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REVIEW

Engineering *Saccharomyces cerevisiae* to produce plant benzylisoquinoline alkaloids

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Abstract   Benzylisoquinoline alkaloids (BIAs) are a diverse family of plant natural products with extensive pharmacological properties, but the yield of BIAs from plant is limited. The understanding of BIA biosynthetic mechanism in plant and the development of synthetic biology enable the possibility to produce BIAs through microbial fermentation, as an alternative to agriculture-based supply chains. In this review, we discussed the engineering strategies to synthesize BIAs in *Saccharomyces cerevisiae* (yeast) and improve BIA production level, including heterologous pathway reconstruction, enzyme engineering, expression regulation, host engineering and fermentation engineering. We also highlight recent metabolic engineering advances in the production of BIAs in yeast.

Keywords Benzylisoquinoline alkaloids (BIAs), *Saccharomyces cerevisiae*, Synthetic biology, Metabolic engineering

INTRODUCTION

Benzylisoquinoline alkaloids (BIAs) are a family of plant natural products produced by *Ranunculales* plants, such as opium poppy (*Papaver somniferum*), California poppy (*Eschscholzia californica*), Japanese goldthread (*Coptis japonica*), and barberry (*Berberis wilsonae*) (Hagel and Facchini 2013). There are approximately 2500 different identified BIAs (Facchini and Luca 2008), many of which exhibit extensive pharmacological properties and have been used in traditional medicine for thousands of years. For example, morphine and codeine derived from opium poppy are potent analgesics (Aragon-Poce et al. 2002; Price et al. 1985). Berberine, the active compound of traditional Chinese medicine Huang Lian, is a widely used antimicrobial; it also exhibits anticancer potential by stabilizing human telomeric DNA (Guamán Ortiz et al. 2014; Bessi et al. 2012) and antidiabetic potential by regulating glucose and lipid metabolism (Yin et al. 2008; Yin et al. 2002). Noscapine, a

phthalideisoquinoline alkaloid derived from opium poppy, is a safe, nonnarcotic cough suppressant and has proven to possess anticancer properties (Dahlström et al. 1982; Landen et al. 2004; Mahmoudian and Rahimi-Moghaddam 2009; Chen et al. 2015). Chemical structures of these pharmaceutical BIAs are shown in Fig. 1.

The supply of medicinal BIAs largely relies on the farming of plants. For example, opium poppy is a major natural source of BIAs, including morphine, codeine, sanguinarine, noscapine and papaverine (Winzer et al. 2015). In 2019, approximately 240, 800 hectares of opium poppy were cultivated and produced 73 tons of pharmaceutical opioids (United Nations publication 2020). However, this supply chain based on agriculture could be costly in terms of land and water resource. It is also susceptible to environmental factors such as pests and climate. The fragile agriculture-based supply chain is also vulnerable to global uncertainties and crisis such as the ongoing COVID-19 pandemic.

The importance of BIAs and the vulnerable supply chain have prompted research into the BIA biosynthetic
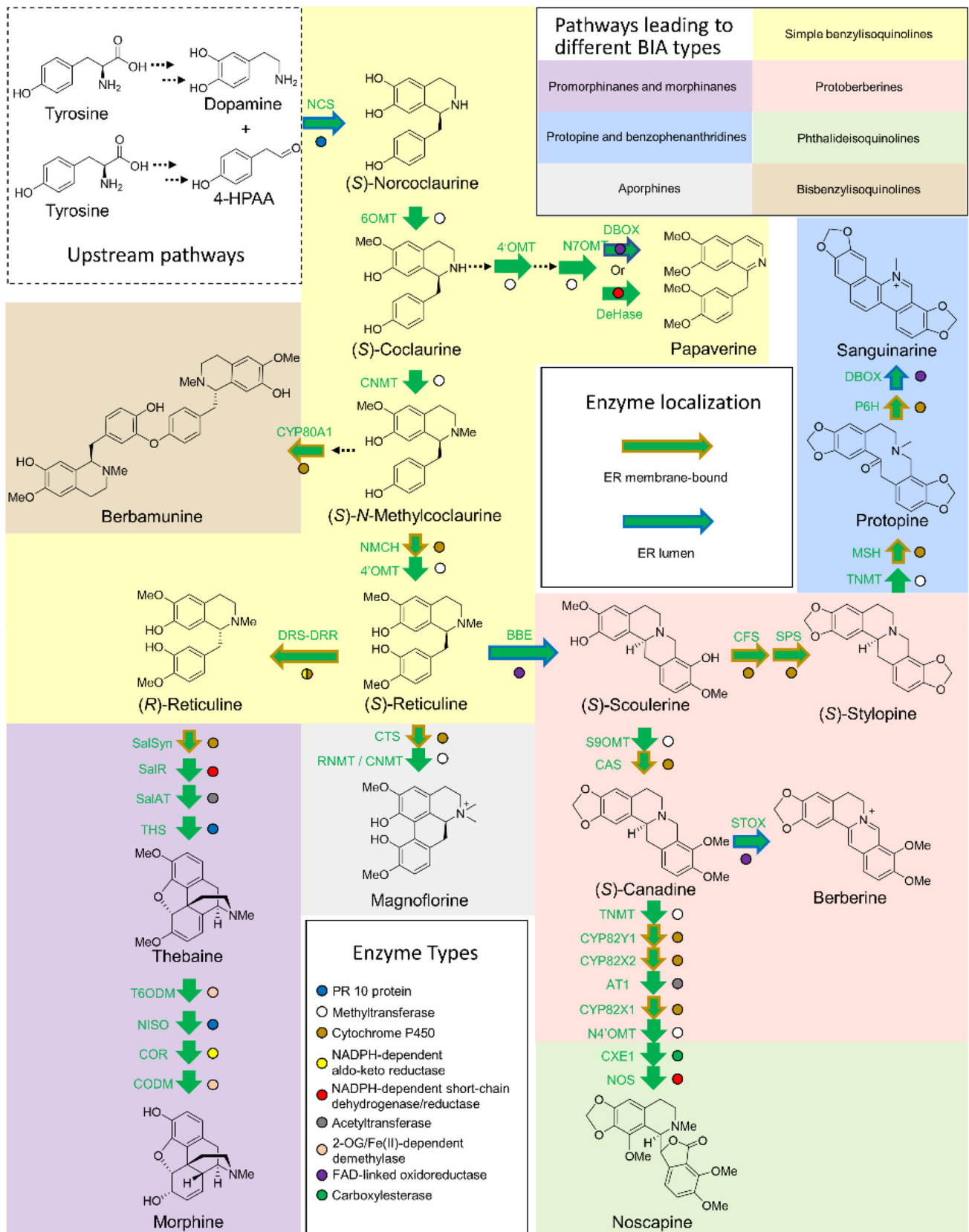
◄**Fig. 1** Representative biosynthetic pathway of BIAs in plant. Key metabolites and enzymes are shown. Block arrows indicate enzymes that have been identified and functionally characterized. Arrow outlines indicate subcellular localization associated with ER: Brown, ER membrane-bound; blue, ER lumen. Enzyme types are identified by colored circles: Blue, PR 10 protein; white, methyltransferase; brown, cytochrome P450; yellow, NADPH-dependent aldo–keto reductase; red, NADPH-dependent short-chain dehydrogenase/reductase; gray, acetyltransferase; pink, 2-OG/Fe (II)-dependent demethylase; purple, FAD-linked oxidoreductase; green, carboxylesterase. Background colors indicate BIA subgroups: Yellow, simple benzylisoquinoline type; purple, promorphinane and morphinane type; pink, protoberberine type; blue, protopine and benzophenanthridine type; green, phthalideisoquinoline type; gray, aporphine type; brown, bisbenzylisoquinoline type. AT1 1,13-dihydroxy-*N*-methylcanadine 13-*O*-acetyltransferase; BBE berberine bridge enzyme; CAS canadine synthase; CFS cheilanthifoline synthase; CNMT coclaurine *N*-methyltransferase; CODM codeine *O*-demethylase; COR codeinone reductase; CEX1 3-*O*-acetylpapaveroxine carboxylesterase; CTS corytuberine synthase; CYP80A1 berbamunine synthase; CYP82X1 1-hydroxy-13-*O*-acetyl-*N*-methylcanadine 8-hydroxylase; CYP82X2 1-hydroxy-*N*-methylcanadine 13-*O*-hydroxylase; CYP82Y1 *N*-methylcanadine 1-hydroxylase; DBOX dihydrobenzophenanthridine oxidase; DRS-DRR 1,2-dehydroreticuline synthase-1,2-dehydroreticuline reductase; MSH *N*-methylstylopine 14-hydroxylase; N7OMT norreticuline 7-*O*-methyltransferase; NCS norcoclaurine synthase; NISO neopinone isomerase; NMCH *N*-methylcoclaurine 3′-hydroxylase; NOS noscapine synthase; N4′MT narcotoline 4′-*O*-methyltransferase; P6H protopine 6-hydroxylase; RNMT reticuline *N*-methyltransferase; SalAT salutaridinol 7-*O*-acetyltransferase; SalR salutaridine reductase; SalSyn salutaridine synthase; S9OMT scoulerine 9-*O*-methyltransferase; SPS stylopine synthase; STOX tetrahydroprotoberberine oxidase; T6ODM thebaine 6-*O*-demethylase; THS thebaine synthase; TNMT tetrahydroprotoberberine *N*-methyltransferase; 4′OMT 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase; 6OMT norcoclaurine 6-*O*-methyltransferase; 4-HPAA 4-hydroxyphenylacetaldehyde

machinery in plants. Recent advances in plant genomics, transcriptomics, proteomics and metabolomics have established the foundation that leads to the identification of plant enzymes and the cascaded reactions they catalyze, namely, biosynthetic pathways enabling BIAs (Dang et al. 2012). BIA biosynthetic pathway and enzymes involved in the pathway have been extensively reviewed (Dang et al. 2012; Beaudoin and Facchini 2014; Singh et al. 2019). Briefly, as shown in Fig. 1, the biosynthesis of all known BIAs start from two tyrosine derivatives, 4-hydroxyphenylacetaldehyde (4HPAA) and dopamine. 4-HPAA and dopamine undergo a Pictet–Spengler condensation to form (*S*)-norcoclaurine, the first committed intermediate in BIA synthesis. This reaction is catalyzed by norcoclaurine synthase (NCS), which belongs to the pathogenesis-related (PR)10/Bet v1 protein family (Samanani et al. 2004; Liscombe et al. 2005; Lee and Facchini 2010). Three *S*-Adenosyl

methionine (SAM)-dependent methyltransferases, namely norcoclaurine 6-*O*-methyltransferase (6OMT) (Morishige et al. 2000; Ounaroon et al. 2003), coclaurine *N*-methyltransferase (CNMT) (Choi et al. 2001), and 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase (4′OMT) (Morishige et al. 2000), together with one cytochrome P450 monooxygenase *N*-methylcoclaurine 3′-hydroxylase (NMCH) (Pauli and Kutchan 1998; Frick et al. 2007), catalyze the following steps converting (*S*)-norcoclaurine to (*S*)-reticuline.

(*S*)-reticuline is an important branch point in the BIA biosynthetic pathway. It can be converted to (*R*)-reticuline in opium poppy to produce morphine by a fusion enzyme DRS-DRR that contains a cytochrome P450 domain and a NADPH-dependent aldo–keto reductase domain. The discovery and identification of DRS-DRR was accomplished via mutant characterization (Winzer et al. 2015) or functional transcriptome analysis (Farrow et al. 2015; Galanie et al. 2015). (*R*)-reticuline is then converted to (7*S*)-salutaridinol 7-*O*-acetate by salutaridine synthase (SalSyn) (Gesell et al. 2009), salutaridine reductase (SalR) (Ziegler et al. 2006) and salutaridinol 7-*O*-acetyltransferase (SalAT) (Grothe et al. 2001). The reaction converting (7*S*)-salutaridinol 7-*O*-acetate to thebaine was considered to be spontaneous in the past. However, a recent study isolated and characterized another PR10 protein from opium poppy, namely thebaine synthase (THS), as the key enzyme that catalyzes the reaction (Chen et al. 2018). The final step that produces morphine from thebaine is catalyzed by thebaine 6-*O*-demethylase (T6ODM) (Hagel and Facchini 2010), codeine *O*-demethylase (CODM) (Hagel and Facchini 2010), codeinone reductase (COR), and neopinone isomerase (NISO), which is the third PR10 protein involved in morphine pathway and was recently discovered via virus-induced gene silencing (VIGS) (Dastmalchi 2019a).

(*S*)-reticuline can also be converted to protoberberine-type BIAs and then lead to the synthesis of sanguinarine, noscapine, or berberine. Such biosynthetic routes start with the production of (*S*)-scoulerine catalyzed by berberine bridge enzyme (BBE), a FAD-linked oxidoreductase (Winkler et al. 2008). The downstream pathway converts (*S*)-scoulerine to sanguinarine via four cytochrome P450s, including cheilanthifoline synthase (CFS) (Chávez et al. 2011), stylopine synthase (SPS) (Chávez et al. 2011), (*S*)-*cis*-*N*-methylstylopine 14-hydroxylase (MSH) (Beaudoin and Facchini 2013), protopine 6-hydroxylase (P6H) (Takemura et al. 2013), one methyltransferase, tetrahydroprotoberberine *cis*-*N*-methyltransferase (TNMT), and one FAD-linked oxidoreductase, dihydrobenzophenanthridine oxidase (DBOX) (Hagel et al. 2012). Another major downstream

pathway starting from (*S*)-reticuline via (*S*)-scoulerine leads to the synthesis of berberine and noscapine. Comparative transcriptomics analysis between noscapine-producing and non-producing opium poppy variants has identified a 10-gene noscapine cluster in opium poppy chromosome (Winzer et al. 2012) including scoulerine 9-*O*-methyltransferase (SOMT) (Dang and Facchini 2012), canadine synthase (CAS, CYP719A) (Dang and Facchini 2014), *N*-methylcanadine 1-hydroxylase (CYP82Y1), 1-hydroxy-*N*-methylcanadine 13-*O*-hydroxylase (CYP82X2), 1,13-dihydroxy-*N*-methylcanadine 13-*O*-acetyltransferase (AT1), 1-hydroxy-13-*O*-acetyl-*N*-methylcanadine 8-hydroxylase (CYP82X1), 3-*O*-acetylpapaveroxine carboxylesterase (CXE1), narcotoline-4′-*O*-methyltransferase (N4′OMT), and a short-chain dehydrogenase/reductase noscapine synthase(NOS). In particular, narcotoline-4′-*O*-methyltransferase (N4′OMT) is a heterodimer of two methyltransferase (Li and Smolke 2016). (*S*)-canadine, the product of SOMT and CAS can also be converted to berberine by a FAD-linked oxidoreductase, (*S*)-tetrahydroxyprotoberberine oxidase (STOX) (Amann et al. 1988; Gesell et al. 2011), or via a spontaneous reaction (Galanie and Smolke 2015).

Biosynthesis of aporphines, for example, magnoflorine, also starts from (*S*)-reticuline. In *C. japonica*, a cytochrome P450, corytuberine synthase (CTS, CYP80G2) catalyzes the intramolecular C–C phenol bond formation of (*S*)-reticuline, leading to corytuberine, and then CNMT catalyzes the conversion from corytuberine to magnoflorine (Ikezawa et al. 2008). While CNMT is a multi-functional enzyme that also catalyzes the *N*-methylation of coclaurine, a reticuline *N*-methyltransferase (RNMT) (Morris and Facchini 2016) was recently isolated from opium poppy root as a specific enzyme for the conversion of corytuberine to magnoflorine.

While (*S*)-reticuline is the central branch point of BIA biosynthetic pathway, metabolites upstream of (*S*)-reticuline can also serve as the branch point for other kinds of BIAs. For example, biosynthesis of papaverine starts from (*S*)-coclaurine via a partially characterized pathway with only a few enzymes identified. 4′OMT and norreticuline 7-*O*-methyltransferase (N7OMT) was proved to be involved in the synthetic pathway of tetrahydropapaverine (Pienkny et al. 2009), while a proposed 3′-hydroxylase and a proposed 3′OMT still remain uncharacterized. The conversion from tetrahydropapaverine to papaverine can be catalyzed by DBOX, but with only 43% efficiency (Hagel et al. 2012). A short-chain dehydrogenase/reductase, referred as DeHase, was recently discovered in opium poppy, which may be the putative specific enzyme for catalyzing this step, indicated by expression analysis and VIGS (Agarwal et al. 2020). Bisbenzylisoquinolines are another subgroup of BIAs. The synthesis of bisbenzylisoquinolines starts from the conversion from *N*-methylcoclaurine to berbamunine, catalyzed by berbamunine synthase (CYP80A1) (Kraus and Kutchan 1995). Berbamunine can be further converted to other bisbenzylisoquinolines, but the downstream pathway has not been identified yet.

The understanding of BIA biosynthetic pathways in BIA-producing plants such as opium poppy and the development of synthetic biology makes it feasible to produce BIAs in heterologous hosts, especially in microorganisms. Compared with sourcing BIAs from cultured plants, fermentation in a tractable microbial host such as *Saccharomyces cerevisiae* or *Escherichia coli* is more rapid, cost-effective, efficient (Rathbone and Bruce 2002) and environmentally friendly (Cordell 2011). However, the complexity of plant BIA pathway hinders its reconstruction in a bacterial host such as *E. coli*. BIA biosynthesis involves multiple membrane-bound cytochrome P450 enzymes, and several other enzymes that are also associated with endoplasmic reticulum (ER) lumen (Hagel and Facchini 2012), such as NCS, BBE and DBOX (Fig. 1). For example, to produce thebaine in *E. coli*, the reaction catalyzed by DRS-DRR has to be circumvented because of the low activity of this fusion enzyme in bacterium (Nakagawa et al. 2016). Instead, *S. cerevisiae* (baker's yeast), as a unicellular eukaryotic model microorganism, can provide the endomembrane system for the functional expression of membrane-bound plant enzymes. The well-studied yeast metabolism also makes it a tractable host for metabolic engineering. The development of synthetic biology provides a variety of genetic tools and elements to reconstruct heterologous pathways and enables dynamically regulation of the expression of diverse proteins respectively (Redden and Alper 2015). Finally, reconstructing de novo BIA biosynthetic pathways in yeast enables the production of novel BIA derivatives. The broad substrate specificity of some enzymes in BIA pathway allows us to produce BIA derivatives by feeding modified simple molecules such as tyrosine derivatives to yield new structures and concomitant bioactivities. In this review, we discuss the engineering strategies to reconstruct BIA synthetic pathway in yeast and to improve the production of BIAs, as well as major achievements in the production of diverse BIAs in yeast.

STRATEGIES FOR PRODUCING BIAS IN YEAST

Non-native pathway construction

Despite the research progress in understanding the BIA biosynthetic pathway, several enzymes catalyzing certain reactions remain unidentified. A strategy to fill in these gaps is to introduce non-native enzymes from other organisms that can catalyze the same reactions, especially those catalyzing the upstream reactions leading to dopamine and 4-HPAA synthesis that are common in different organisms. For example, the production of dopamine that enables de novo BIA biosynthetic pathway reconstruction in yeast has remained challenging for long, as the enzymes for dopamine synthesis in BIA-producing plant have not been fully elucidated yet (Singh et al. 2019). Instead, a bacterial dihydroxyphenylalanine (DOPA) decarboxylase (DoDC) from *Pseudomonas Putida* (Koyanagi 2012) was reported to convert DOPA to dopamine (Fig. 2A). Engineered yeast expressing PpDoDC can produce dopamine and the downstream product norcoclaurine with a titer of

49 μg/L, when fed with 2 mM DOPA in the cultural medium (Trenchard et al. 2015). Then the next step is to find an enzyme (tyrosine 3-hydroxylase, TyrH) that can catalyze the reaction from tyrosine to DOPA in yeast. Two different approaches were employed here (Fig. 2A). An enzyme-coupled biosensor was developed to screen for heterologous tyrosine hydroxylase that are active in yeast (DeLoache et al. 2015). The DOPA produced by the candidate TyrH was converted by a plant DOPA dioxygenase to betaxanthin in yeast. Betaxanthin is a yellow pigment that is produced by plants in *Caryophyllales* in nature, the accumulation of which leads to yeast colorimetric change and corresponding TyrH screening (DeLoache et al. 2015). With this biosensor, a tyrosine hydroxylase (CYP76AD1) from *Beta vulgaris* (sugar beets) was identified to possess high activity in yeast and produced 1.3 mg/L DOPA (DeLoache et al. 2015). Reconstruction of the downstream pathway led to the de novo production of (*S*)-reticuline with a titer of 80.6 μg/L (DeLoache et al. 2015). Instead of plant TyrH, another alternative candidate is a mammalian tyrosine hydroxylase TyrH
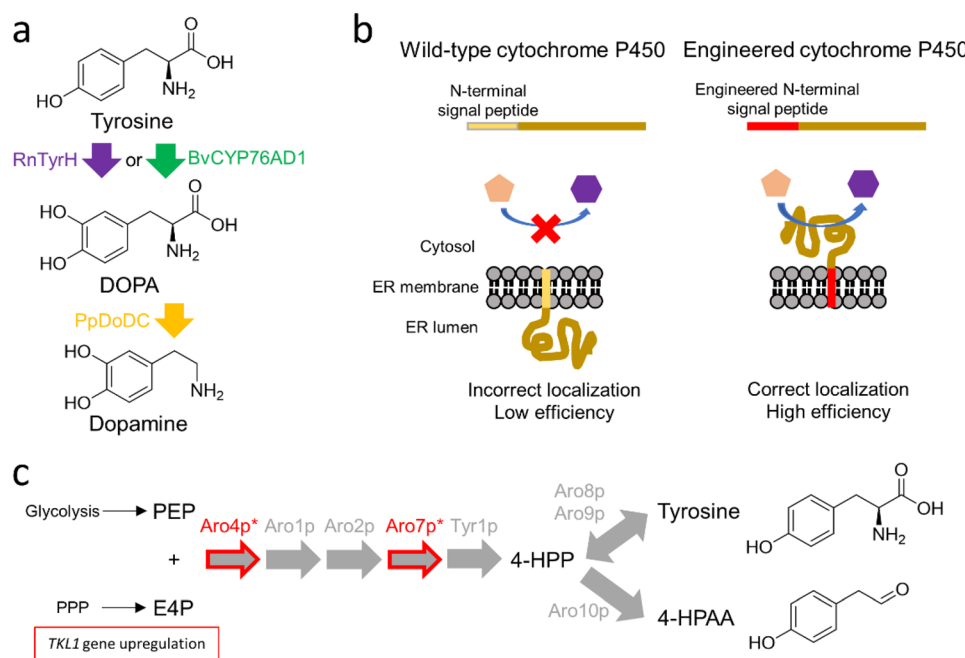
Fig. 2 Representative strategies and examples to produce BIAs in yeast. **A** Reconstruction of dopamine biosynthetic pathway via non-native enzymes. Orange arrow, bacterial enzyme; purple arrow, mammalian enzyme; green arrow, plant enzyme. DOPA, dihydroxyphenylalanine; PpDODC, DOPA decarboxylase from *Pseudomonas Putida*; RnTyrH, tyrosine 3-hydroxylase from *Rattus norvegicus* (Brown rat); BvCYP76AD1, tyrosine 3-hydroxylase from *Beta vulgaris* (sugar beets). **B** Schema of protein engineering on N-terminal signal peptide to guide the correct localization and enhance the activity of plant cytochrome P450 enzymes. **C** Engineering of yeast endogenous pathway to produce 4-HPAA. Red outlines indicate engineered enzymes. *Aro10p* phenylpyruvate decarboxylase; *Aro1p* pentafunctional arom enzyme; *Aro2p* bifunctional chorismate synthase and flavin reductase; *Aro4p** DAHP synthase with feedback inhibition-resistant mutation Q166K; *Aro7p** chorismate mutase with feedback inhibition-resistant T266I; *Aro8p* aromatic aminotransferase I; *Aro9p* aromatic aminotransferase II; *E4P* erythrose-4-phosphate; *PEP* phosphoenolpyruvate; *4-HPP* 4-hydroxyphenylpyruvate; *4-HPAA* 4-hydroxyphenylacetaldehyde

from *Rattus norvegicus* (Brown rat) (Trenchard et al. 2015). RnTyrH exhibits higher substrate and product specificity compared with other kinds of tyrosine hydroxylase from plants or bacteria. However, it relies on the electron carrier tetrahydrobiopterin (BH4) that is absent in wild-type yeast (Trenchard et al. 2015). Therefore, four additional mammalian enzymes were introduced into engineered yeast to enable BH4 synthesis pathway (Trenchard et al. 2015). Eventually, 19.2 μg/L (*S*)-reticuline titer was achieved by the additional introduction of DODC and other five downstream enzymes (Trenchard et al. 2015). These yeast strains with non-native dopamine biosynthetic pathways provide dopamine as the essential building block molecules for diverse BIA synthesis.

Enzyme engineering

Protein directed evolution is a widely used strategy to increase an enzyme's catalytic activity, substrate specificity, or to decrease product feedback inhibition. For example, in addition to the tyrosine hydroxylase activity, wild-type CYP76AD1 also exhibits DOPA oxidase activity (Hatlestad et al. 2012), which is undesirable for BIA production. From an enzyme variant library containing approximately 200,000 members, researchers obtained a W13L F309L variant of CYP76AD1 associated with 2.8-fold higher DOPA production and correspondingly decreased DOPA oxidase activity (DeLoache et al. 2015). The mammalian TyrH was also engineered via site-directed mutagenesis with W166Y R37E R38E mutations to relieve the product feedback inhibition and increase the norcoclarine titer by ninefold over the strain with wild-type TyrH enzyme (Trenchard et al. 2015).

The subcellular localization of enzymes is another important factor that can influence the function of enzymes. A variety of cytochrome P450 enzymes are involved in BIA synthetic pathway, all of which need to localize to the endoplasmic reticulum (ER) membrane and to face the cytosolic surface of ER membrane correctly. N-terminal signal peptide that guides the correct localization of ER-bound protein is critical in this process, and therefore becomes the key target in enzyme localization engineering (Fig. 2B). For example, in morphine biosynthetic pathway, salutaridine synthase (SalSyn) is a key enzyme that used to show low activity in yeast, as a number of nascent proteins were incorrectly localized on ER membrane surface facing the lumen (Galanie et al. 2015). Replacing the N-terminal signal peptide of SalSyn with another cytochrome P450 signal peptide led to a chimeric SalSyn that could be anchored on ER membrane correctly and improved the production of salutaridine by sixfold (Galanie et al. 2015).

Meanwhile, the function of some other enzymes that are localized into ER in plant does not rely on ER in microorganisms. For example, the localization of NCS in ER lumen may even have a negative effect on its catalytic efficiency in yeast. Hence, a noscapine-producing yeast strain that expressed an NCS variant with the first 24 amino acids deleted increased the end-product noscapine production by eightfold compared with the strain with wild-type NCS (Li et al. 2018).

Regulation of gene expression

Expression of heterologous enzymes may bring extra expression burden to the engineered yeast cells, especially for those ER membrane-bound cytochrome P450 enzymes. Overexpression of cytochrome P450 enzymes can trigger a stress response in yeast that leads to abnormal proliferation of ER (Sandig et al. 1999). As a result, the expression of these enzymes needs to be tuned to yield optimal productivity. In yeast, a variety of promoters have been characterized as standard genetic elements to drive gene expression at different levels. For example, five different yeast promoters (P_{GPD}, P_{TEF1}, P_{PGK1}, P_{TPI1}, and P_{HXT7}) were used to drive the expression of cheilanthifoline synthase (CFS) where P_{PGK1} provided a tenfold improvement in cheilanthifoline production compared to the other promoters (Trenchard and Smolke 2015). The process of tuning expression level can become easier with the use of inducible promoters, such like the GAL promoters that can be activated by galactose. The GAL promoters have been used to regulate the expression of three methyltransferases (6OMT, 4′OMT and CNMT) in engineered yeast. With these tunable strains, researchers characterized the relationship between galactose concentration, enzyme expression levels and reticuline production, and thus determined the optimal expression level of these enzymes (Hawkins and Smolke 2008). Gene copy number is another factor that can influence the expression level. For example, 4′OMT has been long believed to be the bottleneck of the conversion from (*S*)-norcoclaurine to (*S*)-reticuline. It has been recently proven by the integration of an additional copy of 4′OMT that resulted in a 45% improvement in (*S*)-reticuline titer (Pyne et al. 2020). Moreover, the ratio of gene copy number is also important. In the reconstruction of morphine pathway in yeast, the highest morphine production was achieved when the copy number of three genes encoding enzymes (T6ODM, COR and CODM) was 2:1:3, increasing twofold compared with the original strain with enzyme copy number in 1:1:1 ratio (Thodey et al. 2014).

Host engineering

The endogenous pathways in yeast also play an important role in BIA synthesis. The production of 4-HPAA in yeast is closely related to the central metabolism. In yeast, 4-HPAA is an endogenous metabolite that can be synthesized from two central metabolites, phosphoenolpyruvic acid (PEP) in glycolysis and erythrose-4-phosphate (E4P) in pentose phosphate pathway (Fig. 2C). Incorporation of Q166K feedback inhibition-resistant mutant of 3-deoxy-arabino-hepturosonate-7-phosphate synthase (DHAP synthase, Aro4) (Fukuda et al. 1992) resulted in 14-fold increase in norcoclaurine production (Trenchard et al. 2015). Deletion of the endogenous *zwf1* gene encoding glucose-6-phosphate and upregulation of *TKL1* gene encoding transketolase further increased norcoclaurine production by 60-fold (Trenchard et al. 2015), as these modifications directed the flux in pentose phosphate pathway to favor E4P. However, the deletion of *zwf1* decreased the production of downstream compound such like noscapine (Li et al. 2018). One possible reason is that *zwf1* deletion decreased NADPH level, which is an important cofactor for cytochrome P450 enzymes (Li et al. 2018). Additionally, 4-HPAA can be rapidly converted to alcohols via the Ehrlich pathway. Seven genes (*ari1, adh6, ypr1, ydr541c, aad3, gre2* and *hfd1*) are involved in the consumption of 4-HPAA; knocking out of these genes increased norcoclaurine and (*S*)-reticuline production in yeast (Pyne et al. 2020).

Fermentation engineering

Parameters in fermentation such as pH and temperature, have a remarkable impact in the production of BIAs. pH can significantly affect the activity of yeast in microbial fermentation and thus influence the production of BIAs. For example, in the production of canadine, maintaining higher pH conditions at 5–5.7 improved the titer by approximately threefold compared with the unbuffered media at pH 3.5–5.7 (Galanie and Smolke 2015). Temperature is another key factor in BIA production due to the enhanced activity and stability of many plant enzymes at lower temperature. In a protoberberine-producing strain, stylopine production increased by 3.4-fold when the strain was grown at 25 °C compared to 30 °C. However, the effect of temperature does not always take place on all enzymes. For example, in a berberine-producing strain, temperature showed no influence on the production of berberine and canadine (Galanie and Smolke 2015).

The production of BIAs can also be influenced by the primary carbon source in the cultural medium.

Galactose was shown to have a positive effect on the production of stylopine (Trenchard and Smolke 2015), canadine and berberine (Galanie and Smolke 2015), possibly due to its regulatory effect on the expression of several yeast endogenous pathways. In noscapine production, trehalose was found to be the best source, increasing the titer by 50-fold (Li et al. 2018).

Additionally, sufficient cofactor level is essential to enzyme functions. For example, as mentioned above, BH4 synthesis pathway was introduced into yeast when mammalian TyrH was selected for DOPA production. Furthermore, adding 2 mM ascorbic acid to media can increase DOPA production by 1.5-fold as ascorbic acid prevents the oxidation of BH4 and increase the activity of TyrH (Trenchard et al. 2015). For morphine production, two demethylases, T6ODM and CODM, require 2-oxoglutarate as the cofactor. In the production of semisynthetic opioid drug hydrocodone, 50 mM 2-oxoglutarate was added to media to support T6ODM activity (Galanie et al. 2015).

APPLICATIONS

High-level production of (*S*)-reticuline in yeast

As most BIAs are derived from (*S*)-reticuline, a yeast platform with high level of (*S*)-reticuline production is essential to produce other downstream BIAs. The first yeast strain for (*S*)-reticuline production was reported in 2008 (Table 1) (Hawkins and Smolke 2008), with the integration of 6OMT, CNMT and 4′OMT from *P. somniferum*, in which norlaudanosoline (NLDS) was used as the substrate. Reconstruction of non-native dopamine synthetic pathway in yeast has overcome the obstacle of the de novo production of (*S*)-reticuline. In 2015, as is mentioned above, heterologous expression of plant TyrH (DeLoache et al. 2015) or mammalian TyrH (Trenchard et al. 2015) lead to 80.6 or 19.2 μg/L of de novo (*S*)-reticuline production from tyrosine, respectively (Table 1). However, the production is still not comparable with that in bacteria like *E. coli*, which has reached 46 mg/L in 2011 (Nakagawa et al. 2011) and 160 mg/L in 2018 (Matsumura et al. 2018), yet bacteria are not suitable hosts for the downstream BIA pathways due to the lack of membrane-bound organelles for the functional expression of cytochrome P450 enzymes. A great breakthrough that tremendously enhanced (*S*)-reticuline production in yeast was achieved in 2020. The production of (*S*)-reticuline in yeast reached 4.6 g/L (Table 1), by introducing a new TryH ortholog (CYP76AD5), adjusting the gene copy number, and

Table 1 Major advances in BIA production in yeast

Substrate	Product	Titer	Comments	Ref
(R, S)-NLDS	(S)-reticuline	150 mg/L	First synthesis of BIAs in yeast	Hawkins and Smolke (2008)
	Salutaridine		Intermediate to morphine	
	Canadine	20 mg/L	Intermediate to berberine and noscapine	
		30 mg/L	Titers in this article are estimated based on percentage substrate conversion	
De novo	(S)-reticuline	80.6 μg/L	First de novo synthesis of reticuline in yeast with plant TyrH	DeLoache et al. (2015)
De novo	(S)-reticuline	19.2 μg/L	De novo synthesis of reticuline in yeast with mammalian TyrH	Trenchard et al. (2015)
De novo	(S)-reticuline	4.6 g/L	Highest production of reticuline in yeast	Pyne et al. (2020)
Thebaine	Codeine	7.7 mg/L		Thodey et al. (2014)
	Morphine	4.7 mg/L		
	Hydrocodone	51 mg/L		
	Oxycodone	70 mg/L		
(R)-reticuline	Thebaine	0.31 mg/L		Fossati et al. (2015)
De novo	Thebaine	6.4 μg/L	DRS-DRR was identified to catalyze the key step from (S)- to (R)-reticuline	Galanie et al. (2015)
	Hydrocodone	0.3 μg/L		
(R, S)-NLDS	Thebaine	0.7 mg/L	Improved titer by incorporating THS	Chen et al. (2018)
(R, S)-NLDS	Canadine	1.8 mg/L		Galanie and Smolke (2015)
	Berberine	6.5 μg/L		
(R, S)-NLDS	Noscapine	0.68 mg/L		Li and Smolke (2016)
De novo	Noscapine	2.2 mg/L		Li et al. (2018)
(R, S)-NLDS	Sanguinarine	80 μg/L		Trenchard and Smolke (2015)
Dopamine	Magnoflorine	7.25 mg/L	Coculture with reticuline-producing E. coli	Minami et al. (2008)

NLDS norlaudanosoline; *THS* thebaine synthase; *TyrH* tyrosine 3-hydroxylase

deleting seven host oxidoreductase that competitively consume precursor 4-HPAA (Pyne et al. 2020).

Production of morphinan alkaloids in yeast

Among the reconstruction of downstream BIA pathways, the branched pathway towards morphinan alkaloids is the most attracting due to the high pharmaceutical value of products like morphine and codeine and semisynthetic opioids like oxycodone, hydrocodone, and hydromorphone. Before the identification of the enzyme for the synthesis of (R)-reticuline from (S)-reticuline, it was impossible to engineer a recombinant host for the de novo production of morphinan alkaloids. Using (R)-norlaudanosoline as the substrate, (R)-reticuline was produced via 6OMT, CNMT and 4′OMT with the titer of about 150 mg/L, and further salutaridine was produced for the first time with the titer of approximately 20 mg/L (Hawkins and Smolke 2008), which is the first morphinan alkaloid

produced in yeast. In 2014, high-value morphinan alkaloids including codeine (7.7 mg/L), morphine (4.7 mg/L), hydrocodone (51 mg/L) and oxycodone (70 mg/L) were successfully produced from thebaine in yeast (Table 1) (Thodey et al. 2014). The synthetic pathway of thebaine from (R)-reticuline was subsequently reconstructed in yeast, with a thebaine titer of 0.31 mg/L (Table 1) (Fossati et al. 2015). Finally, with the identification of DRS-DRR that catalyzes the conversion from (S)-reticuline to (R)-reticuline, the de novo synthesis of morphinan alkaloids was achieved in 2015, with the production of 6.4 μg/L thebaine and 0.3 μg/L hydrocodone (Table 1) (Galanie et al. 2015). The engineered yeast strains were further optimized based on new discovery of synthetic mechanism in plant. For example, in 2018, thebaine synthase (THS), which was newly discovered to catalyze the formation of thebaine from 7-(S)-salutaridinol 7-O-acetate, was incorporated in engineered yeast and increased thebaine production by 24-fold compared with the strain that relied on

spontaneous reaction for thebaine formation (Table 1) (Chen et al. 2018).

Production of berberine, noscapine, sanguinarine, and magnoflorine in yeast

Berberine, noscapine and sanguinarine are three representative pharmaceutical BIAs in addition to morphinan alkaloids. In 2008, based on the first (S)-reticuline-producing yeast, canadine, as the important precursor of berberine and noscapine, was produced in yeast (Hawkins and Smolke 2008). The strain was optimized by enzyme variant screening and genetic copy number variation, leading to a 1.8 mg/L canadine titer and 6.5 µg/L berberine production (Table 1) (Galanie and Smolke 2015). Noscapine synthetic pathway from norlaudanosoline was reconstructed in yeast in 2016 (Li and Smolke 2016), and optimized in 2018 (Table 1) (Li et al. 2018). The yeast strain harboring complete de novo noscapine synthetic pathway reached 2.2 mg/L titer of noscapine production (Li et al. 2018). Moreover, sanguinarine synthetic pathway was reconstructed and engineered in yeast, producing 80 µg/L sanguinarine (Table 1) (Trenchard and Smolke 2015). Although the sanguinarine titer is far below the need for commercial uses, the reconstruction of sanguinarine synthetic pathway provided valuable experiences and engineering strategies for the functional expression of multiple plant cytochrome P450 enzymes, as there are four cytochrome P450 enzymes involved in this pathway. Heterologous production of magnoflorine was achieved in the consortium fermentation of engineered *E. coli* and yeast: Engineered *E. coli* harboring a 5-gene pathway including monoamine oxidase (MAO), NCS, 6OMT, CNMT, and 4'OMT, provides reticuline to an engineered yeast that co-expressed CTS and CNMT. Engineered *E. coli* can efficiently produce (S)-reticuline from dopamine but lack the intercellular membrane structure to support the functional expression of cytochrome P450 enzymes in the downstream pathway. The co-culture system combines the advantages of higher reticuline production in *E. coli* and functional expression of CTS in yeast, and resulted in a titer of 7.2 mg/L (Minami et al. 2008).

Production of novel BIA derivatives

One of the goals of synthetic biology is to expand the diversity of natural products by engineering chemical modifications and structural scaffolds that have not ever existed in nature (Smanski et al. 2016). Owing to the broad substrate specificity of enzymes, engineered yeast is able to convert modified simple molecules, such as tyrosine derivatives, to novel BIA derivatives. Among the chemical modifications, halogenation of BIAs received the most attention. Halogens (fluorine, chlorine, bromine, and iodine) are highly prevalent in licensed drugs, and their benefits on pharmacokinetic properties have been thoroughly reviewed (Fejzagić et al. 2019; Bradley et al. 2020). In engineered yeast, 8-fluororeticuline and 8-chloro-reticuline were produced when feeding 3-fluoro-tyrosine and 3-chloro-tyrosine, respectively, and 8-iodo-(S)-N-methylcoclaurine was produced when feeding 3-iodo-tyrosine (Li et al. 2018). The diversity of BIA structural scaffold can also be expanded by feeding exogenous amino acid analogs, due to the promiscuity of NCS. Sulfur-containing isoquinoline scaffold was formed in yeast when feeding methionine (Pyne et al. 2020). The production of ethyl-, propyl-, butyl-, and pentyl-substituted isoquinoline scaffolds in yeast were also achieved by feeding 2-aminobutyrate, norvaline, norleucine, and 2-aminoheptanoic acid, respectively, and these novel isoquinoline scaffolds can be further methylated by 6OMT and CNMT (Pyne et al. 2020). The biosynthesis of these novel BIA derivatives with various chemical modifications and structural scaffolds can dramatically broaden the diversity of BIAs for human to screen new drugs with novel pharmaceutical properties.

CONCLUSION AND FUTURE PERSPECTIVE

With the rapid development of synthetic biology, yeast has been engineered as a versatile microbial factory to produce a variety of high-value BIAs (e.g., morphine, codeine, berberine, and noscapine), which evinces profound potential to replace the traditional agriculture-based supply chain. In this review, we focus on the reconstruction, optimization, and engineering of BIA biosynthetic pathways that have been elucidated. Regarding the start-of-the-art strategies and progress identifying unknown pathways or missing steps in plant BIA metabolism (e.g., the missing enzymes in bisbenzylisoquinoline and papaverine synthesis), we refer the readers to a number of reviews (Dang et al. 2012; Beaudoin and Facchini 2014; Singh et al. 2019) outlining the discovery and characterization of BIA metabolism in plant.

Throughout this review, we have highlighted the synthetic biology strategies that have been leveraged in yeast to reconstruct the BIA biosynthetic pathway, to optimize the production, and to synthesize diverse unnatural BIA derivatives. However, great efforts are still needed both at laboratory and at industrial scale to achieve efficient and/or profitable biomanufacturing, levels, host backgrounds, and fermentation conditions.

However, for commercial production, further engineering efforts are still required to meet the minimal titer (~ 5 g/L) for yeast production of BIAs to be competitive with poppy farming (Galanie et al. 2015). Although the recent highest titer to date has reached 4.6 g/L for (S)-reticuline (Pyne et al. 2020), high-level production of downstream pharmaceutical BIAs still remains challenging for future work.

Functional expression of plant enzymes in yeast remains a bottleneck in BIA biosynthetic pathway reconstruction, even if the corresponding genes and enzymes have been identified and characterized *in planta* (Beaudoin and Facchini 2014; Singh et al. 2019). This gap hindering successful BIA pathway reconstruction relates back with the intrinsic structural and functional differences between plant and yeast cells. Lack of plant-specific organelles or space in yeast (e.g., chloroplasts and plant cell apoplast) might lead to an unfavorable chemical environment where the recombinant enzymes are inactive or less active. We anticipate that relocalization of plant enzymes into an appropriate yeast organelle can tackle this challenge: For example, although plant NCS is localized in ER lumen, engineered NCS that is relocalized in yeast cytosol increased BIA titer (Li et al. 2018), probably due to the enhanced transportation of metabolites. Intriguingly, a very recent study further revealed that high-level expression of cytosolic NCS is also toxic to yeast, and managed to enhanced the BIA titer by relocalizing the recombinant NCS into peroxisome (Grewal et al. 2021). Therefore, protein engineering for the correct localization of plant enzymes in yeast is important to improve their catalytic activity and to increase the titer of BIAs.

Furthermore, we anticipate that novel strategies to optimize the multi-enzyme pathway in a synchronized manner are key to enhance the production of BIAs toward scalable biomanufacturing. In addition to the metabolic engineering and genome editing approaches that optimize target BIA production in a unicellular environment, co-culture of multiple types of cells, or consortia fermentation, shows the potential of addressing the expression burden in the long, complex BIA biosynthetic processes. Lower metabolic burden may increase the substrate and energy utilization in individual strains and therefore increase the overall efficiency of the co-culture system. It has become a widely used strategy in biotechnology, especially when producing complex chemicals from simple small molecules, which has been extensively reviewed recently (Diender et al. 2021). Intercellular transportation is always an important concern in co-culture system. The recent discovery of a BIA-specific transporter family (Dastmalchi 2019b) may broaden the application of co-culture system in BIA synthesis.

Another exciting application of microbial biomanufacturing is the biosynthesis of "unnatural" BIAs that have tailormade modifications towards enhanced pharmaceutical activities, which is difficult to pursue using traditional methods. While modified substrate fed into BIA-producing yeast (e.g., tyrosine derivatives) may lead to novel BIA derivative production, the BIA biosynthetic pathways that are composed of enzymes similar or identical to the wild-type plant enzymes usually do not exhibit high promiscuity or efficiency in unnatural BIA derivative synthesis. Aiming at a more flexible and efficient microbial platform, it is essential to employ protein engineering methods to create engineered enzymes that have higher promiscuity and efficiency for BIA derivatives (e.g., halogenated noscapines) synthesis in yeast. This dilemma between substrate promiscuity and catalytic efficiency poses a new challenge to protein engineering, as low substrate specificity of an enzyme usually reduces the production of desired compound due to the competition of multiple substrates. De novo rational design and structural identification of the enzymes in BIA metabolism will be a promising approach to develop such tailormade enzymes for novel BIA derivative synthesis. Discovery and engineering of halogenases may provide an alternative way to produce BIA analogous by modifying BIAs directly. Although only a small number of halogenases were characterized and with limited substrate range, enzyme engineering efforts to exploit the substrate promiscuity (Brown and O'Connor 2015) based on the understanding of their mechanism will make it possible to incorporate them in the BIA synthetic pathways,

Availability of data and material Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest All authors declare that they have no conflict of interest.

Ethics approval Not applicable.

Consent to participat Not applicable.

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