Saponins in Food, Feedstuffs and Medicinal Plants

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Chapter 13

OAT ROOT SAPONINS AND ROOT-INFECTING FUNGI

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1. Antimicrobial Compounds and Plant Defence

Plants produce a diverse array of secondary metabolites, many of which can inhibit the growth of microbes *in vitro*, leading to speculation that such molecules may protect plants against attack by pathogens (for review see [1]). Investigations of the contribution of antimicrobial compounds to plant defence have focussed mainly on phytoalexins, because these molecules are actively synthesized in and around the site of attempted infection as part of the array of induced defence responses associated with disease resistance. By definition, phytoalexins are absent from healthy plants, and accumulate only in response to pathogen attack or stress [2,3]. Recent evidence indicates that some phytoalexins can indeed act as antimicrobial phytoprotectants. For example, the ability of pea- and chickpea-infecting isolates of the fungus *Nectria haematococca* to detoxify host plant phytoalexins has been shown to be important for full virulence [4,5], and experiments in which levels of phytoalexins in plants have been altered, either by the generation of mutants or by transformation-mediated manipulation of gene expression, have provided evidence to link phytoalexins with disease resistance [6-9].

Many plants also synthesize antimicrobial compounds as part of their normal growth and development. Such compounds are known as pre-formed antimicrobial compounds, or "phytoanticipins" [10-12]. The role of pre-formed antimicrobial compounds in plant defence has attracted relatively little attention, even though these molecules are likely to represent the first chemical barriers to infection. However, there is increasing evidence to indicate that such compounds are likely to be important for disease resistance, as demonstrated by the enhanced disease susceptibility of maize mutants defective in the ability to synthesize 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) [13,14], and of transgenic tobacco lines in which the accumulation of pre-formed phenylpropanoids has been suppressed [15]. Saponins (glycosylated triterpenoid, steroid or steroidal alkaloid molecules that occur constitutively in many plant species) comprise an important class of pre-formed antimicrobial compounds. Because many saponins have potent antifungal activity and are often present at high levels in healthy plants, these molecules have been implicated as antimicrobial phytoprotectants [1,16,17]. We have pursued a variety of

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routes to investigate the role of saponins in interactions between plants and microbes, with particular emphasis on the triterpenoid saponins found in oat roots.

2. Oat Root Saponins

Oats (*Avena* spp.) synthesize a family of four related antifungal triterpenoid saponins, avenacins A-1, B-1, A-2 and B-2, that accumulate in the roots [18,19]. The major oat root saponin avenacin A-1 (Fig. 1) is localized in the root epidermis [20], and is likely to present a protective barrier to infection of oats by saponin-sensitive fungi.

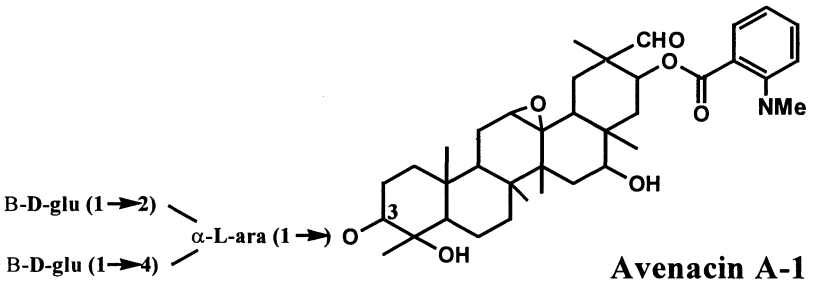

Figure 1. The major oat root triterpenoid saponin, avenacin A-1

2.1. INFLUENCE OF OAT ROOT SAPONINS ON ROOT COLONISING FUNGI

The resistance of oats to the root infecting fungus *Gaeumannomyces graminis* var. *tritici*, the causal agent of take-all disease of wheat, has been associated with avenacins [21]. Consistent with this is the demonstration that the ability of an oat-attacking variant of the take-all pathogen *(G. graminis* var. *avenae*) to infect this host is dependent on production of the saponin-detoxifying enzyme avenacinase, which hydrolyses D-glucose molecules from avenacin A-1 [22]. Detoxification of avenacin A-1 is not unique to *G. graminis* var. *avenae* and has also been described for another oat pathogen, *Fusarium avenaceum* [23]. The antifungal activity of saponins is associated with their ability to form complexes with membrane sterols, and so is relatively non-specific [24-28]. We therefore decided to investigate whether avenacins are likely to have a general effect on the colonisation of oat roots by rhizosphere fungi.

A collection of 161 fungal isolates was obtained from the surface sterilized roots of field-grown oat and wheat plants [29]. The isolates were initially grouped according to their colony morphology. Further characterisation was carried out by rDNA sequence analysis, since identification of root colonizing fungi on the basis of morphological criteria is often problematic, especially for the sterile, darkly pigmented fungi that commonly occur in this habitat [30,31]. Molecular analysis indicated that the collection contained a wide range of Ascomycetes, and also some Basidiomycete fungi (Figure 2) [29].

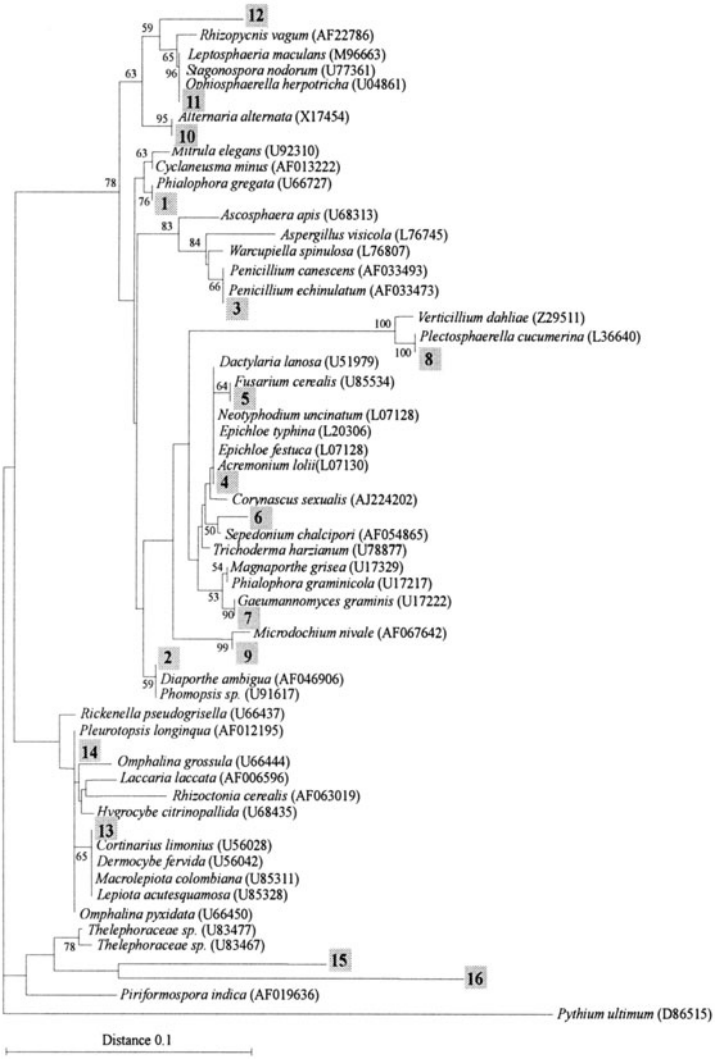

Figure 2. Unrooted phylogenetic tree of fungal 5.8S ribosomal DNA (rDNA) sequences from the EMBL and GenBank databases (accession numbers given in brackets), and from representative isolates recovered from field-grown plants in our study (indicated by the numbers 1-16).

The fungi were subsequently assessed for their ability to tolerate and degrade the antifungal oat root saponin, avenacin A-1. Nearly all the fungi obtained from oat roots were avenacin A-1 resistant, while both avenacin-sensitive and avenacin-resistant fungi were isolated from the roots of the non-saponin producing cereal, wheat. The majority of the avenacin-resistant fungi were able to degrade avenacin A-1, apparently by a similar

mechanism to that of *G. graminis* var. *avenae*, although it is not clear whether this enzymatic degradation represents the primary mode of saponin resistance in these other fungi. Collectively these observations suggest that resistance to avenacin A-1 is required for pathogenicity to oats, as has been shown to be the case for *G. graminis* var. *avenae*, and that avenacin A-1 can have a major influence on the development of fungal communities within oat roots [29]. Avenacin A-1 has also been extracted from the soil around roots at concentrations which would be expected to be inhibitory to many fungi, and so is likely to influence the development of microbial communities not only within the root, but also in a zone around it. There is evidence for other plant-fungus interactions that tolerance of plant antibiotics may be a prerequisite for infection. For example, the ability of a variety of fungi to infect tomato has been associated with resistance to the steroidal glycoalkaloid α-tomatine [32, 33].

2.2. ISOLATION OF SAPONIN-DEFICIENT MUTANTS OF OAT

The generation of avenacin-deficient oat mutants would allow a direct genetic test of the importance of these compounds in the regulation of the development of microbial populations in and around oat roots, and also in plant defence against potential pathogens. The isolation of plant mutants that are defective in the production of antimicrobial compounds is often hindered by the lack of suitable screening procedures. However we have been able to exploit the UV-fluorescence of the major oat root saponin avenacin A-1 [18], a property that is extremely rare amongst saponins, to isolate a collection of sodium-azide generated mutants of the diploid oat species *Avena strigosa* that are deficient in their ability to synthesise avenacins. Ten independent saponin-deficient (*sad*) mutants have been isolated after screening seedlings representing 1,289 M_2 families [34]. To our knowledge this represents the first report of the isolation of saponin-deficient mutants for any plant. These *sad* mutants are compromised in their resistance to *G. graminis* var. *tritici* (Table 1) and a variety of other fungal pathogens.

TABLE 1. Disease scores for infection of *Avena strigosa* wild type and mutant lines with *Gaeumannomyces graminis* var. *tritici*.

Oat line	Disease rating (% seedlings)			
	0	+	++	+++
Wild type	100	0	0	0
610	0	25	12	63
109	28	28	28	16
791[a]	62	38	0	0
1027	31	44	6	19
616	12	71	17	0
376	27	40	33	0
825	6	44	50	0
9[ab]	74	21	5	0
1139[b]	56	38	6	0

[a] Mutants containing reduced levels of avenacins
[b] Mutants with growth defects

The association between compromised disease resistance and saponin deficiency has been confirmed by genetic analysis, providing compelling evidence to indicate that avenacins do indeed act as antimicrobial phytoprotectants in oats.

2.3. HOW ARE AVENACINS SYNTHESISED?

Very little is known about the biosynthetic pathway for avenacins, or indeed about saponin biosynthesis in plants in general. The first committed step in avenacin biosynthesis is likely to be the cyclization of 2,3-oxidosqualene to β-amyrin, mediated by β-amyrin cyclase (Fig. 3) [17,35]. Recently we have cloned the cDNAs predicted to encode cycloartenol cyclase and β-amyrin cyclase from *A. strigosa*. The intermediates in the biosynthetic pathway between β-amyrin and the avenacins have not been characterised, nor have any of the cognate genes been cloned. A number of enzymes are likely to be involved in the elaboration of β-amyrin into avenacins, and these will include cytochrome P450-dependent monooxygenases, benzoate- and *N*-methyl anthranilate CoA ligases, glycosyl transferases and other enzymes. The *sad* mutants that we have isolated represent a valuable resource for the dissection of the avenacin biosynthetic pathway and its control, and biochemical and genetic approaches are now being employed to isolate the genes that are required for avenacin synthesis in oat. At least six different genetic complementation groups have been identified in our mutant collection. The respective loci have been designated *Sad1* to *Sad6*. Mutants 610 and 109 belong to the same genetic complementation group, and represent different mutant alleles at the *Sad1* locus. These mutants differ from the other *sad* mutants and from the wild type oat line in that they accumulate radiolabelled 2,3-oxidosqualene but not β-amyrin when fed with ^{14}C-labelled mevalonic acid, suggesting that the triterpenoid pathway is blocked between 2,3-oxidosqualene and β-amyrin [35]. Furthermore, roots of these mutants lack detectable β-amyrin cyclase activity, and the levels of the corresponding mRNA transcript are substantially reduced. Thus 109 and 610 are likely to be mutated either in the gene encoding β-amyrin cyclase (the first committed enzyme of the triterpenoid biosynthetic pathway), or in a factor involved in the regulation of expression of β-amyrin cyclase. The other *Sad* loci are represented by mutants 1027 and 791 (*Sad2*), mutant 1139 (*Sad3*), mutant 9 (*Sad4*), mutants 616 and 376 (*Sad5*), and mutant 825 (*Sad6*). Mutant 1243 is yet to be assigned to a complementation group. The defects in these other *sad* mutants are not fully understood, although the *sad3* and *sad4* mutations both result in accumulation of incompletely glycosylated avenacins (minus a *D*-glucose molecule), and so may be affected in glucosyltransferase activity. Assays to confirm this are currently being developed.

The recent finding that in maize five genes that are necessary and sufficient to confer the ability to synthesise DIBOA are all clustered on the same arm of one maize chromosome represents the first example of clustered genes for secondary metabolism in plants, and offers potential for metabolic engineering for improved disease resistance in cereals and other plants [14]. Interestingly, genetic analysis of F_2 progeny derived from crosses between different saponin-deficient oat mutants indicates that at least five of the *Sad* genes that we have identified are linked, although it is not possible to establish map distances from these data. The ability to produce avenacins is restricted to the genus *Avena* [19,20], and saponins of any kind do not appear to be well represented in the

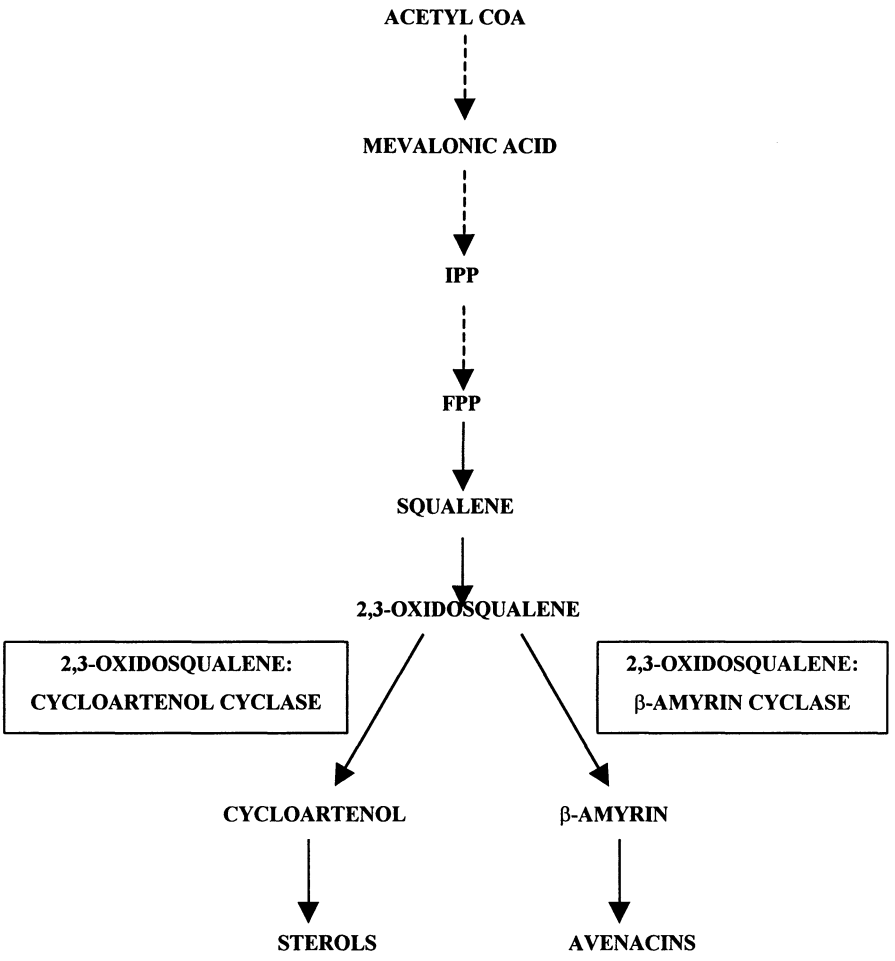

Figure 3. Avenacin biosynthesis in oat.

cereals. Cereals other than oat may lack the entire avenacin biosynthetic pathway, or alternatively may be deficient in only a few steps. The isolation of genes required for avenacin biosynthesis from oat will ultimately allow us to investigate the presence, distribution and function of gene homologues in other members of the Gramineae.

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