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# Silent constraints: the hidden challenges faced in plant metabolic engineering

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Metabolic engineering is embraced as a method to sustainably enhance production of valuable phytochemicals with beneficial properties. However, successful production of these compounds in plants is not always predictable even when the pathways are fully known, frequently due to the lack of comprehensive understanding of plant metabolism as a whole, and interconnections between different primary, secondary, and hormone metabolic networks. Here, we highlight critical hidden constraints, including substrate availability, silent metabolism, and metabolic crosstalk, that impair engineering strategies. We explore how these constraints have historically been manifested in engineering attempts and propose how modern advancements will enable future strategies to overcome these impediments.

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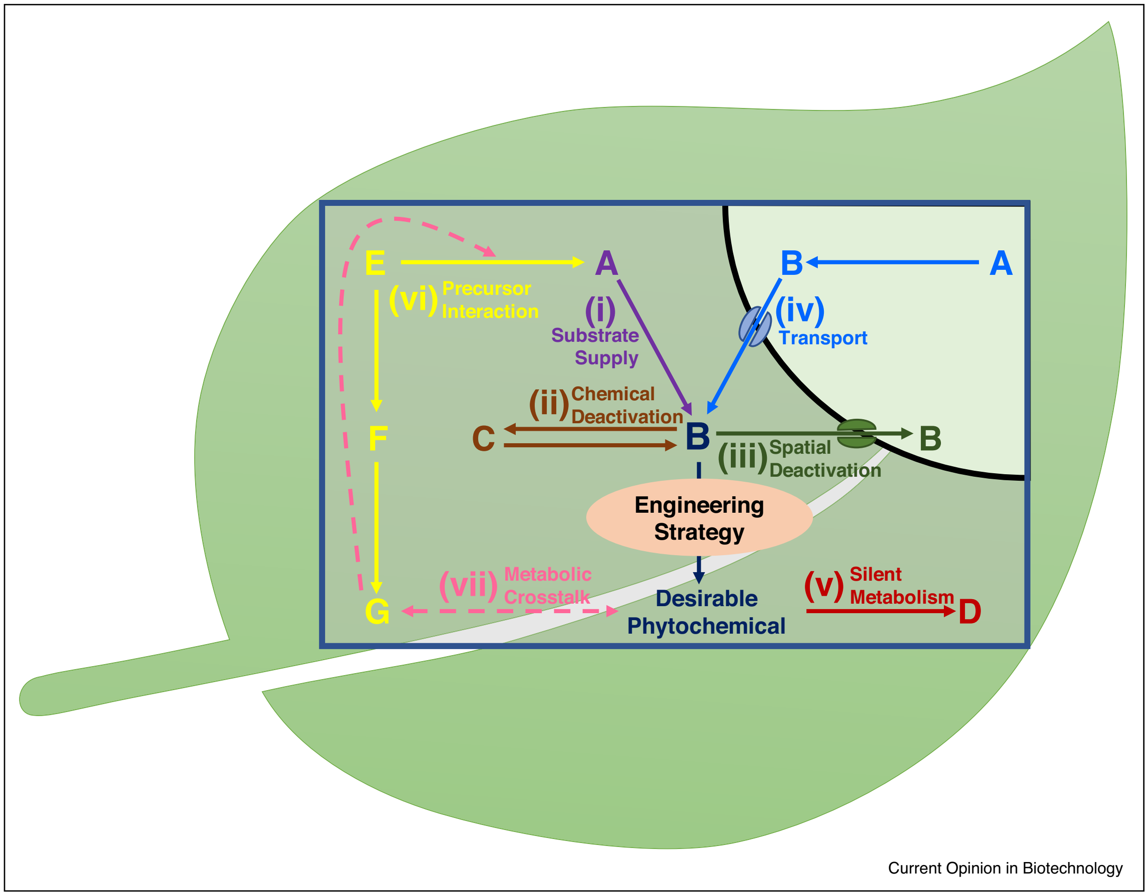
## Introduction

Plants have adapted over the past billion year to survive and thrive across diverse habitats and ecological niches by evolving the metabolic machinery to produce a dizzying array of metabolites, thus sustaining their fitness across varying environments [1–3]. A subset of these phytochemicals is of distinct benefit to humanity, either for the agronomic traits they confer on crops, or for their nutraceutically, pharmaceutically, or industrially beneficial properties [4]. Complex chemistry, the necessity for sustainability, and consumer preference for naturally derived compounds has precluded chemical synthesis of many of these products, and yet in many cases, natural production is insufficient to meet demand. Classical breeding could be used to improve yields of some target compounds; however, metabolic engineering provides more rapid strategy for increased production of beneficial phytochemicals in both native and heterologous plant systems. There are numerous examples of successes in this field with the most prominent being 'Golden Rice,' which was genetically engineered for high carotenoid levels with the aim of decreasing nutritional deficiencies [5]. Recent reviews have summarized some of the more successful engineering outcomes [6,7•]. However, the outcome of metabolic engineering is not always predictable due to lack of knowledge about (1) availability of substrate in relevant subcellular compartment of targeted tissues; (2) hidden biochemical activities ('silent metabolism') of engineered cells and (3) hidden crosstalk between different metabolic networks. In this review, we discuss this general concept of hidden constraints that are not readily apparent in the natural setting yet restrict metabolic engineering and propose future directions to overcome these constraints.

## Substrate availability as a constraint for metabolic engineering

Although one of the most obvious potential constraints on metabolic engineering is substrate supply (Figure 1), the number of factors that influence this potentially limiting factor is more complex than generally considered. Substrate has to be not only present in engineered tissue, but be also readily available for the introduced enzyme and present at sufficient levels for high production of target compound(s). These could be achieved by three common strategies, via: (*i*) targeting enzymes to the compartment with the highest precursor pool; (*ii*) increasing flux toward precursor formation or (*iii)* relocation of a precursor-producing pathway to another compartment allowing high level of substrate [8–10]. Good examples for these general strategies could be easily found in the terpenoid biosynthetic network. In plants, two core isoprenoid biosynthetic pathways provide five-carbon building blocks for terpenoid biosynthesis in cytosol, plastids and mitochondria [11]. Present at different levels in these compartments, the precursors differentially support biosynthesis of terpenoids. Indeed, overexpression of patchoulol synthase (PTS) in tobacco plastids along with farnesyl diphosphate (FPP) synthase, which produces the sesquiterpene precursor FPP, resulted in 100-fold higher accumulation of patchoulol relative to its cytosolic production [12]. Similarly, directing only committed step of the entire introduced pathway for production of the antimalarial drug artemisinin into tobacco mitochondria achieved almost eightfold increase in artemisinin levels

**Figure 1**



Graphical representation of silent constraints imposed during an attempt to increase production of a hypothetical desirable phytochemical. (i) insufficient precursor production; (ii) chemical substrate deactivation; (iii) spatial substrate deactivation; (iv) substrate transport; (v) silent metabolism modifying product; (vi) pathway interaction via precursors; (vii) higher order of metabolic interactions. Engineering strategy can include individual enzymes or multi-step pathways.

over its cytosolic-driven production [8]. In the latter case, in addition to the artemisinin-specific genes, deregulated 3-hydroxy-3-methylglutaryl-coenzyme A reductase, catalyzing a rate limiting step in the cytosol-localized mevalonic acid (MVA) pathway, was also introduced to tobacco to increase supply of substrate for artemisinin production. Moreover, even higher yield of artemisinin, equivalent to the average levels in *Artemisia annua*, was obtained in tobacco by pyramiding artemisinin biosynthetic genes with the whole MVA pathway in chloroplasts [9,13]. While these strategies lead to increased production of target compounds, they can have unwanted and sometimes detrimental effects on primary metabolism thus affecting plant growth and development [13]. However, splitting the MVA pathway and artemisinin biosynthetic genes between chloroplast and nuclear genomes not only further increased the level of artemisinin, but also resulted in plants with no morphological abnormalities [14]. To date, coordination between primary and secondary metabolism is still a relatively unexplored area of study and the assumption that primary metabolic pathways are always capable of providing sufficient level of substrate to support an excess of secondary metabolite biosynthesis without consequences on normal plant function is overly simplistic.

Recently it has been shown that plant cells have a metabolically inactive pool of compounds, which can be reactivated and used to increase precursor pool for phytochemical formation (Figure 1). These 'deactivated' pathway substrates can act as either unanticipated sinks for metabolic flux, or as unanticipated sources for metabolic potential. This was clearly demonstrated in terpenoid production with the discovery of the isopentenyl phosphate kinase/isopentenyl pyrophosphate hydrolase enzyme pair [15,16•]. These enzymes catalyze a futile cycle, yet their manipulation – either naturally through plant regulatory mechanisms or synthetically – alter the availability of an irreplaceable substrate in terpenoid metabolism, isopentenyl pyrophosphate (IPP), by either siphoning it to the inactive form (isopentenyl phosphate, IP), or conversely drawing it from the IP pool to increase IPP availability. Despite both enzymes of this futile pair being cytosolic, they also influence production of plastidial terpenoids [16•], thus highlighting general existence of underappreciated intercompartmental connectivity that permits organelles to function not as isolated boxes with their own unique metabolism, but rather in the full context of the total cellular metabolic network.

The existence of intercompartmental interactions was recently emphasized by increasing cellular levels of phenylalanine (Phe) in petunia flowers by downregulation of phenylalanine ammonia lyase (PAL) [17]. Despite prior model prediction [18] of enhanced production of phenylacetaldehyde and 2-phenylethanol, which are directly derived from Phe via a PAL-independent pathway, an increase in overall cellular Phe level did not result in elevated synthesis of these compounds [17]. Instead excess of Phe was spatial deactivated by shuttling it from the cytosol to the vacuole by an endogenous transporter [17] (Figure 1). Thus, even though the metabolic perturbation did result in increased total Phe levels in the tissue, this did not have the expected impact on target metabolites due to previously unknown localization-related metabolic constraints. Future understanding of whole metabolic and transport capacities of engineered cells will help to overcome these deactivation constraints.

The impact of transport extends beyond spatial deactivation. In general, plant metabolism is highly compartmentalized as biochemical steps of a single pathway can be distributed between multiple subcellular locations [11,16•,19,20] or substrate biosynthesis and its utilization can occur in different compartments (Figure 1) as happens, for example, for Phe/phenylpropanoids and Trp/auxin. Phenylpropanoid metabolism is dependent on sufficient Phe precursor availability in the cytosol. However, Phe is predominantly synthesized in the plastids, requiring its transport through the plastidial membrane to be utilized [21]. Indeed, increased expression of only the plastidial aromatic amino acid transporter in petunia flowers, without any perturbations of enzymatic steps, was sufficient to increase production of Phe-derived volatiles [22]. Likewise, overexpression of the gene encoding a plastidial pyruvate transporter in algae was found to increase biomass production and lipid content for biofuel engineering [23]. In addition, inter-cellular transport can be limiting, as a recent study demonstrated that tissue-specific overexpression of sucrose transporters in *Pisum sativum* could be used to enhance source-to-sink carbon movement, thereby increasing sucrose loading in developing seeds and enhancing nitrogen loading efficiency [24••].

Even when there is apparently sufficient substrate available, biosynthetic flux towards target compounds may be limiting due to inefficient substrate utilization by the introduced enzyme. One example of this comes from the famous metabolic engineering of rice. The phytoene synthase (PSY) is thought to catalyze the limiting step in carotenoid biosynthesis [5]. Overexpression of daffodil PSY, which enables high carotenoid levels in daffodil flowers, along with bacterial carotene desaturase led in carotenoid production in rice endosperm, resulting in Golden Rice [25]. However, replacing of daffodil PSY by maize PSY increase carotenoid accumulation by >10-fold, suggesting that daffodil enzyme although increasing metabolic flux, was not catalytically efficient enough in engineered species [5].

## Impact of silent metabolism on metabolic outcome

One of the many reasons that metabolic engineering is not always predictable is due to a phenomenon generally known as ‘silent metabolism’. First defined by Lewinsohn and Gijzen [26], silent metabolism refers to an underlying natural metabolic potential of a plant cell that becomes apparent only upon perturbation of the metabolic system. In general, reactive moieties such as carboxyl and hydroxyl groups are more susceptible to silent metabolism catalyzed by endogenous glycosyltransferases and methyltransferases, as a consequence of the high reactivity of these functional groups and substrate promiscuity of endogenous enzymes [27]. Multiple examples have been published to date showing the impact of silent metabolism on targeted metabolic engineering (Figure 1). A classic example comes from attempts to engineer production of linalool, an acyclic monoterpene alcohol with pleasant floral, sweet scent, which is the direct product of linalool synthase (LIS). Unexpectedly, introduction of the *Clarkia breweri LIS* gene in petunia plants did not lead to an increase in either emission or storage of free linalool despite accumulating *LIS* transcripts [28]. Instead, it was efficiently converted to a non-volatile metabolite *S*-linalyl-β-D-glucopyranoside by the action of a yet unknown endogenous glucosyltransferase [28]. Similarly, when three enzymes involved in the biosynthesis of the monoterpene acid moiety (chrysanthemic acid) of natural pyrethrin insecticides in *Tanacetum cinerariifolium* were transiently co-expressed in *Nicotiana benthamiana* leaves, more than 50% of produced chrysanthemic acid was converted to its ester derivatives including *trans*-chrysanthemic acid malonylglucoside conjugate [29]. When overexpressed in tobacco, *Valeriana officinalis* geraniol synthase was targeted to different subcellular compartments, including plastids, cytosol, and mitochondria, geraniol-derived glycosides were detected in all cases, indicating that active glycosyltransferases are able to utilize heterologously produced geraniol independent of site of its biosynthesis [30]. Also, partial reconstitution of gossypol biosynthetic pathway in tobacco leaves resulted in efficient glycosylation of one of the pathway intermediates, 8,11-dihydroxy-7-keto-δ-cadinene, produced by overexpressed

CYP71BE79, presumably precluding utilization of this precursor by downstream enzyme [31].

In addition to glycosylation, methylation is a common modification driven by silent metabolism. Indeed, introduction of perillyl aldehyde pathway in tobacco leaves resulted in detectable amount of methyl perillate in the headspace, indicating the existence of endogenous methyltransferases which could methylate perillic acid [32]. Moreover, additional classes of endogenous enzymes as a part of silent metabolism, can compromise the targeted outcome. The overexpression of a non-catalytic small subunit of snapdragon geranyl diphosphate (GPP) synthase in tomato fruits unexpectedly increased the accumulation of several monoterpenes, which were not the result of action of endogenous monoterpene synthases, but of downstream modifications of GPP by endogenous phosphatases, reductases and alcohol dehydrogenases [33]. Similarly, expression of *Ocimum basilicum* geraniol synthase gene in ripening tomato fruits led to the accumulation of a set of geraniol-derived compounds including geranial, geranyl acetate, citronellol, citronellal and rose oxide, as well as other unexpected monoterpenes such as limonene, myrcene and *allo*-ocimene, *cis*-$\beta$-ocimene and *trans*-$\beta$-ocimene [34]. In case of tomato fruits, silent metabolism has beneficial outcome by increasing the diversity of flavor compounds that was recognized by the untrained taste panelists, 60% of which preferred the engineered tomato fruits over controls [34].

## Unintended consequences of hidden metabolic crosstalk

Silent metabolism can influence metabolic engineering in more ways than simply modifying a produced target metabolite, but rather impacting crosstalking metabolic pathways (Figure 1). While crosstalk between two compartmentally separated core isoprenoid biosynthetic pathways is well known [9,16•], interconnection between cellular metabolic pathways is more widespread and largely unknown. A dramatic example of this comes from an attempt to increase production of Phe and Phe-derived metabolites in petunia flowers by increasing flux through feedback insensitive cytosolic phenylpyruvate pathway [35••]. Overexpression of the first enzyme of the pathway, chorismate mutase 2 (CM2), indeed successfully increased flux through the entire phenylpyruvate pathway, demonstrating that substrate supply and catalytic capacity were both sufficient to achieve the target goal. However, net Phe production was unexpectedly decreased due to a concomitant decrease in Phe synthesis via the plastidially localized arogenate pathway. The cause of this effect was attributed to the ability of an aminotransferase in a Trp-dependent auxin biosynthetic pathway to use phenylpyruvate instead of pyruvate as a co-substrate. Thus, the increase in production of the Phe biosynthetic intermediate phenylpyruvate due to *CM2* overexpression inadvertently perturbed auxin homeostasis, thereby disrupting plastid development and precluding efficient plastidial metabolism [35••]. The ability to unintentionally perturb metabolism of signaling compounds, and thus trigger a multitude of downstream effects, is well documented [36–41] even as the underlying interconnections are poorly understood.

Metabolic crosstalk can also constrain production of compounds connected by shared upstream biosynthetic steps. For example, the Mitchell cultivar of petunia flowers is nearly devoid of color, despite retaining the entire anthocyanin biosynthetic pathway. Although the absence of anthocyanins in this cultivar had been attributed to a mutation in the controlling transcription factors AN2 and AN4 [42], two independent studies have induced color formation by decreasing expression of genes encoding phenylpropanoid/benzenoid biosynthetic enzymes downstream of their respective split from flavonoid biosynthesis: caffeoyl-coenzyme A O-methyltransferase (CCoAOMT) [43] and benzoyl-CoA thioesterase (TE) [44]. The effect of the TE on anthocyanin synthesis was particularly interesting, since it is not only involved in a separate pathway, but is also compartmentally separated from flavonoid biosynthesis; TE is exclusively localized to the peroxisomes [44], while anthocyanin synthesis occurs entirely in the cytosol [45].

Another level of crosstalk between disparate pathways has also been documented with phenylpropanoid synthesis. A missense mutation in Arabidopsis CYP83B1 can be used to reduce indole glucosinolate levels with simultaneous accumulation of the enzyme's substrate, the Trp-derived indole-3-acetaldoxime [46]. Surprisingly, though, this is accompanied by a decrease in phenylpropanoid metabolism [46]. Analysis of the underlying mechanism of this crosstalk showed that the accumulated glucosinolate intermediates, acting via the Mediator complex, increase production of Kelch Domain F-Box proteins (KFBs) that stimulate proteolytic turnover of the gatekeeping phenylpropanoid biosynthetic enzyme, PAL [47•]. Further crosstalk between Phe-derived and Trp-derived metabolism has been suggested, but still remains uncharacterized [48].

## Future perspectives

Here we have presented a brief overview of metabolic engineering attempts falling short of expectations, with a special emphasis on how our limited knowledge of plant metabolism impedes accurate predictions of engineering outcomes. The complexity of cellular metabolism leads to underlying silent constraints that are undetectable under normal conditions, yet restrain success of engineering endeavors. Thus, all metabolic engineering efforts, even if they are partially successful, set the stage for discovering an unknown biological reality about the plant systems and the obtained outcomes should be embraced as an opportunity to gather valuable information about underlying regulatory principles. As every silent constraint is comprises an underappreciated biological function, their characterization not only provides a foundation for rational designing the next iteration of the engineering strategy, but also yields critical insight into the natural function of plant metabolism. Historically, our ability to uncover the mechanisms underlying silent constraints was limited by ability to systematically generate large metabolite datasets, and by limited computational power for their analysis. However, recent technological advances in omics approaches including genomics, transcriptomics, proteomics, and metabolomics, combined with innovations in synthetic biology and computational tools, and progress in functional characterization of biosynthetic genes are allowing us to overcome the historical impediments and delineate unknown biochemical pathways [49•]. This also enables analysis of plant metabolism in depth and provides more comprehensive description of connectivity of primary, secondary, and hormone metabolic networks [3], as well as potential silent constraints. Implementation of metabolic flux analysis techniques previously used in microbial and mammalian systems, together with the enhancements in analytical capabilities, will help to resolve carbon flux distribution within the metabolic networks and their subcellular compartmentalization, uncover metabolic channeling, and reveal existing inactive metabolite pools [50]. These tools will not only guide the selection of metabolic engineering targets, but also enhance the potential of metabolic modeling, leading to the development of models that are not only explanatory, but predictive, thus ensuring the success of future engineering strategies.

## Conflict of interest statement

Nothing declared.

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