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Research review

Dissecting plant secondary metabolism – constitutive chemical defences in cereals

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Summary

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Collectively plants synthesise a diverse array of secondary metabolites. Secondary metabolites are well known as agents that mediate pollination and seed dispersal. They may also act as chemical defenses that ward off pests and pathogens or suppress the growth of neighbouring plants. The ability to synthesise particular classes of secondary metabolite is commonly restricted to selected plant groups, and the evolution of different pathways in distinct plant lineages is likely to have been key for survival and for the generation of diversity at the organism level. An understanding of the evolution of secondary metabolism requires the characterisation of enzymes and genes for complete pathways in a broad range of plants in addition to the two model species, *Arabidopsis thaliana* and rice. Tracing the ancestry of the pathway components can then unravel the chain of events that led to the creation of individual pathways. This review summarises progress that has been made in the dissection of the pathways for constitutive chemical defences in cereals, namely saponins and benzoxazinoids.

Introduction

Thalecress (*Arabidopsis thaliana*) and rice (*Oryza sativa*) are predicted to have around 25 500 genes (The *Arabidopsis* Genome Initiative, 2000) and 32 000–50 000 genes (Goff *et al.*, 2002; Yu *et al.*, 2002), respectively, while the genetic complement of the fruit fly (*Drosophila melanogaster*) is substantially lower (13 601 predicted genes) (Adams *et al.*, 2000). One explanation for this seemingly incongruous discrepancy in the relationship between biological and genetic complexity may lie in the differences between the ways that plants and animals protect themselves against predators, pests and diseases. Vertebrates have developed sophisticated nervous and immune systems that enable them to detect and respond to danger, and they are capable of fleeing from perilous situations. By contrast, plants cannot take avoidance measures to escape from their attackers and so must stay and fight. The production of chemicals that deter or kill pests and pathogens represents one means of self-protection. Collectively, plants are capable of synthesising a diverse array of secondary metabolites (Wink, 1999). These may be produced constitutively (preformed antimicrobial compounds, or phytoanticipins) or in response to pathogen or herbivore attack or stress (phytoalexins) (Morrissey & Osbourn, 1999; Wittstock & Gershenzon, 2002). In addition to their direct effects on pests and pathogens some secondary metabolites may also be important in defence-related signal transduction (Bouarab *et al.*, 2002). The ability of plants to carry out *in vivo* combinatorial chemistry by mixing, matching and evolving the gene products required for secondary metabolite biosynthetic pathways is likely to have been key for their survival and for the generation of diversity at the organism level.

Genetic potential for production of secondary metabolites – what is a genome capable of?

The total number of plant secondary metabolites for which structures have been elucidated is around 50 000 (De Luca & St Pierre, 2000), and this is likely to be only the tip of the iceberg in terms of the chemical diversity that is represented in nature. Each plant species produces only a small fraction of this spectrum. In *A. thaliana* it is estimated that around 5000 genes (i.e. 25% of the total) are involved in secondary metabolism (The *Arabidopsis* Genome Initiative, 2000) while around 25% of rice genes are predicted to be involved in primary or secondary metabolism collectively (Goff *et al.*, 2002). The functions of only a very limited subset of these genes are understood. These include genes required for the synthesis of phytoprotectants such as glucosinolates and the indole phytoalexin camalexin in *A. thaliana*, and flavanone and diterpene phytoalexins in rice. What is the function of all of the other genes that have been implicated in secondary metabolism? Genes sharing sequence similarity with those required for alkaloid biosynthesis in species such as *Papaver somniferum*, *Berberis stolonifera* and *Catharanthus roseus* (e.g. strictosidine β-glucosidase, berberine bridge enzyme and strictosidine synthase) are present in the genomes of both *A. thaliana* and rice (Goff *et al.*, 2002) although neither of these two model plant species are known to produce alkaloids. However, sequence relatedness is not a reliable indicator of specific biological function, and painstaking biochemical analysis of individual gene products is still required to support predictions based on gene sequences.

A burning question in plant biology is just how much combinatorial chemistry can a single species or accession carry out, with or without the help of man-made genetic modifications? The synthesis of secondary metabolites is often tightly regulated, and is commonly either restricted to specific plant tissues or developmental stages, or induced in response to pathogen attack or treatment with inducing agents (methyl jasmonate and other elicitors). Plant genomes are relatively rich in genes for transcription factors when compared with those of animals (Riechmann *et al.*, 2000), and one explanation for this bias may lie in the need to regulate the myriad of complex processes associated with secondary metabolism (Szathmáry *et al.*, 2001). Interference with transcriptional regulators for genes encoding biosynthetic enzymes can 'wake up' dormant biochemical pathways. For example, activation tagging of a gene for a transcriptional regulator of alkaloid biosynthesis in *C. roseus* circumvented the normal jasmonate requirement for pathway induction and led to constitutive synthesis of these secondary metabolites (van der Fits & Memelink, 2000). Similarly, in *A. thaliana* activation tagging of a MYB transcription factor gene resulted in overproduction of phenylpropanoids (Borevitz *et al.*, 2000) (Fig. 1). Thus the absence of secondary metabolites may simply be a regulatory issue; the necessary genes may be present and have the potential to encode fully functional products but they are not expressed under the commonly studied growth conditions. Differences in the ability of different species and genera to synthesise secondary metabolites are more likely to be explained by the presence or absence of genes for biosynthetic enzymes. The absence of genes required for one or more key steps in the biosynthetic pathway need not be an insurmountable barrier to metabolite engineering for plant defence. For example, *A. thaliana* lacks the genes for cyanogenic glycoside biosynthesis but if provided with three genes from sorghum that are necessary and sufficient for the entire biosynthetic pathway it will synthesise and store large amounts of these compounds (Tattersall *et al.*, 2001). Cyanogenic glycosides have been implicated in defence against pathogens and herbivores (Morrissey & Osbourn, 1999). Consistent with this, transgenic *A. thaliana* plants that produce cyanogenic glycosides display enhanced resistance to flea beetle, a herbivore pest of brassicas (Tattersall *et al.*, 2001).

Fig. 1 Overexpression of the MYB transcription factor gene *Pap1* leads to enhanced phenylpropanoid production in *Arabidopsis thaliana*. (Left, control transformant; right, transformant overexpressing *Pap1*).

Learning from crop plants – dissection of chemical defences in cereals

It is clear that the manipulation of secondary metabolite biosynthesis in plants holds great potential for the development of enhanced pest and disease resistance in economically important crops. The application of genome- and metabolome-based approaches to *A. thaliana* and rice will allow major advances to be made in our understanding of plant secondary metabolism, and is expected to unveil novel

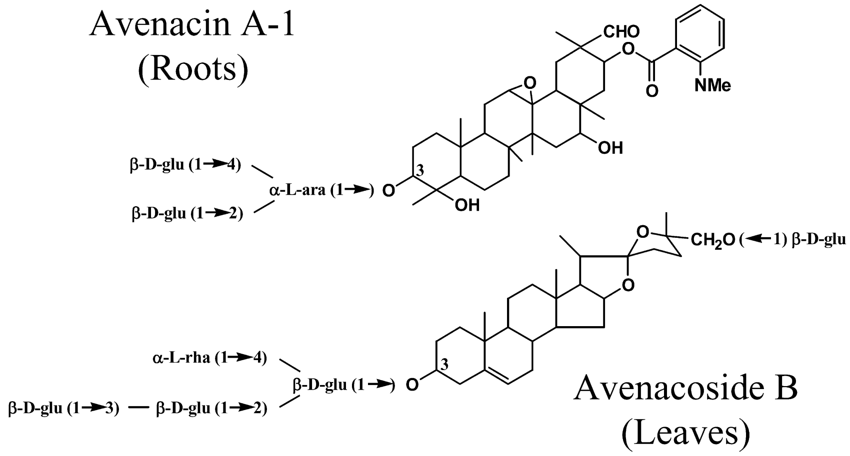

Fig. 2 Representatives of the two different families of saponins synthesised by oat. Top, the triterpenoid saponin avenacin A-1, which is the major saponin present in the roots; bottom, the steroidal saponin avenacoside B, which is found in the leaves.

pathways and complex metabolic networks (Fiehn *et al.*, 2000; Fiehn, 2002; Hadacek, 2002; Weckwerth & Fiehn, 2002). However, a comprehensive picture of the *in vivo* combinatorial chemistry behind the myriad of compounds found in nature will depend on a detailed knowledge of enzymes, genes and pathways required for the synthesis of secondary metabolites in other species in addition to the two 'model' plants. Information of this kind will also be important in assessing the potential of different genetic backgrounds to synthesise novel or altered compounds. Multi-faceted approaches that combine biochemistry, molecular genetics and genomics with classical genetics have proved to be highly effective in the dissection of secondary metabolite biosynthetic pathways. Here the application of such approaches to two classes of constitutive secondary metabolites that confer resistance to pests and diseases in cereals will be considered, namely saponins and benzoxazinoids.

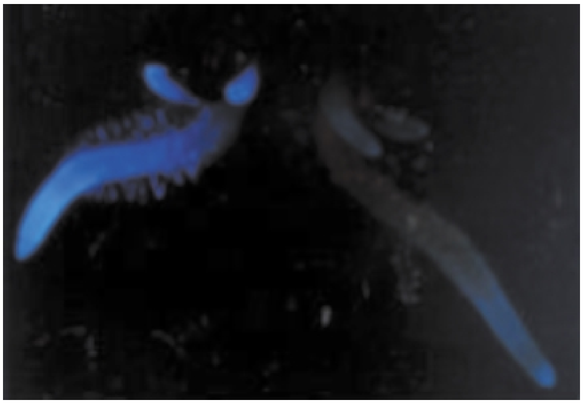

Fig. 3 Identification of saponin-deficient mutants of diploid oat by screening for reduced root fluorescence. Left, wild type; right, a saponin-deficient mutant.

Saponins

Saponins are an important group of plant secondary metabolites consisting of glycosylated triterpenes and steroids. These compounds are widespread in dicotyledonous plant species. Cereals and grasses, however, appear to be generally saponin-deficient with the exception of oats (*Avena* spp.) (Hostettmann & Marston, 1995). Oats synthesise two different families of saponins, the steroidal avenacosides (Tschesche *et al.*, 1969; Tschesche & Lauven, 1971) and the triterpenoid avenacins (Crombie & Crombie, 1986; Crombie *et al.*, 1986), which are produced in the leaves and roots, respectively (Fig. 2). Many saponins have potent antimicrobial activity, suggestive of a role in plant defence (Morrissey & Osbourn, 1999). Detection methods for saponins generally rely on relatively nonspecific chromogenic stains or on HPLC-based methods (Hostettmann & Marston, 1995), making screening for saponin-deficient mutants difficult. However the major oat root saponin avenacin A-1 is esterified with *N*-methyl anthranilic acid and so fluoresces under ultra-violet light (Crombie & Crombie, 1986; Crombie *et al.*, 1986). This property, which is extremely unusual amongst saponins, has facilitated the isolation of a collection of sodium azide-generated mutants of diploid oat that are deficient in their ability to synthesise avenacins (Papadopoulou *et al.*, 1999) (Fig. 3). These saponin-deficient (*sad*) mutants are compromised in resistance to a range of pathogens, confirming a role for saponins in plant defence.

The process of saponin biosynthesis is not well understood for any plant species, despite the considerable interest in this important group of natural products (Haralampidis *et al.*, 2001a). This is due in part to the complexity of the molecules and also to the lack of pathway intermediates for biochemical studies. In plants, synthesis of sterols is initiated by cyclisation of 2,3-oxidosqualene to cycloartenol, mediated by the oxidosqualene cyclase enzyme cycloartenol synthase. The first committed step in avenacin biosynthesis involves the cyclisation of 2,3-oxidosqualene to an alternative product, the triterpene β-amyrin (Fig. 4). This reaction is catalysed by another oxidosqualene cyclase, β-amyrin synthase (Haralampidis *et al.*,

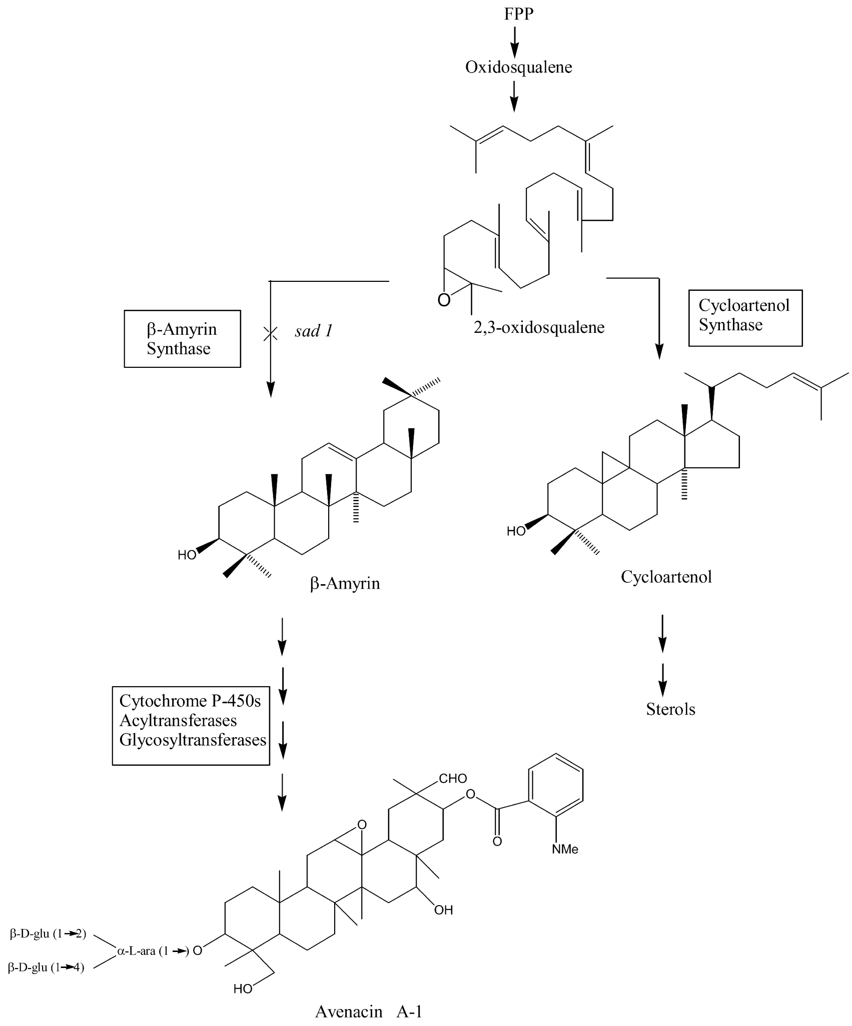

Fig. 4 Synthesis of sterols and triterpenoid saponins in oat. The oxidosqualene cyclase enzymes cycloartenol synthase and β-amyrin synthase are key branchpoint enzymes for the sterol and triterpene pathways, respectively. *sad1* mutants are blocked in the synthesis of β-amyrin. The conversion of β-amyrin to the antimicrobial triterpenoid saponin avenacin A-1 is predicted to involve as yet uncharacterised cytochrome P-450s, acyltransferases and glycosyltransferases.

2001a,b). β-Amyrin does not have substantial antimicrobial activity but is converted to the biologically active saponin avenacin A-1 by a series of modifications that are predicted to involve cytochrome P-450s, acyltransferases and glycosyltransferases (Fig. 4). Avenacin A-1 is concentrated in the tips of young oat roots, and radiolabelled precursor feeding experiments and β-amyrin synthase assays indicate that this is the site of synthesis (Trojanowska *et al.*, 2000; Trojanowska *et al.*, 2001).

Expressed sequence tag (EST) collections derived from plant tissues that are actively synthesising secondary metabolites represent valuable resources for gene discovery (Lange *et al.*, 1999; Ohlrogge & Benning, 2000; White *et al.*, 2000; Gang *et al.*, 2001; Brandle *et al.*, 2002). The exploitation of ESTs derived from oat root tips in combination with the *sad* mutants has proved to be a powerful strategy for the isolation of saponin biosynthetic genes. A cDNA for a predicted oxidosqualene cyclase was identified in an oat root EST collection and was subsequently shown to encode oat β-amyrin synthase (AsbAS1) by expression in yeast (Haralampidis *et al.*, 2001b). *AsbAS1*, which is the first triterpene synthase to be cloned from monocots, defines a new subgroup within the oxidosqualene cyclase superfamily (Haralampidis *et al.*, 2001b) (Fig. 5). A direct link between *AsbAS1*, triterpenoid saponin biosynthesis and disease resistance has emerged from studies of the *sad* mutants. Mutants at *Sad1*, one of the loci defined in the screen for *sad* mutants, accumulate radiolabelled 2,3-oxidosqualene when fed with the ^{14}C-labelled precursor mevalonic acid, suggesting that the triterpenoid pathway is blocked between 2,3-oxidosqualene and β-amyrin (Trojanowska *et al.*, 2001) (Fig. 4). *sad1* mutants also lack β-amyrin synthase activity (Trojanowska *et al.*, 2000) and have substantially reduced levels of the *AsbAS1* transcript (Haralampidis *et al.*, 2001b), indicative of mutations either in the *AsbAS1* gene itself or in a regulator of this gene. These two possibilities have been resolved by DNA sequence analysis of the complete *AsbAS1* genes of two independent *sad1* mutants, which identified single point mutations within the *AsbAS1* coding sequence in each of the mutants (Haralampidis *et al.*, 2001b). These point mutations, which are both predicted to result in premature termination of translation, presumably affect transcript stability. The connection

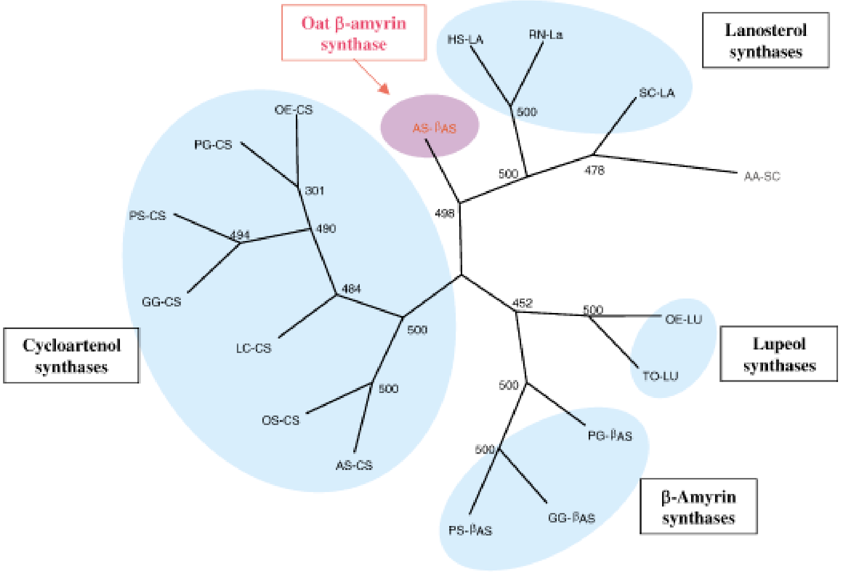

Fig. 5 Amino acid sequence relatedness of members of the oxidosqualene cyclase superfamily. The phylogenetic tree was constructed using the UPGMA method as implemented in the 'Neighbor' program of the PHYLIP package (Version 3.5c). Amino acid distances were calculated using the Dayhoff PAM matrix method 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 sequences represented are as follows: ASβAS, *A. strigosa* AsbAS1 (AJ311789); Lanosterol synthases (LaS): HS-LA, *Homo sapiens* (U22526); RN-LA, *Rattus norvegicus* (U31352); SC-LA, *Saccharomyces cerevisiae* (U04841); AA-SC, *Alicyclobacillus acidocaldarius* squalene-hopene cyclase (AB007002); Lupeol synthases (LuS): OE-LU, *Olea europaea* (AB025343); TO-LU, *Taraxacum officinale* (AB025345); β-amyrin synthases (βAS): PG-βAS, *Panax ginseng* (AB009030); GG-βAS, *Glycyrrhiza glabra* (AB037203); PS-βAS, *Pisum sativum* (AB034802); cycloartenol synthases (CS): AS-CS, *A, strigosa AsCS1* (AJ311790); OS-CS, *Oryza sativa* (AF169966); LC-CS, *Luffa cylindrica* (AB033334); GG-CS, *Glycyrrhiza glabra* (AB025968); PS-CS, *Pisum sativum* (D89619); PG-CS, *Panax ginseng* (AB009029); OE-CS, *Olea europaea* (AB025344).

between *AsbAS1* and *Sad1* was subsequently confirmed by single nucleotide polymorphism analysis of populations segregating for the *sad1* phenotype (Haralampidis *et al.*, 2001b; Qi *et al.*, 2001).

Southern hybridisation and database mining suggests that DNA sequences that are closely related to *AsbAS1* are present only in *Avena* spp. (Haralampidis *et al.*, 2001b), although other members of the Gramineae are capable of producing β-amyrin (Ohmoto & Ikuse, 1970; Heupel & Nes, 1985; Taton *et al.*, 1986). The *sad* mutant collection defines at least six additional loci that are required for avenacin biosynthesis (Papadopoulou *et al.*, 1999). These loci may encode biosynthetic enzymes required for the conversion of β-amyrin into avenacins (cytochrome P450s, acyltransferases and glycosyltransferases) or regulatory components (Papadopoulou *et al.*, 1999; Haralampidis *et al.*, 2001a,b). The characterisation of more enzymes and genes for saponin biosynthesis from oat and comparative analysis with other members of the Gramineae should reveal why saponin biosynthesis is restricted to *Avena* spp. One explanation may be that other cereals have lost components of the pathway during the selective processes leading to the development of modern cultivars. Alternatively the pathways for avenacin and avenacoside biosynthesis may have evolved after the divergence of oats from other cereals.

Benzoxazinoids

Benzoxazinoids occur constitutively as glucosides in a number of members of the Gramineae (Niemeyer, 1988; Sicker *et al.*, 2000). In rye the main benzoxazinoid is a glucoside of 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), whereas in maize and wheat it is the glucoside of the methoxylated form, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA). The glucosides are hydrolysed in response to infection or physical damage to produce DIBOA and DIMBOA, which are antimicrobial and also have pesticidal and allelopathic activity. The complete molecular pathway for benzoxazinoid biosynthesis has been elucidated in maize using an innovative combination of gene expression analysis, reverse genetics, transposon tagging and genetic mapping (Gierl & Frey, 2001).

The first committed step towards DIBOA and DIMBOA biosynthesis is the conversion of indole-3-glycerol phosphate to indole, which is catalysed by the tryptophan synthase α (TSA) homologue BX1 (Fig. 6). The subsequent conversion of indole into DIBOA is catalysed by four related but highly substrate-specific cytochrome P450s (Bx2-Bx5) belonging to the CYP71C subfamily. The *Bx1* gene was cloned from maize using the *Mutator* transposon tagging system (Frey *et al.*, 1997). *Bx2–5* were cloned using an approach based on differential

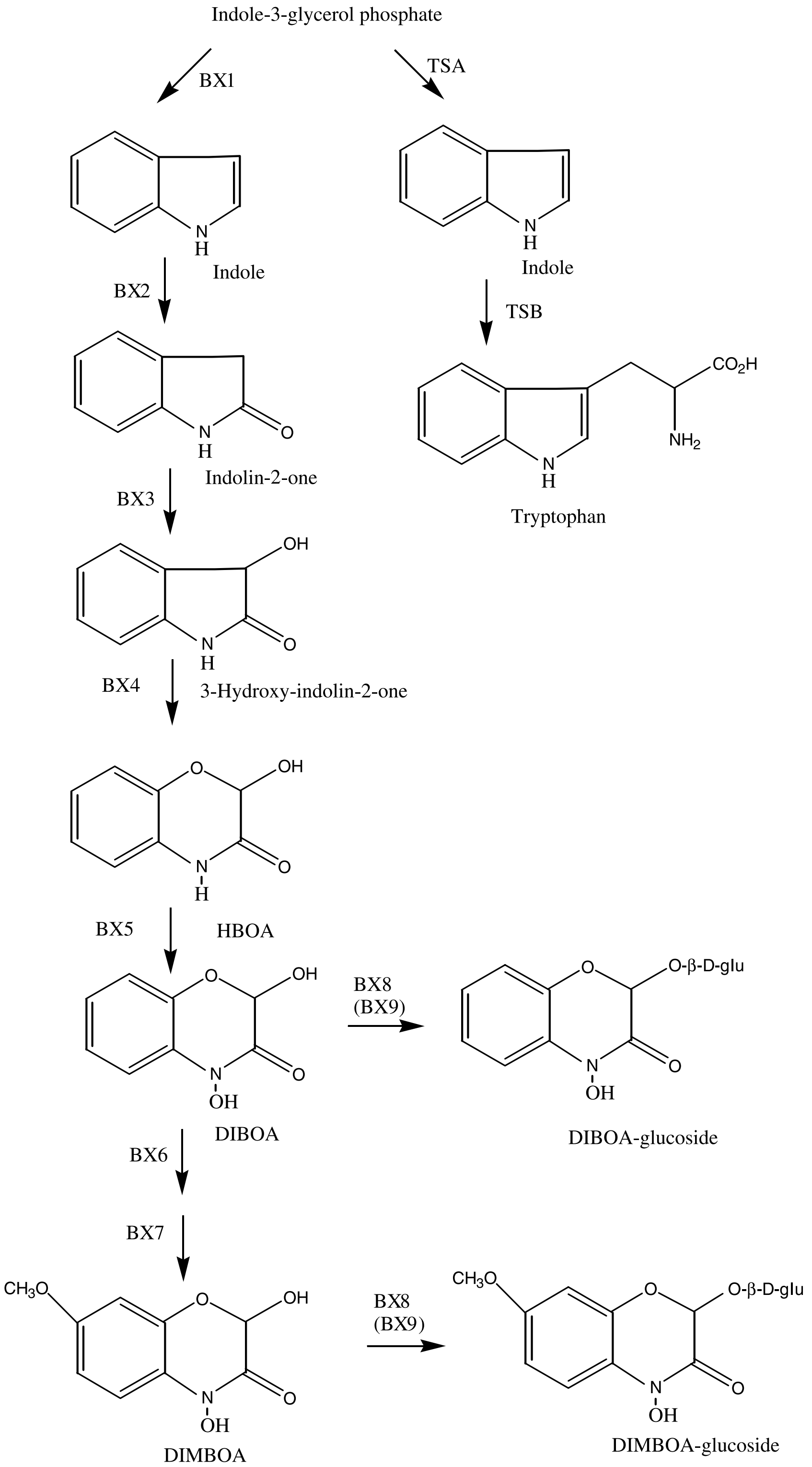

Fig. 6 Synthesis of benzoxazinoids. In primary metabolism tryptophan is synthesised from indole-3-glycerol phosphate via indole. This conversion is catalysed by the sequential action of the tryptophan synthase subunits α and β (TSA and TSB, respectively). The first committed step in the benzoxazinoid pathway is catalysed by the TSA homologue BX1. The subsequent conversion of indole to DIBOA is mediated by the cytochrome P-450-dependent monooxygenases BX2-BX5. The enzymes that convert DIBOA to DIMBOA (BX6 and 7) have not yet been characterised. Glucosylation of DIBOA and DIMBOA is carried out by the glucosyltransferases BX8 and BX9 (adapted from Gierl & Frey, 2001).

cDNA screening with high and low DIMBOA content maize lines (Frey *et al.*, 1995). Direct evidence for the involvement of one of these enzymes (BX3) in benzoxazinoid biosynthesis was obtained by isolation of a mutant allele, again using the *Mutator* transposon tagging system (Frey *et al.*, 1997). Two further genes (*Bx8* and *Bx9*) encoding benzoxazinoid glucosyltransferases, the enzymes that catalyse the last step in benzoxazinoid biosynthesis, were also recently isolated using a reverse genetics approach that relied on protein purification (von Rad *et al.*, 2001). The glucosides of DIBOA and

DIMBOA have reduced chemical reactivity when compared with the aglycones, suggesting that glucosylation may reduce phytotoxicity and so be important for storage (von Rad *et al.*, 2001).

Gene sequence comparisons and genetic mapping have yielded a glimpse of the evolutionary events that have led to the creation of the benzoxazinoid pathway (Gierl & Frey, 2001). *Bx1* presumably arose either directly or indirectly by duplication of the maize gene encoding TSA, which is required for primary metabolism (Frey *et al.*, 1997) (Fig. 6). The evolution of the *Bx1* gene has involved not only modification of the enzymatic properties of the product but also a change in the gene expression pattern (Gierl & Frey, 2001). Remarkably, all of the other characterised *Bx* genes, with the exception of *Bx9*, are clustered within 6 cM of *Bx1* on the short arm of chromosome 4, *Bx1* and *Bx2* being separated by 2.5 kb (Frey *et al.*, 1997; von Rad *et al.*, 2001). The sequence similarity, similar exon/intron structures and clustering of the cytochrome P450 genes *Bx2–Bx5* is indicative of gene duplication from a common precursor (Frey *et al.*, 1997). The glucosyltransferase genes *Bx8* and *Bx9* share similar gene structures and the gene products have around 90% amino acid similarity, again suggesting a common origin (von Rad *et al.*, 2001).

The distribution of benzoxazinoids across the Gramineae is sporadic. Maize, wheat, rye and certain wild barley species are capable of the synthesis of these compounds, while oats, rice and cultivated barley varieties are not (Niemeyer, 1988; Sicker *et al.*, 2000). All of the cytochrome P-450-mediated enzyme activities required for DIBOA synthesis are present in rye, and functional orthologues of *Bx2–5* have been found in wild barley species. By contrast cultivated barley lacks these genes and enzyme activities (Glawischnig *et al.*, 1999; Gierl & Frey, 2001). Similarly, benzoxazinone glycosyltransferase activity has been detected in some wild barley varieties but is absent in other varieties that do not contain benzoxazinoids (Leighton *et al.*, 1994). If the *Bx* gene cluster does turn out to be conserved in distantly related cereals such as maize and rye (members of the *Panicoideae* and *Pooideae*, respectively) then its origins must lie way back in the evolution of the Gramineae and possibly earlier, since some dicotyledonous species also produce benzoxazinones (Sicker *et al.*, 2000; Gierl & Frey, 2001). Analysis of the *A. thaliana* and rice genomes should shed light on this. The failure of cultivated barley to synthesise these compounds is presumably due to loss of components of the pathway during the plant breeding process.

The genes for benzoxazinoid biosynthesis represent the first example of clustered genes for a secondary metabolite biosynthetic pathway in plants. The reasons for clustering are unclear. In fungi, clusters of genes that encode distinct classes of enzymes required for synthesis of secondary metabolite biosynthetic pathways are common. One suggestion is that 'selfish' gene clusters persist because clustering favours transmission by horizontal gene transfer, a process that may be significant in the evolution of fungal genomes (Walton, 2000). The *Bx* gene cluster may be a consequence of horizontal gene transfer from a microbe, although this is an unlikely scenario. Presumably there are benefits to the plant in maintaining the genes as a cluster. Clustering may have advantages for coregulation of gene expression, while disruption of the cluster may lead to failure to produce phytoprotectants and possibly also accumulation of deleterious intermediates (Gierl & Frey, 2001).

Concluding remarks

The secondary metabolites that plants produce – chemical phytoprotectants, scents, colours and flavours – represent a snapshot of the ongoing processes of evolution and diversification in the Plant Kingdom. We are only just beginning to understand how particular secondary metabolite biosynthetic pathways have arisen in different plant lineages. Many of the genes for biosynthetic enzymes are likely to have been recruited from primary metabolism. New pathways may be generated in different ways, for example by the formation of new enzymes or by changes in the spatial distribution of existing ones (Pichersky & Gang, 2000). The components of these new pathways may then be recruited for the next round of biosynthetic diversification. An understanding of the evolution of secondary metabolism requires the characterisation of enzymes and genes for complete pathways. This will enable the chain of events that led to the creation of individual pathways to be unravelled by tracing the ancestry of the pathway components. The story of the *Bx* gene cluster is an elegant example of this. The *A. thaliana* and rice genomes, and other plant genome sequences that emerge in the future, will serve as key reference templates for studies of this kind.

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