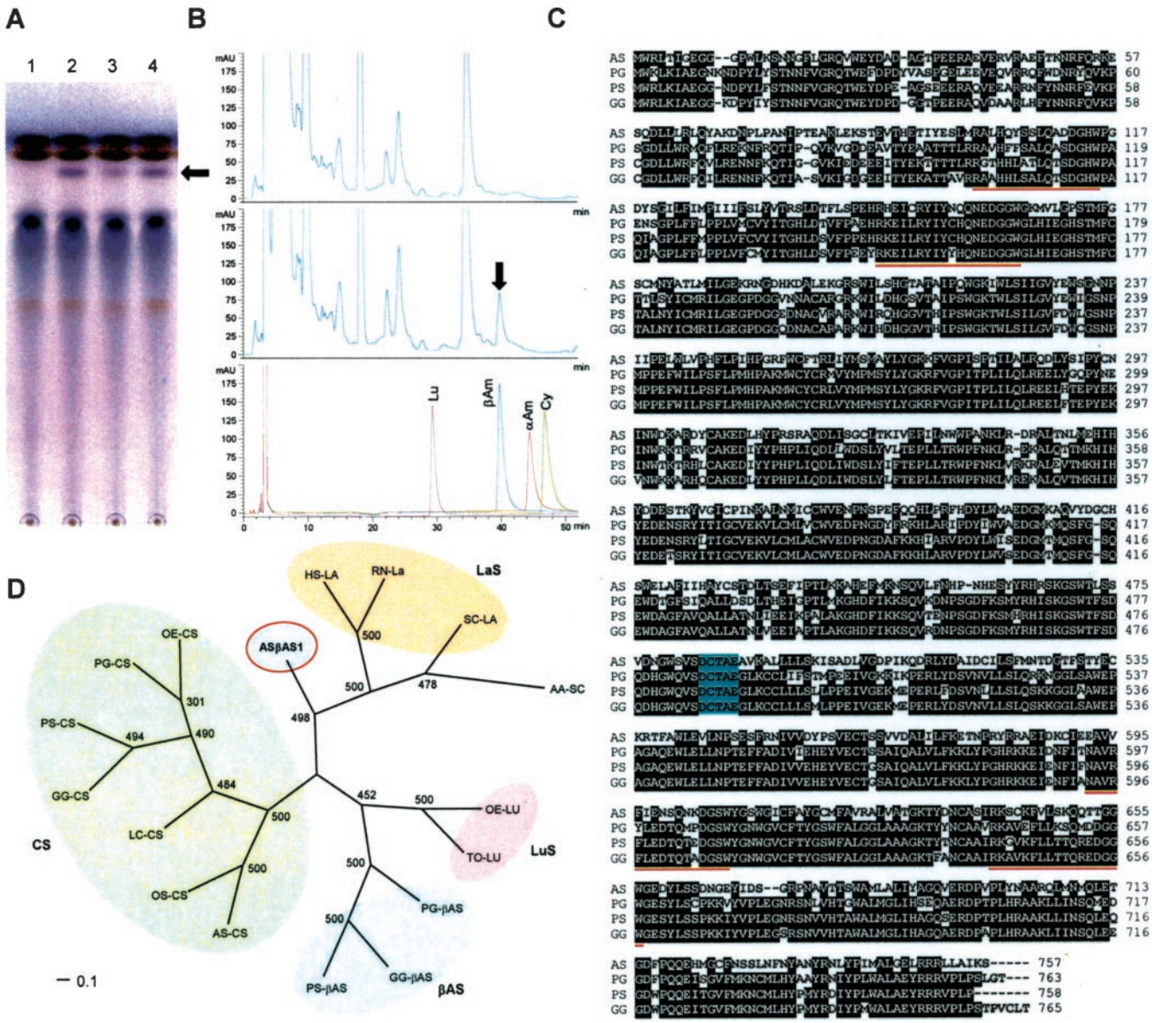

Fig. 1. Characterization of *A. strigosa* β-amyrin synthase. (*A* and *B*) Expression in yeast. (*A*) TLC analysis of yeast cell extracts. Lane 1, expression vector alone; lanes 2 and 3, expression vector containing full-length *A. strigosa* β-amyrin synthase cDNA clones (with 59 and 36 bp of 5′ untranslated leader sequence, respectively); and lane 4, *P. ginseng* β-amyrin synthase cDNA clone pOSC$_{PNY}$ (8). The migration position of a β-amyrin standard is indicated by the arrow. (*B*) HPLC analysis of yeast cell extracts. (*Top*) Expression vector alone; (*Middle*) expression vector containing the *A. strigosa* β-amyrin synthase cDNA; (*Bottom*) standards: Lu, lupeol; βAm, β-amyrin; αAm, α-amyrin; Cy, cycloartenol. (*C*) Alignment of the deduced amino acid sequence of *AsbAS1* with those of other β-amyrin synthase genes. PG, *P. ginseng* (GenBank accession no. AB009030); PS, *P. sativum* (GenBank accession no. AB034802); GG, *Glycyrrhiza glabra* (GenBank accession no. AB037203). Conserved amino acid residues are boxed in black, the QW motifs are underlined in red, and the DCTAE substrate-binding site is indicated in blue. (*D*) Amino acid sequence relatedness of AsbAS1 and other members of the oxidosqualene cyclase superfamily. ASβAS, *A. strigosa* AsbAS1 (GenBank accession no. AJ311789); LaS, lanosterol synthases: HS-LA, *Homo sapiens* (GenBank accession no. U22526); RN-LA, *Rattus norvegicus* (GenBank accession no. U31352); SC-LA, *Saccharomyces cerevisiae* (GenBank accession no. U04841); AA-SC, *Alicyclobacillus acidocaldarius* squalene-hopene cyclase (GenBank accession no. AB007002); LuS, lupeol synthases: OE-LU, *O. europaea* (GenBank accession no. AB025343); TO-LU, *Taraxacum officinale* (GenBank accession no. AB025345); βAS, β-amyrin synthases: AS PG-βAS, *P. ginseng* (GenBank accession no. AB009030); GG-βAS, *G. glabra* (GenBank accession no. AB037203); PS-βAS, *P. sativum* (GenBank accession no. AB034802); CS, cycloartenol synthases: AS-CS, *A. strigosa AsCS1* (GenBank accession no. AJ311790); OS-CS, *O. sativa* (GenBank accession no. AF169966); LC-CS, *Luffa cylindrica* (GenBank accession no. AB033334); GG-CS, *G. glabra* (GenBank accession no. AB025968); PS-CS, *P. sativum* (GenBank accession no. D89619); PG-CS, *P. ginseng* (GenBank accession no. AB009029); OE-CS, *O. europaea* (GenBank accession no. AB025344). The phylogenetic tree was constructed by using the unweighted pair group method with arithmetic mean (UPGMA) method as implemented in the NEIGHBOR program of the PHYLIP package (Version 3.5c; ref. 29). Amino acid distances were calculated by using the Dayhoff PAM matrix method of the PROTDIST program of PHYLIP. The numbers indicate the numbers of bootstrap replications (of 500) in which the given branching was observed. The protein parsimony method (the PROTPARS program of PHYLIP) produced trees with essentially identical topologies.

Sad1. Taken together these data provide compelling evidence that *Sad1*, which is required for avenacin biosynthesis and disease resistance, encodes AsbAS1.

***AsbAS1*-Like Sequences Are Not Conserved in Other Cultivated Cereals.** The ability to produce avenacins is restricted to the genus *Avena* (17, 33). There is little natural variation in avenacin

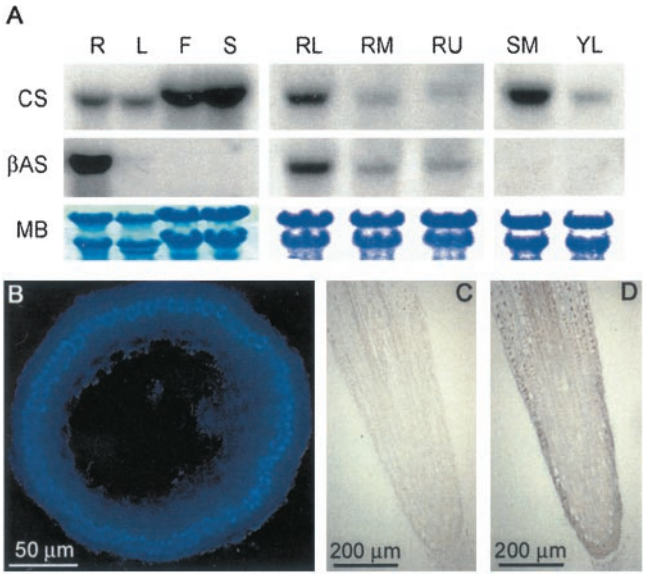

Fig. 2. Expression of *AsbAS1* in oat. (*A*) Northern blot analysis with *AsbAS1* (βAS) and *AsCS1* (CS) as probes. (*Left*) R, root; L, leaf; F, flower, S, stem. (*Center*) *Bottom* (RL), *Middle* (RM), and *Top* (RU), 1-cm segments of root from the tip upwards. (*Right*) SM, shoot meristem; YL, young leaves. Ten micrograms of total RNA was loaded per lane. RNA levels were monitored by using methylene blue (MB) dye. (*B*) Cross-section showing localization of avenacin A-1 in the epidermal cells of young oat roots. Avenacin A-1 is naturally autofluorescent when visualized under UV illumination (excitation and emission wavelengths 365 and 397 nm, respectively). (*C* and *D*) *In situ* mRNA hybridization of longitudinal sections of young oat roots hybridized with digoxigenin-labeled riboprobes for *AsbAS1* expression (*C*, sense; *D*, antisense).

content within the genus *Avena*, although one diploid species that does not produce avenacins (*Avena longiglumis*) has been identified (33). Other cultivated cereals seem to be unable to synthesize saponins of any kind. Northern blot analysis of total RNA from roots of different cereals under high stringency conditions revealed transcripts that hybridized strongly to the *AsbAS1* cDNA probe in other *A. strigosa* accessions and in *A. longiglumis* (Fig. 4). Thus, *A. longiglumis* may produce a functional β-amyrin synthase enzyme, although this requires verification by enzyme assays. No transcript was detectable in RNA preparations from roots of the wheat, barley, rice, and maize accessions that we examined by using a range of different

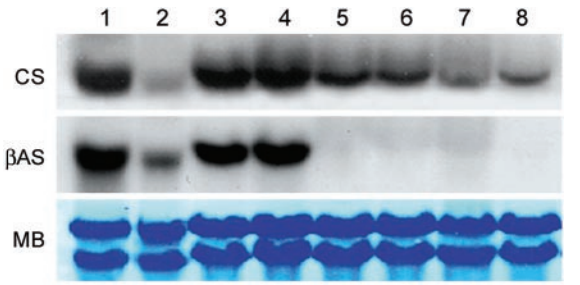

Fig. 4. Northern blot analysis of RNA from roots of different cereals. Probes were *AsCS1* (CS) and *AsbAS1* (βAS). Ten micrograms of total RNA was loaded per lane. RNA levels were monitored by using methylene blue (MB) dye. Hybridization was performed in Church buffer (500 mM phosphate buffer/7% SDS/1 mM EDTA/1% BSA) for 16 h at 65°C. The filters were washed with 40 mM phosphate buffer, 5% SDS, and 1 mM EDTA (3 × 15 min), followed by 40 mM phosphate buffer, 2% SDS, and 1 mM EDTA (3 × 15 min), and then 40 mM phosphate buffer, 1% SDS, and 1 mM EDTA (2 × 15 min). All washes were carried out at 65°C. Lane 1, *A. strigosa* S75 (21); lane 2, *A. longiglumis* (33); lanes 3 and 4, *A. strigosa* accession nos. CI1994 and CI13815 (40); lane 5, *Triticum aestivum* (cultivar Riband); lane 6, *Hordeum vulgare* (cultivar Golden Promise); lane 7, *O. sativa* (accession M12); and lane 8, *Zea mays* (accession P10).

stringency conditions, although the *A. strigosa* cycloartenol synthase cDNA probe gave good signals with these species (Fig. 4). Southern blot analysis with genomic DNA of these other cereals, using *AsbAS1* or *P. ginseng* β-amyrin synthase cDNA probes, failed to reveal strongly hybridizing sequences (data not shown), and database searches for related sequences in cereals identified only the rice cycloartenol synthase sequence (see Fig. 2*D*). Thus, *AsbAs1* may have evolved after the divergence of oats from other cereals or alternatively the gene may have been lost from these other species during cultivation. It is also possible that the gene may have been introduced from a wild grass.

In summary, we have shown that the first committed step in the synthesis of the antimicrobial avenacin saponins in oat roots is mediated by the novel oxidosqualene cyclase AsbAS1. The cyclization of 2,3-oxidosqualene to triterpenoids forms a branch-point between primary and secondary metabolism and has been implicated as the rate-limiting step for triterpenoid formation (5, 41–43). The *AsbAS1* gene therefore represents an important tool for the investigation of the regulation of triterpenoid and sterol synthesis in cereals and potentially for metabolic engineering for improved disease resistance by means of enhanced saponin

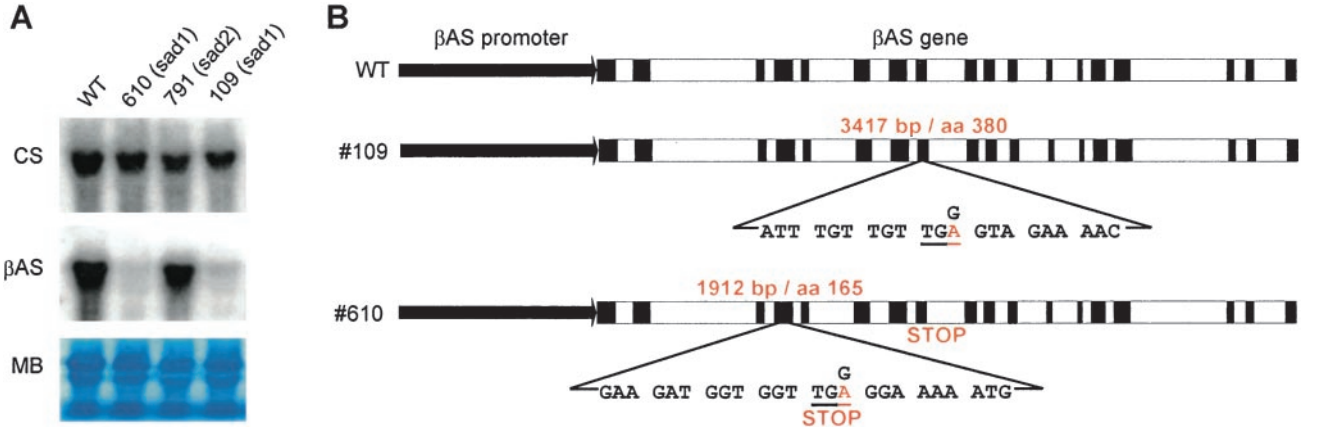

Fig. 3. Characterization of *sad1* mutants. (*A*) Expression of *AsbAS1* (βAS) and *AsCS1* (CS) in wild-type and saponin-deficient lines of *A. strigosa*. Ten micrograms of total RNA was loaded per lane. RNA levels were monitored by using methylene blue (MB) dye. (*B*) Identification of premature termination codons in the *AsbAS1* genes of *sad1* mutants by DNA sequence analysis. Black boxes represent exons and white boxes represent introns.

content. Because AsbAS1 shares greater sequence similarity with lanosterol synthases from animals and fungi than with other plant oxidosqualene cyclases, it may also shed new light on mechanisms of 2,3-oxidosqualene cyclization and on the amino acid residues that determine product specificity.

We have shown by a combination of molecular and classical genetics that the two independent *sad1* mutants isolated in our earlier screen for saponin-deficient mutants (21) are mutated in the *AsbAS1* gene. As far as we are aware, triterpene synthase mutants have not yet been described for any other plant species. Because *sad1* mutants are not obviously affected in root morphology, growth, tillering, flowering time, or seed production, it seems that β-amyrin is not a critical component of plant membranes. Thus, although AsbAS1 is essential for saponin biosynthesis and disease resistance, it is not required for normal growth and development.

The absence of sequences that are closely related to *AsbAS1* in the other cultivated cereals that we have examined suggests that this gene may have potential applications for metabolic engineering for improved disease resistance in other crops. The collection of saponin-deficient oat mutants that we have isolated defines at least six additional loci that are required for synthesis of avenacins, a number of which may be linked to *AsbAs1* (ref. 21 and K.P. and A.O., unpublished results). *AsbAS1* may therefore also aid in the cloning of genes for subsequent steps in the pathway. The isolation of additional genes for saponin biosynthesis from *A. strigosa* and the use of linked genetic markers will allow us to use comparative analysis to investigate why other cultivated cereals are deficient in triterpenoid saponins, and may identify strategies for enhancement of disease resistance through metabolic engineering.

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1. Hostettmann, K. & Marston, A. (1995) *Saponins* (Cambridge Univ. Press, Cambridge, U.K.).
2. Price, K. R., Johnson, I. T. & Fenwick, G. R. (1987) *CRC Crit. Rev. Food Sci. Nutr.* **26,** 27–133.
3. Morrissey, J. P. & Osbourn, A. E. (1999) *Microbiol. Mol. Biol. Rev.* **63,** 708–724.
4. Abe, I., Rohmer, M. & Prestwich, G. D. (1993) *Chem. Rev.* **93,** 2189–2206.
5. Baisted, D. J. (1971) *Biochem. J.* **124,** 375–383.
6. Goad, L. J. (1983) *Biochem. Soc. Trans.* **11,** 548–552.
7. Abe, I., Sankawa, U. & Ebizuka, Y. (1992) *Chem. Pharm. Bull.* **40,** 1755–1760.
8. Kushiro, T., Shibuya, M. & Ebizuka, Y. (1998) *Eur. J. Biochem.* **256,** 238–244.
9. Kushiro, T., Shibuya, M. & Ebizuka, Y. (1998) in *Towards Natural Medicine Research in the 21st Century*, eds. Ageta, H., Aimi, N., Ebizuka, Y., Fujita, T. & Honda, G. (Elsevier Science, Amsterdam), pp. 421–427.
10. Morita, M., Shibuya, M., Kushiro, T., Masuda, K. & Ebizuka, Y. (2000) *Eur. J. Biochem.* **267,** 3453–3460.
11. Shibuya, M., Zhang, H., Endo, A., Shishikura, K., Kushiro, T. & Ebizuka, Y. (1999) *Eur. J. Biochem.* **266,** 302–307.
12. Herrera, J. B. R., Bartel, B., Wilson, W. K. & Matsuda, S. P. T. (1998) *Phytochemistry* **49,** 1905–1911.
13. Husselstein-Muller, T., Schaller, H. & Benveniste, P. (2001) *Plant. Mol. Biol.* **45,** 63–80.
14. Kushiro, T., Shibuya, M., Masuda, K. & Ebizuka, Y. (2000) *Tetrahedron Lett.* 7705–7710.
15. Haralampidis, K., Trojanowska, M. & Osbourn, A. E. (2001) *Adv. Biochem. Eng./Biotechnol.*, in press.
16. Crombie, L., Crombie, W. M. L. & Whiting, D. A. (1986) *J. Chem. Soc. Perkin Trans. 1*, 1917–1922.
17. Crombie, W. M. L. & Crombie, L. (1986) *Phytochemistry* **25,** 2069–2073.
18. Turner, E. M. (1953) *J. Exp. Bot.* **4,** 264–271.
19. Trojanowska, M. R., Osbourn, A. E., Daniels, M. J. & Threlfall, D. R. (2000) *Phytochemistry* **54,** 153–164.
20. Trojanowska, M. R., Osbourn, A. E., Daniels, M. J. & Threlfall, D. R. (2001) *Phytochemistry* **56,** 121–129.
21. Papadopoulou, K., Melton, R. E., Leggett, M., Daniels, M. J. & Osbourn, A. E. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 12923–12928.
22. Gish, W. & States, D. J. (1993) *Nat. Genet.* **3,** 266–272.
23. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
24. Gollub, E. G., Liu, K. P., Dayan, J., Adlersberg, M. & Sprinson, D. B. (1977) *J. Biol. Chem.* **252,** 2846–2854.
25. Church, G. M. & Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1991–1995.
26. Shu, G. G., Baum, D. A. & Mets, L. J. (2000) *Biochem. Newsl.* **1,** 37–38.
27. Syvanen, A. C., Aaltosetala, K., Harju, L., Kontula, K. & Soderlund, H. (1990) *Genomics* **8,** 684–692.
28. Ohlrogge, J. & Benning, C. (2000) *Curr. Opin. Plant Biol.* **3,** 224–228.
29. Felsenstein, J. (1996) *Methods Enzymol.* **266,** 418–427.
30. Venkatramesh, M. & Nes, W. D. (1995) *Arch. Biochem. Biophys.* **324,** 189–199.
31. Abe, I. & Prestwich, G. D. (1995) *Lipids* **30,** 231–234.
32. Poralla, K., Hewelt, A., Prestwich, G. D., Abe, I., Reipen, I. & Sprenger, G. (1994) *Trends Biochem. Sci.* **19,** 157–158.
33. Osbourn, A. E., Clarke, B. R., Lunness, P., Scott, P. R. & Daniels, M. J. (1994) *Physiol. Mol. Plant Pathol.* **45,** 457–467.
34. Nes, W. D. & Heftmann, E. (1981) *J. Nat. Prod.* **44,** 377–400.
35. Hilleren, P. & Parker, R. (1999) *Annu. Rev. Genet.* **33,** 229–260.
36. Jofuku, K. D., Schipper, R. D. & Goldberg, R. B. (1989) *Plant Cell* **1,** 427–435.
37. Voelker, T. A., Moreno, J. & Chrispeels, M. J. (1990) *Plant Cell* **2,** 255–261.
38. vanHoof, A. & Green, P. J. (1996) *Plant J.* **10,** 415–424.
39. Petracek, M. E., Nuygen, T., Thompson, W. F. & Dickey, L. F. (2000) *Plant J.* **21,** 563–569.
40. Yu, G. X. & Wise, R. P. (2000) *Genome* **43,** 736–749.
41. Threlfall, D. R. & Whitehead, I. M. (1990) in *Plant Lipid Biochemistry*, eds. Quinn, P. J.& Harwood, J. L. (Portland, London), pp. 344–346.
42. Van der Deijden, R., Threlfall, D. R., Verpoorte, R. & Whitehead, I. M. (1989) *Phytochemistry* **28,** 2981–2988.
43. Henry, M., Rahier, A. & Taton, M. (1992) *Phytochemistry* **31,** 3855–3859.