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Omics-guided metabolic pathway discovery in plants: resources, approaches, and opportunities

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Abstract
Plants produce a vast array of metabolites, the biosynthetic routes of which remain largely undetermined. Genome-scale enzyme and pathway annotations and omics technologies have revolutionized research to decrypt plant metabolism and produced a growing list of functionally characterized metabolic genes and pathways. However, what is known is still a tiny fraction of the metabolic capacity harbored by plants. Here, we review plant enzyme and pathway annotation resources and cutting-edge omics approaches to guide discovery and characterization of plant metabolic pathways. We also discuss strategies for improving enzyme function prediction by integrating protein 3D structure information and single cell omics. This review aims to serve as a primer for plant biologists to leverage omics datasets to facilitate understanding and engineering plant metabolism.

Introduction
Plants harbor tremendous metabolic diversity, which is essential to cope with many of the world's challenges, including food security, drug development, and ecosystem functioning [1–3]. The social and economic importance of plant metabolites has motivated research to elucidate how they are biosynthesized. Yet, the overall understanding of the genetic basis for plant metabolism is still limited [4]. Plants are predicted to synthesize over 1 million metabolites [5], but only about 0.1% of the biosynthetic pathways have been functionally elucidated [6]. Molecular genetics and analytical biochemistry are classic approaches to uncover the biochemical functions of individual metabolic genes and pathways [7,8]. However, this process tends to be labor intensive and limits the functional study of enzymatic genes to species that are amenable for genetic analysis [8].

Advances in metabolic pathway mapping infrastructure and omics approaches facilitate the discovery of novel pathways in diverse plant species. Several publicly available knowledge bases provide annotations for enzymes and pathways. The widely cited resources for enzyme annotation include UniProt, BRENDA, and Rhea [9–11] (**Table 1**).The infrastructure for metabolic pathway mapping is available at MetaCyc, Kyoto Encyclopedia of Genes and Genomes (KEGG), PlantReactome, and the Plant Metabolic Network (PMN) [12–16] (**Table 1**). Besides resources for genome-scale enzyme annotation and gene-to-pathway mapping, tools are available to predict metabolic gene clusters (MGCs), which are formed by physically co-

localized genes catalyzing reactions in the same biosynthetic pathway [6,17,18]. These resources and tools enable the initial identification of metabolic pathways and gene clusters in the species of interest. Omics technologies (e.g. genomics, transcriptomics, proteomics, and metabolomics) can further enhance characterization of metabolic pathways by prioritizing candidate genes responsible for producing metabolites of interest in certain tissue types and conditions (**Fig. 1**). Here, we discuss recent advances in enzyme and pathway annotation resources and omics approaches to guide novel pathway characterization. First, we summarize widely applied pathway annotation resources and tools that can systematically identify metabolic genes, pathways, and metabolic gene clusters. Then, we discuss cutting-edge approaches for leveraging omics datasets to guide novel pathway discovery. We also provide perspectives on future directions for metabolic gene function prediction to enhance high throughput discovery of pathways important for agriculture, healthcare, and the environment.

Plant enzyme function annotation and pathway mapping

Effective omics-guided metabolic pathway discovery requires accessing enzyme annotations and gene-to-pathway mapping infrastructure. The growing list of experimentally characterized enzymes expands the understanding of the genetic basis underlying the biosynthesis of diverse groups of metabolites. Several public databases continue to expand the inventory of enzymes with experimental evidence by curating the data from the literature (**Table 1**) [6,9,11,13,15,16]. These resources are valuable for inferring the function of unknown genes based on sequence homology to generate genome-scale annotations for all enzymes in a species [16].

Once enzyme annotations are established, pathways can be inferred by connecting enzymes involved in making the same metabolite. A widely applied strategy is to assemble enzymes into pathways by searching against “gold standard” databases that contain all experimentally characterized pathways, such as MetaCyc and PlantCyc [4,6,15,16,19]. Then a validation step is followed to compute whether a pathway is present in each species based on the fraction of its constituent reactions predicted in that species. This approach can systematically map enzymes into pathways in sequenced genomes (**Fig. 1A**). The prediction of metabolic gene clusters requires a different strategy to capture physically co-localized metabolic genes that might be involved in a biosynthetic pathway [17,18,20*–22*]. Several tools have been developed to predict MGCs in sequenced plant genomes. Most predict MGCs based on sequence homology to known clusters and local metabolic gene density [20*,22*]. PlantiSMASH represents a homology-based prediction tool, which compares protein sequence similarity for the co-localized metabolic genes within a genomic region to the enzyme families associated with all characterized clusters [23]. In contrast, PlantClusterFinder (PCF) is a *de novo* prediction method based on local enzyme density [6]. PCF scans the genome to find regions that are highly enriched with metabolic genes and the loci containing at least three enzymes catalyzing two different reactions are identified as MGCs. The inferred metabolic pathway and gene clusters can be integrated with omics datasets to further dissect functional patterns of metabolism and accelerate novel pathway discovery.

Leveraging co-expression and gene-metabolite correlation

Genes participating in the same biological process often show coordinated expression patterns in response to genetic or environmental perturbations [21,24,25]. Built upon this foundation, co-expression analysis has been widely applied to identify candidate genes associated with pathways of interest (**Fig. 1B**). For example, co-expression analysis facilitates identifying a triterpenoid metabolic network derived from the metabolites produced by known MGCs [26**]. In *Arabidopsis,* four MGCs have been characterized to produce triterpenoids and genes within each cluster show high co-expression [27,28]. Interestingly, several metabolic genes scattered in the genome are also highly co-expressed with terpene synthases in the clusters, but their function remains characterized [26**]. Co-transformation assays in *N. benthamiana* showed that these metabolic genes can use terpenoids produced by the gene clusters as substrates to synthesize new metabolites [26**]. Disrupting the biosynthesis of terpenoid-derived metabolites using *Arabidopsis* mutants resulted in shifted microbe communities in the rhizosphere, which may affect plant-microbe interactions [26**]. This study shows how genes catalyzing novel reactions can be discovered based on co-expression analysis with known pathways.

Besides co-expression, correlation between gene expression and metabolite accumulation has been used to prioritize candidate genes involved in synthesizing metabolites of interest (**Fig. 1B**) [29*]. A prominent example is the discovery of the falcarindiol biosynthesis pathway in tomato. Falcarindiol is a highly modified lipid, which can be induced by different biotic stresses in tomato to promote resistance against fungal and bacterial pathogens [30**]. Based on the chemical structure of falcarindiol, an acetylenase was hypothesized to catalyze early steps of the biosynthesis using linoleic acid as the precursor [31]. To identify the acetylenase involved in falcarindiol biosynthesis, correlation analysis was applied to identify the candidate enzyme whose gene expression pattern showed the highest similarity to falcarindiol accumulation under diverse biotic stress conditions [30**]. The top candidate identified from this analysis showed expected enzymatic activity based on experimental validation in a heterologous system and native plants [30**]. This study demonstrates the advantage of correlation analysis between gene expression and metabolite accumulation in elucidating previously unknown pathways.

Exploiting metabolic diversity of natural populations

Genome-wide association studies (GWAS) have been widely applied to dissect genetic architectures underlying phenotypes of interest [32]. Combining GWAS with metabolic profiling facilitates identifying genes underpinning metabolic diversity using the content of metabolites as phenotypic traits [33,34]. Metabolite GWAS (mGWAS) can discover various types of genes, such as transcription factors and biosynthetic genes, associated with a metabolic trait (**Fig. 1C**) [34–38]. For example, a mGWAS was conducted using kernels of seven hundred maize genotypes grown at multiple locations and resulted in over 1,000 associations between genomic loci and metabolic traits. Two candidate genes highly associated with phenolamides, *PHT* (*Putrescine Hydroxycinnamoyl-Transferase*) and *CCoAOMT* (*Caffeoyl-CoA O-MethylTransferase*), were tested for function using mutants in rice and maize. Metabolite composition analysis confirmed that these two enzymes catalyzing early reactions involved in phenolamide biosynthesis using both arginine and putrescine as substrates [35**]. Despite the usefulness of mGWAS, it can be challenging to functionally validate causal genes for the trait of interest as multiple SNPs can be associated with the same metabolic feature [38]. Co-

expression and gene-metabolite correlation analysis can serve as orthogonal approaches to mGWAS to prioritize causal genes and eliminate false positives, which facilitates downstream functional validation using molecular genetics [29*,39].

Harnessing evolutionary diversification of metabolism

Plant metabolism diversification arises from gradual modification of existing enzymes, which is mainly caused by gene duplication followed by sub- or neo-functionalization. This can lead to the emergence of new metabolic pathways and metabolites in specific lineages [40–42]. Combining the taxonomic distribution of a metabolite and evolutionary patterns of enzymes help prioritize candidates involved in synthesizing the compound of interest in specific lineages (**Fig. 1D**). This approach was used to discover the biosynthesis of various economically important metabolites showing lineage specific distribution [43,44**]. A prominent example is the identification of the enzyme catalyzing the first committed step of anthraquinone biosynthesis in a medicinal plant *Senna tora*. *S. tora* belongs to the Fabaceae family and accumulates high levels of anthraquinones, which are a group of aromatic polyketides that have been used as a traditional herbal medicine to treat various diseases [44**]. To elucidate anthraquinone biosynthesis in *S. tora*, phylogenomics was used to distinguish the two hypothesized routes to produce this compound in plants. This approach identified a group of chalcone synthase-like proteins that have the catalytic capacity to generate anthraquinone scaffold and showed lineage-specific expansion in *S. tora*. Combining transcriptomics and *in vitro* enzymatic assays, CHS-L9 was identified as the enzyme candidate for catalyzing the initial step of anthraquinone biosynthesis via the polyketide pathway [44**]. This study demonstrates the power of leveraging evolutionary diversification to discover pathways.

Future directions

Fruitful progress has been made in discovering metabolic pathways, yet, this only represents a small fraction of the metabolic capacity in plants [16]. To accelerate metabolic pathway characterization, high quality enzyme function annotation is essential. Sequence similarity serves as the major criterion to propagate function annotation between homologs. This strategy has limited power to distinguish enzymes with high sequence similarity in large protein families or polyploid genomes. To establish accurate homologous relationships between species, gene expression patterns can serve as an additional feature besides sequence similarity [45]. With the advances of single cell technologies in plants, high resolution gene expression maps are becoming available across diverse cell types, which can further enhance ortholog prediction and enzyme function annotation [45,46*]. Besides sequence homology, developing a holistic understanding of enzymes can improve function prediction, especially for orphan genes with limited prior knowledge. This goal can be achieved by integrating diverse types of information to infer function. For example, protein 3D structures generated by AlphaFold provide new opportunities to infer substrate-enzyme pairs using protein sequences [47]. Taken together, integrating various types of omics resources facilitates toolset innovation and functional characterization of novel metabolic pathways.

Conclusion

Massive amounts of omics datasets can help delineate novel metabolic pathways in non-model species, which represent major sources for economically significant metabolites [8,30**,44**]. In this review, we summarized publicly available knowledge bases that enable the identification of metabolic genes and pathways and discussed cutting-edge omics-guided approaches for elucidating the biosynthetic routes for metabolites of interest. The growing list of genome-scale resources can help guide traditional molecular genetics and biochemical studies. Future advances in enzyme annotation and integrated omics will inform the characterization and engineering of plant metabolism, which promotes sustainable agriculture, healthcare innovation, and climate stabilization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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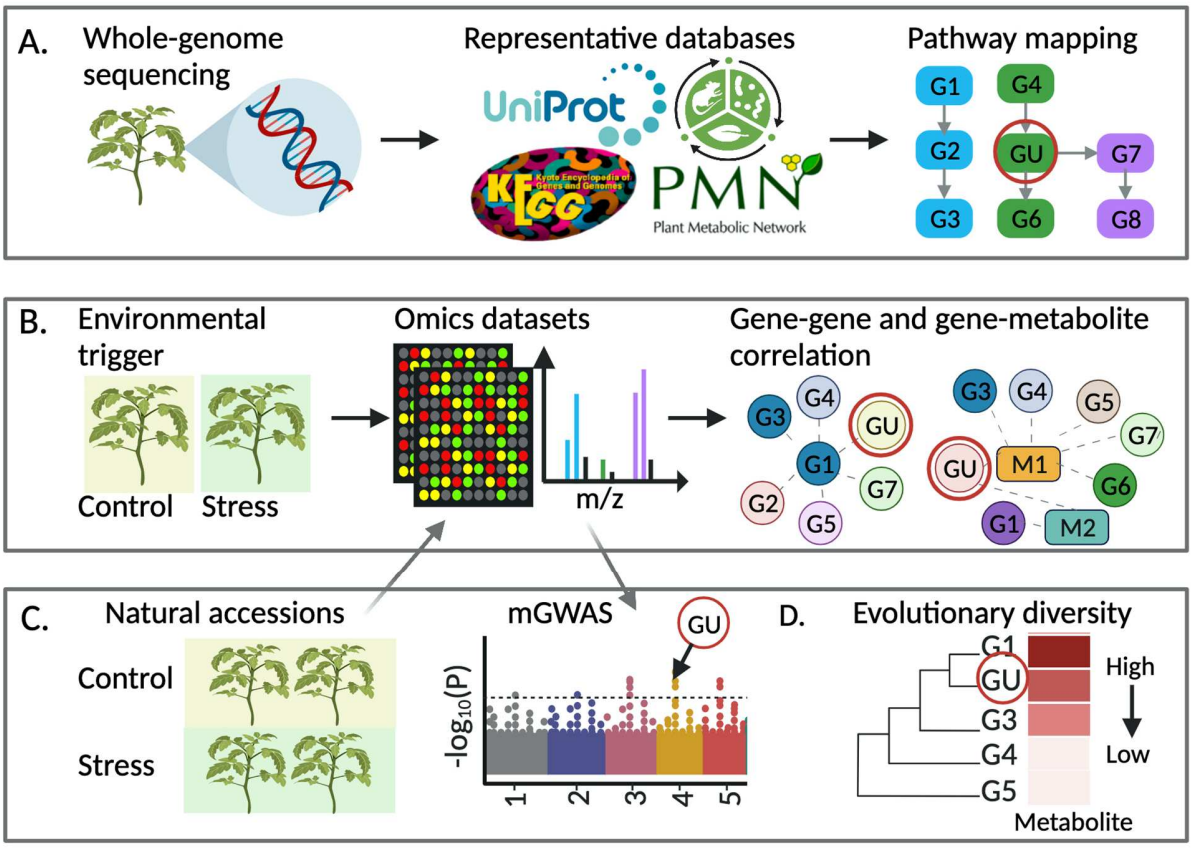

Figure 1. Omics-guided strategies to elucidate novel metabolic pathways: A. Plant metabolic pathway databases, B. Correlation analysis, C. metabolic genome wide association studies (mGWAS), D. phylogenomics. G1 to G8 represent metabolic genes. Red circle highlights the gene of unknown function (GU) discovered by each omics approach. M1 and M2 represent metabolites.

Table 1. Summary of publicly available knowledge bases that provide enzyme annotation and gene to pathway mapping infrastructure.

Database	Resources available	Number of curated enzymes	Number of tested pathways	Plant specific?	Metabolism specific?
UNIPROT[9]	Enzyme annotation	107,868	NA	No	No
BRENDA[11]	Enzyme annotation	113,179	NA	No	No
Rhea[10]	Enzyme annotation	Unavailable	NA	No	Yes
MetaCyc[15]	Enzyme annotation and pathway mapping	13,540	2,980	No	No
KEGG[12]	Enzyme annotation and pathway mapping	Unavailable	543	No	No
Plant Reactome[14]	Enzyme annotation and pathway mapping	1,824	298	Yes	No
PMN[16]	Enzyme annotation and pathway mapping	3,769	1,163	Yes	Yes

Box 1. Outstanding questions that can be addressed to further leverage omics resources to investigate plant metabolism

1. What new infrastructures are required to provide high-resolution annotation for genes and pathways from leveraging single cell or single-molecule level omics datasets?
2. What resources need to be developed (e.g. data generation standards, data processing tools, repository databases, benchmarking data) to make publicly available omics datasets Findable, Accessible, Interoperable, and Reusable (FAIR) [48]?
3. What are the best methods of integrating different types of omics datasets (e.g. transcription factor binding, epigenomics, chromatin accessibility assays) to provide a holistic view of information flow from genes to phenotypes in the context of metabolism?

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