Elevated CO$_2$ concentration promotes photosynthesis of grape (Vitis vinifera L. cv. ‘Pinot noir’) plantlet in vitro by regulating RbcS and Rca revealed by proteomic and transcriptomic profiles

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Abstract

Background: Plant photosynthesis can be improved by elevated CO$_2$ concentration (eCO$_2$). In vitro growth under CO$_2$ enriched environment can lead to greater biomass accumulation than the conventional in micropropagation. However, little is know about how eCO$_2$ promotes transformation of grape plantlets in vitro from heterotrophic to autotrophic. In addition, how photosynthesis-related genes and their proteins are expressed under eCO$_2$ and the mechanisms of how eCO$_2$ regulates RbcS, Rca and their proteins have not been reported.

Results: Grape (Vitis vinifera L. cv. ‘Pinot Noir’) plantlets in vitro were cultured with 2% sucrose designated as control (CK), with eCO$_2$ (1000 μmol·mol$^{-1}$) as C0, with both 2% sucrose and eCO$_2$ as Cs. Here, transcriptomic and proteomic profiles associated with photosynthesis and growth in leaves of V. vinifera at different CO$_2$ concentration were analyzed. A total of 1814 genes (465 up-regulated and 1349 down-regulated) and 172 proteins (80 up-regulated and 97 down-regulated) were significantly differentially expressed in eCO$_2$ compared to CK. Photosynthesis-antenna, photosynthesis and metabolism pathways were enriched based on GO and KEGG. Simultaneously, 9, 6 and 48 proteins were involved in the three pathways, respectively. The leaf area, plantlet height, qP, ΦPSII and ETR increased under eCO$_2$, whereas Fv/Fm and NPQ decreased. Changes of these physiological indexes are related to the function of DEPs. After combined analysis of proteomic and transcriptomic, the results make clear that eCO$_2$ have different effects on gene transcription and translation. RbcS was not correlated with its mRNA level, suggesting that the change in the amount of RbcS is regulated at their transcript levels by eCO$_2$. However, Rca was negatively correlated with its mRNA level, it is suggested that the change in the amount of its corresponding protein is regulated at their translation levels by eCO$_2$.

Conclusions: Transcriptomic, proteomic and physiological analysis were used to evaluate eCO$_2$ effects on photosynthesis. The eCO$_2$ triggered the RbcS and Rca up-regulated, thus promoting photosynthesis and then advancing transformation of grape plantlets from heterotrophic to autotrophic. This research will helpful to understand the influence of eCO$_2$ on plant growth and promote reveal the mechanism of plant transformation from heterotrophic to autotrophic.

Keywords: Vitis vinifera, iTRAQ labeling, RNA-Seq, Elevated CO$_2$ concentration, Photosynthesis, Photoautotrophic
Background
Increasing atmospheric CO₂ concentration influences plant growth [1, 2]. Photosynthesis, respiration and water relations are the three primary physiological processes influenced by elevated CO₂ concentration (eCO₂) in plants [3]. CO₂ concentration inside the culture vessels decreased when plantlets grown in vitro, which limits the photosynthetic rate of the plants [4, 5]. The biomass accumulation of the in vitro cultured plants increased under photoautotrophic and CO₂ enrichment conditions, also affected nutrient absorption and secondary metabolism [6, 7].

Plantlet grown vigorously under CO₂ enriched photoautotrophic and photomixotrophic conditions, with high photosynthetic photon flux density [8]. Photosynthetic response to light and CO₂ increased with Rubisco activities and proteins of plantlets grown in vitro [7]. Rubisco, the main catalytic enzyme determines photosynthetic rate [8], would respond to eCO₂ [9] and increase carboxylation efficiency under eCO₂ [10]. Succinctly, the synthesis of the Rubisco holoenzyme is mainly affected by ribulose bisphosphate carboxylase small chain (RbcS) [11]. The activity of Rubisco is related to Rubisco activase (Rca) and other proteins [12, 13].

In some species, it is reported that the transcript levels of RbcS are differentially regulated by red and blue light or growth temperature [14]. The abundance of the RbcS multigene family transcript has been researched in many plants [15]. RbcS regulates Rubisco through coordinated expression of RbcL and RbcS in plants [11]. In addition to the folded RbcL subunits assemble [16], RbcS could combine more CO₂ than the RbcL in all Rubiscos [17]. The detailed mechanism of RbcS mediated assembly of RbcL under different environment and how the expression of RbcS and its protein responds to eCO₂ remains to be investigated. The Rca could gain energy from ATP hydrolysis to remodel Rubisco inhibitors and activate Rubisco [18]. Inhibit expression of Rca on some plants results in severe photoautotrophic growth defects [19]. Rca proteins belong to a subgroup of the ATPases associated with various cellular activities (AAA+) called AAA + [20]. There are two Rca forms both can activate Rubisco [21]. Rca is regulated by the intracellular ATP/ADP ratio [22] or the C-terminal extension of the α-isofrom of Rca in some plants [18]. Some research indicated that Rca could reduce the effects of abiotic stresses on plants, such as high temperature, drought, salt [23–25] and heavy metal [26]. The expression of Rca is regulated by trans-acting factors in soybean [27]. The actual change mechanism of Rca expression and whether Rca related to other proteins under eCO₂ is less studied.

‘Pinot Noir’ is a wine grape variety widely planted in worldwide and its growth influenced by various environmental factors [28]. The increasing CO₂ concentration could promote plant growth. Although, many studies have focused on the effects of CO₂ on grape ripening [29] and postharvest [30]. It is unclear the mechanism of how eCO₂ affects the plant growth and photosynthesis. Additionally, there are a few reports on the analysis of transcriptome combined with proteome to study the effects of eCO₂ on grape growth and development. In light of this situation, the experiment was conducted based on the hypothesis that eCO₂ will enhance photosynthesis by regulating the expression of related genes and proteins in grape plantlets. Therefore, grape plantlets grown in vitro cultured with eCO₂ were used in this study based on transcriptome, proteome and photosynthetic physiology analysis.

Results
Effects of eCO₂ on growth and chlorophyll fluorescence
Grape plantlets were cultured for 25 days at 1000 μmol·mol⁻¹ of CO₂ and compare with control conditions. The results showed that the leaf area, plantlet height and shoot fresh weight increased significantly in Cs and C0 compared with CK (Additional file 1: Table S1). In addition, the number of adventitious roots in tubers also was increased in Cs and C0 (Fig. 1a).

In compared to CK, the Fv/Fm decreased in Cs and C0, and significantly lower in C0 than that of CK (Fig. 1b, c). The qP and ETR rised in Cs and C0 (Fig. 1d, e). The ETR of C0 was significantly higher than the Cs and CK. The qP of C0 was significantly higher than Cs and CK (Fig. 1d). The NPQ was in the following order: Cs < C0 < CK (Fig. 1d). The decrease of NPQ indicated that eCO₂ enhanced the efficiency of PSII and reduced the damage caused by biotic and abiotic stress. The ΦPSII of Cs and C0 were significantly higher than CK (Fig. 1d). These results suggested that eCO₂ improved photosynthesis and reflected by chlorophyll fluorescence parameters, including Fv/Fm, ETR, qP, NPQ and ΦPSII.

Transcriptome and proteome differences expression in eCO₂
In the transcriptome project, three RNA-Seq groups with three replications were sequenced, 29.5Gb clean bases were generated from the 9 libraries. After data processing, 46.49–47.46 million high-quality reads were obtained (Table 1). Through transcriptome analysis, a total of 1814 DEGs were observed by comparing with CK, of which 116 up-regulated and 632 down-regulated DEGs were identified in Cs versus CK, 349 up-regulated and 717 down-regulated DEGs were identified in C0 versus CK (Fig. 2a). According to SDS-PAGE analysis, protein sample could be tested in the next step (Additional file 2: Figure S1). After analysis of proteomic profiling, a total of 177 DEPs were observed from the pooled data for above two comparison groups. Among them, 48 up-regulated DEPs and 67 down-regulated DEPs were
identified in Cs versus CK, 32 up-regulated DEPs and 30 down-regulated DEPs were identified in C0 versus CK (Fig. 2b).

**GO analysis of DEGs and DEPs**

Of the 25,679 genes identified in the transcriptome analysis, 17,750 genes (69.12%) were annotated via GO analysis. Compared with CK, 748 DEGs identified in Cs were enriched in the biological process (BP), cellular component (CC), and molecular function (MF) categories. In the cellular components category, most of DEGs were involved in integral component of membrane (99 genes) and cytoplasm (94 genes). In the biological process category, most of DEGs were involved in defense response (70 genes) and transcription, DNA-templated (59 genes). In the molecular function category, most of DEGs were involved in transcription factor activity, sequence-specific DNA binding (54 genes) and ATP binding (48 genes) (Additional file 3: Table S2 A).

The 1066 DEGs of C0 versus CK were detected. Most DEGs mainly enriched in cytoplasm (98 genes) and integral component (97 genes) of cellular components. In the biological process category, most of DEGs were involved in defense response (53 genes) and transcription, DNA-templated (48 genes). In the molecular function category, most of DEGs were involved in metal ion binding (52 genes) and transcription factor activity, sequence-specific DNA binding (45 genes) (Additional file 3: Table S2 B).

From the pooled data for Cs versus CK, 115 DEPs were enriched in cell part (68 proteins) of cellular components. In the biological process category, most of DEPs were involved in metabolic process (81 proteins). In the molecular function category, most of DEPs were involved in catalytic activity (59 proteins) (Additional file 4: Table S3 A).

### Table 1

|                      | CK          | C0          | Cs          |
|----------------------|-------------|-------------|-------------|
| Total reads          | 46,888,137  | 47,455,710  | 46,499,479  |
| Total Mapped         | 42,923,887  | 43,235,905  | 42,843,192  |
| Multiple mapped      | 1,017,694   | 1,009,825   | 982,852     |
| Uniquely mapped      | 41,906,193  | 42,226,081  | 41,860,340  |
| Read-1               | 20,981,121  | 21,144,642  | 20,980,375  |
| Read-2               | 20,925,072  | 21,081,439  | 20,879,965  |
| Reads map to ‘+’     | 20,973,998  | 21,131,755  | 20,957,526  |
| Reads map to ‘-’     | 20,932,195  | 21,094,326  | 20,902,814  |
| Non-splice reads     | 25,189,915  | 25,355,802  | 25,331,236  |
| Splice reads         | 16,716,278  | 16,870,279  | 16,529,104  |
| Reads mapped in pairs| 40,350,237  | 40,714,512  | 40,408,286  |
Among the top 10 up-regulated DEPs, there were 3 proteins with abundance change related to photosynthesis: oxygen-evolving enhancer protein 3 (PsbQ; XP_002275624.1), chlorophyll a-b binding protein CP26 (Lhcb5; XP_002264295.1), photosystem II protein V (PsbE; YP_567093.1) (Table 2). But ribulose bisphosphate carboxylase/oxygenase activas (Rca; XP_002282979.1) was the 11th top up-regulated DEPs. Among the top 10 down-regulated DEPs, 2 proteins related to secondary metabolites: aspartokinase 2 (XP_010660689.1) and protein luteindeficient 5 (XP_002279984.3); one protein related to polysaccharide catabolic process: inactive beta-amylase 9 (XP_002276777.1); one protein related to stress tolerance: acid phosphatase 1 (XP_003632911.1) (Table 2).

A total of 62 DEPs were detected in C0 versus CK. DEPs were annotated and enriched in the three categories. In the cellular components, most proteins were involved in cell part (36 proteins). In the biological process category, most proteins were involved in metabolic process (45 proteins). In the molecular function category, most proteins were involved in catalytic activity (28 proteins) (Additional file 4: Table S3 B). Among the top 10 up-regulated DEPs, 5 of the top 10 DEPs were photosynthesis proteins: oxygen-evolving enhancer protein 3 (PsbQ; XP_002275624.1), Plastocyanin (Pc; XP_002285904.1), Chlorophyll a-b binding protein (Lhcb6; XP_002263201.1), chlorophyll a-b binding protein CP26 (Lhcb5; XP_002264295.1) and chlorophyll a-b binding protein of LHCII type 1 (Lhcb1; XP_002283566.1) (Table 3). The Rca (XP_002282979.1) was the 13th top up-regulated DEPs. Three of the top 10 DEPs down-regulated proteins: bifunctional 3-dehydroquinate dehydratase/shikimate dehydrogenase (XP_002270232.1, XP_002270188.1) and beta-glucosidase 13 (XP_002270422.2) (Table3) were associated with biosynthesis of secondary metabolites.

KEGG pathway analysis for DEPs
To further investigate the plant reaction to eCO2, DEPs were identified by searching the KEGG database. The 115 DEPs of Cs were assigned to 52 KEGG pathways, and the top 5 pathways with the highest rich factor were photosynthesis-antenna proteins, metabolic pathways, biosynthesis of secondary metabolites, carbon metabolism, biosynthesis of amino acids (Additional file 5: Table S4 A). The 62 DEPs of C0 were assigned to 20 KEGG pathways, and the top 5 pathways with the highest rich factor were photosynthesis-antenna proteins, photosynthesis, metabolic pathways, phenylpropanoid biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis (Additional file 5: Table S4 B).
The common pathways with the highest rich factor of Cs versus CK and C0 versus CK were photosynthesis-antenna proteins, photosynthesis and metabolic pathways. Simultaneously, 9, 6 and 48 proteins were involved in the three pathways, respectively. Moreover, 12 proteins involved in metabolic pathway were overlaps with photosynthesis (Table 4).

There were 8 chlorophyll a-b binding proteins: Chlorophyll a-b binding protein (Lhcb6, XP_002263201.1), chlorophyll a-b binding protein 151 (Lhcb2; XP_002271687.1),
| Accession       | Description                                                                 | Fold Change(C0/CK) | KEGG term                                         | P value     |
|-----------------|------------------------------------------------------------------------------|--------------------|---------------------------------------------------|-------------|
| XP_002274242.1  | PREDICTED: major allergen Pru av. 1 [Vitis vinifera]                        | 2.00               | none                                              | 4.049E-07   |
| XP_002275624.1  | PREDICTED: oxygen-evolving enhancer protein 3, chloroplastic [Vitis vinifera] | 1.91               | Photosynthesis (ko00195)                          | 2.24E-08    |
| XP_002285904.1  | PREDICTED: plastocyanin [Vitis vinifera]                                    | 1.88               | Photosynthesis (ko00195)                          | 6.998E-08   |
| XP_002263201.1  | PREDICTED: chlorophyll a-b binding protein CP24 10A, chloroplastic [Vitis vinifera] | 1.75               | Photosynthesis - antenna proteins (ko00196)       | 3.512E-07   |
| XP_002279607.1  | PREDICTED: sec-independent protein translocase protein TATA, chloroplastic [Vitis vinifera] | 1.70               | Bacterial secretion system/Protein export (ko03070; ko03060) | 0.0005      |
| XP_002267428.1  | PREDICTED: patellin-3 [Vitis vinifera]                                       | 1.69               | none                                              | 5.44E-06    |
| XP_010653784.1  | PREDICTED: uncharacterized protein LOC100245204 isoform X1 [Vitis vinifera]  | 1.66               | none                                              | 0.0001      |
| XP_019076764.1  | PREDICTED: metal transporter Nramp3 isoform X2 [Vitis vinifera]             | 1.66               | Ferroptosis/Lysosome (ko04216;ko04142)            | 2.638E-06   |
| XP_002263064.1  | PREDICTED: plasma membrane-associated cation-binding protein 1 [Vitis vinifera] | 1.61               | none                                              | 2.445E-06   |
| XP_002264295.1  | PREDICTED: chlorophyll a-b binding protein CP26, chloroplastic [Vitis vinifera] | 1.59               | Photosynthesis-antenna proteins (ko00196)         | 1.856E-09   |
| XP_002277053.2  | PREDICTED: GDSL esterase/lipase At1g09390 [Vitis vinifera]                  | 0.55               | none                                              | 3.675E-05   |
| XP_019073045.1  | PREDICTED: S05 ribosomal protein L24, chloroplastic [Vitis vinifera]         | 0.59               | Ribosome (ko03010)                                | 6.23E-07    |
| XP_002283566.1  | PREDICTED: chlorophyll a-b binding protein of LHCI type 1 [Vitis vinifera]   | 0.62               | Photosynthesis - antenna proteins (ko00196)       | 0.0009      |
| XP_002274561.1  | PREDICTED: limonoid UDP-glucosyltransferase [Vitis vinifera]                | 0.63               | none                                              | 0.00012     |
| XP_002270232.1  | PREDICTED: bifunctional 3-dehydroquininate dehydratase/shikimate dehydrogenase, chloroplastic [Vitis vinifera] | 0.63               | Phenylalanine, tyrosine and tryptophan biosynthesis (ko00400) | 6.293E-05   |
| XP_002270188.1  | PREDICTED: bifunctional 3-dehydroquininate dehydratase/shikimate dehydrogenase, chloroplastic [Vitis vinifera] | 0.65               | Phenylalanine, tyrosine and tryptophan biosynthesis (ko00400) | 3.948E-07   |
| XP_003634206.1  | PREDICTED: thaumatin-like protein [Vitis vinifera]                           | 0.65               | none                                              | 8.466E-05   |
| NP_001268023.1  | lipoxygenase [Vitis vinifera]                                                | 0.65               | Linoleic acid metabolism/alpha-Linolenic acid metabolism (ko00591; ko00592) | 4.482E-07   |
| XP_002285526.1  | PREDICTED: transmembrane 9 superfamily member 9 [Vitis vinifera]            | 0.65               | none                                              | 1.907E-05   |
| XP_002270422.2  | PREDICTED: beta-glucosidase 13 [Vitis vinifera]                              | 0.65               | Phenylpropanoid biosynthesis/ Starch and sucrose metabolism/Cyanoamino acid metabolism (ko00940; ko00500; ko00460) | 0.0001      |
| UniProt ID       | Accession      | Description                                                                 | Fold Change | Cs/CK | CO/ CK | P value        | Cs/CK | CO/ CK |
|------------------|----------------|------------------------------------------------------------------------------|-------------|--------|--------|----------------|--------|--------|
| **Photosynthesis** |                |                                                                              |             |        |        |                |        |        |
| A5ASW8           | XP_002263201.1 | chlorophyll a-b binding protein CP24 10A, chloroplastic [Vitis vinifera]     | 1.61        | 1.75   | 2.56E-07 | 3.51E-07      |        |        |
| A5BA4            | XP_003633024.1 | Chlorophyll a-b binding protein, chloroplastic [Vitis vinifera]              | 0.94        | 1.39   | 0.0321  | 0.0001         |        |        |
| A5ASG6           | XP_002271687.1 | chlorophyll a-b binding protein 151, chloroplastic [Vitis vinifera]          | 1.46        | 0.94   | 9.56E-06 | 0.0177         |        |        |
| F6ISI9           | XP_002273201.1 | photosystem I chlorophyll a/b-binding protein 3-1, chloroplastic [Vitis vinifera] | 1.50        | 1.50   | 5.03E-08 | 4.30E-08      |        |        |
| A5BW14           | XP_002274150.2 | chlorophyll a-b binding protein 13, chloroplastic [Vitis vinifera]           | 1.49        | 1.49   | 1.40E-07 | 1.07E-07      |        |        |
| F6HMH7           | XP_002275075.1 | chlorophyll a-b binding protein of LHCII type 1 [Vitis vinifera]             | 1.45        | 1.5    | 6.43E-08 | 3.28E-07      |        |        |
| F6H2E4           | XP_002284493.1 | chlorophyll a-b binding protein 13, chloroplastic [Vitis vinifera]           | 1.42        | 1.46   | 2.60E-06 | 5.47E-06      |        |        |
| A5SC4U9          | XP_002285646.1 | chlorophyll a-b binding protein of LHCII type 1 [Vitis vinifera]             | 1.62        | 0.66   | 0.0006  | 0.0003         |        |        |
| A5SPB2           | XP_002283566.1 | chlorophyll a-b binding protein of LHCII type 1 [Vitis vinifera]             | 1.36        | 0.62   | 0.0014  | 0.0009         |        |        |
| **Metabolic pathway** |            |                                                                              |             |        |        |                |        |        |
| D0VBC1           | NP_001268000.1 | 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase 03 [Vitis vinifera]     | 0.73        | 0.85   | 1.32E-05 | 0.0001         |        |        |
| A5ASW8           | XP_002263201.1 | chlorophyll a-b binding protein, chloroplastic [Vitis vinifera]              | 1.61        | 1.71   | 2.56E-07 | 3.51E-07      |        |        |
| Q22519           | NP_001268064.1 | chalcone synthase [Vitis vinifera]                                           | 1.16        | 0.66   | 0.0027  | 3.91E-05       |        |        |
| A5BA4            | XP_003633024.1 | Chlorophyll a-b binding protein, chloroplastic [Vitis vinifera]              | 0.94        | 1.39   | 0.0321  | 0.0001         |        |        |
| D7T1Y1           | XP_002264311.2 | Threonine dehydratase [Vitis vinifera]                                       | 0.67        | 0.79   | 4.02E-05 | 0.0004         |        |        |
| F6HTH9           | XP_002267374.1 | bifunctional riboflavin biosynthesis protein RIBA 1, chloroplastic [Vitis vinifera] | 0.68        | 0.87   | 4.02E-06 | 0.0002         |        |        |
photosystem I chlorophyll a-b-binding protein 3–1 (Lhcb3; XP_002273201.1), chlorophyll a-b binding protein 13 (Lhcb3; XP_002274150.2), chlorophyll a-b binding protein of LHClI type 1 (Lhcb1; XP_002275075.1), chlorophyll a-b binding protein 13 (Lhcb3; XP_002284493.1), chlorophyll a-b binding protein of LHClI type 1 (Lhcb1; XP_002285646.1) and chlorophyll a-b binding protein of LHClI type 1 (Lhcb1; XP_002283566.1) significantly up-regulated in Cs and C0 compared with those in CK, only 1 protein, Chlorophyll a-b binding protein (Lhcb3; XP_003633024.1) was descend in Cs (Fig. 3). There were 4 subunits of PSII: PsbQ (XP_002227562.1), PsbE (YP_567093.1), photosystem II protein D2 (PsbD; YP_567071.1) and photosystem I reaction center subunit N (PsaN; XP_003631913.1) significantly up-regulated in Cs and C0, but 1 subunit Pc (XP_002285904.1) of PSI was down-regulated (Fig. 3). Ribulose bisphosphate carboxylase small chain (RbcS; XP_002276967.1) and ATP synthase delta chain (XP_002274963.1) were up-regulated in Cs and C0 (Figs. 3, 4). Other proteins: beta-glucosidase 13 (XP_002270422.1), beta-amylase 1 (XP_002276777.1), threonine dehydratase (XP_003631913.1) and GDP-L-galactose phosphorylase 2 (XP_002278339.1) involved in metabolic were down-regulated in Cs and C0 compared with those in CK, and also involved in polysaccharide catabolic process and biosynthesis of secondary metabolites (Table 4).

### Combined analysis of transcriptome and proteome data

To reveal eCO2 regulates photosynthesis gene via transcript and protein levels, the transcript data were used to analyze 18 DEPs associated with photosynthesis and metabolic pathways. Seven DEPs: Lhcb6 (XP_0022273201.1), Lhcb3 (XP_0022273201.1, XP_002284493.1), Lhcb1 (XP_002275075.1, XP_002285646.1, XP_002283566.1) and RbcS (XP_002276967.1) and their mRNA expression showed up-regulated in Cs and C0. However, there were 3 DEPs, PsbQ (XP_0022275624.1), Rca (XP_002282979.1)

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| UniProt ID | Accession | Description | Fold Change | P value |
|------------|-----------|-------------|-------------|---------|
| F6HE54     | XP_002278339.1 | GDP-L-galactose phosphorylase 2 [Vitis vinifera] | 0.65 | 4.62E-07 |
| F6HDW1     | XP_002279975.1 | pyruvate kinase isozyme A, chloroplastic [Vitis vinifera] | 0.66 | 2.26E-06 |
| A5AGN5     | XP_002280094.1 | ketol-acid reductoisomerase, chloroplastic-like [Vitis vinifera] | 0.68 | 3.24E-08 |
| F6I397     | XP_002280760.1 | transketolase, chloroplastic [Vitis vinifera] | 0.71 | 1.22E-06 |
| F6HS21     | XP_002281731.1 | peroxidase P7 [Vitis vinifera] | 1.48 | 0.0002 |
| F6HO42     | XP_002283364.1 | geranylgeranyl pyrophosphate synthase, chloroplastic [Vitis vinifera] | 0.66 | 0.0002 |
| F6HE24     | XP_002284493.1 | chlorophyll a-b binding protein 13, chloroplastic [Vitis vinifera] | 1.42 | 5.47E-06 |
| E0CSP0     | XP_002284769.2 | protochlorophyllide reductase, chloroplastic [Vitis vinifera] | 0.70 | 0.0008 |
| A5BZY3     | XP_002285583.1 | glutamyl-tRNA reductase 1, chloroplastic-like [Vitis vinifera] | 0.62 | 3.96E-05 |
| A5C4U9     | XP_002285646.1 | chlorophyll a-b binding protein of LHClI type 1 [Vitis vinifera] | 1.62 | 0.0003 |
| D7SYQ0     | XP_010646454.1 | acetalactate synthase small subunit 2, chloroplastic [Vitis vinifera] | 0.69 | 4.67E-05 |
| F6HA90     | XP_010651495.1 | serine--glyoxylate aminotransferase [Vitis vinifera] | 1.47 | 5.81E-08 |
| D75V29     | XP_010652823.1 | inositol-3-phosphate synthase [Vitis vinifera] | 1.42 | 4.29E-08 |
| A5C6H7     | XP_002271896.1 | sucrose synthase 2 [Vitis vinifera] | 1.01 | 0.6544 |
| F6HWQ2     | XP_010656841.1 | aspartokinase 1, chloroplastic [Vitis vinifera] | 0.86 | 0.0002 |
| E0CUM8     | XP_010662621.1 | plastidial pyruvate kinase 2 [Vitis vinifera] | 0.68 | 0.0002 |
| A5CAL1     | XP_003632860.1 | hydroxyphenylpyruvate reductase [Vitis vinifera] | 0.99 | 5.87E-06 |
| D7UCDO     | XP_019081328.1 | bifunctional L-3-cyanoalanine synthase/cysteine synthase 1, mitochondrial [Vitis vinifera] | 1.45 | 4.39E-06 |
| Q0ZJ25     | YP_567071.1 | photosystem II protein D2 [Vitis vinifera] | 1.48 | 6.80E-09 |
| Q0ZJ03     | YP_567093.1 | photosystem II protein V.chloroplast [Vitis vinifera] | 1.67 | 4.26E-07 |
| D7T2U5     | XP_002270422.2 | beta-glucosidase 13 [Vitis vinifera] | 0.87 | 0.0001 |
| F6HW3     | XP_002274963.1 | ATP synthase delta chain, chloroplastic [Vitis vinifera] | 1.30 | 3.35E-09 |
| A5BPT8     | XP_002285277.1 | phenylalanine ammonia-lyase-like [Vitis vinifera] | 0.93 | 0.0002 |
| F6ID0D     | XP_003631913.1 | photosystem I reaction center subunit N, chloroplastic [Vitis vinifera] | 1.37 | 3.98E-09 |
| F6HO8O     | XP_003634480.1 | cationic peroxidase 1 [Vitis vinifera] | 1.11 | 6.57E-06 |
and Lhcb5 (XP_002264295.1) involved in photosynthesis up-regulated in Cs and C0, but their mRNA down-regulated. Other 8 DEPs were not correlated with genes expression under eCO2 (Figs. 3, 4).

The RbcS (XP_002276967.1) and its corresponding gene were up-regulated in Cs and C0. ATP synthase delta chain (XP_002274963.1) and Rca (XP_002282979.1) were up-regulated but their corresponding genes were down-regulated in Cs and C0 (Figs. 3, 4).

The results make clear that eCO2 have different effects on gene transcription and translation. RbcS was not correlated with its mRNA level, suggesting that the change in the amount of RbcS is regulated at their transcript levels by eCO2. However, Rca was negatively correlated with its mRNA level, it is suggested that the change in the amount of its corresponding protein is regulated at their translation levels by eCO2.
Confirmation of qRT-PCR
In order to evaluate our transcriptome-sequencing data, 18 genes in the photosynthesis and metabolic pathway were selected for qRT-PCR. The results analyse indicated that 15 genes (83.33%) showed similar trends in the relative expression levels, which suggested that the gene expression changes detected by transcriptome-sequencing analysis were reliable. But 3 genes (16.67%) analyzed by qRT-PCR, i.e., *PsbE* (4025054), *PsbD* (4025083) and *LHCB3* (LOC100252004) were not consistent with our RNA-seq data (Fig. 5).

Discussion
Proteins involved in photosynthesis were regulated by eCO₂
Light-harvesting complexes (LHC) of photosynthetic plant bind pigments essential for augmenting light capture and photoprotection [31]. LHCl and LHCCI belong to photosystem I (PSI) and photosystem II (PSII), respectively. LHCCI is a trimeric light-harvesting complex (Lhc) composed of a combination of the *Lhcb* gene products and others [32]. Plants can develop strategies of acclimating varies light conditions during seasons and can rapidly adjust photosynthesis antenna sizes in case of excess light, avoiding over excitation and formation of harmful by products [33]. CO₂ concentration could affect the primary light reaction of photosynthesis in soybean leaves [34]. In our research, 8 proteins of LHCCI were up-regulated in eCO₂ (Table 4), this change indicated that eCO₂ could induce more light-harvesting proteins (Fig. 3), and cause an increase in the size of the PSI and PSII antenna. The light-harvesting complex II (LHCII) could convert most photons to biochemical energy and biomass [35]. With the increase of LHCII in eCO₂, more light energy can be absorbed and converted into photosystem. The increase of qP and decrease of NPQ confirming that leaves could absorb more light energy under eCO₂ (Fig. 1D). In present study, the number of up-regulated light-harvesting proteins of PSII was more than that of PSI, which showed that the eCO₂ had a great influence on PSII. Additionally, the LHCCI conditions would migration from PSII to PSI under deficient CO₂ environment [36]. CP24 was up-regulated in eCO₂, it was essential for connecting LHCII to the PSII complex [37, 38]. Lack of the light-harvesting complex CP24 affects the structure and function of the grana membranes of higher plant chloroplasts [37]. Overall, these proteins, which were up-regulated under eCO₂, could absorb and convert more light energy into the photosystem.

In photosynthesis pathway, the expression of PetE (XP_002285904.1) and Chlorophyll a-b binding protein (XP_003633024.1) were descend in Cs. Interestingly, the expression of *PetE* (LOC100248911) and *LHCB3* (LOC100252004) were ascend in Cs and C0. These results indicated that most of the DEPs and their corresponding genes expression were inconsistent. The eCO₂ may cause various modifications of related proteins after translation and needs to be study for further.

eCO₂ regulates metabolic protein expression
There were 48 DEPs involved in metabolic pathway, while 12 of them were overlaps with photosynthesis.

![Fig. 5 qRT-PCR validation of the relative expression levels of 18 selected genes from Cs, C0 and CK in leaves](imageURI)
This might indicate that eCO2 would affect other metabolic
through adjusting photosynthesis. Our results indicated
many of down-regulated DEPs were enriched in metabolic
pathway in eCO2, which were related to biosynthesis of sec-
ondary metabolites (Table 4). This change is suggesting that
eCO2 probably decreased biosynthesis of secondary metab-
olites [39]. Therefore, plant could accumulation more pri-
mary metabolism products to encourage growth.

The eCO2 could ameliorate the effects caused by
drought [40], high temperatures [41], and maintaining
higher photosynthetic rates. This may be linked to the
reduction in stomatal conductance [42]. Moreover, in-
creasing photosystem antenna size must inevitably cause
structural changes needed to ensure high efficiency of its
functioning [43]. There were 4 DEPs (PsbQ, PsbE, PsbD
and PsaN; Fig. 3) up-regulated in eCO2. Those proteins
could maintain the stability of the photosystem reaction
center [44, 45]. By analyzing changes of those proteins
in eCO2, we can conclude that eCO2 could trigger some
proteins to maintain the stability of the photosynthesis
system. Therefore, eCO2 could ameliorate the adverse
effect under abiotic stress. PsbQ can increase PSII activ-
ity and stability of oxygen release complexes (OECs)
[45]. It is also the water decomposition subunit [46].
The PsbQ was up-regulated in eCO2, this means eCO2
could promote water decomposition and maintain stabil-
ity in OECs by regulating PsbQ. The other 3 proteins
(PsbE, PsbD and PsaN) related to photosynthetic elec-
tron transport and accumulation of photosynthetic sub-
stances [44]. Those proteins (LHCs, PsbQ, PetE, PsbD,
PsaN) increased in eCO2 (Fig. 3), resulting in absorbing
more light energy and promoting more photosynthetic
electron transport. This is causing the advance of qP
and ETR, and the reduction of NPQ (Fig. 1d, e).

ATP synthase delta chain is CF1 subunit (8) belongs to
the F-type ATPase, which utilizes the energy of a trans-
membrane electrochemical gradient to generate ATP by
rotary catalysis [47]. F-type ATPase products would pro-
vide energy for photosynthesis carbon fixation [48]. ATP
synthesis in the hydrophilic α3β3δ head (CF1) is powered
by the CF0 rotary motor in the membrane [49]. Previous
studies have shown that the ATP synthase delta chain is
mainly related to the component linkage of the F-type
ATPase sector [50, 51]. In our study, ATP synthase delta
chain protein was up-regulated in eCO2 (Fig. 3), this
indicated that eCO2 can affect leaf redox pathways by
changing the F-type ATPase subunit accumulation. Our
results confirmed that ATP synthase delta chain act as a
stator to prevent unproductive rotation of CF1 with CF0,
this is consistent with previous study [49].

eCO2 promotes up-regulation of RbcS and Rca
Rubisco is L8S8 hexadecamer complex [52] and ineffi-
cient [53]. RbcS regulates Rubisco through coordinated
expression of RbcL and RbcS in plants [11]. RbcS is
linked to the folded RbcL subunits assemble [54] and as a
‘reservoir’ for CO2 storage [17]. In our results, RbcS
was up-regulated in eCO2 (Fig. 4), this indicated that
RbcS not only has high affinity with CO2, but also re-
sponds to eCO2 in the environment. It has reported that
RbcS mRNA levels and RbcS synthesis simultaneously
increased in RbcS-sense plants [11]. The RbcS transcript
was found to be inhibited in source of sugar (sucrose or
glucose) in the media of photoautotrophic Chenopodium
callus and some plants, but over-expression of RbcS was
found in low CO2 [55]. Interestingly, RbcS mRNA level
was up-regulated in C0, and down-regulated in Cs and
CK, which indicate that the medium with sugar inhibits
the expression of RbcS, this is consistent with previous
studies. The amount of RbcS synthesize was tightly cor-
related with RbcL mRNA level [11]. In our research,
large amounts of RbcS accumulated under eCO2 but
there was no significant change in RbcL mRNA level.
This result showed that RbcS accumulated not only asso-
ciated with RbcL mRNA level, but also related to CO2
concentration. It has reported that long-term growth of
Arabidopsis at high CO2 (1000 μmol·mol−1) resulted in
nonstructural carbohydrates increased and an even
greater decline in mRNA of RbcS [56]. Nevertheless, the
mechanism of eCO2 regulates RbcS accumulated would
research in future.

Sugar phosphate inhibits Rubisco activity [57], such as
RuBP, CATP and Xu5P [12]. Rca catalyzes the remodel-
ing of inactive Rubisco, releases it’s bound sugar phos-
phate and activate Rubisco [20]. Heat [23], drought [24]
and salt [25] could increase Rca. In our results, Rca was
up-regulated under eCO2. Through the previous ana-
lysis, LHCII, PsbQ, PsbE, PsbD, PsaN and ATP synthase
delta chain were up-regulated, indicating that these pro-
teins would absorb more energy and produce more ATP,
which could change ATP/ADP ratio. Rca uses the hy-
drosis of ATP to facilitate the dissociation of RuBP
bound as an inhibitor at the active site of uncarbamy-
lated and inactive Rubisco [58]. Therefore, the activity of
Rca was affected by ADP/ATP ratio [22]. We speculated
that eCO2 affect Rca activity by up-regulating the ex-
pression of light-harvesting proteins and F-type ATPase,
and all of those changes ultimately affect the activation
of Rubisco. Galmés et al. [39] reported that Rubisco con-
tent reduced was the primary driver in the regulation of
Rubisco activity to eCO2. At normal conditions, Rca
negatively affects the Rubisco content [60]. However,
Rca level is a major limiting factor of non-steady-state
photosynthesis [61]. Therefore, Rca up-regulated to
adjust the non-steady-state photosynthesis caused by
eCO2. Overproduction of Rubisco does not enhance
photorespiration as well as CO2 assimilation probably
due to partial deactivation of Rubisco [62]. Rca was
negatively correlated with mRNA levels, it is suggested that changes in the expression of these proteins are regulated at their translation levels by eCO₂.

**Conclusions**

The detailed analysis of transcriptome and proteome of grape (V. vinifera L. cv. ‘Pinot Noir’) plantlets in vitro under differential concentration of CO₂ revealed crucial molecular mechanism difference in transformation from heterotrophic to autotrophic. The results indicated that eCO₂ triggers the RbcS and Rca up-regulated, then promoting photosynthesis and then advancing transformation of grape plantlets from heterotrophic to autotrophic. The study provided deep refinements into the existing knowledge of plantlets in vitro response to eCO₂, and the molecular mechanism was revealed through identification and comparative analysis of genes and proteins from photosynthesis-antenna, photosynthesis and metabolism pathways. The expression level of RbcS was not related to protein expression and the expression of Rca was highly inverse correlated with protein expression. Consequently, these data provide clues as to the fundamental regulatory network targeted by eCO₂, and will lead to future functional analyses that may be valuable for both agronomic improvement and our understanding of the means by which new phenotypes may arise.

**Methods**

**Plant materials**

‘Pinot Noir’ (V. vinifera L.) grape plantlets, which was kept in the Fruit Tree Physiology and Biotechnology Laboratory, College of Horticulture, Gansu Agricultural University, were used as test materials in an in vitro experiment. The grape plantlets were propagated in advance and were vigorous in growth without contamination. Each nodal segment (approximately 2.0 cm long) with two bud was cultured on modified B5 solid medium + IAA (0.1 mg·L⁻¹) (50 mL of medium was taken in 150 mL Erlenmeyer flasks). Plantlets were grown in controlled climate chamber (PQX-430D) at a day/night regime of 16 h/8 h (light/dark), an irradiance of 120 μmol·m⁻²·s⁻¹, temperatures of 26 °C day and night. One climate chamber (PQX-430D-CO₂) have TC-5000 (T) intelligent CO₂ controller to regulate CO₂ concentration. The CO₂ concentration treatments were as follows: environmental atmospheric CO₂ concentrations (380 ± 40 μmol·mol⁻¹); and elevated CO₂ concentrations (1000 μmol·mol⁻¹). The grape plantlets were cultured with 2% sucrose designated as control (CK), with eCO₂ while without sucrose as C0, with both 2% sucrose and eCO₂ as Cs. Each treatment had three biological replicates with 15 plantlets per replicate. Plantlet leaves were harvested at 25 days after inoculation. The leaf samples were transferred immediately to liquid nitrogen and stored at −80°C for subsequent analysis. Different treatments were simultaneously sampled from three comparable plants used as three biological replications.

**Chlorophyll fluorescence parameters**

Chlorophyll fluorescence parameters of functional leaves were measured using the IMAG-PAM fluorometer (MAXI Imaging-PAM, Walz, Effeltrich, Germany). All daytime measurements were carried out between 10:00 and 12:00 in the morning. After the dark adaptation, minimal fluorescence (F0), steady fluorescence (Fs) and maximum fluorescence (Fm) were respectively measured under light irradiation (0.1110 and 2700 μmol·m⁻²·s⁻¹). The optimal photochemical efficiency of PSII (Fv/Fm), effective quantum yields of PSII (ΦPSII), photochemistry quenching (qP) and photosynthetic electron transport (ETR) were calculated according to previous equations [63].

**RNA isolation and library preparation for transcriptome analysis**

Total RNA samples were extracted using the mirVana miRNA Isolation Kit (Ambion). The RNA samples were evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), with RNA Integrity Number (RIN) ≥ 7 were subjected to the subsequent analysis. The libraries were constructed using TruSeq Stranded mRNA LTSample Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Then these libraries were sequenced on the Illumina sequencing platform (HiSeq™ 2500 or Illumina HiSeq X Ten) and 125 bp/150 bp paired-end reads were generated.

**Analysis of RNA-sequencing data**

Raw data (raw reads) were filtered into clean reads using NGS QC Toolkit. The reads containing ploy-N and the low quality reads were removed to obtain the clean reads. Then the clean reads were mapped to reference genome sequence (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/) using HISTA 2. Briefly, the number of mapped reads for each transcript was normalized into a reads per kb per million reads value (RPKM) to calculate level of differential expression for each transcript. In analysis, a criterion of P value < 0.05 and fold change >2 or fold change <0.5 was used to identify DEG. Functional gene classification was performed using UniProtKB/Swiss-Prot database. GO enrichment and KEGG pathway enrichment analysis of DEGs were performed using the R programming language based on the hypergeometric distribution, respectively.
qRT-PCR analysis

One micrograms total RNA was subjected to reverse transcription using SYBR Green PCR Master Mix (TaKaRa) Kit with gDNA Eraser (Perfect for Real Time). Real-time PCR was carried out by using SYBRs Premix Ex Taq II (TaKaRa) in ABI StepOne™ Plus Real-Time PCR System (Roche, Switzerland). All primers used for qRT-PCR were listed in Additional file 6: Table S5.

Protein extraction

Fresh leaves (0.5 g) from each biological replicate were ground into powder in liquid nitrogen and dissolved (vortex blending) with 500 μL extraction buffer (0.7 M sucrose, 0.1 M NaCl, 0.5 M Tris-HCl (pH 7.5), 50 mM EDTA and 0.2% DTT). The samples were grinded at the power of 60 Hz for 2 min. Then supplemented with extraction buffer for 1 mL and mixed and added with Tris-phenol buffer and mixed for 30 min at 4 °C. The mixtures were centrifuged at 7100 g for 10 min at 4 °C. Collect phenol supernatants and added for 5 volumes of 0.1 M cold ammonium acetate-methanol buffer and precipitated at −20 °C overnight. The samples were centrifuged at 12,000 g for 10 min to collect precipitations. The precipitations were dried and dissolved in lysis buffer (1% DTT, 2% SDS, 10% glycerinum, 50 mM Tris-HCl (pH 6.8) for 3 h. The samples were centrifuged at 12000 g for 10 min to collect supernatants. The supernatants were centrifuged again to remove precipitations completely. The protein concentration was quantified by BCA method [64] and the protein purity was detected by SDS-PAGE [65], 15μg proteins of each sample were separated on 12% SDS-PAGE gel.

Protein digestion and iTRAQ labeling

Protein digestion was performed according to the FASP procedure [66]. Briefly, protein sample (100 μg) was subjected with 120 μL reducing buffer (10 mM DTT, 8 M Urea, 100 mM TEAB, pH 8.0) on 10 K ultrafiltration tube and the solution was incubated at 60 °C for 1 h. IAA was added to the solution with the final concentration of 50 mM in the dark at room temperature for 40 min. The solutions were centrifuged on the filters at the solution of 50 mM in the dark at room temperature for 40 min. The solutions were collected and lyophilized. The collected solutions were transferred into new tubes, add TEAB (100 μL, 50 mM) and 40 μL of each sample was transferred into new tubes for labeling. Each sample add iTRAQ label reagent (iTRAQ® Reagents-8plex kit, Sigma) following the manufacturer’s protocol (Applied Biosystems, Foster City, CA, USA). All labeled peptides were pooled together.

RP chromatography separation

iTRAQ labeled peptides were fractionated by RP chromatography separation using the 1100 HPLC System (Agilent). RP separation was performed on the Agilent Zorbax Extend RP column (5 μm, 150 mm × 2.1 mm). Mobile phases A (2% acetonitrile in HPLC water) and B (98% acetonitrile in HPLC water) were used for RP gradient. The solvent gradient was set as follows: 0~8 min, 98% A; 8.00~8.01 min, 98%~ 95% A; 8.01~38 min, 95%~75% A; 38~50 min, 75%~60% A; 50~50.01 min, 60~10% A; 50.01~60 min, 10% A; 60~60.01 min, 10~98% A; 60.01~65 min, 98% A. Tryptic peptides were separated at an eluent flow rate of 300 μL·min⁻¹ and monitored at 210 and 280 nm. Dried samples were harvested from 8 min to 50 min and elution buffer were collected in every minute and numbered from 1 to 10 with pipeline. The separated peptides were lyophilized for MS detection.

Mass spectrometry analysis

All LC-MS/MS analyses were performed on a Q-Exactive mass spectrometer (Thermo, USA) equipped with a Nanospray Flex source (Thermo, USA). The peptides mixtures were loaded by a capillary C18 trap column (3 cm × 100 μm, C18, 3 μm, 150 Å) and separated by a C18 column (15 cm × 75 μm, C18, 3 μm, 120 Å) on an ChromXP Eksigent system (AB Sciei). The flow rate was 300 nL·min⁻¹ and linear gradient was 70 min (0~0.5 min, 95%~92% A; 0.5~48 min, 92%~74% A; 48~61 min, 74%~62% A; 61~61.1 min, 62%~15% A; 61.1~67 min, 15% A; 67~67.1, 15%~95% A; 67.1~70 min, 95% A. mobile phase A = 2% ACN/0.1% FA and B = 95% ACN/0.1% FA). Full MS scans were acquired in the mass range of 300~1600 m/z with a mass resolution of 70,000 and the AGC target value was set at 1,000,000. The 10 most intense peaks in MS were fragmented with higher-energy collisional dissociation (HCD) with collision energy of 30. MS/MS spectra were obtained with a resolution of 17,500 with an AGC target of 200,000 and a max injection time of 50 ms. The Q-E dynamic exclusion was set for 15.0 s and run under positive mode.

Protein identification and function annotation

Raw data of iTRAQ-labeled proteins by was search against V. vinifera (Grape) genome protein database in National Center for Biotechnology Information (NCBI) using the Proteome DiscovererTM 2.2 (Thermo, USA). Database searches were performed with trypsin digestion specificity, and the cysteine alkylation was considered as parameters in the database searching. For protein quantification method, iTRAQ8-plex was selected. For protein identification, a decoy database search approach
was used to determine the false discovery rate (FDR) with acceptance if their FDR < 1.0% while protein identification containing at least two peptides.

The molecular functions of the identified proteins were classified according to their gene ontology annotations and their biological functions. Only the proteins identified with at least two different peptides and P value < 0.05, and quantified with a ratio of fold change > 1.4 or fold change < 5/7 and P value < 0.05, were considered. The NCBI and Uniprot databases were chosen to the validation and annotation of the protein sequences. Gene Ontology (GO) annotation for the identified proteins was assigned according to Uniprot database (http://www.uniprot.org).

Statistical analysis
The control and treatment groups were analyzed for statistical significance of differences between multiple groups using one-way ANOVA followed by Duncan’s multiple comparisons test. All calculations were performed using SPSS software (version 21; IBM, Armonk, NY, USA). All results are presented as mean ± SD from 3 independent biological replications. Treatment means were separated by the Duncan multiple range test at P value less than 0.01. We use min-max normalization method through the R programming language (3.4.3, pheatmap) to analysis transcriptional and proteomic represent expression values of heat map.

Additional files

Additional file 1: Table S1. Effect of eCO2 on fresh weight, dry weight, leaf area and plant height. (DOC 29 kb)

Additional file 2: Figure S1. The protein sample analysis by SDS-PAGE. (TIF 508 kb)

Additional file 3: Table S2. The category with the most DEGs of Cs and C0 compare with CK. (DOC 112 kb)

Additional file 4: Table S3. The category with the most DEPs of Cs and C0 compare with CK. (DOC 100 kb)

Additional file 5: Table S4. The top KEGG pathways of Cs versus CK, C0 compare with CK. (DOC 90 kb)

Additional file 6: Table S5. Sequences of primer employed in qRT-PCR analysis. (DOC 59 kb)

Abbreviations
DEGs: Differentially expressed genes; DEPs: Differentially expressed proteins; eCO2: Elevated CO2 concentration; GO: Gene ontology; iTRAQ: Isobaric Tag for Relative Absolute Quantitation; KEGG: Kyoto encyclopedia of genes and genomes; qRT-PCR: Quantitative real-time PCR; RbcS: Ribulose bisphosphate carboxylase/oxygenase small chain; Rca: Rubisco activase; Ribul: Ribulose-1, 5-bisphosphate carboxylase/oxygenase

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Availability of data and materials
The datasets supporting the conclusions of this article are included within the article and additional files.

Author’s contributions
BHC and JM designed the experiments. XZ and YW conducted the experiments. WFL and XZ analyzed the data. ZHM, SJY and QZ performed the research. WFL and XZ wrote the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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