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Cytochromes P450 in the biosynthesis of glucosinolates and indole alkaloids

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Abstract Characteristic of cruciferous plants is the synthesis of nitrogen- and sulfur-rich compounds, such as glucosinolates and indole alkaloids. The intact glucosinolates have limited biological activity, but give rise to an array of bioactive breakdown products when hydrolysed by endogenous β-thioglucosidases (myrosinases) upon tissue disruption. Both glucosinolates and indole alkaloids constitute an important part of the defence of plants against herbivores and pathogens, with the difference that a basal level of glucosinolates is ever-present in the plant whereas indole alkaloids are true phytoalexins that are de novo synthesised upon pathogen attack. With the completion of the genome sequence of the model plant, *Arabidopsis thaliana*, which is a crucifer, many genes involved in the biosynthesis of glucosinolates and indole alkaloids have been identified and cytochromes P450 are key players in these pathways. In the present review, we will focus on the cytochromes P450 in the biosynthesis of both groups of compounds. Their functional roles and regulation will be discussed.

Keywords *Arabidopsis thaliana* · Brassicales · CYP79 family · CYP83 · Oximes

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Introduction

Plants synthesise a vast number of complex molecules to use in their interactions with the surroundings, e.g. as defence compounds against herbivores and pathogens and as attractants for pollinators. One of the chemical defence strategies is the rapid induction of low molecular weight defence compounds, phytoalexins. Plants accumulate phytoalexins in response to several biotic and abiotic factors and chemically diverse phytoalexins from many plant families have been identified (Hammerschmidt 1999). Typically, the production of specific secondary metabolites is restricted to certain taxonomically related species. In the present review, we will focus on two groups of nitrogen- and sulfur-rich secondary metabolites produced within species from the Brassicales order. This includes glucosinolates, which are found throughout the Brassicales, and the indole alkaloids, which are restricted to the Brassicaceae family, and which were shown to be evolutionarily related to indole glucosinolates (Glawischnig et al. 2004). The Brassicaceae family comprises

the economically important rape (*Brassica napus*), the Brassica vegetables (*Brassica oleracea*), and the condiment mustards (e.g. *Sinapis alba*). Fortunately, the model plant *Arabidopsis thaliana* is a crucifer, and therefore a wealth of genomic tools has been available for elucidation of the biosynthetic pathway of these compounds. Cytochromes P450 have been found to be key biosynthetic enzymes in both pathways. Their functional roles and regulation will be discussed.

Glucosinolates

Glucosinolates, also known as mustard oil glucosides are organic anions characteristic of having a thioglucose moiety, a (*Z*)-*N*-hydroximinosulfate ester, plus a variable side group derived from amino acids. Glucosinolates are stored in the vacuole, and upon rupture of the cells, they come into contact with myrosinases, which are β-thioglucosidases that specifically cleave the thioglucosidic bond. This results in the release of the aglucone and its subsequent rearrangement into bio-active compounds in the form of, e.g. isothiocyanates, nitriles, and oxazolidine-2-thiones. The type of breakdown compounds formed depends on the structure of the original glucosinolate, the reaction conditions and the presence of co-factors. For humans, glucosinolates have attracted attention and interest both due to the flavour they release in, e.g. condiments, their reported beneficial effects as cancer-preventive agents in foods (Fahey et al. 2001; Mithen et al. 2000), their antinutritional effects in fodder such as *Brassica napus* (Mawson et al. 1994) and as bio-pesticides (Kirkegaard et al. 2000). There is a strong interest in being able, tissue-specifically, to regulate the levels of individual glucosinolates to improve the nutritional value and pest resistance of crops. Much of the glucosinolate pathway has been elucidated within recent years and cytochromes P450 have turned out to play an important part in the process.

The biosynthetic pathway of glucosinolates

The synthesis of the core glucosinolate structure takes its starting point in an amino acid that is converted into an aldoxime and proceeds via an oxidation and conjugation with cysteine to an *S*-alkyl-thiohydroximate. This is subsequently cleaved by a *C–S* lyase to a thiohydroximic acid which undergoes glycosylation to form a desulfoglucosinolate that is finally sulfated to form the glucosinolate. Different amino acids constitute the starting point for the synthesis and the glucosinolate end products are divided into three groups according to their origin—aliphatic (methionine, alanine, valine, leucine and isoleucine), aromatic (tyrosine and phenylalanine) and indole glucosinolates (tryptophan). The sequenced ecotype Columbia of *Arabidopsis thaliana* naturally contains methionine-, phenylalanine- and tryptophan-derived glucosinolates (Kliebenstein et al. 2001b). The further diversification of glucosinolates—more than 120 different have been found (Fahey et al. 2002)—is derived from chain elongation of methionine and phenylalanine, which takes place before the compounds enter the core synthesis of glucosinolates (Graser et al. 2000) as well as from secondary modifications of the amino acid side chain. Recently two enzymes, MAM1 and MAML, catalysing the first reaction of the chain-elongating step, were identified and characterised (Field et al. 2004). The secondary modifications include oxidations, hydroxylations, methoxylations, desaturations, sulfations as well as glucosylations.

At present, only two enzymes involved in secondary modifications in *Arabidopsis* have been identified. Kliebenstein et al. (2001a, b) used the extensive natural variation of aliphatic glucosinolates to identify the two α-ketoglutarate-dependent dioxygenases *AOP2* and *AOP3*, which control the production of alkenyl and hydroxyalkyl glucosinolates, respectively. Similar approaches are likely to result in the identification of genes responsible for other secondary modifications.

Cytochromes P450 in the biosynthesis of the core structure

The first committed step in the synthesis of the core structure is taken when a precursor amino acid is converted into the corresponding aldoxime,

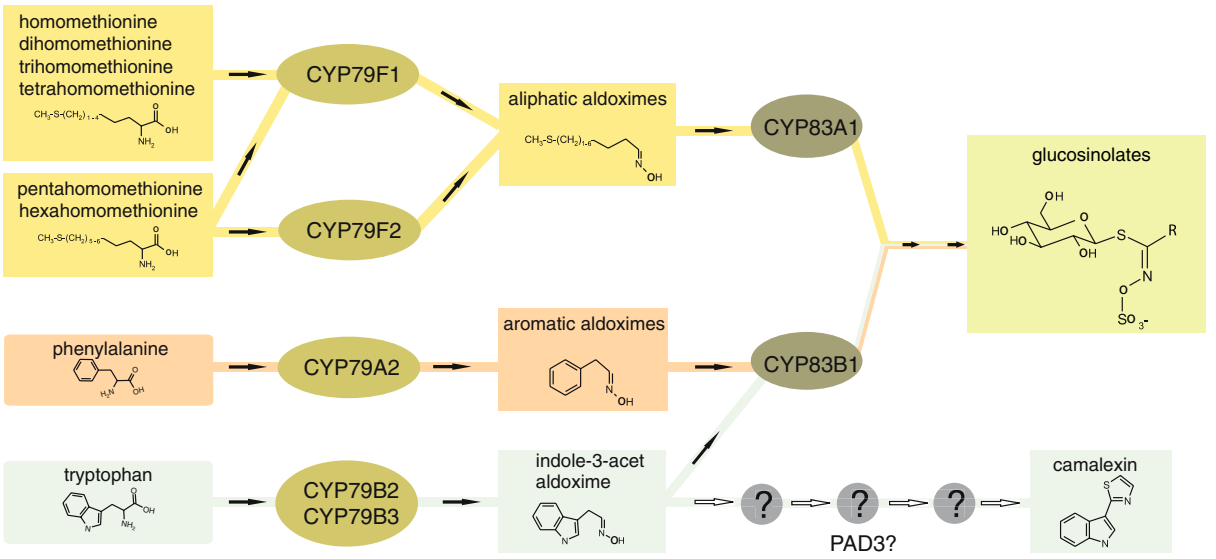

Fig. 1 Cytochrome P450 enzymes involved in the biosynthesis of the glucosinolate core structure as well as in the biosynthesis of indole alkaloids. The amino acids are metabolised by members of the CYP79 family whereas the oximes channelled into glucosinolate biosynthesis are metabolised by CYP83s. No oxime metabolising enzyme in the camalexin pathway has been identified. The glucosinolate core structure is depicted where R denotes the site group derived from the indicated amino acids. The amino acids and the corresponding oximes are shown in boxes while the P450s catalysing the reaction are shown in circles. Broken lines indicate proposed pathways

which is catalysed by cytochromes P450 belonging to the CYP79 family (Fig. 1). This discovery was made on the basis of sequence comparison to the CYP79 family found to catalyse the same type of reaction in the pathway of the cyanogenic glucosides (Mikkelsen et al. 2002). Seven members of this family are present in *Arabidopsis* (Col-0). Heterologous expression of the CYP79s in typically *E. coli*, followed by biochemical characterisation of the recombinant proteins has identified the substrate specificity of five of the seven CYP79s (Fig. 1). The enzymes are highly substrate-specific and therefore represent the entrance point for biosynthesis of the core structure of glucosinolates.

It was found that CYP79A2 converts phenylalanine to its corresponding aldoxime (Wittstock and Halkier 2000). Furthermore, CYP79B2 and CYP79B3 catalyse the conversion of tryptophan to indole-3-acetaldoxime (IAOx) (Hull et al. 2000; Mikkelsen et al. 2000). Likewise, both CYP79F1 and CYP79F2 can convert the chain-elongated methionine-derivatives into the corresponding aldoximes (Chen et al. 2003). However, their preferences differ so that CYP79F2 exclusively metabolises the long-chained methionine-derivatives (penta- and hexahomomethionine), whereas CYP79F1 can take both short- and long-chained methionine derivatives (Chen et al. 2001). The substrate specificity of CYP79C1 and CYP79C2 remains to be determined, but due to their low expression (Chen et al. 2001), it has been hypothesised that they may participate in the synthesis of the low-abundant glucosinolates such as the tyrosine-, methionine- or homophenylalanine-derived glucosinolates (Mikkelsen et al. 2002), or that they simply constitute pseudogenes that have lost their function.

The oxidation of the aldoxime is performed by another family of cytochromes P450, namely two members of the CYP83 family, CYP83B1 and CYP83A1 (Bak and Feyereisen 2001; Barlier et al. 2000) (Fig. 1) that share 65% amino acid sequence identity (Paquette et al. 2000). The substrate specificity of the two enzymes has been tested in vitro and chain-elongated aliphatic aldoximes are substrates for CYP83A1 and to a much lesser extent for CYP83B1. In fact, the latter occurs at such a low rate that the reaction is hypothesised not to be physiologically relevant (Naur et al. 2003b).

However, the presence of residual levels of alkyl glucosinolates in the *cyp83a1* knockout mutant implies that this genetic background creates conditions under which CYP83B1 is able to partly substitute for CYP83A1 (Hemm et al. 2003). CYP83A1, on the contrary, is able to metabolise the preferred substrates for CYP83B1, the aromatic aldoximes. However, CYP83B1 obtains a much lower K_m for the aromatic aldoximes in vitro—in particular for IAOx (Bak and Feyereisen 2001; Naur et al. 2003b). Both enzymes are able to metabolise phenylacetaldoxime and *p*-hydroxyphenylacetaldoxime although at a higher K_m. This demonstrates that *Arabidopsis* can produce Tyr- and Phe-derived glucosinolates (Bak et al. 1999; Wittstock and Halkier 2000).

Other enzymes in the biosynthesis of the core structure

Recently, the *C–S* lyase catalysing the step from *S*-alkylthiohydroximate to thiohydroximic acid was identified (Mikkelsen et al. 2004). It turned out to be identical to *SUR1/AFL1/RTY/HLS3* (hereafter called *SUR1*), whose knockout mutant had previously been described as an auxin-overproducing mutant based on its excessive root proliferation (Boerjan et al. 1995; Celenza et al. 1995; King et al. 1995; Lehman et al. 1996). Although the absence of glucosinolates in the *sur1* mutant suggests that *SUR1* is a single gene family member, one can not exclude that other *C–S* lyases are expressed in tissue that is not represented in the morphologically stressed *sur1* mutant.

Recently, a putative tyrosine aminotransferase gene (At5g36160) was found to be clustering with the known glucosinolate biosynthetic genes when integrating metabolomics and transcriptomics data (Hirai et al. 2005). Since *SUR1* was previously annotated as a tyrosine aminotransferase, it is tempting to suggest that this other putative tyrosine aminotransferase may also constitute a *C–S* lyase in the glucosinolate synthesis. Piotrowski et al. (2004) proposed another *C–S* lyase, CORI-3, as a potential player in the biosynthesis of indole glucosinolates based on specific upregulation of indole-3-ylmethyl and *N*-methoxyindole-3-methyl-glucosinolate combined with the concurrent upregulation of *CORI-3* upon treatment with coronatine, a phytotoxin produced by many pathovars of *Pseudomonas syringae*.

Based on sequence similarity to an in vivo confirmed thiohydroximic acid *S*-glucosyltransferase (S-GT) in *Brassica napus*, the glycosyltransferase UGT74B1 was identified and afterwards shown to glucosylate phenylacetothiohydroximic acid in vitro (Douglas et al. 2004). The knockout mutant, *ugt74b1*, displays a phenotype similar to auxin overproduction and is severely reduced in total amount of glucosinolates. The fact that ugt*74b1* contains glucosinolates makes it evident that other glucosyltransferases are involved in the glucosylation process (Douglas et al. 2004). Recently, on the basis of a hierarchical clustering of publicly available gene expression data, wherein *UGT74B1* was also pulled out, *UGT74C1* was proposed to be a candidate *S*-glucosyltransferase as it co-regulated with the genes in the synthesis of aliphatic glucosinolates (Gachon et al. 2005)

The final sulfation step was shown to be catalysed by three desulfoglucosinolate:PAPS sulfotransferases (STs) in *Arabidopsis*, ST5a, ST5b and ST5c (Klein et al. 2006; Piotrowski et al. 2004). The STs from the ecotype Colombia displayed a broad substrate specificity in vivo, although ST5a appears to prefer aromatic and indole desulfoglucosinolates and ST5b and ST5c the chain-elongated aliphatic ones (Piotrowski et al. 2004), whereas the same STs from another ecotype, C24, had slightly different substrate specificities (Klein et al. 2006).

Subcellular localisation of the glucosinolate metabolon

Enzymes involved in secondary biosynthetic pathways have been suggested to be organised in metabolons in order to ensure efficient channelling of intermediates (Winkel 2004). What are the indications of such a metabolon in glucosinolate biosynthesis? One indication is that in vivo feeding of radiolabelled amino acid precursors to, e.g. *Sinapis alba* or *Arabidopsis* appears to result in the accumulation of only intact glucosinolates (Chen et al. 2001; Du and Halkier 1998; Klein

et al. 2006; Piotrowski et al. 2004). With identification of the biosynthetic genes, knowledge of the subcellular organisation of the metabolon is emerging. Subcellular localisation of GFP fusions of CYP79F1 and CYP79F2 transformed into tobacco BY2 cells showed that both enzymes are localised to the endoplasmic reticulum (ER) (Reintanz et al. 2001). Presently, it has not been shown where MAM1 and MAML, involved in short-chain and long-chain elongation of methionines, respectively, are located. It has been indicated that they are located in the chloroplast as is the chain elongation machinery that converts valine to leucine (Kroymann et al. 2001). If correct, the chain-elongated methionine derivatives have to be translocated across the chloroplast membrane to CYP79F1 and CYP79F2 in the ER. The efficient channelling of chain-elongated methionine derivatives in wild-type plants suggests that the ER-located CYP79F1 and CYP79F2 are in close proximity with the chloroplast-located transporters. Further studies are required to understand the regulation at the branching point between continuations of chain elongation of precursor amino acids and channelling of precursor amino acids into the biosynthetic pathway of the core glucosinolate structure.

The cytochromes P450 CYP79B2 and CYP79B3 have been suggested to be localised in the chloroplast as evidenced by prediction programs (Hull and Celenza 2000) and promoter-GUS data (Ljung et al. 2005). However, CYP83A1 and CYP83B1, are likely to be localised on the ER which is the typical location for cytochromes P450 (Schuler and Werck-Reichhart 2003), and which at least in the case of CYP83A1 is where its substrates, the aliphatic aldoximes, are produced by CYP79F1 and CYP79F2 (Reintanz et al. 2001). Chloroplast prediction programs for the major P450s in the glucosinolate biosynthetic pathway and the NADPH cytochrome P450 reductases suggests that only CYP79B2 and CYP79B3 are localised to the chloroplast (Table 1) as has also been suggested by Celenza and coworkers (Hull et al. 2000; Hull and Celenza 2000). Although, the NADPH cytochrome P450 reductase 2 (ATR2) does not give a value above 0.5 in the chloroP prediction program, it is hypothesised to be targeted to the chloroplast based on its poly(Ser/Thr) N-terminal extension (Urban et al. 1997). Accordingly, ATR2 could function as the in vivo partner for the CYP79Bs. However, further experimental documentation is necessary to determine conclusively the subcellular localisation of CYP79B2 and CYP79B3, as well as CYP83A1 and CYP83B1. The remaining enzymes in the biosynthesis of the core glucosinolate structure, the *C–S* lyase, the thioglucosyltransferase, and the sulfotransferases, are soluble enzymes likely to be associated to the membrane-bound enzymes to form the glucosinolate metabolon, in a similar fashion to the soluble enzyme UDPG glucosyltransferase, UGT85B1, in the cyanogenic pathway. Bioimaging of the metabolon of the biosynthetic pathway of the cyanogenic glucoside dhurrrin, that consists of three genes, *CYP79A1*, *CYP71E1* and *UGT85B*, has shown that not until all three genes are introduced into a heterologous host plants does the metabolon assemble itself (K.A. Nielsen and B.L. Møller, personal communication). Anchoring of operational soluble enzymes to membrane-associated complexes have also been observed for enzymes involved in the phenylpropanoid and flavonoid biosynthetic pathways (Achnine et al. 2004; Burbulis and Winkel-Shirley 1999; Liu and Dixon 2001; Saslowsky and Winkel-Shirley 2001).

Channelling of intermediates along the glucosinolate biosynthetic highway appears to be a dynamic and highly controlled process that (re)organise the biosynthetic units of each of the

Table 1 Chloroplast predictions of major CYPs in the glucosinolate pathway and the two NADPH cytochrome P450 reductases

Protein	Length	Prediction score
CYP79B2	541	0.521
CYP79B3	543	0.542
CYP79F1	423	0.497
CYP79F2	537	0.488
CYP83B1	499	0.496
CYP83A1	502	0.480
ATR1	692	0.467
ATR2	712	0.469

The predictions were made using the ChloroP 1.1 server at http://www.cbs.dtu.dk. A prediction score >0.5 suggests that the protein has a N-terminal chloroplast transit peptide (Emanuelsson et al. 1999)

major phases (chain elongation, core structure biosynthesis and secondary modifications). An interesting challenge for the future is to understand how pathway organisation at the subcellular level, in combination with translocation (and turnover?) of glucosinolates at the whole plant level generate specific glucosinolate profiles to release specific active hydrolysis products in response to external as well as endogenous cues. Advancing from understanding a two-dimensional to a three dimensional organisation may provide insights into the regulation of metabolism beyond gene expression and into engineering alteration of pathway fluxes at critical branch points.

Mutations in glucosinolates biosynthetic genes interfere with hormone balances

''High auxin'' mutants

Many of the genes involved in the glucosinolate synthesis were already taken notice of before they were identified as players in the pathway. Mutations in some of the genes conferred imbalance in the total amount of plant hormones such as auxin and cytokinin, and as a consequence thereof created mutants with severe phenotypes. As an example, a mutant that contained a mutation in *CYP83B1* was first identified as the auxin-overproducing mutant *superroot 2 (sur2)* allelic to *rnt1/red1/atr4* (Bak et al. 2001; Hoecker et al. 2004; Smolen and Bender 2002) (hereafter referred to as *sur2*), due to its extensive root growth and elevated auxin levels (Delarue et al. 1998). Today, it is generally accepted that the phenotype of the *cyp83b1* knockout mutant is caused by IAOx being channelled into the IAA pathway when the homolog CYP83A1 can not efficiently enough process all the IAOx due to its very high K_m value for IAOx (Bak et al. 2001; Bak and Feyereisen 2001). Considering that glucosinolates are present in the millimolar range in plants whereas IAA acts in the micromolar range, it is understandable that such a shift would stress the plant. Another *cyp83b1* mutant,*red1*, was identified in a screen for suppressors of the enhanced de-etiolation response to continuous red light observed in transgenic *Arabidopsis* seedlings overexpressing the photoreceptor phytochrome B (Hoecker et al. 2004; Wagner et al. 1997). It seems likely that this effect evolves as a consequence of the enhanced amounts of IAA and thus can be considered a secondary rather than a primary effect of the mutation. Yet another *cyp83b1* mutant, *atr4*, was isolated in a screen for mutants in *Arabidopsis* with altered tryptophan regulation (*atr*) (Bender and Fink 1998; Smolen and Bender 2002). The authors show that the mutant has constitutively activated expression of the *ATR1* Myb factor gene, a positive regulator of tryptophan genes, and that the mutant has lesion-mimic phenotypes. The latter suggests that multiple stress pathways are activated by loss of CYP83B1 function.

Another glucosinolate biosynthetic mutant with ''high-auxin'' phenotype is *sur1*, which is mutated in the *C–S* lyase in the glucosinolate biosynthetic pathway (Mikkelsen et al. 2004). In *sur1* knockout mutants, the phenotype is believed to be due to IAOx accumulation as in *sur2*, but as opposed to the *cyp83b1* knockout mutant, the homozygous *sur1* mutant has a more severe phenotype and never reaches a reproductive stage (Mikkelsen et al. 2004), possibly due to the additional accumulation of *C–S* lyase substrates as well as aliphatic aldoximes. The knockout mutant *ugt74b1-1*, of the *S*-glucosyltransferase in the glucosinolate pathway, exhibited a phenotype reminiscent of auxin overproduction such as elongated hypocotyls, epinastic cotyledons and excessive adventitious root formation in addition to being dwarfed and partially sterile. The severe phenotype of *ugt74b1-1* is more likely to reflect pleiotropic effects due to the toxicity of accumulating thiohydroximic acid than a possible auxin activity of thiohydroximic acids (Douglas et al. 2004).

''High cytokinin'' mutants

Mutations in the aldoxime-metabolising P450 enzymes are not the only ones that have an influence on hormone homeostasis. Mutations in *CYP79F1* results in a plant with massive proliferation of shoots; thus the name of the isolated knockout mutants *bushy1* (Hansen et al. 2001b;

Reintanz et al. 2001) and *supershoot1* (Tantikanjana et al. 2001). Measurement of hormone levels in the mutant showed that the cytokinin level was upregulated 3-9-fold (Tantikanjana et al. 2001) and the auxin level was upregulated ~2-fold (Reintanz et al. 2001). Using hormone reporter genes, it was shown that the increased levels of cytokinin, but not auxin, correlated well with the expression of the *CYP79F1* gene in the site of bud initiation. It is an open question whether this reflects that a gene encoding a biosynthetic enzyme activity for aliphatic aldoximes also plays a role in cytokinin homeostasis or whether the co-localisation is a coincidence (Tantikanjana et al. 2004). Similarly, the observed phenotypes of *cyp79f1*, with massive proliferation of shoots, and *cyp79f2*, with only 65% root growth compared to wild-type, may reflect that CYP79F1 and CYP79F2 play distinct roles in plant growth and development or possibly be secondary metabolical and physiological effects of the accumulation of short- and long-chained derivatives of methionine in the mutants (Hansen et al. 2001b). Further studies are needed to elucidate whether CYP79F1 and CYP79F2 play a direct role in growth and development.

''Phenylpropanoid'' mutants

A knockout mutant of *CYP83A1*, that primarily metabolises aliphatic aldoximes, was unexpectedly identified in a screen for plants having altered fluorescence under UV-light. This was due to ~3-fold reduction in sinapate esters such as sinapoyl malate and sinapoyl glucose (Hemm et al. 2003). Accumulation of aliphatic aldoximes was hypothesised to interfere with the biosynthesis of phenylpropanoids, possibly by inhibition of *O*-methyltransferases in lignin synthesis. The hypothesis was supported by the fact that the *sur2* knockout mutant exhibited a similar reduction in sinapoyl malate. As expected the *cyp83a1* mutant showed a reduction in aliphatic glucosinolates in leaves, but also, somewhat surprisingly, increased contents of indole glucosinolates (Hemm et al. 2003). Similarly, the *cyp79f1* knockout mutant showed the expected abolishment of short-chain glucosinolates and increase in long-chain aliphatic glucosinolates, but also an increase in indole glucosinolates (Hansen et al. 2001b; Reintanz et al. 2001). Hemm et al. (2003) have suggested that a cross-talk mechanism between aliphatic and indole glucosinolate pathways exists, whereby one type of glucosinolate substitutes for the lack of the other. This argument was based on the observation that indole glucosinolate levels rose in plants reduced in aliphatic glucosinolates independent of whether the reduction was due to mutations in pre-aldoxime (CYP79F1) or post-aldoxime (CYP83A1) aliphatic enzymes. However, both chain-elongated methionine derivatives and the corresponding aldoximes accumulate in *cyp79f1* and *cyp83a1*, respectively, which suggest that they are metabolically stressed. Accordingly, the boost in indole glucosinolates could comprise a stress response rather than an actual cross-talk as it has been demonstrated that *CYP79B2* and *CYP79B3* can be induced by stress (Brader et al. 2001; Mikkelsen et al. 2003).

Regulation

ATR1

Now that most of the genes in the biosynthetic pathway of the core glucosinolate structure have been elucidated, attention has turned towards the discovery of regulators of the pathway, which is a relatively unexploited area. It is clear from above that imbalance in the products of the glucosinolate pathway can cause serious disturbances in plant metabolism, which emphasises the necessity for regulation of the pathway. The first transcription factor to be identified as a regulator of the glucosinolate pathway was *ATR1* (altered tryptophan regulation), which was originally identified in a screen of EMS mutants for regulators of enzymes in the tryptophan biosynthesis (Bender and Fink 1998). *ATR1* is the AtMYB34 member of the Myb family of transcription factors, one of the three largest families of transcription factors in *Arabidopsis*, with an estimated number of 136 members (Riechmann and Ratcliffe 2000). Myb proteins have been shown to be involved in as diverse processes as the control of secondary metabolism, the regulation of cellular morpho-

genesis and as players in the downstream signal transduction pathway of plant growth regulators (Martin and Paz-Ares 1997).

The role of *ATR1* was shown to include a specific impact on the amount of indole glucosinolates present in the plant (Celenza et al. 2005). The originally identified mutant, *atr1-D*, was a dominant overexpression allele of the Myb transcription factor (Bender and Fink 1998). Interestingly, this dominant mutation produced more leaf indole glucosinolates than a *CaMV* 35S overexpresser of the gene in spite of the larger amount of transcript levels in the leaves of this transformant in comparison to the *atr1-D* (Celenza et al. 2005). This emphasises the importance of expressing a transcription factor at the right time and in the right place in order to generate as much of the desired secondary metabolite as possible.

The *atr1-D* mutation resulted in elevated transcripts of *CYP79B2*, *CYP79B3* and *CYP83B1* as well as elevated levels of IAA, whereas neither *CYP79F1* transcript nor the amount of methionine-derived glucosinolates were affected. Correspondingly, a T-DNA insertion allele of *ATR1*, *atr1-2*, accumulated reduced levels of indole glucosinolates in the leaves (Celenza et al. 2005). (Narusaka et al. 2004) performed a promoter analysis on *CYP79B2* and *CYP83B1* 1 kb upstream of the open reading frame. They identified five Myb recognition sites in *CYP79B2* and six Myb recognition sites in *CYP83B1* as well as recognition sites for MYC, ACGT, W-boxes, P-boxes, DRE-core and G-boxes in the genes. Since indole glucosinolates were still present in the *atr1* knockout mutant, ATR1 is not the sole transcription factor responsible for indole glucosinolate production and these recognition sites might be a clue to other transcription factors involved in the process.

IQD1

Other transcription factors have been connected to glucosinolate biosynthesis although on a less specific level than *ATR1*. IQD1 is a calmodulin-binding nuclear-localised protein that was shown to alter the composition and amounts of glucosinolates in plants both when it was down- and up-regulated (Levy et al. 2005). An *iqd1* knockout mutant has decreased amounts of aliphatic glucosinolates (up to 75% decrease), whereas indole glucosinolates are affected to a lesser extent (up to 40% decrease). An overexpression mutant accumulated about twice as much glucosinolates as the wild-type. In this mutant, the expression of *CYP79B3* and *CYP83B1* was particularly enhanced, whereas the expression of *CYP79F1* and *CYP79F2* was reduced to 10–25% of wild-type levels (Levy et al. 2005). Another indication of the involvement of IQD1 in glucosinolate biosynthesis is the fact that the tissue-specific expression pattern of the gene overlaps with *CYP79F1*, *CYP79F2*, *CYP79B2* and *UGT74B1*(Levy et al. 2005). Due to the role of calcium and calmodulin in signalling, IQD1 has been suggested to play a role in the integration of signalling of pathogen attack and a corresponding glucosinolate production response.

Other regulators

Alterations in the amounts of glucosinolates have been shown to be present in mutants with an insertion in another transcription factor, *OBP2*, a member of the Dof transcription factor family in *Arabidopsis*. OBP2 has been shown to function in the regulation of *CYP83B1* in the glucosinolate pathway, as overexpression of *OBP2* upregulates *CYP83B1* and as in RNA interference *OBP2* was downregulated along with *CYP83B1* (manuscript in submission). These data provide evidence that OBP2 is part of a regulatory network that regulates glucosinolate biosynthesis in *Arabidopsis*.

Other studies have exploited the natural variation of glucosinolates in *Arabidopsis* ecotypes to identify loci that control the amounts of glucosinolates. These loci could be actual regulators of the glucosinolate pathway or represent allelic differences in biosynthetic enzymes either conferring higher expression or different enzyme efficiencies. Kliebenstein et al. (2001a) took a QTL (quantitative trait loci) approach to identify loci that influenced the total amount of glucosinolates (quantitative as opposed to qualitative). Their data suggested that accumulation of the

different types of glucosinolates may be independently regulated as evidenced by the absence of overlapping QTLs controlling aliphatic and indolic glucosinolate levels in both leaves and seeds in the recombinant inbred lines, Landsberg and Cvi, used in this study (Kliebenstein et al. 2001a). Further studies are needed to identify specific regulators of aliphatic, aromatic and indolic glucosinolates.

Cruciferous phytoalexins—the sulfur-rich indole alkaloids

Phytoalexins are characterised by being absent in an unstressed plant and only produced in response to pathogen attack or abiotic stress (Hammerschmidt 1999). Several of the cruciferous indole alkaloids have been demonstrated, in vitro, to have anti-fungal activity towards certain pathogens (Pedras et al. 2000; Pedras et al. 2003). In addition, epidemiological studies suggest that cruciferous phytoalexins have antiproliferative activities against cancer cell lines similar to the glucosinolate-derived isothiocyanate, sulphoraphane (Mezencev et al. 2003; Pilatova et al. 2005; Sabol et al. 2000). About 30 phytoalexin structures have been identified in cruciferous plants (Pedras et al. 2003). The individual phytoalexins are usually only produced in a limited number of species and can there almost, but not quite, be regarded as species-specific (Pedras et al. 2000; Pedras et al. 2003). *Arabidopsis thaliana* accumulates the phytoalexin, camalexin, which is the only indole alkaloid for which biosynthetic genes have been identified. This part of the review will focus on cytochromes P450 in the biosynthesis of camalexin.

Biosynthesis of camalexin

The role of CYP79Bs in camalexin biosynthesis

The structure of camalexin was identified in 1991 (Browne et al. 1991) and the presence of camalexin seems to be limited to a few closely related crucifer species: *Arabidopsis thaliana*, *Arabis lyrata*, *Capsella bursa-pastoris* and *Camelina sativa* (Browne et al. 1991; Jimenez et al. 1997; Tsuji et al. 1992; Zook et al. 1998). In *Arabidopsis*, only the unmodified form of camalexin has been reported whereas 6-methoxycamalexin can be found in *Capsella bursa-pastoris* and *Camelina sativa* (Pedras et al. 2000) and 1-methylcamalexin has been identified in *Capsella bursa-pastoris* (Jimenez et al. 1997).

Initially, camalexin was thought to be derived from indole, and not tryptophan, as indole was a better precursor for camalexin than tryptophan in feeding experiments (Zhao and Last 1996; Zook 1998). Furthermore, heterotrophic tryptophan biosynthetic mutants accumulated wild-type levels of camalexin when treated with pathogens (Zhao and Last 1996), suggesting that tryptophan was an unlikely intermediate in the biosynthesis. It has also been proposed that at least some of the indole alkaloids were derived from indole glucosinolates which will require that the alkaloids were derived from tryptophan. Recently, it was shown unequivocally that camalexin is derived from tryptophan as it was demonstrated that the cytochrome P450 enzymes CYP79B2 and CYP79B3 were involved in camalexin biosynthesis (Glawischnig et al. 2004). These enzymes catalyse the conversion of tryptophan to IAOx (Hull et al. 2000; Mikkelsen et al. 2000), which is a precursor for indole glucosinolate as well as auxin biosynthesis (Bak et al. 2001; Hansen et al. 2001a; Zhao et al. 2002). The importance of IAOx was demonstrated genetically by the complete lack of camalexin in a *cyp79b2/cyp79b3* background (Glawischnig et al. 2004). Furthermore, it was proven that camalexin does not result from the breakdown of indole glucosinolates as the glucosinolate-free *sur1* mutant, which is mutated in a *C–S* lyase in the glucosinolate pathway, accumulated camalexin in response to silver nitrate (Glawischnig et al. 2004).

At present, the last identified common intermediate between indole glucosinolates and camalexin is IAOx. Therefore, one can not exclude that the branch point between the two pathways is located between IAOx and the *C–S* lyase substrate. No IAOx-metabolising enzyme in the biosynthesis of camalexin has been identified, but if IAOx is the branching point between the two pathways, it may

be a cytochrome P450 enzyme as the only oxime-metabolising enzymes in plants identified to date are CYP83B1 and CYP83A1, both involved in glucosinolate biosynthesis (Bak and Feyereisen 2001; Hansen et al. 2001a; Naur et al. 2003b) and CYP71E1 involved in the biosynthesis of cyanogenic glucosides (Bak et al. 1998).

Other enzymes in camalexin biosynthesis

In a genetic screen, phytoalexin deficient (*pad*) mutants were isolated based on their accumulation of reduced amounts of camalexin in response to *Pseudomonas syringae* infection (Glazebrook et al. 1996; Glazebrook and Ausubel 1994; Rogers et al. 1996). Of the five *pad* mutants isolated, *PAD3* and *PAD5* may be candidate genes for post-aldoxime enzymes in the biosynthetic pathway of camalexin, whereas *PAD1*, *PAD2* and *PAD4* seem to have a regulatory role (Glazebrook et al. 1997). The *PAD3* gene was cloned by a map-based approach and found to encode the P450 enzyme CYP71B15 (Zhou et al. 1999). The substrate for PAD3 has yet to be identified, but recently, when metabolic profiling was made on pathogen-inoculated roots from wild-type plants and *pad3* mutants, the compound, 2-(indol-3-yl)-4,5-dihydro-1,3-thiazole-4-carboxylic acid (CA), accumulated in pathogen inoculated *pad3* roots but not in wild-type (Bednarek et al. 2005). This suggests that CA could be the substrate for PAD3. The identity of *PAD5* is still unknown, but CA was also found to accumulate in inoculated roots of a *pad5* knockout mutant, which points to a similar function of PAD3 and PAD5 (Bednarek et al. 2005).

The thiazole ring of camalexin was demonstrated to be derived from cysteine as evidenced by incorporation of labeled cysteine, but not methionine, into camalexin (Zook and Hammerschmidt 1997). It has been proposed that cysteine condenses with indole-3-carboxaldehyde to form a product that subsequently cyclizes to form the thiazole ring (Browne et al. 1991). So far, the involvement of indole-3-carboxaldehyde as an intermediate in camalexin biosynthesis is purely theoretical as there are no experimental data to verify this hypothesis. In addition, there is no proposed mechanism for the conversion of IAOx into indole-3-carboxaldehyde.

It has generally been assumed that secondary modifications of phytoalexins take place after the biosynthesis of the phytoalexin core structure. Interestingly, in vivo experiments in kohlrabi (*Brassica*) and rutabaga (*Brassica napus* L. ssp. *Rapifera*) showed that 1-methoxyindol-3-acetaldoxime, rather than brassinin, is the precursor of the phytoalexin 1-methoxybrassinin (Pedras and Montaut 2004). These results suggest that methoxylations of IAOx-derived metabolites may take place already at the aldoxime level. This may be possible not only for indole alkaloids, but also for glucosinolates. The occurrence of 1-methoxyindol-3-acetaldoxime in *Arabidopsis* or 6-methoxyindol-3-acetaldoxime in *Camelina sativa*, or other Brassica species, has never been reported, but neither has the occurrence of IAOx, which is probably due to an efficient channelling of the compound.

Induction of camalexin

Camalexin accumulates in response to a wide range of biotic and abiotic factors, e.g. pathogen attack, UV-B light, ozone and silver nitrate (Kliebenstein 2004). This regulation of induction seems complex and is currently not known in details. Despite the classification of camalexin as a defence compound, its induction seems to be largely independent of defence hormones such as jasmonic acid and salicylic acid (Glazebrook 2005; Heck et al. 2003; Thomma et al. 1999), although ethylene seems to be partly important, as the ethylene insensitive mutants *ein2* and *etr1* accumulate lower camalexin levels than wild-type plants in response to pathogens (Heck et al. 2003; Thomma et al. 1999).

Many of the factors that induce camalexin accumulation generate reactive oxygen species (ROS), e.g. UV-B (Baier et al. 2005), ozone (Brosche and Strid 2003) and the chemicals paraquat and aciflourfen (Bowler et al. 1992; Matringe et al. 1989). In addition, mutants with deregulated ROS responses, e.g. *esa1* and *ups1*, have distorted camalexin accumulation in

response to pathogens (Denby et al. 2005; Tierens et al. 2002), indicating that ROS signaling regulates camalexin accumulation. It appears as if the only requirement for camalexin induction is the production of reactive oxygen species or cell death. The observation that various *Arabidopsis* ecotypes accumulate camalexin in different amounts in response to the same pathogen and unrelated to the level of resistance (Denby et al. 2004; Kagan and Hammerschmidt 2002; Mert-Turk et al. 2003) suggests that camalexin is regulated by non-specific stress-stimulated networks that overlap with a number of pathogen induced networks (Kliebenstein 2004).

Although indole glucosinolates and camalexin share a biogenetic relationship and both are considered defense compounds, they seem to be regulated by distinct mechanisms. For instance, the application of exogenous jasmonic acid or methyl jasmonate increases the accumulation of indole glucosinolates, whereas camalexin cannot be induced by this treatment (Brader et al. 2001; Mikkelsen et al. 2003; Thomma et al. 1999). The difference in induction and regulation between indole glucosinolates and camalexin indicates a tight regulation of the genes in the biosynthetic pathways, especially of *CYP79B2* and *CYP79B3* as they are involved in both pathways. In a study where production of indole glucosinolate, and not camalexin, was highly induced following treatment with *Erwinia carotorova*, *CYP79B3* and not *CYP79B2*, was induced (Brader et al. 2001). When induction of camalexin was measured in *cyp79b2* and *cyp79b3* single knockout mutants, the *cyp79b3* knockout mutant accumulated wild-type levels of camalexin, whereas the *cyp79b2* knockout mutant only accumulated approximately 50% of wild-type level (Glawischnig et al. 2004). Furthermore, *CYP79B2* and not *CYP79B3* expression was upregulated following induction of camalexin production (Glawischnig et al. 2004) as has also been observed in a number of microarray experiments (Glazebrook et al. 2003; Narusaka et al. 2003; van Wees et al. 2003). These data suggest that CYP79B2 and CYP79B3 have a differential function in camalexin biosynthesis.

Biosynthesis of other indole alkaloids

The importance of IAOx in phytoalexin biosynthesis was further highlighted when labeling experiments in *Brassica juncea* and *Brassica rapa* showed that IAOx was incorporated in vivo into brassilexin, brassinin, spirobrassinin and cyclobrassinin whereas the proposed precursor, glucobrassinin, failed to be incorporated into these phytoalexins (Pedras et al. 2001). Feeding experiments have demonstrated that brassinin is a precursor for cyclobrassinin, which furthermore is a precursor for rutalexin and brassilexin (Pedras et al. 1998; Pedras et al. 2004). In addition, brassinin is a precursor of brassicanate A, dioxybrassinin, brassicanal A and spirobrassinin (Fig. 2) (Pedras et al. 2003).

The incorporation of IAOx into rather structurally different phytoalexins points to a general role of IAOx in the biosynthesis of crucifer phytoalexins. A requirement for this hypothesis is the presence of IAOx-producing enzymes in all phytoalexin accumulating crucifers. In *Arabidopsis*, tryptophan is converted to IAOx by the functionally redundant CYP79B2 and CYP79B3 enzymes (Hull et al. 2000; Mikkelsen et al. 2000). However, other sources of IAOx might exist; the flavin-mono-oxygenase-like enzyme YUCCA has been reported in vitro to catalyze N-hydroxylation of tryptamine, which may subsequently be converted to IAOx (Hansen and Halkier 2005; Zhao et al. 2001). The IAOx pool derived from tryptamine does not seem to be channeled into phytoalexin biosynthesis as *cyp79b2/b3* mutants are devoid of camalexin (Glawischnig et al. 2004). In addition, deuterium labeled tryptamine was not incorporated into IAOx-derived phytoalexins isolated from rutabaga tubers (Pedras et al. 2004). The latter could reflect a problem with uptake of tryptamine or the presence of a tryptamine-metabolising enzyme that has a higher affinity for tryptamine than YUCCA, as tryptamine was metabolised in rutabaga (Pedras et al. 2004). These results suggest that CYP79B homologs are responsible for synthesis of the IAOx that is precursor for phytoalexin synthesis in other crucifers. This is supported by the enzymatic

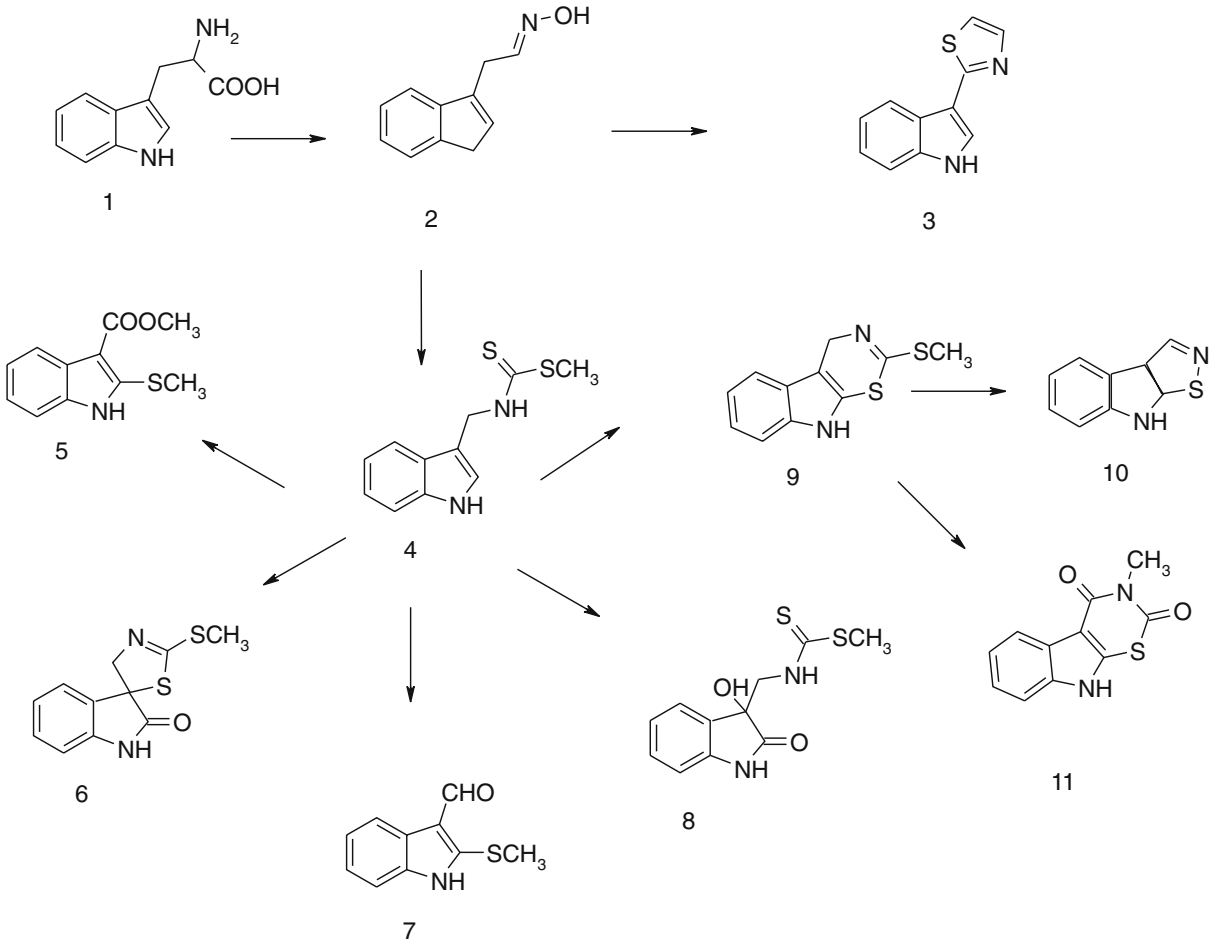

Fig. 2 Structure of IAOx-derived phytoalexins. IAOx **2**, derived from tryptophan **1**, is the direct precursor of camalexin **3** and brassin **4**. Feeding studies have demonstrated that brassinin is the precursor of brassicanate A **5**, spirobrassinin **6**, brassicanal A **7** and dioxybrassinin **8**. In addition, brassinin is the precursor for cyclobrassinin **9**, which is a closer precursor for rutalexin **10** and brassilexin **11**

conversion of tryptophan to IAOx by CYP79B1 from *Sinapis alba* (Naur et al. 2003a).

Conclusion

Since the completion of the *Arabidopsis* genome in 2000, there has been a significant increase in our knowledge of glucosinolate biosynthesis, as well as the biosynthesis of camalexin. Identification of biosynthetic genes has shown that cytochromes P450 are key players. Particularly, the *CYP79* genes have become important molecular tools for metabolic engineering of plants with altered glucosinolate profiles to increase our understanding of the physiological role of glucosinolates, e.g. in plant–herbivore interactions and as storage compounds. More knowledge about the factors that regulate cytochromes P450 and thereby the flux through the biosynthetic pathways is necessary if we are to successfully metabolically engineer these biosynthetic pathways.

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