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The use of genetics to dissect plant secondary pathways

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Plant secondary metabolism comprises an enormous diversity in compounds and enzymes, and wide spectra of mechanisms of gene regulation and of transport of metabolites and enzymes. Genetic approaches using the model plant *Arabidopsis thaliana* have contributed importantly to recent progress in understanding glucosinolate biosynthesis and its intricate linkage with auxin homeostasis. *Arabidopsis* genetics have also caused revolutionary changes in the existing views on the metabolic intermediates and enzyme activities that are involved in phenylpropanoid biosynthesis. Some progress has been achieved in understanding the transcriptional regulation of the flavonoid pathway. Transcriptional regulators have also been identified for glucosinolate and terpenoid indole alkaloid biosynthesis.

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Introduction

Plant secondary metabolism is a paradigm for the metabolic diversity found in nature. The plant kingdom collectively contains a bewildering number and array of structures, produced by a similarly large number of mostly substrate-specific enzymes. Enormous diversity also exists in the functions of the compounds, in their tissue-specific or signal-responsive production, and in transport mechanisms for metabolites, metabolic intermediates and enzymes. Because of this diversity, it has been difficult to define unifying concepts in the description of plant secondary metabolism. The basic networks of metabolic intermediates and enzymatic activities have been worked out for several secondary metabolite pathways, mainly through biochemical approaches. Genetic approaches have also been successful in unraveling the metabolic network for phenylpropanoid and flavonoid metabolism, as well as in finding transcriptional regulators of structural genes in the genetically tractable plant species maize, petunia, snapdragon and, more recently, *Arabidopsis* [1–3].

Classical genetics is a phenotype-driven approach to the dissection of biological processes, which does not require prior knowledge of the molecular mechanisms or information about genes, proteins, enzyme activities or metabolic intermediates. Using appropriate mutagens, such as transposons or T-DNA, it is possible to quickly connect the mutant phenotype to a certain gene. Reverse genetics comprises the engineering of changes in the genome using existing information about the targeted gene. Common approaches are ectopic overexpression of the gene and, as a complementary approach, post-transcriptional silencing by antisense RNA or RNA interference (RNAi) [4]. The introduction of targeted genetic changes by homologous recombination is still impossible in plants and, as an alternative, TILLING (Targeting Induced Local Lesions IN Genomes) has been developed [5]. In a classical genetic approach, the selection of mutants relies on a screenable phenotype. In secondary metabolism, changes in the levels of metabolites that absorb specific light wavelengths are easily screenable phenotypes. The classic example is the screening with visible light for changes in the levels of anthocyanin pigments, which has allowed the isolation of mutants that are affected in many structural genes, in regulatory genes and in genes that encode transporters [1–3]. Successful mutant screens have also been conducted for metabolites that absorb UV light [6]. In addition, more elaborate screens using high-throughput metabolite detection methods are possible.

In petunia, maize and snapdragon, endogenous transposons have been used to generate mutations, allowing the rapid recovery of mutated genes. In *Arabidopsis*, which lacks naturally active transposons, foreign transposons have been introduced. Gene cloning after ethylmethanesulfonate (EMS) mutagenesis in *Arabidopsis* is facilitated by rapid advances in map-based cloning techniques. In addition to mutant selection, the natural genetic variation that occurs among ecotypes or cultivars can be exploited to discover quantitative trait loci (QTL) [7]. Isolating the genes that correspond to QTLs is a major challenge, however, which in *Arabidopsis* is facilitated by map-based cloning approaches. Knocking out known genes in *Arabidopsis* using reverse genetics approaches is now facilitated enormously by the development of saturated collections of transposon and T-DNA insertion lines. Genetic tools have not been established for the large majority of plant species, and this causes difficulties in the

mapping and subsequent cloning of mutated genes. Reverse genetics, on the other hand, is applicable to all plant species that can be genetically transformed. In addition, reverse genetic techniques that introduce dominant traits are applicable to plant cell cultures, which cannot be taken through meiosis.

Glucosinolate biosynthesis in *Arabidopsis*

In the past couple of years, remarkable advances in the understanding of glucosinolate biosynthesis in *Arabidopsis* have been achieved [8]. The research has deployed phenotype-driven classical genetics as well as gene-based reverse genetic studies that use mutants from transposon- and T-DNA-tagged *Arabidopsis* collections. As such, recent work on glucosinolate biosynthesis forms a showcase for the power of both *Arabidopsis* genetics and the available genetic tools for studying secondary metabolism. For example, piling up insertional gene knockouts in a single plant line allows the analysis of functionally redundant genes, which escape classical genetic screening, for involvement in a process of interest.

The individual functions of two cytochromes P450 that are involved in aliphatic glucosinolate biosynthesis have been unraveled using transposon knockouts [9]. CYP79F1 turns out to be involved in the biosynthesis of short-chain and long-chain aliphatic glucosinolates from (n)homomethionine, whereas CYP79F2 is involved only in long-chain aliphatic glucosinolate biosynthesis. In the double-knockout mutant, aliphatic glucosinolate biosynthesis is completely abolished [10]. The *ref2* mutant, which had reduced levels of phenylpropanoid end products, was isolated using a screen for reduced epidermal fluorescence under UV light [11]. Surprisingly, this mutant turned out to be affected in the gene that encodes CYP83A1, which is involved in aliphatic glucosinolate biosynthesis one enzymatic step downstream of the CYP79-catalyzed step. Indeed, the *ref2* mutant had reduced levels of both short-chain and long-chain aliphatic glucosinolates. It is not well understood how CYP83A1 affects phenylpropanoid product levels. A detailed characterization of the *superroot1* (*sur1*) mutant, which is affected in a gene with hitherto unknown function, showed that SUR1 is the *C-S* lyase that produces the thiohydroximic acid in glucosinolate biosynthesis [12]. The *sur1* mutant is completely devoid of aliphatic and indole glucosinolates. The super-root phenotype is attributed to increased auxin levels resulting from the accumulation of indole-3-acetaldoxime, the common upstream precursor for indole glucosinolates and auxin. This finding indicates an intricate linkage between glucosinolate biosynthesis and auxin homeostasis. A similar linkage was revealed by an insertional loss-of-function mutant that is affected in the gene encoding the glucosyltransferase UGT74B1 [13]. This knockout mutant had reduced glucosinolate levels, showing that UGT74B1 catalyzes the glucosylation reaction that occurs one enzymatic step downstream from the SUR1-catalyzed step in glucosinolate biosynthesis. The mutant had increased auxin levels and a corresponding phenotype.

Further analysis of a double-knockout mutant for the genes encoding CYP79B2 and CYP79B3, which catalyze the first step in indole glucosinolate biosynthesis from tryptophan (Trp), showed a total lack of the indole phytoalexin camalexin [14]. This finding demonstrates that the product of the CYP79-catalyzed reaction, indole-3-acetaldoxime, is a key branching point between indole glucosinolate, camalexin and auxin biosynthesis. Analysis of a knockout mutant of the gene *ATR1* (*ALTERED TRYPTOPHAN REGULATION1*, also known as *AtMYB34*) combined with the study of a transformant that overexpressed ATR1 showed that this MYB-type transcription factor controls the expression of genes that are involved in Trp biosynthesis, as well as of the *CYP79B2*, *CYP79B3* and *CYP83B1* genes, which are involved in Trp-derived indole glucosinolate biosynthesis [15•]. Interestingly, in a loss-of-function mutant affected in the *CYP83B1* gene, which encodes the cytochrome P450 that channels indole-3-acetaldoxime into indole glucosinolate biosynthesis, expression of the *ATR1* gene is upregulated. Probably as a consequence of this upregulation, the Trp biosynthesis gene *ANTHRANILATE SYNTHASE1* (*ASA1*) and the *CYP79B2* gene are also upregulated [16]. This indicates that the super-root phenotype of the *cyp83b1* mutant, which is also known as *sur2* [17], could be caused by increased auxin levels that occur both as a result of the accumulation of indole-3-acetaldoxime (because of lack of further processing) and because of increased production of this auxin precursor (because of the upregulation of the biosynthesis genes). The molecular mechanism behind the effect of the *cyp83b1* mutation on gene expression is not clear because *ATR1* gene expression was not induced, but rather was repressed, by exogenous auxin application [16].

Phenylpropanoid biosynthesis in *Arabidopsis*

Arabidopsis genetics has also facilitated progress in the understanding of phenylpropanoid biosynthesis, which has brought about some revolutionary changes in the existing views. Using bioinformatics-guided gene discovery and biochemistry, the postulated enzyme activity *p*-coumarate 3-hydroxylase (C3H), which introduces the hydroxyl group at the 3-position of the phenylpropanoid ring, was identified as a cytochrome P450 that uses the shikimate and quinate esters of *p*-coumarate as substrates [18]. The *C3H* gene was also isolated by positional cloning from the *ref8* mutant, which has reduced epidermal fluorescence under UV light because of a reduced level of sinapoylmalate [19]. The previously widely accepted view did not attribute the 3-hydroxylation to a cytochrome P450 activity, and envisaged free *p*-coumarate as the substrate.

Phenylpropanoid metabolism has long been thought to involve a series of reactions in which deamination of phenylalanine and a series of hydroxylation and *O*-methylation reactions generate hydroxycinnamic acids, which are converted to their corresponding hydroxycinnamaldehydes and hydroxycinnamyl alcohols. The *ref1* mutant shows reduced epidermal fluorescence under UV light because of a reduced content of sinapic acid and ferulic acid. The *REF1* gene has been found to encode an aldehyde dehydrogenase, which can convert coniferaldehyde and sinapaldehyde to ferulate and sinapate, respectively [20•]. Therefore, this finding has caused a complete reversal in the previously accepted view of intermediate to end-product relationships in phenylpropanoid metabolism. In the current revised view, ferulate and sinapate feature as end products rather than as intermediates.

Condensed tannins or proanthocyanidins are flavonoid oligomers that are synthesized by a branch of the flavonoid pathway [3]. The brown color of the seed coat of *Arabidopsis* seeds is determined by these compounds; in the *banyuls* (*ban*) mutant, the seeds have a transparent testa (tt). The BAN enzyme was proposed to be a leucoanthocyanidin reductase [21]. Using BAN from *Arabidopsis* and *Medicago truncatula*, Xie *et al*. [22] demonstrated that BAN is in fact a reductase of anthocyanidin compounds located one step downstream from the leucoanthocyanidins. In accordance with this finding, an *Arabidopsis* mutant in which anthocyanidin synthase is defective lacks proanthocyanidins in the seed coat. The accumulation of proanthocyanidins in the *Arabidopsis* seed coat is also dependent on the MYB transcription factor TT2 [23], the basic-helix–loop–helix transcription factor TT8 [24], and the WD-repeat protein TRANSPARENT TESTA GLABRA1 (TTG1) [24]. By expressing glucocorticoid receptor fusion proteins in the corresponding mutant backgrounds, Baudry *et al*. [25] demonstrated that TT2, TT8 and TTG1 regulate expression of the *BAN* gene by direct interaction with its promoter. Yeast two- and three-hybrid experiments indicate that these proteins form a complex on the *BAN* promoter. Analysis of the new *tt19* mutant allowed the isolation of a glutathione S-transferase-encoding gene, which is involved in anthocyanin and proanthocyanidin accumulation [26].

Phenotype-driven genetics in non-model species

Attempts to dissect secondary metabolism using classical genetics in non-model plant species are still scarce. In one example, a mutant screen was conducted in opium poppy following EMS mutagenesis [27]. Analysis of the latex of 8000 plants derived from EMS-treated seed led to the identification of the *thebaine oripavine poppy1* (*top1*) mutant, which does not contain morphine or codeine but instead has elevated levels of the precursors thebaine and oripavine. Identification of the single mutated gene was attempted by analyzing global changes in gene expression with a 17 000-gene microarray, which led to the identification of 10 genes that were underexpressed in the *top1* mutant. None of these genes was an obvious candidate for being related to the *top1* phenotype. This demonstrates the difficulties in connecting a mutant phenotype with a gene in plant species for which genetic tools are lacking.

A powerful tool in phenotype-driven genetics is gain-of-function mutagenesis with a strong constitutive promoter that is carried on an insertion element such as *Agrobacterium tumefaciens* T-DNA. T-DNA activation tagging causes dominant gain-of-function mutations that are caused by the overexpression of the genes that flank the T-DNA insertion. It allows the recovery of mutant phenotypes that are conferred by functionally redundant genes. In addition, the T-DNA tag allows gene recovery from the primary transformant without a need for genetic crosses. T-DNA activation tagging has been successfully used in the model species *Arabidopsis* to identify the transcriptional regulator PAP1 (PRODUCTION OF ANTHOCYANIN PIGMENT1) [28]. Overexpression of this MYB-type protein upregulates multiple genes that are involved in anthocyanin biosynthesis and gives the transformant a purple color.

The T-DNA activation tagging technique has also been successfully applied to unravel secondary metabolism in non-model species. In tomato, T-DNA activation tagging allowed the identification of the MYB-type transcriptional regulator ANTHOCYANIN1 (ANT1). Overexpression of ANT1 upregulated multiple genes that are involved in the early and later steps of anthocyanidin biosynthesis, as well as genes that are involved in glycosylation and transport into the vacuole [29•]. This overexpression caused an intense purple pigmentation throughout development [29•].

In *Catharanthus roseus* cells, T-DNA activation tagging of the octadecanoid-derivative-responsive *Catharanthus* APETALA2 (AP2)-domain3 (ORCA3) transcription factor conferred resistance to a toxic derivative of the metabolic intermediate tryptamine in the terpenoid indole alkaloid (TIA) pathway, which is detoxified by the TIA biosynthesis enzyme tryptophan decarboxylase [30]. Overexpression of ORCA3 upregulated several other genes in TIA biosynthesis besides that encoding tryptophan decarboxylase, as well as genes in precursor pathways. Additional evidence indicates that ORCA3 normally controls the jasmonic acid (JA)-responsive expression of TIA biosynthesis genes [31].

Reverse genetics in non-model species

Reverse genetics forms an integral part of the normal classical genetic cycle needed to confirm the gene–

phenotype relationship by complementing a mutant phenotype with the cloned candidate gene or by recapitulating a phenotype with a gene cloned from a T-DNA activation-tagged mutant. In the absence of mutants, however, reverse genetics is useful in uncovering processes in which a cloned gene is involved. One reverse genetic approach is the post-transcriptional silencing of gene expression using a variety of techniques, of which the most recent and successful is RNAi [4].

Reverse genetic approaches have been used to change the nicotine content in *Nicotiana attenuata*. One study involved virus-induced gene silencing in which constructs that expressed sense, antisense or inverted-repeat fragments corresponding to conserved parts of the two *PMT* genes, which encode putrescine methyltransferase, were introduced by *Agrobacterium*-mediated inoculation of the leaves. This led to a reduction in the corresponding transcript levels in the roots (the main site of nicotine biosynthesis) and a reduction of the basal and methyl jasmonate-induced nicotine content in the plants [32]. In a second study, silencing of *PMT* genes by a similar inverted repeat expressed from the CaMV 35S promoter led to reduced transcript levels in roots and reduced nicotine contents in leaves [33]. In contrast to wildtype plants, the silenced plants contained anatabine, which is presumably formed because of the accumulation of nicotinic acid, which is normally coupled to a downstream product of *PMT* to form nicotine. A similar observation of elevated levels of anatabine at the expense of nicotine was made in *Nicotiana tabacum* plants expressing antisense *PMT* [34].

In opium poppy, codeinone reductase (COR), a terminal enzyme in morphine biosynthesis, has been knocked out by RNAi using a chimeric inverted repeat that targets all seven members of the gene family, leading to a significant reduction in the morphine and codeine levels of the transgenic poppy latex [35•]. Surprisingly, none of the morphine-type precursors that are normally present in the latex were detectable. Instead, the transgenic latex accumulated rare alkaloids. These included the upstream precursor reticuline, which is located seven enzymatic steps before the COR-mediated reaction in the biosynthesis pathway, and several methylated derivatives of reticuline. The reasons for the apparent shutdown of the complete morphine-specific branch, resulting in a drastic switch in the alkaloid pattern, are not clear.

Conclusions

In the past couple of years, genetic approaches have brought about tremendous advances in our understanding of secondary metabolism in *Arabidopsis*. Studies have overturned existing views on the metabolic intermediates and enzyme activities that are involved in general phenylpropanoid metabolism, a ubiquitous and well-studied secondary metabolite pathway in plants. The revolution in views on phenylpropanoid metabolism demonstrates the power of genetics and shows that long-term and worldwide research efforts that employed biochemical approaches have failed to correctly describe the basic network of intermediates and enzyme activities in this pathway. Enormous advances have also been made in understanding the basic network of glucosinolate biosynthesis, and its relationship to auxin homeostasis, in *Arabidopsis*.

In non-model plant species, the use of genetic approaches (with the exception of some reverse-genetic RNAi approaches) appears to have come to an almost complete standstill. It has been demonstrated that some genetic approaches, such as T-DNA activation tagging, can be successful with non-model species but this has not led to the expected explosion of similar approaches in a variety of plant species. It is possible that the timeframe is too short to expect results from such approaches, and that eventually such studies will be conducted successfully.

Understanding the basic network of metabolic intermediates and enzymes is a first step in unraveling secondary metabolism. Subsequent steps should aim at dissecting the regulatory mechanisms that control the enzyme-encoding genes, the mechanisms that control the activities of transcriptional regulators, and the mechanisms of intra- and inter-cellular transport of metabolites and enzymes. Some progress has been achieved in understanding the transcriptional regulation of the phenylpropanoid and flavonoid pathways. Transcriptional regulators have also been identified in glucosinolate and TIA biosynthesis. The picture is far from complete, however, and how the expression and the activities of these transcription factors are regulated remains largely mysterious [36]. The mechanisms of intra- and intercellular transport of metabolic intermediates and enzymes remain poorly understood aspects of secondary metabolism, but *Arabidopsis* genetics and genetic tools clearly offer promising opportunities for studying this neglected part of plant secondary metabolism.

Acknowledgements

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