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Abstract: L-malate is an important platform chemical that has extensive applications in the food, feed, and wine industries. Here, we synergistically engineered the carbon metabolism and redox metabolism in the cytosol and mitochondria of a previously engineered *A. oryzae* to further improve the L-malate titer and decrease the byproduct succinate concentration. First, the accumulation of the intermediate pyruvate was eliminated by overexpressing a pyruvate carboxylase from *Rhizopus oryzae* in the cytosol and mitochondria of *A. oryzae* and consequently, the L-malate titer increased 7.5%. Then, malate synthesis via glyoxylate bypass in the mitochondria was enhanced and citrate synthase in the oxidative TCA cycle was downregulated by RNAi, enhancing the L-malate titer by 10.7%. Next, the exchange of byproducts (succinate and fumarate) between the cytosol and mitochondria was regulated by the expression of a dicarboxylate carrier Sfc1p from *Saccharomyces cerevisiae* in the mitochondria, which increased L-malate titer 3.5% and decreased succinate concentration 36.8%. Finally, an NADH oxidase from *Lactococcus lactis* was overexpressed to decrease the NADH/NAD$^+$ ratio, and the engineered *A. oryzae* strain produced 117.2 g/L L-malate and 3.8 g/L succinate, with an L-malate yield of 0.9 g/g corn starch and a productivity of 1.17 g/L/h. Our results showed that synergistic engineering of the carbon and redox metabolisms in the cytosol and mitochondria of *A. oryzae* effectively increased the L-malate titer, while simultaneously decreasing the concentration of the byproduct succinate. The strategies used in our work may be useful for the metabolic engineering of fungi to produce other industrially important chemicals.

Key words: L-malate; *Aspergillus oryzae*; redox metabolism; mitochondrial engineering

Large-scale production of platform and bulk chemicals from renewable resources instead of

petroleum has gained attention in the last years, especially with the growing concerns regarding

global warming and environmental pollution[1-2]. Malate, as an essential intermediate of cell

metabolism, is widely used in the food and beverage industries as a new generation acidulant and

flavor enhancer. Malate can also improve the uptake rate of amino acids and can find applications in

the pharmaceutical industry for the treatment of liver disease, anemia, hypertension, and other

diseases[3-4]. In addition, malate can also be used as a feed additive, industrial cleaning agent, resin

curing agent, and feedstock for the chemical synthesis of polymalic acid[5].

In the past few decades, several studies have investigated the fermentative production of

L-malate, and many natural or genetically engineered microbes have been used to produce malate,

including *Escherichia coli* [6-8], *Saccharomyces cerevisiae*[9-12], *Zygosaccharomyces rouxii*[13], *Torulopsis*

glabrata[14], *Aspergillus flavus*[3], *Aspergillus oryzae*[15-17], *Rhizopus delemar*[18], and *Ustilago*

trichophora[19-20]. As a generally regarded as safe (GRAS) microorganism, *A. oryzae* has been commonly

used in food fermentation and is also an advantageous cell factory to produce fine chemicals, such as

malate.

As shown in Fig. 1, there are four metabolic pathways for malate biosynthesis from glucose in *A.*

oryzae. The first is the reductive tricarboxylic acid (rTCA) pathway in the cytoplasm, which starts with

the carboxylation of pyruvate to oxaloacetate (OAA) by pyruvate carboxylase (PYC), and the

subsequent reduction of OAA to L-malate by malate dehydrogenase (MDH). This reductive pathway

can fix 1 mol CO_2/mol malate and has a maximum theoretical yield of 2 mol malate/mol glucose[15, 21].

The other three pathways are located in the mitochondria. The second pathway is the oxidative TCA

cycle, which releases two molecules of CO_2, and the third pathway is the cyclic glyoxylate shunt.

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Oxidative decarboxylation of pyruvate produces acetyl-CoA and thus, the theoretical maximum yield

of these two pathways is 1 mol malate/mol glucose. The fourth pathway is the noncyclic glyoxylate

bypass, where OAA is replenished by pyruvate carboxylation, instead of malate; this pathway has a

theoretical maximum yield of 1.33 mol malate/mol glucose[22].

Thus far, all metabolic engineering strategies for malate synthesis have focused on engineering

metabolic pathways in the cytosol, and there are no reports about mitochondrial engineering in *A.*

oryzae to improve malate production, even though three of the four malate synthesis pathways are

located in the mitochondria. In fact, the TCA cycle plays a very important role in catabolism, and

mitochondria-compartmentalized substrate utilization pathways can offer better performance for

product synthesis[23]. Mitochondrial engineering has been successfully used to produce alkenes[23],

branched-chain alcohols[24-25], aconitic acid[26-27], and acetoin[28] in yeast or *Aspergillus*. Succinate is the

main byproduct of malate production in *A. oryzae*[16-17]. As the synthesis of succinate from fumarate in

the cytosol and the synthesis of fumarate from succinate in the mitochondria share the same enzyme,

succinate dehydrogenase (SDH) [22], the formation of succinate in the cytosol cannot be directly blocked

by deleting the SDH gene. Therefore, a simultaneous increase in the malate titer and decrease in the

succinate concentration remains an unsolved challenge for malate synthesis in *A. oryzae*.

In our previous work, we engineered *A. oryzae* by engineering the metabolic pathways in the

cytoplasm, and the engineered strain produced 93.2 g/L malate from glucose in shake flask culture[16].

In addition, by promoter engineering and improvement of starch hydrolytic capability, the engineered

strain *A. oryzae* GAAF41 produced 88.5 g/L malate directly from corn starch[17]. In this work, we

achieved a substantial increase in the malate titer and decrease in the succinate concentration by

synergistically engineering the carbon and redox metabolisms in the cytosol and mitochondria of *A.*

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oryzae. We first reduced the accumulation of the intermediate in cytosol and mitochondria. Then we

engineered mitochondria pathways to channel more metabolic flux towards malate and reduced the

formation of by-products. Finally redox balance was optimized to maximize the production of Malate.

Together, the final strain increased the production of malate and decreased succinate formation.

RESULTS AND DISCUSSION

Kinetic analysis of key metabolites in cytosol and mitochondria of *A. oryzae*

To determine the potential metabolic bottleneck of malate synthesis, we first analyzed the

metabolic kinetics of key metabolites (malate, pyruvate, succinate, and fumarate) in the cytosol and

mitochondria of *A. oryzae* GAAF41, which was constructed in our previous work[17]. Fig. 2A and Fig. 2B

show that malate, pyruvate, succinate, and fumarate were detected in the cytosol, whereas only

malate, pyruvate, and fumarate accumulated in the mitochondria, suggesting that the byproduct

succinate is mainly produced in the cytosol, not mitochondria, or it is quickly transported to the

cytosol. The results also showed that the concentrations of pyruvate, malate, fumarate, and succinate

in the cytosol were substantially higher than those in the mitochondria. The mitochondrial

concentrations of malate and fumarate peaked at 65 h (0.233 g malate/g dry cell weight (DCW); 0.055

g fumarate/g DCW) and then decreased slightly, whereas in the cytosol, the malate concentration

increases continually and fumarate disappeared at the end of fermentation. The pyruvate

concentrations in the cytosol and mitochondria remained relatively stable (about 1.0 g pyruvate/g

DCW and 0.023 g pyruvate/g DCW, respectively). Pyruvate is an important intermediate that connects

glycolysis in the cytosol to the TCA cycle in the mitochondria and is also the precursor for malate

synthesis. As the accumulation of pyruvate indicated an overflow metabolic in the engineered malate

synthesis pathway, we next overexpressed a PYC from *R. oryzae* in the cytosol and mitochondria of *A.*

oryzae to convert the accumulated pyruvate to OAA, the precursor for malate synthesis.

Effects of overexpressing PYC in cytosol and mitochondria on malate synthesis

As a crucial enzyme in the synthesis of L-malate, PYC links the glycolytic, TCA, and rTCA pathways, and is found in both the mitochondrial and cytosolic fractions of *A. oryzae.*[30] To reduce the accumulation of pyruvate, we localized PYC from *R. oryzae* (ROPYC) in the cytoplasm and mitochondria of *A. oryzae*. The promoter of translation elongation factor 1 alpha gene (*tef*) and the terminator of tryptophan C gene (*tryC*) were used for the construction of the gene cassette. Due to the most of pyruvate carboxylase of fungi were found in the cytosol,[30-31] for localization of ROPYC in the mitochondrial matrix, an N-terminal 32-amino acid sequence of cytochrome c oxidase polypeptide 4 from *A. oryzae* was fused with *pyc*. This sequence is the first exon in the cytochrome c oxidase subunit 4 gene, and the last two amino acids in the sequence were neutral amino acids, in accordance with the characteristic of this type of signal peptide.[32]

The parent strain *A. oryzae* GAAF41 produced 88.5 g/L malate, 9.2 g/L succinate, and 0.79 g/L fumarate.[17] Based on the heterologous expression of ROPYC, as expected, the malate titers in the *A. oryzae* strain CP (localized in the cytoplasm) and *A. oryzae* strain MP (localized in the mitochondria) increased to 92.3 g/L and 90.5 g/L, respectively. When ROPYC was localized in both, the cytoplasm and mitochondria of *A. oryzae* (*A. oryzae* strain CMP), the malate titer was further improved to 95.1 g/L. In addition, lower succinate (6.9 g/L) and fumarate (0.6 g/L) concentrations were observed in *A. oryzae* CMP.

The concentrations of the main intracellular metabolites in the cytosol and mitochondria of *A. oryzae* CMP are shown in Fig. 2C and Fig. 2D. Compared with the parent strain *A. oryzae* GAAF41, the pyruvate concentration decreased significantly, and only trace amounts of pyruvate could be

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determined in the cytosol and mitochondria of *A. oryzae* CMP. Additionally, the succinate

concentration in the cytosol showed a slight decrease. In contrast, the malate titers in the cytosol and

mitochondria of *A. oryzae* CMP increased significantly. The fumarate concentrations in the cytosols of

both strains were almost the same, while unexpectedly, the fumarate concentration in the

mitochondria of *A. oryzae* CMP increased significantly. The results demonstrated that the localization

of PYC in the cytosol and mitochondria of *A. oryzae* was effective in increasing malate concentration

and decreasing the intermediate pyruvate concentration simultaneously. The elimination of pyruvate

from the cytosol indicated pyruvate carboxylation is a key node in malate synthesis in the rTCA

pathway. Surprisingly, earlier studies have mainly focused on the malate synthesis pathway in the

cytosol and little attention has been given to the malate synthesis pathways in the mitochondria,

even though three of the four malate synthesis pathways are located in the mitochondria. In addition,

succinate and fumarate, the main byproduct difficult to avoid is also synthetized in the mitochondria.[3,]

[7, 15] Therefore, next, we decided to engineer the malate synthesis pathways in the mitochondria.

Engineering the oxidative branch of TCA cycle and glyoxylate bypass in mitochondria

There are three pathways for malate synthesis in the mitochondria: the oxidative TCA cycle, and

the cyclic and noncyclic glyoxylate bypasses[22] (Figure 1). We first improved the oxidative branch of the

TCA cycle in the mitochondria by simultaneous overexpression of four enzymes: citrate synthase (CIS),

isocitrate dehydrogenase (ISD), a-oxoglutarate dehydrogenase (OXD), and aconitate hydratase (ACH)

(Figure 1). The engineered strain was named *A. oryzae* CMPT. Surprisingly, the rates of spore

germination, mycelial growth, and sporulation of *A. oryzae* CMPT were lower than those of *A. oryzae*

CMP, and the DCW of *A. oryzae* CMPT increased by 18.9%. Table 2 shows that the enhanced oxidative

TCA cycle was unfavorable for malate synthesis (as the malate titer decreased from 95.1 to 83.7 g/L),

while it had little influence on succinate and fumarate syntheses. Time course profile of dry cell

weight (DCW) and malate production during fermentation of main engineered strains are shown in

Fig. S1A and S1B, respectively. These results indicated that strengthening the oxidative TCA route

directed more carbon flux to biomass formation, but spore formation and malate production in *A.

oryzae* were diminished.

At a branch point in the TCA cycle, isocitrate lyase (ICL) catalyzes the first unique step of the

glyoxylate pathway, which diverts isocitrate through a carbon-conserving bypass.[33] ICL and malate

synthase (MAS) are two key enzymes in the glyoxylate bypass. ICL catalyzes the conversion of

isocitrate to succinate and glyoxylate reversibly, with a $\Delta G_0'$ of 8.6 kJ/mol.[22] MAS catalyzes the

synthesis of malate from glyoxylate and acetyl-CoA with a $\Delta G_0'$ of -32.7 kJ/mol, which is

thermodynamically favorable.[22] As the yield of malate in the non-cyclic glyoxylate bypass (1.33 mol

malate/mol glucose) was higher than that of the oxidative TCA pathway (1 mol malate/mol glucose),

we up-regulated the activity of the glyoxylate branch by co-overexpression of *icl1* and *icl2* genes

encoding ICL and *mas* gene encoding MAS.

The transcriptional levels of the *icl1*, *icl2*, and *mas* genes in the parent strain (CMP) and

transformants (CMPIM) were analyzed by qRT-PCR. In CMPIM, the transcriptional levels of the *icl1*,

icl2, and *mas* genes were respectively, 2.13-, 2.64-, and 3.58-fold higher than those of the parent

strain CMP. As expected, the up-regulation of the glyoxylate bypass produced more malate (the

malate titer increased from 95.1 g/L in CMP to 99.8 g/L in CMPIM). Since ICL catalyzes the conversion

of isocitrate to succinate and glyoxylate, as expected, the succinate concentration of CMPIM (8.3 g/L)

was higher than that of CMP (6.9 g/L). Since the improved oxidative TCA cycle was unfavorable for

malate synthesis, we further studied the effects of weakening the oxidative TCA cycle on malate

synthesis.

Effects of down-regulating the oxidative TCA cycle by RNA interference (RNAi) on malate synthesis

The reaction step involving the condensation of OAA and acetyl-coA to synthesize citrate in the

TCA cycle was inhibited by RNAi to weaken the oxidative TCA cycle. Compared to other gene

expression regulation methods, RNAi has a simple antisense mechanism, which allows a facile and

tunable gene knockdown that depends on the hybridization between the injected RNA and the

endogenous messenger RNA.[34-35] pAgsCa, a constructed expression cassette that expressed a

self-complementary RNA homologous to the *cis* gene (Fig. 3A), was transformed into the engineered

strain CMPIM to generate the derivative strain CMPIMI. The pAgsCa comprises a 301-bp fragment

from the *A. oryzae cis* gene (the seventh exon) and is used to generate the inverted repeat, flanked by

a 67-bp loop region (the seventh intron of *cis*). The linear plasmid was under the control of the

superoxide dismutase (*sodM*) promoter.[36-37]

Five positive mitotic stable transformants and CMPIM were isolated and cultivated in shake flasks

for 30 h with 0.1% hydrogen peroxide as the inducer. As shown in Table 2, CMPIMI was the highest

producer of malate (105.3 g/L malate), showing a 5.5% increase in malate concentration compared to

CMPIM. However, with the increase in malate concentration, the concentrations of the byproducts

succinate and fumarate also increased from 8.3 to 9.5 g/L and 1.02 to 1.31 g/L, respectively, in

CMPIMI.

To compare the transcription levels of the key genes in the reductive and oxidative TCA pathways

with RNAi and with no induction, RT-PCR analyses of cells before induction, cells without induction,

and cells with induction after fermentation for 50 h were performed. As shown in Fig. 3B, compared

with the levels in control strain, the transcription levels of *pyc*, *mdh*, *cis*, *isd*, *oxd*, *ach*, and *fuh* in TCA

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and rTCA pathways increased after fermentation for 30 h and 50 h without induction in CMPIMI. After

induction (for 50 h), the transcription levels of genes in the TCA cycle were down-regulated, but the

expression of *pyc* (in rTCA) was up-regulated. These results suggested that appropriate interference of

the TCA cycle can increase the carbon flux to the reductive pathway by down-regulating the oxidative

pathway of the TCA cycle. However, the increase in byproduct concentrations of succinate and

fumarate was unexpected. Hence, we further engineered the dicarboxylate carriers in the

mitochondria to reduce the accumulation of succinate and fumarate.

Engineering the exchange of succinate and fumarate between cytosol and mitochondria

In eukaryotes, mitochondria are the major locations of energy transduction and metabolism.

Various transporters, such as dicarboxylate carriers, [38-39] coenzyme A transporters, [40] and

tricarboxylate and dicarboxylate-tricarboxylate carriers, [41] located in the inner or outer membrane of

the mitochondria control the transport of metabolites into and out of the mitochondria. Dicarboxylate

carriers, including fumarate/malate, succinate/malate, and fumarate/succinate antiporters, selectively

transport dicarboxylates across the inner mitochondrial membrane. [42]

As shown in Fig. 2A, succinate is mainly present in the cytosol, while fumarate and malate are

present in the mitochondria. A dicarboxylate carrier, Sfc1p from *S. cerevisiae*, which transports

succinate into and fumarate out of the mitochondria, [38, 43] was overexpressed in the mitochondria of

CMPIMI. The malate concentration of the derived strain CMPIMIS increased from 105.3 to 109.1 g/L,

while the succinate and fumarate concentrations decreased from 9.5 to 6.0 g/L and from 1.31 to 0.7

g/L, respectively. In the mitochondria, 1.89 g succinate/g DCW was obtained, malate increased from

0.67 g/g DCW to 0.89 g/g DCW, and fumarate decreased from 0.0056 g/g DCW to 0.0024 g/g DCW.

And in the cytosol, the malate and fumarate yields increased from 10.55 to 16.30 g/g DCW and from

0.18 to 0.31 g/g DCW, respectively, and the succinate yield decreased from 2.54 to 1.50 g/g DCW.

Succinate synthesis is NADH-driven and hence, we engineered the redox potential to further reduce

the accumulation of succinate.

Engineering redox metabolism for improved L-malate production

In theory, the complete oxidation of 1 mole of glucose by glycolysis generates 2 moles of NADH,

which can be used for the syntheses of malate and succinate. These reactions are redox-balanced and

do not require an additional pathway for NADH regeneration.[22] In the rTCA pathway, 2 moles of

malate can be produced with an input of 1 mole of NADH (Glucose + $2CO_2$ + NADH = 2Malate + NAD +

H).[44] Furthermore, it was confirmed that succinate was mainly derived from the rTCA pathway

(Glucose + $2CO_2$ + 2NADH = 2Succinate + 2NAD), instead of the oxidative branch of the TCA cycle, and

from the glyoxylate bypass (3Glucose + $2H_2O$ + 8NAD = 4Succinate + $2CO_2$ + 8NADH).[45] We

hypothesize that the surplus NADH from glycolysis induced the accumulation of succinate and

therefore, it may be possible to down-regulate succinate synthesis by decreasing the NADH supply.

In order to validate this, the intracellular levels of NADH and NAD^+ were determined after the

cells were cultivated and collected. As shown in Fig 4A, in the initial stage of fermentation, NAD^+ was

a bit higher than NADH. However, during acid-producing stage, more NADH was accumulated, which

may be explained due to a strengthened glycolysis[16] and a reduced concentration of succinate in the

cytoplasm, indicating that the provision of reducing power may be sufficient for succinate synthesis.

Overexpression of NOX is a useful method to reduce the NADH/NAD ratio.[46] To determine

whether the decrease in the NADH/NAD ratio was favorable in decreasing the succinate

concentration, a codon-optimized water-forming LINOX[47] was expressed in *A. oryzae*. Fig. 4B shows

the NOX activity in the cytosol and mitochondria of the engineered strain CMPIMIS and four mutants

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with LlNOX overexpression. Owing to the varying locations of the insertion loci and the copy number

of inserted gene, the NOX activity was different and in the cytosol were much higher than that in the

mitochondria. With the overexpression of LlNOX, the NAD concentration obviously increased and

accordingly, the NADH/NAD ratio significantly decreased (Fig. 4C). The malate titer of the engineered

strain CMPIMISN-3 increased from 105.3 to 117.2 g/L, and as expected, the byproduct succinate

concentration decreased from 6.0 to 3.8 g/L (Fig. 4D). However, it seems that the decrease in the

NADH/NAD ratio had little influence on the synthesis of fumarate. It should also be noted that very

low NADH/NAD ratio was not favorable for malate synthesis because low NADH concentration cannot

provide sufficient NADH for malate synthesis, as it is the case of the clone CMPIMISN-1 (Fig. 4D).

CONCLUSIONS

To our knowledge, this work is the first attempt to improve malate production by synergistically

engineering the carbon and redox metabolisms in the cytosol and mitochondria of microbial cells, and

the malate production was significantly increased, with simultaneous decrease of the byproduct

succinate concentration. The kinetic analysis of key metabolites in the cytosol and mitochondria

indicated that succinate was mainly localized in the cytosol, and not the mitochondria. The

accumulation of pyruvate in the cytosol and mitochondria was also observed. The overexpression of

PYC in the cytosol and mitochondria directed more carbon flux from pyruvate to malate synthesis,

and the accumulation of pyruvate was eliminated.

Besides rTCA engineering, the malate synthesis pathways in the mitochondria were also

systematically engineered. The improved oxidative TCA pathway was favorable for cell growth, but

unfavorable for malate synthesis. Based on this result, the oxidative TCA pathway was

down-regulated by the inhibition of citrate synthase using RNAi, and as expected, an increase in

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malate production was achieved. Due to in the glyoxylate shunt, the theoretical yield of 1.33 mole

malate/mole glucose was obtained, which is higher than that obtained from the oxidative TCA

pathway (1.0 mole malate/mole glucose), the co-overexpression of ICL and MAS in the glyoxylate

bypass resulted in an obvious increase in the malate titer, while the succinate and fumarate

concentrations also increased.

To reduce succinate accumulation, two strategies were developed. In the first strategy, the

expression of the dicarboxylate carrier Sfc1p from *S. cerevisiae*, which transports succinate from the

cytosol into the mitochondria,[39] resulted in a considerable decrease in the succinate concentration.

This approach can also be useful for the metabolic engineering of other fungus and yeasts to increase

the production of other organic acids. The second strategy involved the engineering of the redox

potential in the cytosol and mitochondria. Glycolysis and the TCA pathway generate NADH, which is

an essential cofactor in malate and succinate syntheses. Adequate NADH supply may accelerate

succinate synthesis. By engineering the redox potential with the expression of water-forming LlNOX,

the malate titer was further increased and the succinate concentration was significantly decreased. It

was also observed that a very low level of NADH was unfavorable for malate synthesis.

In summary, we simultaneously achieved significant increases in the malate titer and decreases

in the byproduct succinate concentration in metabolically engineered *A. oryzae* by the synergistic

engineering of the carbon and redox metabolisms in the cytosol and mitochondria. To the best of our

knowledge, this was the first report demonstrating enhancement of malate production through

engineering of the mitochondria and metabolic pathways of byproducts synthesis and transport.

Although the malate titer of 117.2 g/L was lower than the titer of some other engineered strains,[15, 48]

the concentration of the byproduct succinate obtained in this study is the lowest ever reported for

malate production, especially directly from raw material, signifying the effectiveness of the metabolic engineering approaches used in this work. Our results strongly suggest that only cytosolic or mitochondrial engineering is insufficient to simultaneously achieve an increase in the malate titer and a decrease in the succinate concentration in *A. oryzae*.

Compared with our previous work, the yield and productivity of malate improved by 32.4% and 46.3%, respectively. It should be noted that although a comprehensive metabolic engineering strategy was developed in this study to decrease the byproduct succinate concentration, a complete blocking of succinate production in the fermentation broth remains an unresolved challenge. Therefore, future studies on the catalytic characterization of SDH are necessary to clarify its molecular mechanism. Further investigations involving the molecular engineering of SDH can facilitate in developing successful strategies to decrease succinate concentrations even further.

MATERIALS AND METHODS

Strains, plasmids, and transformation

The strains used in this study are listed in Table 1. *E. coli* JM109 was used as the expression host for plasmid constructions. The genetically engineered strain *A. oryzae* GAAF41[17] was used as the initial host. The genes *icl1*, *icl2*, *mas*, *cis*, *isd*, *oxd*, and *ach* were amplified from the cDNA of *A. oryzae* GAAF41, the *Sfc1p* gene was amplified from the genome of *S. cerevisiae*, and the codon-optimized LlNOX gene was synthesized from Genewiz (Suzhou, China) and cloned using the primers listed in Table S1. All of the promoters and terminators, including P*tef*/T*tryC* for PYC, P*pgk*/T*glaA* for regulation of oxidative branch of TCA cycle and glyoxylate bypass, P*sodM*/T*amyB* for RNAi, and P*eno*/T*eno* for Sfc1p, were obtained from the genome of *A. oryzae*. The PCR products were purified with a TaKaRa MiniBEST Agarose Gel DNA Extraction Kit (TaKaRa, Dalian, China). The resulting plasmids were

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constructed using the pMD19-T vector (TaKaRa Bio Inc., Otsu, Shiga, Japan), and the plasmid

pY15TEF1-RoPYCP474N was provided by Dr. Xu of Jiangnan University, Wuxi, China.[49]

All plasmids were constructed as vectors designed to integrate randomly into the *A. oryzae*

genome. The procedures for protoplast transformation and the isolation of transformants were

performed as described previously.[16]

Cultivation conditions

A. oryzae conidia were plated onto potato dextrose (PDA) agar and incubated at 34 °C for 3 days,

and 0.05% Tween-80 solution was used for the collection of spores. Shake flask fermentation included

two steps: seed culture and acid production. The seed medium contained 30 g/L glucose, 6 g/L

peptone from fish, 1.05 g/L $NaH_2PO_4 \cdot 2H_2O$, 2.07 g/L $Na_2HPO_4 \cdot 12H_2O$, 560 mg/L KH_2PO_4, and 734 mg/L

$K_2HPO_4 \cdot 3H_2O$. All the fermentations were done in corn starch, and the acid-production medium was

composed of 130 g/L corn starch, 12 g/L peptone from fish, 150 mg/L KH_2PO_4, 196 mg/L $K_2HPO_4 \cdot 3H_2O$,

and 90 g/L $CaCO_3$. The final spore concentration in the seed medium was 1.5×10^6 spores/mL, and it

was incubated at 30 °C with shaking at 220 rpm for 14 h. Acid production cultures were then prepared

by inoculation of 10% of the seed cultures and fermentation at 30 °C with shaking at 200 rpm until the

glucose was consumed.

Purification of mitochondria

The broth was filtered to collect the pellets and the cells were flash-frozen using liquid nitrogen

for the termination of cell metabolism. The mitochondria were extracted according to the instructions

provided with the Mitochondria Isolation kit (Sigma-Aldrich, Shanghai, China) for profiling cultured

cells, and most of the isolated mitochondria contained intact inner and outer membranes.

Metabolite analysis

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To remove the acid precipitations and undissolved calcium, the same volumes of 2N HCl were added to the culture broth. For mitochondrial metabolism analysis, 1 mL lysis buffer from Mitochondria Isolation kit was added to the purified mitochondria. The analysis of organic acid concentrations was performed by reverse phase high-performance liquid chromatography (HPLC) using an Agilent 1260 Series Binary LC System equipped with an HPX-87H column (Bio-Rad, Hercules, CA, USA), and 5 mM H_2SO_4 was used as the mobile phase with a flow rate of 0.6 mL/min at 40 °C. The glucose concentration was determined by an SBA_40E Glucose Biosensor (Bosheng, Jinan, China).

RNA extraction and transcription analysis

Cell pellets were collected in the mid-exponential phase and immediately transferred into liquid nitrogen to terminate cell metabolism. Then, the frozen samples were ground in to a powder using a sterilized pre-chilled mortar and pestle. Total RNA was extracted using the RNApure Plant kit (CoWin Biosciences, Beijing, China), according to the manufacturer's recommendations instructions. To remove genomic DNA from total RNA, all samples were treated with DNase and quantification was performed at 260/280 nm in a Nano Drop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Total RNA was reverse-transcribed into cDNA using the PrimeScript RT reagent kit (Perfect Real Time) (TaKaRa Bio-Inc). Quantitative real-time (qRT)-PCR analysis was conducted in a LightCycler 480 II real-time instrument (Roche Diagnostics, Mannheim, Germany), as described previously.[16] The actin protein-encoding gene was used as the reference gene for normalization, and the primers used for qRT-PCR are listed in Table S2. All experiments were performed with at least three biological replicates.

Enzyme activity assay and determination of NADH/NAD$^+$ concentrations

The samples of cells in different stages of fermentation and diverse transformants were

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harvested and dried. The NADH oxidase (NOX) activity was determined using the NOX Assay kit

(Solarbio, Shanghai, China). The intracellular concentrations of NAD^+ and NADH were determined

using the NAD/NADH Quantification kit (Sigma-Aldrich).

Author Contributions

J. L., Y. L., G.D. and L. L. designed the experiments, J. L. performed the experiments, J.L., H.S., L.L.,

G.D. and J.C. conceived the project and analyzed the data, J. L., R. L. A., L. L. wrote the paper.

Competing financial interests

The authors declare that they have no competing financial interests.

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Table 1 Strains used in this study

Strains	Characteristics	Source or reference
GAAF41	overexpression of amylolytic enzymes and *S. cerevisiae* fumarase	(Liu et al., 2017a)
CMP	GAAF41 derivate, cytoplasm and mitochondria-localized ROPYC	This study
CMPT	CMP derivate, strengthen the TCA pathway	This study
CMPIM	CMP derivate, enhance the glyoxylate bypass	This study
CMPIMI	CMPIM derivate, RNAi with *cis* gene	This study
CMPIMIS	CMPIMI derivate, overexpression of Sfc1p	This study
CMPIMISN-3	CMPIMIS derivate, overexpression of LlNOX	This study

Table 2. Effects of metabolic engineering on L-malate production in this study.

Strains (*A. oryzae*)	MA (g L^{-1})	SUC (g L^{-1})	FUM (g L^{-1})	Time (h)	Yield (g/g)	Productivity (g L^{-1} h^{-1})
GAAF41	88.5	9.2	0.79	110	0.68	0.80
CP	92.3	7.2	0.68	107	0.71	0.86
MP	90.5	7.9	0.71	108	0.70	0.84
CMP	95.1	6.9	0.60	105	0.73	0.91
CMPT	83.7	6.8	0.65	115	0.64	0.73
CMPIM	99.8	8.3	1.02	103	0.77	0.97
CMPIMI	105.3	9.5	1.31	103	0.81	1.02
CMPIMIS	109.1	6.0	0.70	102	0.84	1.07
CMPIMISN-3	117.2	3.8	0.75	100	0.90	1.17

Yields are given in gram L-malate per gram corn starch unless otherwise indicated.

Fermentations were carried out in 250 mL shake flasks.

Abbreviations: MA, L-malate; SUC, succinate; FUM, fumarate.

The data shown are mean values from three independent fermentation.
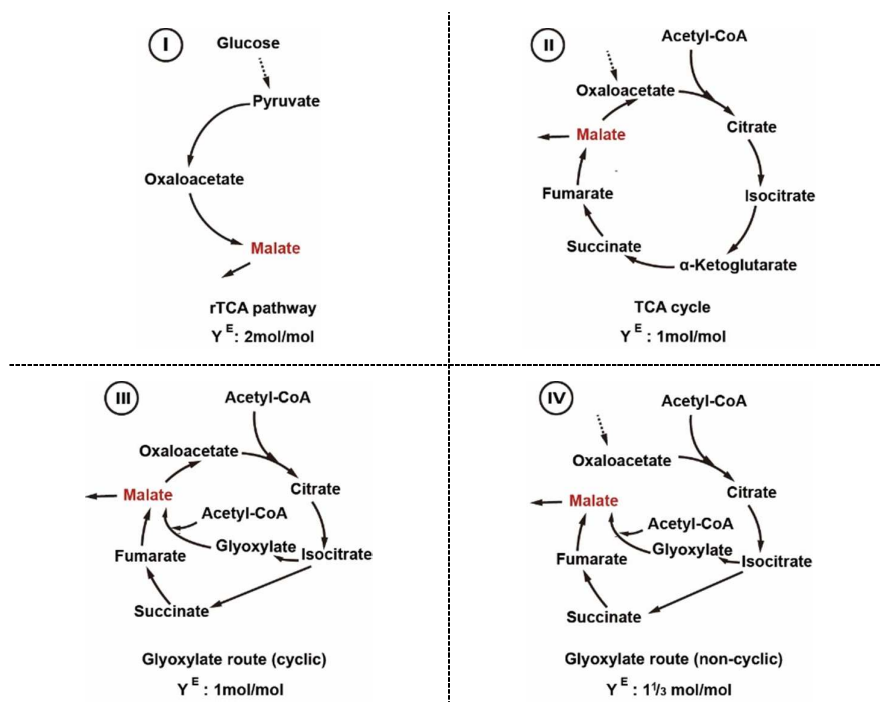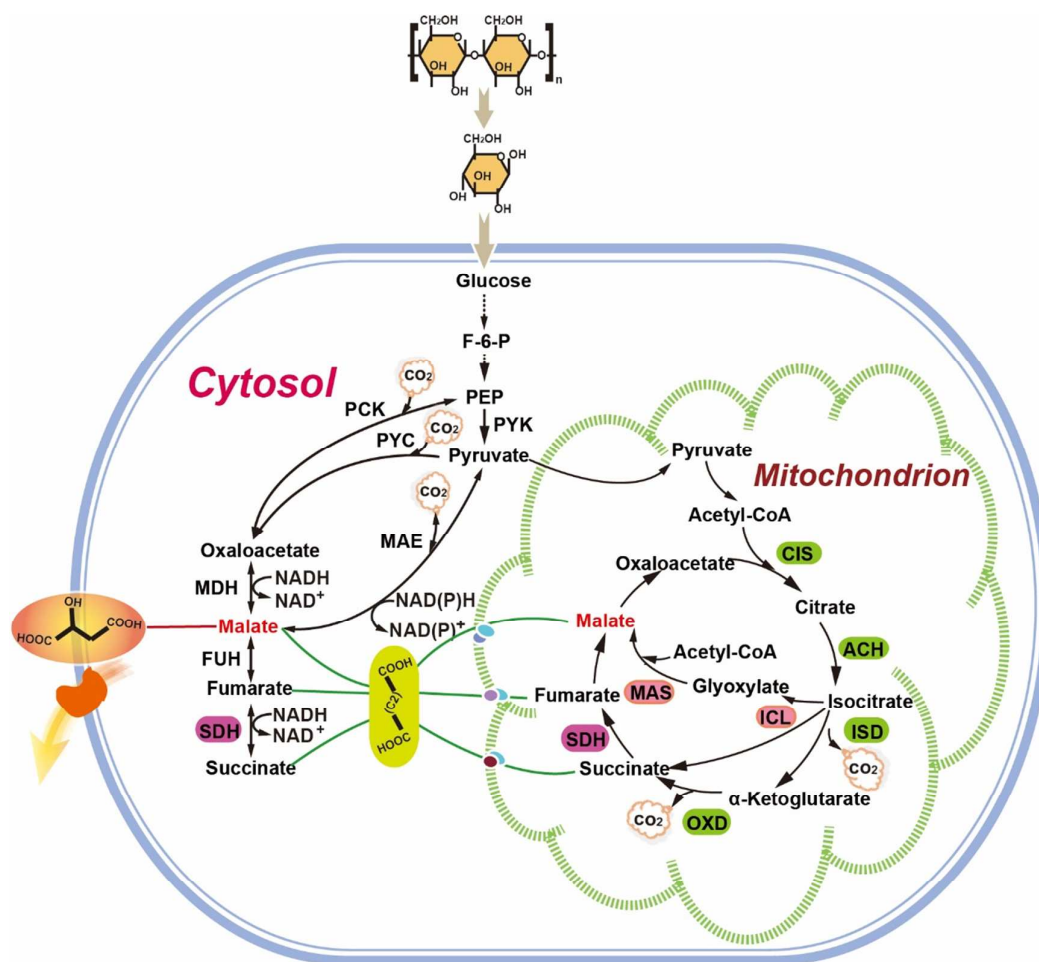
Figure captions

Fig. 1. Metabolic pathways of L-malate from starch in *Aspergillus oryzae*. F-6-P: fructose-6-phosphate; PEP: phosphoenolpyruvate; PYK: pyruvate kinase; PPC: phosphoenolpyruvate carboxylase; PYC, pyruvate carboxylase; MAE: malic enzyme; MDH: malate dehydrogenase; FUH: fumarate hydratase; SDH: succinate dehydrogenase; CIS: citrate synthase; ACH: aconitate hydratase; ISD: isocitrate dehydrogenase; OXD: a-oxoglutarate dehydrogenase; ICL: isocitrate lyase; MAS: malate synthetase. (I) reductive tricarboxylic acid (rTCA) pathway; (II) oxidative TCA cycle; (III) cyclic glyoxylate route; (IV) noncyclic glyoxylate route. Y^E: maximum theoretical yield (in mol malate per mol glucose).
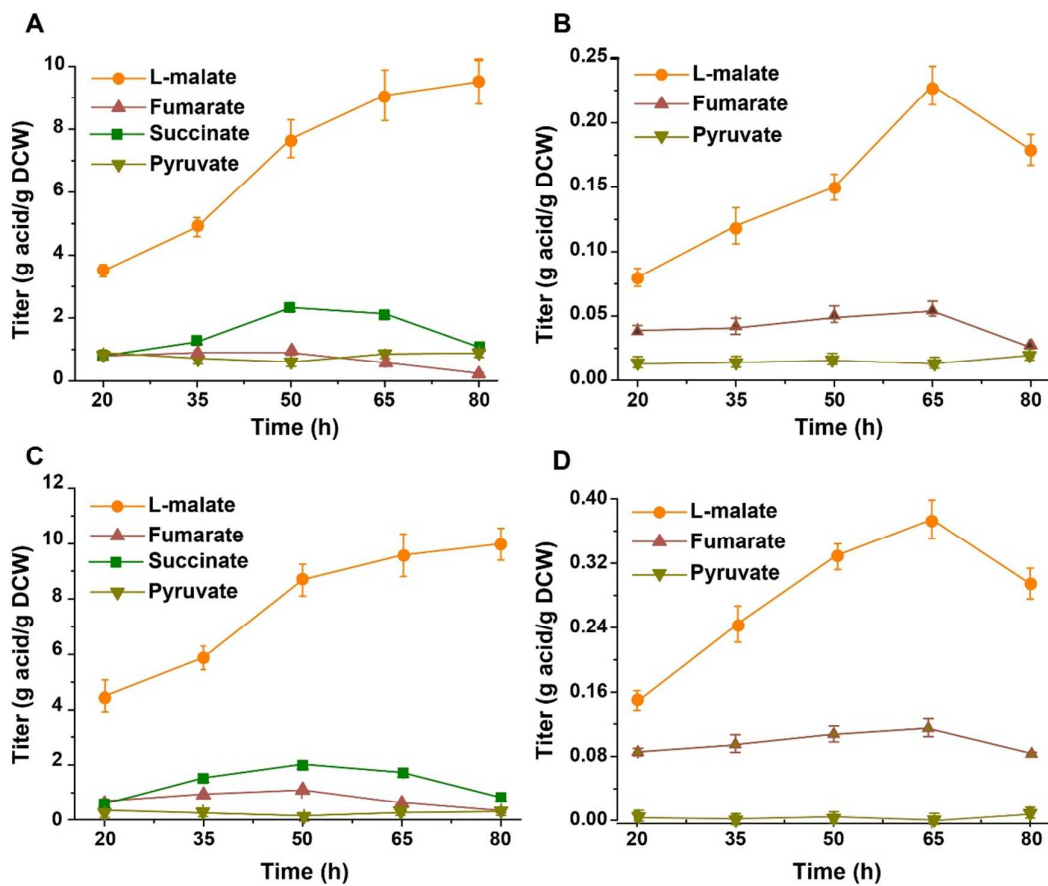
Fig. 2. The main intracellular metabolites of *Aspergillus oryzae*. (A) in cytosol of GAAF41 (B) in mitochondria of GAAF41 (C) in cytosol of CMP (D) in mitochondria of CMP. The data shown are mean values from triplicates with error bars indicating the standard error.

Fig. 3. (A) Plasmid construction for down-regulation of *cis* gene expression. The inverted repeats corresponding to the 7th exon of the *cis* gene and separated by the 7th intron of the same gene were placed in the plasmid. (B) The change of expression of key genes in rTCA and TCA pathway in CMPIMI compared with the CMPIM. The data shown are mean values from triplicates with error bars indicating the standard error. *pyc*: pyruvate carboxylase; *mdh*: malate dehydrogenase; *cis*: citrate synthase; *isd*: isocitrate dehydrogenase; *oxd*: a-oxoglutarate dehydrogenase; *ach*: aconitate hydratase; *fuh*: fumarate hydratase.
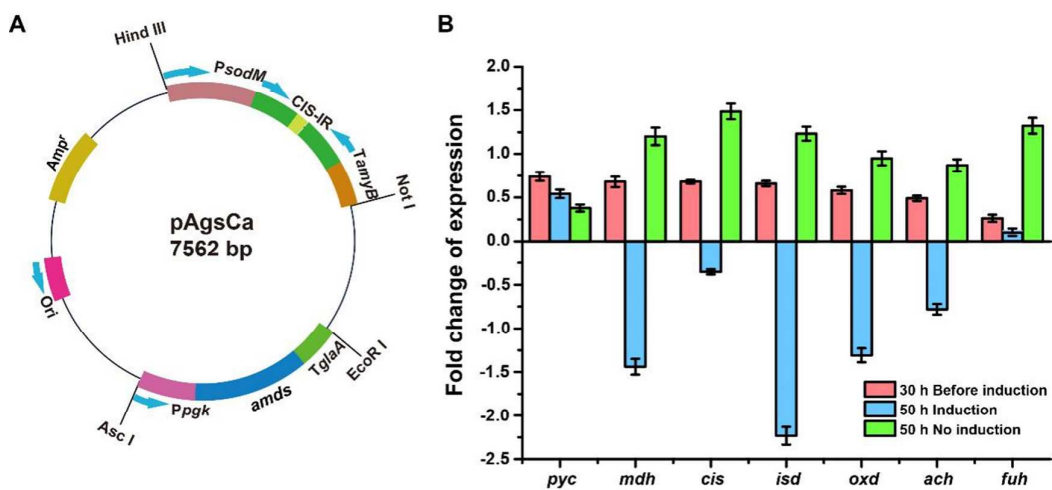
Fig. 4. (A) The intracellular NAD^+ and NADH concentrations of CMPIMIS during the fermentation. (B) The NOX activity in CMPIMIS and four LlNOX transformants. (C) The intracellular NAD^+ and NADH concentrations in CMPIMIS and four LlNOX transformants. (D) The concentrations of malate, succinate and fumarate in CMPIMIS and four LlNOX transformants. Orange: L-malate; green: succinate; pink: fumarate. The data shown are mean values from triplicates with error bars indicating the standard error.
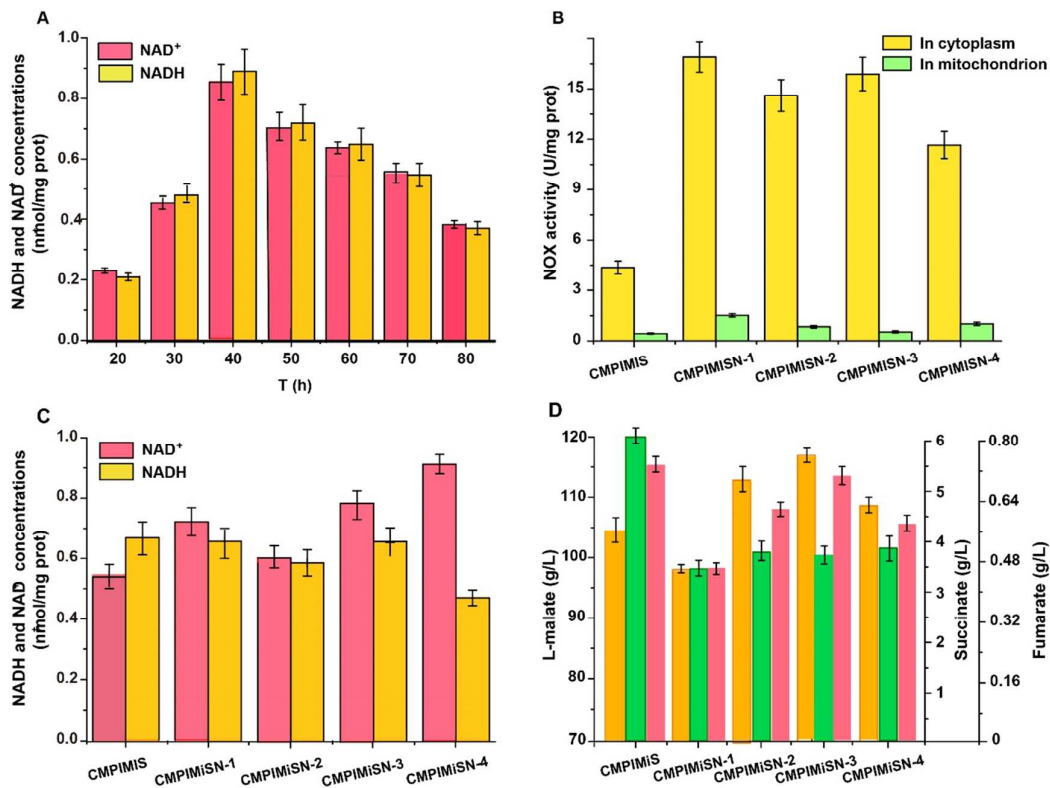
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(Fig. 1)

(Fig. 2)

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(Fig. 3)

(Fig. 4)

Synergistic rewiring of carbon metabolism and redox metabolism in cytoplasm and

mitochondria of *Aspergillus oryzae* for increased L-malate production

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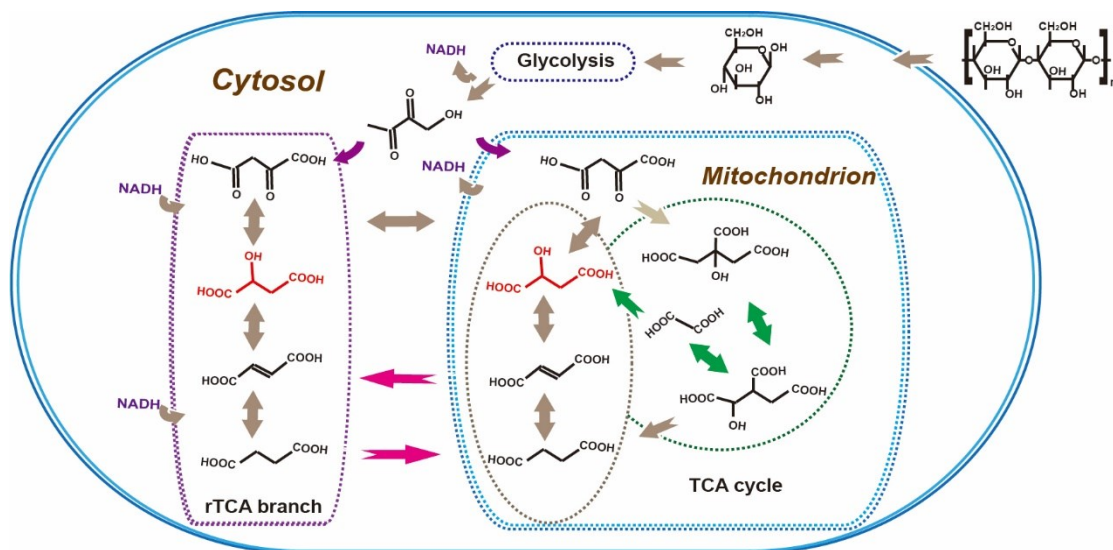Long Liu, Tel: +86-510-85918312, Fax: +86-510-85918309, E-mail: longliu@jiangnan.edu.cn

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