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REVIEW ARTICLE

Heterologous biosynthesis of artemisinic acid in *Saccharomyces cerevisiae*

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Summary

Artemisinic acid is a precursor of antimalarial compound artemisinin. The titre of biosynthesis of artemisinic acid using *Saccharomyces cerevisiae* platform has been achieved up to 25 g l^{-1}; however, the performance of platform cells is still industrial unsatisfied. Many strategies have been proposed to improve the titre of artemisinic acid. The traditional strategies mainly focused on partial target sites, simple up-regulation key genes or repression competing pathways in the total synthesis route. However, this may result in unbalance of carbon fluxes and dysfunction of metabolism. In this review, the recent advances on the promising methods *in silico* and *in vivo* for biosynthesis of artemisinic acid have been discussed. The bioinformatics and omics techniques have brought a great prospect for improving production of artemisinin and other pharmacal compounds in heterologous platform.

Introduction

The artemisinin is isolated from the leaves of *Artemisia annua* in the 1980s by Chinese scientists. It is acknowledged to be an effective pharmaceutical compound for the treatment of malaria (Barbacka and Baer-Dubowska 2011). However, the content of artemisinin in plant tissue is extremely low, which results in low production of artemisinin. The medicine of artemisinin is still expensive (Barbacka and Baer-Dubowska 2011). Scientists have been endeavoured to produce artemisinin in alternative ways (Kong *et al.* 2013), such as extraction from *Artemisia annua*, chemical synthesis in *de novo*, biosynthesis by plant genetic engineering and metabolic engineering of micro-organisms. In term of the cost and feasibility in industry (Nair *et al.* 1986; Paniego and Giulietti 1996; Liu *et al.* 1997; Delabays *et al.* 2001; Weathers *et al.* 2005), *Saccharomyces cerevisiae* is recognized as a better platform to synthesize artemisinic acid, the precursor of artemisinin (Kirby and Keasling 2009; Farhi *et al.* 2011; Kampranis and Makris 2012), since the yield of artemisinic acid has been achieved up to 25 g l^{-1}. However, the conversion of artemisinic acid to artemisinin has to be processed with the aid of chemical methods (Paddon and Keasling 2014). The final yield of artemisinin is still too low to be cheap.

To improve the production of artemisinic acid in yeast cells, many strategies have been established, such as the overexpression of key genes, the repression of competing pathways (Kong *et al.* 2013; Wang *et al.* 2013; Paddon and Keasling 2014). All these traditional strategies mainly focused on increasing carbon flux towards heterologous biosynthesis pathway in host cells. However, the simple up-regulation or down-regulation of key genes may result in dysfunction of metabolism, subsequently, the growth of the cell could be inhibited by the accumulation of toxic intermediates (Sun *et al.* 2014).

In this review, the recent developments in the heterologous biosynthesis of artemisinic acid in yeast cells have been discussed. At the same time, the application of promising methods *in silico* and *in vivo* in metabolic engineering has been summarized. Here, this review

demonstrated not only the technical progresses in the biosynthesis of artemisinin acid, but also the lessons we can learn from past experiences.

Heterologous biosynthesis of artemisinic acid

Biosynthesis pathway of artemisinin in *Artemisia annua*

In *A. annua*, the glandular secretory trichomes (GSTs) are the main sites for the synthesis of artemisinin (Nguyen *et al.* 2011). The GSTs consists of 10 cells and the synthesizing process occurs in apical cells which are the only cell types possessing chloroplasts (Olsson *et al.* 2009). The whole synthesis process in GSTs includes three steps (Brown 2010): (i) the combination of isopentenyl diphosphate (IPP) and its isomer (dimethylallyl pyrophosphate, DMAPP) to form farnesyl diphosphate (FPP) that converts into amorphadiene (AD) subsequently; (ii) the oxidation of AD to synthesize the precursor of artemisinin (dihydroartemisinic acid or artemisinic acid); (iii) the synthesis of artemisinin through the conversion of its precursors (Fig. 1).

In higher plants, the carbon skeleton of artemisinin stems from FPP which derives from 2 IPP and 1 DMAPP (Ku *et al.* 2005). Although both methylerythritol phosphate (MEP) pathway (exist in plastid) and mevalonate (MVA) pathway (exist in cytoplasm) attribute to the formation of FPP for the synthesis of AD in *A. annua*, the MVA pathway is dominant (approx. 80%) (Ram *et al.* 2010). In GSTs, the synthesis of artemisinin commences with the formation of FPP. MEP pathway in chloroplast of apical cells provides one molecule IPP to form the core of FPP which subsequently combines with IPP and DMAPP derived from MVA pathway to form the FPP (Schramek *et al.* 2009; Nguyen *et al.* 2011). Amorphadiene synthase (*ADS*) further catalyses the transformation of FPP to AD which immediately generates two routes to synthesis the precursor of artemisinin: one is for artemisinic acid synthesis, and the other is for dihydroartemisinic acid formation (Weathers *et al.* 2005) (Fig. 1).

The 3-hydroxy-3-methyl-glutaryl CoA (HMG-CoA) reductase (*HMGR*) in MVA pathway is one of the principal targets of complex regulation in artemisinin synthesis (Ye and Bhatia 2012). It catalyses the conversion of HMG-CoA to MVA, followed by successive steps leading to the synthesis of IPP and DMAPP (Ye and Bhatia 2012). The farnesyl diphosphate synthase (FPPS, encoded by *ERG20*) is another important enzyme catalysing the formation of FPP (Covello *et al.* 2007; Paddon 2013). The level and activity of FPPS are likely important for the yield of end-product.

However, the conversion mechanism of precursor compounds to artemisinin is still not clear (Liu *et al.* 2013). It is reported that artemisinic acid is the precursor of artemisinin (Sangwan *et al.* 1993). However, recently it is pointed out that dihydroartemisinic acid may be the necessary precursor for artemisinin synthesis (Brown 2010). Dihydroartemisinic acid can react with reactive oxygen species (ROS) in cells to withstand the outside stress coupled with the production of artemisinin (Liu *et al.* 2013). It is indicated that artemisinin synthesis in GSTs depends on the spontaneous oxidation of dihydroartemisinic acid in sunlight (Weathers *et al.* 2005; Ro *et al.* 2006; White 2008). This process may be also involved in the reaction between dihydroartemisinic acid and ROS (Liu *et al.* 2013).

Artemisinic acid biosynthesis in *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a suitable platform for expressing plant genes (Kirby and Keasling 2009; Farhi *et al.* 2011). The MVA pathway in *S. cerevisiae* is identical to that in *A. annua* and those in almost all other plants (Fig. 2) (Zeng *et al.* 2007; Zeng and Bao 2011). *Saccharomyces cerevisiae* uses MVA as the substrate to produce isoprenoids. The synthesis of MVA commences with acetoacetyl-CoA in cytosol. Isoprenoids are converted into IPP via successive enzymatic reactions. The isomerase is responsible for catalysing the conversion of IPP to DMAPP. The IPP and DMAPP are then combined to form FPP catalysed by FPPS (Covello *et al.* 2007; Paddon 2013). The heterologous biosynthesis route of artemisinic acid commencing with FPP is similar to that described by Paddon *et al.* (Paddon and Keasling 2014). The regulation targets include amorphadiene synthase (*ADS*) (Brown 2010), cytochrome P450 monooxygenase (*CYP71AV1*) (Bouwmeester *et al.* 1999; Shiba *et al.* 2007; Ajikumar *et al.* 2008), artemisinic alcohol dehydrogenase (*ADH1*) (Paddon *et al.* 2013) and artemisinic aldehyde dehydrogenase (*ALDH1*) (Paddon *et al.* 2013). The functions of these enzymes are shown in Fig. 3.

Although the nearly complete biosynthesis route has been established, the final step, the conversion of artemisinic acid to artemisinin, has to be processed with aid of chemical methods. Interestingly, as mentioned above, the precursor for artemisinin synthesis is dihydroartemisinic acid rather than artemisinic acid. Here, we mainly introduce the synthesis pathway of artemisinic acid in yeast cells, because the conversion of artemisinic acid to artemisinin is commercial and industrial feasible (Paniego and Giulietti 1996). In addition, as Paddon described, artemisinin acid can be extracted from fermentation broth after synthesis and secretion, and the subsequent chemical conversion is doable (Paddon *et al.* 2013).

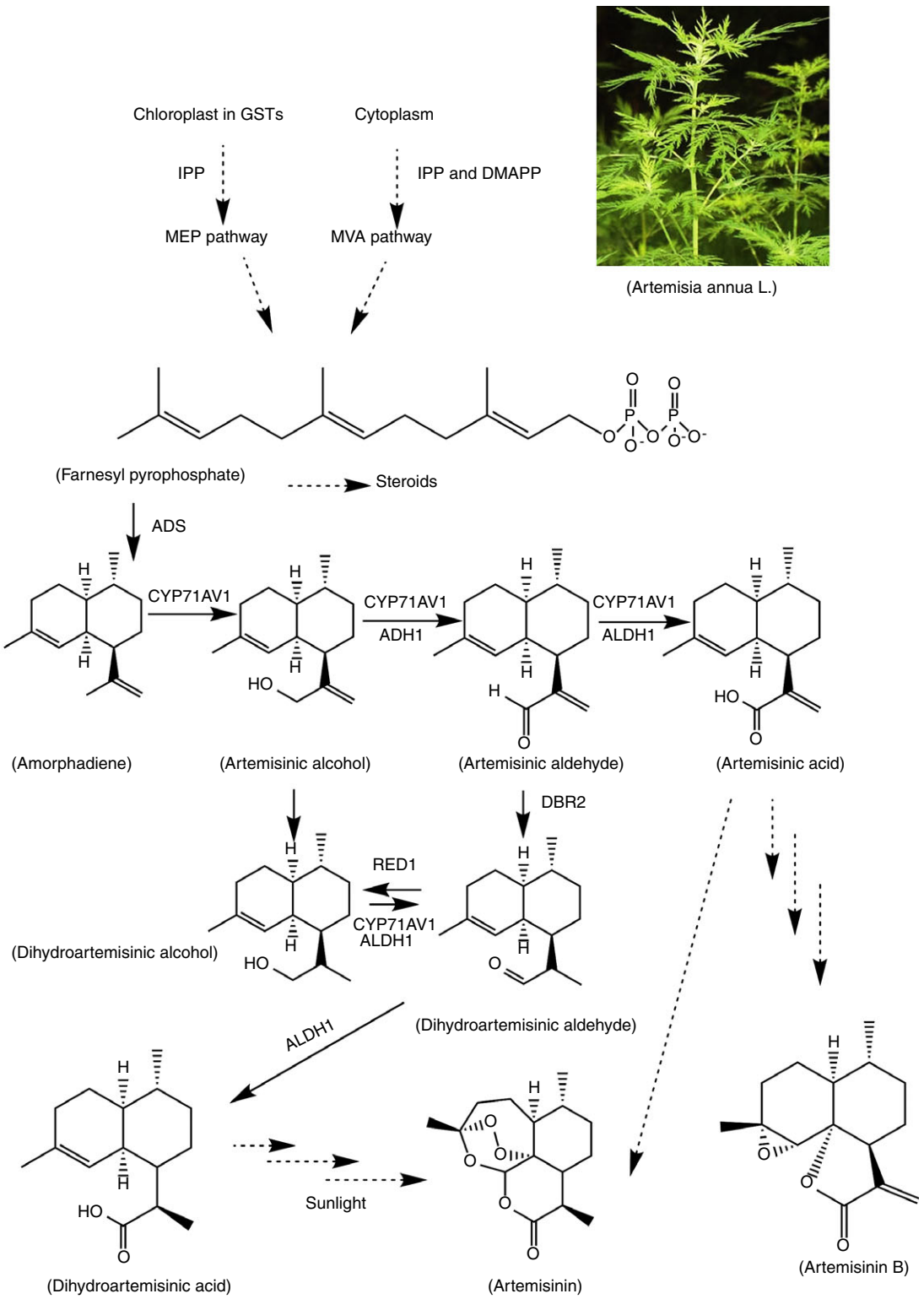

Figure 1 The biosynthesis pathway of artemisinin in the GSTs of *Artemisia annua* (Based on literatures of Paddon *et al.* (2013), Ye and Bhatia (2012) and Li *et al.* (2012). *ADS*, amorphadiene synthase; *CYP71AV1*,cytochrome P450 enzyme; *ADH1*,NAD-dependent artemisinic alcohol dehydrogenase; *ALDH1*, artemisinic aldehyde dehydrogenase; *DBR2*, artemisinic aldehyde D11(13) double bond reductase; *RED1*, reductase 1; The enzymes involved in MVA pathway is described in Fig. 2.

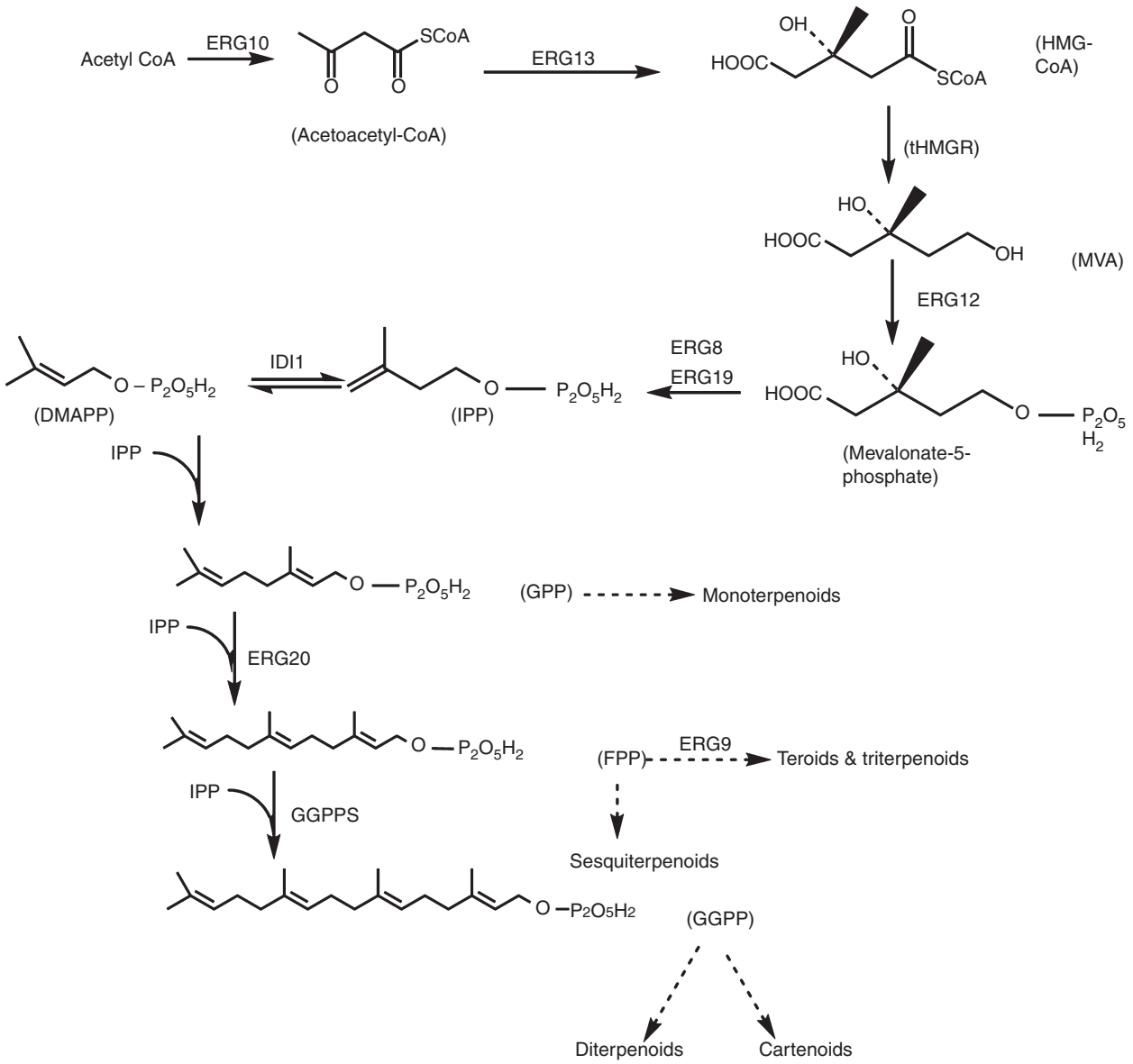

Figure 2 The mevalonate (MVA) pathway (Based on the literature of Wu (2009)). *EGR10*, Acetoacetyl-CoA thiolase; *ERG13*, HMG-CoA synthase; *HMGR*, HMG-CoA reductase; *ERG12*, MVA kinase; *ERG8*, phospho MVA kinase; *ERG19*, MVA pyrophosphate decarboxylase; *IDI1*, IPP isomerase; *ERG20*, FPP synthase; GGPPS, GGPP synthase; *ERG9*, squalane synthase.

The progress of heterologous biosynthesis in *Saccharomyces cerevisiae*

Improving amorphadiene titre

The intermediate in the MVA pathway of yeast cells, farnesyl diphosphate (FPP), is responsible for producing ergosterols, dolichol and ubiquinone, and its analogues (Keasling 2012). The concentration of FPP in cells may dominate the level of AD (even the titre of artemisinic acid) due to the whole synthesis of artemisinic acid in yeast cells commencing with FPPs. Thus, the crucial strategy is to increase FPP production and to control it towards AD synthesis pathway rather than other competing routes such as sterol synthesis. Most of genes involved in the synthesis of FPP in yeast have been cloned, for example, *HMGR* and *ERG20* (Wang *et al.* 2013). The increase in the expression of *HMGR* and *ERG20* can improve the titre of FPP (Souret *et al.* 2002; Kong *et al.* 2007; Han *et al.* 2008; Ram *et al.* 2010; Peng *et al.* 2011; Zhang *et al.* 2012b). The expression of amorphadiene synthase gene (*ADS*) using *GAL1* promoter in BY4742 strain (a derivative of S288C strain of *S. cerevisiae*) yields low titre of AD (4·4 mg l^{-1}) (Ro *et al.* 2006). It is reported that carbon flux increases towards

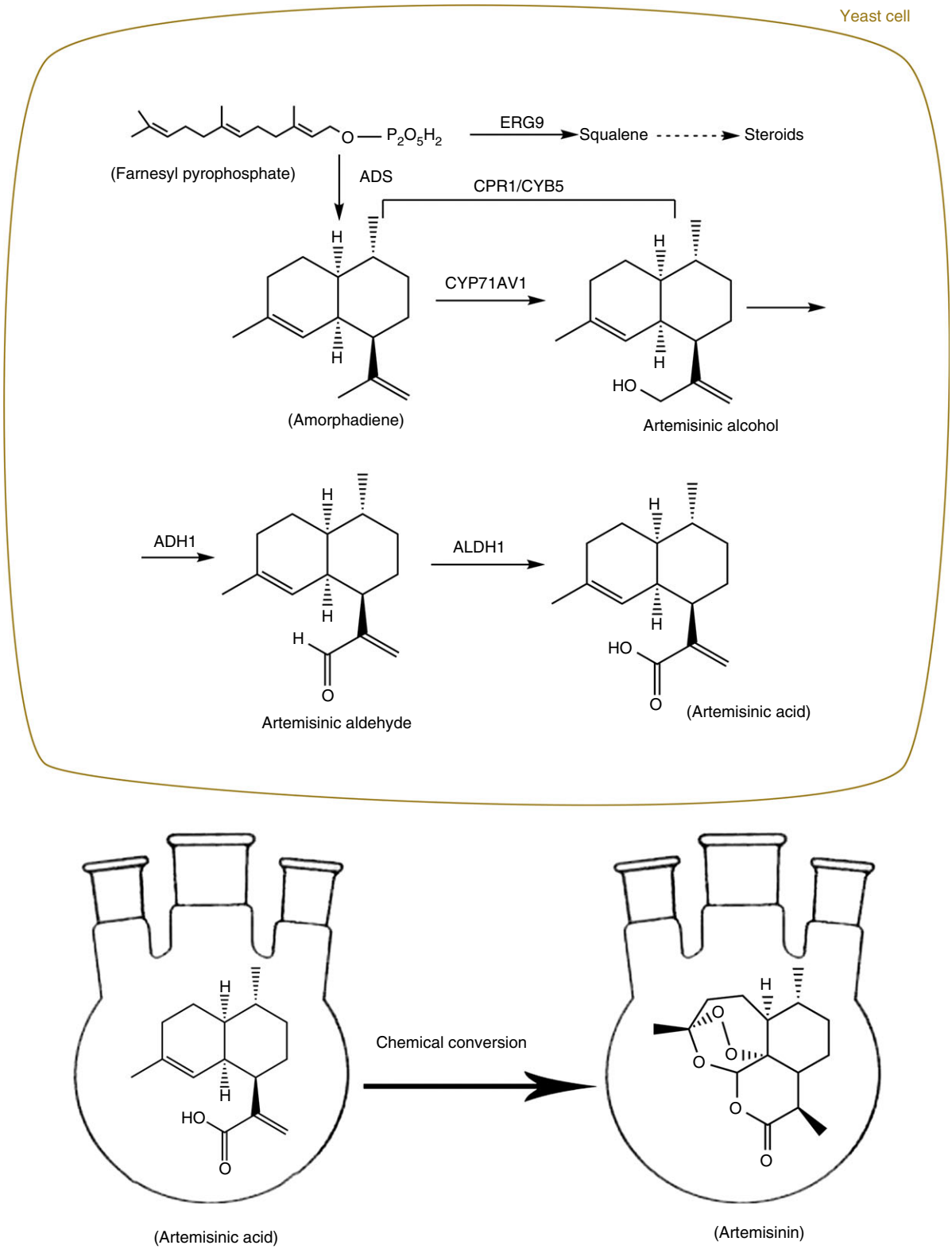

Figure 3 The synthesis routes of artemisinic acid in *Saccharomyces cerevisiae* (Based on the literature of Paddon *et al*. (2013)). *ERG9*, squalane synthase; *ADS*, amorphadiene synthase; *CPR1*, cytochrome P450 reductase; *CYB5*, cytochrome b_5; *CYP71AV1*, cytochrome P450 enzyme;*ADH1*, NAD-dependent artemisinic alcohol dehydrogenase;*ALDH1*, artemisinic aldehyde dehydrogenase.

isopentenyl diphosphate when the N-terminal regulatory region of HMG-CoA reductase is deleted (Donald *et al.* 1997). In the WHT strain of *S. cerevisiae*, the expression of *ADS*, *ERG20* and *tHMGR* (a truncated, soluble form of 3-hydroxy-3-methylglutaryl-coenzyme A reductase) using *GAPDH1*, *ADH1* and *GAPDH1* promoter, respectively, enhances the productivity of AD (23·6 mg l^{-1}) (Kong *et al.* 2007). This confirms the conclusion proposed by Donald *et al.* (Donald *et al.* 1997). The attempt carried out by Kong *et al.* also suggests that the key enzymes of MVA pathway may dominate the production of end-products. Of course, the difference may result from the genetic background of engineering strains. Because of several competing pathways commencing with common FPP, the overexpression of *tHMGR* and *ERG20* genes may not increase the carbon flux towards the synthesis of AD efficiently (Paddon *et al.* 2013). The synthesis of squalene catalysed by squalane synthase (SQS, encoded by *EGR9*) in the whole process is the principal competing site (Paradise *et al.* 2008). The down-regulation of *ERG9* gene combined with the expression of *ERG20* and *ADS* in MTCC3157 strain raises AD production to 25·06 mg l^{-1} (Baadhe *et al.* 2013). The accumulation of FPP is observed, which demonstrates that the carbon flux towards the synthesis of squalene decreases (Baadhe *et al.* 2013). This phenomenon may result from the low expression level of ADS gene (Baadhe *et al.* 2013). Although the down-regulation of *ERG9* gene decreases the consumption of FPP, the disruption of *ERG9* expression may seriously affect the energy metabolism and leads to the low growth rate (Huang *et al.* 2013), and inhibits cell proliferation (Baadhe *et al.* 2013).

Besides *ERG9*, another target for weakening the synthesis pathway of sterol is UPC2, a transcription factor regulating the expression of sterol biosynthesis gene *ERG2* (Vik and Rine 2001). The expression of mutant *upc2-1* allele inhibits sterol biosynthesis and uptakes the exogenous sterol for growth (Paddon and Keasling 2014). The overexpression of *upc2-1* along with down-regulation of *ERG9* using *MET3* promoter combined with the expression of *ADS* increases the yield of amorphadiene to 105 mg l^{-1} (Ro *et al.* 2006). However, the overexpression of *upc2-1* results in cell growth arrest because of acetate accumulation in the growth broth (Paddon and Keasling 2014).

The further up-regulation of *ERG20* and *tHMGR* genes (two copies of fragments are integrated into chromosome) and down-regulation of *ERG9* and *ERG2* in BY4742 strain raise the titre of AD to 153 mg l^{-1} (Ro *et al.* 2006). The overexpression of all genes involved in MVA pathway coupled with promoter modification and fermentation process optimization increases the production of AD to 36 g l^{-1} (Westfall *et al.* 2012). This suggests that the overexpression of key genes involved in MVA pathway may increase the production of precursors for the synthesis of artemisinic acid in *S. cerevisiae* (Table 1).

High-level artemisinic acid production in Saccharomyces cerevisiae

In spite of some achievements in improving amorphadiene production, the titre of artemisinic acid, an important precursor for chemical conversion *in vitro*, remains low or nearly undetectable (Ro *et al.* 2006; Kong *et al.* 2009; Baadhe *et al.* 2012). The absence of enzymes that are responsible for the oxidation of AD may result in FPP accumulation and low artemisinic acid productivity. The cytochrome P450 enzyme (*CYP71AV1*) derived from *A. annua* is recognized to be involved in AD oxidation (Bertea *et al.* 2005). The expression of *CYP71AV1* and its cognate reductase (*CPR1*) does raise the production of artemisinic acid. Coupled with ADS expression, up-regulation of *ERG20*, *upc2-1*, *tHMGR*(×2) and down-regulation of *ERG9*, the yield of artemisinic acid achieves up to 115 mg l^{-1} (Ro *et al.* 2006). The further optimization increases the titre of artemisinic acid to 1·8 g l^{-1}

Table 1 Achieved yield of amorphadiene in previous studies

Strains	Genotype/Modifications	Amorphadiene (mg l^{-1})	Reference
EPY201	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0, pRS425ADS*	4·4	Ro *et al.* (2006)
EPY213	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0, pRS425ADS* P_{GAL1}*-tHMGR* P_{GAL1}*-upc2-1erg9::*P_{MET3}*-ERG9 (ura*$^-$*)*	105	Ro *et al.* (2006)
YCF-006	*MATα his3Δ1leu2Δ0lys2Δ0ura3Δ0, erg9::pMET3-ERG9, pY01ADS-FPPS*	25·06	Baadhe *et al.* (2013)
Y293	*MATα erg9Δ::kan*^{r} P_{MET3}*-ERG9, leu2-3,112::HIS* P_{GAL1}*-MVD1* P_{GAL10}*-ERG8his3Δ1::HIS* P_{GAL1}*-ERG12* P_{GAL10}*-ERG10ade1Δ::*P_{GAL1}*-tHMG1* P_{GAL10}*-IDI1 ADE1ura3-52::*P_{GAL1}*-tHMG1* P_{GAL10}*-ERG13 URA3trp1-289::*P_{GAL1}*-tHMG1* P_{GAL10}*-ERG20 TRP1, pAM426 [2μ-leu2d* P_{GAL1}*-ADS (codon opt)], gal80Δ::natr,* pure ethanol feed fermentation	36000	Westfall *et al.* (2012)
WHT-2	*MATα, ade2-1; his3-11, -15; leu2-3, -112; ura3-1;trp1-1,* *pGBT9 -*P_{ADH1}*-tHMGR-*P_{GAPDH} *-ERG20; pYeDP60-*P_{GAPDH}*-ADS*	23·6	Kong *et al.* (2007)

(Westfall *et al.* 2012). Fermentation optimization also contributes to the improvement of artemisinic acid production (2·5 g l^{-1}) (Lenihan *et al.* 2008).

Although the expression of *CYP71AV1* and *CPR1* in yeast cells accelerates amorphadiene oxidation to form artemisinic acid, the viability of cells decrease because yeast cells with *CYP71AV1* expression undergo severe oxidative stress (Paddon and Keasling 2014). The stress does not occur in strains lacking *CYP71AV1*. The poor coupling between P450 cytochromes and their reductases is observed to release ROS (Zangar *et al.* 2004). The repression of *CPR1* increases the cell health and viability but decreases artemisinic acid production (Paddon *et al.* 2013). Three genes encoding cytochrome b5 (*CYB5*) (Schenkman and Jansson 2003; Zhang *et al.* 2007), artemisinic aldehyde dehydrogenase (*ALDH1*) (Teoh *et al.* 2009) and NAD-dependent artemisinic alcohol dehydrogenase (*ADH1*) (Covello *et al.* 2007) also contribute to artemisinic acid production. Integration of all above five genes (*ADH1*, *CYP71AV1*, *CPR1*, *CYB5* and *ALDH1*) into the chromosome of yeast cells plus fermentation optimization improve artemisinic acid yield up to 25 g l^{-1} (Paddon *et al.* 2013) (Table 2).

Strategies for the improvement of target molecules in *Saccharomyces cerevisiae* heterologous biosynthesis

To raise the productivity of artemisinic acid, many strategies have been tested, such as the overexpression of key genes, the repression of competing pathways, fermentation optimizing, and codon optimizing etc. (Wang *et al.* 2013). Although the titre of artemisinic acid is improved to some extent by these efforts (Lenihan *et al.* 2008; Westfall *et al.* 2012; Paddon *et al.* 2013), the productivity of heterologous host cells still remains unsatisfied for large-scale industrial production. In addition, although optimizing heterologous synthesis based on overexpression of key genes or repression of competing pathway improves the performance of host cells. The crucial point of synthesis optimization is to improve enzyme property rather than simply increasing enzymes content (Hong 2012). The overexpression of genes may bring excessive stress to host cells; therefore, the production improvement of end-products cannot be achieved. Deep understanding of the mechanism of the synthesis pathways for artemisinin and its regulations in *A. annua*, for example, discovering new genes and regulation factors, may help improve artemisinic acid production. On the other hand, developing new platforms which are superior to *S. cerevisiae* also is necessary. Here, we proposed some potential means *in silico* and *in vivo* to solve these problems.

Increase the content of enzymes via fusion protein technique

In terms of the heterologous synthesis in yeast cells, the low metabolic flux towards to synthesis may result from the diffusion and degradation of enzymes (Jorgensen *et al.* 2005; Conrado *et al.* 2008) or low catalysis activity of enzymes such as ADS (Picaud *et al.* 2005). The technique of fusion protein (Kourtz *et al.* 2005) and proxim-

Table 2 Achieved yield of artemisinic acid in previous studies

Strains	Genotype/Modifications	Artemisic acid (g l^{-1})	Reference
EPY224	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0, pRS425ADS* P_{GAL1}*-tHMGR* P_{GAL1}*-upc2-1erg9::*P_{MET3}*-ERG9* P_{GAL1}*-tHMGR* P_{GAL1}*-ERG20 (ura⁻), pESCURA::CPR/pESCURA::CPR/CYP71AV1*	0·115	Ro *et al.* (2006)
Y51	*MATα his3D1 leu2D0 lys2D0 ura3D0 d1::*P_{GAL1}*-tHMGR d2::*P_{GAL1}*-UPC2-1 d3::*P_{GAL1}*-tHMGR d4::*P_{GAL1}*-ERG20erg9::*P_{MET3}*-ERG9::HIS3, pAM179-LEU2d*P_{GAL1}*-ADS-*P_{GAL10}*-CYP71AV1-*P_{GAL1}*-CPR1*, galactose as the carbon source and inducer, addition of methionine, oxygen-stat algorithm	2·5	Lenihan *et al.* (2008)
Y973	*gal1,gal10,gal7ΔPGAL3-AaCPR1_natA,ndt80Δ:hphAP*$_{GAL7}$-AaALDH1, *erg9::dsdA_*P_{CTR3}*-ERG9,leu2::kanAP*$_{GAL7}$*-AaCYB5P*$_{GAL1}$*-ERG19_PGAL10-ERG8, his3Δ1::hisMXP*$_{GAL1}$*-ERG12P*$_{GAL10}$*-ERG10,ade1Δ::*P_{GAL1}*-tHMG1P*$_{GAL10}$*-IDI1ADE1, ura3-52::*P_{GAL1}*-tHMG1P*$_{GAL10}$*-ERG13_URA3,trp1-289::*P_{GAL1}*-tHMG1P*$_{GAL10}$*-ERG20TRP1, pAM552:2μ-leu2dP*$_{GAL1}$*-ADSP*$_{GAL10}$*-CYP71AV1*	7·7	Paddon *et al.* (2013)
Y1284	*gal1,gal10,gal7ΔP*$_{GAL3}$*-AaCPR1natA,erg9Δ::dsdAP*$_{CTR3}$*-ERG9, leu2−kanAP*$_{GAL7}$*-AaCYB5P*$_{GAL1}$*-ERG19P*$_{GAL10}$*-ERG8,ade1::*P_{GAL1}*-tHMG1P*$_{GAL10}$*-IDI1ADE1, his31::hphAP*$_{GAL7}$-AaALDH1P_{GAL1}*-ERG12P*$_{GAL10}$*-ERG10, ura3−5P*$_{GAL1}$*-tHMG1P*$_{GAL10}$*-ERG13hisG,trp1-289::*P_{GAL1}*-tHMG1P*$_{GAL10}$*-ERG20TRP1, ndt80ΔP*$_{TDH1}$*-HEM1HIS3P*$_{PGK1}$*-CTT1,gal80Δ::URA3P*$_{GAL7}$-AaADH1, *pAM552:2μ-LEU2dP*$_{GAL1}$*- ADSP*$_{GAL10}$*-CYP71AV1*	25	Paddon *et al.* (2013)

ity effect (Tokuhiro *et al.* 2009; Ohto *et al.* 2010; Albertsen *et al.* 2011; Dai *et al.* 2012; Zhou *et al.* 2012) could be applied to increase the titre of enzymes. However, the expression of fusion protein may be affected by the length and flexibility of linker and the sequential order (Lu and Feng 2008; Albertsen *et al.* 2011). The interference may occur if the linker is too short, and this may damage the structure and function of enzymes (Albertsen *et al.* 2011). Besides, the sequential order may affect the catalysis activity (Ohto *et al.* 2010; Zhou *et al.* 2012). The effects are determined by the nature of two linked enzymes (Albertsen *et al.* 2011). When FPP synthase and amorphadiene synthase are expressed with sequential order of *ADS-ERG20* in MTCC3157 strain (Baadhe *et al.* 2013), the yield of amorphadiene only is about 12·08 mg l^{-1}. However, when these two enzymes are expressed with the order of *ERG20-ADS*, approximately two-fold amorphadiene is achieved. It seems that the expression of fusion protein is another regulation site for improving end-products. However, this conclusion is inferred according to the phenotype of engineering strains. Fusion protein technique could be used to increase the concentration of enzymes. To employ this technique, analysis of metabolic flux in cells is necessary.

Metabolic flux and metabolic pathway analysis contribute to develop metabolic strategies

Metabolic flux analysis can evaluate the cell physiological condition, and reveal key factors affecting end-product production (Hong 2012). The regulation of metabolic fluxes occurs at various levels of regulations, for example, transcription, translation, modifications of post-translation, degradation of mRNA, activation of enzymes and allosteric regulation of enzymes etc. The capacity of end-product production and distribution of metabolic fluxes in cells can be inferred by the modelling metabolic reactions and line programme. This can provide information to control the expressions of key genes in heterologous biosynthesis (Hong 2012). In addition, flux balance analysis is used to design engineering strain (Libourel and Shachar-Hill 2008). Metabolic pathway analysis covers the distribution of metabolic flux, the topology of metabolic network, and the control flow of metabolism. It is more comprehensive than metabolic flux analysis. Many other approaches are useful to get more accurate data for metabolic pathway and flux analysis, for example, C^{13}-isotope tracer technique, NMR and GC-MS (Hong 2012). With the system-biology methods, we can reveal the distribution of metabolic flux, the interrelation of genes, enzymes and metabolic pathways, and design the total synthesis pathway of artemisinic acid in yeast cells accurately.

Feedback regulation is a promising approach to control carbon fluxes

To achieve high end-product production in metabolic engineering, competing pathways are required to be repressed or deleted if they are not essential for host cells (Choi and Lee 2013). Unfortunately, in yeast platform cells, most competing pathways fulfil many functions that are important to host cells (Yuan and Ching 2015), for example, the biosynthesis of ergosterols. Although the repression of *ERG 9* improves the production of AD in the surface, these down-regulations result in various problems such as inhibition of cell proliferation, growth arrest and accumulation of toxic intermediates (Baadhe *et al.* 2013; Paddon and Keasling 2014). To address this issue, Yuan *et al.* proposed a dynamic regulation strategy to control the expression of *ERG 9* gene. According to feedback regulation, the ergosterol-responsive promoters are used to control carbon fluxes towards ergosterol synthesis pathway (Yuan and Ching 2015). This strategy has been applied to improve production of natural products in heterologous host cells (Zhang *et al.* 2012a; Dahl *et al.* 2013; Xie *et al.* 2015). Thus, the future work may focus on utilizing analogous responsive promoters to control gene expressions that are responsible for competing pathways.

Searching knockout targets for improving end-product yield

As mentioned above, the traditional strategies result in dysfunction of metabolism (Sun *et al.* 2014). To address this issue, iMM904, a genome-scale metabolic model of *S. cerevisiae* has been developed (Zomorrodi and Maranas 2010). It is a promising tool to aid the design of metabolic strategies in yeast cells. Combining with flux balance analysis (FBA) and minimization of metabolic adjustment, the knockout sites can be predicted. Sun *et al.* (2014) predicted some knockout sites that can improve the titre of terpenoids but not inhibit cell growth *in silico* and *in vivo*. Another attempt identifies the knockout sites for increasing dihydroartemisinic acid production based on *in silico* FBA and minimization of metabolic adjustment on a genome-scale model (Misra *et al.* 2013). Therefore, the knockout of nontrivial genes may be promising for improving the titre of artemisinic acid.

Identifying new genes or transcriptional factors that are responsible for biosynthesis of artemisinin in *Artemisia annua*

Despite of many achievements in micro-organism, the mechanism of biosynthesis of artemisinin in *A. annua* is

still poorly understood (Zhang *et al.* 2015). To further improve the titre of artemisinic acid, the detailed mechanism needs to be explored. In recent years, some new phenomena have been observed and help us to further understand the mechanism of biosynthesis of artemisinin in *A. annua*. In native tissue, the promoter of *ADS* and *CYP71AV1* containing E-box elements can be bind by a helix-loop-helix transcriptional factor AabHLH1 (Ji *et al.* 2014). The overexpression of AabHLH1 in *A. annua* increases the expression level of *ADS*, *CYP71AV1* and *HMGR* genes (Ji *et al.* 2014). In native tissue basic leucine zipper family transcription factor AabZIP1 is recognized to be important for expression of *ADS* and *CYP71AV1* genes (Zhang *et al.* 2015). The overexpression of AabZIP1 significantly increases the accumulation of artemisinin in *A. annua* (Zhang *et al.* 2015).

Construct new platform based on inverse metabolic engineering

Although the titre of artemisinic acid has been achieved to 25 g l^{-1} in CEN.PK2 strain of *S. cerevisiae* (Paddon *et al.* 2013), the yield remains industrial unsatisfactory. The applications of various engineering strains indicate that strains with different genetic backgrounds, such as W303-1A, S288C, WHT, MTCC3157, have different performance on the yield of target products (Ro *et al.* 2006; Kong *et al.* 2007; Farhi *et al.* 2011; Baadhe *et al.* 2012). Thus, constructing new engineering strains superior to *S. cerevisiae* is likely an important research direction. The inverse metabolic engineering provides a great means to design engineering stains with satisfied performance (Bailey *et al.* 2002).

Bioinformatics tools and omics data contribute to design metabolic strategies

The traditional methods of metabolic engineering focus on changing partial pathway to increase the titre of artemisinic acid. In contrast, recent attentions have been transferred to the global synthesis route (Tae *et al.* 2008). The genome-scale cell model is a great tool to design and reform strategies to alter cell properties (Hong 2012; Lu *et al.* 2014; Basler 2015; Diken *et al.* 2015; Imam *et al.* 2015; Kavscek *et al.* 2015; King *et al.* 2015; Parambil and Sarkar 2015). Bioinformatic tools for digging genome data and system-biology approaches contribute to the research of metabolic engineering (Nielsen and Jewett 2008) such as MetaFluxNet (Sang *et al.* 2005) and TICL tool (Alexey *et al.* 2009). The MetaFluxNet program is used to build the genome-scale model and construct metabolic network. Coupled with the metabolic flux analysis, it is possible to design a superior metabolic strategy (Sang *et al.* 2005). The TICL is designed for automatic parse the transformation model of compounds (Alexey *et al.* 2009). The integration of bioinformatics annotation data and metabolome data to reconstruct the metabolic network has been proved doable in metabolic engineering (Patrick *et al.* 2008).

With the techniques *in silico*, both the metabolic network of artemisinin synthesis in *A. annua* and the metabolic network of yeast cells can be reconstructed. The information is useful to design heterologous synthesis pathway of artemisinic acid. To get more accuracy information, the integration of various omics data into reconstruction of the synthesis pathway is necessary due to the complex regulation of artemisinin synthesis in *A. annua* cells (De *et al.* 2006; Kazuyuki 2009). The data produced by high-throughput technology can be integrated into genome-scale constraint model, which describe the metabolic network from genetic and biochemical aspects (Radhakrishnan *et al.* 2005). This model can be used to analyse interrelation of genes, proteins and reactions, and the relationships of genes, proteins and catalysis reactions carried out by proteins (Radhakrishnan *et al.* 2005). In addition, it become easier for metabolic process analysis since next generation sequencing accelerates the expansion of public database, such as KEGG.

Conclusion

Although great progress has been made in the heterologous synthesis of artemisinic acid in *S. cerevisiae*, the yield of artemisinic acid is too low to meet industrial requirement. The low titre of artemisinic acid may be result from: (i) the absence of the regulation mechanism of artemisinin synthesis in *A. annua*; or (ii) the deficiency of host cells in the respect of expressing plant genes. The overexpression or down-regulation of some genes may disrupt the balance of energy metabolism, and bring excessive stress to cells. The strategies based on system-biology studies are useful to further improve the production of artemisinic acid. Many factors are related to the production of artemisinic acid, such as the distribution of metabolic flux, the energy parameter, and the interrelation of various compounds both in *A. annua* and host strains. Thus, more attention should be paid on global metabolic pathway of cells, rather than simple up-regulation or down-regulation of genes. Above information is useful for the design of heterologous synthesis pathway and fermentation control. Developments of bioinformatics tools make the reconstruction of metabolic pathway easier than before. Bioinformatics analysis will provide more information to understand the regulation mechanism of artemisinin synthesis in *A. annua* and to design new engineering strains.

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Conflict of Interest

The authors declared that they have no conflicts of interest in this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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