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Review

Genome-wide analysis of phenylpropanoid defence pathways

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SUMMARY

Phenylpropanoids can function as preformed and inducible antimicrobial compounds, as well as signal molecules, in plant–microbe interactions. Since we last reviewed the field 8 years ago, there has been a huge increase in our understanding of the genes of phenylpropanoid biosynthesis and their regulation, brought about largely by advances in genome technology, from whole-genome sequencing to massively parallel gene expression profiling. Here, we present an overview of the biosynthesis and roles of phenylpropanoids in plant defence, together with an analysis of confirmed and predicted phenylpropanoid pathway genes in the sequenced genomes of 11 plant species. Examples are provided of phylogenetic and expression clustering analyses, and the large body of underlying genomic data is provided through a website accessible from the article.

INTRODUCTION

Among their many functions in plants, phenylpropanoid compounds play important roles in resistance to pathogen attack (Dixon *et al.*, 2002). Plant defence compounds have been classified into three main groups, signalling molecules, constitutive or preformed phytoanticipins, and phytoalexins, which are synthesized *de novo* in response to microbial attack (VanEtten *et al.*, 1994); phenylpropanoid compounds are found among all three groups.

Flavonoids (C_6–C_3–C_6) represent one of the largest classes of phenylpropanoid-derived, plant-specialized metabolites (with an estimated 10 000 structurally different members) (Tahara, 2007). They consist of two main groups: the 2-phenylchromans (flavonoids) and the 3-phenylchromans (isoflavonoids) (Fig. 1). The structural diversity of flavonoids is derived by complex substitution of these carbon skeletons through hydroxylation, glycosylation, methylation, prenylation, etc. The enzymes that catalyse these reactions often belong to large gene families, which can be recognized in expressed sequence tag (EST) and genome datasets through family-specific conserved sequence motifs.

In our earlier review (Dixon *et al.*, 2002), the available knowledge on the genomics of phenylpropanoid metabolism was limited primarily to EST expression data and information from the Arabidopsis and rice genome sequencing projects. During the past 8 years, there has been a major increase in such information, brought about largely by advances in genome technology, from whole-genome sequencing to massively parallel gene expression profiling. Here, we provide an update of the field by first reviewing our current understanding of how phenylpropanoid compounds are synthesized and function in plant defence, followed by a discussion of phenylpropanoid biosynthesis across whole plant genomes, with an emphasis on flavonoids. Because of the massive amount of data now available, we have generated a website (http://bioinfo.noble.org/manuscript-support/mpp/) to present the information in a readily accessible manner. It is important to note that genes identified on the basis of bioinformatics approaches alone require subsequent functional identification by biochemical or genetic approaches; genes with proven functions are annotated as such on the website.

FUNCTIONS OF PHENYLPROPANOID COMPOUNDS IN PLANT DEFENCE

Lignin and lignans

Lignin and lignans share monolignols as common precursors (Figs 1 and 2). Lignification makes the cell wall more resistant to mechanical pressure applied during fungal penetration (Bechinger *et al.*, 1999). A lignified cell wall is water resistant and thus less accessible to cell wall-degrading enzymes from plant pathogens.

Natural lignin contains three major units: *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S). The structural analysis of lignins induced by elicitor from *Rhizosphaera kalkhoffii* in spruce revealed that the defence lignins are significantly different from

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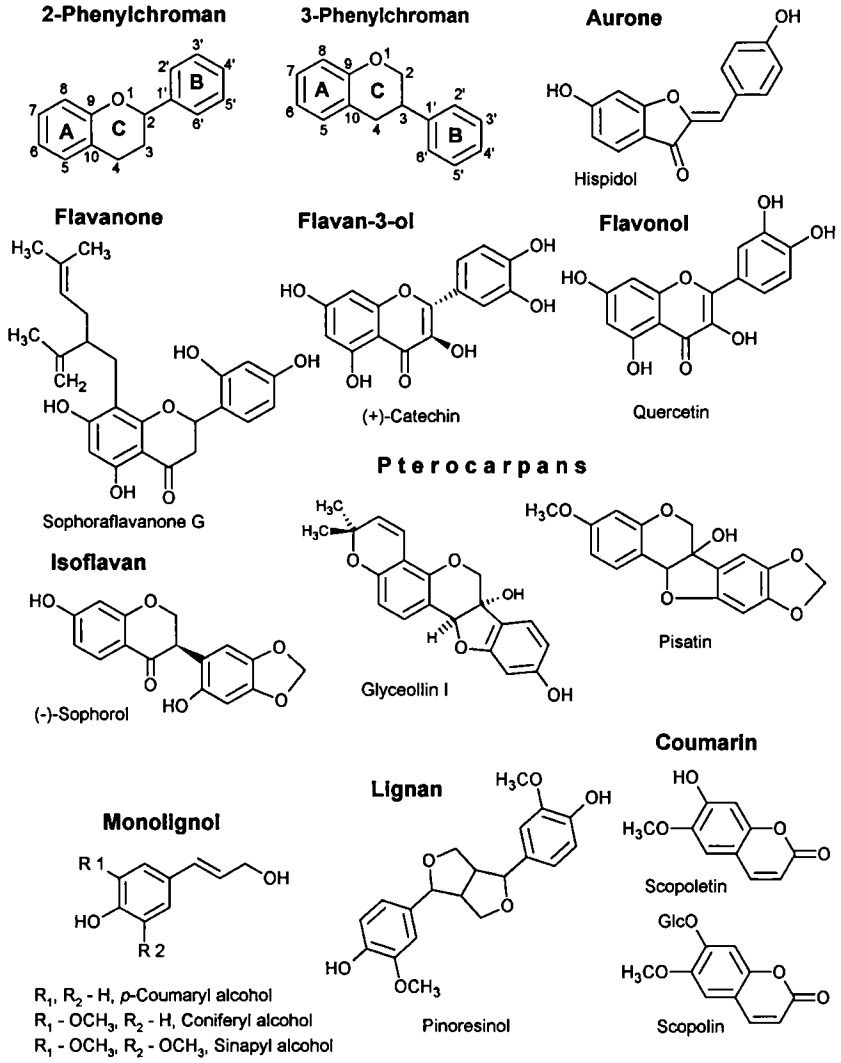


Fig. 1 Basic structures of the phenylpropanoid classes described in this article.

the lignins that are laid down during plant development (Lange *et al.*, 1995). The specific activation of an S lignin biosynthesis pathway in elicitor-treated wheat leaves was suggested by the induction of a sinapyl alcohol-specific isoform of cinnamyl alcohol dehydrogenase (CAD) (Mitchell *et al.*, 1999). Wheat leaves infected with stem rust fungus or exposed to a derived fungal elicitor subsequently accumulated S lignin (Menden *et al.*, 2007).

The function of lignification during plant defence was originally assumed to be as a physical barrier (Moerschbacher *et al.*, 1990), although lignins or their precursors might also function in chemical defence. Induction of monolignol biosynthesis genes by pathogen infection has been reported in a number of plant species (Bhuiyan *et al.*, 2009; Dixon and Paiva, 1995; Koutaniemi *et al.*, 2007; Lauvergeat *et al.*, 2001; Menden *et al.*, 2007). For example, 5-hydroxyguaiacyl 3-*O*-methyltransferase [also known as caffeic acid 3-*O*-methyltransferase 1 (COMT1)] expression increased on infiltration of Arabidopsis leaves with flagellin (flg22) or harpin (HrpZ), or with the necrosis-inducing *Phytophthora* protein 1 (NPP1) (Engelhardt *et al.*, 2009; Fellbrich *et al.*, 2002; Zipfel *et al.*, 2004); thus, transcription of *COMT1* appears to correlate with the onset of the plant's pathogen-associated molecular pattern (PAMP)-triggered immune (PTI) response.

Cinnamoyl-CoA reductase (CCR), the first enzyme specific for monolignol synthesis, was shown to be involved in pathogen defence signalling in rice (*Oryza sativa*). *OsCCR1* is an effector of OsRac1, a small guanosine triphosphatase (GTPase) from the Rac/Rop family, which is known to play a critical role in defence responses and to which OcCCR1 binds through a guanosine triphosphate (GTP)-dependent physical interaction (Kawasaki *et al.*, 2006).

In some cases, the downregulation of monolignol biosynthesis genes not only compromises resistance to host pathogens, but also allows the penetration of nonhost pathogens. For instance,

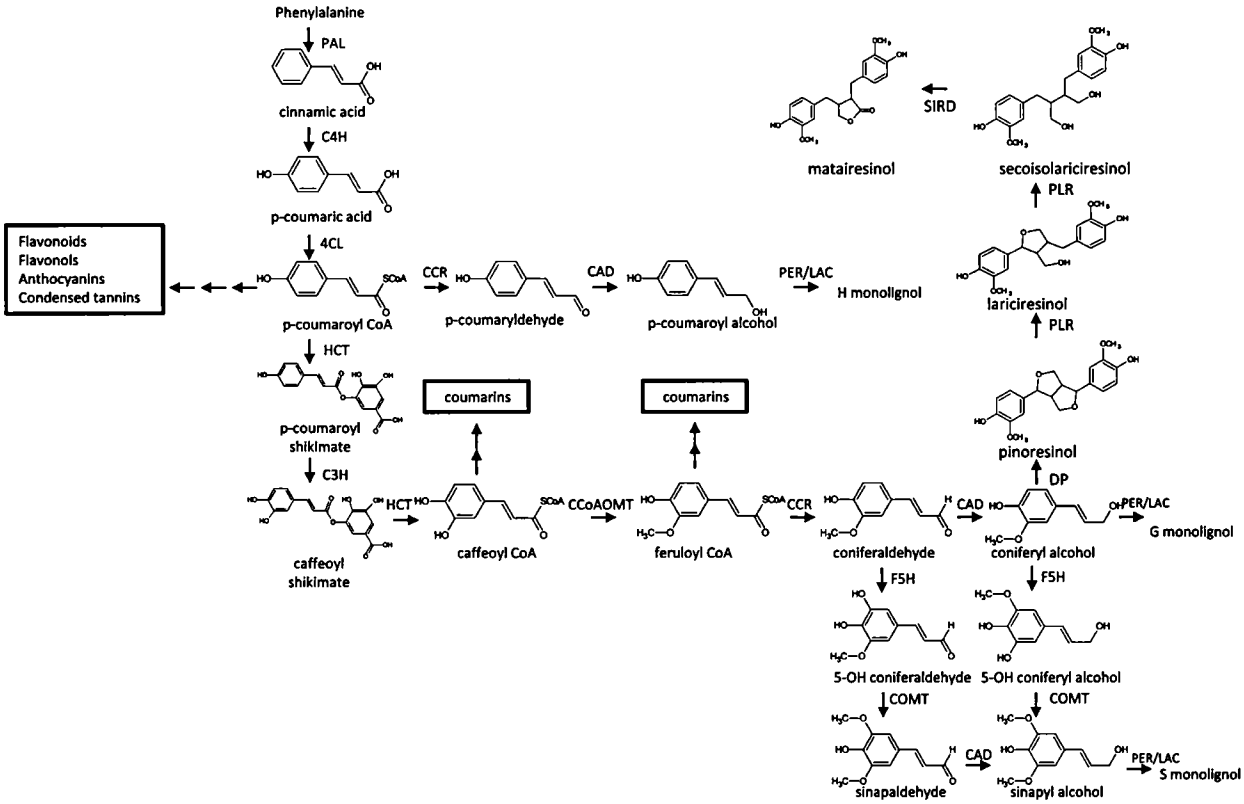


Fig. 2 The monolignol biosynthetic pathway. The enzymes are as follows: CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl CoA 3-*O*-methyl transferase; CCR, cinnamoyl CoA reductase; C3H, 4-coumaroylshikimate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; COMT, caffeic acid *O*-methyltransferase; DP, dirigent protein; F5H, ferulic acid 5-hydroxylase; HCT, hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase; LAC, laccase; PAL, L-phenylalanine ammonia-lyase; PER, peroxidase; PLR, pinoresinol/lariciresinol reductase; SIRD, secoisolariciresinol dehydrogenase.

RNA interference (RNAi)-mediated gene silencing of *COMT* in wheat led to successful penetration of *Blumeria graminis* f. sp. *hordei*, a nonhost pathogen, in leaf cells, whereas no penetration was observed in control plants (Bhuiyan *et al.*, 2009). However, the downregulation of lignin biosynthesis genes does not necessarily impair plant defence and, in some cases, reduced lignin plants have increased resistance to plant pathogens (Funnell and Pedersen, 2006). The mechanisms for this are not always clear. Arabidopsis *Comt1* mutants accumulated more soluble 2-*O*-5-hydroxyferulol-L-malate than did wild-type plants, and this, rather than changes in lignin, accounted for the *Comt1* mutant's enhanced resistance to *Hyaloperonospora arabidopsidis* (Quentin *et al.*, 2009).

The lignans (Figs 1 and 2) represent a structurally diverse class of plant-specialized metabolites which, unlike lignins, are not found ubiquitously in all land plants. The primary physiological role of lignans *in planta* is probably in defence, and several lignans have been reported to have antibacterial and antifungal activities (Akiyama *et al.*, 2007; Carpinella *et al.*, 2005; Ralph *et al.*, 2006). Lignans can act as both phytoanticipins and phytoalexins. The heartwood of many tree species, such as loblolly pine and western red cedar, contains significant levels of lignans, and constitutive deposition of lignans reinforces durability, longevity and resistance of the heartwood of many tree species against wood-rotting fungi. On the other hand, *de novo* formation of lignans in response to fungal attack has been reported in both woody and nonwoody species (Gang *et al.*, 1999).

Coumarins

Scopoletin and its glucosylated form scopolin (Fig. 1), which are induced under various stress conditions (Gachon *et al.*, 2004; Shimizu *et al.*, 2005), have antimicrobial and antioxidative activities and appear to play an important role in disease resistance (Chong *et al.*, 2002). In addition to effects on fungi and bacteria, scopoletin accumulation may also be part of a mechanism for virus restriction *in planta*. Exogenously applied scopoletin inhibits *Tobacco mosaic virus* (TMV) replication in tobacco BY2 protoplasts (Chong *et al.*, 2002), and transgenic tobacco plants overaccumulating scopoletin show enhanced resistance

against infection with *Potato virus Y* (PVY) (Matros and Mock, 2004).

Scopoletin may be involved in the regulation of reactive oxygen intermediate (ROI) accumulation, one of the earliest changes underlying hypersensitive cell death (Dangl *et al.*, 1996). Tobacco plants expressing an antisense construct to a coumarin-specific glycosyltransferase exhibited reduced levels of scopolin and scopoletin, and showed more intense ROI accumulation relative to control plants after virus infection (Chong *et al.*, 2002).

Flavonoids

Crude extracts from plants rich in flavonoids have been used for centuries to treat microbial diseases. Different compounds may target different components and functions of the bacterial cell, although individual flavonoids may target multiple sites (Cushnie and Lamb, 2005; Tsuchiya and Iinuma, 2000). The flavonol quercetin (Fig. 1) binds to the GyrB subunit of *Escherichia coli* DNA gyrase and inhibits the enzyme's adenosine triphosphatase (ATPase) activity (Plaper *et al.*, 2003). Quercetin from leaves of *Lotus* has been described as a major antimicrobial compound against five microorganisms, namely *Actinobacillus actinomycetemcomitans*, *Actinomyces viscosus*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Actinomyces naeslundii* (Li and Xu, 2008).

Some flavonoids inhibit bacterial cytoplasmic membrane function. The antibacterial activity of the prenylated flavanone sophoraflavanone G (SFG, Fig. 1) involves the reduction of bacterial membrane fluidity (Tsuchiya and Iinuma, 2000). Catechins (flavan 3-ols, Fig. 1) from tea can damage bacterial membranes by directly penetrating them and disrupting their barrier function. Alternatively, catechins may promote membrane fusion, a process that results in leakage of intramembranous materials and aggregation (Tamba *et al.*, 2007).

Different modifications of flavonoid skeletons have a significant impact on their antimicrobial activity. For example, prenylation generally increases inhibitory activity against bacteria and fungi (Sohn *et al.*, 2004); glycosylation of flavonols from carnation increases their activity against *Fusarium oxysporum* (Galeotti *et al.*, 2008); methoxylation drastically decreases the antibacterial activity of flavonoids (Alcaraz *et al.*, 2000).

Pterocarpans, such as medicarpin from alfalfa (*Medicago sativa*) (Fig. 3), pisatin from pea (*Pisum sativum* L.) (Fig. 1) and maackiain from chickpea (*Cicer arietinum*), constitute a major class of antimicrobial isoflavonoid phytoalexins in legumes. Genetic evidence now supports the hypothesis that pisatin production plays an important role in disease resistance of pea. For instance, pea hairy roots expressing an antisense construct to 6a-hydroxymaackiain *O*-methyltransferase (HMM, the last enzyme in pisatin biosynthesis) exhibit a large reduction in (+)-pisatin content, and are more susceptible than controls to damage caused by the fungus *Nectria haematococca* (Wu and VanEtten, 2004). Furthermore, detoxification of pisatin by the cytochrome P450 enzyme pisatin demethylase (PDA) is a virulence factor for *N. haematococca* in pea (Funnell *et al.*, 2002). *PDA* transcripts are induced in infected pea tissues or by *in vitro* application of pisatin to the fungus, and other genes necessary for pathogenesis on pea display similar temporal patterns to *PDA* gene expression both *in planta* and *in vitro*, consistent with a coordinated regulation of these genes by pisatin during pathogenesis (Liu *et al.*, 2003b). A Zn(2)–cysteine(6) zinc binuclear DNA-binding protein is involved in pisatin-responsive activation of *PDA* gene expression (Khan *et al.*, 2003).

RECENT ADVANCES IN OUR UNDERSTANDING OF PHENYLPROPANOID BIOSYNTHESIS

Lignin and lignans

Lignins are synthesized by the oxidative coupling of hydroxycinnamyl alcohol monomers. Although some researchers have proposed that a variety of units can be incorporated into the lignin polymer, and that lignin assembly is essentially combinatorial, this is still a matter of some controversy (Davin *et al.*, 2008; Ralph *et al.*, 2008).

Monolignols share the general phenylpropanoid biosynthetic pathway with other phenolic compounds, such as anthocyanins, condensed tannins and isoflavones (Fig. 2). This pathway includes the entry-point enzyme L-phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate:CoA ligase (4CL). The specific portion of the monolignol synthesis pathway includes the enzymes hydroxycinnamoyl CoA:shikimate hydroxycinnamoyltransferase (HCT), 4-coumaroylshikimate 3-hydroxylase (C3H), CCR, CAD, COMT, caffeoyl CoA 3-*O*-methyltransferase (CCoAOMT) and ferulic acid 5-hydroxylase (F5H) (Fig. 2). Several reviews have described the monolignol pathway in much detail (Boerjan *et al.*, 2003; Raes *et al.*, 2003). Recent evidence has suggested that simple phenolic acids are derived by reduction of aldehyde intermediates of monolignol biosynthesis; thus, ferulic acid is now believed to originate from the reduction of coniferaldehyde rather than sequential ring modification of cinnamic acid, and sinapic acid may be derived similarly (Nair *et al.*, 2004). Esterification with malate leads to the formation of the sinapate esters found in the seeds and leaves of the Brassicaceae, and recently implicated in the modulation of pathogen reproduction *in planta* (Quentin *et al.*, 2009).

After synthesis, monolignols are transported to the cell wall by mechanisms yet unknown. They then undergo oxidative polymerization, catalysed by peroxidases and/or laccases (Gabaldón *et al.*, 2006; Sato *et al.*, 2006). There are 73 peroxidase genes in the Arabidopsis genome (Tognolli *et al.*, 2002), and laccases are

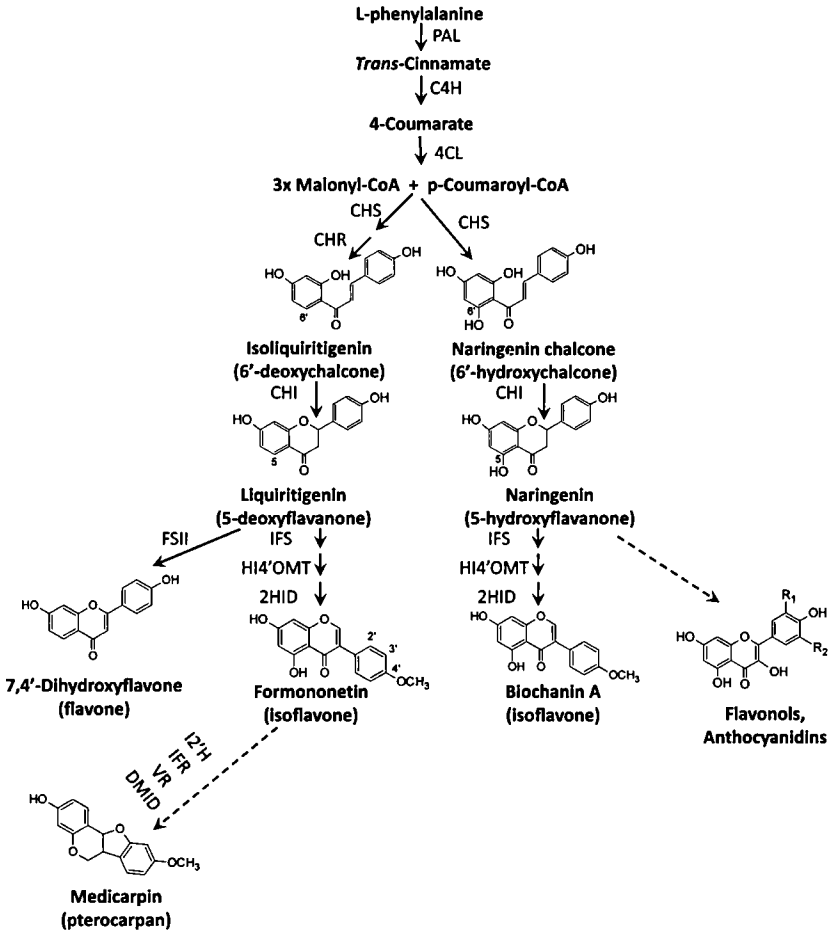


Fig. 3 Biosynthesis of flavonoids and isoflavonoids. The enzymes are as follows: C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; CHR, chalcone reductase; CHS, chalcone synthase; 4CL, 4-coumarate:CoA ligase; DMID, 7,2′-dihydroxy-4′-methoxy-isoflavonol dehydratase; FSII, flavone synthase II; 2HID, 2-hydroxyisoflavanone dehydratase; HI4′OMT, 2-hydroxyisoflavanone 4′-*O*-methyltransferase; IFR, isoflavone reductase; IFS, isoflavone synthase; I2′H, isoflavone 2′-hydroxylase; PAL, L-phenylalanine ammonia-lyase; VR, vestitone reductase.

also encoded by multigene families in plants, making it difficult to identify isoforms that are specifically involved in monolignol oxidation. Some peroxidases can cause ectopic lignification when overexpressed *in planta* (Elfstrand *et al.*, 2002). However, solid genetic evidence is still missing to identify unambiguously the specific isoforms of peroxidases and/or laccases involved in lignification.

Because monolignol biosynthesis is a metabolically costly process and its carbon investment is not reversible, the activation of this pathway must be tightly regulated. Many of the genes encoding lignin biosynthetic enzymes, such as *PAL*, *C4H*, *COMT*, *CCR* and *CAD*, share a conserved motif, known as the AC element, in their promoters that binds lignin pathway regulatory transcription factors (Chen *et al.*, 2000; Hatton *et al.*, 1995; Lauvergeat *et al.*, 2002). A network of transcription factors is responsible for the regulation of secondary wall biosynthesis in Arabidopsis. In this network, two NAC transcription factors, NST1 and NST3, act as a master switch to turn on the entire process of secondary wall biosynthesis, including monolignol biosynthesis (Mitsuda *et al.*, 2007; Zhong *et al.*, 2007, 2008). Two MYB transcription factor genes, *MYB58* and *MYB63*, are directly regulated by NST1 and NST3, and can specifically activate the monolignol biosynthesis pathway (Zhou *et al.*, 2009). Some lignin gene family members are involved in constitutive lignification, whereas other gene family members are only expressed in response to pathogen infection (Lauvergeat *et al.*, 2001). Therefore, it is likely that lignin biosynthesis in vascular tissue development is regulated differentially from that occurring during plant defence responses.

Dimeric lignans result from the stereoselective dirigent protein-directed oxidative coupling by laccases of two coniferyl alcohol units (Davin *et al.*, 1997) (Fig. 2), a process that has been well characterized in flax (*Linum usitatissimum*) (Ford *et al.*, 2001). In recent years, a large number of dirigent protein genes have been identified in various plant species (Kim *et al.*, 2002; Ralph *et al.*, 2006). Many complex substituted lignan structures exist. In one commonly found pathway, dimerization of two coniferyl alcohol units produces pinoresinol, which is then reduced to lariciresinol and secoisolariciresinol by pinoresinol/lariciresinol reductase (PLR), and finally into matairesinol by secoisolariciresinol dehydrogenase (SIRD) (Hano *et al.*, 2006; von Heimendahl *et al.*, 2005) (Fig. 2). Secoisolariciresinol is

mainly found in its diglucoside form (SDG), and is stored as a hydroxymethyl glutaryl ester-linked complex (Ford *et al.*, 2001).

Although the dirigent protein determines the stereoselectivity of the oxidative free radical coupling reaction, the enantiomeric composition of pinoresinol differs in various plants (Umezawa, 2003). However, the fact that some lignans derived from pinoresinol, such as matairesinol, are optically pure indicates that PLR and SIRD, which catalyse subsequent metabolic steps, also contribute to the enantiomeric composition of lignans (von Heimendahl *et al.*, 2005; Nakatsubo *et al.*, 2008). PLR normally reduces both pinoresinol and lariciresinol efficiently (Hemmati *et al.*, 2007; von Heimendahl *et al.*, 2005). However, the two Arabidopsis PLRs show strict substrate preference towards pinoresinol and no activity towards lariciresinol, in contrast with conventional PLRs of other plants (Nakatsubo *et al.*, 2008).

Coumarins

Coumarins share hydroxycinnamoyl CoA intermediates with the monolignol pathway. Recently, the production of scopoletin and scopolin in *Arabidopsis* roots has been shown to require the activity of C3H; in C3H T-DNA insertion mutants, the content of scopoletin and scopolin decreased to around 3% of that in the wild-type (Kai *et al.*, 2006).

The role of glucosyltransferase that catalyses the glucosylation of scopoletin has been demonstrated by both loss- and gain-of-function approaches in transgenic tobacco. The down-regulation of tobacco scopoletin glucosyltransferase (TOGT) activity was accompanied by decreased virus resistance (Chong *et al.*, 2002). Conversely, the overexpression of TOGT increased virus resistance (Matros and Mock, 2004).

Flavonoids

Chalcone synthase (CHS) catalyses the first committed enzyme of flavonoid biosynthesis (Fig. 3). CHSs are type III polyketide synthases (PKSs) with two independent active sites that catalyse a series of decarboxylation, condensation and cyclization reactions (Jez *et al.*, 2002). Structural biology has recently become an important tool for understanding the mechanisms and evolution of enzymes involved in the biosynthesis/modification of phenylpropanoid skeletons (Noel *et al.*, 2005; Ritter and Schulz, 2004; Shao *et al.*, 2005). Guided by the three-dimensional structure of CHS, substitution of the active site phenylalanine-215 (Phe215) by serine (Ser) yielded a mutant CHS that preferentially accepts the CoA-thioester substrate to generate a novel alkaloid, namely *N*-methylanthraniloyltriacetic acid lactone (Jez *et al.*, 2002). Thus, a single point mutation in *CHS* dramatically shifts the molecular selectivity of this enzyme.

5-Deoxy(iso)flavonoids, which are predominantly found in legumes, have strong antimicrobial properties and also act as signals in plant–microbe interactions. Chalcone reductase (CHR) is the critical enzyme for the 5-deoxyflavonoid pathway, reducing an unstable intermediate of the CHS reaction to ultimately form 6′-deoxychalcone (isoliquiritigenin, Fig. 3).

Chalcone isomerases (CHIs) catalyse the stereospecific isomerization of chalcones into their corresponding flavanones (Shimada *et al.*, 2003). Type I CHIs isomerize only 6′-hydroxychalcone to 5-hydroxyflavanone, and type II CHIs, generally found in legumes, act on both 6′-deoxychalcone and 6′-hydroxychalcone, yielding 5-deoxyflavanone and 5-hydroxyflavanone, respectively (Fig. 3).

The conversion of flavanones to isoflavones is catalysed by 'isoflavone synthase' (IFS), a cytochrome P450 enzyme of the CYP93C subfamily. In the first step, IFS catalyses the 2-hydroxylation of flavanone, followed by 1,2-aryl migration to yield a 2-hydroxyisoflavanone. All the P450s of the CYP93 family so far functionally characterized are involved in flavonoid/isoflavonoid biosynthesis. For example, the CYP93Bs flavanone 2-hydroxylase (Akashi *et al.*, 1998b) and flavone synthase II (Martens and Forkmann, 1999) catalyse the hydroxylation at C-2 or introduction of a double bond between C-2 and C-3 of the flavanone substrate, respectively. CYP93A is a soybean pterocarpan 6a-hydroxylase (Schopfer *et al.*, 1998).

To date, there are no crystal structures available for plant membrane-associated cytochrome P450s. However, molecular modelling studies have suggested that two active site residues of licorice IFS, Ser310 and lysine-375 (Lys375), are critical for the aryl migration of the flavanone substrate (Sawada and Ayabe, 2005; Sawada *et al.*, 2002). The Lys375Thr mutant (Thr, threonine) produced only 3-hydroxyflavanone, a byproduct of the IFS reaction. Thus, introduction of Lys375 was a critical event in the development of the unique catalytic function of IFS which was then fixed in the Leguminosae because of the selective advantage of the presence of isoflavonoids.

Pea, alfalfa, licorice and Lotus produce their isoflavonoid phytoalexins from a 4′-methoxyisoflavone, formononetin, whereas soybean synthesizes 4′-hydroxy-type isoflavonoid phytoalexins (glyceollins) from daidzein (Fig. 3). The dehydration (1,2-elimination) step from 2-hydroxyisoflavanone to isoflavone is now known to be enzyme-catalysed rather than a spontaneous process (Akashi *et al.*, 2005; Hakamatsuka *et al.*, 1998). Licorice 2-hydroxyisoflavanone dehydratase (HID) is specific for 2,7-dihydroxy-4′-methoxyisoflavanone, whereas soybean HID has broader specificity for both 4′-hydroxylated and 4′-methoxylated 2-hydroxyisoflavanones, reflecting the structures of the isoflavones in each species. Site-directed mutagenesis studies and phylogenetic analysis revealed that HID proteins evolved from the plant carboxylesterase family (Akashi *et al.*, 2005). HID appears to be critical for isoflavone biosynthesis in hairy root cultures of *Lotus japonicus* (Shimamura *et al.*, 2007). In contrast,

isoflavones are formed in Arabidopsis expressing IFS in the absence of HID (Liu *et al.*, 2002).

Hydroxylation of flavonoid skeletons by NADPH-dependent cytochrome P450 monooxygenases is important in the biosynthesis of complex flavonoids. Hydroxylation at the 2′ or 3′ position of the B-ring is essential for the formation of pterocarpans or coumesterol (Guo *et al.*, 1994; Hinderer *et al.*, 1987). 6a-Hydroxylation of pterocarpans occurs in the biosynthesis of the glyceollins in soybean and of pisatin in pea. The first isoflavone 2′-hydroxylase (I2′H; CYP81E1) was cloned and characterized from licorice (Akashi *et al.*, 1998a). Subsequently, three CYP81E subfamily members were identified in *Medicago truncatula* by mining of EST databases (Liu *et al.*, 2003a). Two of the corresponding enzymes, when expressed in yeast, utilized the same isoflavone substrates, but produced different products hydroxylated at the 2′ and/or 3′ positions of the B-ring. Unique tissue-specific expression patterns suggested the involvement of these enzymes in different defence processes in Medicago (Liu *et al.*, 2003a).

Isoflavone reductase (IFR) is a key enzyme in the latter part of the pterocarpan phytoalexin biosynthetic pathway (Paiva *et al.*, 1991). In species producing medicarpin, it catalyses the NADPH-dependent reduction of 2′-hydroxyformononetin to the isoflavanone (3*R*)-vestitone. An engineered IFR lacking residues 39–47, which constitute a flexible region, was crystallized, and the structure of Δ39–47 IFR was shown to comprise two domains (N- and C-terminal) which form a cleft in which both NADPH and 2′-hydroxyformononetin are presumed to bind (Wang *et al.*, 2006). The study provides a structural basis for understanding the enzymatic mechanism and substrate specificity of IFRs, as well as the functions of IFR-like proteins (Wang *et al.*, 2006).

Enzymatic *O*-methylation of (iso)flavonoids is catalysed by *O*-methyltransferases (OMTs), which transfer a methyl group from *S*-adenosyl-L-methionine (SAM) to a hydroxyl moiety of the acceptor molecule. Two different groups of small-molecule OMTs can be distinguished from plants: proteins of group I do not require a metal ion for activity, whereas OMTs of group II are Mg^{2+} dependent (Noel *et al.*, 2003). 4′-*O*-Methylation of the isoflavonoid B-ring by a SAM-dependent 2-hydroxyisoflavanone 4′-*O*-methyltransferase (HI4′OMT) is a critical step at the entry point into the formation of isoflavonoid phytoalexins in pea, alfalfa, *M. truncatula* and chickpea (Akashi *et al.*, 2003; Dixon, 1999). However, among these four species, only pea catalyses A-ring 3-*O*-methylation that converts the pterocarpan 6a-hydroxymaackiain to pisatin (Fig. 1). The HI4′OMT from *M. truncatula* shares 90% amino acid sequence identity with (+)-6a-hydroxymaackiain 3-*O*-methyltransferase from pea, which is responsible for the final step in the biosynthesis of (+)-pisatin. Although (+)-6a-hydroxymaackiain is not synthesized in *M. truncatula*, cell suspension cultures accumulate pisatin when fed with 6a-hydroxymaackiain (Liu *et al.*, 2006). Recombinant HI4′OMT of *M. truncatula* shows comparable activity towards both 2,7,4′-trihydroxyisoflavanone and the unnatural substrate (+)-6a-hydroxymaackiain. Protein X-ray crystal structures of HI4′OMT substrate complexes revealed similar bound conformations for the seemingly distinct chemical substrates, explaining how leguminous plants can use homologous enzymes for two different biosynthetic reactions (Liu *et al.*, 2006).

Vestitone reductase (VR) is the central enzyme for the conversion of nonenantiomeric isoflavones to enantiomeric pterocarpans. It is a member of the short-chain dehydrogenase/reductase (SDR) superfamily and catalyses the NADPH-dependent reduction of (3*R*)-vestitone in the biosynthesis of medicarpin. It acts with high stereochemical specificity, and recognizes only the 3*R* conformation of vestitone, not (3*S*)-vestitone (Shao *et al.*, 2007). A VR homologue, sophorol reductase (SOR), was identified for the biosynthesis of pisatin in pea. SOR prefers (–)-sophorol (Fig. 1) over (+)-sophorol as substrate, converting it to (–)-7,2′-dihydroxy-4′,5′-methylenedioxyisoflavanol for the production of (+)-pisatin (DiCenzo and VanEtten, 2006). Homologues of VR and SOR have recently been described in additional species, including soybean (*Glycine max*) (Liu, 2009). The three final steps in pisatin biosynthesis are catalysed by SOR, pterocarpan 6a-hydroxylase and (+)-6a-hydroxymaackiain-3-*O*-methyltransferase (HMM). The catalytic specificity of two genetically linked HMMs in pea suggested gene duplication for (+)-pisatin biosynthesis (Akashi *et al.*, 2006).

The prenylation of flavonoids enhances their antibacterial, antifungal and other biological activities by increasing their lipophilicity and membrane permeability (Sohn *et al.*, 2004). Recently, Sasaki and colleagues isolated the first cDNA of a flavonoid-specific plant prenyltransferase, SfN8DT-1, involved in SFG (Fig. 1) biosynthesis in *Sophora flavescens* (Sasaki *et al.*, 2008). Phylogenetic analysis revealed that the naringenin dimethylallyltransferase SfN8DT-1 has the same evolutionary origin as prenyltransferases for vitamin E and plastoquinone biosynthesis (Sasaki *et al.*, 2008). This discovery facilitated the subsequent identification of the pterocarpan 4-dimethylallyltransferase (G4DT) catalysing the critical prenylation step in glyceollin biosynthesis in soybean (Akashi *et al.*, 2009).

Family 1 uridine diphosphate (UDP) glycosyltransferases (UGTs) transfer sugars to a wide range of acceptors. Plants contain very large *UGT* gene families: over 100 putative *UGT* genes have been identified in Arabidopsis and more than 300 in *M. truncatula*. Significant progress has been made during recent years in the isolation and structural characterization of (iso)flavonoid UGTs from legume species (He *et al.*, 2006; Modolo *et al.*, 2007; Noguchi *et al.*, 2007; Shao *et al.*, 2005). Most of the identified UGTs to date prefer UDP-glucose as sugar donor. Relationships between the structure and function in *M. truncatula*

(iso)flavonoid UGTs have been explored by X-ray crystallography and site-directed mutagenesis (He *et al.*, 2006; Li *et al.*, 2007; Shao *et al.*, 2005).

Genome-wide analysis of phenylpropanoid metabolism

The last decade has seen remarkable advances in the structural analysis of model plant genomes. Post-sequence, functional genomics approaches are increasingly being applied to the discovery of new gene functions, and comprehensive web-based software tools have become available for biologists to explore gene expression across a wide variety of biological contexts. Reference expression databases for the meta-analysis of transcriptomes, such as Expression Profiler (http://www.ebi.ac.uk/expressionprofiler) or the *Medicago truncatula* Gene Expression Atlas (http://bioinfo.noble.org/gene-atlas/v2), have provided new tools for the analysis of the expression of genes in many different contexts, for the clustering of genes into expression modules, and for the modelling of expression responses in the context of metabolic and regulatory networks.

Because of the exponential increase in plant genome information since the publication of our previous review (Dixon *et al.*, 2002), it is no longer possible to summarize this field in hard copy text and tables. We have therefore created a database of the genes involved in the biosynthesis of phenylpropanoid natural products from 11 plant species with fully or near-fully sequenced genomes; the moss *Physcomitrella patens*, the spike moss *Selaginella moellendorffii*, three monocots (*Oryza sativa*, *Sorghum bicolor*, *Zea mays*) and six dicots (*Arabidopsis thaliana*, *Populus trichocarpa*, *Vitus vinifera*, *Glycine max*, *Medicago truncatula* and *Lotus japonicus*). For gene annotation, proteins predicted from genome sequences were searched by BLASTX against the uniprot trembl database (http://www.ebi.ac.uk/uniprot/) (e-value < 1e-04), and the top five hits with meaningful annotations were retrieved as annotation. Genes were sorted as representing proteins involved in the various branches of the phenylpropanoid pathway, including flavonoid, isoflavonoid, lignin, etc., based on predicted function. The database is available online (http://bioinfo.noble.org/manuscript-support/mpp/); the information includes the source of genome sequences, enzyme name, gene identification, redundancy, Affymetrix probeset ID, location on chromosome (if possible), length of sequence, five top hit annotations and the sequence itself. It is critical to note that this analysis uses informatics approaches to predict function; annotations should be viewed as tentative unless true biochemical functions have been ascribed to a particular gene. For example, many genes are now being given '-like' annotations, such as CCR-like or IFR-like, highlighting the fact that they have sequence identity to functionally characterized genes, but that their protein products have not been shown to catalyse the predicted reaction. Gene products with proven function are indicated in the database and, where appropriate, in the following figures in the text.

Genomic evidence for the evolution of flavonoids as protective compounds

To illustrate how genomic data can be mined to reveal details of phylogenetics, chromosomal organization and expression patterns of relevance to plant defence, we have chosen the flavonoid pathway as an example. It is believed that flavonoids initially evolved as protective compounds against UV irradiation (Koes *et al.*, 1994; Markham, 1988; Shirley, 1996), or as hormone regulators (Stafford, 1991). During evolution, plants adopted flavonoids for many different functions, including defence against pathogens.

Mosses are one of the oldest plant groups that produce flavonoids, including chalcones, flavonols and flavones (Fig. 1) (Markham, 1988). Flowering plants extended the array of flavonoids, also producing aurones, anthocyanidins, proanthocyanidins and isoflavonoids (the latter mainly in legume species). *CHS*, encoding the initial enzyme of the pathway, exhibits strong sequence similarity to bacterial genes encoding fatty acid PKSs. PKSs are classified into three types according to their architectural configurations (Fischbach and Walsh, 2006); type III (which includes CHS-related enzymes) is present in bacteria, plants and fungi (Austin and Noel, 2003; Funa *et al.*, 2007; Seshime *et al.*, 2005).

Gene duplication followed by sequence divergence has been used as a model to explain how novel biochemical functions have arisen from CHSs. Multiple copies of *CHS*-related genes are found in many species, including the 11 analysed in the present work (Fig. 4). In general, the numbers of *CHS*-related genes in the genomes correlate with total genome size (Fig. 4A,B). However, soybean, with double the genome size of *M. truncatula*, rice and sorghum, contains fewer *CHS*-related genes (Fig. 4A,B). *CHS*-related genes are scattered throughout the genomes; however, they form clusters of up to five to seven genes in rice, *M. truncatula* and soybean that may reflect ongoing duplication events (Fig. 4C). The *Zea mays* genome (the largest genome analysed in this work) is not fully sequenced, and so we do not yet know how many *CHS*-related genes exist.

Four residues, cysteine-164 (Cys164), Phe215, histidine-303 (His303) and asparagine-336 (Asn336), are highly conserved in CHS proteins (Fig. S1, see Supporting Information; conserved residues are highlighted by blue rectangles) and define the active site (Ferrer *et al.*, 1999). Stilbene synthases (STSs) are among the closest related enzymes to CHSs. They likewise condense one coumaroyl- and three malonyl-CoA molecules, but the reaction involves a decarboxylation leading to the formation of resveratrol. STS shows high amino acid sequence identity to

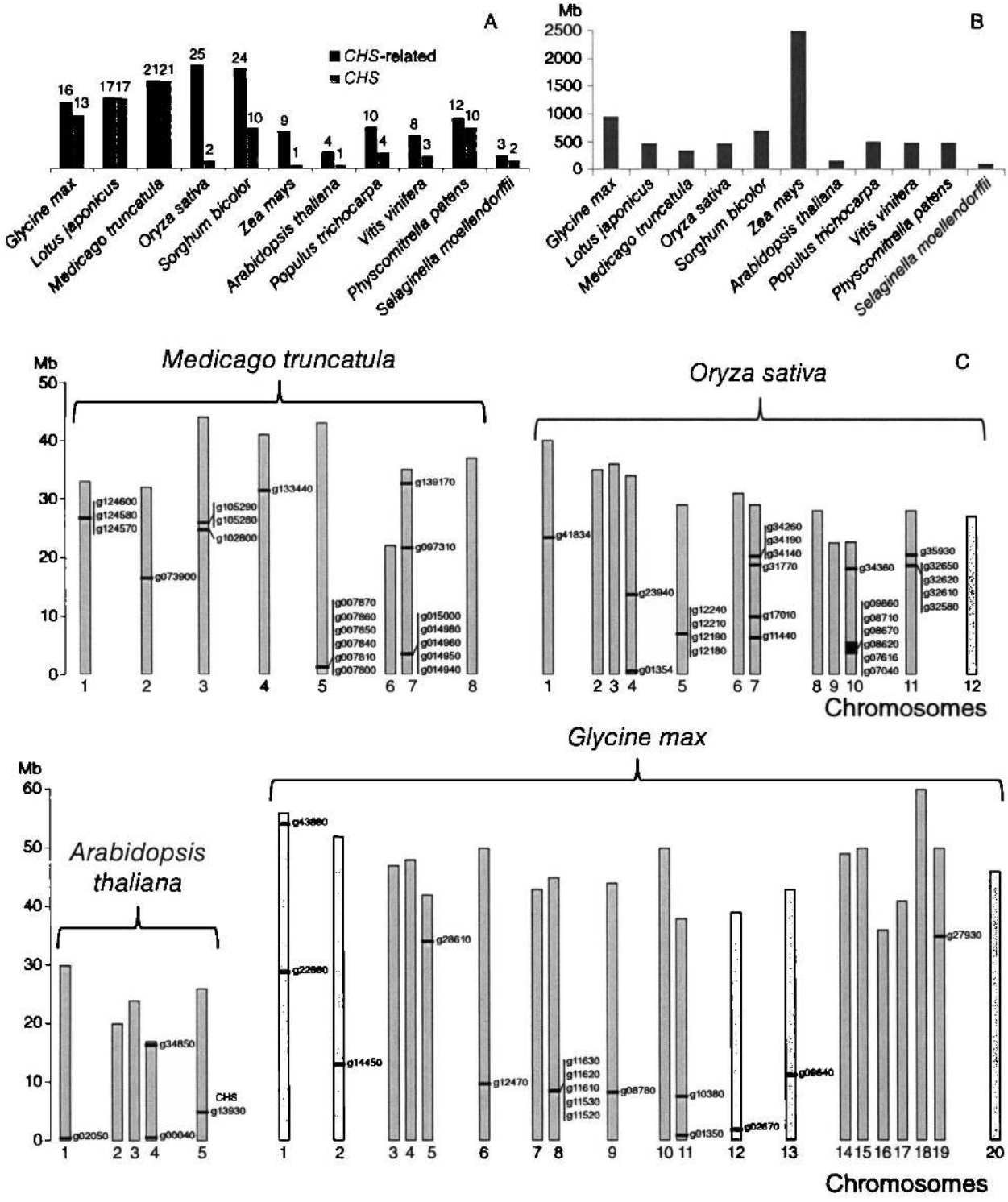
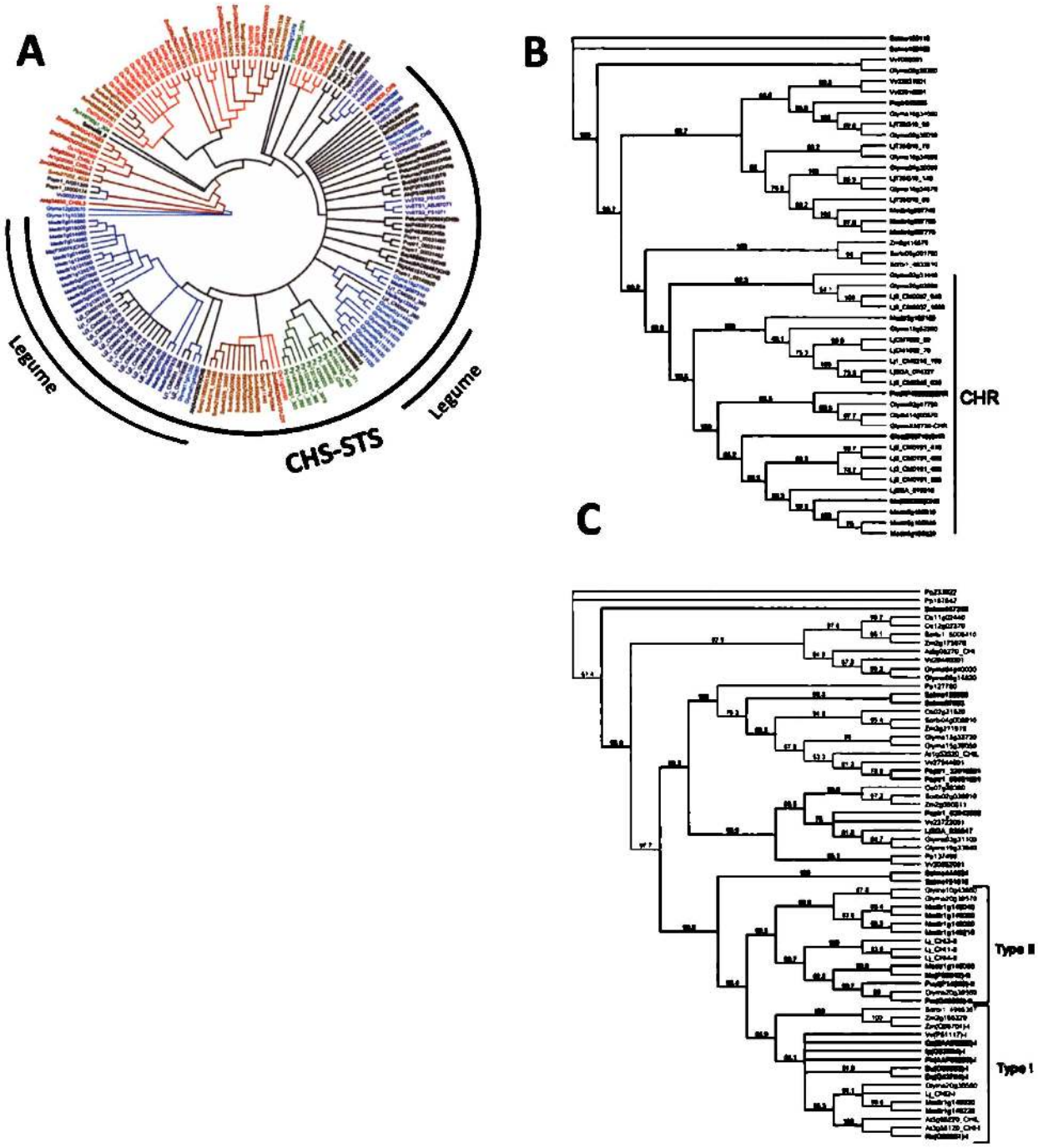


Fig. 4 Distribution of chalcone synthase *(CHS)*-related genes in multiple sequenced genomes. (A) The number of *CHS*-related and true *CHS* genes in the genomes of 11 species. (B) Size of the genomes of the 11 species in (A). (C) Map positions of *CHS*-related genes on chromosomes of *Medicago truncatula*, *Oryza sativa*, *Arabidopsis thaliana* and *Glycine max*.



CHS; however, it differs in several critical amino acids, such as aspartic acid-255 (Asp255), His266 and leucine-268 (Leu268) in the β1d–β2d region of the CoA-binding tunnel (Ferrer *et al.*, 1999) (see alignment in Fig. S1; critical amino acids highlighted by red rectangles and STSs by yellow rectangles). It has been suggested that *STS*s evolved from *CHS*s several times independently, as they group phylogenetically with *CHS*s from the same or related plants (Tropf *et al.*, 1994). Our analyses indicate that *STS* distributes into the *CHS* cluster, but as a separate subcluster (Fig. 5A).

Cloning of *CHS* from the moss *P. patens* revealed an important transition from the *CHS*s present in microorganisms to those present in higher plants (Jiang *et al.*, 2006). Of the 12 *CHS*-related genes found in the *P. patens* genome, 10 are distributed

Fig. 5 Phylogenetic trees of CHS-related genes (A), aldo-keto reductases (B) and chalcone isomerases (C). Deduced amino acid sequences were aligned using the L-INS-i method (Katoh and Toh, 2008) (the MAFFT program is available online at http://align.bmr.kyushu-u.ac.jp/mafft/software/) and subsequently inspected by eye. Neighbour-joining analyses were performed on the aligned sequences using Geneious v4.7 software (Drummond *et al.*, 2009). The bootstrap replicates were 1000 (support thresholds greater than 50% are given at the nodes). Abbreviations: CHI, chalcone isomerase; CHIL, chalcone isomerase-like; CHR, chalcone reductase; CHS, chalcone synthase; CHSL, chalcone synthase-like; STS, stilbene synthase. Genes encoding enzymes from 11 species are colour-coded as follows: *Physcomitrella patens* (Pp), light green; *Selaginella moellendorffii* (Selmo), dark green; *Populus trichocarpa* (Poptr), grey; *Arabidopsis thaliana* (At), maroon; *Vitis vinifera* (Vv), dark violet; *Medicago truncatula* (Medtr), blue; *Lotus japonicus* (Lj), cobalt; *Glycine max* (Glyma), light blue; *Oryza sativa* (Os), red; *Sorghum bicolor* (Sorbi), yellow ochre; *Zea mays* (Zm), orange. Bootstrap values for the nodes of the *CHS*-related gene tree (A) are provided in Fig. 2 (see Supporting Information). Protein sequences from the 11 species are available online (http://bioinfo.noble.org/manuscript-support/mpp/). Gene names from additional different species are colour-coded in black. Accession numbers for genes encoding enzymes from additional species: for CHSs, *Gerbera hybrida* cultivar, GerberaCHS1 (P48390), GerberaCHS3 (P48392); *Ipomoea purpurea*, IpCHSA (P48397), IpCHSB (P48398), IpCHSE (O22047); *Petunia hybrida*, PetuniaCHSB (P22924), PetuniaCHSD (P22925), PetuniaCHSG (P22927); *Arachis hypogaea*, AhCHS (AAO32821), AhSTS1 (P20178), AhSTS3 (P51069); *Bauhinia variegate*, BvSTS (ABF59517); *Medicago sativa*, MsCHS2 (P30074); *Cannabis sativa*, CsCHS (AAL92879); *Rosa hybrida* cultivar, RosaCHS (BAC66467); *Rubus idaeus*, RiCHS (AAK15174); *Sorbus aucuparia*, SaCHS (ABB89213); for STSs, *Vitis vinifera*, VvSTS1 (ABJ97071), VvSTS2 (P51070), VvSTS3 (P51071); for CHRs, *Glycyrrhiza echinata*, GlecCHR (D83718); *Medicago sativa*, MsCHR (X82368); *Pueraria montana* var. *lobata* PmCHR (AF462632); for CHIs type I, *Zea mays* (Q08704); *Citrus sinensis* (BAA36552); *Dianthus caryophyllus* (Q43754); *Elaeagnus umbellate* (O65333); *Ipomoea purpurea* (O22604); *Petunia hybrida* (AAF60296); *Raphanus sativus* (O22651); for CHIs type II, *Medicago sativa* (P28012); *Pueraria montana* var. *lobata* (Q43056); *Phaseolus vulgaris* (P14298).

into the true *CHS* cluster, but form a separate subcluster with the *CHS*-related genes from *Selaginella moellendorffii* (Fig. 5A, moss CHSs coloured green). Moss CHSs showed more than 60% amino acid identity to CHSs from higher plants, although one of the critical amino acids in the CoA-binding tunnel, Leu268, is substituted with methionine, which probably does not affect the enzyme performance (Fig. S1). Mosses typically exhibit a simple flavonoid profile, and the physiological significance of the presence of multiple CHSs in moss requires further investigation.

Although the rice genome contains the largest number of *CHS*-related genes, only two encode true CHSs (Fig. 5A). With a few exceptions, *CHS*s from mosses, monocots (rice, sorghum and maize) and legumes form separate subclusters (Fig. 5A; Fig. S2, see Supporting Information). Importantly, there has been a notable expansion of *CHS* genes in the legumes: of 17 *CHS*-related genes, 13 are true *CHS*s in soybean, and only true *CHS*s are present in the genomes of *M. truncatula* and *L. japonicus* (Fig. 4A). The presence of multiple copies of *CHS* in separate clusters reflects massive duplication events after speciation, indicative of selective evolutionary pressure in favour of flavonoids in legume species.

CHR originated from the aldo/keto reductases (Bomati *et al.*, 2005). Sixteen *CHR* genes are found in *L. japonicus*, seven in *M. truncatula* and 11 in soybean (see database online). *CHR*-like genes are also found in the genomes of nonlegumes, but their functions in these species are obscure. Phylogenetic analysis of putative CHRs with functionally characterized enzymes clearly places only legume enzymes in the true CHR cluster (Fig. 5B).

CHI is also encoded by a multigene family which forms gene clusters in some legume species. For example, a cluster of genes encoding type I and II CHIs is found in *L. japonicus* (Shimada *et al.*, 2003); a cluster of seven genes encoding both types of CHI is observed at the same locus on chromosome 1 of *M. truncatula*. In general, more *CHI* genes are present in the genomes of legumes than nonlegumes (see database online), probably as a result of the occurrence of type II *CHI*s in legumes (Fig. 5C). It has been suggested that type II *CHI*s evolved from an ancestral *CHI* by gene duplication, and the resultant production of 5-deoxyflavonoids paralleled the establishment of the Fabaceae (Shimada *et al.*, 2003).

Three later enzymes of flavonoid biosynthesis, flavanone 3β-hydroxylase (F3βH), flavonol synthase (FLS) and anthocyanidin synthase (ANS), are all members of the family of 2-oxoglutarate-iron-dependent oxygenases. They are closely related by sequence and all catalyse the oxidation of the flavonoid 'C-ring'; these enzymes also exhibit overlapping substrate and product specificity (Turnbull *et al.*, 2004), making annotation of their genes confusing. In our database, we placed these genes in the flavonoid 2-oxoglutarate-dependent dioxygenase group, with putative annotation of some of them based on phylogenetic analysis with functionally characterized enzymes [see database and Fig. S3 (Supporting Information)].

Genes encoding the entry enzyme into isoflavonoid biosynthesis, IFS, are present in low copy number in legume genomes (see database online); furthermore, in Medicago, only two of the five are true *IFS* genes (Fig. 6). Isoflavonoid OMTs are more abundant: 17, 11 and three *IOMT* genes were identified in the genomes of soybean, Medicago and Lotus, respectively (see database online). A previous phylogenetic analysis (Deavours *et al.*, 2006) divided the *M. truncatula IOMT*s into two distinct clades, with homology to either I7OMT enzymes, methylating the A-ring 7-hydroxyl of the isoflavone daidzein, or 4'OMT enzymes, methylating the B-ring 4'-hydroxyl of 2,7,4'-trihydroxyisoflavanone.

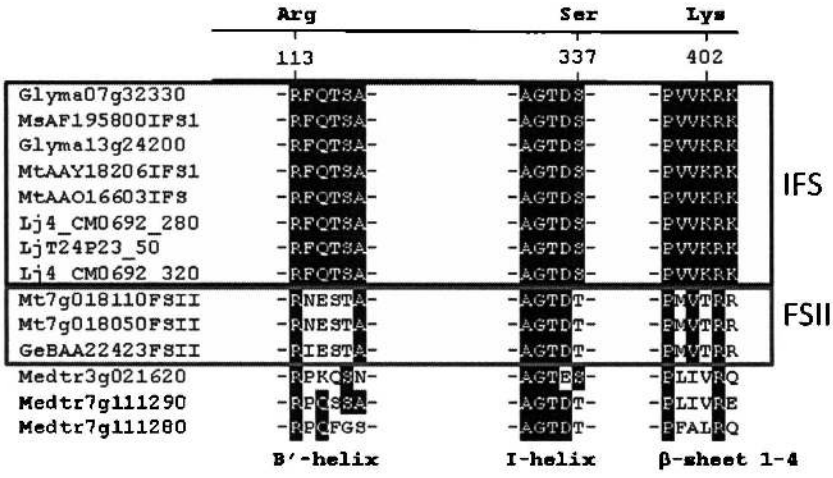


Fig. 6 Putative positions of the substrate recognition sites of CYP93 family enzymes in soybean, Medicago and Lotus. The figure was generated based on the study of Sawada *et al.*, 2002. FSII, flavone synthase II; IFS, isoflavone synthase.

Expression profiling of flavonoid defence responses

To evaluate the involvement of flavonoid pathway genes in defence responses, we downloaded Affymetrix microarray data specifically related to stress or pathogen studies. At present, Affymetrix microarray chips have been developed for 12 plant species: Arabidopsis, barley, citrus, cotton, grape, maize, Medicago, poplar, rice, soybean, tomato and wheat (http://www.affymetrix.com/). Numerous microarray experiments are available for Arabidopsis, rice, Medicago and soybean, and we therefore selected these species for further analysis. Microarray data for rice and soybean were obtained from Expression Profiler (EP, http://www.ebi.ac.uk/expressionprofiler), a web-based platform for microarray gene expression and other functional genomics-related data analysis (Kapushesky *et al.*, 2004). Arabidopsis and Medicago expression data were obtained from AtGenExpress (http://www.weigelworld.org/resources/microarray/AtGenExpress/) and Gene Expression Atlas database version 2 (MtGEAv2, http://bioinfo.noble.org/gene-atlas/v2/), respectively. Affymetrix probesets were mapped to the corresponding genes at individual Affymetrix probe level, using a position-weighted scoring index in which mismatches near the middle of a probe were most heavily penalized as follows: [1,1,1,1,1,2,2,2,2,2,3,3,3,3,3,2,2,2,2,2,1,1,1,1,1] for each 25-mer Affymetrix probe. Therefore, a perfect match for a 25-mer probe yields a score of 45. Matches were declared when at least eight of 11 total probe sets had scores of 43 or higher for each gene (see database online). For better visualization of differential gene expression in response to pathogen or stress, treatment/control fold-change values were calculated. The fold-change values were converted into a log2 scale (see database online) and data were subjected to clustering analysis.

As summarized in Fig. 7 and Figs S4 and S5 (see Supporting Information), different numbers of flavonoid pathway genes responded to pathogen or elicitor treatment in each species. In Arabidopsis, putative *ANS* (At2g38240) was highly induced by *Pseudomonas* and *Phytophthora* treatments, whereas expression of functionally characterized *CHS* (At5g13930; Shirley *et al.*, 1995) was reduced in most experiments (Fig. 7A). The weak response of flavonoid pathway genes to infection in Arabidopsis is consistent with the deployment of indole (i.e. nonflavonoid) phytoalexins in this species (Rogers *et al.*, 1996). In rice, *F3βH* (Os04g49194) was highly expressed in response to infection with *Xanthomonas oryzae* pv. *oryzicola*, and a cluster of genes, including *IFR-Likes*, *DFRs* and some *CHS-Likes*, was expressed in response to infection by the blast fungus *Magnaporthe oryzae*; *CHS* (Os11g32650) was only slightly induced under these conditions (two-fold change) (Fig. S4).

In soybean, eight of the 10 available *CHS* genes were induced either in response to cyst nematode infection (experiment accession: E-MEXP-808) or to Asian soybean rust (Fig. S5; accession: E-TABM-230) (van de Mortel *et al.*, 2007). Many genes involved in isoflavonoid biosynthesis in soybean, including three *CHRs*, *CHIs*, two *I2'Hs*, seven *IFRs*, two *IFSs* and eight *IOMTs*, were induced (Fig. S5). Notably, the expression patterns of at least seven *CHSs* tightly correlated with those of isoflavone biosynthetic genes (*P* value less than 0.05, bottom subcluster in the dendrogram highlighted by the red rectangle in Fig. S5), suggesting their neofunctionalization via *cis*-regulatory evolution into isoflavonoid biosynthesis. It should be noted, however, that a similar set of flavonoid genes is activated in the susceptible soybean genotype EM (Embrapa-48) and in the Rpp2-resistant genotype PI (PI230970) in response to fungal infection, suggesting the involvement of flavonoids in basal soybean defences (Fig. S5).

As in soybean, many flavonoid genes are induced in Medicago by fungal infection (*Phymatotrichopsis omnivora*) or elicitors [methyl jasmonate, which mimics wound signals, and yeast elicitor (YE), which mimics pathogen signals] (Fig. 7B). In contrast, only two of this set of genes, *IFS-Like* (Medtr7g111290) and isoflavone 3'-hydroxylase (*I3'H*, GB 33521519, CYP81E9; Liu

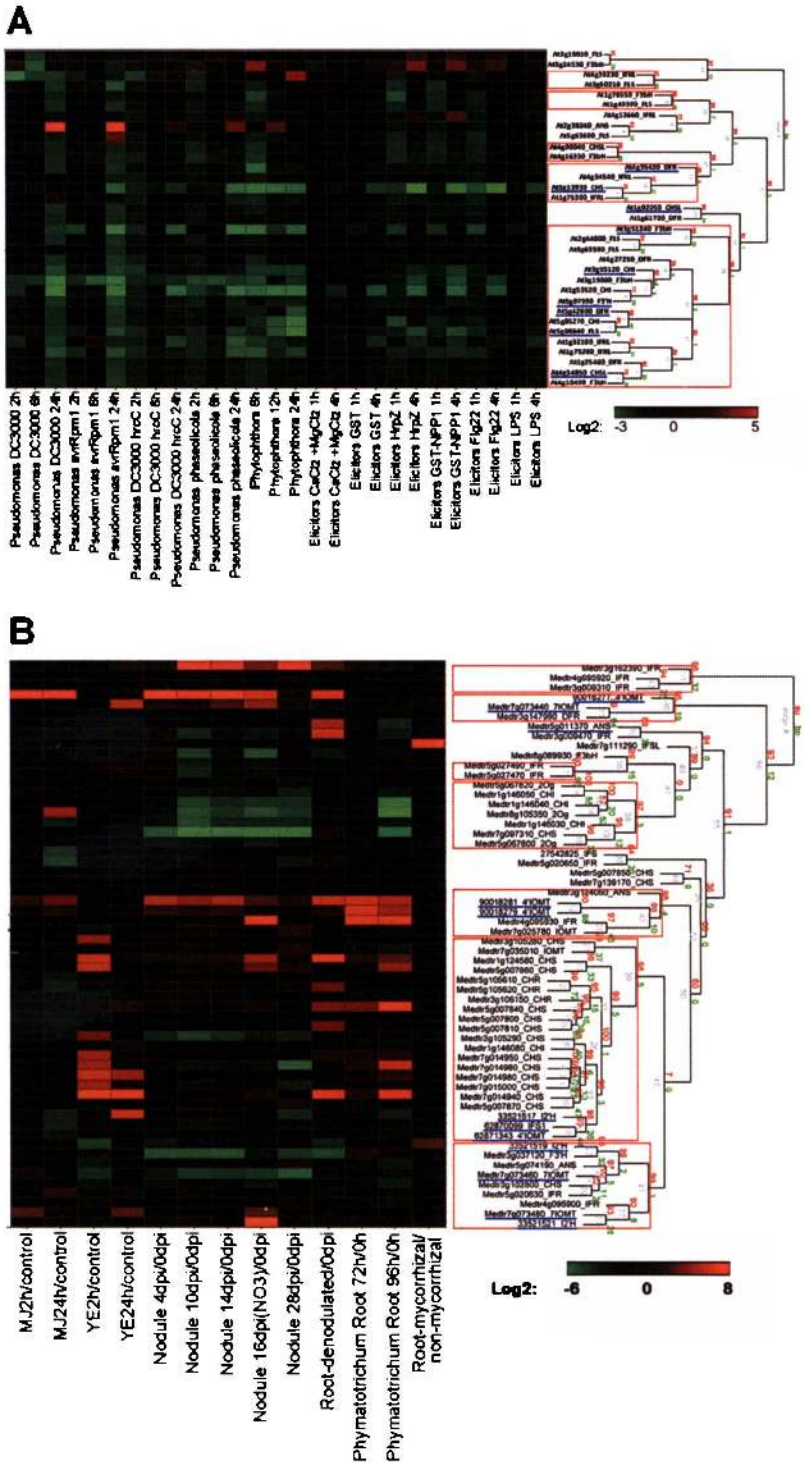


Fig. 7 Hierarchical clustering analysis of expression patterns of flavonoid biosynthesis genes in microarray experiments involving exposure of plants to pathogens or pathogen-derived elicitors. (A) *Arabidopsis thaliana*; (B) *Medicago truncatula*. Microarray data were obtained for *A. thaliana* at AtGenExpress (http://www.weigelworld.org/resources/microarray/AtGenExpress/) and for *M. truncatula* from MtGEAv2 (http://bioinfo.noble.org/gene-atlas/v2/). Treatment/control fold-change values were calculated, converted into a log2 scale (see website online) and subjected to hierarchical cluster analysis (pvclust; Suzuki and Shimodaira, 2006). Clusters with approximately unbiased (AU) *P* value probability of more than 95% are indicated by the red rectangles. Functionally characterized genes are underlined in blue; their National Center for Biotechnology Information (NCBI) gene accession numbers and primary literature citations are available at http://bioinfo.noble.org/manuscript-support/mpp/.

et al., 2003a), were induced in response to colonization by a symbiotic mycorrhizal fungus. Twelve of the 17 available Medicago *CHS* genes were induced by either *Phymatotrichopsis omnivora* or YE (Fig. 7B). Expansion of the legume *CHS* gene family is directly related to the functional diversity of flavonoids, in this case the evolution of isoflavonoids as protective phytoalexins. Individual *CHS* genes in legumes often exhibit differential expression patterns that can be correlated with the types of flavonoid compounds being synthesized (Dixon and Paiva, 1995).

Future perspectives

Functional genomics approaches are powerful tools to facilitate the understanding of secondary metabolism in plants. Enzymes involved in the same function will be temporally and spatially coexpressed, and clustering analysis of transcript profiles, as described above, can be used as a discovery tool to define gene networks associated with plant defence responses. Metabolomics is fast developing as a powerful tool for the analysis of gene function and natural genetic variation (Dixon *et al.*, 2006; Fridman and Pichersky, 2005; Morrell *et al.*, 2006). Combining transcript and metabolite profiles (either global or targeted) is even more informative, and is becoming an important new approach for the identification of candidate genes in complex plant secondary metabolic pathways (Saito *et al.*, 2008; Shulaev *et al.*, 2008; Yonekura-Sakakibara *et al.*, 2007, 2008).

Genes encoding enzymes functioning in secondary metabolism are generally more divergent than those involved in primary metabolism. Although genes for most metabolic pathways in plants are not organized into gene clusters, a small but increasing number of 'operon-like' gene clusters have been identified for the synthesis of plant defence compounds (Field and Osbourn, 2008; Frey *et al.*, 1997; Qi *et al.*, 2004; Shimura *et al.*, 2007), although to date these are mainly in the area of terpenoid metabolism in monocots (oats and rice), and have yet to be seen for consecutive functional enzymes of flavonoid biosynthesis. Investigation of the genomic organization of metabolic pathways may, nevertheless, be a useful additional approach to ascribe potential function to candidate pathway genes, as well as to shed further light on the evolution of chemical diversification in plants.

As shown by the above examples, genomic approaches have huge potential to aid our understanding of how plants have elaborated various chemical defence mechanisms against pathogen attack during evolution. They do not, however, prove causality, which will still require careful biochemical, genetic and physiological analysis of the plant–pathogen interaction. It is a sobering thought that the almost exponential increase in genomic information since our earlier review is likely to accelerate over the next 5–10 years, and data handling and visualization may then become the major challenges to the biologist. The effort will be worth it, however, as being able to visualize plant–microbe interactions at the level of the whole genomes (and metabolomes) of both organisms will open up unprecedented opportunities to understand metabolic and regulatory cross-talk and the evolution of defence mechanisms, and to develop novel antimicrobial strategies.

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Fig. S1 Alignment of the active site defined by Ferrer *et al.* (1999) of chalcone synthase (*CHS*)-related enzymes from 11 species.

Fig. S2 Detailed phylogenetic tree of chalcone synthase (*CHS*)-related genes.

Fig. S3 Phylogenetic analysis of flavonoid 2-oxoglutarate-iron-dependent oxygenases.

Fig. S4 Hierarchical clustering analysis of expression patterns of **flavonoid biosynthesis genes in microarray experiments involv**ing exposure of rice to pathogens.

Fig. S5 Hierarchical clustering analysis of expression patterns of **flavonoid biosynthesis genes in microarray experiments involv**ing exposure of soybean to pathogens.

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