Cell type-specific control and post-translational regulation of specialized metabolism: opening new avenues for plant metabolic engineering

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Abstract

Although plant metabolic engineering enables the sustainable production of valuable metabolites with many applications, we still lack a good understanding of many multi-layered regulatory networks that govern metabolic pathways at the metabolite, protein, transcriptional and cellular level. As transcriptional regulation is better understood and often reviewed, here we highlight recent advances in the cell type-specific and post-translational regulation of plant specialized metabolism. With the advent of single-cell technologies, we are now able to characterize metabolites and their transcriptional regulators at the cellular level, which can refine our searches for missing biosynthetic enzymes and cell type-specific regulators. Post-translational regulation through enzyme inhibition, protein phosphorylation and ubiquitination are clearly evident in specialized metabolism regulation, but not frequently studied or considered in metabolic engineering efforts. Finally, we contemplate how advances in cell type-specific and post-translational regulation can be applied in metabolic engineering efforts *in planta*, leading to optimization of plants as metabolite production vehicles.

Keywords

Specialized metabolism, cell-specific regulation, post-translational regulation, metabolic engineering, single-cell omics, enzyme inhibition

Introduction

Plants contribute to a vast wealth of metabolic diversity with many applications. From medicine to cosmetics, we use and consume plant specialized metabolites every day. With an increasing demand for such compounds, several engineering approaches for increasing specialized metabolite production can be forwarded. To engineer a system in a predictable manner, we must know the basic parts of the system and understand how they function together. In the case of plant metabolic pathways, the basic parts are the metabolites, the pathway enzymes (and their co-factors) that catalyse the reactions of the metabolites, and the regulators [1]. With a detailed blueprint of a specific pathway, such as the enzymes involved, their subcellular and cellular distribution, the regulators driving this distribution, and the metabolic precursors and intermediates, we would be able to reliably engineer the system to increase the production of a certain metabolite. However, due to the multi-layered regulation that governs plant specialized metabolism, this is currently not entirely feasible.

Most of our research efforts are currently focused on the first, and the most important step – discovery of the metabolic enzymes. In several cases, we have also moved to the next step – understanding the transcriptional regulation. What is still lacking for most pathways is our understanding of post-translational regulation as well as of the (sub)cellular distribution of the enzymes and the metabolites. Separation of the pools of substrates and the enzymes seems to be important for metabolic engineering, and using this knowledge can result in large increases in metabolite yields. Therefore, in this review, we non-exhaustively highlight some current developments in research on the spatial distribution of metabolites and their regulation, as well as post-translational regulation of metabolic enzymes and their potential to improve plant metabolic engineering.

Cell type-specific regulation of plant specialized metabolism

It is evident that compartmentalization at the cellular and subcellular level is important for proper functioning of metabolic networks in plants and other multicellular organisms [2]. To understand how plant metabolic pathways function, we ultimately need to determine the cellular distribution of metabolites and of the transcriptional regulation of their biosynthetic enzymes and regulators across plant tissues.

At the metabolite level, it is now possible to measure and visualize the compound of interest in intact tissues or at single-cell resolution [3-5]. This approach includes technologies such as mass spectrometry imaging (MSI) and single-cell metabolomics (scMet) to identify the abundance and location of compounds in various plant species, such as tomato (*Solanum lycopersicum*) [6], *Catharanthus roseus* [7-9], *Camelia sinensis* [10], *Ginkgo biloba* [11], *Derris elliptica* [12] and opium poppy (*Papaver somniferum*) [13]. While these technologies provide valuable and direct evidence about where metabolites accumulate in plant tissues, they are not widely and/or routinely adopted yet due to complexity in sample preparation, high cost of specialized equipment and low concentrations of target compounds in native tissues. We direct interested readers to reviews that cover the promises and challenges of these technologies more extensively [3-5,14].

At the transcriptional level, organ- and cell type-specific expression of enzymes and their regulators guide the metabolic production to specific cells. Techniques such as *in situ* hybridization [15,16] and cell type-

specific cell sorting [17] have been crucial for elucidating cell-type expression of such genes. With the advancement of new omics technologies, such as single-cell and spatial transcriptomics, it is now possible to unravel the intricate regulatory networks in plants (Figure 1). In Arabidopsis, Nguyen, et al. [18] used single-cell RNA sequencing (scRNAseq) on jasmonic acid (JA)-treated plants to identify novel regulators of the triterpene thalianol in the root tip. It is known that *THALIANOL SYNTHASE* (*THAS*), encoding the enzyme that eventually synthesizes thalianol, is predominantly expressed in the outer part of the root tip [19]. Using this information and co-expression data from the scRNAseq experiment, the authors identified several basic helix-loop-helix (bHLH) transcriptional co-activators of the triterpene gene clusters, as well as transcriptional repressors with cell type-specific expression that is opposite to that of *THAS* [18].

In tomato, Cantó-Pastor, et al. [20] used scRNAseq to study the regulation of suberin biosynthesis in the exodermis. The authors integrated previous knowledge from Arabidopsis, a weighted matrix of waterlogged tomato plants with increased suberin biosynthesis and introgression lines between tomato cv. M82 and the drought-resistant/high suberin-containing wild relative of tomato (*S. penellii*), with the developmental trajectory for exodermal maturation information from the scRNAseq dataset. This enabled the identification of two activator transcription factors that are co-expressed with suberin biosynthetic genes at the exodermis and regulate their biosynthesis.

In *C. roseus*, two groups independently investigated the cell type-specific regulation of monoterpene indole alkaloid (MIA) biosynthesis, which produces compounds with anti-cancer properties [21]. Sun, et al. [22] created a high-resolution map of *C. roseus* leaves, and corroborated previous literature showing that different steps of MIA biosynthesis take place in three different cell types [16,23]. The transportation of the intermediate compounds within cell clusters is still elusive. To address that gap, the authors mined the single-cell expression data to identify several transporters at the cell types expressing the biosynthetic enzymes, which are prime candidates for follow-up studies. Li, et al. [9] combined genomics, 3D chromatin organization, scRNAseq and scMet data to provide new insights into the regulation of MIA biosynthesis. To establish biosynthetic gene clusters and have the complete genomic information to identify new candidates in the pathway, the authors generated a chromosome-scale genome assembly of *C. roseus* and used Hi-C to capture the 3D genome organization. Interestingly, they identified a gene encoding a MATE transporter (*CrMATE1/SLTr*) in the biosynthetic gene cluster, which was also a candidate from the data of Sun, et al. [22]. Transcriptional silencing of *CrMATE1/SLTr* increased the build-up of secologanin, suggesting that *CrMATE1/SLTr* is likely involved in the transport of secologanin. Moreover, the authors used two separate scRNAseq technologies to characterize the expression of the 38 genes encoding MIA biosynthetic enzymes and concluded that they are sequentially expressed in three distinct leaf cell types, which corroborated previous literature [16,23]. Finally, authors used a custom single-cell metabolomics platform to obtain metabolic profiles of leaf cells producing MIAs and detected a high concentration of catharanthine (100-mM range) in idioblast cells and not in epidermal cells, where catharanthine is synthesized. This observation was in line with previous publications [8,24] suggesting that catharanthine is transported between cell types by CrTPT2 [25]. To conclude, these works highlight the potential of single-cell and multi-omics approaches to shed light on the complex regulation of plant specialized metabolism.

Post-translational regulation

Most of the work on specialized metabolite regulation in plants is focused on transcriptional regulation. This is not surprising since, for instance, elicitation of specialized metabolism by hormones such as JA is

primarily mediated by transcriptional reprogramming of pathways [26]. This point of view does not imply that specialized metabolism is solely regulated transcriptionally; yet it rather reflects that the other ways of regulation are less investigated. In this review, we distinguish two levels of regulation, metabolite- and protein-based.

Metabolite-based regulation

In addition to transcriptional feedback regulation [27], there are instances of enzymatic feedback (Figure 2A) and feedforward inhibition in specialized metabolism. The reports of this type of regulation are not numerous, and almost all examples come from the phenylpropanoid metabolism. In tomato, the first enzyme of the phenylpropanoid pathway, phenylalanine ammonia-lyase (PAL), is feedback inhibited to varying degrees by phenolic acids and flavonoids, such as naringenin and quercetin [28]. In a comprehensive study of phenylpropanoid biosynthetic enzymes in *Populus trichocarpa*, it was demonstrated that five out of ten enzyme families involved in monolignol biosynthesis show enzyme inhibition [29]: Five PAL enzymes all display feedback inhibition, whereas two 4-coumarate:CoA ligases (4CLs), the 5-hydroxyconiferaldehyde O-methyltransferase (AldOMT2), both tested cinnamyl alcohol dehydrogenases (CADs) and coniferaldehyde 5-hydroxylases (CAld5Hs) are substrate inhibited. Unlike the substrate-inhibited 4CLs from *P. trichocarpa*, a tomato 4CL is feedback inhibited by a downstream product, the flavanone naringenin [30]. *Medicago truncatula* cinnamoyl-CoA reductase (CCR) enzymes – CCR1 and CCR2 – have differential substrate preferences, with CCR2 being inhibited by the preferred substrates of CCR1, feruloyl-CoA and sinapoyl-CoA, presumably providing an alternative, non-redundant pathway for monolignol biosynthesis [31]. Since poplar CCRs are not substrate-inhibited, it is evident that the inhibition networks differ between organisms. In the coumarin branch of the phenylpropanoid pathway of *Ruta graveolens*, p-coumaroyl CoA 2'-hydroxylase (C2'H) involved in umbelliferone biosynthesis is feedback-inhibited by psoralen [32]. The authors propose this to be a mechanism to prevent excessive build-up of toxic psoralen, distinct from transcriptional regulation mechanisms found in species with inducible psoralen biosynthesis. Substrate inhibition also seems to be widespread among UDP-dependent glycosyltransferases (UGTs) involved in phenylpropanoid metabolism, such as UGTs involved in flavonoid metabolism in black soybean [33], strawberry [34] and grapevine [35]. Most recently, *Nicotiana benthamiana* NbUGT72AY1 involved in monolignol and coumarin glycosylation was found to be allosterically inhibited by its substrate. This enzyme has two sites where the coumarin scopoletin binds – the catalytic site and the second, allosteric binding site involved in inhibition [36]. The allosteric site is opened upon substrate binding in the active site, suggesting an uncompetitive inhibition mechanism. It is yet unclear whether this mechanism is common to all substrate-inhibited UGTs involved in specialized metabolism, but it provides a clear direction for future research on these enzymes. It is clear that enzyme inhibition in specialized metabolism is present and complex, but not yet well researched, as reflected in the fact that all of the examples covered here come from the phenylpropanoid pathway.

Protein-based regulation

Enzymes in specialized metabolism are not only post-translationally regulated by metabolites, but also by direct modifications through common cellular regulation mechanisms, involving phosphorylation (Figure 2B), calcium signalling, and ubiquitination (Figure 2C). *P. trichocarpa* PAL is phosphorylated when treated with kinase extracts of French bean explants elicited with *Colletotrichum lindemuthianum* elicitors [37]. The same protein can be phosphorylated by the Arabidopsis calcium-dependent protein kinase (CDPK)

AtCPK1 [38]. Phosphorylation leads to protein instability [37], but further research on this has not been done yet. Calcium-binding regulatory protein calmodulin binds strawberry FvUGT1 and modulates its enzymatic activity by partially alleviating the substrate inhibition [34], suggesting a role for Ca^{2+} in the regulation of specialized metabolism. The aforementioned poplar substrate-inhibited AldOMT2 involved in monolignol biosynthesis was also found to be phosphorylated, resulting in loss of enzymatic activity [39]. Since FvUGT1 and AldOMT2 demonstrate that an enzyme can be regulated by metabolites as well as by protein modifications and interactions, one can imagine that there might be numerous unexplored multi-level regulation mechanisms in specialized metabolism.

Another post-translational modification regulating specialized metabolism enzymes is ubiquitination leading to proteasomal degradation (Figure 2C), a common cellular protein turnover mechanism. The best-studied proteins in this respect are again the PALs. In Arabidopsis, four PAL isozymes are ubiquitinated by three Kelch motif-containing F-box (KFB) proteins, targeting them for proteasomal degradation [40]. The KFB proteins show differential expression in tissues, suggesting regulation through protein degradation as one of the mechanisms for tissue-specific specialized metabolism regulation. It was recently demonstrated that the KFB-dependent turnover of PAL is part of the mechanism behind the cross-talk between the glucosinolate and phenylpropanoid pathways [41]. Seven rice PALs interact with an E3 ubiquitin ligase OsFBK16, and three (OsPAL1, OsPAL5, and OsPAL6) were shown to be degraded in its presence in immunoassays [42]. Another KFB protein, KFB^{CHS}, was found to regulate proteasomal degradation of the chalcone synthase (CHS) enzyme in Arabidopsis [43], regulating anthocyanin biosynthesis through the turnover of this key enzyme. In maize, cinnamyl alcohol dehydrogenase, an enzyme further downstream in the monolignol pathway, is degraded upon ZmFBL41-mediated ubiquitination [44], showing that not only enzymes involved in the committed pathway steps are regulated in this manner. Ubiquitome analysis of rice after treatment with the pathogen elicitors chitin and flagellin 22 demonstrated that many enzymes in the phenylpropanoid pathway, including flavonoid biosynthesis enzymes, are ubiquitinated [45]. Since the enrichment was done with peptides, it is unclear whether these modifications are due to polyubiquitination, and are thus related to proteasomal degradation. Nevertheless, it seems that polyubiquitination and proteasomal degradation are a common mechanism for the phenylpropanoid pathway regulation. For other plant specialized metabolic enzymes and pathways, knowledge of pathway-flux regulation by ubiquitination and proteasomal degradation is scarce to non-existing, with the exception of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) [46]. The HMGR enzyme is catalysing the rate-limiting step in the biosynthesis of triterpene precursors feeding the primary metabolic sterol pathway but also species-specific specialized bioactive triterpenes. In the legume *M. truncatula*, a RING membrane-anchor E3 ubiquitin ligase was found to target HMGR, thereby specifically restraining accumulation of the species-specific triterpene saponins, but not of its sterols [47]. Notably, this control apparatus is equivalent to the system that regulates sterol synthesis in yeasts and mammals but that uses distinct E3 ubiquitin ligases. Hence, this finding suggests that molecular systems to post-translationally regulate specialized metabolism can be recruited from existing systems in primary metabolism and eventually put under divergent regulatory cues.

Conclusions and outlook

Many plant specialized metabolite pathways remain yet (mostly) unexplored due to the large abundance and diversity existing in nature. Currently, most efforts go into elucidating the pathways, and little is done

to investigate their regulation. With technological advances in *in-situ* imaging and single-cell profiling of both transcripts and metabolites, we can now refine our searches for missing biosynthetic enzymes and start exploring regulation mechanisms at the cellular level. Yet, extracting meaningful data at single-cell resolution for proteins and metabolites in plants still remains challenging. Novel technological advances in these areas will be imperative to fully grasp the organisation of plant specialized metabolism. As our understanding of biosynthesis improves, we can focus more on understanding regulation mechanisms at the transcriptional and post-translational level. Eventually, such knowledge can be applied to metabolic engineering in plants, and, over time, consolidate plants as superior organisms for bioactive metabolite production.

Elucidation of cell type-specific regulation of specialized metabolism holds the potential to unlock precise engineering programs in plants. Integration of synthetic biology tools such as logic gates, chemical/light induction and CRISPR activation/interference [48,49], with cell type-specific regulatory elements from the blueprint of a given pathway, could allow one to fine-tune the spatiotemporal regulation of the pathway components to achieve higher yields. In plants we have not explored the available metabolic design space yet [50], with over 5 million biochemical reactions predicted to be possible [51].

Furthermore, feedback inhibition is an important aspect of specialized metabolism to consider during metabolic engineering, because the production of a metabolite of interest might inhibit some of the enzymes in the pathway, limiting the yields. It was already demonstrated that enzymes can be directly engineered to remove allosteric inhibition [30,36], showing that our understanding of enzyme inhibition can be used in engineering (Figure 2D). An alternative approach could be subcellular or cellular separation of precursors and the metabolite of interest to prevent inhibition [2]. Modulation of phosphorylation or ubiquitination events can be achieved by editing of the amino acids corresponding to the phosphorylation (Figure 2E) or ubiquitination sites (Figure 2F) [52] or by mutating kinases, phosphatases or ubiquitin E3 ligases if they are known. Such approaches have not yet been widely tested in plant metabolic engineering, but might be indispensable in endeavours to increase metabolite yields when using plants as hosts. In conclusion, a successful plant metabolic engineering program can be split in separate but co-dependent layers of complexity, starting from identifying all components, elucidating and adapting the (cell type-specific) regulation and compartmentalization of the pathway, and engineering the enzymes and regulators to finally improve the yield.

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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Papers of particular interest, published within the period of review, have been highlighted as: * of special interest

[18] Nguyen *et al.* (2023)

In this work, the redundant cell type-specific regulation of the Arabidopsis thalianol biosynthetic gene cluster was elucidated in the root tip. Upon JA treatment, thalianol biosynthesis is induced specifically in the outer root tip by MYC2 and several bHLH co-activators, while DAG1 acts as a repressor for the cluster in the inner root tip.

[9] Li *et al.* (2023)

Several single-cell multi-omics were used to create a high-resolution map of *C. roseus* monoterpene indole alkaloid (MIA) biosynthesis. The work validates previous literature regarding the compartmentalization of the MIA metabolic pathway, provides new insights into the accumulation of metabolites in single leaf cells and showcases the power of single-cell multi-omics approaches for plant specialized metabolism.

[20] Cantó-Pastor et al (2024)

The regulation of suberin biosynthesis was characterized in the tomato root. Unlike Arabidopsis, suberin is deposited specifically in the exodermis, where cell-type specific regulators activate the expression of suberin biosynthetic genes. This work showcases how traditional approaches (e.g., the analysis of introgression lines with related wild species possessing desirable traits) can be combined with cutting-edge tools such as scRNAseq to answer biological questions.

[36] Liao *et al.* (2023)

Nicotiana benthamiana and *Solanum tuberosum* UGT enzymes that glycosylate coumarins and monolignols are substrate-inhibited through a second, allosteric binding site outside the active site, which forms upon substrate binding in the active site. The inhibition occurs only with one substrate, scopoletin, but not with sinapyl aldehyde. The authors identified a region of the *N. benthamiana* protein involved in substrate inhibition and achieved partial removal of enzyme inhibition by altering amino acids in it.

[42] R. Wang *et al.* (2022)

A high-quality library of over 1,500 rice ubiquitin E3 ligases was cloned by the authors. The library was then used to successfully identify an E3 ligase ubiquitinating three different PAL enzymes, resulting in their degradation. This provides a powerful tool for future identification of ubiquitination-based regulation mechanisms of metabolic enzymes in rice.

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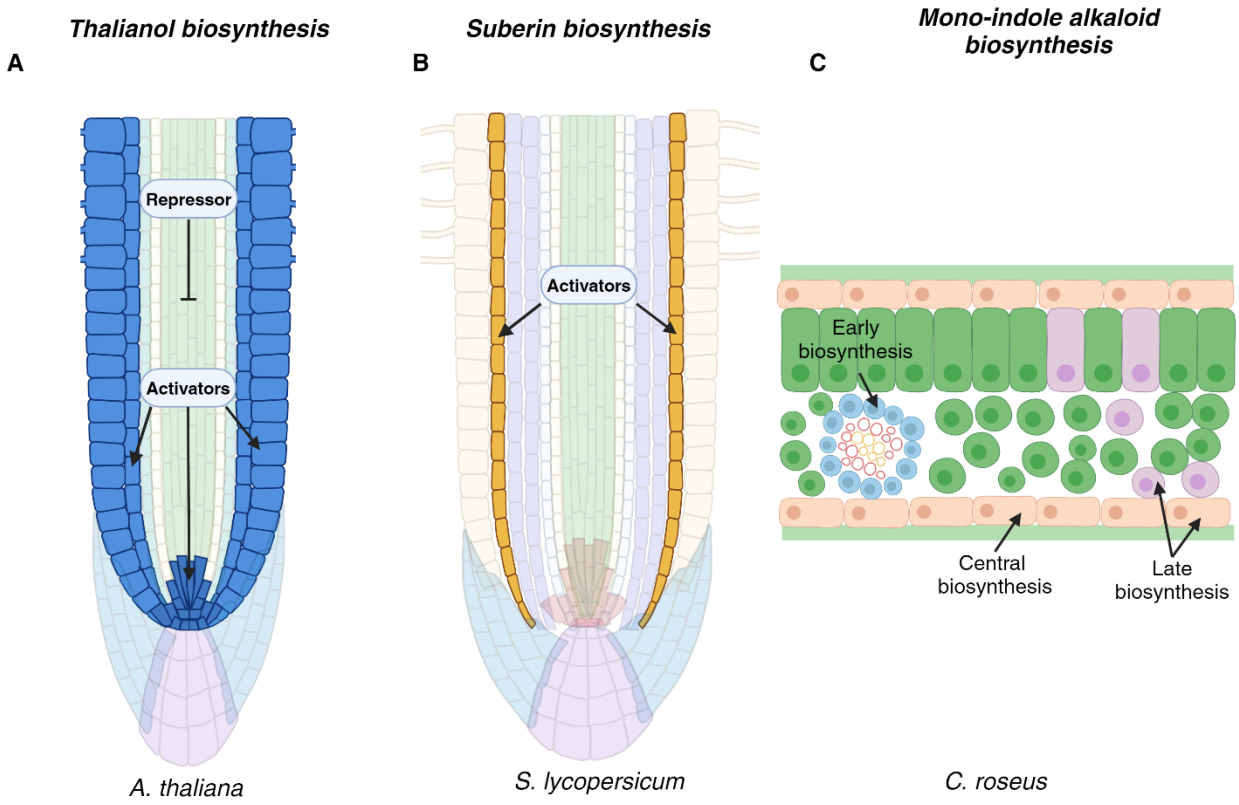

Figure 1. Cell type-specific transcriptional regulation of plant specialized metabolism. A) Transcriptional regulation of thalianol biosynthesis in an *A. thaliana* root tip. Thalianol biosynthesis genes are activated by MYC2 and co-activators in the outer root tip (blue), whereas repressors prevent pathway expression in the inner root tip. B) Transcriptional activation of suberin biosynthesis in tomato. Suberin deposition is observed in the tomato root exodermis (yellow) only, where several transcription factors activate the expression of suberin biosynthesis. C) Pathway compartmentalization in monoterpene indole alkaloid (MIA) biosynthesis in the *C. roseus* leaf. Biosynthesis of MIA compounds is compartmentalized between three cell types, while intermediate compounds are transported between cell types.

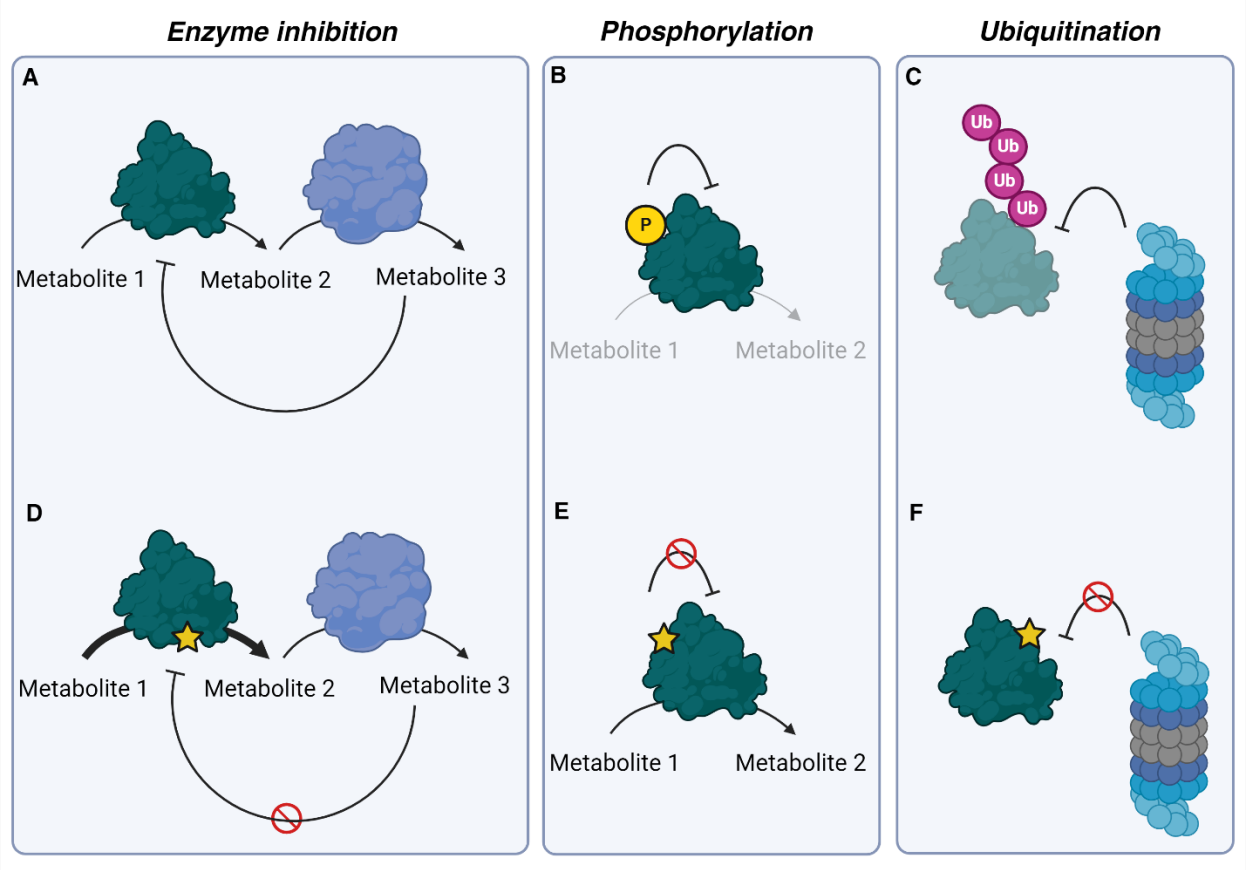

Figure 2. Post-translational regulation of specialized metabolism and corresponding examples of potential engineering approaches. (A – C) Possible mechanisms of post-translational regulation in specialized metabolism. (A) The product of an enzyme downstream (metabolite 3) inhibits the activity of the first enzyme (shown in teal). (B) Enzyme phosphorylation inhibiting its function (biosynthesis of metabolite 2 from metabolite 1). (C) (Poly)ubiquitination of an enzyme leads to its degradation by the 26S proteasome (blue-gray barrel). (D – F) Proposed protein-level engineering approaches utilizing mutagenesis of the sites (marked by a yellow star) involved in post-translational regulation mechanisms. (D) Mutating the binding site of metabolite 3 in the first enzyme prevents its inhibition, leading to higher metabolite 2 production (thick arrow). (E) Removal of phosphorylation site(s) prevents inhibition of activity of the enzyme. (F) Removal of ubiquitination site(s) prevents proteasomal degradation of the enzyme.