Chapter Five

TRITERPENOID SAPONIN BIOSYNTHESIS IN PLANTS

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INTRODUCTION

Plants synthesize a diverse array of secondary metabolites.[1] Since the ability to synthesize particular classes of secondary metabolites is restricted to certain plant groups, these compounds are clearly not essential for survival. However, evidence is accumulating to indicate that they confer selective advantages by protecting against pests, pathogens, and stress.[1,2] Some of these molecules may also have subtle physiological roles in plants that are as yet uncharacterized. In addition to their natural roles in plants, secondary metabolites also represent a vast resource of complex molecules that are valued and exploited by man for pharmacological and other uses.[1]

Saponins are glycosylated secondary metabolites that are widely distributed in the Plant Kingdom.[3,4] They are a diverse and chemically complex family of compounds that can be divided into three major groups depending on the structure of the aglycone, which may be a steroid, a steroidal alkaloid, or a triterpenoid. These molecules have been proposed to contribute to plant defense.[3-6] Saponins are also exploited as drugs and medicines and for a variety of other purposes.[4] Despite the considerable commercial interest in this important group of natural products, little is known about their biosynthesis. This is due in part to the complexity of the molecules, and also to the lack of pathway intermediates for biochemical studies.

A more detailed understanding of the biochemical pathways and enzymes involved in saponin biosynthesis will facilitate the development of plants with altered saponin content. In some cases, enhanced levels of saponins or the synthesis of novel saponins may be desirable (for example, for drug production [4] or improved disease resistance[3,5,6]), while for other plants, reduction in the content of undesirable saponins would be beneficial (for example, for legume saponins that are associated with antifeedant properties in animal feed[7]). This chapter is concerned with recent progress that has been made in the characterization of the enzymes and genes involved in the synthesis of these complex molecules and focuses on triterpenoid saponins.

CYCLIZATION OF 2,3-OXIDOSQUALENE – THE FIRST COMMITTED STEP IN TRITERPENOID BIOSYNTHESIS

Triterpenoid saponins are synthesized via the isoprenoid pathway.[4] The first committed step in triterpenoid saponin biosynthesis involves the cyclization of 2,3-oxidosqualene to one of a number of different potential products (Fig. 5.1).[4,8] Most plant triterpenoid saponins are derived from oleanane or dammarane skeletons although lupanes are also common.[4] This cyclization event forms a branchpoint with the sterol biosynthetic pathway in which 2,3-oxidosqualene is cyclized to cycloartenol in plants, or to lanosterol in animals and fungi.

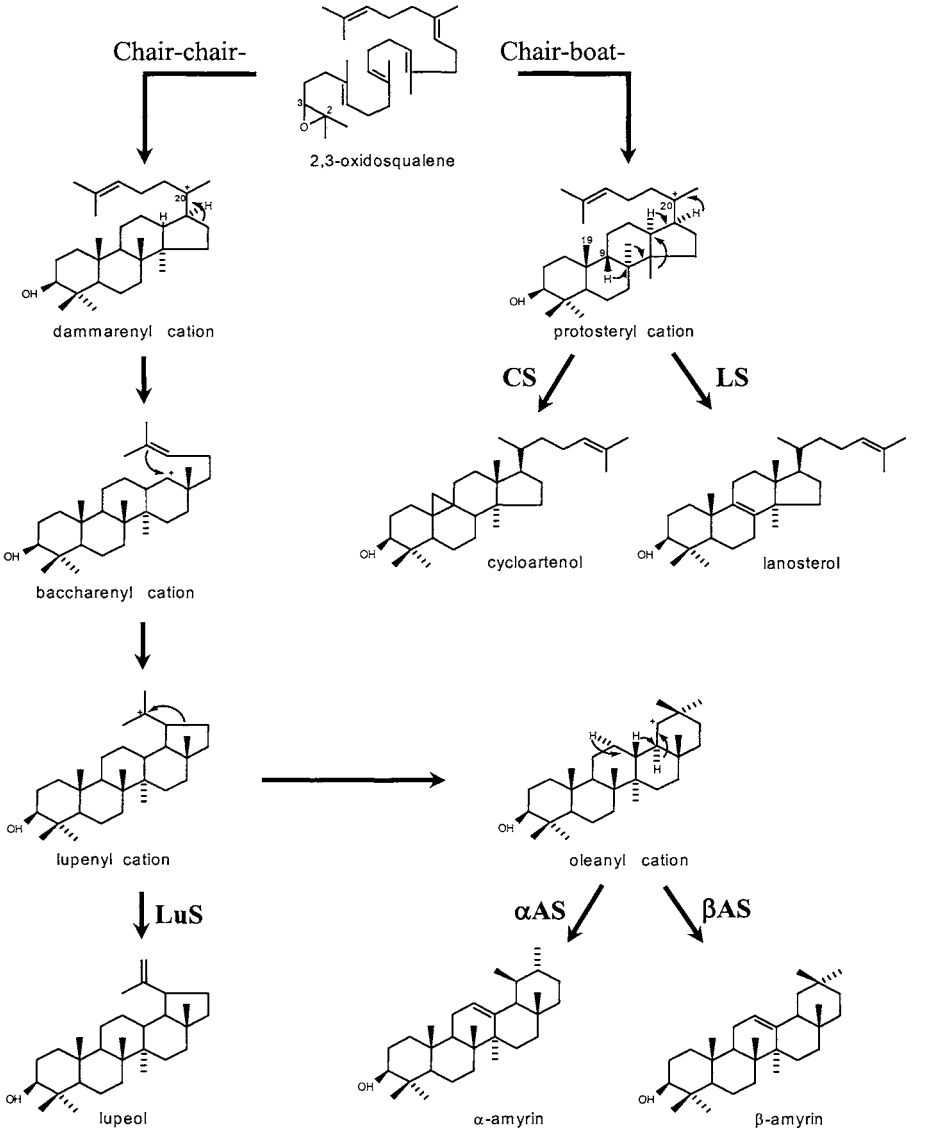

Fig. 5.1: Cyclization of 2,3-oxidosqualene to sterols and triterpenoids. The 2,3-oxidosqualene cyclase enzymes that catalyse the formation of the different products are indicated: LS, lanosterol synthase; CS, cycloartenol synthase; LuS, lupeol synthase; βAS, β-amyrin synthase; αAS, α-amyrin synthase.

Considerable advances have recently been made in the area of 2,3-oxidosqualene cyclization, and a number of genes encoding the oxidosqualene cyclase (OSC) enzymes that give rise to the diverse array of plant triterpenoid skeletons have been cloned. These include α-/β-amyrin synthase enzymes from *Panax ginseng*,[9,10] *Pisum sativum*,[11] and *Glycyrrhiza glabra*,[12] lupeol synthases from *Olea europaea* and *Taraxacum officinale*,[13] and multifunctional triterpene synthases from *Arabidopsis thaliana*.[14-16] These triterpene synthases all share sequence similarity with OSCs required for sterol biosynthesis but form discrete subgroups within the OSC superfamily that reflect the nature of their products.[8-16] It is clear that while some triterpenoid OSCs are highly specific in the products that they generate, others are multifunctional and form a number of different products, at least when expressed in heterologous expression systems.[9-11,13,14,16,17] Oleanane, lupane, and dammarane skeletons are the most common triterpenoid structures associated with saponin biosynthesis, but plants are likely to produce over 80 different oxidosqualene cyclase products.[18] Given the multifunctional properties of some triterpene synthases[14-16] and the fact that single amino acid changes can alter the nature of the product,[19,20] it seems likely that this structural diversity may be generated by a core set of "flexible" enzymes.

OSCs represent attractive tools for investigating the regulation of synthesis and the physiological role of triterpenoids, and potentially for manipulation of sterol and triterpenoid content.[21-24] Several lines of evidence indicate that manipulation of OSCs is likely to alter the flux through the isoprenoid pathway leading to the sterol and triterpenoid pathways.[21,22] OSCs have also been implicated as key regulatory steps in the synthesis of sterols and triterpenes in *Gypsophila paniculata*, *Saponaria officinalis*,[24] and *Tabernaemontana divaricata*.[22,23] Also, chemicals that inhibit lanosterol and cycloartenol synthases but that are not effective against β-amyrin synthases cause blocking of sterol biosynthesis and accumulation of β-amyrin at the expense of cycloartenol and methylene-24-cycloartenol.[25-28]

TRITERPENOID SAPONIN BIOSYNTHESIS IN MONOCOTS

Avenacins – Antimicrobial Phytoprotectants in Oat

Work in our laboratory has focussed on avenacins, a family of antifungal triterpenoid saponins that accumulate in the roots of oat (*Avena* spp.) (Fig. 5.2).[29,30] These secondary metabolites accumulate in the root epidermis[31,32] and have been implicated as chemical defenses against attack by soil fungi.[30,31] We have isolated a collection of sodium azide-generated saponin-deficient (*sad*) mutants of diploid oat that are defective in avenacin biosynthesis, and we have demonstrated that these mutants are impaired in their resistance to fungal pathogens,[6] providing good

evidence to indicate that these molecules do indeed play a role in plant defense. *sad* Mutants show enhanced susceptibility to the root pathogen *Gaeumannomyces graminis* var. *tritici*, which causes "take-all" disease of cereals, and also to other fungal pathogens such as *Fusarium* spp. Saponin deficiency and disease susceptibility were inseparable in segregating F_2 progeny, indicating that saponin deficiency is likely to be the cause of enhanced disease susceptibility.[6] Interestingly, other cereals and grasses do not appear to synthesize avenacins and are generally deficient in antifungal saponins.[3,4,30,32] Thus, the isolation of genes for avenacin biosynthesis may offer potential for the development of improved disease resistance in cultivated cereals.[6] Our efforts have, therefore, focussed on molecular genetic and biochemical dissection of this secondary metabolite pathway.

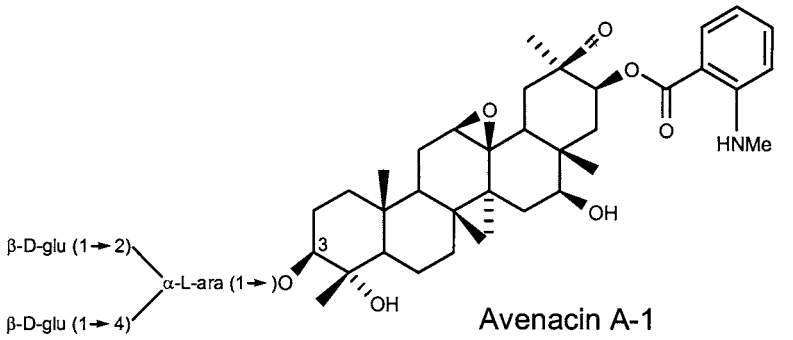

Fig. 5.2: The oat root triterpenoid saponin avenacin A-1.

Characterization of β-Amyrin Synthase from Avena strigosa – A Novel Oxidosqualene Cyclase

Avenacins are synthesized via β-amyrin.[4,33,34] These saponins are found primarily in young oat roots and do not occur in the foliar parts of the plant.[31] Incorporation of radioactivity from *R* [2-^{14}C]MVA into β-amyrin and avenacins occurs primarily in the root tips, and β-amyrin synthase activity is also highest in this region,[33] indicating that this is the site of synthesis. The subsequent conversion of β-amyrin into antifungal avenacins has not been biochemically characterized but is predicted to be a multi-step process involving cytochrome P450-dependent monooxygenases, glycosyltransferases, and other enzymes.[6,35]

Expressed sequence tag (EST) analysis of cDNAs from specific plant tissues has proved to be a valuable tool for the identification of genes for secondary metabolite biosynthesis.[36] We have used this approach to identify two distinct sequences predicted to encode OSCs from cDNA libraries from roots of diploid oat (*Avena strigosa*).[35] One of these sequences is highly homologous to cycloartenol

synthases from other plants (e.g., amino acid sequence identities of 87% with *Oryza sativa* CS (AF169966) and 75% with *Arabidopsis thaliana* CS (U02555)), and represents the *A. strigosa* CS gene *AsCS1*. The second shares 55% amino acid identity with *AsCS1*, and was shown by expression in yeast (*Saccharomyces cerevisiae*) to encode β-amyrin synthase (AsbAS1).[35] *AsbAS1* is present as a single copy gene in the *A. strigosa* genome. The gene is expressed strongly in the epidermal cell layer of the root tips with little or no detectable transcript in the leaves, flowers, and shoots,[35] consistent with the organ-specific accumulation of the saponins[31,32] and also with the biochemical information indicating that the root tips are the site of synthesis.[33]

The deduced amino acid sequence of *AsbAS1* contains the conserved DCTAE motif implicated in substrate binding in OSCs,[37] and also four conserved QW motifs that are characteristic for the OSC superfamily.[38] Remarkably, AsbAS1 is clearly distinct from the other cloned βAS enzymes that have been characterized to date from other plant species, and is more closely related to lanosterol synthases from animals and fungi than to triterpenoid synthases or cycloartenol synthases from plants (Fig. 5.3).[35] There are substantial mechanistic differences in the processes of

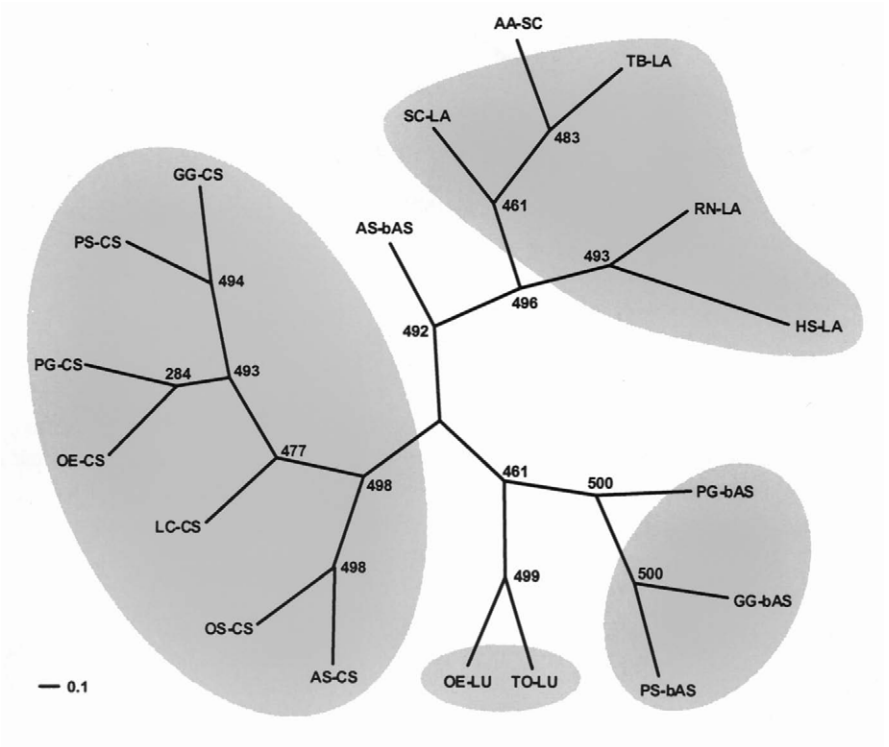

Fig. 5.3: Phenogram showing the relatedness between deduced amino acid sequences of members of the OSC superfamily. Clockwise from the top: AS-bAS, *A. strigosa* AsbAS1 (AJ311789); SC-LA, *Saccharomyces cerevisiae* lanosterol synthase (U04841); AA-SC, *Alicyclobacillus acidocaldarius* squalene-hopene cyclase (AB007002); TB-LA, *Trypanosoma brucei* lanosterol synthase (AF226705); RN-LA, *Rattus norvegicus* lanosterol synthase (U31352); HS-LA, *Homo sapiens* lanosterol synthase (U22526); β-amyrin synthases: PG-bAS, *Panax ginseng* (AB009030); GG-bAS, *Glycyrrhiza glabra* (AB037203); PS-bAS, *Pisum sativum* (AB034802); lupeol synthases: TO-LU, *Taraxacum officinale* (AB025345); OE-LU, *Olea europaea* (AB025343); cycloartenol synthases: AS-CS, *A, strigosa* AsCS1 (AJ311790); OS-CS, *Oryza sativa* (AF169966); LC-CS, *Luffa cylindrica* (AB033334); OE-CS, *Olea europaea* (AB025344); PG-CS, *Panax ginseng* (AB009029); PS-CS, *Pisum sativum* (D89619); GG-CS, *Glycyrrhiza glabra* (AB025968). The phylogenetic tree was constructed by using the UPGMA method as implemented in the "Neighbor" program of the PHYLIP package (Version 3.5c).[48] Amino acid distances were calculated using the Dayhoff PAM matrix method of the "Protdist" program of PHYLIP. The numbers indicate the numbers of bootstrap replications (out of 500) in which the given branching was observed. The protein parsimony method (the "Protpars" program of PHYLIP) produced trees with essentially identical topologies.

cyclization of 2,3-oxidosqualene to sterols and triterpenoids. Although these processes are catalyzed by related subgroups of enzymes, cyclization to yield sterols proceeds with the substrate in the "chair-boat-chair" conformation, while triterpenoid synthesis involves cyclization of the "chair-chair-chair" conformation of 2,3-oxidosqualene.[39] The amino acid residues that are required for sterol and triterpenoid determination in oxidosqualene cyclases have not yet been fully resolved, but the closer relatedness of AsbAS1 to lanosterol synthases rather than to triterpenoid synthases raises intriguing questions about the enzymology and evolution of this superfamily of enzymes.[35]

sad1 Mutants of A. strigosa Are Specifically Defective in AsbAS1

Genetic analysis indicates that two of the 10 *sad* mutants of *A. strigosa* that we isolated represent different mutant alleles at the *Sad1* locus.[6] These mutants accumulate radiolabelled 2,3-oxidosqualene but not β-amyrin when the roots are fed with ^{14}C-labelled precursor mevalonic acid, suggesting that the triterpenoid pathway is blocked between 2,3-oxidosqualene and β-amyrin.[34] The roots of these mutants also lack detectable β-amyrin synthase activity, but, like the wild type and the other mutants, are unimpaired in cycloartenol synthase (CS) activity and sterol biosynthesis.[34] The transcript levels for *AsbAS1* are substantially reduced in roots of *sad1* mutants, while *AsCS1* transcript levels are unaffected,[35] suggesting that the *sad1* mutants are either mutated in the *AsbAS1* gene itself or in a gene involved in its regulation.

DNA sequence analysis of the *AsbAS1* genes of both *sad1* mutants has resolved this by identifying single point mutations in the *AsbAS1* gene in each mutant that would be predicted to give rise to premature termination of translation.[35] The reduced *AsbAS1* transcript levels in the *sad1* mutants may, therefore, be due to nonsense-mediated mRNA decay.[40-44] Genotyping of F_2 populations that segregate for the saponin-deficient phenotype indicated that there was no recombination between *AsbAS1* and *Sad1*. Taken together, the evidence that *Sad1* encodes AsbAS1 is compelling.[35] To our knowledge the *sad1* mutants are the first βAS mutants to be isolated for any plant. Although it has been suggested that β-amyrin may act as a structural component of plant membranes,[45,46] the *sad1* mutants were not obviously affected in root morphology, growth, tillering, flowering time, or seed production.[6,35] Thus, while AsbAS1 is essential for saponin biosynthesis and disease resistance, it is not required for normal plant growth and development.

FUTURE PROSPECTS

There have been a number of exciting recent advances in the molecular characterization of the OSC enzymes that catalyze the first committed step in the synthesis of triterpenoid plant secondary metabolites. Further advances in this area will give a comprehensive insight into the mechanistic enzymology and evolution of this fascinating superfamily of enzymes, and should enable the development of new enzymes that generate novel products by "accelerated evolution". The oat enzyme AsbAS1 is the first triterpene synthase to be characterized from monocots.[35] This enzyme is clearly distinct from other β-amyrin synthases that have been cloned from other plants, and defines a new class of triterpene synthases within the OSC superfamily. Orthologs of *AsbAS1* are absent from modern cereals,[35] raising the possibility that this gene could be exploited to enhance disease resistance in crop plants. The availability of cloned genes for plant triterpene synthases will now enable

the regulation of triterpenoid biosynthesis to be investigated in more detail, and the effects of altering the levels of these enzymes on sterol and triterpenoid synthesis to be assessed via the generation of transgenic plants.

The synthesis of saponins from the cyclization product of 2,3-oxidosqualene involves a series of further modifications, including a variety of oxidation and substitution events and glycosylation.[4,8] Little is known about the enzymes and genes involved in the elaboration of the triterpenoid skeleton, although genetic and biochemical analysis of saponin-deficient mutants of plants is likely to accelerate the dissection of these processes. Progress has been made in the characterization of saponin glucosyltransferases (primarily for steroidal and steroidal alkaloid saponins) (reviewed in 8), and the first of these enzymes has recently been cloned from potato.[47] Since glycosylation at the C-3 hydroxyl position confers amphipathic properties on the molecule and is normally critical for biological activity,[2-5] this is clearly an important area in which to invest effort in the future.

SUMMARY

Saponins are an important group of glycosylated plant secondary metabolites. Their natural role is likely to be in the protection of plants against attack by pests and pathogens.[3-6,35] They are also exploited commercially as drugs and medicines, and for a variety of other purposes.[4] Saponins are synthesized via the isoprenoid pathway from 2,3-oxidosqualene,[4] which is then cyclized to sterol or triterpenoid products by different 2,3-oxidosqualene cyclases. We have recently cloned and characterized the novel oxidosqualene cyclase *AsbAS1,* which catalyzes the synthesis of the triterpenoid saponin precursor β-amyrin in oat.[35] This enzyme is unusual in that it is more closely related to lanosterol synthases from animals and fungi than to other oxidosqualene cyclases (triterpenoid synthases or cycloartenol synthases) from plants. *AsbAS1* is required for the synthesis of triterpenoid avenacin saponins and for resistance to a variety of pathogens, indicating that avenacins contribute to disease resistance in oat. *AsbAS1* and other as yet uncharacterized genes required for saponin biosynthesis have potential for the development of plants with altered saponin content through metabolite engineering. In some cases, enhanced levels of saponins or the synthesis of novel saponins may be beneficial (for drug production or improved disease resistance, for example), while in others the objective may be to reduce the content of undesirable saponins (such as those associated with antinutritional effects in legumes).

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