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Review Article

Transportation engineering for enhanced production of plant natural products in microbial cell factories

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ABSTRACT

Plant natural products (PNPs) exhibit a wide range of biological activities and have essential applications in various fields such as medicine, agriculture, and flavors. Given their natural limitations, the production of high-value PNPs using microbial cell factories has become an effective alternative in recent years. However, host metabolic burden caused by its massive accumulation has become one of the main challenges for efficient PNP production. Therefore, it is necessary to strengthen the transmembrane transport process of PNPs. This review introduces the discovery and mining of PNP transporters to directly mediate PNP transmembrane transportation both intracellularly and extracellularly. In addition to transporter engineering, this review also summarizes several auxiliary strategies (such as small molecules, environmental changes, and vesicles assisted transport) for strengthening PNP transportation. Finally, this review is concluded with the applications and future perspectives of transportation engineering in the construction and optimization of PNP microbial cell factories.

1. Introduction

Plant natural products (PNPs) represent a class of functional secondary metabolites, mainly including terpenes, alkaloids, and flavonoids [1]. These compounds endow plants with stress resistance and exhibit a wide range of pharmacological and biological activities, such as anti-oxidation, immune enhancement, and anti-cancer properties. The market demand for PNPs is steadily rising, prompting large-scale production through plant extraction or chemical synthesis due to limitations in natural plant growth and the low content of PNPs. However, both methods present challenges. Plant extraction requires substantial amounts of plant raw materials, posing the risk of vegetation destruction. In addition, the intricate structure of PNPs, often containing multiple chiral carbon atoms, results in a relatively low selective yield for chemical synthesis. In response to these limitations, researchers have recently explored hetero-biosynthesis of PNPs in microbial cell factories. Notable PNPs, such as artemisinic acid, amorphadiene [2], vinblastine [3], vindoline, catharanthine [4], nootkatone [5], and santalol [6], have been successfully synthesized in *Escherichia coli*, *Saccharomyces cerevisiae*, *Pichia pastoris*, and other chassis cells.

Despite these advancements, challenges persist in maximizing PNP yield [7]. Most PNPs encounter difficulty in efficient transport out of cells, leading to product accumulation within cells and subcellular organelles. Product accumulation imposes a significant metabolic burden, detrimentally affecting normal cell growth and product formation of host cells, thereby impeding efficient synthesis and practical applications of PNPs [8,9]. Therefore, it is particularly important to strengthen the transmembrane transport process of PNPs. In addition, the intricate PNP biosynthetic pathways generally involve multiple organelles and tissues in plants. The lack of transports for metabolite transportation between intracellular compartments impedes efficient biosynthesis and accumulation of the desired products in microbial cell factories [10,11]. Therefore, the exploration of suitable transporters from plants and microorganisms to overcome challenges associated with PNP

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transportation is expected to provide new ideas for the design of microbial cell factories for efficient production of value-added PNPs [12].

This review summarizes the strategies and applications of enhancing transmembrane transportation in PNP biosynthesis, including 1) transmembrane transport between intracellular organelles; 2) direct efflux facilitated by transporters; and 3) small molecule and low temperature-assisted efflux. The review is concluded with future perspectives in the mining of PNP transporters, new strategies to enhance PNP transportation, and the applications of transporter engineering in microbial cell factories.

2. Discovery and classification of PNP transporters

2.1. ATP binding cassette (ABC) transporters

The systematic and precise regulation of PNP secretion often involves the pivotal role of ABC transporters, representing the most widely used protein family. Most ABC transporters can transport substrates directly using the energy released from ATP hydrolysis. The structural representation of an ABC transporter is shown in Fig. 1, which generally contains two domains: the nucleotide binding domain (NBD) and the transmembrane domain (TMD). TMD generally contains 4–6 α-helices, forming transmembrane channels. NBD consists of WalkerA box [GX_4GK (ST)], WalkerB box [(RK)X_3GX_3L (hydrophobic)$_3$], and characteristic motif [(LIVMFY) S (SG) GX_3 (RKA) (LIVMYA) *X* (LIVMF) (AG)] [13,14].

According to the homology and domain organization, ABC transporters in higher plants can be divided into 8 subfamilies (ABCA-I, except ABCH). There are three subfamilies of common full-molecular ABC transporters: 1) multidrug resistance (MDR) subfamily, 2) pleiotropic drug resistance (PDR) subfamily, and 3) multidrug resistance-associated protein (MRP) subfamily. The difference among these subfamilies lies in the sequence arrangement of TMDs and NBDs (Fig. 1A and B). The MDR and MRP subfamilies are arranged in positive orders, while the PDR subfamily is arranged in reverse orders [15] (Fig. 1C).

As a model organism, *Arabidopsis thaliana* serves as a reservoir for the identification of numerous plant ABC transporters. The first reported ABC transporter, AtPGP1 [16,17], belongs to the *A. thaliana* phosphoglycoprotein family and is involved in the transmembrane transport of auxin. Subsequent findings revealed that ABCC1, ABCC2 [18], and ABCB subfamily were also involved in auxin transport, collectively regulating stem tissue lignification. Additional plant ABC transporters have been identified, such as the PDR subfamily SpTUR2 [19] from the aquatic plant *Spirodella polyrhiza*, playing a role in plant stress resistance, especially cold resistance and salt tolerance. Noteworthy examples also include the MDR subfamily transporter CrMDR1 (associated with terpenoids and alkaloids) [20] and the ABCG subfamily transporter CrTPT2 (involved in the production of the anticancer drug precursor catharanthine) [21] from *Catharanthux roseus*.

In *Coptis japonica*, several ABC transporters have been characterized. ABCB subfamily transporters CjABCB1 and CjABCB2 [22] are involved in the intracellular accumulation of the alkaloid berberine. Additionally, a PDR subfamily of transporter, AaPDR3 [23], has been identified in the hairy roots of *Artemisia annua*, facilitating the transport of β-caryophyllene, a sesquiterpene compound used in anesthesia. Another PDR subfamily transporter, PDR12, has also been found in tobacco plants, playing a key role in transporting the alkaloid nicotine accumulated in root tissues to vacuoles, conferring resistance against diseases and insect pests.

2.2. Multidrug and toxic compound extrusion (MATE) transporters

MATE transporters, initially identified as drug-resistant bacterial efflux pumps, have emerged as crucial players in various biological processes, including plant nutrient absorption, PNP transportation, and detoxification of harmful substances. For example, the absorption of chloride ions by plants is involved in the detoxification of aluminum and the regulation of iron homeostasis [24]. Most MATE transporters consist of 12 transmembrane helices with approximately 363–1141 amino acid residues, featuring a typical MATE conserved domain (Fig. 1D). The potential energy of the transport process is provided by ATP hydrolysis, and the MATE transporter is fueled by H^+ or Na^+. Three-dimensional structure predictions of the MATE transporters indicate the

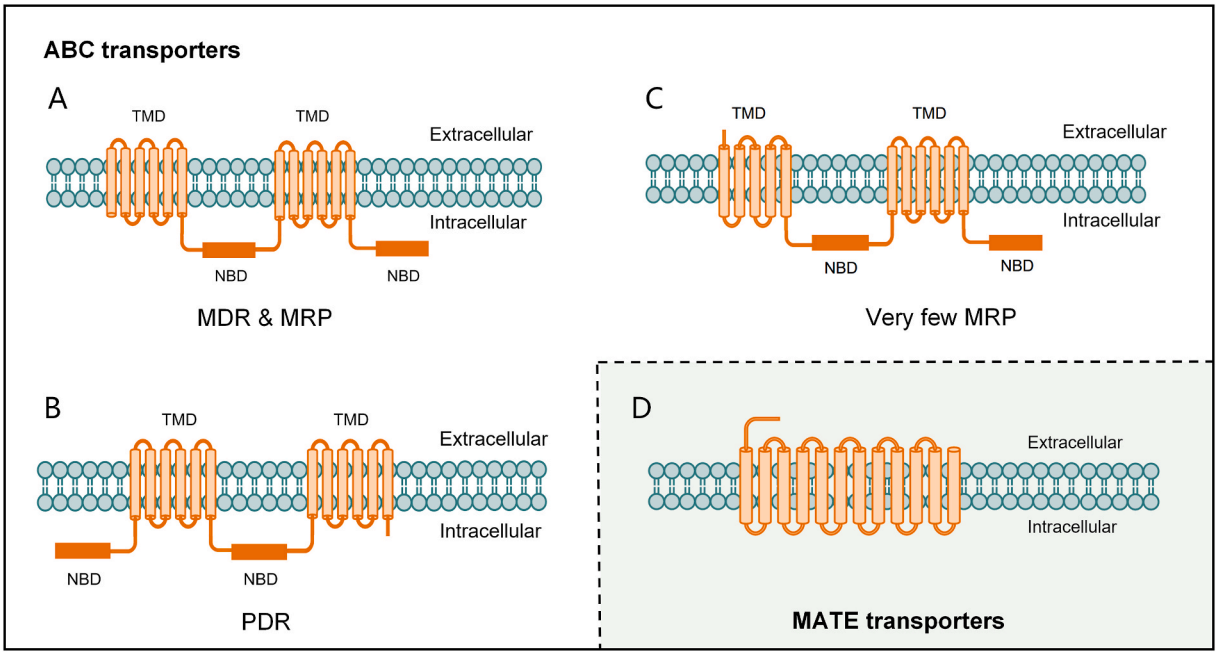

Fig. 1. The structure of ABC transporters and MATE transporters in plants. (A) The structure of plant ABC transporters, featuring positively arranged two TMDs and two NBDs in MDR subfamily and MRP subfamily transporters. (B) The structure of plant ABC transporter PDR subfamily, displaying two TMDs and two NBDs arranged in reverse. (C) The structure of a small number of MRP subfamily transporters in plant ABC transporters, characterized by five α-helices at the *N*-terminal. (D) The structure of a typical plant MATE transporter. ABC: ATP binding cassette; MATE: multidrug and toxic compound extrusion; TMD: transmembrane domain; NBD: nucleotide-binding domain; MDR: multidrug resistance; MRP: multidrug resistance-associated protein; PDR: pleiotropic drug resistance.

dependence of the transport process on protonation-induced conformational change.

MATE transporters have been found in a variety of plants for the transport and storage of PNPs. The MATE family transporter AtDTX41, also known as TT12, is located on the vacuole membrane of *A. thaliana* seed coat. Heterologous expression in yeast revealed that TT12 specifically transported epicatechin 3′-*O*-glucoside and cyanidin 3-*O*-glucoside, contributing to the formation of proanthocyanidins. Homologous proteins of TT12 in many higher plants, such as BnTT12-1 and BnTT12-2 in *Brassica napus* [25], exhibited similar characteristics in terms of subcellular localization, transmembrane helices, and phosphorylation sites. In grapevines, transcriptomic sequencing uncovered two MATE family transporters, VvAM1 and VvAM3 [26], involved in anthocyanin transport and located in the vacuole membrane, exhibiting hydrogen ion-dependent acylated vacuole anthocyanin transport properties [27]. Comparative genomics identified *MdMATE1* and *MdMATE2* in apples, functioning similarly to *A. thaliana* MATE genes, encoding vacuole flavonoid/H^+ antiporters that facilitate flavonoid accumulation in apple fruit [28]. In addition, MATE transporters MtMATE1 [29] and MtMATE2 [30] from *Medicago truncatula* and FaTT12-1 [31] from *Fragaria ananassa* were involved in the transport and accumulation of flavonoids.

Some alkaloids with cytotoxicity should be excreted from cells or transported to vacuoles for detoxification, facilitated by vesicular transport or MATE transporter-mediated transportation processes [32]. On one hand, many vacuolar membrane-localized MATE transporters have been identified to contribute to the accumulation of alkaloids in vacuoles. The MATE transporters NtMATE1 [33] and NtJAT2 [32] are both involved in alkaloid transport and play tissue-specific roles. NtMATE1 is specifically expressed in the root of tobacco and is responsible for transporting alkaloids synthesized in the root to vacuoles for storage. NtJAT2 is specifically expressed in leaf tissues and is responsible for nicotine transport. Takanashi et al. [34] found that MATE protein CjMATE1 was preferably expressed in the rhizome and localized in the vacuole membrane to participate in the transport of berberine. On the other hand, some plasma membrane-localized MATE transporters have been reported to transport alkaloids out of cells. Dobrizsch et al. [35] overexpressed *A. thaliana* MATE transporter gene *AtDTX18* in *Solanum tuberosum* led to the accumulation of a significant amount of hydroxy-cinnamic acid amide with antibacterial activity in the extracellular space, which could inhibit the germination of potato late blight spores and prevent the pathogen infection on the surface of leaves. The membrane-localized MATE transporter CrMATE1 is implicated in the transport of tryptamine, contributing to the accumulation of vindoline and vinblastine in the leaves of *C. roseus*. The transport of value-added PNPs mediated by these transporters provides valuable insights for biosynthesis and drug development. Understanding the relationship between transporters and specific substrates paves the way for innovative approaches in chassis cell engineering.

Table 1
Summary of several types of PNPs transporters.

PNPs		Transporters	Location	Transporter type	Source	Effects	Reference
Terpenoids	Glycyrrhetinic acid	BPT1	Vacuole membrane	ABCB	*S. cerevisiae*	Increase glycyrrhetinic acid production by 1.23-fold	[36]
	Crocins	CsABCC4a; CsABCC2	Vacuole membrane	ABCC	*C. sativus*	Crucial for crocin accumulation in *C. sativus* stigmas	[52]
	Cucurbitacin B	CmMATE1; ClMATE1	Plasma membrane	MATE	*C. melo*; *C. lanatus*	Potential cucurbitacin transporters	[51]
	β-Carotene	SNQ2	Plasma membrane	ABCG	*S. cerevisiae*	Best endogenous transporter for β-carotene efflux	[56]
	Rubusoside	PDR11	Plasma membrane	ABCG	*S. cerevisiae*	Increase rubusoside production by 129.8 %	[8]
	Amorphadiene	AcrB; TolC	Plasma membrane	RND	*E. coli*	Increase amorphadiene production of by 63 %	[40,41]
	Kaurene	AcrA; AcrB; TolC	Plasma membrane	RND	*E. coli*	Increase kaurene production by 82 %	[40,41]
	α-Bisabolene	ABC-G1	Plasma membrane	ABCG	*E. coli*	Decrease cytotoxicity and increase α-bisabolene production by 88 %	[42]
	(−)-α-Bisabolol	PDR15	Plasma membrane	ABCC	*S. cerevisiae*	Increase extracellular (−)-α-bisabolol production by 138.9 %	[43]
	Tocotrienol	PDR11; YOL075C	Plasma membrane	ABCG	*S. cerevisiae*	Increase extracellular tocotrienol by 1.34- and 1.36-fold	[44]
Alkaloids	Reticuline	AtDTX1	Plasma membrane	MATE	*A. thaliana*	Increase reticuline secretion by 11-fold	[45]
	Dopamine; (*S*)-reticuline; codeine	BUP1	Plasma membrane	BUP	*P. somniferum*	Improve the uptake of dopamine, reticuline, and codeine by 300-, 10-, and 30-fold	[46]
	Betaxanthins	QDR1; QDR2	Plasma membrane	MFS	*S. cerevisiae*	Increase the intracellular proportion of betaxanthins	[54]
	Caffeine	SNQ2; PDR5	Plasma membrane	ABCG; ABCC	*S. cerevisiae*	Involve in caffeine efflux transport	[55]
	Coclurine	SNQ2; PDR5	Plasma membrane	ABCG; ABCC	*S. cerevisiae*	Increase tetrahydropapaverine titer by 15-fold	[47]
	Tropine	NtJAT1; NtMATE2	Vacuole membrane	MATE	*N. tabacum*	Facilitate tropine import into vacuoles	[11]
	Littorin	AbPUP1	Vacuole membrane	PUP	*A. belladonna*	Facilitate littorine from vacuoles to the cytosol	[11]
Flavonoids	Resveratrol	AraE	Plasma membrane	–	*E. coli*	Increase resveratrol production by 2.44-fold	[49]
	p-Coumaric acid	PDR12	Plasma membrane	ABCG	*S. cerevisiae*	Decrease intracellular accumulation of *p*-coumaric acid	[48]
Phenylpropanoids	Caffeic acid	YcjP	Plasma membrane	ABC	*E. coli*	Be identified as a caffeic acid transporter	[57]
Others	Cannabigerolic acid	BPT1	Vacuole membrane	ABCB	*S. cerevisiae*	Facilitate cannabigerolic acid import to vacuoles	[38]

3. Transporter-mediated intracellular and extracellular transportation of PNPs

3.1. Engineering of intracellular PNP transportation

PNP biosynthesis in plants is intricate and involves multiple organelles and tissues. When employing engineered unicellular hosts, the absence of an efficient transport mechanism can significantly impede metabolite transport for biocatalysis and corresponding to the accumulation of the desired products. Some typical PNP transporters are summarized in Table 1.

The reconstruction of metabolic transport strategies between organelles is crucial. For example, tropane alkaloids (TAs), found in nightshade plants (*Solanaceae*), are neurotransmitter inhibitors with therapeutic potential for neuromuscular diseases. The biosynthesis of TAs involves various intracellular compartments, including the cytosol, mitochondria, chloroplasts, peroxisomes, endoplasmic reticulum (ER), and vacuoles. Srinivasan and Smolke [10,11] engineered a yeast strain for tropane alkaloid production, utilizing *Nicotiana tabacum* MATE transporters (NtJAT1 and NtMATE2) to facilitate tropine import into vacuoles. The introduction of NtJAT1 resulted in 74 % and 18 % increase in the production of hyoscyamine and scopolamine, respectively. However, exporting the synthesized littorine from vacuoles to the cytosol posed a grand challenge. To overcome this, the authors identified two transporters AbPUP1 and AbLP1 from *Atropa belladonna*, located on the vacuole membrane, to alleviate littorine transport limitations. Specifically, AbPUP1 overexpression increased the accumulation of hyoscyamine and scopolamine by 2.4-fold and 1.5-fold, while

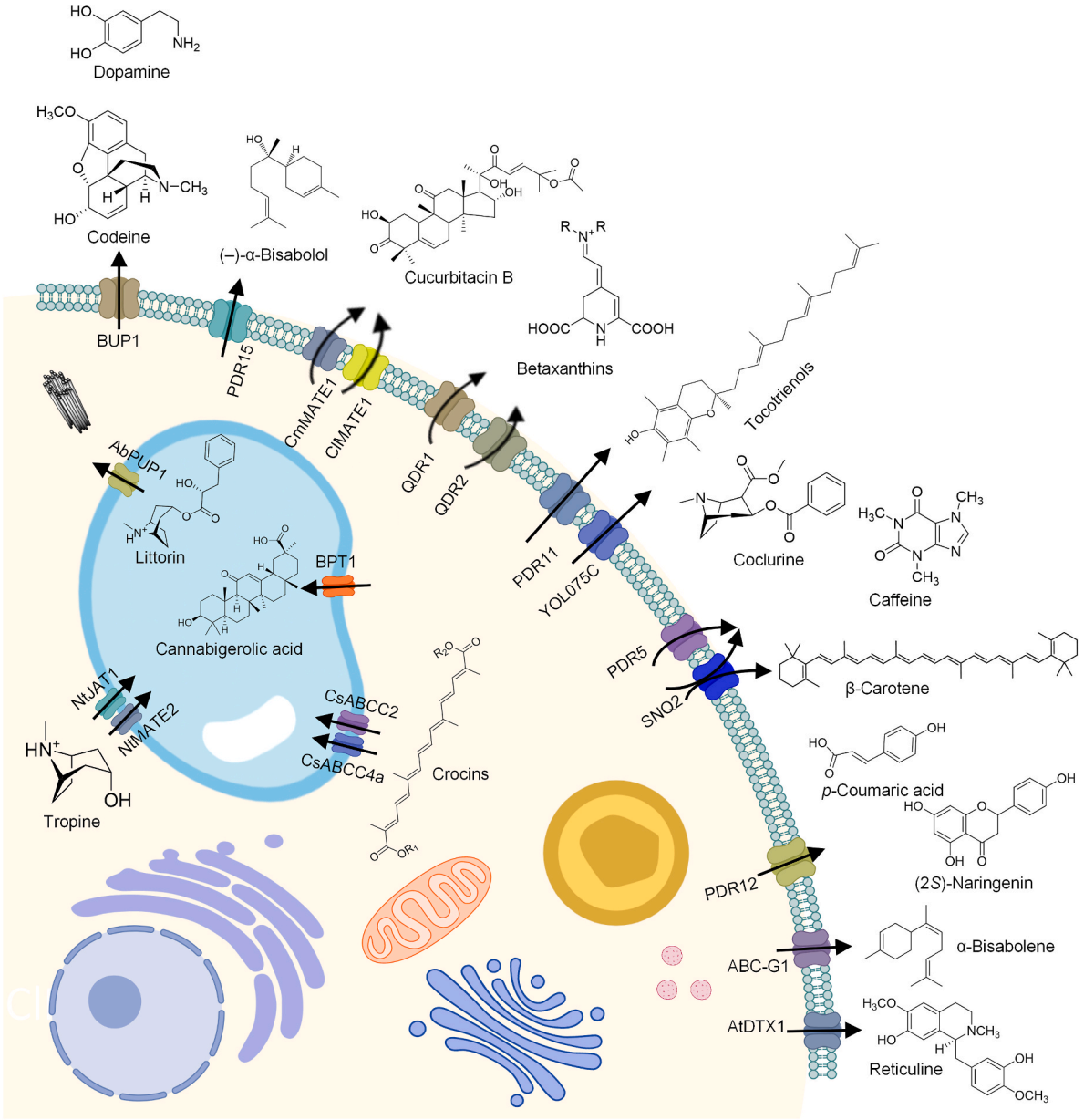

Fig. 2. Intracellular and extracellular transport process of PNPs. The figure illustrates typical transport processes of PNPs in eukaryotic cells and sub-cellular organelles. Arrows indicate the direction of PNPs transfer. BUP1: BIA uptake permease; PDR5/11/12/15: pleiotropic drug resistance transporter 5/11/12/15; YOL075C: an endogenous ABC transporter of *S. cerevisiae*; CmMATE1: *C. melo* MATE transporter 1; ClMATE1: *C. lanatus* MATE transporter 1; QDR1/2: quinidine resistance transporter 1/2; SNQ2: sensitivity to 4-nitroquinoline-*N*-oxide transporter 2; ABC-G1: *G. clavigera* ABC transporter; AtDTX1: *A. thaliana* detoxification 1; BPT1: bile pigment transporter 1; AbPUP1: *A. belladonna* purine uptake permease-like transporter 1; NtJAT1: *N. tabacum* jasmonate-inducible alkaloid transporter 1; NtMATE2: *N. tabacum* MATE transporter 2; CsABCC2/4a: *C. sativus* stigma ABC transporter 2/4a.

AbLP1 overexpression increased the production of these products by 2.0-fold and 1.3-fold, respectively.

In addition to the introduction of plant-derived PNP transporters, endogenous transporters can also be employed to enhance PNP transportation. For example, in the yeast genome, there are more than 30 ABC transporters with diverse functions and subcellular localizations. Alkhadrawi et al. [36] investigated the involvement of vacuolar ABC transporters in glycyrrhetinic acid (GA) production. Molecular docking studies with GA and its precursors revealed the crucial role of vacuolar ABC transporters, particularly bile pigment transporter BPT1 and vacuolar multidrug resistance transporter VMR1. Overexpression of *BPT1* increased GA production by 1.23-fold, may be associated with detoxification mechanisms under nutritional stress, highlighting their essential role in engineered *S. cerevisiae*. Cannabidiol (CBD) from *Cannabis sativa*, known for its various pharmacological activities, was successfully synthesized in yeast [37]. However, low titer and physical barrier between substrates (cytoplasmic localization of cannabigerolic acid, CBGA) and enzymes (vacuolar localization of cannabidiol acid synthase, CBDAS) impeded CBGA catalysis. Thus, Qiu et al. [38] overexpressed ABC transporters and enhanced the CBD titer by 159.6 %. Specifically, BPT1, located in the vacuole membrane of *S. cerevisiae*, facilitated CBGA transportation from cytoplasm to vacuoles, eliminating physical barriers and providing insights for the construction of high-yield CBD strains.

3.2. Transporter engineering for enhanced efflux of PNPs

In addition to facilitate intracellular transport of intermediates, a more potent application of transporter engineering is to pump PNPs out of the producing cells. While extensive pathway optimization strategies have been established to maximize PNP yields [7], inefficient efflux of most PNPs result in product accumulation within cells and subcellular organelles. Intracellular product accumulation exerts not only significant metabolic burdens and cytotoxicity, but also feedback inhibition of the biosynthetic pathways [8]. Therefore, transporter engineering is crucial to achieve efficient biosynthesis and practical applications of PNPs. Some typical intracellular and extracellular PNP transport processes are summarized in Fig. 2.

Widely distributed in nature, terpenoids serve as key components in the flavors, resins, and pigments of various plants and exhibit a wide array of biological activities, including cough suppressants, insect repellents, and analgesics [39]. While most terpenoids are highly hydrophobic, efflux pumps are crucial for discharging from microbial cell factories. With amorphadiene (AD) and kaurene as examples, Wang et al. [40] enhanced the efflux pump system of *E. coli* by adjusting the copy number of multidrug-resistant pump encoding genes (such as *tolC*) and the resistance-nodulation cell division (RND) superfamily *acrB* and *acrA*, which increased the production of amorphadiene and kaurene by 63 % and 82 %, respectively. Additionally, the authors found the outer membrane protein TolC played a critical role in the secretion of artemisinin precursor AD in *E. coli* [41]. Zhao et al. [42] engineered the production of α-bisabolene, a sesquiterpene that can be used as a fragrance and anticancer drug, in the peroxisome of *Yarrowia lipolytica*. The introduction of *Grosmania clavigera* ABC transporter family member ABC-G1 resulted in the secretion of α-bisabolene, which decreased the cytotoxicity and increased the titer by 88 %. Jiang et al. [43] engineered a *de novo* (−)-α-bisabolol biosynthesis strain, with the overexpression of *PDR15* increased the production of extracellular (−)-α-bisabolol by 138.9 %. Jiao et al. [44] engineered *S. cerevisiae* to produce tocotrienol, the important component of vitamin E, where the overexpression of *PDR11* and *YOL075C* increased extracellular tocotrienol by 1.34- and 1.36-fold, respectively.

Alkaloids, a diverse class of nitrogen-containing compounds, find applications as anticancer drugs, vascular disorder medications, pain relievers, antimalarials, and novel antimicrobial therapies against drug-resistant bacteria. Addressing issues with low productivity and cytotoxicity, transporter engineering has emerged as a strategy to enhance alkaloid biosynthesis. Yamada et al. [45] engineered *E. coli* to produce reticuline, a crucial intermediate in alkaloid synthesis, utilizing the MATE transporter AtDTX1 from *A. thaliana*. AtDTX1 increased the secretion of reticuline into the culture medium by 11-fold. Dastmalchi et al. [46] investigated benzylisoquinoline alkaloid (BIA) transporters in opium poppy (*Papaver somniferum*). Introducing BIA uptake enzymes (BUPs) into cocultured yeast strains significantly improved the uptake of early intermediates (300- and 25-fold for dopamine and norcoclaurine), central pathway metabolites (10-fold for reticuline), and end products (30-fold for codeine). Jamil et al. [47] constructed an engineered yeast strain for *de novo* synthesis of tetrahydropapaverine (THP), an antispasmodic drug. The disruption of MDR transporters sensitivity to 4-nitroquinoline-*N*-oxide transporter 2 (SNQ2) and PDR5 increased THP titers by 15-fold, revealing their role in exporting the pathway intermediate coclurine from the cytosol to the medium.

Flavonoids, prominent plant secondary metabolites with diverse biological characteristics, including antioxidant, coronary heart disease prevention, anticancer, antibacterial, and liver disease prevention properties. (2*S*)-Naringenin, a crucial scaffold for various flavonoid subclasses, exhibits beneficial effects on human conditions such as obesity, cancer, diabetes, and Alzheimer's disease. Mao et al. [48] adopted a *de novo* biosynthesis approach for (2*S*)-naringenin. Unfortunately, the key intermediate *p*-coumaric acid (*p*-CA) was mainly accumulated extracellularly, limiting (2*S*)-naringenin biosynthesis. Transporter engineering via *PDR12* overexpression decreased *p*-CA accumulation and increased metabolic fluxes towards (2*S*)-naringenin production. Overexpression of *PDR12* in two yeast strains with different downstream pathway fluxes both significantly increased total (2*S*)-naringenin production, with a slight decrease in extracellular *p*-CA accumulation.

Resveratrol, a polyphenolic compound with protective effects against diseases like Parkinson's, typically accumulates in plant vacuoles. Wang et al. [49] introduced the low-affinity arabinose transporter gene *araE* from *E. coli* into yeast and increased resveratrol production by 2.44-fold. Although AraE showed no affinity for resveratrol in import and export analyses conducted in *Xenopus* oocytes, the study suggested that, beyond a certain threshold, AraE might enhance resveratrol permeability.

4. Strategies for identifying PNP transporters

The application of transporter engineering provides a direction to debottleneck PNP biosynthesis in microbial cell factories [50]. However, the current application of transporter engineering is not mature, mainly due to the lack of efficient transporters for specific compounds and generally high substrate specificity of transporters, which brings great obstacles to the application of transporter engineering. Therefore, it is crucial to obtain efficient transporters. Here, we summarize several strategies for identifying transporters, with a hope to provide references for the application of transporter engineering.

Mining from plants is the most direct and effective method to obtain specific PNP transporters. The most classic strategy is to compare gene expression levels in different plant tissues by transcriptomic tools. This co-expression method usually uses linear regression or hierarchical clustering to screen candidate genes and analyzes the transcriptional abundance and similarity of "bait" genes in the eradication pathway to mine transporters (Fig. 3A). Zhong et al. [51] used this strategy to identify potential cucurbitacin transporters (CmMATE1 and ClMATE1 from *Cucumis melo* and *Citrullus lanatus*, respectively), revealing their role in cucurbitacin B (CuB) and cucurbitacin E (CuE) transport in melon and watermelon. This strategy also applies to the identification of crocin transporters. Crocins, the expensive spice found in *Crocus sativus* stigmas, is known for its potent anti-oxidant activity. Due to the polar nature, crocins in the cytosol should be transported with the aid of one or more tonoplast transporters to reach vacuoles for storage. Demurtas

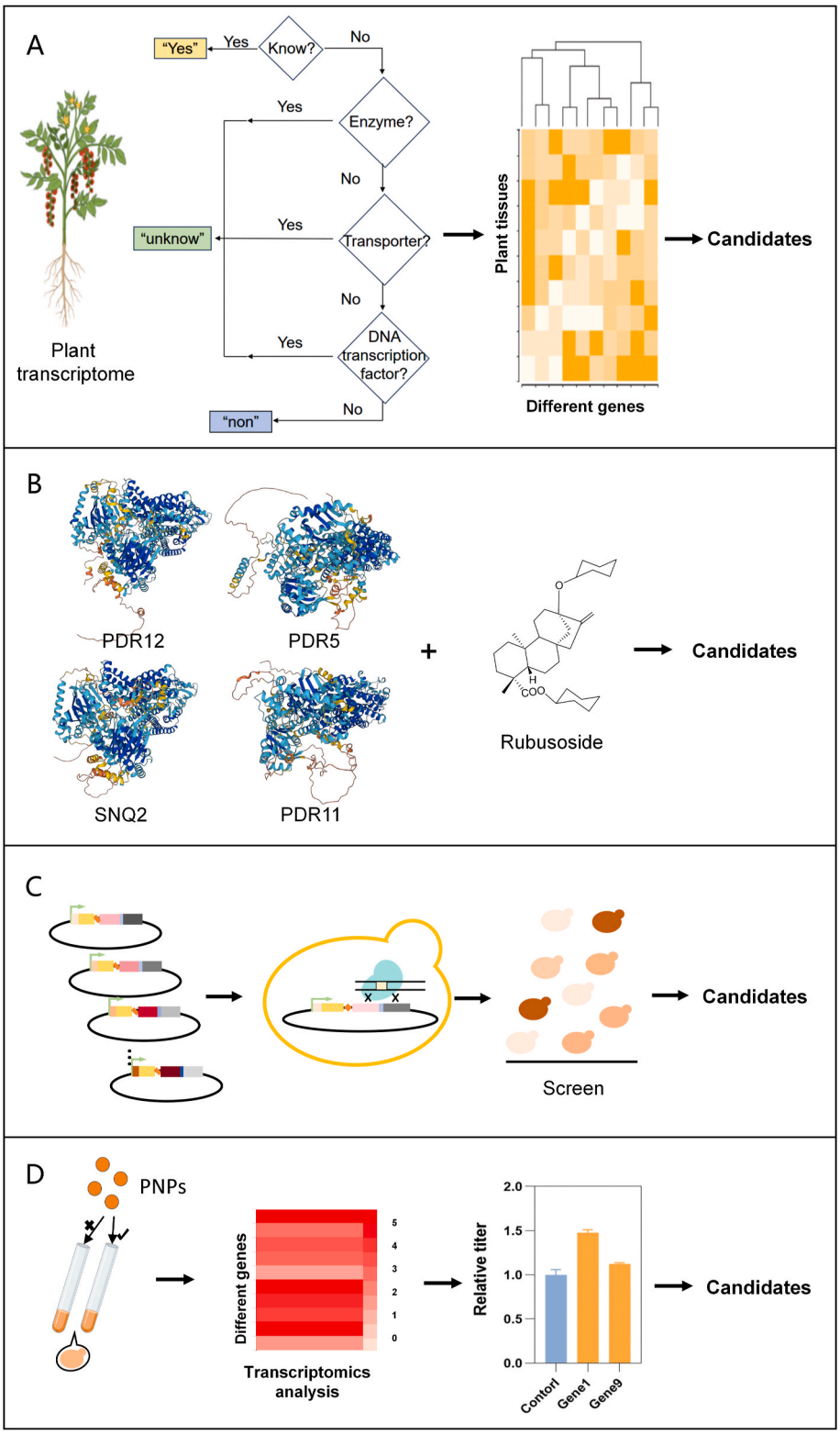

Fig. 3. Strategies for identifying PNP transporters. (A) General processes for the mining of PNP transporters, involving the establishment of a suitable screening model and bioinformatics analysis to identify specific PNP transporters from different plant tissues. (B) Molecular docking strategies to identify the best transporter candidates for a specific substrate. (C) Transportom-wide engineering and high-throughput screening processes for identifying specific PNP transporters. (D) Transcriptomic analysis with or without exogenous supplementation of PNPs for the identification of candidate transporters. PDR5/11/12: pleiotropic drug resistance transporter 5/11/12; SNQ2: sensitivity to 4-nitroquinoline-*N*-oxide transporter 2.

et al. [52] employed a methodology based on transcriptomic data, heterologous expression in *S. cerevisiae*, and *in vitro* transportomic analyses to identify ABC transporters CsABCC4a and CsABCC2, crucial for crocin accumulation in *C. sativus* stigmas.

The specific design between transporters and PNP structures is another strategy for identifying potential transporters. This method involves molecular docking of potential transporters with the target compounds. According to the docking results, some potential transporters for the specific compound were identified, followed by transportation verification via gene knockout or overexpression experiments. This strategy has been employed to identify transporters for the sweetness-enhancing sterol glycoside rubusoside. Xu et al. [8] engineered *S. cerevisiae* to produce rubusoside, and the efflux pump PDR11 was found to be involved in its secretion. The disruption of *PDR11* resulted in decreased rubusoside production, while overexpression increased the titer by 129.8 %, indicating the active role of PDR11 in rubusoside transportation (Fig. 3B).

Considering the limited understanding of the interaction between transporters and PNPs, genome-scale or transportome-wide engineering has been established as a powerful strategy to identify potential PNP transporters in a non-intuitive manner [53]. Wang et al. [54] developed a CRISPR/Cas9-based genome-wide transporter disruption method, combined with metabolite biosensors, to identify transporters affecting target metabolite production in yeast (Fig. 3C). This method revealed two major facilitator superfamily (MFS) transporters (quinidine resistance transporters QDR1 and QDR2) influencing the production of betaxanthins in *S. cerevisiae*, which increased the intracellular proportion of betaxanthins from 40 % to 70 % and 66 %, respectively. Following this concept, further exploration of endogenous transporters of *S. cerevisiae* can be conducted to facilitate the transmembrane transport of PNPs. Nevertheless, it is imperative to acknowledge that genome-scale or transportome-wide engineering should be complemented by high-throughput screening methods. Based on the assumption that PNP efflux is associated with its detoxification, a more general strategy can be established by coupling PNP transportation with cell growth under the stress of specific PNP. Tsujimoto et al. [55] screened a genome-scale transporter overexpression library with the identification of *SNQ2* and *PDR5* as caffeine resistance genes in *S. cerevisiae*. Further experimental verification demonstrated the involvement of SNQ2 and PDR5 in caffeine efflux transportation and accordingly caffeine resistance.

Another popular strategy for identifying PNP-specific transporters in host cells is based on transcriptomic analysis of the exogenous added specific compounds. The implementation of this strategy requires the addition of specific compounds to the culture medium to compare and analyze the transcriptome data under stress, followed by experimental verification of the candidate genes with significantly up-regulated transcription levels (Fig. 3D). For example, Bu et al. [56] discovered the substrate-inducing ability of endogenous transporters and identified five potential ABC transporters (PDR5, PDR10, SNQ2, YOR1, and YOL075C) involved in β-carotene exocytosis in *S. cerevisiae*. Similarly, this strategy is also employed to identify caffeic acid transporters. Caffeic acid, a natural phenylpropanoid compound with antioxidant properties, is employed as an active agent in treating lung cancer and promoting hematopoiesis and hemostasis. Wang et al. [57] identified YcjP, a sugar ABC transporter permease, as a caffeic acid transporter based on transcriptomic data under caffeic acid and ferulic acid stress. The overexpression of *ycjP*, encoding an efflux transporter, extended production cycle and increased caffeic acid production by 24.5 % in engineered *E. coli*.

The use of the above mentioned four PNP transporter identification strategies should comprehensively consider the properties of host cells and PNPs. Transporter mining from plants is straightforward and effective, but this strategy requires the analysis of large amounts of genomic and transcriptomic data and the test of candidate genes. On the other hand, the identification strategy based on molecular docking can focus on a certain type of transporters, which narrows the screening scope. Unfortunately, due to the lack of transporter structures, the molecular docking results are still not satisfying, which directly affects the subsequent knockout or overexpression tests. Genome-scale transporter library screening is suitable for identifying PNP transporters in chassis cells, but only for PNPs with high-throughput screening methods. Finally, transcriptome analysis based on exogenously added compounds is suitable for PNPs that have a significant effect on cell growth or cellular metabolism.

5. Small molecule and low temperature assisted PNP efflux

In addition to transporter-mediated direct enhancement of PNP transportation, small molecules (such as biocompatible organic solvents) [58] and environmental changes (such as low temperature) [59] have also employed as auxiliary strategies to facilitate PNP efflux. Small molecule-assisted PNP efflux is a cell culture technique integrating cell culture with product separation. This method involves the addition of biocompatible organic solvents or adsorptive polymers to the cell culture system, creating an upper and lower phase based on different distribution coefficients. The culture system promotes cell growth and product synthesis in the aqueous phase, while products are captured and accumulated in the organic phase. This approach streamlines upstream and downstream processes, optimizing production processes, and gaining popularity in PNP biosynthesis (Fig. 4A). Commonly used second-phase liquids include alkanes, organic acids, alcohols, and esters.

Initially, Kim et al. [60] found that the addition of hexadecane significantly increased shikonin synthesis by 7.4-fold. Brennan et al. [58] extended this method to engineered bacteria fermentation for PNP production, demonstrating that a biphasic system effectively decreased terpene toxicity. Various organic solvents, such as isopropyl myristate (IPM), *n*-dodecane, and olive oil, have been employed in fermentation production strategies. Depending on the molecular properties of PNPs, different organic solvents should be carefully selected. For instance, Lei et al. [61] modified *E. coli* for resveratrol and borneol production, achieving 966.55 mg/L resveratrol and 87.20 mg/L borneol using the IPM biphasic fermentation strategy. Ye et al. [62] engineered *S. cerevisiae* and combined with IPM biphasic fermentation strategy to produce (+)-valencene with a titer of 16.6 g/L. Zuo et al. [63] modified *P. pastoris* combined with *n*-dodecane biphasic fermentation to obtain 21.5 g/L α-santalene in 1-L fermenter by fed-batch fermentation. Cheng et al. [64] used a biphasic fermentation strategy of *n*-dodecane to obtain 1.9 g/L β-elemene in fed-batch fermentation of engineered *P. pastoris*. The combination of physical methods, such as ultrasound, further enhances product biphasic extraction efficiency [65].

Olive oil and oleyl alcohol are commonly used in biphasic fermentation of phenols and organic acids. Jiao et al. [66] studied *δ*-tocotrienol fermentation in *S. cerevisiae*, adding 2-hydroxypropyl-β-cyclodextrin (β-CD) and olive oil, resulting in 181.12 mg/L production, with 85.6 % being extracellular. Combes et al. [67] studied the heterologous synthesis of *p*-CA in *S. cerevisiae*, adding oleyl alcohol as a biphase extraction agent, which significantly improved the production rate of *p*-CA. In addition, to decrease the cost of β-CD for biomanufacturing, Zhu et al. [68] designed a β-CD in situ synthesis system using yeast surface display technology to realize β-CD assisted β-amyrin efflux in *S. cerevisiae*.

While small molecule-assisted PNP efflux strategies have seen widespread use, a recent study suggested that altering fermentation temperature could also enhance terpenoid secretion and production (Fig. 4B). Lower temperature affects the phospholipid bilayer composition and increases fatty acid chain unsaturation, resulting in 5.5-fold higher GA yield in engineered yeast strain at 22 °C than that at 30 °C [59]. Future investigations may explore additional environmental factors, such as pressure, pH, and light, for their impact on efficient PNP efflux and production.

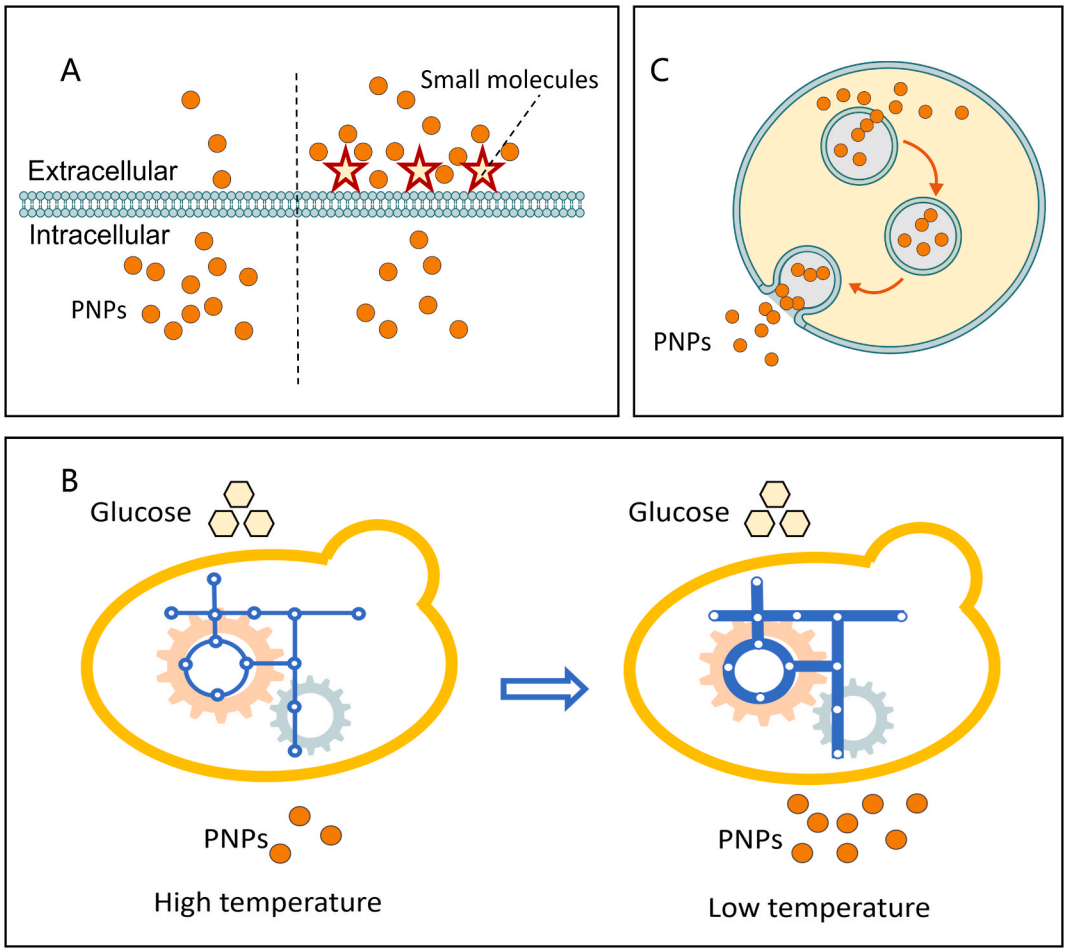

Fig. 4. Small molecule and low temperature assisted PNP efflux. (A) Small molecule-assisted PNP efflux, demonstrating the addition of organic solvents or adsorptive polymers to the cell culture system. (B) Environmental changes, such as low temperature, for enhanced efflux of PNPs. (C) Vesicular transport of PNPs, showcasing the efflux of PNPs encapsulated in vesicles.

6. Conclusions and future perspectives

In recent years, significant strides have been made in understanding the transmembrane transport processes of PNPs, coupled with advancements in the study of transporters and auxiliary transmembrane strategies for bio-manufacturing. Notably, ABC and MATE family transporters play pivotal roles in the transmembrane transport of PNPs, alongside emerging transporter families like PUP (purine uptake permease) and NRT (Nitrate-peptide transporters). Unveiling the specificity between transporters and PNP structures is crucial for optimizing the transmembrane process, fostering large-scale production of scarce PNPs using microbial cell factories.

Despite considerable progress, the research and engineering applications of transporters are still in their infancy, and the transmembrane transport processes of certain PNPs, especially those with medicinal significance like artemisinin, vinblastine, sanguinarine, and camptothecin, remain elusive [69]. The complexity arises from the multitude of transporters in plant genomes, coupled with challenges in characterizing the function of transporters linked to specific PNPs. Strategies such as bioinformatics and metabolomics analysis can be combined to identify potential transporters from plants or the chassis cells. On one hand, it is necessary to establish effective high-throughput screening methods for specific PNPs, and use high-integration, high-throughput, and automated devices (such as BioFoundry platforms developed in recent years) for large-scale data collection and verification [70]. On the other hand, the combination with artificial intelligence and machine learning can help to identify the optimal combination of transporter-substrate interactions in massive data and predict transporter candidates for specific PNPs [71,72].

The small-molecule-assisted transmembrane transport process relies on the coupling of cell culture and product separation. However, challenges persist, particularly in finding in-situ extractants for PNP transmembrane processes. Currently, the selection of organic phases appears to be somewhat arbitrary, with an urgent need to rationally design an effective two-phase fermentation system [58]: 1) minimal cytotoxicity of the organic phase; 2) ease of product dissolution in the organic phase; 3) straightforward separation of the two phases; 4) no reactivity between the organic phase and medium components. The selection of a suitable biphasic fermentation system necessitates extensive experimental and cytotoxicity testing, an aspect that is underreported, highlighting the need for more in-depth mechanistic research.

The latest method involves the efflux of PNPs encapsulated in vesicles, akin to the efflux of secreted proteins, although it has only been reported for a limited number of PNPs [73]. The accumulation of hydrophobic molecules (such as carotenoids) significantly affects chassis cell anabolism, and extracellular secretion becomes crucial for efficient production in microbial cell factories. While the lipid droplet localization strategy effectively stores carotenoids [74], to overcome the upper limit of lipid droplet capacity remains challenging. While it has been reported that the use of ABC transporter MsbA for the efflux of carotenoids [75], the transportation efficiency is very low. Wu et al. [76] introduced a novel artificial membrane vesicle transport system (AMVTS) in *E. coli*, resulting in a 24-fold increase in β-carotene secretion and a 61 % increase in production, addressing the issue of limited

excretion through the natural transportation system (Fig. 4C). Although not widely applied in other chassis cells, this strategy holds promise for diverse microbial cell factories producing hydrophobic PNPs.

In conclusion, approaches combining various methods hold promise for exploring transportation candidate genes as potential targets for metabolic engineering of high-value PNPs. Enhancing the transmembrane transport process of PNPs not only presents an appealing platform, but also facilitates efficient production of PNPs in microbial cell factories.

Declaration of competing interest

None.

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References

[1] Sawai S, Saito K. Triterpenoid biosynthesis and engineering in plants. Front Plant Sci 2011;2:25. https://doi.org/10.3389/fpls.2011.00025.
[2] Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, McPhee D, Leavell MD, Tai A, Main A, Eng D, Polichuk DR, Teoh KH, Reed DW, Treynor T, Lenihan J, Fleck M, Bajad S, Dang G, Dengrove D, Diola D, Dorin G, Ellens KW, Fickes S, Galazzo J, Gaucher SP, Geistlinger T, Henry R, Hepp M, Horning T, Iqbal T, Jiang H, Kizer L, Lieu B, Melis D, Moss N, Regentin R, Secrest S, Tsuruta H, Vazquez R, Westblade LF, Xu L, Yu M, Zhang Y, Zhao L, Lievense J, Covello PS, Keasling JD, Reiling KK, Renninger NS, Newman JD. High-level semi-synthetic production of the potent antimalarial artemisinin. Nature 2013;496(7446):528–32. https://doi.org/10.1038/nature12051.
[3] Gao JC, Zuo YM, Xiao F, Wang YL, Li DF, Xu JH, Ye CF, Feng LJ, Jiang LJ, Liu TF, Gao D, Ma B, Huang L, Xu ZA, Lian JZ. Biosynthesis of catharanthine in engineered *Pichia pastoris*. Nat. Synth. 2023;2(3):231–42. https://doi.org/10.1038/s44160-022-00205-2.
[4] Gao D, Liu TF, Gao JC, Xu JH, Gou YW, Pan YJ, Li DF, Ye CF, Pan RH, Huang L, Xu ZA, Lian JZ. *De novo* biosynthesis of vindoline and catharanthine in *Saccharomyces cerevisiae*. Biodes. Res. 2022;2022:2. https://doi.org/10.34133/bdr.0002.
[5] Wriessnegger T, Augustin P, Engleder M, Leitner E, Müller M, Kaluzna I, Schürmann M, Mink D, Zellnig G, Schwab H, Pichler H. Production of the sesquiterpenoid (+)-nootkatone by metabolic engineering of *Pichia pastoris*. Metab Eng 2014;24:18–29. https://doi.org/10.1016/j.ymben.2014.04.001.
[6] Zha WL, Zhang F, Shao JQ, Ma XM, Zhu JX, Sun PH, Wu RB, Zi JC. Rationally engineering santalene synthase to readjust the component ratio of sandalwood oil. Nat Commun 2022;13(1):2508. https://doi.org/10.1038/s41467-022-30294-8.
[7] Li JH, Meng HL, Wang Y. Synbiological systems for complex natural products biosynthesis. Synth. Syst. Biotechnol. 2016;1(4):221–9. https://doi.org/10.1016/j.synbio.2016.08.002.
[8] Xu YM, Wang XL, Zhang CY, Zhou X, Xu XH, Han LY, Lv XQ, Liu YF, Liu S, Li JH, Du GC, Chen J, Ledesma-Amaro R, Liu L. *De novo* biosynthesis of rubusoside and rebaudiosides in engineered yeasts. Nat Commun 2022;13(1). https://doi.org/10.1038/s41467-022-30826-2.
[9] Belew ZM, Poborsky M, Nour-Eldin HH, Halkier BA. Transport engineering in microbial cell factories producing plant-specialized metabolites. Curr Opin Green Sustainable Chem 2022;33:100576. https://doi.org/10.1016/j.cogsc.2021.100576.
[10] Srinivasan P, Smolke CD. Biosynthesis of medicinal tropane alkaloids in yeast. Nature 2020;585(7826):614–9. https://doi.org/10.1038/s41586-020-2650-9.
[11] Srinivasan P, Smolke CD. Engineering cellular metabolite transport for biosynthesis of computationally predicted tropane alkaloid derivatives in yeast. Proc. Natl. Acad. Sci. U.S.A 2021;118(25):e2104460118. https://doi.org/10.1073/pnas.2104460118.
[12] Liu XN, Ding WT, Jiang HF. Engineering microbial cell factories for the production of plant natural products: from design principles to industrial-scale production. Microb Cell Factories 2017;16:125. https://doi.org/10.1186/s12934-017-0732-7.
[13] Higgins CF. ABC transporters: from microorganisms to man. Annu Rev Cell Dev Biol 1992;8:67–113. https://doi.org/10.1146/annurev.cellbio.8.1.67.
[14] Verrier PJ, Bird D, Buria B, Dassa E, Forestier C, Geisler M, Klein M, Kolukisaoglu Ü, Lee Y, Martinoia E, Murphy A, Rea PA, Samuels L, Schulz B, Spalding EP, Yazaki K, Theodoulou FL. Plant ABC proteins - a unified nomenclature and updated inventory. Trends Plant Sci 2008;13(4):151–9. https://doi.org/10.1016/j.tplants.2008.02.001.
[15] Nuruzzaman M, Zhang R, Cao HZ, Luo ZY. Plant pleiotropic drug resistance transporters: transport mechanism, gene expression, and function. J Integr Plant Biol 2014;56(8):729–40. https://doi.org/10.1111/jipb.12196.
[16] Grotz N, Fox T, Connolly E, Park W, Guerinot ML, Eide D. Identification of a family of zinc transporter genes from *Arabidopsis* that respond to zinc deficiency. Proc. Natl. Acad. Sci. U.S.A 1998;95(12):7220–4. https://doi.org/10.1073/pnas.95.12.7220.
[17] Kaneda M, Schuetz M, Lin BSP, Chanis C, Hamberger B, Western TL, Ehlting J, Samuels AL. ABC transporters coordinately expressed during lignification of *Arabidopsis* stems include a set of ABCBs associated with auxin transport. J Exp Bot 2011;62(6):2063–77. https://doi.org/10.1093/jxb/erq416.
[18] Behrens CE, Smith KE, Iancu CV, Choe JY, Dean JV. Transport of anthocyanins and other flavonoids by the *Arabidopsis* ATP-binding cassette transporter AtABCC2. Sci Rep 2019;9:437. https://doi.org/10.1038/s41598-018-37504-8.
[19] Larsen ES, Alfenito MR, Briggs WR, Walbot V. A carnation anthocyanin mutant is complemented by the glutathione *S*-transferases encoded by maize *Bz2* and petunia *An9*. Plant Cell Rep 2003;21(9):900–4. https://doi.org/10.1007/s00299-002-0545-x.
[20] Jin HB, Liu DH, Zuo KJ, Gong YF, Miao ZQ, Chen YH, Ren WW, Sun XF, Tang KX. Molecular cloning and characterization of *Crmdr1*, a novel MDR-type ABC transporter gene from *Catharanthus roseus*. DNA Sequence 2007;18(4):316–25. https://doi.org/10.1080/10425170701207018.
[21] Yu F, De Luca V. ATP-binding cassette transporter controls leaf surface secretion of anticancer drug components in *Catharanthus roseus*. Proc. Natl. Acad. Sci. U.S.A 2013;110(39):15830–5. https://doi.org/10.1073/pnas.1307504110.
[22] Shitan N, Dalmas F, Dan K, Kato N, Ueda K, Sato F, Forestier C, Yazaki K. Characterization of *Coptis japonica* CjABCB2, an ATP-binding cassette protein involved in alkaloid transport. Phytochemistry 2013;91:109–16. https://doi.org/10.1016/j.phytochem.2012.02.012.
[23] Fu XQ, Shi P, He Q, Shen Q, Tang YL, Pan QF, Ma YA, Yan TX, Chen MH, Hao XL, Liu P, Li L, Wang YL, Sun XF, Tang KX. AaPDR3, a PDR transporter 3, is involved in sesquiterpene β-caryophyllene transport in *artemisia annua*. Front Plant Sci 2017;8:723. https://doi.org/10.3389/fpls.2017.00723.
[24] Upadhyay N, Kar D, Datta S. A multidrug and toxic compound extrusion (MATE) transporter modulates auxin levels in root to regulate root development and promotes aluminium tolerance. Plant Cell Environ 2020;43(3):745–59. https://doi.org/10.1111/pce.13658.
[25] Chai YR, Lei B, Huang HL, Li JN, Yin JM, Tang ZL, Wang R, Chen L. *TRANSPARENT TESTA 12* genes from *Brassica napus* and parental species: cloning, evolution, and differential involvement in yellow seed trait. Mol Genet Genom 2009;281(1):109–23. https://doi.org/10.1007/s00438-008-0399-1.
[26] Gomez C, Terrier N, Torregrosa L, Vialet S, Fournier-Level A, Verriès C, Souquet JM, Mazauric JP, Klein M, Cheynier V, Ageorges A. Grapevine MATE-type proteins act as vacuolar H^+-dependent acylated anthocyanin transporters. Plant Physiol 2009;150(1):402–15. https://doi.org/10.1104/pp.109.135624.
[27] Gomez C, Conejero G, Torregrosa L, Cheynier V, Terrier N, Ageorges A. *In vivo* grapevine anthocyanin transport involves vesicle-mediated trafficking and the contribution of anthoMATE transporters and GST. Plant J 2011;67(6):960–70. https://doi.org/10.1111/j.1365-313X.2011.04648.x.
[28] Frank S, Keck M, Sagasser M, Niehaus K, Weisshaar B, Stracke R. Two differentially expressed *MATE* factor genes from apple complement the *Arabidopsis TRANSPARENT TESTA12* mutant. Plant Biol 2011;13(1):42–50. https://doi.org/10.1111/j.1438-8677.2010.00350.x.
[29] Zhao J, Dixon RA. MATE transporters facilitate vacuolar uptake of epicatechin 3′-*O*-glucoside for proanthocyanidin biosynthesis in *Medicago truncatula* and *Arabidopsis*. Plant Cell 2009;21(8):2323–40. https://doi.org/10.1105/tpc.109.067819.
[30] Zhao J, Huhman D, Shadle G, He XZ, Sumner LW, Tang YH, Dixon RA. MATE2 mediates vacuolar sequestration of flavonoid glycosides and glycoside malonates in *Medicago truncatula*. Plant Cell 2011;23(4):1536–55. https://doi.org/10.1105/tpc.110.080804.
[31] Chen SY, Tang YM, Hu YY, Wang Y, Sun B, Wang XR, Tang HR, Chen Q. *FaTT12-1*, a multidrug and toxin extrusion (MATE) member involved in proanthocyanidin transport in strawberry fruits. Sci Hortic 2018;231:158–65. https://doi.org/10.1016/j.scienta.2017.12.032.
[32] Shitan N, Minami S, Morita M, Hayashida M, Ito S, Takanashi K, Omote H, Moriyama Y, Sugiyama A, Goossens A, Moriyasu M, Yazaki K. Involvement of the leaf-specific multidrug and toxic compound extrusion (MATE) transporter Nt-JAT2 in vacuolar sequestration of nicotine in *Nicotiana tabacum*. PLoS One 2014;9(9):e108789. https://doi.org/10.1371/journal.pone.0108789.
[33] Shoji T, Inai K, Yazaki Y, Sato Y, Takase H, Shitan N, Yazaki K, Goto Y, Toyooka K, Matsuoka K, Hashimoto T. Multidrug and toxic compound extrusion-type transporters implicated in vacuolar sequestration of nicotine in tobacco roots. Plant Physiol 2009;149(2):708–18. https://doi.org/10.1104/pp.108.132811.
[34] Takanashi K, Yamada Y, Sasaki T, Yamamoto Y, Sato F, Yazaki K. A multidrug and toxic compound extrusion transporter mediates berberine accumulation into vacuoles in *Coptis japonica*. Phytochemistry 2017;138:76–82. https://doi.org/10.1016/j.phytochem.2017.03.003.
[35] Dobritzsch M, Lübken T, Eschen-Lippold L, Gorzolka K, Blum E, Matern A, Marillonnet S, Böttcher C, Dräger B, Rosahl S. MATE transporter-dependent export of hydroxycinnamic acid amides. Plant Cell 2016;28(2):583–96. https://doi.org/10.1105/tpc.15.00706.
[36] Alkhadrawi AM, Xue H, Ahmad N, Akram M, Wang Y, Li C. Molecular study on the role of vacuolar transporters in glycyrrhetinic acid production in engineered *Saccharomyces cerevisiae*. Biochim Biophys Acta, Biomembr 2022;1864(6):183890. https://doi.org/10.1016/j.bbamem.2022.183890.

[37] Luo XZ, Reiter MA, d'Espaux L, Wong J, Denby CM, Lechner A, Zhang YF, Grzybowski AT, Harth S, Lin WY, Lee H, Yu CH, Shin J, Deng K, Benites VT, Wang G, Baidoo EEK, Chen Y, Dev I, Petzold CJ, Keasling JD. Complete biosynthesis of cannabinoids and their unnatural analogues in yeast. Nature 2019; 567(7746):123–6. https://doi.org/10.1038/s41586-019-0978-9.
[38] Qiu J, Hou KX, Li Q, Chen JL, Li XW, Hou HP, Wang LQ, Liu J, Xue Q, Wang CX. Boosting the cannabidiol production in engineered *Saccharomyces cerevisiae* by harnessing the vacuolar transporter BPT1. J Agric Food Chem 2022;70(38): 12055–64. https://doi.org/10.1021/acs.jafc.2c05468.
[39] Moses T, Pollier J, Thevelein JM, Goossens A. Bioengineering of plant (tri) terpenoids: from metabolic engineering of plants to synthetic biology in vivo and in vitro. New Phytol 2013;200(1):27–43. https://doi.org/10.1111/nph.12325.
[40] Wang JF, Xiong ZQ, Li SY, Wang Y. Enhancing isoprenoid production through systematically assembling and modulating efflux pumps in *Escherichia coli*. Appl Microbiol Biotechnol 2013;97(18):8057–67. https://doi.org/10.1007/s00253-013-5062-z.
[41] Zhang CQ, Chen XX, Stephanopoulos G, Too HP. Efflux transporter engineering markedly improves amorphadiene production in *Escherichia coli*. Biotechnol Bioeng 2016;113(8):1755–63. https://doi.org/10.1002/bit.25943.
[42] Zhao B, Zhang Y, Wang Y, Lu Z, Miao L, Wang S, Li Z, Sun X, Han Y, He S, Zhang Z, Xiao D, Zhang C, Foo JL, Wong A, Yu A. Biosynthesis of α-bisabolene from low-cost renewable feedstocks by peroxisome engineering and systems metabolic engineering of the yeast *Yarrowia lipolytica*. Green Chem 2023;25(20):8145–59. https://doi.org/10.1039/d3gc01936e.
[43] Jiang YK, Xia L, Gao S, Li N, Yu SQ, Zhou JW. Engineering *Saccharomyces cerevisiae* for enhanced (-)-α-bisabolol production. Synth. Syst. Biotechnol. 2023;8(2): 187–95. https://doi.org/10.1016/j.synbio.2023.01.004.
[44] Jiao X, Shen B, Li M, Ye LD, Yu HW. Secretory production of tocotrienols in *Saccharomyces cerevisiae*. ACS Synth Biol 2022;11(2):788–99. https://doi.org/10.1021/acssynbio.1c00484.
[45] Yamada Y, Urui M, Oki H, Inoue K, Matsui H, Ikeda Y, Nakagawa A, Sato F, Minami H, Shitan N. Transport engineering for improving the production and secretion of valuable alkaloids in *Escherichia coli*. Metab. Eng. Commun. 2021;13: e00184. https://doi.org/10.1016/j.mec.2021.e00184.
[46] Dastmalchi M, Chang L, Chen R, Yu L, Chen X, Hagel JM, Facchini PJ. Purine permease-type benzylisoquinoline alkaloid transporters in opium poppy. Plant Physiol 2019;181(3):916–33. https://doi.org/10.1104/pp.19.00565.
[47] Jamila OK, Cravens A, Payne JT, Kim CY, Smolk CD. Biosynthesis of tetrahydropapaverine and semisynthesis of papaverine in yeast. Proc. Natl. Acad. Sci. U.S.A 2022. https://doi.org/10.1073/pnas.
[48] Mao JW, Mohedano MT, Fu J, Li XW, Liu QL, Nielsen J, Siewers V, Chen Y. Fine-tuning of *p*-coumaric acid synthesis to increase (*2S*)-naringenin production in yeast. Metab Eng 2023;79:192–202. https://doi.org/10.1016/j.ymben.2023.08.003.
[49] Wang YC, Halls C, Zhang J, Matsuno M, Zhang YS, Yu O. Stepwise increase of resveratrol biosynthesis in yeast *Saccharomyces cerevisiae* by metabolic engineering. Metab Eng 2011;13(5):455–63. https://doi.org/10.1016/j.ymben.2011.04.005.
[50] Zhu Y, Zhou C, Wang Y, Li C. Transporter engineering for microbial manufacturing. Biotechnol J 2020;15(9):1900494. https://doi.org/10.1002/biot.201900494.
[51] Zhong Y, Xun WB, Wang XH, Tian SW, Zhang YC, Li DW, Zhou Y, Qin YX, Zhang B, Zhao GW, Cheng X, Liu YG, Chen HM, Li LG, Osbourn A, Lucas WJ, Huang SW, Ma YS, Shang Y. Root-secreted bitter triterpene modulates the rhizosphere microbiota to improve plant fitness. Nat Plants 2022;8(8):887–96. https://doi.org/10.1038/s41477-022-01201-2.
[52] Demurtas OC, de Brito Francisco R, Diretto G, Ferrante P, Frusciante S, Pietrella M, Aprea G, Borghi L, Feeney M, Frigerio L, Coricello A, Costa G, Alcaro S, Martinoia E, Giuliano G. ABCC transporters mediate the vacuolar accumulation of crocins in saffron stigmas. Plant Cell 2019;31(11):2789–804. https://doi.org/10.1105/tpc.19.00193.
[53] Saez-Saez J, Munro LJ, Moller-Hansen I, Kell DB, Borodina I. Identification of transporters involved in aromatic compounds tolerance through screening of transporter deletion libraries. Microb Biotechnol 2024;17(4):e14460. https://doi.org/10.1111/1751-7915.14460.
[54] Wang G, Møller-Hansen I, Babaei M, D'Ambrosio V, Christensen HB, Darbani B, Jensen MK, Borodina I. Transportome-wide engineering of *Saccharomyces cerevisiae*. Metab Eng 2021;64:52–63. https://doi.org/10.1016/j.ymben.2021.01.007.
[55] Tsujimoto Y, Shimizu Y, Otake K, Nakamura T, Okada R, Miyazaki T, Watanabe K. Multidrug resistance transporters Snq2p and Pdr5p mediate caffeine efflux in *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 2015;79(7):1103–10. https://doi.org/10.1080/09168451.2015.1010476.
[56] Bu X, Lin JY, Cheng J, Yang D, Duan CQ, Koffas M, Yan GL. Engineering endogenous ABC transporter with improving ATP supply and membrane flexibility enhances the secretion of β-carotene in *Saccharomyces cerevisiae*. Biotechnol Biofuels 2020;13(1):168. https://doi.org/10.1186/s13068-020-01809-6.
[57] Wang L, Li N, Yu S, Zhou J. Enhancing caffeic acid production in *Escherichia coli* by engineering the biosynthesis pathway and transporter. Bioresour Technol 2023; 368:128320. https://doi.org/10.1016/j.biortech.2022.128320.
[58] Brennan TCR, Turner CD, Krömer JO, Nielsen LK. Alleviating monoterpene toxicity using a two-phase extractive fermentation for the bioproduction of jet fuel mixtures in *Saccharomyces cerevisiae*. Biotechnol Bioeng 2012;109(10):2513–22. https://doi.org/10.1002/bit.24536.
[59] Qin L, Ma DS, Lin GY, Sun WT, Li C. Low temperature promotes the production and efflux of terpenoids in yeast. Bioresour Technol 2024;395:130376. https://doi.org/10.1016/j.biortech.2024.130376.
[60] Kim DJ, Chang HN. Enhanced shikonin production from *Lithospermum erythrorhizon* by in situ extraction and calcium alginate immobilization. Biotechnol Bioeng 1990;36(5):460–6. https://doi.org/10.1002/bit.260360505.
[61] Lei DW, Qiu ZT, Wu JH, Qiao B, Qiao JJ, Zhao GR. Combining metabolic and monoterpene synthase engineering for *de novo* production of monoterpene alcohols in *Escherichia coli*. ACS Synth Biol 2021;10(6):1531–44. https://doi.org/10.1021/acssynbio.1c00081.
[62] Ye ZL, Huang YL, Shi B, Xiang ZL, Tian Z, Huang M, Wu LL, Deng ZX, Shen K, Liu TG. Coupling cell growth and biochemical pathway induction in *Saccharomyces cerevisiae* for production of (+)-valencene and its chemical conversion to (+)-nootkatone. Metab Eng 2022;72:107–15. https://doi.org/10.1016/j.ymben.2022.03.005.
[63] Zuo YM, Xiao F, Gao JC, Ye CF, Jiang LH, Dong C, Lian JZ. Establishing *Komagataella phaffii* as a cell factory for efficient production of sesquiterpenoid α-santalene. J Agric Food Chem 2022;70(26):8024–31. https://doi.org/10.1021/acs.jafc.2c02353.
[64] Cheng JT, Zuo YM, Liu GF, Li DF, Gao JC, Xiao F, Huang L, Xu ZA, Lian JZ. Development of a *Pichia pastoris* cell factory for efficient production of germacrene A: a precursor of β-elemene. Bioresour. Bioprocess. 2023;10(1):38. https://doi.org/10.1186/s40643-023-00657-0.
[65] Yamada R, Ando Y, Mitsui R, Mizobata A, Yoshihara S, Tokumoto H, Matsumoto T, Ogino H. Improving carotenoid production in recombinant yeast, *Saccharomyces cerevisiae*, using ultrasound-irradiated two-phase extractive fermentation. Eng Life Sci 2022;22(1):4–12. https://doi.org/10.1002/elsc.202100051.
[66] Jiao X, Bian Q, Feng TT, Lyu X, Yu HW, Ye LD. Efficient secretory production of *δ*-tocotrienol by combining pathway modularization and transportation engineering. J Agric Food Chem 2023;71(23):9020–30. https://doi.org/10.1021/acs.jafc.3c01743.
[67] Combes J, Imatoukene N, Couvreur J, Godon B, Brunissen F, Fojcik C, Allais F, Lopez M. Intensification of *p*-coumaric acid heterologous production using extractive biphasic fermentation. Bioresour Technol 2021;337:125436. https://doi.org/10.1016/j.biortech.2021.125436.
[68] Zhu Y, Zhou C, Liu X, Li XY, Shi CF, Zhang YP, Wang Y, Li C. Aided-efflux and high production of β-amyrin realized by β-cyclodextrin in situ synthesized on surface of *Saccharomyces cerevisiae*. Biotechnol Bioeng 2023;120(4):1147–58. https://doi.org/10.1002/bit.28327.
[69] Gani U, Vishwakarma RA, Misra P. Membrane transporters: the key drivers of transport of secondary metabolites in plants. Plant Cell Rep 2021;40(1):1–18. https://doi.org/10.1007/s00299-020-02599-9.
[70] Hillson N, Caddick M, Cai YZ, Carrasco JA, Chang MW, Curach NC, Bell DJ, Le Feuvre R, Friedman DC, Fu XF, Gold ND, Herrgård MJ, Holowko MB, Johnson JR, Johnson RA, Keasling JD, Kitney RI, Kondo A, Liu CL, Martin VJJ, Menolascina F, Ogino C, Patron NJ, Pavan M, Poh CL, Pretorius IS, Rosser SJ, Scrutton NS, Storch M, Tekotte H, Travnik E, Vickers CE, Yew WS, Yuan YJ, Zhao HM, Freemont PS. Building a global alliance of biofoundries. Nat Commun 2019;10: 2040. https://doi.org/10.1038/s41467-019-10079-2.
[71] Kong XR, Lin KX, Wu GL, Tao XF, Zhai XH, Lv LL, Dong DS, Zhu YN, Yang SL. Machine learning techniques applied to the study of drug transporters. Molecules 2023;28(16):5936. https://doi.org/10.3390/molecules28165936.
[72] Fiamenghi MB, Bueno JGR, Camargo AP, Borelli G, Carazzolle MF, Pereira GAG, dos Santos LV, José J. Machine learning and comparative genomics approaches for the discovery of xylose transporters in yeast. Biotechnol. Biofuels Bioprod. 2022;15 (1):57. https://doi.org/10.1186/s13068-022-02153-7.
[73] Jin K, Xia HZ, Liu YF, Li JH, Du GC, Lv XQ, Liu L. Compartmentalization and transporter engineering strategies for terpenoid synthesis. Microb Cell Factories 2022;21(1). https://doi.org/10.1186/s12934-022-01819-z.
[74] Tian M, Bin S, Ziling Y, Xiaowei L, Min L, Yun C, Jiang X, Nielsen J, Zixin D, Tiangang L. Lipid engineering combined with systematic metabolic engineering of *Saccharomyces cerevisiae* for high-yield production of lycopene. Metab Eng 2019; 52:134–42. https://doi.org/10.1016/j.ymben.2018.11.009.
[75] Doshi R, Nguyen T, Chang G. Transporter-mediated biofuel secretion. Proc. Natl. Acad. Sci. U.S.A 2013;110(19):7642–7. https://doi.org/10.1073/pnas.1301358110.
[76] Wu T, Li SW, Ye LJ, Zhao DD, Fan FY, Li QY, Zhang BL, Bi CH, Zhang XL. Engineering an artificial membrane vesicle trafficking system (AMVTS) for the excretion of β-carotene in *Escherichia coil*. ACS Synth Biol 2019;8(5):1037–46. https://doi.org/10.1021/acssynbio.8b00472.