Proteomic and metabolic profile analysis of low-temperature storage responses in Ipomoea batata Lam. tuberous roots

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Abstract

Background: Sweetpotato (Ipomoea batatas L.) is one of the seven major food crops grown worldwide, with 70-75% of production in China. Cold stress often can cause protein expression pattern and substance contents variations for tuberous roots of sweetpotato during low-temperature storage. Recently, we developed proteometabolic profiles of the fresh sweetpotatoes (cv. Xinxiang) in an attempt to discern the cold stress-responsive mechanism of tuberous root crops during post-harvest storage.

Results: For roots stored under 4℃ condition, the CI index, REC and MDA content in roots were significantly higher than them at control temperature (13℃). The activities of SOD, CAT, APX, O2.- producing rate, proline and especially soluble sugar contents were also significantly increased. Most of the differentially expressed proteins (DEPs) were implicated in pathways related to metabolic pathway (~22%), especially phenylpropanoids (~10%) and followed by starch and sucrose metabolism (~3%). Proteins including L-ascorbate peroxidase 3 and catalase were down-regulated during low temperature (4℃) storage. α-amylase, sucrose synthase and fructokinase were significantly up-regulated in starch and sucrose metabolism, while β-glucosidase, glucose-1-phosphate adenylyl-transferase and starch synthase were opposite. Furthermore, metabolome profiling revealed that glucosinolate biosynthesis, tropane, piperidine and pyridine alkaloid biosynthesis as well as protein digestion and absorption played a leading role in metabolic pathways of sweetpotato roots. More importantly, leucine, tryptophan, tyrosine, isoleucine and valine were all significantly up-regulated in glucosinolate biosynthesis. Data are available via ProteomeXchange with identifier PXD017728.

Conclusions: Our proteomic and metabolic profile analysis of sweetpotato roots stored at low temperature reveal that the antioxidant enzymes activities, proline and especially soluble sugar content were significantly increased. Most of the DEPs were implicated in phenylpropanoids and followed by starch and sucrose metabolism. The discrepancy between proteomic (L-ascorbate peroxidase 3 and catalase) and biochemical (APX/CAT enzyme activity) data may be explained by higher H2O2 levels and increased glutathione and ascorbate redox states, which enhanced the CAT/APX enzyme activity indirectly. Glucosinolate biosynthesis played a leading role in metabolic pathways of sweetpotao roots. More importantly, leucine, tryptophan, tyrosine, isoleucine and valine were all significantly up-regulated in glucosinolate biosynthesis.

Background

Sweetpotato (*Ipomoea batatas* L.), a dicotyledonous plant which belongs to the *Convolvulaceae* family, ranks as the seventh-most important food crop in the world. As a major nutrition and harvesting organs, storage root (SR) of sweetpotatoes possessed a mass of starch and photoassimilate. Starch accounts for 50-80% proportion of the dry matter in the SR[1,2]. Since soluble sugar content is very low in freshly harvested sweetpotatoes during general production process, a certain time of post-harvest storage at 13-15°C is imperative to facilitate starch-sugar interconversion and boost the sweetness to increase the tuberous food quality before sale. It is noticeable, however, that exposure to low temperature (5°C) for 20

d has been observed to increase sweetness of ‘Kokei 14’ roots, this treatment also caused freezing injury such as rottenness and high rate of carbohydrate loss[3]. Therefore, a better understanding of the biochemical and molecular response mechanisms to chilling stress is essential for extending tuberous crops storage time under low temperature condition.

Compared with model plants, it is more difficult for sweetpotatoes to find out genes implicated in various stress tolerance because of its complicated genetic background. Although some genomic[4, 5] and proteomic[6, 7, 8] resources of sweetpotatoes have been available now, these pieces of information are still limited to explicate the molecular mechanism of chilling resistant. With the development of sequencing technique, metabolomics has been considered as a powerful complementary tool to acquire the biological information associated with the metabolites. Metabolites are not only the end-products of expressions of some genes, but also the consequence of interaction between the genome and its milieu. Therefore, it is probable to envisage the functional genomics assembly by connecting gene expression to the metabolomic knowledge[9].

It is now generally accepted that the normal storage temperature of sweetpotatoes is 13-15°C. As a chilling-sensitive tropical crop, sweetpotatoes can be irreparably damaged when the temperature drops below 10°C. A main reason for this is oxidative injuries caused by an increased accumulation of reactive oxygen species (ROS)[10-16]. In plants, stress-induced ROS scavenging is usually implemented by both enzymatic and non-enzymatic low molecular metabolic antioxidants[17, 18]. As we all know, sweetpotato is a tuberous crop which is rich in starch. The starch content in fresh roots of sweetpotatoes is about 15-30%[8]. Soluble sugar not only serve as substrates for starch production, but also may also function as a signal involved in chilling defense for tuberous roots during low-temperature storage.

To better explore the proteins and metabolic pathways under chilling condition, we carried out the proteometabolomic profile of fresh sweetpotatoes to clarify the cold stress-responsive mechanism. Integration of proteomic and metabolomic profiles information resources would give new insights into the molecular functions of tuberous root crops during post-harvest storage. This would provide a basis for future comparative proteomic efforts for this important crop including gene discovery and improvement of chilling stress tolerance.

Results

Morphological variations under cold storage

To investigate the effect of chilling stress on the storage of sweetpotatoes, freshy harvested ones (cv. Xinxiang) were stored in the storage chamber of 13°C (CK) and 4°C for 14 days (d). As shown in Fig. 1 and Table 1, roots at 13°C after storage for 14 d showed no chilling injury (CI) symptoms, while the epidermis of roots exposed to 4°C (Fig. 1B) were significantly spotted and shriveled than those stored at 13°C (Fig. 1A). The CI index was also significantly higher than that of control roots. In addition, the water

content exhibited significantly decrease under 13°C after storage of 14 d, and no differences were found under low temperature (4°C).

Table 1 CI index and water content of sweetpotatoes after storage at different temperatures

Storage time (d)	CI index		Water content (%FW)	
	13°C	4°C	13°C	4°C
0	0.0±0.0a	0.0±0.0b	64.5±2.5a	64.5±3.1a
14	0.0±0.0a	0.7±0.1a	60.7±1.6b	64±2.7a

Effects of low-temperature storage on oxidative stress

The relative electrical conductivity (REC) level and malondialdehyde (MDA) content were significantly higher in the roots exposed to cold storage condition (4℃) than that at 13℃ (Fig. 2). The activities of SOD, CAT, APX, $O_2^{\cdot-}$ producing rate, proline and soluble sugar contents have been shown in Fig. 3. Similarly, the low temperature (4℃) significantly increased the activities of antioxidant enzymes (Fig. 3A, B, C) and the production rate of $O_2^{\cdot-}$ (Fig. 3D) as compared to the control roots (13℃). Not only that, chilling stress also enhanced the proline (Fig. 3E), glucose, fructose and sucrose (Fig. 3F) contents. It's worth mentioning that three types of soluble sugar contents were increased most among above of physiological indexes, by 112.4%, 145.6% and 139.4%, respectively, as compared to the specific control under freezing temperature storage condition.

Segregation and identification of proteins

Compared to the control roots, 266 and 158 proteins were found significantly up- and down-regulated by >1.5 fold, respectively in sweet potato roots under 4℃ storage (Supplementary Table S2, Additional file 1 and Additional file 2). 30 μg protein were loaded into SDS-PAGE and separated by 1-DE. The protein bands showed that clear, uniform and not degraded in each lane (Supplementary Figure S1). The molecular masses of identified proteins were distributed between 5 and 275 kDa, with majority of proteins (96%) distributed in the range of <100 kDa (Supplementary Figure S2). These results showed that extracted proteins were suitable for LC-MS/MS analysis when lacerated from the gel and subjected to trypsin lysis.

Annotation of differentially expressed proteins in GO classification, subcellular localization and pathway enrichment

Annotation of differentially expressed protein (DEP) function and their cellular location is necessary to understand their roles at molecular level and therefore, the identified tuberous roots proteins under 4°C and 13°C were subjected to Blast2Go annotation to understand their molecular roles (Additional file 3).

The analysis results demonstrated that they were grouped into 15 distinct categories. These proteins were mainly implicated in metabolic processes, cellular components, catalytic activities and binding (Fig. 4A, B, C). Most of them were associated with catalytic activities (~47%), followed by binding (~43%), metabolic process (~40%), cell (~34%) and organelle (~23%). Proteins whose function could not be ascertained in chilling storage condition, were designated as unknown or uncharacterized proteins (~4%) as compared to room temperature.

In addition, wolfpsort software was used to predict subcellular localization of the proteins. The proteins were delegated based on their presence in a particular compartment (Additional file 4). Most of them were localized in the chloroplast/cytoplasm (~30%), followed by nucleus (~15%) and plasma membrane (~5%) (Fig. 4D).

The identified proteins were further analyzed via KEGG database for interpretation of their involvement in different metabolic pathways (Additional file 5). Most of the proteins were implicated in pathways related to metabolic pathway (~22%), followed by biosynthesis of secondary metabolites (~16%), and phenylpropanoid biosynthesis. Starch and sucrose metabolism ranked the sixth (Fig. 4E).

Differentially expressed proteins involved in phenylpropanoid biosynthesis

As previously mentioned, most of proteins were involved in metabolic pathway and biosynthesis of secondary metabolites. Phenolic compounds regulated by differentially expressed proteins (DEPs) such as phenylalanine ammonia lyase (PAL), cinnamyl alcohol dehydrogenase (CAD), Hydroxycinnamoyl transferase (HCT) were listed in Table 2. The *p* value of these proteins was negatively corelated with their significances in phenylpropanoid biosynthesis pathway. Hence, the significance order of the DEPs was shikimate and peroxidase4>4-coumarate-CoA ligase>Cytochrome P450 (cytochrome P450 monooxygenases)>PAL>CAD.

Table 2. Part of DEPs participated in phenylpropanoid biosynthesis

Differentially expressed proteins	*p* value
Phenylalanine ammonia lyase	5.6×10^{-9}
Cinnamyl alcohol dehydrogenase	4.3×10^{-8}
Peroxidase 4	1×10^{-32}
Cytochrome P450	3.7×10^{-13}
4-coumarate-CoA ligase	1.1×10^{-16}
shikimate O-hydroxycinnamoyl transferase	1×10^{-32}

Differential multiple of the DEP participated in starch and sucrose metabolism

As compared to the roots stored at 13℃, there were 11 differentially expressed proteins (DEPs) participated in starch and sucrose metabolism of roots under 4℃ (Fig. 5). The filtered *p* value matrix ($p<0.05$) transformed by the function x=-lg (*p* value) was conduct to evaluate the celesius4/celesius13 ratio, which was positively corelated with the differential multiple of DEP. Three proteins (x>1.5) were up regulated, while others (x<1.5) presented an opposite trend in this metabolic pathway. The ratio of sucrose synthase (P11) and β-glucosidase (P3) was 7.19 and 0.56, significantly higher and lower than other proteins, respectively. These results appeared to indicate that these two proteins play a more important role in starch and sucrose metabolism (Fig. 5).

Functional network of the DEP in starch and sucrose metabolism

Two-tailed Fisher's exact test was conducted to reveal proteins association network. The network for tuberous roots under chilling stress are illustrated in Fig. 6 as compared to the controls in room temperature. There are three up- and three down-regulated differentially expressed proteins (DEP). EC: 3.2.1.1 (red) protein, α-amylase which associated with starch metabolism and carbohydrate digestion or absorption, was significantly up-regulated when maltodextrin or starch was hydrolyzed to maltose. Furthermore, it was homologous with K01177 (β-amylase: EC: 3.2.1.2), K05992 (maltogenic α-amylase: EC:3.2.1.133) in terms of the orthology analysis. Similarly, both of EC: 2.4.1.13 (sucrose synthase) and EC: 2.7.1.4 (fructokinase) protein played a significantly up-regulated role in amino and nucleotide sugar metabolism in the chilling tolerance of sweetpotato roots. On the other hand, EC: 3.2.1.21, EC: 2.7.7.27 and EC: 2.4.1.21 proteins (green) were significantly down-regulated in starch and sucrose metabolism of roots stored at 4℃, which was named as β-glucosidase, glucose-1-phosphate adenylyl-transferase and starch synthase, respectively. They were mainly involved in phenylpropanoid biosynthesis, biosynthesis of starch and secondary metabolites as well as polysaccharide accumulation. These findings thus showed that the degradation of starch into soluble sugar can not only boost the sweetness, but also significantly improve the resistance of sweetpotato roots to chilling stress.

Metabolome profiling and its fold change analysis

The metabolome profiling of sweetpotato tubers led to the identification of 76 differentially expressed metabolites (DEMs) in the roots stored at 4℃ as compared to them at 13℃. There were 31 up- and 45 down-regulated metabolites (Supplementary Table S3 and Additional file 6). PLS-DA classification method was applied to screen out the difference variables among these different metabolic components. The absolute value level of fold change (FC) was closely related to significance of the metabolic component. The results (Fig. 7) showed that in the up-regulated metabolites, the absolute Log_2FC value of 4 components were more than 10, including glutaric acid (16.69), 3-hydroxy-3-methylpentane-1,5-dioic acid (14.97), apigenin O-malonylhexoside (14.1) and apigenin 7-O-glucoside (cosmosiin) (13.56). Nevertheless, 9 components were more than 10 in down-regulated DEM, namely sinapoylcholine (14.38), followed by D-glucoronic acid (14.08), N-acetyl-5-hydroxytryptamine (14.5), 5-Methylcytosine (13.32) etc. We can speculate that the metabolic activities of a large proportion of identified components may drop off for tuberous roots under low temperature storage (Fig. 7).

Screening and distribution of DEMs in tuberous roots under chilling stress

Metabolites among different varieties or organizations can be preliminarily screened based on the OPLS-DA (Partial Least Squares-Discriminant Analysis) statistic results. However, it is more feasible to screen out metabolites accurately with the combined application of fold change and VIP (Variable Importance in Project) value obtained from OPLS-DA model. Compared to the absolute value level of fold change, VIP value (>1) was extremely associated with the significance of metabolic compound in the corresponding class. All the identified DEMs were categorized into 20 classes. Most of them (~33%) were the members of nucleotide and its derivates and amino acid derivatives group. The screened metabolic compounds on the basis of VIP and Log_2FC value were demonstrated in Table 3. The results illustrated that most of components were down-regulated except glutaric acid and 3-hydroxy-3-methylpentane-1,5-dioic acid. The VIP and Log_2FC value of glutaric acid, which belonged to organic acids, were the highest (4.01 and 16.69, respectively), then followed by D-glucoronic acid (3.69 and 14.08), N-acetyl-5-hydroxytryptamine (3.66 and 14.05) and 5-Methylcytosine (3.58 and 13.32) (Table 3 and Fig. 8A). Carbohydrates were represented by D-glucoronic acid, which was an important member of sugar metabolism. Organic acids and nucleotide/amino acid derivates played a vital role in sweetpotato roots response to chilling stress.

Furthermore, KEGG pathway enrichment was conducted to in terms of their P-value and rich factor. P-value and rich factor had negative and positive correlation with enrichment significance of metabolic compounds, respectively. As Table 4 and Fig. 8B shown, the P-value of glucosinolate biosynthesis, tropane, piperidine and pyridine alkaloid biosynthesis (9.94×10^{-3}) was obviously lower than protein digestion and absorption (3.56×10^{-2}). Therefore, these three metabolic pathways may play a key role for sweetpotato roots in the response improvement to chilling stress.

Network of the differential metabolic compounds in glucosinolate biosynthesis

As previously mentioned, glucosinolate biosynthesis, comprised of amino acid such as Leucine, Tryptophan, Tyrosine, Isoleucine and Valine, was significant in metabolic pathways for increasing the chilling tolerance of sweetpotato roots. The glucosinolate can be synthesized from methionine, branched-chain amino acids or aromatic amino acids process (Fig. 9). Leucine, Isoleucine and Valine were involved in branched-chain amino acids. Tryptophan and Tyrosine were imperative for aromatic amino acids pathway. All these amino acids were significantly up-regulated in glucosinolate biosynthesis (Fig. 9).

Table 3. Screening of differential expressed metabolic components

Compounds	Class	VIP	Log_2FC	Type
Glutaric acid	Organic acids	4.01	16.69	up
D-glucuronic acid	Carbohydrates	3.69	14.08	down
N-acetyl-5-hydroxytryptamine	Tryptamine derivatives	3.66	14.05	down
5-Methylcytosine	Nucleotide and its derivates	3.58	13.32	down
Esculin	Coumarins	3.26	11.67	down
3-Hydroxy-3-methylpentane-1,5-dioic acid	Amino acid derivatives	2.66	14.97	up
O-sinapoyl quinic acid	Quinate and its derivatives	2.61	2.75	down
Acetyl tryptophan	Amino acid derivatives	2.45	13.08	down
Sinapic acid	Hydroxycinnamoyl derivatives	2.39	1.72	down
L-Epicatechin	Catechin derivatives	2.35	11.84	down
Protocatechuic aldehyde	Catechin derivatives	2.26	10.67	down
Pantetheine	Vitamins	2.21	5.40	down
D-arabitol	Alcohols and polyols	2.15	9.97	down

Table 4. KEGG pathway enrichment of significantly DEMs

KEGG pathway enrichment	P-value	Compounds
glucosinolate biosynthesis	9.94×10^{-3}	Leu; Try; Tyr; Ile; Val
tropane, piperidine and pyridine alkaloidbiosynthesis	9.94×10^{-3}	Putrescine; piperidine; pipecolic acid; Ile; Lys
protein digestion and absorption	3.56×10^{-2}	Putrescine; piperidine; Indole; Val; Ile; Tyr; Try; Arg; Lys; Leu

Abbreviation: Leu (Leucine), Try (Tryptophan), Tyr (Tyrosine), Ile (Isoleucine), Val (Valine), Arg (Arginine) and Lys (Lysine)

Discussion

ROS scavenging and osmotic adjustment substances

Higher plants growth, productivity and distribution are severely limited by environmental stresses including freezing, drought and salinity. MDA content and ion leakage are indicators of membrane damage caused by chilling stress. Gill et al. (2015) described that excess ROS resulted in rise of MDA, membrane leakage and DNA breakdown which cause severe damage to plant cell[19]. Plants have evolved

in the presence of ROS and have acquired dedicated pathways to protect themselves against oxidative damage and fine modulation of low levels of ROS for signal transduction[20-25]. The enzymatic systems of ROS scavenging mechanisms mainly include SOD, POD, CAT, APX and GPX[26]. Furthermore, the expressions of intracellular genes *CuZnSOD* and *swAPX1* were significantly correlated with low temperature stress (4℃)[27]. Among the enzymatic systems, SOD is able to rapidly convert ·OH to H_2O_2, and the generated H_2O_2 is then converted to water and dioxygen by CAT and APX[28-30]. APX requires an ascorbic acid (AsA) and/or a glutathione (GSH) regenerating cycle involved monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR)[31]. APX genes are widely found in rice, including two cytosolic APXs (*OsAPX1* and *OsAPX*2), two peroxisomal APXs (*OsAPX3* and *OsAPX4*)[32]. However, rice mutants double silenced for cytosolic APXs (APX1/2s) exhibit significant changes in the redox status, triggering alterations in the ROS signaling networks and making the mutants able to cope with abiotic stress[33]. In our research, proteins including L-ascorbate peroxidase 3 and catalase were down-regulated during low-temperature (4℃) storage (Additional file 2), nevertheless the CAT and APX enzyme activities (Fig. 3B, C) were increased. This discrepancy between proteomic and biochemical data may be explained by higher H_2O_2 levels and increased glutathione and ascorbate redox states, which enhanced the CAT/APX enzyme activity indirectly.

Induction of osmoprotectants biosynthesis is another type of the plant response to low temperature. Several studies have attributed an antioxidant feature to proline, suggesting ROS scavenging activity and proline acting as a singlet oxygen quencher[34]. Increasing amounts of data suggested that increased abiotic stress tolerance was obtained by introducing simple metabolic traits from other organisms such as the production of trehalose and proline[35] to plants. Furthermore, proline can protect and stabilize ROS scavenging enzymes and activate alternative detoxification pathways. In salt-stressed tobacco cells, proline increased the activities of methylglyoxal detoxification enzymes, enhanced POD, SOD and CAT activities[36]. Recent data suggested that overexpression of *DlICE1* (*Dimocarpus longan* L.) in transgenic *Arabidopsis* conferred enhanced cold tolerance via increased proline content and antioxidant enzyme such as SOD, CAT, APX[37]. In our research, the low temperature (4℃) significantly increased the activities of antioxidant enzymes (Fig. 3A, B, C), the producing rate of $O_2^{\cdot-}$ (Fig. 3D) and proline content (Fig. 3E) as compared to the control (13℃). Thus, less damage from membrane lipid peroxidation enabled the sweetpotato roots to continue normal metabolism under low-temperature condition, contributing to their higher cold tolerance.

The role of endogenous phenolics compounds and glucosinolate biosynthesis under abiotic stress

Phenylpropanoids are a group of secondary metabolites synthesized from the amino acid phenylalanine[38-40]. In plants, the phenylpropanoid pathway underlying abiotic stress tolerance is tightly connected with physiological and molecular mechanisms. Phenolic accumulation is usually activated when plants face multiple abiotic stresses[41]. Increased phenolic levels play crucial role in plants protection against chilling stress[42]. Gao et al (2016) confirmed that stimulated phenolic biosynthesis

was owing to the enhanced expression of PAL, CAD and HCT by carrying out the experiments with peach under low-temperature stress[43]. In our research, the phenylpropanoid biosynthesis, a main metabolic pathway, were regulated by lots of proteins, especially HCT, PAL and CAD (Fig. 4E and Table 2). Thus, our results were coincidence with former research. More importantly, the thickness of plant cell walls is enhanced due to phenolic accumulation, which is beneficial for the prevention of chilling injury[44, 45].

Glucosinolates, also known as mustard oil glucosides, are nitrogen- and sulfur-containing compounds found in the Arabidopsis and oilseed rape (*Brassica napus* L.). They mainly function as defense molecules[46]. Over 100 glucosinolates are found in plants [47]. Depending on their precursor amino acids, glucosinolates can be categorized into indole glucosinolates derived from Trp, aliphatic glucosinolates from Ala, Leu, Ile, Val, or Met, and aromatic glucosinolates from Phe and Tyr[47]. These results were consistent with our research (Table 4 and Fig. 9). Studies with two Arabidopsis mutants, reduced epidermal fluorescence2 (ref2) and ref5, demonstrated that the accumulation of glucosinolate biosynthetic intermediates can limit the production of phenylpropanoids[48, 49]. However, it seems like that there was no obviously crosstalk between phenylpropanoids and glucosinolates biosynthesis, which may be needed to confirm with further experiments.

Sugar as antioxidants in sweetpotato roots

Sweetpotato has been known as one of the highest starch producing crops due to their higher sink strength[50]. Starch was synthesized from photoassimilate sucrose, which is the most common form of storage of carbohydrates in cells[51]. The metabolism of sucrose and other soluble sugars thus not only acted as a key indicator to evaluate the quality of sweetpotatoes, but also affected various metabolic processes[52]. This protection by soluble sugars may occur if sugar fluxes can be maintained or targeted towards cellular zones of ROS stress[53, 54]. Soluble sugars were linked with the production rates of ROS by regulating its producing metabolic pathways, such as mitochondrial respiration or photosynthesis[55]. It was known that starch content has significantly negative relationship with sucrose in tuber crops, because the catabolism of starch been impaired with respiratory rate decrease in low storage temperature. In our study, three types of soluble sugar contents were obviously increased (Fig. 3F). Moreover, α-amylase, β-amylase, sucrose synthase and fructokinase were significantly up-regulated, which boosted the sweetness of the tuber roots under cold temperature as compared to the control roots (Fig. 5 and Fig. 6). These results confirmed that sweetness could enhance the chilling stress tolerance of sweetpotato roots.

Nevertheless, the concept ‘sugar as antioxidant’ has been put forward in recent years[56], and it is increasingly clear that water-soluble carbohydrates (glucose, fructose and sucrose) are regarded as key regulators in plant responses under abiotic or biotic stress. Sperdouli and Moustakas (2012)[57] reported an accumulation of increased soluble sugars maintaining a high antioxidant protection in *Arabidopsis thaliana* leaves under drought stress. Furthermore, some genes encoding different sugar compounds has been confirmed to enhance freezing tolerance in petunia, tobacco and rice[58-62]. Hence, by definition,

soluble carbohydrates are synthesized in response to osmotic stress, acting as osmoprotectants that stabilize cellular membranes and maintain turgor[63]. Both simple sugars and polysaccharides are able to protect cellular membranes, which is a prerequisite for survival under stress conditions[64, 65].

Conclusions

In summary, our proteomic and metabolic profile analysis of sweetpotato roots stored at low temperature reveal that the CI index, REC and MDA content in roots stored at 4℃ were much higher than them at 13℃. Furthermore, low-temperature storage condition enhanced the activities of SOD, CAT, APX, $O_2^{\cdot-}$ producing rate, proline and especially soluble sugar contents. Most of the proteins were implicated in pathways related to metabolic pathway, especially phenylpropanoids and followed by starch and sucrose metabolism. α-amylase, sucrose synthase and fructokinase were significantly up-regulated in starch and sucrose metabolism, while β-glucosidase, glucose-1-phosphate adenylyl-transferase and starch synthase were opposite. Interestingly, there was discrepancy between proteomic (L-ascorbate peroxidase 3 and catalase) and biochemical (APX/CAT enzyme activity) data may be explained by higher H_2O_2 levels and increased glutathione and ascorbate redox states, which enhanced the CAT/APX enzyme activity indirectly. Furthermore, glucosinolate biosynthesis played a leading role in metabolic pathways of sweetpotao roots. More importantly, leucine, tryptophan, tyrosine, isoleucine and valine were all significantly up-regulated in glucosinolate biosynthesis. These results would expand our understanding of the proteome as well as metabolome and give new insights into how to resist the chilling stress for sweetpotato tubers.

Methods

Plant materials and storage condition

Sweetpotatoes (*Ipomoea batatas* L. cv. Xinxiang), obtained from Zhejiang Academy of Agricultural Sciences of China (supplementary Table S1), were grown in the greenhouse at 25-30℃ under a long-day photoperiod (16/8 h, light/dark) according to standard agricultural practices in 2018. Mature tuberous roots were chosen for uniform in size and appearance (average weight about 100-120g) without physical injury or infection at harvest time. They were divided randomly into two groups (12 per group) and each group was comprised of three replicates of 4 roots. The two groups were stored in Temperature Humidity Chamber of 4℃ and 13℃ (CK) for 14 days, respectively (Laifu MJX-280H, China). After storage, the tubers were sliced to 1 mm thickness, then put into liquid nitrogen and stored at -80℃ for further analysis.

Estimation of chilling injury index

Typical CI symptoms of tuberous roots were characterized by surface pitting, dark watery patches, and internal tissue browning[66]. CI was evaluated visually for 10 roots for each replicate. The severity score of CI ranged from 0 to 4. Among them, 0, 1, 2, 3, and 4 represents no harm, very slight injury, minor injuries,

moderate injury and serious injury, respectively. The CI index was calculated according to CI = $\sum$(a×b)/T. In this formula, a, b and T represents CI level (0-4), the number of roots at the corresponding level and the number of roots in the treatment, respectively.

Relative electrical conductivity and malondialdehyde content assays

The REC was measured as previously described[67]. Ten root discs (10 mm diameter and 2 mm thickness) were placed in a glass tube containing 10 ml of deionized water, and shaken at 100 cycles min^{-1} for 30 min under normal conditions. The initial electrical conductivity (C1) was measured with a conductivity meter (Mettler Toledo FE30, Switzerland). Then, the glass tube containing root discs in distilled water was boiled for 30 min. The final electrical conductivity (C2) was measured after cooling the tube to ambient temperature. The electrical conductivity (C0) of the deionized water was used as the blank. REC was calculated as (C1−C0)/(C2−C0)×100%.

The level of MDA was determined using the thio-barbituric acid method[68]. Root samples were ground with 5 ml of ice-cold 10% trichloroacetic acid (TCA). Absorbance was recorded at 450, 532, and 600 nm using an Infinite M200 microplate reader (Tecan, Switzerland)[69].

Determination of antioxidant enzyme, the rate of $O_2^{\cdot-}$ production and proline content

For enzyme activities, the fresh roots (0.1-0.5g) were homogenized in 10 ml of 50 mM precooled potassium phosphate buffer (PBS; pH 7.8) under ice cold conditions. Homogenate was centrifuged at 8,000 rpm for 20 min at 4°C and the supernatant was remained for the determination of following enzyme activities. Superoxide anions ($O_2^{\cdot-}$) producing rate (μmol·min^{-1}·g) was determined according to Jiang & Zhang (2001)[70] method with some modifications. Superoxide dismutase (SOD, EC 1.15.1.1) (U·min^{-1}·g), catalase (CAT, EC 1.11.1.6) (U·min^{-1}·g) and ascorbate peroxidase (APX, EC1.11.1.11) (U·min^{-1}·g) activities was determined according to Dhindsa & Matowe (1981)[71], Aebi (1984)[72], and Nakano & Asada (1981)[73], respectively. Moreover, proline content (μg·g^{-1}) was measured according to Bates et al. (1973)[74].

Determination of soluble sugar composition

The composition of soluble sugars (glucose, fructose and sucrose) (mg·g^{-1}) in roots was determined by high performance liquid chromatography (HPLC). Fresh root tissues (1-5 g) were homogenized in 50 ml of 80% (v/v) ethanol. The homogenate was placed in a water bath at 45°C and magnetically stirred for 30 min, then centrifuged at 10000 rpm for 15 min. 80% ethanol (10 ml) was added to the residue in the tube and stirred for 20 min in a water bath at 45°C. This procedure was repeated three times for each sample. The pooled supernatants volume was quantitatively adjusted to a total volume of 100 ml with 80% ethanol and filtered through a 0.22-μm filter. The filtrate (10 μl) was applied to HPLC system (Agilent 1200, Agilent Technologies, CA, USA) fitted with a refractive index detector (RID-1260, Agilent) and an

Agilent ZORBAX Carbohydrate Analysis Column (4.6mm×250mm, Agilent). Soluble sugars were separated in 75% (v/v) acetonitrile using a flow rate of 1.0 ml min^{-1} at 30°C.

Protein extraction and 1-DE SDS-PAGE

The flesh of sweetpotato was grinded by liquid nitrogen into fine powder and four volumes of lysis buffer (8 M urea, 1% Triton-100, 10 mM dithiothreitol and 1% Protease Inhibitor Cocktail) were added to sonication extract three times on ice using a high intensity ultrasonic processor. The ultrasonic time was three seconds of ultrasound and six seconds of pause, with a total of 20 cycles. The remaining debris was removed by centrifugation at 12,000 g at 4°C for 10 min. Then, the supernatant was collected and the protein concentration was determined with Enhanced BCA kit (Beyotime, P0009) according to the manufacturer's instructions. The aliquots of 30 μg protein samples were denatured for 5 mins with 5 μl 4×loading buffer and 2% SDS to 20 μl final volume. The electrophoresis was performed with 12% SDS PAGE gels and stained by Coomassie Blue R-250.

LC-MS/MS of digested peptides

The tryptic peptides were loaded onto a home-made reversed-phase analytical column (15 cm length, 75 μm i.d.). The mass ratio of trypsin-to-protein was 1:50 and 1:100 for the first digestion overnight and a second 4 h-digestion, respectively. The digested peptide was subjected to NSI source followed by tandem mass spectrometry (LC-MS/MS) in Q Exactive™ Plus (Thermo Scientific) coupled online to the UPLC. Peptides were then selected for MS/MS using NCE setting as 28 and the fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans with 15.0s dynamic exclusion. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD01772.

Database searching of proteins

The resulting MS/MS data were processed using Maxquant search engine (v.1.5.2.8) in uniport Toxoplasma gondii database. Tandem mass spectra were searched against Vert_tom_20141002 database (117,248 entries). Trypsin/P was specified as cleavage enzyme allowing up to 2 missing cleavages. The mass tolerance for precursor ions was set as 20 ppm in First search and 5 ppm in Main search, and the mass tolerance for fragment ions was set as 0.02 Da. Carbamidomethyl on Cysteine was specified as fixed modification and oxidation on Met was specified as variable modifications.

Bioinformatics analysis

The Gene Ontology (GO) annotation proteome was derived from the UniProt-GOA database (http://www.ebi.ac.uk/GOA/). Identified proteins domain functional description were annotated by InterProScan (http://www.ebi.ac.uk/interpro/) based on protein sequence alignment method used InterPro domain database. The KEGG (Kyoto Encyclopedia of Genes and Genomes) database was used

to annotate protein pathway. Localization of proteins was predicted with wolfpsort software (PSORT/PSORT II). For functional enrichment, a two-tailed Fisher's exact test was conducted to test the enrichment of the differentially expressed protein (DEP) against all identified proteins.

Metabolome profiling of tubers

The tubers sliced to 1 mm thickness was crushed using a mixer mill (MM400, Retsch) with a zirconia bead for 1.5 min at 30 Hz. 100 mg flesh powder was extracted overnight at 4°C with 1.0 ml 70% aqueous methanol. The sample extracts were analyzed using an LC-ESI-MS/MS system (HPLC, Shim-pack UFLC SHIMADZU CBM30A, MS, Applied Biosystems 6500 Q TRAP). LIT and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (Q TRAP), equipped with an ESI Turbo Ion-Spray interface, operating in a positive ion mode and controlled by Analyst 1.6.1 software (AB Sciex). The ESI source operation parameters were as follows: ion source, turbo spray; source temperature 500°C; ion spray voltage (IS) 5500 V; ion source gas I (GSI), gas II (GSII), curtain gas (CUR) were set at 50, 60, and 25 psi, respectively. Instrument tuning and mass calibration were performed with 10 and 100 μmol/l polypropylene glycol solutions in QQQ and LIT modes, respectively. QQQ scans were acquired as MRM experiments with collision gas (nitrogen) set to 5 psi. DP and CE for individual MRM transitions was done with further DP and CE optimization. A specific set of MRM transitions were monitored for each period according to the metabolites eluted within this period.

Protein and metabolite data analysis

Proteins absent in two storage treatments of six (3×2) samples were filtered out prior to analysis. Each metabolite was searched on metware database (MWDB) (https://www.metware.cn) and PubChem (https://pubchem.ncbi.nlm.nih.gov/) was used for their classifications. Significance analysis of possible comparisons (*p* value) were tested at $p<0.05$. The Filtered *p* value matrix ($p<0.05$) transformed by the function x=-lg (*p* value) was used to evaluate the ratio of celesius4 to celesius13, which was positively corelated with the expression multiple of the DEP (x>1.5). A set of significant proteins in each combination were further analyzed for Gene Ontology Enrichment of Biological Process (BP). The analysis of variance and mean separation of all metabolites were performed with Analyst 1.6.1, Partial Least Squares-Discriminant Analysis (PLS-DA) and Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) model. Figures were drawn using origin2018.

Abbreviations

APX: ascorbate peroxidase; Arg: Arginine; CAD: cinnamyl alcohol dehydrogenase; CAT: catalase; DEMs: differentially expressed metabolites; DEPs: differentially expressed proteins; FC: fold change; GO: Gene Ontology; HCT: Hydroxycinnamoyl transferase; Ile: Isoleucine; KEGG: Kyoto Encyclopedia of Genes and Genomes; Leu: Leucine; Lys: Lysine; MWDB: metware database; OPLS-DA: Orthogonal Partial Least Squares-Discriminant Analysis; PAL: phenylalanine ammonia lyase; PLS-DA: Partial Least Squares-

Discriminant Analysis; ROS: reactive oxygen species; $O_2^{\cdot-}$: Superoxide anions; SOD: Superoxide dismutase; Try: Tryptophan; Tyr: Tyrosine; Val: Valine; VIP: Variable Importance in Project.

Declarations

Ethics approval and consent to participate

The voucher specimen of sweetpotato was deposited in Zhejiang Academy of Agricultural Sciences. Their taxon, variety, voucher, geographic origin and identifier were listed in Supplementary Table S1.

Consent to publication

Not applicable.

Availability of data and materials

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD01772. Please see the raw data on https://www.ebi.ac.uk/pride/login (username: reviewer56768@ebi.ac.uk, password: PxhaZhfV). The data generated during this study are all included in the supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

PC and HY conceived the research plan, analysed the data and wrote the manuscript. CC and YH did the sugar content analysis. YL and GL performed antioxidant enzymes measurements. All authors read and approved this final version of manuscript.

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Figures

Figure 1

Morphological differences in tuber shape and color during storage at 13°C (A) and 4°C (B) for 14 d.

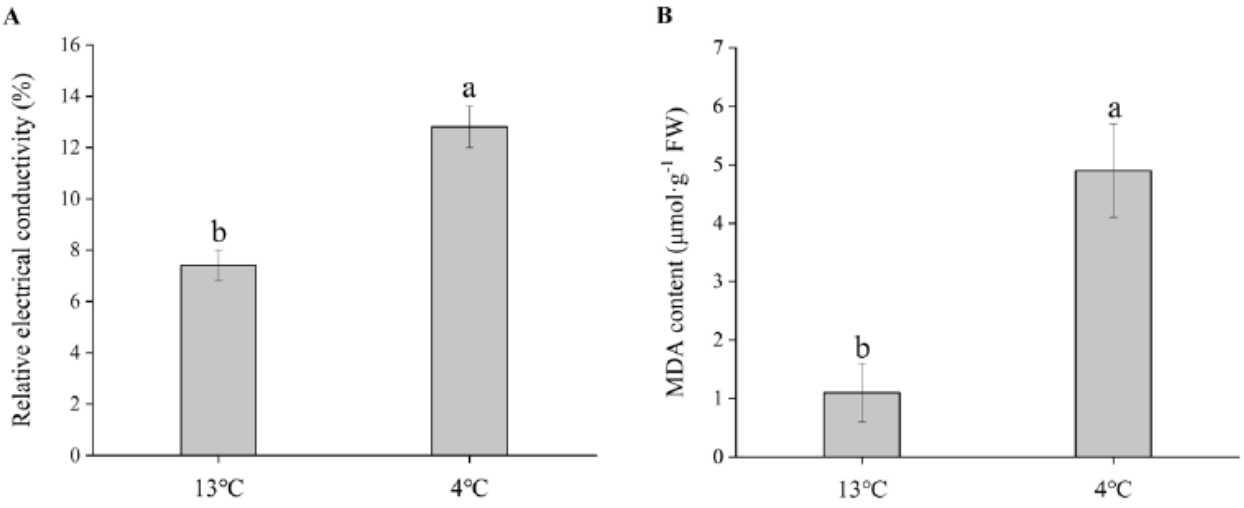

Figure 2

Effects of low-temperature storage on relative electrical conductivity and MDA content in sweetpotato roots for 14 d. A: Relative electrical conductivity. B: MDA content. Vertical bars represent the mean±SE. Different letters indicate statistically significant differences (p□0.05) by the LSD test.

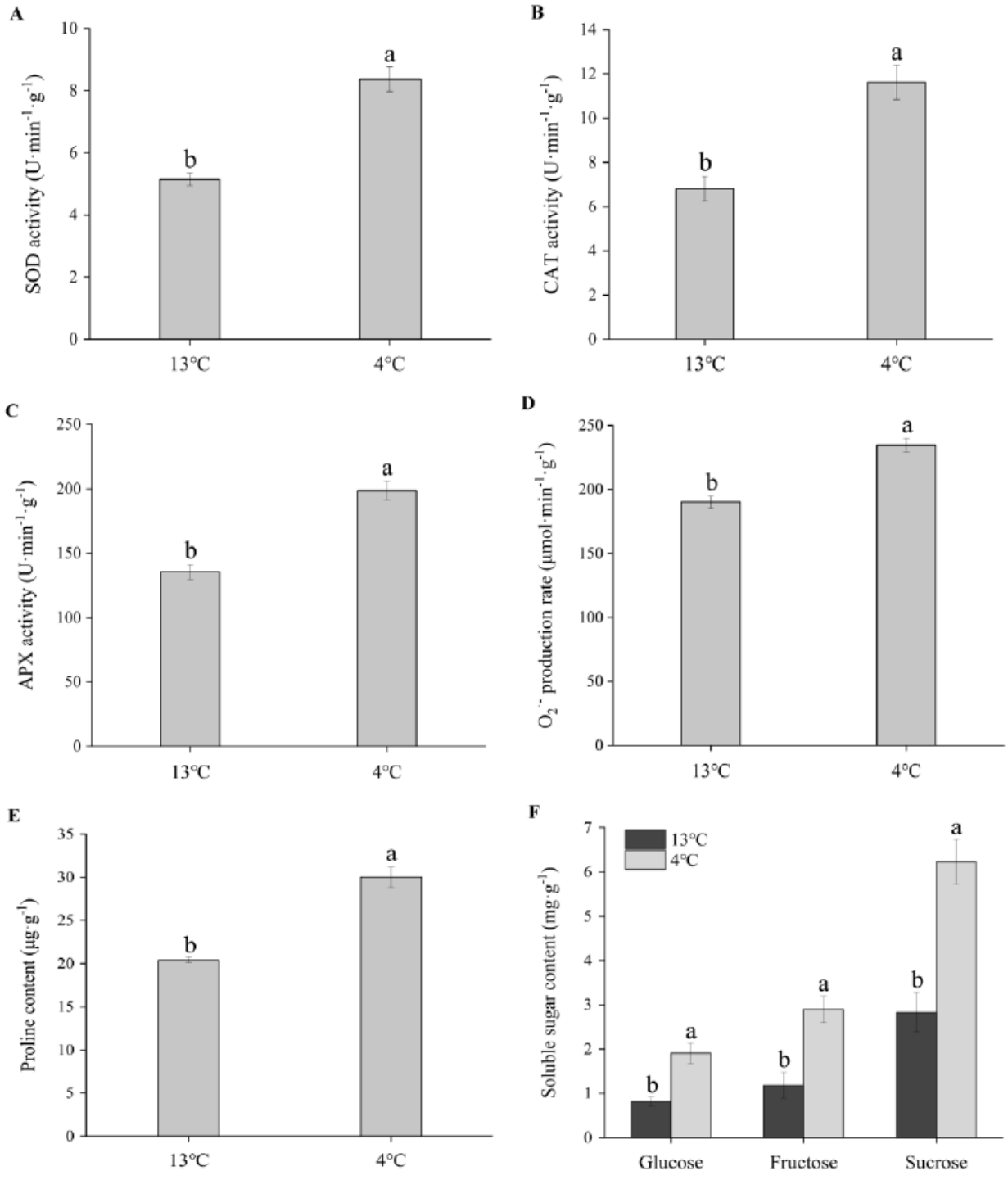

Figure 3

Effect of low-temperature storage on oxidative stress in terms of SOD (A), CAT (B), APX (C) activities, O2-producing rate (D), proline content (E) and soluble sugar content (F) such as glucose, fructose, and

sucrose in sweetpotatoes for 14 d. Vertical bars represent the mean±SE. Different letters indicate statistically significant differences (p≤0.05) by the LSD test.

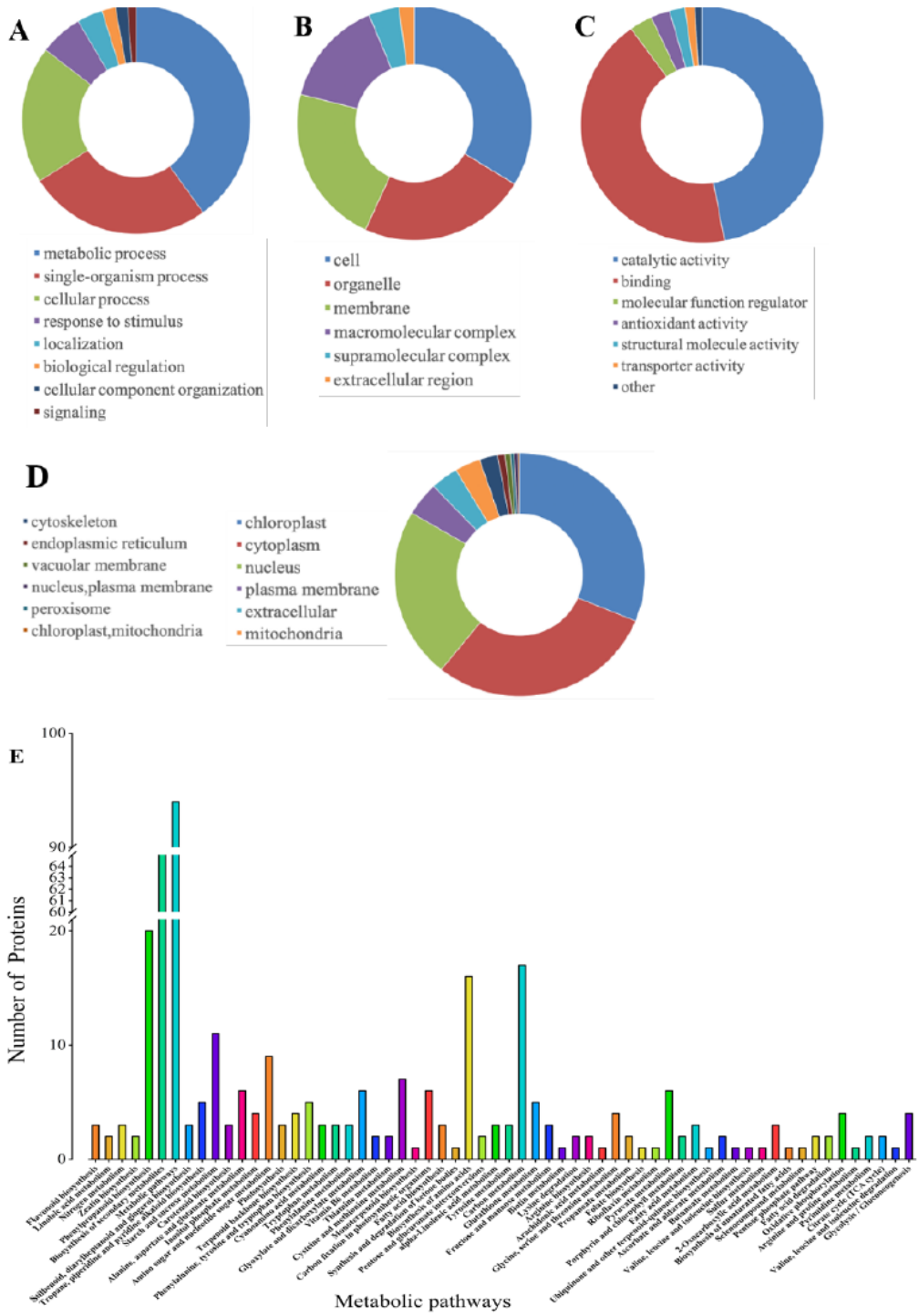

Figure 4

Functional classification, subcellular localization and pathway affiliation of proteins. Identified proteins were categorized according to their gene ontology for their biological processes (A), cellular components

(B), molecular functions (C), subcellular localizations (D) and association with different metabolic pathways (E).

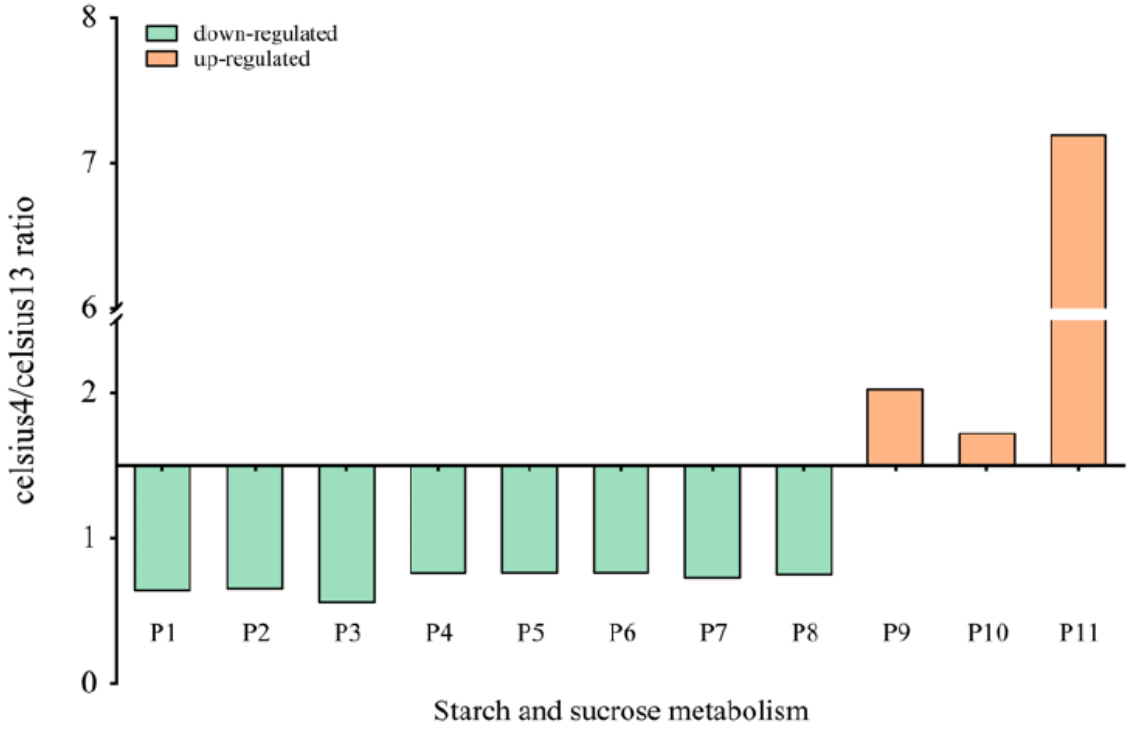

Figure 5

Differential multiple of the differentially expressed proteins (DEP) participated in starch and metabolism. P1: Glucose-1-phosphate adenylyltransferase (large subunit); P2: β-xylosidase/α-arabinofuranosidase 2; P3: β-glucosidase 12; P4: Glucose-1-phosphate adenylyltransferase (small subunit); P5: Sucrose synthase 6; P6: Glucan endo-1,3-β-glucosidase 6; P7: 4-α-glucanotransferase; P8: Isoamylase 3; P9: α-amylase; P10: Probable fructokinase 7; P11: Sucrose synthase.

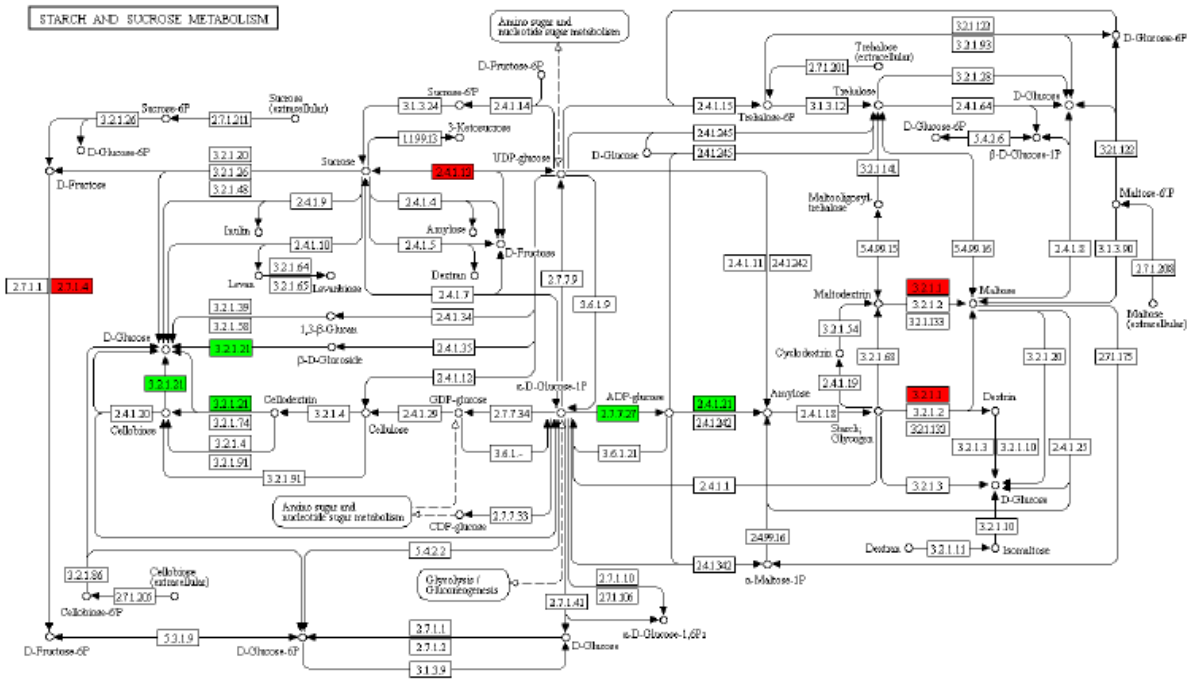

Figure 6

Changes of differentially expressed proteins (DEP) involved in starch and sucrose metabolism of sweetpotato roots under cold stress. The significantly up-(red) and down-regulated (green) expressed proteins are demonstrated. EC: 3.2.1.1, EC: 2.4.1.13 and EC: 2.7.1.4 proteins (red) were α-amylase, sucrose synthase and fructokinase, respectively. EC: 3.2.1.21, EC: 2.7.7.27 and EC: 2.4.1.21 proteins (green) were β-glucosidase, glucose-1-phosphate adenylyl-transferase and starch synthase, respectively.

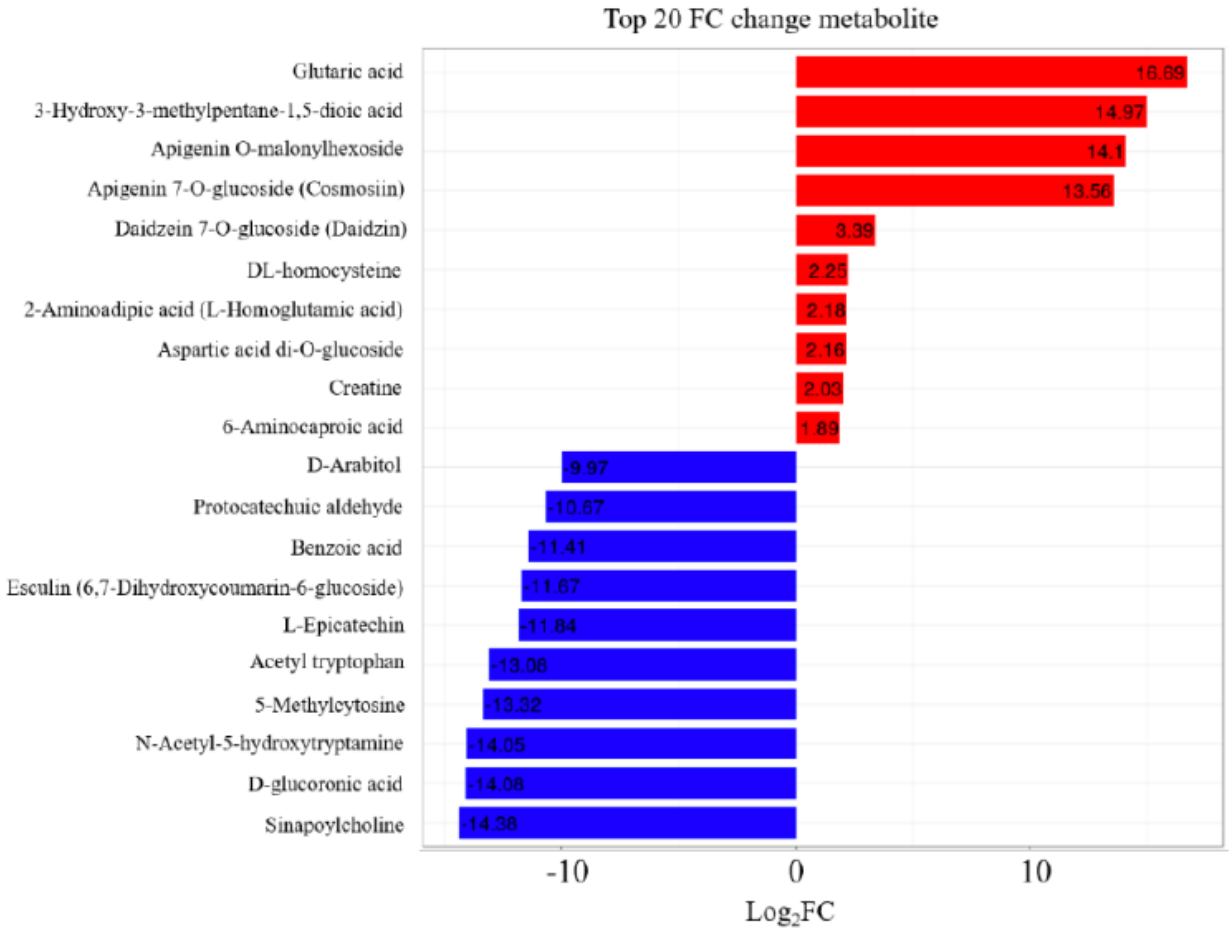

Figure 7

Significant fold change of the metabolites in sweetpotato roots under freezing stress as compared to them in controls. Red and blue lines represent up- and down-regulated metabolites, respectively.

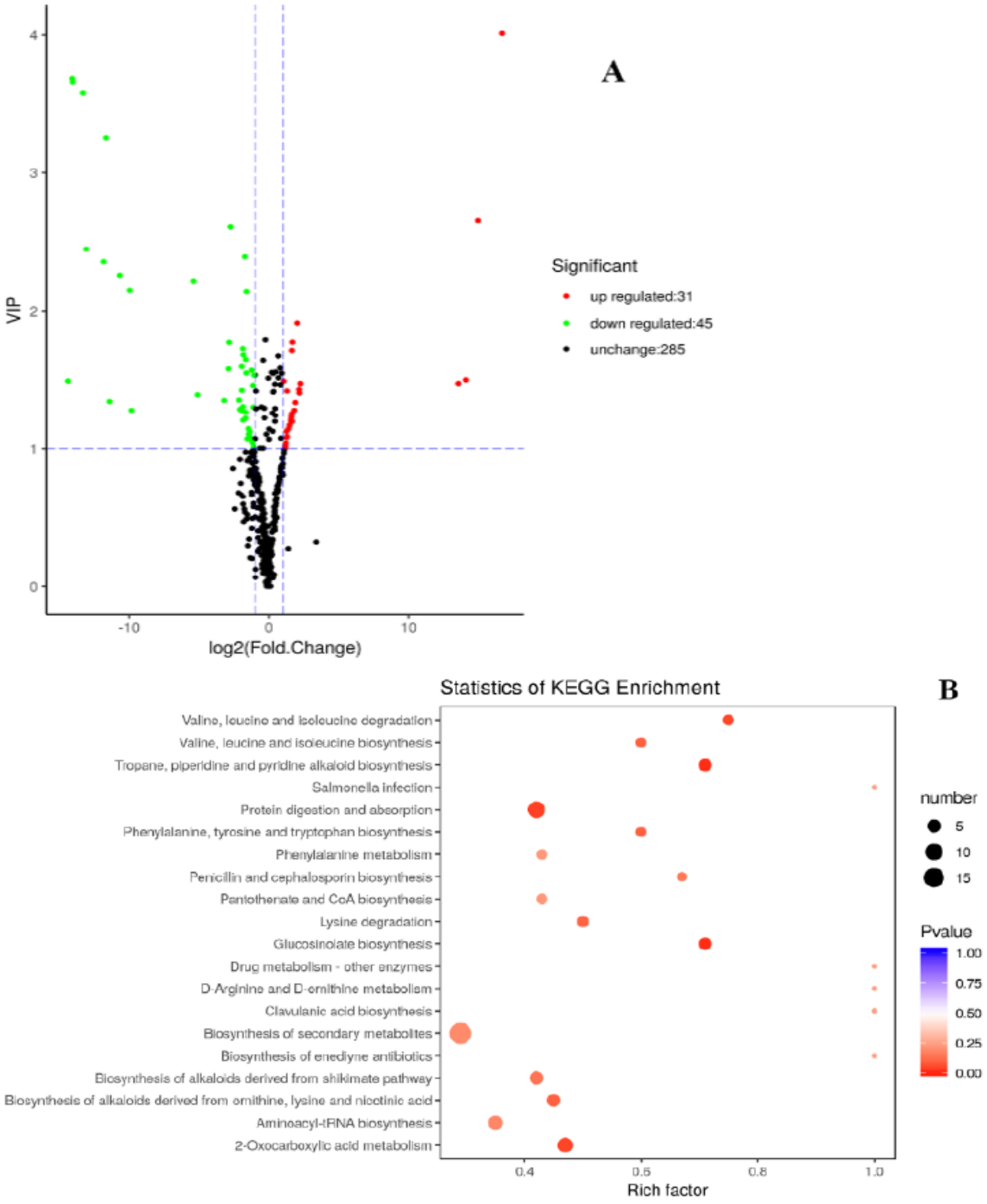

Figure 8

The volcano plots and statistics of KEGG pathway enrichment of significantly differential expressed metabolites (DEMs) were demonstrated. In the volcano plots, red, green and black dots represent up-, down-regulated and insignificant changed metabolites, respectively (A). The dimension of dots indicates the amount of the DEMs. The color (P-value) explained the significance of DEM. Rich factor means the ratio of the number of the DEMs to the total number of them detected in the corresponding pathway (B).

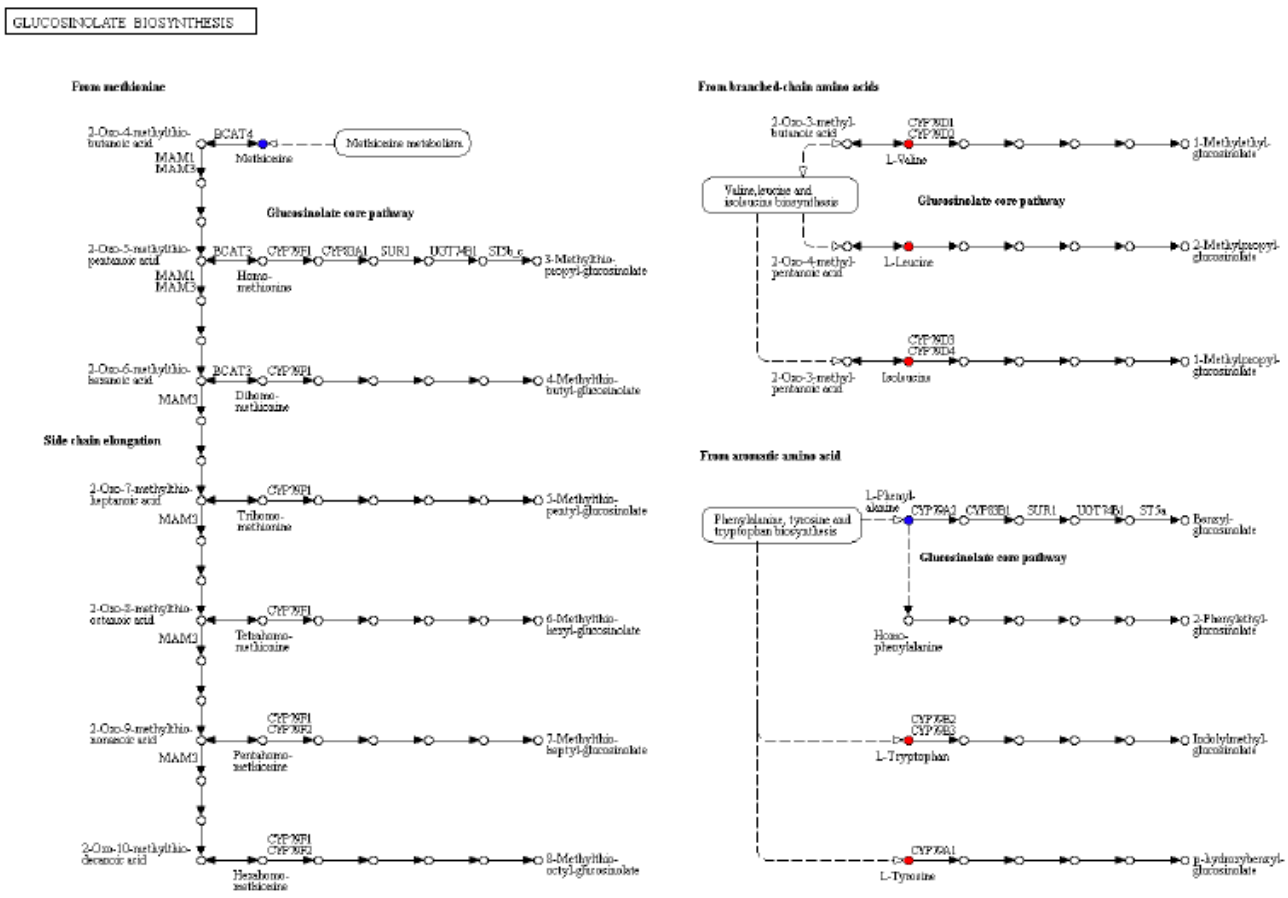

Figure 9

Differential expressed metabolic components in glucosinolate biosynthesis. Red and blue dots represent up-regulated and insignificant changed compounds, respectively.

Supplementary Files

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

- Additionalfile1.xlsx
- Additionalfile2.xlsx
- Addtionalfile3.xlsx
- Addtionalfile4.xlsx
- Additionalfile5.xlsx
- Additionalfile6.xlsx
- Supplementarydata.pdf