Discovery of a super-strong promoter enables efficient production of heterologous proteins in cyanobacteria

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Cyanobacteria are oxygenic photosynthetic prokaryotes that play important roles in the global carbon cycle. Recently, engineered cyanobacteria capable of producing various small molecules from CO2 have been developed. However, cyanobacteria are seldom considered as factories for producing proteins, mainly because of the lack of efficient strong promoters. Here, we report the discovery and verification of a super-strong promoter P_{pcp560}, which contains two predicted promoters and 14 predicted transcription factor binding sites (TFBSs). Using P_{pcp560}, functional proteins were produced at a level of up to 15% of total soluble protein in the cyanobacterium Synechocystis sp. 6803, a level comparable to that produced in Escherichia coli. We demonstrated that the presence of multiple TFBSs in P_{pcp560} is crucial for its promoter strength. Genetically transformable cyanobacteria neither have endotoxins nor form inclusion bodies; therefore, P_{pcp560} opens the possibility to use cyanobacteria as alternative hosts for producing heterogeneous proteins from CO2 and inorganic nutrients.

O2 is one of the main greenhouse gases. Cyanobacteria can convert CO2 into organic compounds through oxygenic photosynthesis with an efficiency higher than that of terrestrial plants1,2. Cyanobacteria account for 20–30% of earth’s photosynthetic productivity; that is, the conversion of solar energy into chemical energy3,4. The use of engineered cyanobacteria capable of producing fuels and other chemicals could reduce greenhouse gas emission and help to address the shortages of energy and resources5. As nonpathogenic aquatic photoautotrophs with a high nutritional value, some cyanobacteria are attractive hosts for producing heterologous proteins that can be used in the health, food, fodder, fertilizer, and environment sectors6–8.

Proteins of industrial or environmental interest that have been produced in cyanobacteria include a metallothionein protein capable of absorbing heavy metals in wastewater7, a larvicidal protein that can kill mosquito eggs in water8, and a human CuZn superoxide dismutase that can be used in health products9. The human CuZn superoxide dismutase was expressed at a level of 3% of total soluble protein in the cyanobacterium Anacystis nidulans 63019. This expression level was achieved by using a native strong promoter, Prbc343, and an optimized SD sequence. Because this expression cassette was delivered by a replicative plasmid, the constant addition of antibiotics was required to maintain the expression level9.

Several heterologous genes have been expressed in cyanobacteria in an effort to create novel biosynthetic pathways to produce chemicals. Heterologous strong promoters such as P_{tn}, and P_{lac} and native promoters such as P_{pcp}, P_{bch} and P_{psbA2} have been used in integrated cyanobacterial expression systems. Lan et al.7 used P_{trc} to express trans-enoyl-CoA reductase (Ter) for butanol production, and Guerrero et al.10 used P_{lac} to express ethylene-forming enzyme (EFE) for ethylene production. Lindberg et al.11 and Bentley et al.12 used P_{psbA2} to drive expression of isoprene synthase (IspS) for isoprene production in cyanobacteria. Although there was successful production of the target chemicals, the expression levels of the proteins encoded by the heterologous genes were not reported7,10. Recently, Angermay et al.13 experimented with various promoters (P_{repB}, P_{psbA2}, and P_{trc}) to drive expression of lactate dehydrogenase in cyanobacteria, and found that even powerful native and artificial promoters from Synechocystis were not strong enough to produce the enzyme in sufficient quantities.

The aim of this study was to create novel expression elements that lead to strong expression of heterologous genes in cyanobacteria. We discovered a super-strong promoter, P_{pcp560}, consisting of two promoters from the cpcB gene and 14 predicted transcription factor binding sites (TFBSs). Using P_{pcp560} two heterologous genes were...
expressed in the cyanobacterium *Synechocystis* sp. 6803 (hereafter, *S*. 6803) to a level comparable to that obtained using the *Escherichia coli* expression system, demonstrating the strength and efficiency of P<sub>pcp560</sub>. We further showed that the presence of multiple TFBBS is crucial for the promoter strength of P<sub>pcp560</sub>. The discovery of this super-strong promoter will stimulate research on using cyanobacteria as hosts to produce valuable recombinant proteins from CO<sub>2</sub> and inorganic nutrients.

**Results**

**Discovery of the super-strong promoter P<sub>pcp560</sub>.** Efficient expression elements are required to increase the expression level of heterologous genes. Since c-phycocyanin and ribulose-bisphosphate carboxylase/oxygenase (Rubisco) are the major soluble proteins in cyanobacteria, expression elements from genes encoding these two proteins have been used widely to construct cyanobacterial expression vectors<sup>6,7,14–16</sup>. For example, a metallothionein gene was expressed in the cyanobacterium *Synechococcus* sp. 7942 using the promoter from *cpcB*, the gene encoding the c-phycocyanin beta subunit<sup>7</sup>, while a human CuZn superoxide dismutase gene was expressed in *Synechococcus* sp. 7002 using the promoter and terminator from the *rbc* operon, which encodes Rubisco<sup>6</sup>.

RBDSigner<sup>7</sup> predicted that the RBS in the *cpcB* promoter is approximately 10-times more efficient than that in the *rbc* promoter in terms of the predicted translation efficiency. Therefore, we chose the *cpcB* promoter for further investigations. The genomic sequence 1,000 bp upstream of the initiation codon of the *cpcB* gene (sll1577) was subjected to promoter analysis using Virtual Footprint<sup>14</sup>. This analysis revealed two promoters located at 135 bp and 374 bp upstream of the initiation codon of the *cpcB* gene. Scanning the genomic sequence 1,000 bp upstream of the initiation codon of the *cpcB* gene also revealed 14 TFBBS located between 381 bp and 556 bp from the initiation codon of the *cpcB* gene<sup>18</sup> (Supplementary Table S1). Negative TFBBS are usually located between 500 bp and 1,000 bp upstream of the transcription start site<sup>19</sup>, and some mammalian proteins such as soluble adenylyl cyclase<sup>20</sup> are highly similar to these proteins in cyanobacteria. Therefore, we chose the genomic sequence of 560 bp upstream of the initiation codon of *cpcB* as the new promoter sequence; this sequence was designated as P<sub>pcp560</sub>. P<sub>pcp560</sub> contains two predicted promoters and 14 predicted TFBBS (Fig. 1).

No terminator was found in the genomic region 300 bp downstream of the stop codon of the *cpcB* gene. This may be because *cpcB* is the first gene of the *cpc* operon. Since the *rbc* terminator was used in a previous study that achieved high-level gene expression (yielding approximately 10-times more efficient than that in the *rbc* promoter in terms of the predicted translation efficiency. Therefore, we chose the *cpcB* promoter for further investigations. The genomic sequence 1,000 bp upstream of the initiation codon of the *cpcB* gene (sll1577) was subjected to promoter analysis using Virtual Footprint<sup>14</sup>. This analysis revealed two promoters located at 135 bp and 374 bp upstream of the initiation codon of the *cpcB* gene. Scanning the genomic sequence 1,000 bp upstream of the initiation codon of the *cpcB* gene also revealed 14 TFBBS located between 381 bp and 556 bp from the initiation codon of the *cpcB* gene<sup>18</sup> (Supplementary Table S1). Negative TFBBS are usually located between 500 bp and 1,000 bp upstream of the transcription start site<sup>19</sup>, and some mammalian proteins such as soluble adenylyl cyclase<sup>20</sup> are highly similar to these proteins in cyanobacteria. Therefore, we chose the genomic sequence of 560 bp upstream of the initiation codon of *cpcB* as the new promoter sequence; this sequence was designated as P<sub>pcp560</sub>. P<sub>pcp560</sub> contains two predicted promoters and 14 predicted TFBBS (Fig. 1).

**High-level expression of crotonyl-CoA-specific trans-enoyl-CoA reductase gene in *S*. 6803.** To validate the efficacy of the newly discovered promoter P<sub>pcp560</sub>, we used it to drive the expressions of two genes encoding important enzymes involved in metabolism in *S*. 6803. One enzyme was crotonyl-CoA-specific trans-enoyl-CoA reductase (Ter), a key enzyme in increasing the driving force towards butanol biosynthesis<sup>21–23</sup>. *ter* has been overexpressed in *Synechococcus* sp. PCC 7942 with the aim to produce butanol from CO<sub>2</sub><sup>3</sup>. A strong promoter from *E. coli*, P<sub>Ptrc</sub>, was used to express a codon-optimized *ter* in *Synechococcus* sp. PCC 7942<sup>24</sup>. However, the activity of *ter* in the crude cell extract of *Synechococcus* sp. PCC 7942 was 0.057 ± 0.005 µmol/min/mg crude cell extract. This was 64-fold lower than that of *Ter* (3.7 ± 0.5 µmol/min/mg) in *E. coli*, in which the *ter* gene was expressed under the control of a medium-strength promoter, P<sub>L lacO1</sub><sup>25</sup>. This suggests that P<sub>Ptrc</sub> is not an efficient promoter in cyanobacteria in terms of gene expression strength. Therefore, we tested whether the newly discovered promoter P<sub>pcp560</sub> could significantly increase the expression level of *ter* in cyanobacteria.

To express *ter* in *S*. 6803, a codon-optimized *ter* gene (Supplementary Fig. S1) from *T. denticola* flanked by P<sub>pcp560</sub> and T<sub>rbc</sub> expression elements was integrated into the *S*. 6803 chromosome at the *pta* (slr2132) insertion site via homologous recombination. The *pta* gene encodes phosphotransacetylase, the first enzyme in acetate synthetic pathway, and disruption of *pta* does not affect cell growth<sup>26</sup>. Homoplasmy and gene insertion were verified by PCR and sequencing (Fig. 2a and 2b). SDS-PAGE analysis demonstrated that *ter* was strongly overexpressed in the *S*. 6803 mutant Δ*pta*:P<sub>pcp560ter</sub>. The protein expression level was approximately 15% of total soluble protein (Fig. 2c), a surprisingly strong expression for cyanobacteria. The band excised from the position at approximately 43.7 kDa was confirmed as *Ter* from *T. denticola* by MALDI-TOF MS analysis (Fig. 2d).

The specific activity of *Ter* was determined to be 32.22 ± 1.93 µmol/min/mg crude cell extract of *S*. 6803 strain Δ*pta*:P<sub>pcp560ter</sub> (Table 1). In a previous study in which the *ter* gene was expressed in *Synechococcus* sp. PCC 7942 under the control of the P<sub>Ptrc</sub> promoter<sup>6</sup>, the activity of *Ter* was 0.057 ± 0.005 µmol/min/mg crude cell extract. When *ter* was expressed in *E. coli* BW25113 under the control of the P<sub>L lacO1</sub> Promoter, on a medium-copy number plasmid, the *Ter* activity was 3.7 ± 0.5 µmol/min/mg crude cell extract<sup>25</sup>. These activities of *Ter* reported in the literature were measured in crude lysates obtained from different organisms cultured in various different conditions. The activity of *Ter* determined in our study indicates that the *ter* gene was strongly expressed in *S*. 6803 strain Δ*pta*:P<sub>pcp560ter</sub>.

**TFBSs are crucial for P<sub>pcp560</sub> strength.** The newly discovered super-strong promoter P<sub>pcp560</sub> differs from other commonly used promoters in that it contains 14 predicted TFBBS located between −556 bp and −381 bp from the initiation codon of *cpcB*. To investigate whether these TFBBS contribute to *cpcB* promoter strength, *ter* was expressed under the control of P<sub>pcp374</sub>, which contained only the two promoters, in *S*. 6803 Δ*pta*:P<sub>pcp374</sub> (Fig. 2a and 2b). Although RT-PCR confirmed *ter* transcription from P<sub>pcp374</sub> (Supplementary Fig. S2), the level of *Ter* protein was too low for detection by SDS-PAGE, whereas the level of *Ter* protein expressed from *ter* driven by P<sub>pcp560</sub> was up to 15% of total soluble protein (Fig. 2c). The specific activity of *Ter* in strain Δ*pta*:P<sub>pcp374ter</sub> was only 0.12 ± 0.01 µmol/min/mg crude extract (Table 1). This was 268-fold lower than that of *Ter* in the strain Δ*pta*:P<sub>pcp560ter</sub> (32.22 ± 1.93 µmol/min/mg), and only slightly higher than that of *Ter* in *Synechococcus* sp. PCC 7942, in which the *ter* gene was expressed under the control of the P<sub>Ptrc</sub> promoter<sup>6</sup>. These data show that the newly discovered super-strong promoter P<sub>pcp560</sub> is indeed much stronger than P<sub>pcp374</sub> and P<sub>Ptrc</sub>, and the multiple TFBBS present in P<sub>pcp560</sub> are crucial for its promoter strength.

**High-level expression of L-lactate dehydrogenase in *S*.6803.** To investigate whether the newly discovered super-strong promoter
Figure 2 | Heterologous gene insertion into genome of S. 6803 at pta site, and expression of ter and DldhE in S. 6803. (a) For all mutants, complete segregation was demonstrated by whole-cell PCR with primers from recombinant cassette at pta site and a primer from 100-bp outside of recombinant cassette. (b) Whole-cell PCR with primers for each heterologous gene verified insertion of each heterologous gene into genome of corresponding strain. M: DNA marker III (top to bottom; 4.5, 3, 2, 1.2, 0.8, 0.5 kb). (c) Detection of heterologous gene expression in corresponding strain by 12% SDS-PAGE analysis. Each marker protein band contains 2 µg protein; 20 µg protein was loaded into each lane. Crude extracts from each strain were analyzed. Expression levels of Ter or DldhE under control of strong promoter P_cpc560 were approximately 15% of total soluble protein, whereas Ter expressed under control of P_cpc374 in Δpta::P_cpc374ter strain was undetectable on gel. (d) Peptide mass fingerprint of bands at approximately 43.6 and 36.5 kDa. 43.6-kDa band was identified as Ter from T. denticola, 36.5-kDa band was identified as DldhE from E. coli K12. Mascot score greater than 53 (default MASCOT threshold for such searches) was accepted as significant (p value < 0.05). All strains (WT, Δpta, Δpta::P_cpc374ter, Δpta::P_cpc560ter and Δpta::P_cpc560DldhE) were continuously passaged more than 10 times without antibiotics.
P_{pcp560} also drives strong expressions of other genes. We used it to control expression of \(Dldh\), which encodes \(\delta\)-lactate dehydrogenase. The \(Dldh\) gene from \(E. coli\) K12 (designated as \(DldhE\)) flanked by \(P_{pcp560}\) and \(T_{\text{ter}}\) was integrated into the chromosome at the \(pta\) insertion site via homologous recombination. Homoplasy and \(DldhE\) gene insertion were verified by PCR and sequencing (Fig. 2a and 2b). SDS-PAGE analysis demonstrated that \(DldhE\) was strongly overexpressed in the \(S. 6803\) mutant \(\Delta\text{pta}::P_{pcp560}\)DldhE; the protein expression level was approximately 15% of total soluble proteins (Fig. 2c). MALDI-TOF MS analysis confirmed that the band excised from the position at approximately 36.5 kDa was \(DldhE\) from \(E. coli\) (Fig. 2d). The specific activity was determined to be 51.92 ± 2.31 \(\mu\text{mol/min/mg}\) crude extract in strain \(S. 6803\) \(\Delta\text{pta}::P_{pcp560}\)DldhE (Table 1). In a previous study in which the \(DldhE\) gene was expressed under the control of \(P_{\text{tetO-1}}\) on a low-copy number plasmid, the activity of \(DldhE\) in \(E. coli\) K12 MG1655 was 0.96 ± 0.06 \(\mu\text{mol/min/mg}\) crude extract. This result confirmed that the \(DldhE\) gene was strongly expressed in \(S. 6803\) under the control of \(P_{pcp560}\).

We also investigated the genetic stability of the mutant containing the strong integrative expression cassette. The mutants \(\Delta\text{pta}::P_{pcp560}\)TrbcL and \(\Delta\text{pta}::P_{pcp560}\)DldhE were continuously passed 10 times in the absence of antibiotics. The expression levels of both \(\text{ter}\) and \(DldhE\) did not change during this procedure, demonstrating the stability of this expression system.

### Discussion

In this study, we discovered and verified a super-strong promoter \(P_{pcp560}\) for efficient and strong expression of heterologous genes in cyanobacteria. The newly discovered super-strong promoter \(P_{pcp560}\) consists of two predicted promoters from the \(pcp\) gene and 14 predicted TFBSs. Using \(P_{pcp560}\) two heterologous genes were expressed in the cyanobacterium \(S. 6803\) to levels of up to 15% of total soluble protein. Despite the fact that only a single copy of the target gene was inserted into the chromosome of \(S. 6803\), the expression level of the target gene driven by \(P_{pcp560}\) was comparable to that which can be achieved in an \(E. coli\) expression system using low- or medium-copy number plasmids. This demonstrates that \(P_{pcp560}\) has potential applications for efficient production of recombinant proteins in cyanobacteria.

It is generally accepted that the genomic sequence 200–300 bp upstream of the initiation codon of a gene that is constitutively expressed at high levels can be used as a promoter sequence. Previous studies have shown that the thymine at 259 bp upstream of the initiation codon of \(pcpB\) is crucial for \(pcpB\) promoter activity24. Recently, a genomic map of the transcriptional start sites (TSS) of \(S. 6803\) showed that the \(pcpB\) gene is one of the genes with a long distance between its TSS and start codon25. Further examination revealed that most of the \(S. 6803\) genes with a long distance between the TSS and start codon are responsive to environmental factors25. Since the \(pcpB\) gene is a light-and redox-responsive gene25,29, it is plausible that the length of the \(pcpB\) promoter is related to its responsiveness to environmental factors. \(P_{pcp560}\) is unusually long in that it contains only two predicted promoters (at 135 bp and 374 bp), but also 14 predicted TFBSs located between 381 bp and 556 bp upstream of the initiation codon of \(pcpB\). \(P_{pcp560}\) differs from \(P_{pcp374}\) in that it has an extra 186 bp DNA fragment containing the 14 predicted TFBSs. The large-scale difference in the expression levels of \(ter\) under the control of \(P_{pcp560}\) and \(P_{pcp374}\) demonstrated that the extra 186 bp DNA fragment in \(P_{pcp560}\) may contain positive TFBSs and that the presence of these multiple TFBSs is crucial for its promoter strength.

Transcription factors are proteins that play roles in virtually every aspect of the transcription process26. In prokaryotes, RNA polymerase recognizes and binds to the promoter region and initiates transcription, and a sigma factor is required for RNA polymerase to bind to the promoter. In eukaryotes, three types of eukaryotic RNA polymerases all require transcription factors to bind to the promoter sequence before transcription can be initiated27. Therefore, compared with eukaryotic promoters such as the yeast promoter27, the promoter of \(E. coli\) is rather short (approximately 30–50 bp) and TFBSs are usually not required. For instance, when we scanned the sequences of \(E. coli\) strong promoters (including \(P_{\text{lac}}, P_{\text{lac}}\) and \(T7\)) that have been used to drive gene expressions in cyanobacteria previously, we found only one or two TFBSs in each of the promoters. In this study, we found 14 predicted TFBSs located between 381 bp and 556 bp upstream of the initiation codon of \(pcpB\), and verified that the extra 186 bp DNA fragment containing multiple predicted TFBSs is crucial for \(P_{pcp560}\) promoter strength in cyanobacteria. This novel discovery raises the possibility that the lack of TFBSs in \(E. coli\) strong promoters may explain why they perform poorly in driving gene expression in cyanobacteria. Thus, we propose that native positive TFBSs should be considered when designing promoters to drive gene expression in cyanobacteria.

The newly discovered super-strong promoter \(P_{pcp560}\) will be useful for further research on the production of useful substances using transgenic cyanobacteria and the very cheap substrate CO2. The principle of considering TFBS in cyanobacterial promoter design will also contribute to designing strong and controllable promoters to drive the expressions of genes involved in new biosynthetic pathways from CO2 in cyanobacteria.

### Methods

**Materials.** We purchased restriction endonucleases and other nucleases for plasmid construction from New England Biolabs (Beverly, MA, USA). All chemicals for enzymatic analyses were purchased from Sigma-Aldrich (St Louis, MO, USA).

**Construction of expression vectors.** The plasmids used and constructed in this study are listed in Supplementary Table S2. Primers are listed in Table S3. General strategies for constructing expression vectors are shown in Supplementary Fig. S3.

The sequences of \(P_{pcp560}\) and the \(T_{\text{ter}}\) terminator were obtained from Sanogen Biotech (Shanghai, China). \(DldhE\) was obtained by PCR using genomic DNA of \(E. coli\) K12 as the template. Fusion PCR26 was used to ligate DNA fragments, including \(P_{\text{pcp560}}\)-\(ter\), \(P_{\text{pcp374}}\)-\(ter\), and \(P_{\text{pcp560}}\)-\(DldhE\)-\(T_{\text{ter}}\). To construct the expression vectors \(P_{\text{pcp560}}\)-\(pcp434\) and \(P_{\text{pcp560}}\)-\(DldhE\), the fusion PCR products \(P_{\text{pcp560}}\)-\(ter\), \(P_{\text{pcp560}}\)-\(DldhE\)-\(T_{\text{ter}}\) were digested with Xhol and ligated into the Xhol site between two homologous arms of vector \(pSM2\). The \(ter\) gene flanked by \(P_{\text{pcp374}}\) and \(T_{\text{ter}}\) was obtained by PCR from the constructed plasmid \(P_{\text{pcp560}}\)-\(pcp434\) and was inserted into the Xhol site of plasmid \(pSM2\).

**Strains.** The bacterial strains used in these experiments are listed in Supplementary Table S2. We used \(E. coli\) DH5\(\alpha\) to propagate constructs, and \(S. 6803\) wild-type as the starting strain. All mutant strains were constructed by transforming \(S. 6803\) wild-type with the plasmids listed in Supplementary Table S2, followed by subsequent selection until homoplasy was achieved.

Transformation were performed as previously described24. All constructed strains are listed in Supplementary Table S2. Briefly, \(\Delta\text{pta}\) was constructed by integration of the plasmid \(pSM2\) at the \(pta\) locus of \(S. 6803\) via double crossover homologous recombination.

Transforming the wild-type strain of \(S. 6803\) with the plasmids \(pSM2\)-\(pcp560\)-\(ter\) and \(pSM2\)-\(pcp374\)-\(ter\) generated strains \(\Delta\text{pta}::P_{pcp560}\) and \(\Delta\text{pta}::P_{pcp374}\), respectively. In these strains, a part of the \(pta\) gene was replaced with the kanamycin resistance cassette and the \(P_{pcp560}\) or \(P_{pcp374}\)--driven expression cassette of the...
enoyl-CoA reductase.

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Forward primer 5'-CAAGGCTTGTACCGGCT-3' and the reverse primer 5'-
CGATCAAGAGGTTCACACT-3'. Transcript levels of mnpB were analyzed as a
positive control.

Preparation of cell extract and gene expression level analysis. Cell crude extracts
were prepared as described elsewhere, with some modifications. We collected 10-ml
samples of cultures in mid-exponential growth phase, and harvested cells by
centrifugation at 1,000 x g for 2 min. Cell pellets were washed twice and then
resuspended in prechilled buffer from the in vitro essay described below. Cells were
disrupted by bead beating and cell debris and beads were removed by centrifugation at
14,000 x g and 4 °C for 30 min. Total protein concentrations of the crude extract were
determined using the Bradford method with bovine serum albumin as the standard.
To measure the decrease in absorbance at 340 nm, which corresponded to consumption of
NADH, trans-2-enoyl-CoA reductase (Ter) assay

Trans-2-enoyl-CoA reductase (Ter) assay. Ter activity was assayed by monitoring the
decrease in absorbance at 340 nm, which corresponded to consumption of
NADH, trans-2-enoyl-CoA reductase (Ter) assay

D-lactate dehydrogenase (Dldh) assay. Dldh activity was determined by monitoring the
decrease of absorbance at 340 nm as previously reported. The 200 μl reaction mixture
contained 50 mM sodium phosphate buffer (pH 6.2), 200 μM crotonyl-CoA (Sigma), 400 μM NADH, and 0.3 μg crude extract protein from strain ΔptpA ΔterA ter
and ΔptpA ΔterA ter cells using Redzol reagent (Qiagen, Beijing, China). Residual DNA in
the decrease in absorbance at 340 nm as previously reported. The 200 μl reaction mixture
contained 50 mM sodium phosphate buffer (pH 6.2), 200 μM crotonyl-CoA (Sigma), 400 μM NADH, and 0.3 μg crude extract protein from strain ΔptpA ΔterA ter
and Δp tpA ΔterA ter cells using Redzol reagent (Qiagen, Beijing, China). Residual DNA in

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Author contributions
J.Z. designed and performed the research. H.Z., H.M. and Y.Z. assisted with the experiments. Y.Z. and G.B. performed expression elements analyses. Y.L. and Y.M. designed the project and supervised the research. J.Z. and Y.L. wrote the manuscript. All authors analyzed data and discussed the results.

Additional information
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