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Multi-scale engineering of plant cell cultures for promotion of specialized metabolism

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To establish plant culture systems for product synthesis, a multi-scale engineering approach is necessary. At the intracellular level, the influx of 'omics' data has necessitated development of new methods to properly annotate and establish useful metabolic models that can be applied to elucidate unknown steps in specialized metabolite biosynthesis, define effective metabolic engineering strategies and increase enzyme diversity available for synthetic biology platforms. On an intercellular level, the presence of aggregates in culture leads to distinct metabolic sub-populations. Recent advances in flow cytometric analyses and mass spectrometry imaging allow for resolution of metabolites on the single cell level, providing an increased understanding of culture heterogeneity. Finally, extracellular engineering can be used to enhance culture performance through media manipulation, co-culture with bacteria, the use of exogenous elicitors or modulation of shear stress.

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Introduction

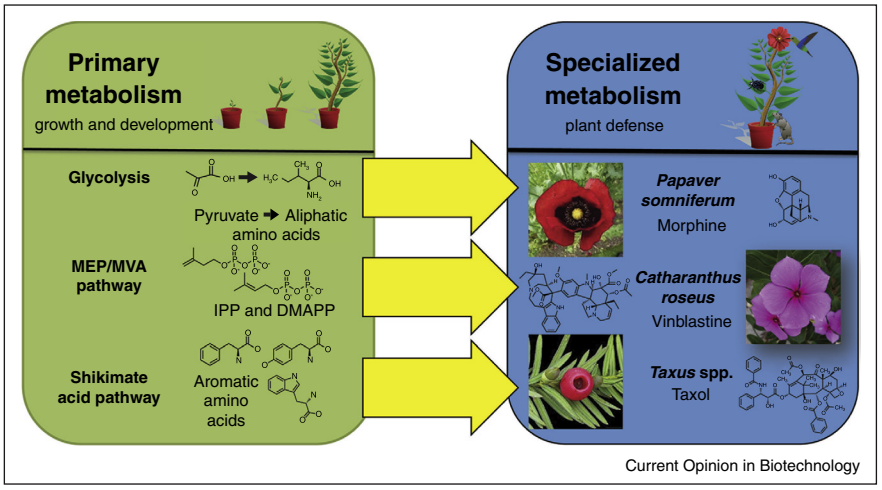
Plant cell culture technology can be used to supply high-value products, obtain fundamental metabolic information, propagate elite species and promote somatic embryogenesis. Plant metabolism is largely divided into primary metabolic processes that contribute to development, growth and maintenance, and specialized metabolic processes (formerly referred to as secondary metabolic processes) that synthesize diverse compounds largely involved in plant defense. To effectively engineer plant cells in culture, strategies must be combined to address the three functional scales of cellular engineering, defined here as intracellular (pathway), intercellular (cell aggregation), and extracellular (external environment). This review focuses on recent advances across these functional scales and presents a new perspective for cellular engineering of plant cells in culture.

Intracellular engineering

Plant metabolic engineering on an intracellular level is a challenge due to the extensive number and diversity of metabolites produced in plant systems, as well as the complex interactions amongst plant primary and specialized metabolic pathways (Figure 1). Although plant primary metabolism is well characterized and well conserved across species, specialized metabolism is species-specific and metabolic pathways are sparsely defined [1,2••]. It has been estimated that there are between 200 000 and 1 000 000 plant metabolites, making identification and characterization of specific metabolites an enormous bottleneck to plant metabolomics [3–5]. In addition, regulation of carbon flux between central and specialized metabolism is poorly understood. With the influx of 'omics' information for many model and non-model plant species, methods must be developed to begin to elucidate these complex pathways and interactions to allow for the design of effective metabolic engineering strategies.

Next-generation sequencing technologies have led to a dramatic increase in the availability of both genomic and transcriptomic datasets [6,7]. In analyzing these complex datasets, homology based annotation is often used, which can lead to overannotation of paralogous proteins such as transporters, multi-domain proteins and large enzyme families [8••]. The use of these data sets to create genomic models [9] and incorporate flux balance analysis has been recently reviewed [10,11]. To aid in the standardization of gene annotation and analysis of complex plant genomic datasets, PlantSEED was developed, which incorporates a manual review of every gene–reaction association to ensure proper annotation of genes involved in 209 pathways of plant primary and specialized metabolism [8••]. This database can be used for reliable annotation and automatic generation of metabolic models for genome sequenced plant systems (e.g. The Model SEED [12]). Recently, genomic datasets were utilized to investigate the evolutionary emergence and maintenance of specialized metabolic pathways in 16 diverse plant species [2••]. Through gene localization studies within these genomes, it was found that up to 30% of genes were located in clusters, and clusters for *Arabidopsis* and soybean were enriched for genes involved in specialized

Figure 1



Whereas plant primary metabolism is conserved across plant species, plant specialized metabolism is species-specific. As a result, the pathways involved in plant primary metabolism (e.g. glycolysis, MEP/MVA pathways, shikimate acid pathway) are well characterized, whereas those involved in specialized metabolism are largely undefined. Additionally, the regulation of carbon flux through primary metabolism and into specialized metabolism is poorly understood.

metabolism. The presence of gene clusters was often related to both the species and the type of compound produced [2••]. For instance, clusters for genes in phenylpropanoid and terpenoid metabolism were found in *Arabidopsis*, whereas genes involved in nitrogen-containing metabolism were unclustered. Conversely, clusters for genes involved in nitrogen-containing metabolism were found in soybean. Similarly, clusters for terpenoid related genes were found in sorghum but phenylpropanoid related genes were unclustered. Gene clusters involved in specialized metabolism have been identified in both model and non-model plant species, such as oat [13,14], Solanaceous crops [15], *Lotus japonicus* [16] and *Papaver somniferum* [17]. Identification of potential biosynthetic gene clusters could aid in the discovery of novel biosynthetic pathways, as well as elucidation of genes involved in the biosynthesis of high value specialized metabolites [18].

For non-model plant systems where genomic information is often unavailable, RNA-sequencing (RNA-seq) can be used to generate a transcriptome. Recently, a data-mining framework was developed to allow for construction and analysis of transcriptomes for 75 non-model medicinal plant species that produce terpenoids, alkaloids and polyketides [7,19]. To understand more about the interactions between primary and specialized metabolism, databases can be developed that take advantage of the more extensive knowledge of primary metabolism that feed into specific specialized metabolic pathways. For example, a metabolic pathway database was recently developed from RNA-seq data for *Catharanthus roseus* that produces terpene indole alkaloids (TIAs) with high pharmaceutical value [20]. This database contains 390 pathways, with a focus on those pathways involved in primary metabolism, as well as incorporating the pathways, precursors and elicitors (e.g. jasmonic hormones) involved in the production of TIAs and triterpenoids. By combining this dataset with existing RNA-seq data from the overexpression of two transcription factors (TFs) involved in TIA biosynthesis, *ORCA2* or *ORCA3*, the remaining four genes in the seco-irodoid biosynthetic pathway were identified [21••]. Through expression of the eight genes within the pathway, as well as two genes for increasing the precursor pool and two genes involved in alkaloid biosynthesis, the production of the monoterpene indole alkaloid strictosidine was achieved in the heterologous plant host *Nicotiana benthamiana*. This study highlights the potential for these datasets to aid in the elucidation of complex metabolic pathways. In addition, the genomic and transcriptomic data will be useful for mining the enzyme diversity of plant systems, increasing the capabilities of synthetic biology for reconstitution of these complex pathways in heterologous systems [22].

Plant cell culture systems provide the opportunity to investigate the effect of metabolic engineering strategies in a high-throughput manner before genetic engineering efforts. For instance, a method was developed to rapidly characterize the effect of multi-gene engineering strategies on the heterologous production of carotenoids in rice embryogenic callus cultures [23]. This system was not only used to identify the function of two uncharacterized genes [23], but also to identify a novel carotenoid, 4-keto-alpha-carotene that was produced through endogenous rice enzyme activity [24]. Similar methods have