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Beyond co-expression: pathway discovery for plant pharmaceuticals

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Plant natural products have been an important source of medicinal molecules since ancient times. To gain access to the whole diversity of these molecules for pharmaceutical applications, it is important to understand their biosynthetic origins. Whilst co-expression is a reliable tool for identifying gene candidates, a variety of complementary methods can aid in screening or refining candidate selection. Here, we review recently employed plant biosynthetic pathway discovery approaches, and highlight future directions in the field.

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Introduction

Plant natural products (NPs) can be used as pharmaceuticals or as the inspiration for synthetic drugs [1]. Discovering the genetic basis of the biosynthetic pathways to these compounds enables their reconstruction and/or modification in heterologous hosts [2]. It also provides access to biocatalysts that can be used in chemoenzymatic or synthetic biology routes to high-value pharmaceuticals [3]. Pathway gene discovery is dominated by RNA-seq-based co-expression analysis, exemplified by field-defining discoveries of enzymes [4] and pathways [5]. Here, we attempt to look beyond co-expression, examining relevant classical, state-of-the-art and emerging methods for plant biosynthetic gene discovery. We examine examples beyond pharmaceuticals as the methods described can be applied broadly across plant NPs.

The genetic basis

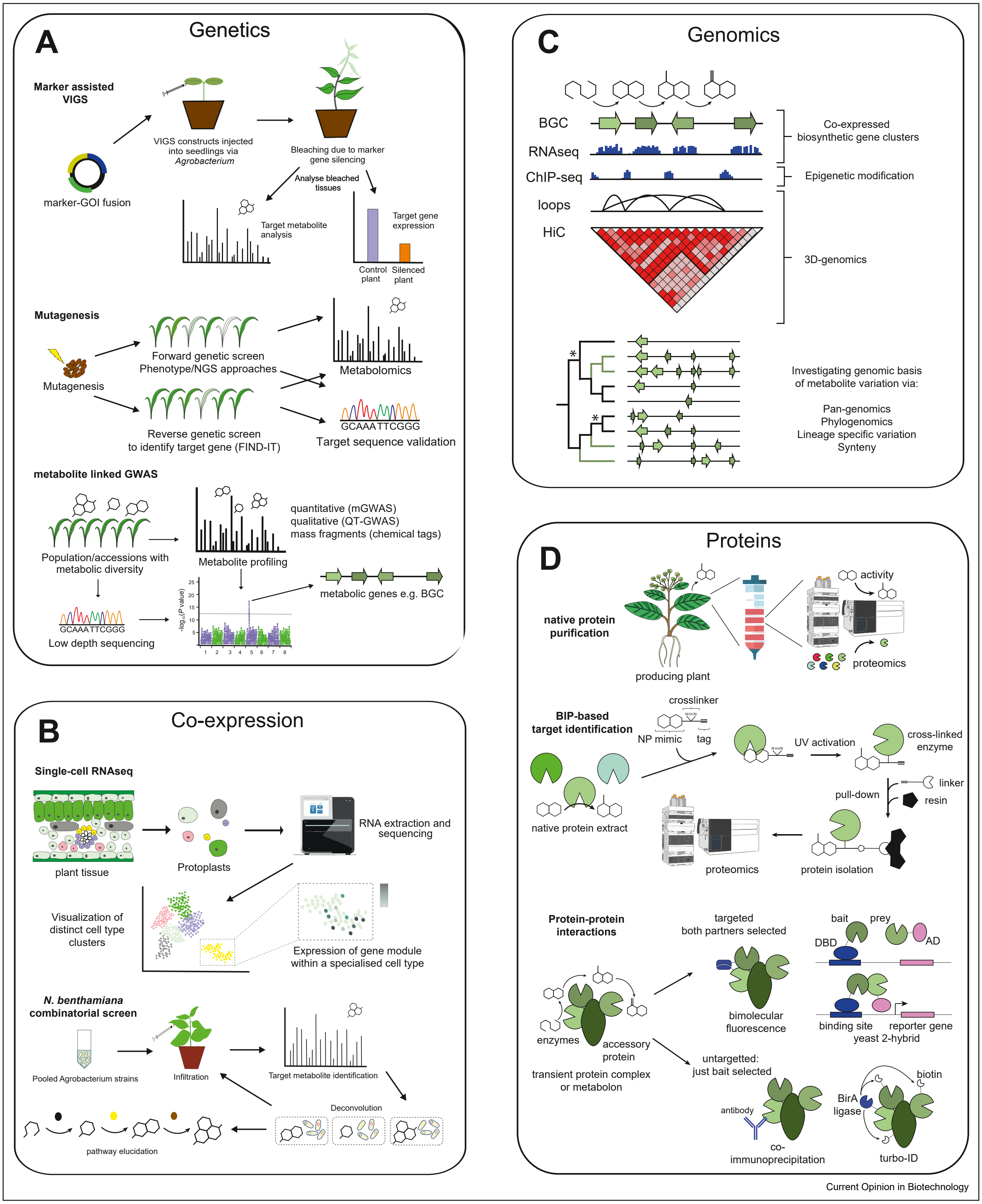
Currently, co-expression and omics-based technologies are the main route for understanding and characterising biosynthetic pathways. However, classical forward and reverse genetics remain valuable tools (Figure 1a). A modified virus-induced gene-silencing (VIGS) system was used to identify a serpentine synthase enzyme in *Catharanthus roseus* wherein the silencing construct also included a marker gene, phytoene desaturase, to pinpoint the silenced tissue [6]. This fusion method was also successfully used in elucidating a branchpoint enzyme in the bioactive diterpenoid pathway for jatrophanes and ingenanes in *Euphorbia peplus* [7]. VIGS can be used to quickly validate related pathway enzymes within plant families [8] to understand the pathways that could help to reconstruct pathways to produce relevant molecules for pharmaceutical applications.

Classical mutagenesis remains valuable where creating genetic variants is feasible. A recently developed approach for screening variant populations, FIND-IT [9], was used to characterise an unexpected acetyltransferase involved in biosynthesis of lupin quinolizidine-type alkaloids [10]. Genome-wide association studies (GWAS), although limited by the availability of population-level data, can be useful for determining the genetic basis of plant-specialized metabolism [11]. In a recent study, a modified metabolic GWAS (mGWAS) was developed where the metabolic fragment enrichment features were used as chemical tags and applied to a natural population of 391 diverse wheat accessions resulting in identification of around 500 potential metabolism-related genes for future analysis [12]. Whilst mGWAS focussed on quantitative metabolite traits, a new method QT-GWAS can detect associations with qualitative metabolite traits [13]. An intriguing 'genetical metabolomics' method has been proposed, which further combines biosynthetic gene cluster (BGC) prediction with metabolite-QTLs, looking for overlaps in signals, which can lead to prioritisation of BGCs for further investigation; though this method is yet to be experimentally validated [14].

Co-expression reimagined

Gene co-expression analysis remains the 'go to' approach for gene mining in biosynthetic gene discovery [5]. The

Figure 1



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Schematic representation of methods for pathway discovery. **(a)** Genetics-based methods. **(b)** Emerging trends in co-expression. **(c)** Methods for aiding gene discovery within a single-genome assembly and across multiple genomes. **(d)** Direct interactions with proteins and metabolites for gene discovery.

spatial organisation of specialised metabolism means pathways may be localised in specific tissues, cells or specialised structures. Therefore, careful assessment of metabolite distribution followed by precision sampling can greatly increase the power of co-expression analysis. This has been demonstrated across plant taxonomic groups, with precision sampling in faba bean [15], daffodil [16] and clubmoss [17,18] being crucial for alkaloid pathway elucidation.

Cellular localisation represents an extreme of metabolite spatial organisation. Single-cell (sc) methods are revolutionising this approach, with expression patterns of specific cell types now available (Figure 1b). scRNA sequencing has been used to reveal the spatial distribution of monoterpene indole alkaloid (MIA) biosynthesis genes in *C. roseus* leaves [19], a pathway in *Nicotiana attenuata* corolla cells [20] and a transcriptional regulatory network of terpenoid biosynthesis in cotton-secretory glandular cells [21]. In *Hypericum perforatum*, scRNA-seq led to the identification of distinct cells responsible for hyperforin biosynthesis, and identification of the pathway genes [22]. A multi-omics investigation into *C. roseus* combined chromosome-scale genome assembly with scRNA sequencing alongside sc metabolomics [23]. Such integrated approaches will greatly improve the precision in identifying candidate genes for bioactive compounds.

An efficient screening system is equally important for functionally characterising the predicted plant biosynthetic genes. *Nicotiana benthamiana* is the current system of choice [24] due to the availability of a highly efficient transient expression toolbox and ease of expression of complex plant enzymes (i.e. membrane-bound, glycosylated). Moreover, *N. benthamiana* can be used for combinatorial screening of large pools of genes rapidly (Figure 1b) [25]. This is particularly useful for when computational prediction methods are unable to narrow down the candidate gene set. The combinatorial approach was used in the solution of the 20-step biosynthetic pathway for QS-21, a potent vaccine adjuvant, with 68 candidate genes pooled in one experiment [26]. Furthermore, the entire 20-step QS-21 pathway was reconstituted in *N. benthamiana* highlighting its use as a production system [27].

Genomics-based approaches

Thanks to the falling costs and increasing availability of long-read and scaffolding sequencing technologies, obtaining chromosome-level genome assemblies for medicinal plants is becoming a routine part of biosynthetic elucidation (e.g. [26]). Selected recent progress here includes the 10-Gb sequences of taxol-producing *Taxus* species [28,29], new or improved assemblies for MIA-producing species [23,30], a genome for *Ginkgo biloba* [31] and improved assemblies for the chassis *N. benthamiana* [32].

The major benefit in obtaining a genome sequence for biosynthetic pathway elucidation is in the chance of identifying a BGC for the pathway of interest (Figure 1c). BGCs are defined as the close genomic association of three or more independently evolved biosynthetic genes [33], though other genomic features can also have biosynthetic importance, including gene pairs and tandem arrays. BGCs can be identified by identifying the location of genes already known to be involved in the pathway. For example, in *G. biloba*, five CYPs proximal to a known diterpene synthase were characterised and found to catalyse the early oxidative steps in ginkgolide biosynthesis [34]. BGCs are especially useful for identifying genes that might not be easily identifiable via homology or functional annotation, for example, the clustered isopiperitenone reductase in *Schizonepeta tenuifolia* monoterpenoid biosynthesis, which is unrelated to the equivalent enzyme in *Mentha* [35].

Bioinformatic tools for mining genomes can identify BGCs [36,37]. For example, PlantiSmash was used to uncover a BGC of TPSs and P450s involved in paclitaxel biosynthesis [28]. Such tools enable a cluster-first approach where predicted but uncharacterised BGCs can be screened to discover new chemistry, akin to methods in prokaryotic systems [38]. This approach has been employed to discover the unusual amide bond-forming capability of chalcone synthase through heterologous expression of a predicted tomato BGC in yeast [39]. However, a proportion of predicted BGCs are likely to be non-functional [40], and the inclusion of data such as co-expression or phylogenomic context can prioritise BGC candidates or identify non-clustered gene partners.

Active and repressed BGCs may have distinct chromosomal conformations, which are also associated with epigenetic markers (i.e. H2A.Z for activation and H3K27me3 for repression) (Figure 1c) [41]. Furthermore, chromatin accessibility, which is related to both DNA conformation and epigenetic markers, may also be modulated in biosynthetic genes [42]. However, the connections between the spatial genome, epigenetics and plant biosynthesis are still emerging and have yet to be widely used for biosynthetic gene discovery, with the exception of a *C. roseus* secologanin transporter that was studied in part as it was present in a topologically associated domain with other biosynthetic genes [23].

Natural variation of metabolism within a species can be used to identify genes and genomic regions responsible for biosynthetic pathways (Figure 1c) [43]. When many genomes are available, it becomes possible to take a pangenomic approach, identifying groups of genes only present in a proportion of a population. For example, in a rice diterpenoid investigation, pangenome (three assemblies) analysis was coupled to the mGWAS (424 samples) to identify a BGC present in *japonica* but absent in *indica* [44]. Pangenomic approaches have been used to investigate evolution and discover variation in *Arabidopsis* triterpene biosynthesis gene clusters [45,46].

As specialised metabolism is taxonomically restricted, comparisons of genetic and genomic content between taxa with different metabolic content can be useful for identifying genes involved in biosynthetic pathways. A phylogenetic approach was used to identify the lineage-specific expansion of *Taxus* CYP725As that are functional in paclitaxel biosynthesis [28,47]. Lineage-specific variation in *Salvia* CYP76AKs has also been explored to investigate diterpenoid diversity [48]. Phylogenetics integrated with metabolite data and computational pathway reconstruction led to the identification of a CYP involved in iridoid biosynthesis [49]. Phylogenomic approaches, looking at genome synteny across families, have identified variations in terpenoid BGCs in Brassicaceae [50] and the mint family [51,52], with implications for gene discovery and exploring chemical variation.

Protein-directed

The methods discussed so far are genetics-based. However, approaches that target the proteins and metabolites can provide a more direct route to the activities of interest (Figure 1d). The classic method of activity-guided isolation of enzymes from plant extracts still has considerable value, especially for isolating enzymes of unknown family type or so-called auxiliary proteins that influence metabolic flux [53]. This approach led to the isolation of thebaine synthase (THS) from *Papaver somniferum*, a PR10 protein that catalyses a reaction that can also occur without the enzyme catalyst present [54]. The identification of THS led to a further to the identification of neopinone isomerase, which also catalyses a so-called spontaneous reaction [55], alongside related proteins that interact with alkaloid metabolites and influence metabolic flux [56].

The binding of metabolites to proteins can be exploited for protein identification through the development of probes. In such chemoproteomics approaches, chemical probes that mimic the enzyme substrate or product can be used to enrich proteins of interest, which can then be identified and quantified via proteomics [57]. This method, biosynthetic intermediate probe (BIP)-based target identification, can be used for identifying the targets of bioactive NPs [58]. A key demonstration of its utility was shown in its role in identifying Diels–Alderases from *Morus alba* that are responsible for catalysing intermolecular cycloadditions to produce complex flavonoids [59]. An equivalent approach was used in *Ophiorrhiza pumila* to discover a CYP able to produce strictosamide epoxide from strictosamide [60].

In some specialised metabolic pathways, biosynthetic enzymes in a single pathway have been shown to physically interact in enzyme–enzyme assemblies. In some specific cases, such multi-protein interactions are termed metabolons, wherein the interactions facilitate substrate channelling [61]. In either case, it may be possible to identify biosynthetic enzymes by probing protein–protein interactions (PPI). Bimolecular fluorescence (biFC), a method that demonstrates whether two labelled proteins are proximal, was used in kratom to prioritise medium-chain dehydrogenase/reductase candidates that interact with strictosidine glucosidase [62]. However, the requirement to clone fusion tags onto candidate genes makes this method less widely applicable than untargeted approaches, which include chemical cross-linking and immunoprecipitation as described in approaches to elucidate indican biosynthesis in *Persicaria tinctoria* [63]. Last, three complementary approaches to assess PPIs in *Arabidopsis thaliana* glucosinolate biosynthesis have been applied: yeast two-hybrid (Y2H), coimmunoprecipitation and BiFC [64].

Protein interactions may be important to identify auxiliary proteins that are not directly responsible for key pathway steps but could aid flux through pathways in heterologous systems. For example, membrane steroid-binding proteins that aid Arabidopsis lignin biosynthesis were discovered through a Y2H system [65]. Similarly, Y2H revealed interactions between cytochrome P450 (CYP)s and distinct electron transfer chain proteins in the biosynthesis of phenolics [66]. Newer methods for identifying PPIs, such as turbo-ID, have yet to be applied for plant biosynthetic pathways but hold much promise [67]. Use of PPIs to detect similar interactions in medicinal plant systems could lead to identification of valuable auxiliary proteins.

AI ahead

Computational approaches for specialised metabolite pathways and gene identification are improving in sophistication and utility [68], and we anticipate machine learning methods will replace classical co-expression approaches for gene candidate identification. Prediction of specific specialised metabolic pathway membership was developed using transcription co-expression datasets, looking at three common strategies (naive, unsupervised and supervised predictions) [69]. The work focussed on tomato, and found that multiple datasets, especially those that are pathway-function-associated, are particularly beneficial for accurate predictions. More specifically, these are datasets with information about biological processes related to the metabolites of interest such as hormone elicitation or pathogen treatment.

Recently, computational/AI methods for gene identification have been used to identify genes that have been subsequently validated. An *Atropa belladonna* alkaloid transporter was discovered by first selecting gene candidates from a transcriptome using supervised classification strategies, with a neural network performing best [70]. This method yielded just three candidates, of which two were functional, compared with over 100 candidates in a typical co-expression and protein homology-determined list. A similar method was used to identify alkaloid alcohol dehydrogenases from the *Rauvolfia tetraphylla* genome, through the classification of sequences as alkaloid-related [30]. The machine learning method added nine candidates that classical co-expression did not identify, including an enzyme later characterised as an ajmalicine/mayumbine synthase.

Tools that can predict gene function based on sequence alone have been used to identify alternative entry points to alkaloid biosynthesis in *Papaver somniferum* [71]. Support-vector-machine-based algorithms were developed, based on sequences, to help find the elusive aromatic aldehyde synthases and phenylpyruvate decarboxylases that contribute to alkaloid production. The inclusion of chemistry into machine learning methods could be useful for biosynthetic pathway discovery and engineering. Pathways for NPs and NP-like compounds can be predicted or designed using chemical logic [72,73]. Enzyme activity prediction is also improving, with some methods providing broad EC classifications that could be used to prioritise candidates [72], or even predict protein ligand interactions that could be used to identify enzyme-substrate pairs [74,75].

Conclusion

As described above, an assortment of tools providing gene-level resolution can be used to aid gene discovery. Improved tools in this regard either enable higher-throughput screening (i.e. combinatorial *N. benthamiana*) or reducing candidate choice through experiment (i.e. PPIs or scRNA-seq) or computation (i.e. sequence-based AI algorithms). Approaches that require high effort or cost for only slight improvements in candidate choice remain more suited to mechanistic investigations of already-identified biosynthetic genes. Within the next five years, we anticipate AI-based computational methods that integrate phylogenetics, genomics, structure/activity prediction and co-expression to become standard. There is rapid progress in methods for untargeted metabolomics that can be applied to plant-specialised metabolism [76]. An integration of metabolomics with plant biosynthetic pathway discovery would unlock the full complexity and diversity of plant NPs and their potential as therapeutic agents.

Author contributions

Both authors conceptualized and wrote the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data Availability

No data were used for the research described in the article.

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