Ethylene signaling transcription factor promote grape growth induced by exogenous carbon

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Abstract

Background The carbon can be converted into sugar which is not only important for plant growth and development, but also for plant signal transduction, especially in plant hormone response. The objective of this work was to build available genomic and proteomic resource to investigate the molecular mechanisms of exogenous carbon regulating plant growth and development. Results Grape (Vitis vinifera L. cv. 'Pinot Noir') plantlets cultured with exogenous carbon (2% sucrose, 1000 μmol·mol-1 CO2 and with both 2% sucrose and 1000 μmol·mol-1 CO2 were designated as S1, C0 and Cs, respectively). We used S0 (without sucrose, ambient CO2) as CK to analyze the differential expression genes and proteins induced by exogenous carbon. Through the transcriptomic and proteomic analysis, with pooled data for Cs, C0 and S1 compared with CK, 70 differentially expressed genes (DEGs) and 65 differentially expressed proteins (DEPs) were identified. Based on biological functions and physiological characteristics, we identified 8 DEGs and 2 DEPs related to ethylene signaling process. Amongst the DEGs we focussed on ERF TFs, including ERF5 (LOC100244353, LOC100247763, LOC100254616 and LOC100261260), ERF105 (LOC100249507 and LOC100259725), ERF2 (LOC100254640) and CTr (CTr7). Also, there were 2 DEPs related to ethylene metabolism, such as S-adenosylmethionine synthase 5 (SAM synthase 5; XP_002280106.1) and 1-aminocyclopropane-1-carboxylic acid oxidase 2 (ACC oxidase 2; NP_001267871.1) were also identified. The transcriptome and proteome results suggested that exogenous carbon inhibits ethylene biosynthesis through ACC oxidase 2. Additionally, CTr7 and ERF5, which were up-regulated, are related to the ethylene signaling pathway. We speculate that exogenous carbon regulates plant growth through ethylene signaling pathways, but which inhibit ethylene biosynthesis. Conclusions Exogenous carbon regulates the expression of ethylene biosynthesis and signaling related genes, which may improve plant growth through the ethylene signaling pathway.

Background

Carbon is one of the vital substances of plant cytoskeleton and plays an irreplaceable role in plant growth and development process. The carbon is fixed by photosynthesis and converted into sugar [1]. The sugar plays pivotal roles in plant nutrient balance, optimum carbon to nitrogen ratio can either promote storage reserve mobilization and photosynthesis [2]. It is not only served as fuel supplying plant growth and a necessary compound for the synthesis of other substances, but also a signal which regulating plant growth and development [3, 4]. Plants use many sugar sensor proteins, such as Hexokinases (HXK), to interrelate light, and hormone signaling networks for controlling growth and development in response to the changing environment [5, 6]. In plants, sugars including sucrose, glucose, fructose, and trehalose, and they have hormone like regulatory activities [7].

Sugar metabolism plays a pivotal role in governing the outcome of various kinds of plant-pathogen interactions and defense signaling [8, 9]. Sugar is also tightly interconnected with hormonal signaling pathways [1, 10]. Gaseous phytohormone ethylene affects many aspects of plant growth. Ethylene is related to the following biological processes: regulation of leaf development, senescence, fruit ripening [11-13], stimulation of germination and plant responses to biotic and abiotic [14, 15]. Ethylene is a growth inhibitory hormone because ethylene sensitivity is negatively correlated with leaf growth [16]. Ethylene signal transduction is mainly related to ethylene receptor (ETR), constitutive triple response (*CTR*), ethylene insensitive (EIN) and Ethylene insensitive-like (EIL) [17-20]. The EIN/EIL proteins bind to upstream regions of ERF transcription factors (ERF TFs) to promote its expression in tissues. ERF TFs had been shown to be involved in various processes of plant development and response to biotic and abiotic stress [11, 21]. Ethylene signal in plants is affected by environmental changes. Previous studies have shown that copper affects ethylene binding growth of ETR1 receptor in Arabidopsis thaliana [22]. Sugars have been proved to act as a signaling molecule to interact with ethylene signal, in regulating plant growth and development. Mutants displaying nonfunctional ethylene receptors (etr1, ein4) or alteration of signal transduction proteins (ein2 and ein3), are hypersensitive to sugar-mediated photosynthesis repression, while constitutive triple response 1 (ctr1), a negative regulator of ethylene signaling, is glucose insensitive [23-25]. ERFs, have been classified into AP2/EREBP-TF family, were identified as regulators of genes which related to plant growth [21, 26, 27].

Molecular connection between ethylene and growth-regulatory pathways has been uncovered, we already know ethylene as inhibitor of leaf growth [16] and ERFs modulate transcription of a wide variety of genes which response to stress [28-30]. However, in higher plants the mechanism of exogenous carbon affects ethylene pathway remains unclear and whether exogenous carbon affects plant growth through ethylene pathway is uncertain. Although, the effects of ethylene on grape mostly focused on fruit ripening and postharvest [31, 32]. For example, postharvest ethylene treatment affects berry dehydration, polyphenol and anthocyanin conten [33].

While basic models have been suggested for regulatory mechanisms among these pathways, but sugar concentration, localization, or the nature of the sugar signal may differentially affect hormone signals and gene [34]. Therefore, this work aims to investigate the changes in ethylene related genes and proteins under the influence of exogenous carbon. Further study on how does ERFs response to exogenous carbon and regulates plant growth.

We have revealed the effect of high CO_2 concentration on photosynthesis of grape plantlets based on previous analysis [35]. However, the expression of genes, proteins and an understanding of plant growth regulated by exogenous carbon at molecular levels are still undisclosed. In this study, we used 2×2 experimental design in which sucrose and eCO_2 were the main factor, to analyze the differential expression of ethylene-related genes and proteins in grape leaves induced by exogenous carbon through comparing with no carbon treatment, further to reveal the regulation of exogenous carbon on plant growth and development.

Results

Exogenous carbon enhances plant biomass

Grape plantlets *in vitro* were cultured for 25 days and exogenous carbon is supplied by eCO_2 and sucrose. Those exogenous carbon treatments were compared with no carbon treatments. In agreement with what is known about the effect of exogenous carbon phenotype, our results showed that the leaf area, plantlet height and shoot fresh weight increased significantly in each treatment compared with CK (Fig. 1). Through the analysis of physiological indicators, exogenous carbon significantly affects plant growth. The fresh weight of the underground part, leaf area and plantlet height of grape plantlet *in vitro* were 0.07g, 0.01g and 4.63cm^2, respectively. These data were significantly lower than other treatments. The exogenous carbon significantly affected the fresh weight of aerial and underground part, but only caused changes in the dry weight of the aerial part of Cs. It had no significant effects on the dry weight of the underground part (Table 1).

Analysis of transcriptomics

To identify the molecular mechanisms responsible for increased plant growth with supply of exogenous carbon, comparison of gene transcription for plants grown with exogenous carbon and deficiency carbon was performed. A robust data set was collected after data processing, 46.50, 47.05, 46.89 and 47.08 million high-quality reads were obtained at Cs, C0, S1 and CK (Table S2). The bases content were 97.81%, 97.86%, 97.54 and 97.04%, respectively. The GC content were 46.33%, 46.00%, 46.00%, 46.33%, respectively (Table S2) .

To elucidate the mechanisms underlying the growth of plant supplied by exogenous carbon, genes expression in Cs, C0 and S1 were compared with CK. From 25,679 compiled genes, we identified 70 differentially expressed genes (DEGs) (*P*-value<0.05, FC≥2) when plants grown in exogenous carbon versus CK. Among those DEGs, 65 were up-regulated and 5 were down-regulated (Table 2). The fold change and *P*-value listed in Table S3.

Annotated DEGs were further categorised into GO terms and KEGG pathways. Through GO analysis of 70 DEGs identified in transcriptome, 58 genes (82.86%) were annotated. Of these 70 DEGs, 12 were no match with SwissProt, KOG, KEGG or GO databases. The other 58 DEGs were able to match these databases and characterized. Those 58 DEGs with known function were further partitioned into 53 up-regulated (UR) and 5 down-regulated (DR). According to functional classification, the 58 DEGs were divided into 8 sections (Fig. 2). They were classified into 9 categories: primary metabolism (15.52%), transcription (15.52%), cell morphogenesis (10.34%), bio-signaling (3.45%), secondary metabolism (18.97%), translation/protein (12.07%), transport (15.52%), transcription/polynucleotide biosynthesis (1.72%) and stress tolerance (6.90%) (Fig. 2).

Analysis of proteomics

To better dissect the molecular regulated network in grape plantlets *in vitro* response to exogenous carbon, we utilized iTRAQ labeling strategy to perform quantitative proteomics and analyze the global protein changes in exogenous carbon supplied plants. From the pooled data for Cs, C0 and S1 compare with CK, 3047 unique proteins were identified. There were 65 differentially expressed proteins (DEPs) identified from Cs, C0 and S1 compare with CK (*P*-value <0.05, FC≥1.4 or FC <5/7), including 7 UR proteins and 58 DR proteins (Table 3). The fold change and *P*-value listed in Table S4.

Among these DEPs, 17 DEPs could not match with the UniParc and RefSeq database. Therefore, the biological functions of these proteins are not clear. The other 48 DEPs matched with proteins of known function to be characterized in the UniProt database, but 7 DEPs functions might still unclear (Fig. 3). Based on biological functions, the 41 DEPs were classified into 7 categories: primary metabolism (25.64%), secondary metabolism (41.03%), energy (2.56%), bio-signaling (7.69%), translation (7.69%) and transport (7.69%) (Fig. 3).

The combined analysis of exogenous carbon affects plant ethylene signaling

Using transcriptome and proteomics analysis, we identified 8 DEGs and 2 DEPs related to ethylene signaling process (Fig. 4). These DEGs were ERF transcription factors: *ERF5* (LOC100244353, LOC100247763, LOC100254616 and LOC100261260), *ERF105* (LOC100249507 and LOC100259725), *ERF2* (LOC100254640) and *CTR* (*CTR7*) (Table 4). Differential expression of ethylene pathway related genes was confirmed by qRT-PCR and their relative expression level was consistent with FPKM values fold change observed from transcriptional analysis, only 2 genes analyzed by qRT-PCR, i.e., *METK5* and *ACO2* under S1 treatment were not consistent with our RNA-seq data (Table 4).

Although previous transcriptome analysis revealed that exogenous carbon associated with ethylene signaling, there were 2 DEPs related to ethylene metabolism: S-adenosylmethionine synthase 5 (SAM synthase; XP_002280106.1) and 1-aminocyclopropane-1-carboxylic acid oxidase 2 (ACC oxidase 2; NP_001267871.1). We identified the SAM synthase 5 up-regulated but ACC oxidase 2 down-regulated in exogenous carbon treatments (Table 4).

DEGs associated with plant growth

DEGs involved in primary metabolism: There were 9 DEGs related to primary metabolism, 7 up-regulated in Cs, C0 and S1 treatments, 2 down-regulated in exogenous carbon treatments. Those 9 DEGs could divide into 5 categories: nucleoside (LOC100242429), carbohydrate catabolic process (LOC100244286, LOC100247598 and LOC100252971), carbohydrate metabolic process (LOC100263433), amino acid metabolic (LOC100254909), lipid (LOC100266479, LOC100257217 and LOC100266419). Two of the 3 genes related to lipids were down-regulated (Fig. 5).

DEGs involved in stress tolerance: DEGs that were associated with plant stress tolerance including oxidative stress (LOC100255112), biotic stress (LOC100255226) and abiotic stress (LOC100262206 and SODCP). Under the influence of exogenous carbon, 3 genes were up-regulated and 1 gene (SODCP) down-regulated (Fig. 5).

DEGs involved in secondary metabolism: Compared with the control, 11 DEGs were differentially expressed in the secondary metabolic pathway. Eleven genes can be clustered into 3 groups: glutathione metabolic (LOC100242506 and LOC109122826), proteolysis (LOC100265220 and LOC100253211) and flavonoid biosynthetic (LOC100250788, LOC100852631, LOC100249367, LOC109121674, LOC100243852, LOC100255939 and LOC100266388). Of these DEGs, only 1 gene related to flavonoid biosynthetic was down-regulated in exogenous carbon treatment, and the others were up-regulated (Fig. 5).

DEPs associated with plant growth

Out of 11DEPs associated with primary metabolism, all those DEPs directly or indirectly catalyze sugar biosynthesis. There were 7 DEPs were related to the carbohydrate metabolic process (NP_001267891.1, NP_001267896.1, NP_001268153.1, XP_002276351.1, XP_002276777.1, XP_002282132.1 and XP_003635074.1). There were 2 DEPs were related to the tricarboxylic acid cycle (XP_002278138.1 and XP_002284064.1). There were 2 DEPs were related to sucrose metabolic process (XP_002271896.1 and XP_002270414.1). However, all the proteins associated with primary metabolism were up-regulated in CK and down-regulated in treatments (Fig. 6).

With exogenous carbon supply, proteins that participate in environmental stress are differential expression. Indeed, 16 DEPs were observed up-regulated in CK, these DEPs could be divided into three categories: defense response (NP_001267956.1, XP_002274242.1, XP_002274535.1, XP_002283780.1, XP_002284278.1, XP_002281607.1, XP_002282917.2 and XP_002283030.1), oxidative stress (NP_001268098.1, XP_002269918.1, XP_010651402.1, XP_010656112.1 and XP_002285723.1) and glutathione metabolic process (XP_002262842.1, XP_002280532.1 and XP_002278339.1) (Fig. 6). Interestingly, 1 DEP in glutathione metabolism pathway was up-regulated in exogenous carbon treatment.

All of 3 DEPs that are associated with secondary metabolism were to be down-regulated under exogenous carbon supply. These DEPs can be classified into three categories: biosynthetic process (NP_001268064.1), mucilage biosynthetic process (XP_002269677.1), phenylpropanoid metabolic process (XP_002281799.1) (Fig. 6).

Discussion

Exogenous carbon promotes plant growth through ethylene signaling

Although SAM synthetase 5 was up-regulated under exogenous carbon treatment, ACC oxidase 2 was down-regulated. The final step in ethylene biosynthesis is catalysised by ACC oxidase [36]. ACC oxidase was referred to as ethylene forming enzyme [37]. However, in sugar-free control, ACC oxidase expression was up-regulated, this change will likely producing additional ethylene, which affected the no normal growth of plants and resulted in plant slower growth (Fig 7). In the absence of exogenous glucose, plant growth is restricted to the seedling stage even after culturing on MS medium [38]. Ethylene is a growth inhibitory hormone [16]. In *Arabidopsis*, excess ethylene would cause plant dwarfism and slows down growth [39, 40]. Therefore, those plantlets which lack of exogenous sugar grows slowly may also be affected by endogenous ethylene.

In the process of ethylene signaling, copper ions likely play a role in ethylene binding and transported by RAN1[22, 41], it serves as a cofactor for ethylene binding and is required for proper biogenesis of the receptors. The results implicate that exogenous carbon, especially eCO_2 could enhance the *CTr7* expression. We speculate that eCO_2 may regulate ethylene signal by affecting the transport of copper ions.

ERF, which is involved downstream of ethylene signaling, is involved in various processes of plant development [11, 21] and different stress responses [42, 43]. However, we observed that *ERF* expression increased with exogenous carbon supply. ERF could promoters of secondary target genes, which contains GCC box, such as chitinase [44]. Therefore, we speculated that exogenous carbon can regulate the expression of other genes through *ERF*. The ERF transcription factors can be classified as activating-or repressing-transcription factors, with ERF2 and ERF4 being activators and ERF3 being a repressor of transcription [45]. ERF5 is an activator of transcription and interacts with multiple proteins, such as ERF6, ERF8, and SCL13 [46, 43]. ERFs belong to the AP2/EREBP transcription factor family [26], which can strongly bind a wide range of cis-regulatory elements, in the promoter of target genes [47, 48]. As the final response gene in ethylene signaling pathway, basic endochitinase precursor (NP_001267891.1) changes, proving its relation to ERF [43]. Overexpression of *ERF* enhances resistance to bacterial and fungal pathogens [49]. Under exogenous carbon treatment, which genes interact with ERF remains to be illustrated. In addition, some ERF enhances the activities of ACC oxidase, thereby promoting ethylene synthesis and signal transduction [50, 51]. However, some ERFs also repress of ACC oxidase activities to prevent ethylene biosynthesis [52-54]. In our study, we speculate that ERF5 exhibits an inhibitory effect on ACC oxidase.

After analysis of transcriptome and proteome data, we speculated that exogenous carbon regulates plant growth through ethylene signaling pathways that inhibit ethylene biosynthesis. The expression of *ERF5* increased under the action of exogenous carbon may further promoting plant growth. However, mechanisms on how exogenous carbon affects *ERF5* and which genes are affected by ERF require further study. ERF TFs likely play a major role in these regulatory pathways. Identification of their direct target genes will be helpful and will improve our understanding of their sometimes contradictory roles in plant growth.

Exogenous carbon affects Primary metabolism

Many DEGs and DEGs are involved in the process of primary metabolism under exogenous carbon treatment. Compared with CK, *beta-glucosidase* was up-regulated but chitinase was down-regulated. However, the mechanism of tyrosine/DOPA decarboxylase and xyloglucan galactosyl transferase MUR3 needs further study. These DEPs can be categorized into carbohydrate metabolic process, sucrose metabolic process and tricarboxylic acid cycle. SUS is a sucrose degrading enzyme in plants [2]. SUS produces more energy than INV during metabolism [55]. Probably because of this reason, the expression of SUS was up-regulated in control and could produce additional energy to supply plants without sugar. Additionally, exogenous fructose significantly reduces leaf and root SUS activity [56], so we speculate that exogenous carbon may be converted into fructose in leaves to reduce SUS activity. Supported by exogenous carbon, the leaves were used as the source organs for energy conversion through photosynthesis. However, under sugar free treatment, SUS activity was high and the leaves sank.

Glyceraldehyde-3-phosphate dehydrogenase is a glycolytic enzyme [57]. Citrate synthase is a key enzyme of the citric acid cycle that provides energy for cellular function [58]. These two proteins were significantly up-regulated under sugar free treatment. This result suggests that carbon fixed by photosynthesis is further metabolized through glycolysis and tricarboxylic acid cycling in the plant of absence sugar. The light-harvesting complex-like protein was up-regulated under exogenous carbon treatment, indicating that exogenous carbon can promote photosynthesis. Carbohydrate repression of photosynthetic gene expression can only be observed under low nitrogen conditions [4].

Exogenous carbon affects second metabolism through ethylene signaling

Plant secondary metabolism and its metabolites are related to plant function and growth [59]. Different environmental conditions regulate the production of secondary metabolites, such as water, flavonoids [60, 61] and others. Under exogenous carbon treatment, the secondary metabolism related genes and proteins expressed differently, especially flavonoid synthesis, phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS).

PAL, the important enzyme linking the secondary metabolism to primary metabolism, participates in the biosynthesis of flavonoids, lignins, stilbenes and many other compounds [62]. PAL can be induced by some environmental conditions, such as sunlight, mechanical wounding, methyl jasmonate and salicylic acid [63-66]. Sugar is also related to PAL activity. El-Awady [67] indicated that sucrose can induce PAL. However, PAL induction is repressed by glucose [68]. Ethylene is involved in the signaling pathways modulating the production of secondary production in plants cells [69]. Under exogenous carbon, ERF up-regulates the expression of *PAL* and PAL increased. This finding suggests that exogenous carbon affecting secondary metabolism in leaves is associated with ethylene signaling.

CHS is the first enzyme of the flavonoid biosynthesis pathway [70]. CHS can be induced by sugar [71]. The expression of *CHS* was not significant compared with CK, but the CHS expression was significantly up-regulated under exogenous carbon treatment. The ethylene antagonist 1-MCP can inhibit CHS [72]. Exogenous ethylene can stimulate genes which are related to anthocyanin biosynthesis increase, such as *CHS* [73]. Ethylene signaling is associated with secondary metabolism [69]. In transcriptome data, the expression of genes related to flavonoid synthesis was up-regulated. This result indicates that exogenous carbon may promote the synthesis of flavonoids in plants by ethylene signaling. The mechanisms by which exogenous carbon cause changes *CHS* and CHS remain to be further studied.

Conclusions

This study reveals that exogenous carbon may regulates plant growth through ethylene pathway. Exogenous carbon affects plant growth by inhibiting ethylene biosynthesis and ethylene signaling through ACC oxidase 2, *CTR and ERF*. However, the increased expression of *ERF5* under the action of exogenous carbon may promote plant growth. Without exogenous carbon supplied, the carbon fixed by photosynthesis will further metabolise through glycolysis and tricarboxylic acid cycling. Exogenous carbon can also promote the synthesis of flavonoids in plant.

Methods

Plant materials and growth conditions

The ‘Pinot Noir’ (*V. vinifera L.*) samples were collected from the main producing area of Gansu Province, northwest China. The plantlets material was propagated from branches of adult mother plants. The voucher specimens of grape were deposited in the Fruit Tree Physiology and Biotechnology Laboratory, College of Horticulture, Gansu Agricultural University. Those plantlets were grown at 26°C, at a 16 h light and 8 h dark cycle. The average photosynthetic photon flux was 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$. One climate chamber (PQX-430D-CO_2), which have TC-5000 (T) intelligent CO_2 controller to regulate CO_2 concentration at approximately 1000 $\mu mol \cdot mol^{-1}$. The other chamber was maintained with current atmospheric CO_2. After conventional propagation, nodal segments (average 20 mm in length) with leaves and with two axillary buds were cultured on modified B5 solid medium containing 0.1 $mg \cdot L^{-1}$ IAA, 50 mL of medium was taken in 150 mL erlenmeyer flasks, which was using gas-permeable membrane sealing. Then put those explants materials into two climate chambers for treatment. Meanwhile, explants were treated by eCO_2 (1000 $\mu mol \cdot mol^{-1}$) and sucrose after inoculation for 25 days. We use sucrose and CO_2 to provide exogenous carbon. Growth occurred under following four conditions: Cs: modified B5 solid medium containing 0.1 $mg \cdot L^{-1}$ IAA with 2% sucrose and eCO_2; C0: modified B5 solid medium containing 0.1 $mg \cdot L^{-1}$ IAA without sucrose but with eCO_2; S1: modified B5 solid medium containing 0.1 $mg \cdot L^{-1}$ IAA with 2% sucrose, ambient CO_2; S0: modified B5 solid medium containing 0.1 $mg \cdot L^{-1}$ IAA without sucrose, ambient CO_2 (380 ± 40 $\mu mol \cdot mol^{-1}$), we use S0 as CK to analyze the differential expression genes and proteins induced by exogenous carbon. Each treatment had three biological replicates with 15 plantlets per replicate.

Plantlet leaves were harvested at 25 days after inoculation. Fully expanded younger leaves (the third and fourth functional leaves) of the cultivars were sampled. Three independent biological replicates were acquired. Each replicate was collected from more than 10 randomly selected plantlets. The leaf samples were transferred immediately to liquid nitrogen and stored at -80°C for transcriptome and iTRAQ analyses.

Growth parameters

The *in vitro* growth characteristics assessed after 25 days were as follows: fresh weight of aerial parts (g), fresh weight of underground part (g), dry weight of aerial parts (g), dry weight of underground part (g), total dry mass (g), average leaf area (cm^2) and plantlet height (cm).

RNA isolation and library preparation for transcriptome analysis

Total RNA was extracted using the mirVana miRNA Isolation Kit (Ambion). Each sample was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) as as described previously [35].

Analysis of RNA-Sequencing data

Raw microarray data was acquired and analyzed as previously described [35]. Raw data (raw reads) were filtered into clean reads using NGS QC Toolkit [74]. Then the clean reads were mapped to reference genome using hisat2 [75]. A differentially expressed gene was defined as a variation in the gene expression test with a *P*-value < 0.05 and a fold change (FC) >2 or FC< 0.5. Functional gene classification was performed using the UniProtKB/Swiss-Prot database.

Protein extraction, digestion and iTRAQ labeling

Total proteins were extracted from the leaf tissue of grape *in vitro* as previously described [35]. The protein concentration was quantified by BCA method [76] and the protein purity was detected by SDS-PAGE [77]. Protein digestion was performed according to the FASP procedure [78].

RP chromatography separation and Mass spectrometry analysis

iTRAQ labeled peptides were fractionated by RP chromatography separation using the 1100 HPLC System (Agilent). The specific process as described previously [35].

Protein identification and function annotation

Protein identification was performed using the Proteome DiscovererTM 2.2 (Thermo, USA) with the *V. vinifera* genome protein database. Search parameters were chosen as reported by Zhao et al. [35]. The NCBI and UniProt databases were selected for validation and annotation of the protein sequences. GO annotation for the identified proteins was assigned according to UniProt database (http://www.uniprot.org).

qRT-PCR analysis

The 10 genes related to ethylene pathways were verified by qRT-PCR. Primer sequences used for qRT-PCR are provided in Table S1.

Statistical analysis

Data are expressed as the mean ± SD from three independent biological replicates. Significance was determined via one-way analysis of variance (ANOVA).

Abbreviations

DEGs: Differentially expressed genes; DEPs: Differentially expressed proteins; eCO_2: Elevated CO_2 concentration; GO: Gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; CTR: Constitutive triple response; ERF: Ethylene response factor; SAM synthase: S-adenosylmethionine synthase; ACC oxidase: 1-aminocyclopropane-1-carboxylic acid oxidase; SUS: Sucrose synthase; IAA: Indole acetic

acid; PAL: phenylalanine ammonia-lyase; CHS: chalcone synthase; ETR: ethylene receptor; EIN: ethylene insensitive; EIL: Ethylene insensitive-like

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publish

Not applicable

Availability of data and materials

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

BHC and JM designed the experiments. XZ and YW conducted the experiments. XZ, WFL and MFL analyzed the data. ZHM and CWZ managed the materials. XZ and WFL wrote the manuscript. MYC and MD previewed and revised the English of manuscript. All authors read and approved the manuscript.

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Not Applicable.

Additional files

Additional files 1: Table S1. Sequences of primer employed in qRT-PCR analysis.

Additional files 2: Table S2. Summary of sequencing data of ‘Pinot Noir’ plantlet *in vitro.*

Additional files 3: Table S3. The description and Fold Change of DEGs from exogenous carbon treatments compared with CK.

Additional files 4: Table S4. The description and Fold Change of DEPs from exogenous carbon treatments compared with CK.

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Tables

Table 1: Phenotypical characteristics changes of ‘Pinot Noir’ plantlet *in vitro*

	Fresh weight of aerial parts (g)	Fresh weight of underground part (g)	Dry weight of aerial parts (g)	Dry weight of underground part (g)	leaf area(cm2)	plantlet height (cm)
CK	0.56±0.02c	0.07±0.04c	0.04±0.02b	0.01±0.003a	2.98±0.04c	4.63±0.04c
S1	0.60±0.03c	0.59±0.06a	0.05±0.03b	0.03±0.016a	4.07±0.07b	5.78±0.33b
C0	0.82±0.07b	0.27±0.03b	0.07±0.02b	0.01±0.004a	6.67±0.04a	6.29±0.19b
Cs	1.02±0.04a	0.50±0.04a	0.16±0.03a	0.02±0.005a	7.97±0.06a	7.56±0.08a

Table 2: The accession and molecular function of DEGs from exogenous carbon treatments compared with CK

Gene ID	Gene name	Molecular Function	Sub-classifications	Categories
CTR7	*CTr7*	Copper ion transmembrane transporter activity	metal ion	Transport
LOC100233051	*MT*	metal ion binding	metal ion	Transport
LOC100242429	*NUDIX 18*	Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	nucleoside	Primary metabolism
LOC100242506	*Glutathione S-transferase F13*	Glutathione transferase activity	glutathione metabolic	Secondary metabolism
LOC100243221	*ABCC10*	ATPase activity, coupled to transmembrane movement of substances/ATP binding	energy(transmembrane transport)	Transport
LOC100243852	*F3H*	Oxidoreductase activity/ metal ion binding	flavonoid biosynthetic	Secondary metabolism
LOC100243915	*ns-LTP 2*	Lipid binding	lipid transport	Transport
LOC100244286	*beta-glu 13*	Vicianin beta-glucosidase activity/hydrolase activity, hydrolyzing O-glycosyl compounds	carbohydrate catabolic process	Primary metabolism
LOC100244353	*ERF5*	DNA binding/DNA-binding transcription factor activity	DNA repair	Transcription
LOC100244913		—	—	Uncharacterized
LOC100245930	*ns-LTP 8*	Lipid binding	lipid transport	Transport
LOC100247598	*beta-glu 12*	Beta-glucosidase activity	carbohydrate metabolic process	Primary metabolism
LOC100247763	*ERF5*	DNA binding/DNA-binding transcription factor activity	DNA-binding transcription factor activity	Transcription
LOC100249012		—	—	Uncharacterized
LOC100249271	*CaBP CML45*	Calcium ion binding	metal ion	Transport
LOC100249367	*CYP 714C2*	Heme binding/monooxygenase activity/iron ion binding/oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	—	Secondary metabolism
LOC100249507	*ERF105*	Transcription factor activity, sequence-specific DNA binding	DNA-binding transcription factor activity	Transcription
LOC100250788	*CYP 706C*	Flavonoid 3'-monooxygenase activity/heme binding/iron ion binding	flavonoid biosynthetic	Secondary metabolism
LOC100252971	*1,3-β-glucanase*	Hydrolase activity, hydrolyzing O-glycosyl compounds/polysaccharide binding	carbohydrate metabolic process	Primary metabolism
LOC100253211	*SCPL7*	Transferase activity, transferring acyl groups other than amino-acyl groups	proteolysis	Secondary metabolism
LOC100253381	*ATL2*	Ubiquitin protein ligase activity	protein ubiquitination	Translation/protein
LOC100253485	*CAF1*	Nucleic acid binding/poly(A)-specific ribonuclease activity	RNA biosynthesis	Transcription/polynucleotide Biosynthesis
LOC100253692	*PE*	Aspartyl esterase activity/pectinesterase	growth	Cell morphogenesis

		activity/pectinesterase inhibitor activity		
LOC100254616	*ERF5*	Transcription factor activity, sequence-specific DNA binding	DNA-binding transcription factor activity	Transcription
LOC100254640	*ERF2*	DNA binding/DNA-binding transcription factor activity	DNA-binding transcription factor activity	Transcription
LOC100254909	*TYDC1*	Aromatic-L-amino-acid decarboxylase activity/tyrosine decarboxylase activity/pyridoxal phosphate binding	amino acid metabolic	Primary metabolism
LOC100255006	*SCPL45*	Carboxypeptidase activity/transferase activity, transferring acyl groups other than amino-acyl groups	protein modification	Translation/protein
LOC100255112	*POD*	heme binding/peroxidase activity/metal ion binding	oxidative stress	Stress tolerance
LOC100255176	*SAUR40*	—	hormone	Bio-signaling
LOC100255226	*HSPRO2*	Heme binding/metal ion binding	biotic stress	Stress tolerance
LOC100255246	*OEP16*	Protein import into chloroplast stroma/protein import into mitochondrial matrix	protein	Transport
LOC100255547	*OsXTH*	Hydrolase activity, hydrolyzing O-glycosyl compounds/xyloglucan:xyloglucosyl transferase activity	growth	Cell morphogenesis
LOC100255800	*DTX 41*	Drug transmembrane transporter activity/solute:proton antiporter activity	metabism transporter	Transport
LOC100255939	*PAL*	Phenylalanine ammonia-lyase activity	flavonoid biosynthetic	Secondary metabolism
LOC100257047		—	—	Uncharacterized
LOC100257500	*GA 20-oxidase*	Gibberellin 20-oxidase activity/metal ion binding	hormone	Bio-signaling
LOC100257695	*PEAMT*	Phosphoethanolamine N-methyltransferase activity/S-adenosylmethionine-dependent methyltransferase activity	protein modification	Translation/protein
LOC100258846	*6-OMT*	O-methyltransferase activity/protein dimerization activity/S-adenosylmethionine-dependent methyltransferase activity	protein modification	Translation/protein
LOC100259725	*ERF105*	DNA binding/DNA-binding transcription factor activity	DNA-binding transcription factor activity	Transcription
LOC100260258		Metal ion binding	—	Uncharacterized
LOC100260626	*AGP31*	—	growth	Cell morphogenesis
LOC100261260	*ERF5*	DNA binding/DNA-binding transcription factor activity	DNA-binding transcription factor activity	Transcription
LOC100262206	*HSPs*	—	abiotic stress	Stress tolerance
LOC100263433	*MUR3*	Transferase activity, transferring glycosyl groups	carbohydrate metabolic process	Primary metabolism
LOC100264526	*bHLH93*	Transcription factor activity,	DNA-binding	Transcription

		sequence-specific DNA binding/DNA binding/	transcription factor activity	
LOC100265220	*SCPL16*	Transferase activity, transferring acyl groups other than amino-acyl groups	proteolysis	Secondary metabolism
LOC100265720	*bHLH51*	Transcription factor activity, sequence-specific DNA binding/DNA binding	DNA-binding transcription factor activity	Transcription
LOC100265969	*4CL5*	Catalytic activity	protein modification	Translation/protein
LOC100266479	*GDSL esterase/lipase*	Hydrolase activity, acting on ester bonds	lipid	Primary metabolism
LOC100267224		—	—	Uncharacterized
LOC100267366		—	—	Uncharacterized
LOC100267812		—	—	Uncharacterized
LOC100852581		—	—	Uncharacterized
LOC100852631	*3GT*	Flavonol 3-O-glucosyltransferase activity/daphnetin 3-O-glucosyltransferase activity/myricetin 3-O-glucosyltransferase activity	flavonoid biosynthetic	Secondary metabolism
LOC100852930		—	—	Uncharacterized
LOC100852969	*BNM2A*	Seed development	growth	Cell morphogenesis
LOC100853024	*S2*	—	differentiation	Cell morphogenesis
LOC100853060	*P4*	—	differentiation	Cell morphogenesis
LOC100854364	*SBT5.3*	Serine-type endopeptidase activity	protein modification	Translation/protein
LOC100854550	*RHG1A*	Ubiquitin protein ligase activity/zinc ion binding	protein modification	Translation/protein
LOC100854991		—	—	Uncharacterized
LOC100855013		—	—	Uncharacterized
LOC104881847		—	—	Uncharacterized
LOC109121674	*GT*	Quercetin 3-O-glucosyltransferase activity/quercetin 7-O-glucosyltransferase activity	—	Secondary metabolism
LOC109122826	*GST*	Glutathione transferase activity	—	Secondary metabolism
LOC100257217	*D14*	Hydrolase activity	lipid	Primary metabolism
LOC100260805	*CaBP CML37*	Calcium:sodium antiporter activity/calcium ion binding	metal ion	Transport
LOC100266388	*CYP 82D47*	—	—	Secondary metabolism
LOC100266419	*D14*	—	lipid	Primary metabolism
SODCP	*SODCP*	Metal ion binding/superoxide dismutase activity	abiotic stress	Stress tolerance

Table 3: The accession and molecular function of DEPs from exogenous carbon treatment compared with CK

Protein Accession	Protein name	Molecular Function	Sub-classifications	Categories
NP_001267871.1	ACC oxidase 2	Metal ion binding/oxidoreductase activity	ethylene biosynthetic process	Bio-signaling
NP_001267891.1	Chitinase	Chitinase activity/chitin binding	carbohydrate metabolic process	Primary metabolism
NP_001267896.1	GluB	Hydrolase activity, hydrolyzing O-glycosyl compounds/polysaccharide binding	carbohydrate metabolic process	Primary metabolism
NP_001267956.1	PR10.3	—	defense response	Stress tolerance
NP_001268064.1	CHS	Transferase activity, transferring acyl groups other than amino-acyl groups	biosynthetic process	Secondary metabolism
NP_001268098.1	GCat	Catalase activity/heme binding/metal ion binding	oxidative stress	Stress tolerance
NP_001268120.1	LTP	Lipid binding	lipid transport	Transport
NP_001268153.1	GluB	Hydrolase activity, hydrolyzing O-glycosyl compounds	carbohydrate metabolic process	Primary metabolism
XP_002262842.1	GST	Glutathione ransferase activity	glutathione metabolic process	Stress tolerance
XP_002263986.1	SYP121	SNAP receptor activity/SNARE binding	—	Transport
XP_002269677.1	CSLA2	Glucomannan 4-beta-mannosyltransferase activity/mannan synthase activity	—	Secondary metabolism
XP_002269908.1		Hydrolase activity	—	Uncharacterized
XP_002269918.1	POD4	Heme binding/metal ion binding/peroxidase activity	oxidative stress	Stress tolerance
XP_002270155.1		Nutrient reservoir activity	—	Uncharacterized
XP_002270970.1	nsLTP	Lipid binding	lipid transport	Transport
XP_002271896.1	SUS	Sucrose synthase activity	sucrose metabolic process	Primary metabolism
XP_002274242.1	PR10.2	—	defense response	Stress tolerance
XP_002274535.1	PR10.2	—	defense response	Stress tolerance
XP_002275501.1		Hydrolase activity	—	Uncharacterized
XP_002276351.1	beta-D-xylosidase X1	Hydrolase activity, hydrolyzing O-glycosyl compounds	carbohydrate metabolic process	Primary metabolism
XP_002276353.1	4CL7	Fatty-acyl-CoA synthase activity	—	Bio-signaling
XP_002276431.1		—	—	Uncharacterized
XP_002276777.1	BAM9	Amylopectin maltohydrolase activity/beta-amylase activity	carbohydrate metabolic process	Primary metabolism
XP_002276965.1	KPHMT	3-methyl-2-oxobutanoate hydroxymethyltransferase activity	pantothenate biosynthetic process	Energy
XP_002278007.1	9S-LOX5	Metal ion bindingoxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen	oxylipin biosynthetic proces	Translation
XP_002278138.1	ACO2	—	tricarboxylic acid cycle	Primary metabolism
XP_002278339.1	GGP2	GDP-D-glucose phosphorylase activity	glucose metabolic process	Stress tolerance
XP_002278643.1		—	—	Uncharacterized

XP_002280106.1	SAM synthetase 5	ATP binding/metal ion binding/methionine adenosyltransferase activity	S-adenosylmethionine biosynthetic process	Translation
XP_002280532.1	GST	Glutathione transferase activity	glutathione metabolic process	Stress tolerance
XP_002281607.1	PHOS32	—	—	Stress tolerance
XP_002281799.1	PAL	Phenylalanine ammonia-lyase activity	phenylpropanoid metabolic process	Secondary metabolism
XP_002282132.1	beta-galactosidase	Beta-galactosidase activity/carbohydrate binding	carbohydrate metabolic process	Primary metabolism
XP_002282836.1		Transferase activity, transferring acyl groups other than amino-acyl groups	—	Uncharacterized
XP_002282917.2	P21	—	—	Stress tolerance
XP_002283030.1	P21	—	—	Stress tolerance
XP_002283150.1		—	—	Uncharacterized
XP_002283780.1	AOS1	Heme binding/iron ion binding/monooxygenase activity/oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	defense response/	Stress tolerance
XP_002284064.1	CS	Transferase activity, transferring acyl groups, acyl groups converted into alkyl on transfer	tricarboxylic acid cycle	Primary metabolism
XP_002284278.1	POD73	Heme binding/metal ion binding/peroxidase activity	defense response/	Stress tolerance
XP_002285653.1	KAT2	Transferase activity, transferring acyl groups other than amino-acyl groups	—	Translation
XP_002285723.1	CPOD	Heme binding/metal ion binding/peroxidase activity	defense response/	Stress tolerance
XP_010651402.1	NRX	Thioredoxin-disulfide reductase activity	oxidative stress	Stress tolerance
XP_010656112.1	ALDH7	Oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	oxidative stress	Stress tolerance
XP_010658590.1	UXS	NAD+ binding/UDP-glucuronate decarboxylase activity	—	Primary metabolism
XP_010660502.1	AOC	allene-oxide cyclase activity	jasmonic acid biosynthetic process	Bio-signaling
XP_002270414.1	GAPDH	—	sucrose metabolic process	Primary metabolism
XP_003635074.1	LHCP	—	carbohydrate metabolic process	Primary metabolism
XP_002272549.1		—	—	Uncharacterized
XP_002277520.2		—	—	Uncharacterized
XP_002278812.3		—	—	Uncharacterized
XP_002280729.1		—	—	Uncharacterized
XP_002282477.2		—	—	Uncharacterized
XP_002284136.1		—	—	Uncharacterized
XP_002284571.1		—	—	Uncharacterized
XP_002284864.1		—	—	Uncharacterized
XP_003633883.1		—	—	Uncharacterized
XP_010644121.1		—	—	Uncharacterized

XP_010648868.2	—	—	Uncharacterized
XP_010654144.1	—	—	Uncharacterized
XP_010654522.1	—	—	Uncharacterized
XP_010658505.1	—	—	Uncharacterized
XP_019075863.1	—	—	Uncharacterized
XP_019077167.1	—	—	Uncharacterized
XP_019082045.1	—	—	Uncharacterized

Table 4: Ethylene metabolism-related genes and proteins that differentially expressed at exogenous carbon versus CK.

Gene	Protein	Transcripts Identified	Proteomics Identified	FPKM Fold Change			Relative expression level		
				Cs/CK	C0/CK	S1/CK	Cs/CK	C0/CK	S1/CK
METK5	SAM synthase 5	√	√	1.30/1.46*	1.19/1.525*	0.85/1.25*	2.63	2.75	2.38
ACO2	ACC oxidase 2	√	√	0.86/0.64*	1.51/0.67*	0.93/0.64*	0.95	4.60	3.73
CTR7	CTR7	√		5.25	2.52	0.23	3.53	3.49	0.71
ERF5	ERF5	√		4.85	4.91	3.76	4.44	4.24	3.17
ERF5	ERF5	√		3.91	3.59	2.91	2.10	4.02	1.71
ERF105	ERF105	√		4.28	3.28	3.10	3.59	2.40	2.93
ERF5	ERF5	√		4.12	3.01	3.66	1.50	5.46	3.58
ERF2	ERF2	√		2.93	2.43	2.22	3.82	5.84	2.36
ERF105	ERF105	√		2.46	2.40	2.21	1.54	2.54	1.26
ERF5	ERF5	√		3.44	2.35	2.51	3.81	5.25	1.57

* The relative protein Fold Change.

Figures

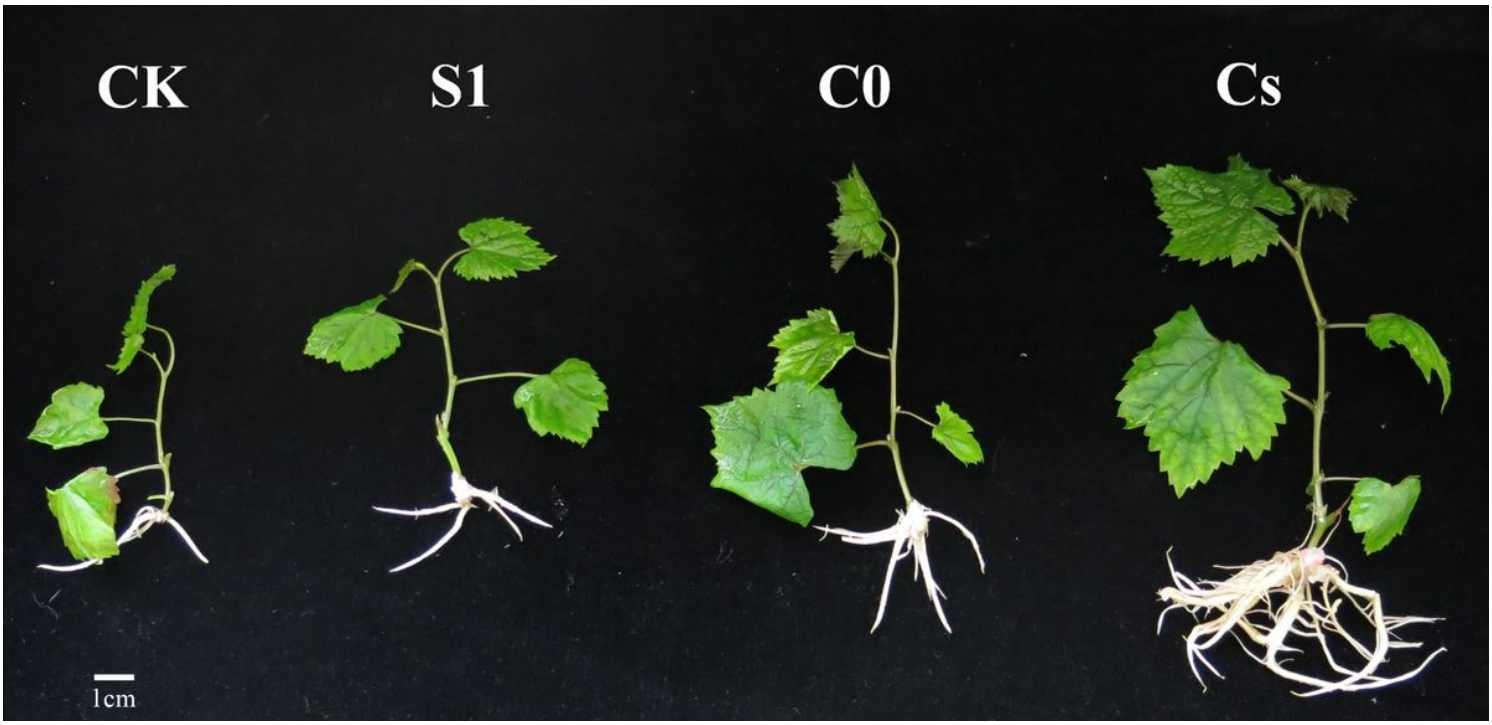

Figure 1

Effects of exogenous carbon on phenotypes of grape plantlets in vitro. The leaf area, plantlet height and shoot increased significantly in exogenous carbon treatment compared with CK.

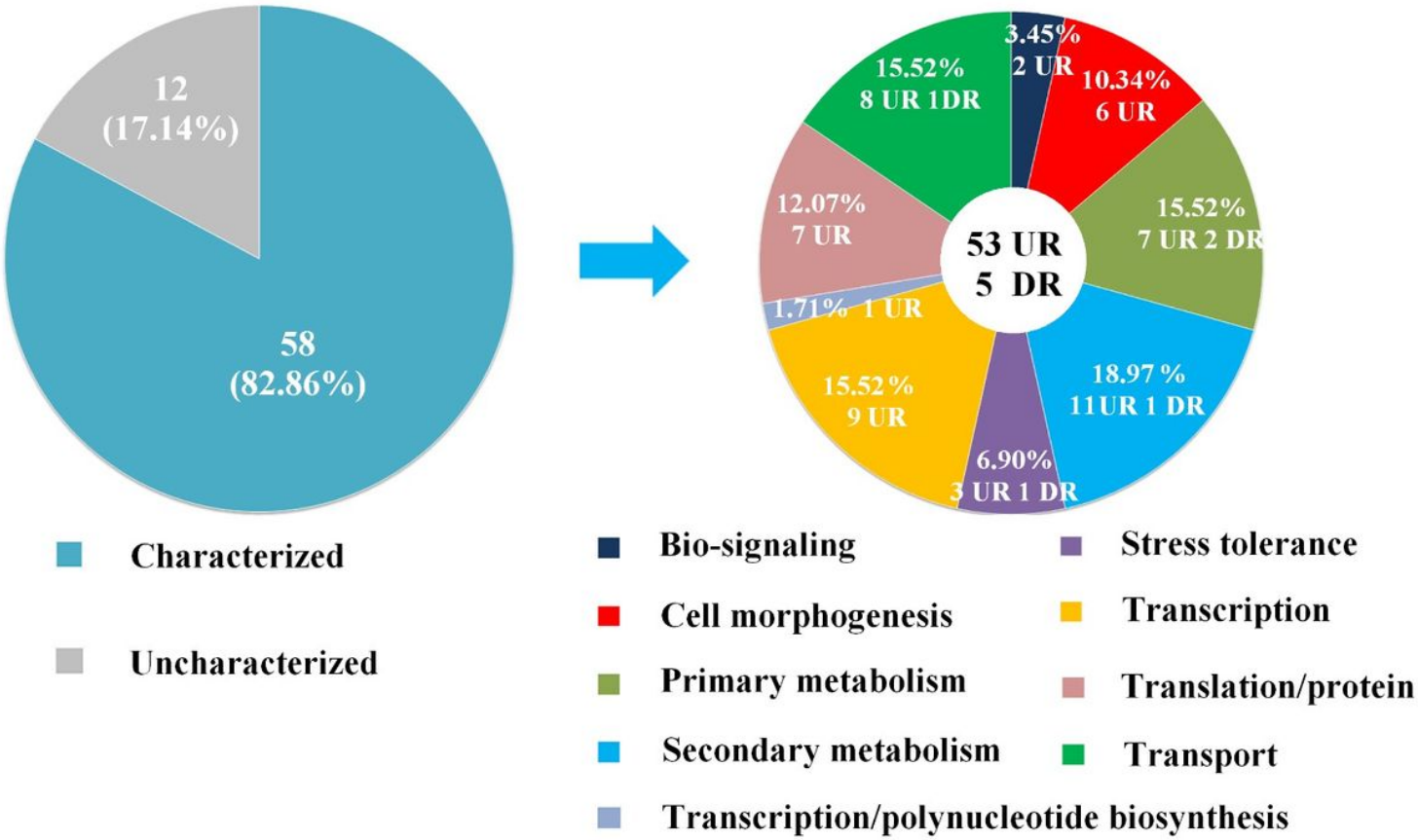

Figure 2

Distribution and classification of differentially expressed genes (DEGs) at Cs, C0 and S1 compared with CK. UR and DR representing up-regulation and down-regulation, respectively.

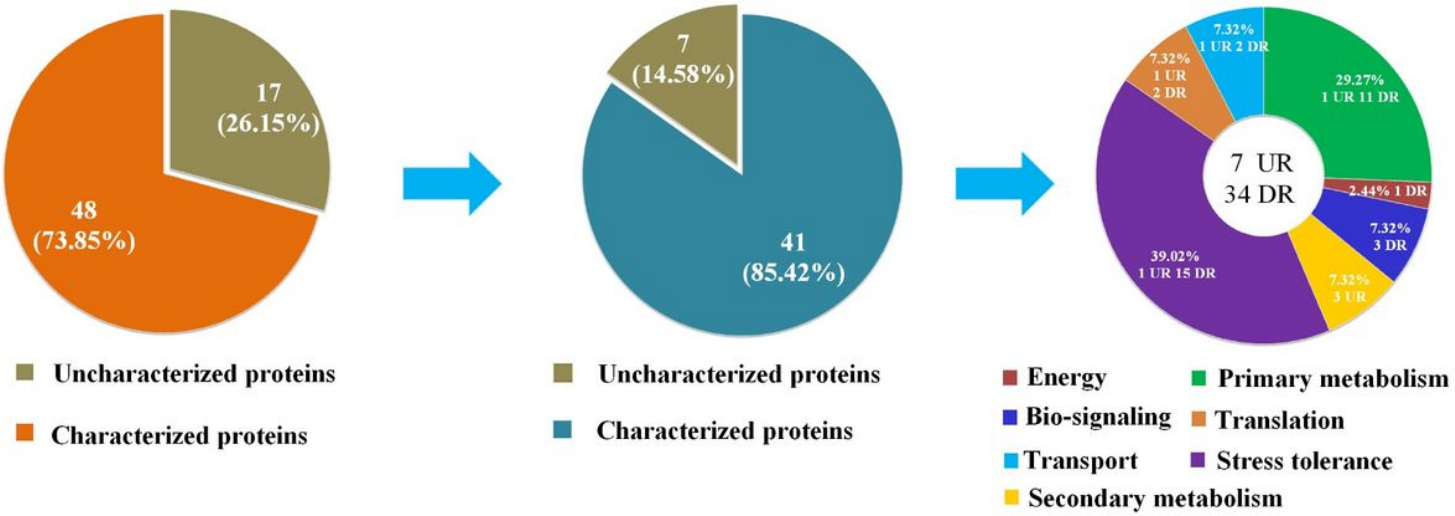

Figure 3

Distribution and classification of differentially expressed protein (DEPs) at Cs, C0 and S1 compared with CK.

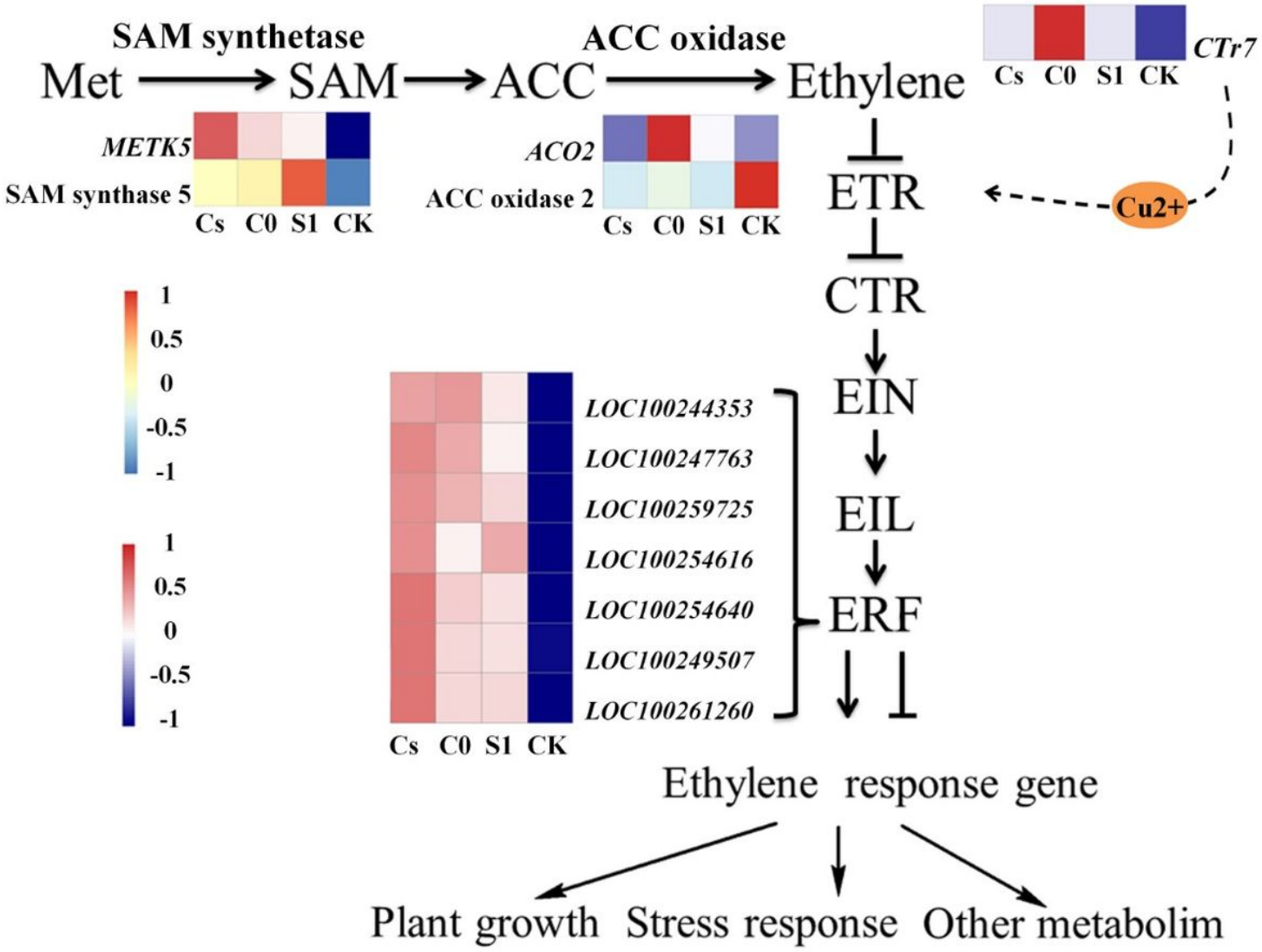

Figure 4

Ethylene metabolism pathway with up-regulated genes and proteins at exogenous carbon versus CK shown in red. The changing DEGs include 7 of ERFs and 1 CTR. The changing DEPs include S-adenosylmethionine synthase 5 (SAM synthase) and 1-aminocyclopropane-1-carboxylic acid oxidase 2 (ACC oxidase 2).

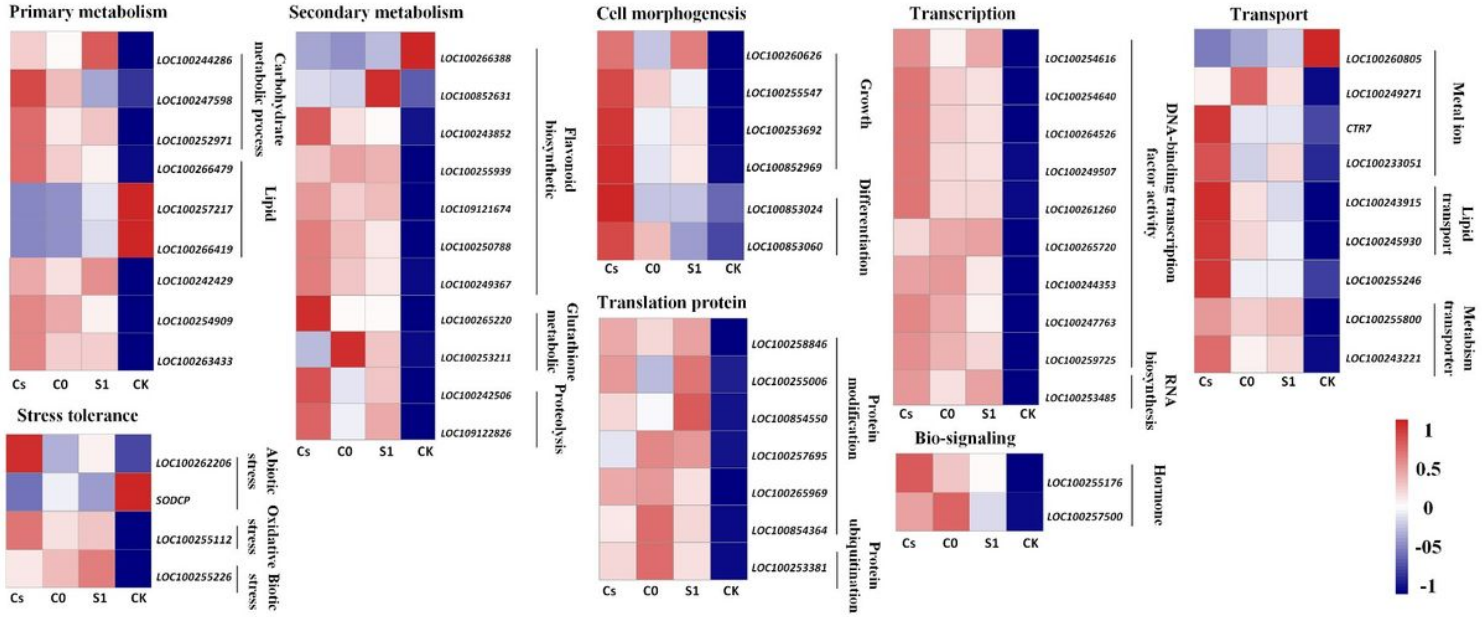

Figure 5

Gene expression heat map shows differential regulation at Cs, C0 and S1 compared with CK based on fragments per kb per million reads (FPKM). Differentially expressed genes have been categorized into primary metabolism; secondary metabolism; cell morphogenesis; bio-signaling; transcription; translation; transport and stress tolerance.

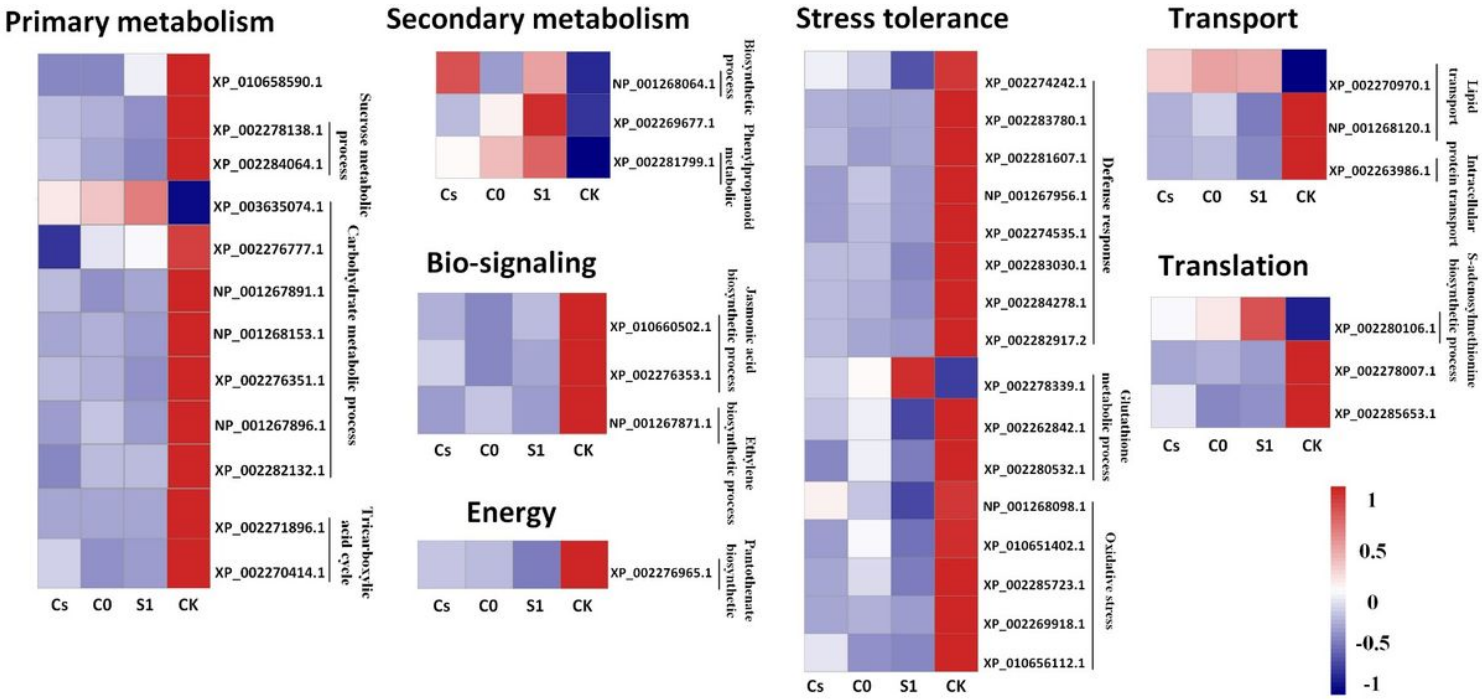

Figure 6

Heat map illustrating the relative protein expression at Cs, C0 and S1 compared with CK based on the DEPs fold change (FC). Differentially expressed proteins have been categorized into primary metabolism; secondary metabolism; energy; bio-signaling; translation; transport as well as stress tolerance.

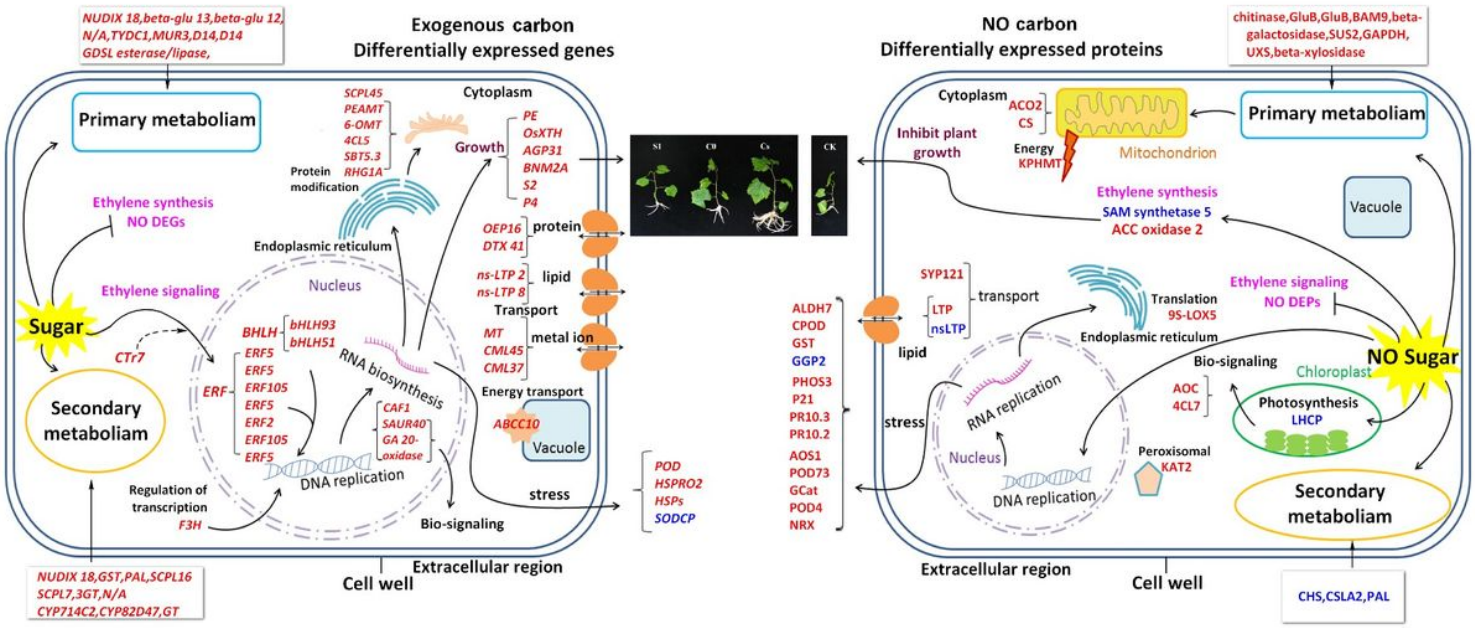

Figure 7

Effects of exogenous carbon on genes and proteins expression in grape plantlets in vitro. The DEGs or DEPs of red was up-regulated and blue was down-regulated.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS4DescriptionandFoldChangeofDEPs.docx
- TableS2Summaryofsequencingdata.docx
- TableS3DescriptionandFoldChangeofDEGs.docx
- TableS1SequencesofprimeremployedinqRTPCRanalysis.doc