

# Natural products and plant disease resistance

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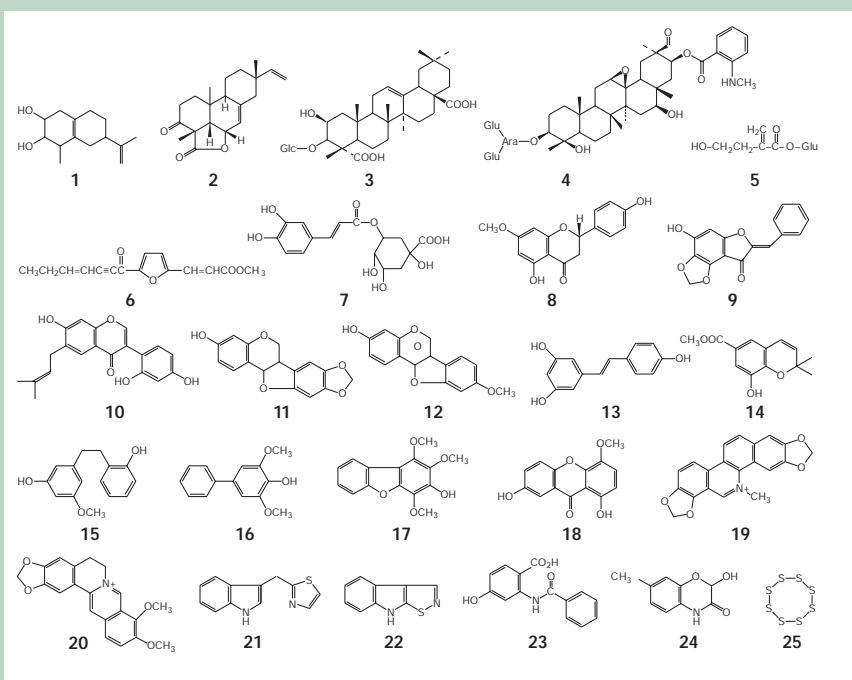
Plants elaborate a vast array of natural products, many of which have evolved to confer selective advantage against microbial attack. Recent advances in molecular technology, aided by the enormous power of large-scale genomics initiatives, are leading to a more complete understanding of the enzymatic machinery that underlies the often complex pathways of plant natural product biosynthesis. Meanwhile, genetic and reverse genetic approaches are providing evidence for the importance of natural products in host defence. Metabolic engineering of natural product pathways is now a feasible strategy for enhancement of plant disease resistance.

Collectively, plants produce a remarkably diverse array of over 100,000 low-molecular-mass natural products, also known as secondary metabolites. Secondary metabolites are distinct from the components of intermediary (primary) metabolism in that they are generally non-essential for the basic metabolic processes of the plant. Most are derived from the isoprenoid, phenylpropanoid, alkaloid or fatty acid/polyketide pathways (Fig. 1). This rich diversity results in part from an evolutionary process driven by selection for acquisition of improved defence against microbial attack or insect/animal predation. However, such diversity has made it difficult to apply conventional molecular and genetic techniques to address the functions of natural products in plant defence,

or to improve plant disease resistance using metabolic pathway engineering.

Related plant families generally make use of related chemical structures for defence (for example, isoflavonoids in the Leguminosae, sesquiterpenes in the Solanaceae), although some chemical classes are used for defensive functions across taxa (for example, phenylpropanoid derivatives). Some species produce a broad range of antimicrobial compounds. For example, cocoa, when infected by the vascular wilt fungus *Verticillium dahliae*, accumulates the pentacyclic triterpene arjunolic acid, two hydroxylated acetophenones and, most unusually, elemental sulphur (Fig. 1, 25), the only known inorganic antimicrobial agent produced by plants<sup>1</sup>. Most antimicrobial plant natural products have relatively broad-spectrum activity, and specificity is often determined by

**Figure 1** Chemical diversity of constitutive and inducible antimicrobial plant natural products. The chemical class of the compound is followed in parentheses by the trivial name (if available), a selected species of origin, and an indication as to whether the compound is produced constitutively (C) or is inducible (I). Terpenoids: **1**, sesquiterpene (rishitin, *Nicotiana tabacum*, I); **2**, diterpene (momilactone A, *Oryza sativa*, I); **3**, saponin (medicagenic acid 3-*O*-glucoside, *Dolichos killimandscharicus*, C); **4**, saponin (avenacin A, *Avena sativa*, C). Aliphatic acid derivatives: **5**, butyrolactone precursor (tuliposide A, *Tulipa* spp., C); **6**, furanoacetylene (wyerone, *Vicia faba*, I). Phenolics and phenylpropanoids: **7**, hydroxycinnamic acid ester (chlorogenic acid, *Nicotiana tabacum*, C); **8**, flavanone (sakuranetin, *Ribes nigrum*, C; *Oryza sativa*, I); **9**, aurone (*Cephalocereus senilis*, I); **10**,



isoflavone (luteone, *Lupinus albus*, C); **11**, pterocarpan (maackiain, *Cicer arietinum*, I); **12**, pterocarpan (medicarpin, *Medicago sativa*, I); **13**, stilbene (resveratrol, *Vitis viniferis*, I); **14**, chromene (*Piper aduncum*, C); **15**, bibenzyl (batatasin IV, *Dioscorea batatas*, C); **16**, biphenyl (aucuparin, *Malus pumila*, I); **17**, benzofuran (*Cotoneaster* spp., I); **18**, xanthone (*Polygala nyikensis*, C). Nitrogen- and/or sulphur-containing compounds: **19**, benzophenanthridine alkaloid (sanguinarine, *Papaver bracteatum*, I); **20**, benzylisoquinoline alkaloid (berberine, *Berberis* spp., I); **21**, indole (camalexin, *Arabidopsis thaliana*, I); **22**, indole (brassicalexin, *Brassica* spp., I); **23**, anthranilamide (*Dianthus caryophyllus*, I); **24**, benzoxazinone (DIMBOA, *Zea mays*, C); **25**, elemental sulphur (*Theobroma cacao*, I).

whether or not a pathogen has the enzymatic machinery to detoxify a particular host product<sup>2</sup>. Accumulation of inducible antimicrobial compounds is often orchestrated through signal-transduction pathways linked to perception of the pathogen by receptors encoded by host resistance genes (see review by Dangl and Jones, pages 826–833).

*Arabidopsis*, rice, corn, soya bean and the model legume *Medicago truncatula*, which have been subject to intensive sequencing efforts, are, collectively, rich sources of antimicrobial indole, terpenoid, benzoxazinone and flavonoid/isoflavonoid natural products. Bioinformatic analysis of large-scale plant genomic and expressed sequence tag (EST) databases<sup>3,4</sup> is beginning to reveal how new enzymes of natural product biosynthesis may have arisen through processes of gene duplication and mutation<sup>5</sup>. This provides the genetic variation that leads to continued elaboration of new chemical structures that will be selected for if they impart a significant advantage in plant defence.

### Phytoalexins and phytoanticipins

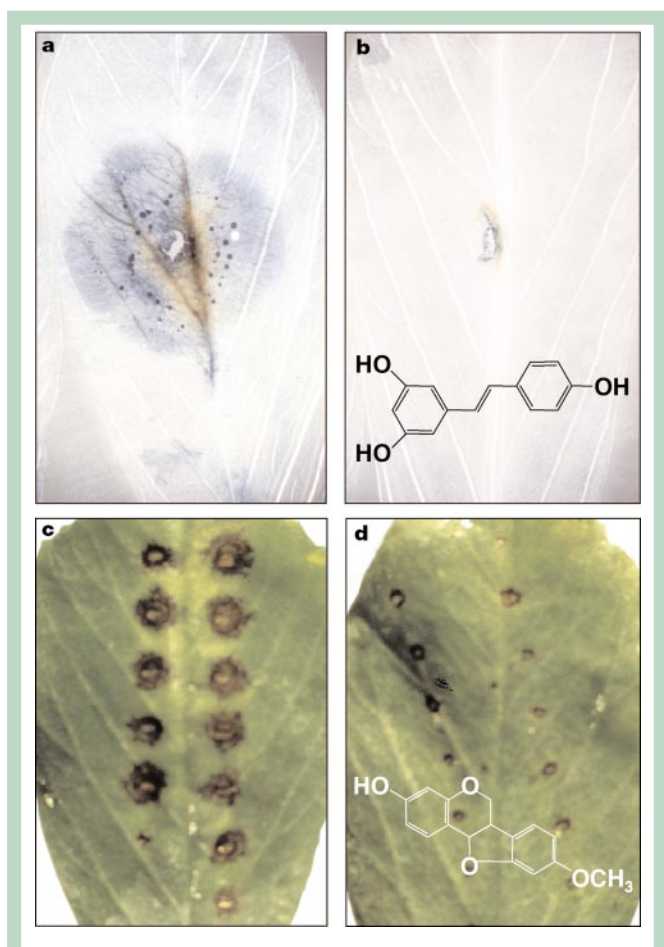
Details of the structures and sources of many antimicrobial plant natural products have been compiled<sup>6,7</sup>, and evidence for the functions of these compounds has also been reviewed<sup>8,9</sup>. The simplest functional definitions recognize phytoalexins as compounds that are synthesized *de novo* (as opposed to being released by, for example, hydrolytic activity) and phytoanticipins as pre-formed infectional inhibitors<sup>10</sup>. However, the distinction between phytoalexin and phytoanticipin is not always obvious. Some compounds may be phytoalexins in one species and phytoanticipins in others. A good example is the methylated flavanone sakuranetin (Fig. 1, 8), which accumulates constitutively in leaf glands of blackcurrant, but which is a major inducible antimicrobial metabolite in rice leaves<sup>11</sup>. In cases where a constitutive metabolite is produced in larger amounts after infection, its status as a phytoalexin would depend on whether or not the constitutive concentrations were sufficient to be antimicrobial.

*In vivo* antimicrobial activity is inherent in the definition of a phytoalexin or phytoanticipin, but it is this feature that has proven most problematical to determine directly in the absence of methods to genetically modify the host plant's natural product profiles. In most cases, concentrations of phytoalexins have not been measured specifically in the cells that are in direct contact with the invading microorganism. One exception is a careful study of the cellular concentrations of sesquiterpenoid phytoalexins in leaves of cotton varieties responding to the bacterial pathogen *Xanthomonas campestris* pv. *malvacearum*, in which it was shown that phytoalexin levels in and around the challenged cells were significantly higher than would be required to effectively inhibit the growth of the pathogen *in vitro*<sup>12</sup>.

### Genetic approaches to natural product function

One approach for addressing phytoalexin or phytoanticipin function *in vivo* has been to take advantage of the power of gene-knockout technology in microbes to disrupt pathogen genes that encode enzymes known to detoxify the host plant's antimicrobial compounds. For example, saponins are widely occurring, constitutively expressed, glycosylated steroids, steroidal alkaloids or triterpenes, many of which have antimicrobial activity *in vitro*. Mutants of the fungal pathogen of oat roots, *Gaeumannomyces graminis*, that had lost the enzyme avenacinase were no longer able to detoxify the triterpene saponin avenacin (Fig. 1, 4); they lost pathogenicity on oats, but retained full pathogenicity on wheat, which does not produce saponins<sup>13</sup>. This indicates that avenacin, which is localized to the epidermal cells that would represent one of the first barriers to infection by *G. graminis*, is a key determinant of resistance in this particular host–pathogen system. Likewise, disruption of the *MAK1* gene in the fungal pathogen *Nectria haematococca* leads to inability to detoxify the pterocarpan phytoalexin maackiain (Fig. 1, 11) and reduced virulence of the fungus on chickpea. In this case the effect was incomplete, and host factors in addition to maackiain are therefore involved in resistance<sup>14</sup>.

In a few cases, a role for plant natural products in host defence has been demonstrated by the increased disease susceptibility of mutants



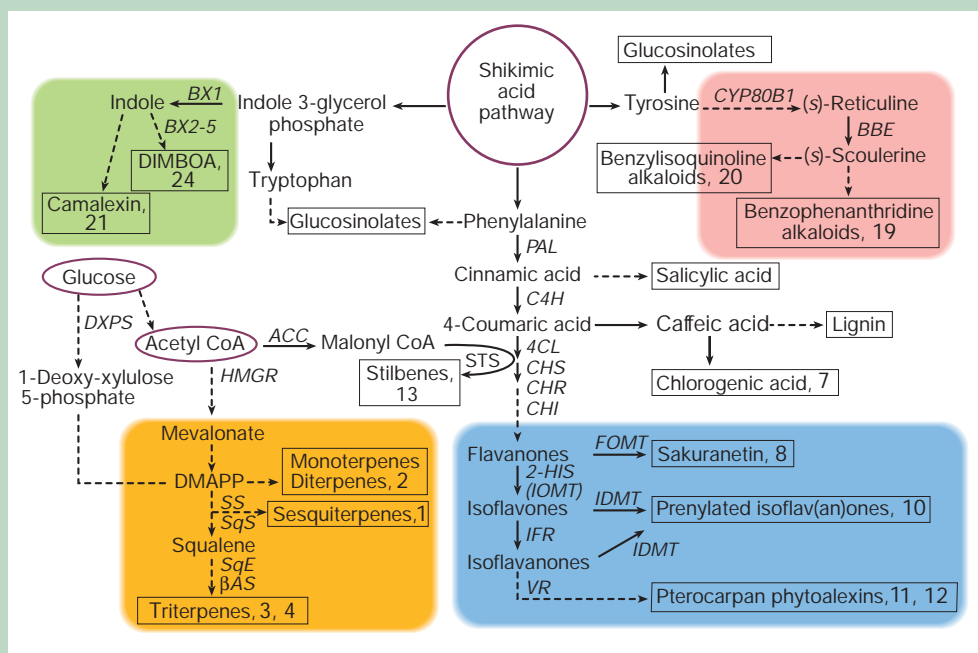
**Figure 2** Metabolic engineering for improved fungal disease resistance in alfalfa.

**a**, Disease lesion on a leaf of an empty-vector control alfalfa plant 10 days after infection with *Phoma medicaginis* (fungal hyphae and reproductive structures (pycnidia) are stained blue against the background of the leaf that has been cleared of chlorophyll). **b**, Lesion of identical age on a plant that had been transformed with a grapevine stilbene-synthase gene under control of the cauliflower mosaic virus 35S promoter. Such plants constitutively accumulate a glucoside of the novel phytoalexin resveratrol (structure indicated) and exhibit vastly reduced fungal development (data of N. L. Paiva, Noble Foundation<sup>21</sup>). **c**, Lesions of *Phoma medicaginis* 5 days post-inoculation on leaves of alfalfa plants transformed with an empty-vector control construct. **d**, Lesions of identical age on plants constitutively expressing an alfalfa *IOMT* transgene from the 35S promoter. The endogenous *IOMT* genes, and the other genes of the isoflavonoid pathway, are not constitutively expressed, but are induced after fungal infection. The high expression of *IOMT* during the early stages of the response to the fungus results in more rapid production of the endogenous phytoalexin medicarpin (structure indicated) and consequent reduction in disease severity<sup>22</sup>.

impaired in production of phytoanticipins or phytoalexins. Thus, the maize mutation *bxi* abolishes DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) biosynthesis, and *bxi* homozygotes are extremely susceptible to northern corn leaf blight (*Helminthosporium turcicum*) and stalk rot (*Diplodia maydis*)<sup>15</sup>. Likewise, saponin-deficient (*sad*) mutants of oats are compromised in resistance to *G. graminis* var. *tritici*, and avenacin content and disease resistance correlate in segregating progeny<sup>16</sup>. This last result confirms the critical role of avenacin in disease resistance in oats, first revealed by disruption of the fungal avenacin detoxifying system.

Several phytoalexin-deficient (*pad*) mutants of *Arabidopsis thaliana* have reduced levels of the indole camalexin (Fig. 1, 21)<sup>17–19</sup>. The *PAD4* gene seems to encode a regulatory factor<sup>18</sup>, whereas *PAD3* encodes a cytochrome P450 that might be directly involved in camalexin biosynthesis<sup>19</sup>. The disease phenotypes of various single

**Figure 3** Biosynthetic relationships between antimicrobial plant natural products. Primary precursors/pathways are circled, and end products are boxed. Important cloned genes or characterized enzymes are in *italic type*. Numbers in bold refer to chemical structures in Fig. 1. Pathways leading to alkaloids are in pink, flavonoids/isoflavonoids in blue, indole derivatives in green, and isoprenoids in orange. BBE, berberine bridge enzyme; PAL, L-phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; CHR, chalcone (polyketide) reductase; CHI, chalcone isomerase; STS, stilbene synthase; FOMT, flavanone 7-*O*-methyltransferase; 2-HIS, 2-hydroxyisoflavone synthase ('isoflavone synthase'); IOMT, isoflavone 4'-*O*-methyltransferase; IDMT, isoflavone or isoflavonone dimethylallyl transferase; IFR, isoflavone reductase; VR, vestifone reductase; BX1, indole-3-glycerol phosphate lyase; BX2-5, four consecutive cytochrome P450 enzymes of DIMBOA biosynthesis; DXPS, 1-deoxy-xylulose 5-phosphate synthase; ACC, acetyl CoA carboxylase; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; SS, sesquiterpene synthase; SqS, squalene synthase; SqE, squalene epoxidase;  $\beta$ AS,  $\beta$ -amyrin synthase. Note that salicylic acid is a signal molecule for plant defence responses (see article in this issue by Stuijver and Custers, pages 865–868).



and double *pad* mutants reveal a complex relationship between phytoalexin production and disease resistance that is highly pathogen-dependent. Thus, the *pad-3* mutant shows increased susceptibility to two fungal pathogens, *Cochliobolus carbonum* and *Alternaria brassicola*, but not to *Botrytis cinerea*, or to the obligate pathogens *Peronospora parasitica* (downy mildew) or *Erysiphe orontii* (powdery mildew)<sup>18,20</sup>. However, in spite of a total lack of camalexin production, *pad-3* does not show increased susceptibility to strains of the bacterial pathogen *Pseudomonas syringae*<sup>17</sup>. In contrast, *pad-1* and *pad-2* show increased susceptibility to disease-causing strains of *P. syringae*, but not to strains to which the wild-type *Arabidopsis* plants were resistant<sup>17,18</sup>. This suggests that camalexin production may limit disease symptoms, but is not responsible for limiting ingress of the pathogen in resistant interactions.

Targeted transgenic approaches allow for evaluation of effects of directly altered phytoalexin profiles, and such approaches have been undertaken in the case of stilbenoids and isoflavonoids. Introduction of a novel phytoalexin, resveratrol (Fig. 1, 13), into alfalfa by constitutive expression of a grapevine stilbene-synthase gene resulted in reduced symptoms following infection by the leaf spot pathogen *Phoma medicaginis*<sup>21</sup> (Fig. 2a, b). Constitutive overexpression of isoflavone *O*-methyltransferase (IOMT) in transgenic alfalfa resulted in more rapid and increased production of the pterocarpan phytoalexin medicarpin (Fig. 1, 12) after infection by *P. medicaginis*, resulting in amelioration of symptoms<sup>22</sup> (Fig. 2c, d).

Taken together, the results of genetic analyses indicate that phytoalexins and phytoanticipins can indeed be effective in contributing to resistance *in vivo*, but more than one individual compound or class of compound may be necessary to impart resistance, which is consistent with the multi-component nature of plant defence responses. Clearly, the diversity of plant natural products and host–pathogen combinations makes it impossible to make any general conclusions in regard to the multitude of systems not yet analysed.

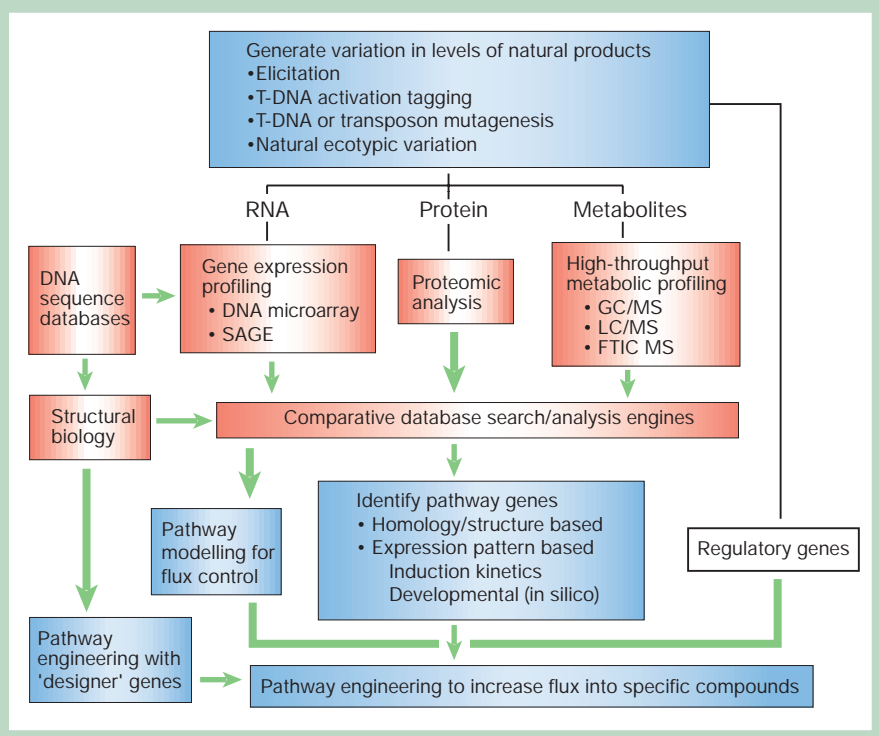
### Enzymology and organization of biosynthetic pathways

The diversity and apparent complexity of biosynthetic pathways of plant natural products was for many years seen as a barrier to progress in advancing the understanding of phytoalexin function, and in

developing technologies to improve resistance by pathway engineering. However, there have recently been a number of successful applications of genetic and genomic approaches to identify genes of plant natural product biosynthesis. For example, comparative EST database mining was used to identify the 2-HIS cytochrome P450 that catalyses the entry-point reaction into isoflavonoid phytoalexin biosynthesis<sup>23,24</sup>, based on the predicted expression of this enzyme for production of isoflavonoids in developing seeds and elicited cell cultures (Fig. 3). Mass sequencing of complementary DNA libraries corresponding to metabolically specialized cells has been used to identify several of the genes of monoterpene biosynthesis and the associated Rhomer pathway for formation of the isoprenoid precursor isopentenyl diphosphate in mint glandular trichomes<sup>25,26</sup>. Tagging with the *Mutator* transposable element was used to map and subsequently clone the first enzyme specific for DIMBOA (Fig. 1, 24) biosynthesis in maize<sup>15</sup> (Fig. 3). Virtually all the enzymes of flavonoid and isoflavonoid biosynthesis<sup>27,28</sup>, the early steps of mono-, sesqui- and diterpene biosynthesis<sup>29</sup>, and the formation of DIMBOA<sup>15</sup> have now been isolated and functionally characterized. Figure 3 presents an overview of these and other pathways leading to phytoalexins or phytoanticipins, indicating how they are inter-related through primary metabolism, and highlighting some of the genes that have been cloned so far.

Although information derived from large-scale genomics initiatives is invaluable, it can be potentially difficult to mine these data to construct whole pathways from gene sequence information alone. With few exceptions (for example, the BX1 indole-3-glycerol phosphate lyase and four subsequent cytochrome P450 enzymes (BX2–BX5) of DIMBOA biosynthesis<sup>15</sup>; Fig. 3), genes encoding enzymes in natural product pathways are not closely linked in plants, making genetic characterization of pathways more difficult than in bacteria or fungi. However, bioinformatic tools are available for identifying genes encoding members of specific classes of enzymes, such as cytochrome P450s<sup>30</sup>, *O*-methyltransferases (OMTs)<sup>31</sup>, terpene cyclases<sup>29</sup> and polyketide synthases<sup>32</sup> from large EST projects. In addition, knowledge of transcript expression patterns (for example, upregulated after pathogen attack) from DNA-microarray or more conventional membrane-hybridization approaches can sufficiently

**Figure 4** A genomics approach to understanding and manipulating complex natural product pathways for plant defence. A current impediment to progress is a lack of understanding of the biosynthetic and regulatory genes of many natural product pathways. Requirements for a genomics-based approach are large DNA sequence (genomic or EST) databases and ability to experimentally manipulate the levels of the metabolites of interest. This can be done by chemical elicitation, which removes the complication of the presence of the infecting organism, by T-DNA activation tagging to upregulate regulatory genes or rate-limiting enzymes of the pathway, or by exploiting variation in metabolite levels or composition among natural ecotypes or cultivar germplasm. The key to this type of analysis will be the development of improved computational methods for comparative analysis of databases housing information on relative changes in metabolites, proteins and gene transcripts, thereby facilitating the linking of sets of genes or gene products to particular metabolic pathways. SAGE, serial analysis of gene expression; GC/MS and LC/MS, gas chromatography or liquid chromatography linked to mass spectrometry; FTIC MS, Fourier transform ion cyclotron mass spectrometry.



reduce the number of candidate EST clones to allow functional identification by heterologous expression in bacteria, yeast or insect cells.

Sequence-based gene annotation for enzymes in natural product pathways can occasionally be misleading because of the evolutionary flexibility of plant secondary metabolism. For example, chalcone synthase is a simple homodimeric polyketide synthase (PKS) catalysing the first committed step in flavonoid biosynthesis using 4-coumaroyl coenzyme A as the starter molecule (Fig. 3). Many genes annotated as chalcone synthase probably encode different PKS enzymes that use different starter molecules or different numbers of malonyl CoA additions to generate different types of polyketide derivatives<sup>32</sup>. Two acyl transferases of natural product biosynthesis have recently been shown to be closely related to serine proteases, including possession of the catalytic triad diagnostic for this class of protease<sup>33,34</sup>, and would have been mistaken for such in the absence of full functional identification.

The methyltransferase (IOMT, Fig. 3) that introduces the 4'-methoxyl function into isoflavonoids *in vivo* methylates the 7-position of isoflavones *in vitro*<sup>22</sup>. In this case, and in other pathways leading to antimicrobial natural products<sup>35,36</sup>, consecutive enzymes are physically associated in complexes through which intermediates are channelled, impacting both the kinetics and the regioselectivity of the reactions. Understanding the molecular architecture of such complexes will have a large impact on our ability to engineer new metabolic pathways for crop protection. At a higher order of sub-cellular organization, some phytoalexins may be synthesized, or at least accumulate, in specialized vesicles that are then delivered to the site of microbial infection, as in the case of the red deoxyanthocyanidin phytoalexins of sorghum<sup>37</sup>.

### Metabolic engineering for plant disease resistance

The concept of improving disease resistance by engineering natural product pathways has met with several objections, despite a generally held belief that many crop plants are susceptible to pathogens because of years of selective breeding leading to removal of natural products found in their more resistant, wild counterparts (see review in this issue by Stuijver and Custers, pages 865–868). The first objection concerns the large numbers of genes that may have to be transferred, and coordinately regulated, to introduce effective

antimicrobial activity. This still represents a technological challenge, although several single-step conversions can generate antimicrobial compounds from ubiquitous or common metabolic intermediates (for example, *O*-methylation of the flavanone naringenin to yield sakuranetin, isoprenylation of isoflavones, or production of stilbenes and other polyketides from malonyl CoA and various starter molecules, as shown in Fig. 3). Increasing production of an endogenous antimicrobial compound through overexpression of a rate-limiting enzyme is conceptually a simple strategy, but in most cases the flux control points in the pathway are not understood. Transferred DNA (T-DNA) activation tagging has recently been applied to the characterization of transcriptional regulators of natural product pathways<sup>38,39</sup>, and this approach, by which regulatory genes can be identified without the need to understand the individual enzymatic steps of the pathway, offers an exciting opportunity to develop new molecular tools for pathway engineering for improved plant defence.

A second problem is the ability of pathogens to overcome effects of antimicrobial compounds by rapidly evolving detoxification systems, often involving cytochrome P450 enzymes<sup>40</sup>. It would now seem possible to circumvent this problem by introduction of more than one unrelated, new antimicrobial compound, using the approaches outlined above. However, such a strategy remains to be tested.

Recently, significant progress has been made in elucidating the three-dimensional structures of several key enzymes involved in the biosynthesis of terpenoid and isoflavonoid phytoalexins<sup>41–43</sup>. These studies open up the possibility of structure-based rational design of enzymes such as terpene cyclases, polyketide synthases and *O*-methyltransferases for transgenic introduction of new natural products for plant defence. For example, alfalfa chalcone synthase has been converted into a pyrone synthase by rational introduction of six point mutations<sup>44</sup>, and the alterations in plant OMT substrate specificities that result in some cases from heterodimer formation<sup>45</sup> could form a basis for 'combinatorial biochemistry' approaches to pathway engineering.

### Future prospects

The concept of phytoalexins as induced antimicrobial compounds in plants was first developed in 1940<sup>46</sup>. It has taken over 60 years to gain a basic understanding of how a small part of nature's plant antimicro-

bial arsenal is manufactured. Much more rapid progress can now be expected. To use genomics to identify genes of plant natural product biosynthesis, it is necessary to be able to perturb the production of these products, whether they be naturally constitutive or inducible. This can be achieved by elicitation with plant or microbial signal molecules<sup>47,48</sup>, or by T-DNA activation tagging. The latter method has the advantage of direct identification of regulatory genes or rate-limiting enzymes for the pathway under consideration<sup>49</sup>. Recent improvements in high-throughput metabolite profiling, using gas chromatography or liquid chromatography linked to mass spectrometry, now make it possible to screen for changes in the levels of several hundred plant metabolites in a single sample<sup>50,51</sup>. Linking changes in metabolite profiles to parallel analysis of gene expression through DNA microarray analysis<sup>52</sup>, and of protein levels through mass spectrometric proteomic analysis<sup>53</sup>, provides possibilities for assigning gene functions based on cellular dynamics. This will also facilitate modelling of pathway flux for determination of rate control points for subsequent metabolic engineering.

The conceptual framework for a genomics approach to natural product pathways is illustrated in Fig. 4. Current technical limitations are the throughput rates for metabolite separation and determination of protein structure, the sensitivity of protein separation methods for proteomic analysis<sup>54</sup>, and the need to develop algorithms for comparisons between the very different types of databases that store gene, protein and metabolite information. Fourier transform ion cyclotron mass spectrometry removes the requirement for chromatographic separations and may revolutionize proteomic<sup>54</sup> and metabolic profile analysis. Given the pace of recent developments in genomics and bioinformatics, we should soon be evaluating plants with 'designer' natural product profiles for their adaptation to both biotic and abiotic environmental stresses. □

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