Comparative Proteomics Reveals Differential Mechanism of Root Cold-resistance between *Vitis. Riparia* × *V. Labrusca* and Cabernet Sauvignon

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Research Article

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Abstract

Grapevines, containing large amounts of bioactive metabolites that offer health benefits, are widely cultivated around the world. The cold damage of growing outside with extreme low temperature during overwintering stage limits the expansion of production. Although the levels of morphological, biochemical and molecular in different *Vitis* species exposure to different temperatures have been investigated, differential expression of proteins in roots is still limited. Here, the roots of cold-resistant (*Vitis. riparia* × *V. labrusca*, T1) and cold-sensitive varieties (Cabernet Sauvignon, T3) at −4°C as well as of the former at −15°C (T2) were measured by iTRAQ-based proteomic analysis, expression levels of genes encoding candidate proteins were validated by qRT-PCR. The results showed that the root activity of cold-resistant variety was stronger than that of cold-sensitive variety, and it declined with the decrease of temperature. A total of 25 proteins were differentially co-expressed at T2 versus (vs) T1 and T1 vs T3, and these proteins were involved in stress response (e.g. DHN1, SHSPCP and USPCP), bio-signaling (e.g. PKCP, S/TPP and nsS/TP), metabolism (e.g. GluP, GluBE and PE), energy (e.g. AAC, AAACP and NADCP), and translation (e.g. rpL14, rpS21 and PPI). The relative expression levels of the candidate 13 genes were consistent with their fold-change values of proteins. The signature translation pattern for the roots at spatio-temporal treatments of varieties and temperatures provides insight into the differential mechanism of cold resistance of grapevines.

Introduction

Grapevines (*Vitis vinifera* L.) are rich in phenolics, flavonoids and resveratrol that have many biological activities such as antioxidant, inhibition of plasma platelet aggregation, and cyclooxygenase [1, 2]. Nowadays grapevines are cultivated in many countries around the world, principally distributed in Europe [3]. In China, the optimum regions for cultivation are mainly distributed in Gansu, Inner Mongolia, Ningxia, Shaanxi, Shandong and Xinjiang [4]. Currently, thirteen varieties of *Vitis* that are large-scale cultivated (≥100,000 hm²) in the world mainly include: Cabernet Sauvignon, Merlot, Chardonnay, Syrah, Sauvignon Blanc and Pinot Noir [5].

In the commercial large-scale cultivation, grapevines are frequently exposed to environmental stresses such as drought, salinity and extreme temperatures [6]. Most cultivated grapevines are suited to grow in temperate and subtropical regions with mild winter conditions [7]. However, grapevines are often grown outside with severe winter conditions characterized by low temperature, which limits the current and future expansion of production [8]. In order to diminish the freeze damage, several efforts including evaluating the cold-resistant species or varieties as well as revealing the mechanism of cold-resistance have been conducted. For the evaluating the cold-resistant species or varieties, *V. riparia* is found to be the most cold-resistant species and *V. labrusca* belongs to medium resistance among of 7 wild *Vitis* species native to America [9]; the cold resistance of *V. riparia* × *V. labrusca* (Beta) and *V. berlandieri* × *V. riparia* (5BB) is stronger than that of Cabernet Sauvignon and Merlot [10]. For the revealing the mechanism of cold-resistance, physiological and biochemical metabolites (e.g. soluble sugars, proteins and hormones) in buds, branches and roots were determined [11–13]; key genes (e.g. *CBF/DREB*, *ICE* and

AP2/ERF) enhancing freezing tolerance were identified [14–16]; and differentially expressed genes among different species and temperatures were analyzed by transcriptomics [8, 17, 18].

Previous literatures have reported that grape branches and buds can survive low temperature of -13°C or lower, but roots have weaker cold resistance than the above-ground parts [19, 20]. To date, although the levels of osmoregulatory metabolites, the activities of antioxidant enzymes, and the expression levels of cold resistance genes between different *Vitis* species or low temperature have been investigated in extensive experiments [11–18], differential expression of proteins in roots between cold-resistant and cold-sensitive species or varieties as well as different low temperatures has not been determined and identified. In this study, the roots of cold-resistant (*V. riparia* $\times$ *V. labrusca*, T1) and cold-sensitive varieties (Cabernet Sauvignon, T3) at -4°C as well as the former at -15°C (T2) were spatio-temporally measured by quantitative iTRAQ-based proteomic analysis. We found that 25 proteins were differentially co-expressed at the three treatments and their biological functions were involved in stress response, bio-signaling, metabolism, energy and translation; the expression levels of related genes were validated by qRT-PCR.

Results

Difference in root activity

As shown in Fig. 1, there was a 1.68-fold decrease of root activity at T2 compared to T1, and a 2.54-fold greater at T1 than T3. Moreover, the root activities presenting at the different treatments suggest that the roots can be used for proteomic analysis.

Analysis of differentially expressed proteins (DEPs)

To reveal the cold resistance of root in grapevines, the DEPs in roots of *V. riparia* $\times$ *V. labrusca* and Cabernet Sauvignon at different treatments were analyzed by iTRAQ. A total of 56 and 143 DEPs were obtained at T2 vs T1 and T1 vs T3, respectively (Fig. 2). The cluster heat maps of the DEPs at T1, T2 and T3 were shown in Fig. 3.

The functions of the DEPs were enriched against the GO database (Fig. 4). At T2 vs T1, 32 proteins were enriched into biological process including: cellular macromolecule metabolic process (14, GO: 0044260), cellular protein metabolic process (13, GO: 0044267), proteolysis involved in cellular protein catabolic process (3, GO: 0051603), iron ion transport (1, GO: 0006826) and cellular iron ion homeostasis (1, GO: 0006879); and 31 proteins were enriched into molecular function including: catalytic activity (26, GO: 0003824), phosphotransferase activity, alcohol group as acceptor (4, GO: 0016773), and ATP:ADP antiporter activity (1, GO: 0005471) (Fig. 4A). At T1 vs T3, 56 proteins were enriched into biological process including: response to stimulus (15, GO: 0050896), response to stress (14, GO: 0006950), response to oxidative stress (6, GO: 0006979), response to biotic stimulus (5, GO: 0009607), defense response (5, GO: 0006952), cation transport (5, GO: 0006812), proteasome-mediated ubiquitin-dependent protein catabolic process (2, GO: 0043161), ATP hydrolysis coupled proton transport (2, GO: 0015991)

and metal ion transport (2, GO: 0030001); 13 proteins were enriched into cellular component including: nucleosome (7, GO: 0000786) and thylakoid (6, GO: 0009579); and 30 proteins were enriched into molecular function including: peroxidase activity (6, GO: 0004601), calcium ion binding (6, GO: 0005509), heme binding (5, GO: 0020037), ion transmembrane transporter activity (4, GO: 0015075), hydrogen ion transmembrane transporter activity (3, GO: 0015078), NADH dehydrogenase (ubiquinone) activity (2, GO: 0008137), triose-phosphate isomerase activity (2, GO: 0004807) and O-methyltransferase activity (2, GO: 0008171) (Fig. 4B).

Among of the 56 and 143 DEPs at T2 vs T1 and T1 vs T3, 25 proteins were differentially co-expressed (Fig. 5). Based on the biological functions annotated against the SwissProt, the 25 DEPs were classified into 5 categories including: stress response (6), bio-signaling (4), metabolism (6), energy (6), and translation (3) (Fig. 5; Table 1).

Table 1

Twenty-five DEPs identified in *V. riparia* × *V. labrusca* and Cabernet Sauvignon at different treatments.

Protein Name (Abbreviation)	SwissProt ID	log ₂ FC (T2 vs T1)	log ₂ FC (T1 vs T3)
Stress response (6)			
Dehydrin (DHN1)	Q4VT48	1.34	1.78
SHSP domain-containing protein (SHSPCP)	F6HJZ4	1.76	2.01
Usp domain-containing protein (USPCP)	F6H727	1.60	2.69
Ferritin (FER)	A5BV73	2.83	1.83
Glutaredoxin-dependent peroxiredoxin (GluDP)	A5ARL2	2.72	1.92
Glutathione peroxidase (GPX)	D7TW03	1.28	1.73
Bio-signaling (4)			
Protein kinase domain-containing protein (PKCP)	A5ALY7	1.44	1.27
Serine/threonine-protein phosphatase (S/TPP)	D7TV73	1.98	2.92
Non-specific serine/threonine protein kinase (nsS/TPK)	F6H1V3	1.75	2.07
Ubiquitin receptor RAD23 (RAD23)	D7T959	1.22	1.35
Metabolism (6)			
Alpha-1,4 glucan phosphorylase (GluP)	D7SXJ4	2.65	1.36
1,4-alpha-glucan branching enzyme (GluBE)	E0CQR2	0.58	1.57
Pectinesterase (PE)	F6HZ64	0.63	2.92
Abhydrolase 3 domain-containing protein (ABHD3CP)	F6HQC6	0.34	1.21
Proline iminopeptidase (ProIP)	D7T3J3	2.28	1.94
Methyltransferase (MT)	A5B620	2.61	1.75
Energy (6)			
ADP/ATP carrier protein (AAC)	A5BVR2	1.63	1.61
AAA domain-containing protein (AAACP)	D7TZI9	1.61	1.59
NAD(P)-bd dom domain-containing protein (NADCP)	F6HL96	1.59	2.06
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 7 (NDUFB7)	A5BAM7	1.82	2.61
Phytocyanin domain-containing protein (PCP)	A5C3C3	1.85	2.83

Protein Name (Abbreviation)	SwissProt ID	log ₂ FC (T2 vs T1)	log ₂ FC (T1 vs T3)
Succinate dehydrogenase [ubiquinone] flavoprotein subunit (SDHFS)	A5BGN3	1.69	1.88
Translation (3)			
Ribosomal protein L14 (rpL14)	B6VJZ7	2.61	2.80
40S ribosomal protein S21 (rpS21)	A5BUA6	2.76	1.67
Peptidylprolyl isomerase (PPI)	D7UDY0	1.61	2.51

DEPs involved in stress response

Six DEPs that are involved in stress response include: dehydrin (DHN1), SHSP domain-containing protein (SHSPCP), Usp domain-containing protein (USPCP), ferritin (FER), glutaredoxin-dependent peroxiredoxin (GluDP), and glutathione peroxidase (GPX) (Table 1). The six proteins were over-expressed based on FC values, with up-regulated (UR) of 1.28- to 2.83-fold at T2 vs T1 and 1.73- to 2.69-fold at T1 vs T3 (Table 1). The relative expression levels (RELs) of the genes *DHN1*, *FER* and *GPX* were consistent with FC values, with UR of 2.77-, 4.83- and 3.62-fold at T2 vs T1 and 3.28, 3.74- and 3.85-fold at T1 vs T3, respectively (Fig. 6A).

DEPs involved in bio-signaling

Four DEPs that are involved in bio-signaling include: protein kinase domain-containing protein (PKCP), serine/threonine-protein phosphatase (S/TPP), non-specific serine/threonine protein kinase (nsS/TPK), and ubiquitin receptor RAD23 (RAD23) (Table 1). The four proteins were over-expressed with UR of 1.22- to 1.98-fold at T2 vs T1 and 1.27- to 2.92-fold at T1 vs T3 (Table 1). The RELs of the genes *S/TPP* and *RAD23* were consistent with FC values, with UR of 3.88- and 2.42-fold at T2 vs T1 and 4.11- and 2.61-fold at T1 vs T3, respectively (Fig. 6B).

DEPs involved in metabolism

Six DEPs that are involved in metabolism include: alpha-1, 4 glucan phosphorylase (GluP), 1, 4-alpha-glucan branching enzyme (GluBE), pectinesterase (PE), abhydrolase 3 domain-containing protein (ABHD3CP), proline iminopeptidase (ProIP), and methyltransferase (MT) (Table 1). The six proteins were differentially expressed at T2 vs T1, with UR of 2.65-, 2.28- and 2.61-fold for GluP, ProIP and MT, and down-regulated (DR) of 0.58-, 0.63- and 0.34-fold for GluBE, PE and ABHD3CP, respectively; while at T1 vs T3, the six proteins were over-expressed with UR of 1.21- to 2.92-fold (Table 1). The RELs of the genes *GluP*, *PE* and *MT* were consistent with FC values, with differential regulation of 4.61-, 0.89- and 4.85-fold at T2 vs T1 and 2.38-, 4.85- and 2.63-fold at T1 vs T3, respectively (Fig. 6C).

DEPs involved in energy

Six DEPs that are involved in energy include: ADP/ATP carrier protein (AAC), AAA domain-containing protein (AAACP), NAD (P)-bd dom domain-containing protein (NADCP), NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 7 (NDUFB7), phytoeyanin domain-containing protein (PCP), and succinate dehydrogenase [ubiquinone] flavoprotein subunit (SDHFS) (Table 1). The six proteins were over-expressed with UR of 1.59- to 1.85-fold at T2 vs T1 and 1.59- to 2.83-fold at T1 vs T3 (Table 1). The RELs of the genes *AAC*, *NADCP* and *PCP* were consistent with FC values, with UR of 3.85-, 3.43- and 3.63-fold at T2 vs T1 and 3.45-, 4.59- and 4.78-fold at T1 vs T3, respectively (Fig. 6D).

DEPs involved in translation

Three DEPs that are involved in translation include: ribosomal protein L14 (rpL14), 40S ribosomal protein S21 (rpS21), and peptidylprolyl isomerase (PPI) (Table 1). The three proteins were over-expressed with UR of 1.61- to 2.76-fold at T2 vs T1 and 1.67- to 2.80-fold at T1 vs T3 (Table 1). The RELs of the genes *rpL14* and *PPI* were consistent with FC values, with UR of 3.52- and 3.45-fold at T2 vs T1 and 3.86- and 5.64-fold at T1 vs T3, respectively (Fig. 6E).

Discussion

Plants are frequently exposed to environmental stresses such as low temperature, which plays a major role in the distribution of plant species. Adaptation and acclimation to cold stress result from integrated events occurring at all levels of organization, from the anatomical and morphological level to the cellular (*e.g.* changes in cell cycle, division and wall architecture), biochemical (*e.g.* producing osmoregulatory compounds such as proline and glycine betaine), and molecular level (*e.g.* linking the perception of a stress signal with the genomic responses) [21]. In this study, we found that there was a stronger root activity in *V. riparia* × *V. labrusca* than Cabernet Sauvignon, and the root activity declined with the decrease of temperature; a total of 25 proteins were differentially co-expressed in *V. riparia* × *V. labrusca* and Cabernet Sauvignon at -4°C and/or -15°C treatments, and these 25 DEPs were classified into 5 categories including: stress response, bio-signaling, metabolism, energy, and translation.

The root system is an important organ absorbing water and minerals from the soil, storing foods (*e.g.* starch, polysaccharides and secondary metabolites), and synthesizing the vital substances (*e.g.* amino acids, hormones and vitamins) for plant growth and development [22, 23]. Here, a stronger root activity at -4°C was observed in *V. riparia* × *V. labrusca* than Cabernet Sauvignon, authenticating that the cold-resistance of *V. riparia* × *V. labrusca* is higher than that of Cabernet Sauvignon in field [10].

For the DEPs involved in stress response, previous literatures have reported that dehydrin (DHN) are highly hydrophilic proteins that are involved in cold acclimation processes [24]. Small heat shock proteins (sHSPs) are ubiquitous stress proteins proposed to act as chaperones and have been ascribed an unusual diversity of functions in the cellular response to environmental stress [25]. Universal stress

proteins (USPs) are stress-responsive proteins that may contain a single USP domain or two tandem repeats of USP domains [26, 27]. Ferritins (FERs) are a broad superfamily of iron storage proteins, exert a fine tuning of the quantity of metal required for metabolic purposes and help plants to protect against oxidative stress [28]. The GluDP plays a role in cell protection against oxidative stress by detoxifying peroxides [29]. Glutathione peroxidases (GPXs) are key enzymes of the cell antioxidant defense system and involved in scavenging oxyradicals [30]. Investigations have found that the genes *DHN* in tomato [31], *shsp16.9* in rice [32], *USPs* in *Arabidopsis* [33], *TaFER-5B* in *Triticum aestivum* [34], and *GluPX* in *Taxus chinensis* [35] were overexpressed in response to cold stress. In this study, the overexpression of the proteins (DHN1, SHSPCP, USPCP, FER, GluDP and GPX) that enhance cold stress are consistent with higher cold-resistance of *V. riparia* × *V. labrusca* than Cabernet Sauvignon.

For the DEPs involved in bio-signaling, both protein kinases (PKs) and protein phosphatases (PPs) play important roles in determining the magnitude and duration of a signaling event, with PKs catalyzing the transfer of a phosphate moiety from ATP to proteins and PPs acting to remove this phosphate group by hydrolysis [36]. Protein serine/threonine phosphatases family from plants constitute PP1, PP2A, PP2B, and novel phosphatases, which have multiple biological functions by regulating a wide variety of cellular signal transduction pathways in response to stresses [37]. The RAD23 is involved in cell cycle regulation, protein quality control, DNA damage response and cellular metabolism [38]. Investigations have found that the genes *GsLRPK* in *Glycine soja* [39], *S/TPP* in rice [40], and *RAD23* in apple [41] were overexpressed in response to cold stress. In this study, the overexpression of the proteins (PKCP, S/TPP, nsS/TPK and RAD23) was also observed in higher cold-resistance of *V. riparia* × *V. labrusca* compared to Cabernet Sauvignon as well as at lower temperature -15°C compared to -4°C .

For the DEPs involved in metabolism, the GluP is an important allosteric enzyme in carbohydrate metabolism [42]. The GluBE is involved in the pathway starch biosynthesis, which is part of glycan biosynthesis [43]. The PE is involved in the pathway pectin degradation and in glycan metabolism [44]. The ABHD family of proteins in plants influences the lipid biosynthesis more towards leaf lipids such as galactolipids and less towards storage lipids [45]. The ProIP catalyzes specifically hydrolysis of N-terminal proline from peptides [46]. The MT is involved in the sterol and steroid biosynthesis [47]. Extensive experiments have demonstrated that there are overexpression and activity of enzymes that participate in soluble sugars biosynthesis and starch degradation, which produce proper metabolites to adjust the metabolism and physiology of the plant to cold stress [48]. In this study, the overexpression of the proteins (GluP, GluBE, PE, ABHD3CP, ProIP and MT) was also observed in *V. riparia* × *V. labrusca* compared to Cabernet Sauvignon at -4°C . While the downexpression of the proteins (GluBE, PE and ABHD3CP) was observed in *V. riparia* × *V. labrusca* at -15°C compared to -4°C . The downexpression at freezing temperature (-15°C) might be a part of the mechanism associated with the delay of senescence and death [49].

For the DEPs involved in energy, the AAC plays a key role in the energetic cell metabolism because it exchanges ATP and ADP, product, and substrate of the mitochondrial ATP synthase, respectively [50]. The AAA protein family is a group of ATPases that are associated with various cellular activities [51]. The

NAD(P) plays crucial roles in pro-oxidant and antioxidant metabolism and the NAD contents are both flexible and potentially important in determining cell fate [52]. The NDUF7 is an accessory subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I), which functions in the transfer of electrons from NADH to the respiratory chain [53]. The phytycyanins (PCs) are a class of plant-specific blue copper proteins and play critical roles in plant growth and development [54]. The SDHFS is involved in complex II of the mitochondrial electron transport chain and is responsible for transferring electrons from succinate to ubiquinone [55]. Extensive experiments have demonstrated that the membranes become less fluid and the proteins components can no longer function normally in chilling-sensitive plants, the result is inhibition of H⁺-ATPase activity, energy transduction and enzyme-dependent metabolism [39]. In this study, the overexpression of the proteins (AAC, AAACP, NADCP, NDUF7, PCP and SDHFS) was observed in the *V. riparia* × *V. labrusca* variety.

For the DEPs involved in translation, the large and small subunits of ribosomal proteins are structural constituents of ribosomes, which perform the essential task of protein synthesis in the cell [56]. The PPI functions in the folding of membranal proteins [57]. Investigations have found that the genes *SOL34* in *Glycine max* [58], *RPS5* in *Arabidopsis* [59], and *OsCYP19-4* that has the PPI activity in rice [60] were overexpressed in response to cold stress. In this study, the overexpression of the proteins (rpL14, rpS21 and PPI) could be required for growth acclimation to cold stress during overwintering stage.

Conclusion

From the above observations, the root activity of cold-resistant *Vitis* species is stronger than that of cold-sensitive species, and it declines with the decrease of temperature. The DEPs observed in roots between the cold-resistant and cold-sensitive species as well as different temperatures suggest translation-based regulation of roots in response to low temperature. The biological function of the 25 DEPs involved in stress response, bio-signaling, metabolism, energy, and translation should be further investigated using transgenic assays.

Methods

Plant material

The one-year-old seedlings (cutting-propagated) of *V. riparia* × *V. labrusca* (cold-resistant variety) and Cabernet Sauvignon (cold-sensitive variety) were planted and grown in field with glass separated (depth 50 cm, width 150 cm) to avoid the roots interfering with each other. Complex fertilizer (N+P₂O₅+K₂O≥500 g/L, Cu+Fe+Mn+Zn+B: 3-30g/L; 100 ml per plant) was applied each year in the soil at the depth from 10 to 30 cm, the soil water content was controlled from 45% to 55%. After three years, the lateral roots of *V. riparia* × *V. labrusca* were collected (n = 9 plants) at the depth of 20 cm when the average temperature of soil surface was at -4°C (T1; December 12) and -15°C (T2; January 12), respectively; and the lateral roots of *V. vinifera* were collected (n = 9 plants) at -4°C (T3; December 12). The collected samples were rapidly frozen in liquid nitrogen for measurement of root activity and analysis of proteomics. The two varieties *V.*

riparia × *V. labrusca* and Cabernet Sauvignon were identified by Prof. Delong Yang (Gansu Agricultural University, China). The samples were collected from the germplasm resources nursery of wine grapes built by our own laboratory, and the voucher specimens were deposited in the herbarium of Gansu Agricultural University, China.

Measurement of root activity

Root activity was measured according to a triphenyl tetrazolium chloride (TTC) method [61]. Briefly, root-tip samples (100 mg) were cut into pieces and then placed into a glass tube (10 mL). The TTC solution (0.4% w/v, 3 mL) and Na₂HPO₄-KH₂PO₄ buffer (0.1 mol/L, 3 mL, pH 7.0) were sequentially added in the tube. After incubated at 37°C for 1 h, H₂SO₄ (1 mol/L, 1.5 mL) was added into the mixture to stop the reaction. The colored samples were transferred into a sealed tube, methanol (15 mL) was added and then the mixture was incubated at 37°C for 4 h to decolor. Absorbance readers were taken at 485 nm and root activity was evaluated based on µg of triphenylformazan (TTF).

Protein extraction, quantification and digestion

Total protein samples were extracted according to a previous protocol [62] with some modifications. Briefly, root samples (0.5 g) were ground into powder in liquid nitrogen and then dissolved in pre-chilled (−20°C) trichloroacetic acid/acetone (10% w/v, 5 mL). After precipitated at −20°C for 12 h, the homogenate was centrifuged at 14,000 g at 4°C for 15 min. After the supernatant removed and the precipitate suspended in acetone at −20°C for 2 h, the suspension was centrifuged at 14,000 g at 4°C for 10 min. Following exhaustive suspension in acetone (× 3), the precipitate was dissolved in triethylammonium bicarbonate (0.5 mol/L, 0.5 mL) at 4°C for 1 h and then centrifuged at 14,000g at 4°C for 10 min. Finally, the supernatant was transferred to a new tube. The quality of the extracted protein was quantified by SDS-PAGE. The protein samples were digested using a Filter aided sample preparation method [63].

iTRAQ labeling and strong cation exchange (SCX) chromatography fractionation

The digested peptides were labeled using an iTRAQ reagents (iTRAQ® Reagents-8plex kit, Sigma Chemical Co., St. Louis, Mo, USA) [64-66]. The labeled samples were pooled and purified using a SCX chromatography on an Agilent 1260 HPLC (Agilent technologies Inc., Santa Clara, CA, USA) [67].

Liquid chromatography (LC)-electrospray ionization (ESI) tandem MS/MS analysis

LC-MS/MS was performed with an Easy nLC (Thermo Fisher Scientific, Waltham, MA, USA) coupled to Q Exactive MS (Thermo Finnigan, San Jose, CA, USA) [68]. Briefly, the iTRAQ-labeled peptides (5 µg) were separated by a Thermo Scientific Easy C₁₈ column (75 µm × 100 mm, 3 µm) with gradient elution from 2% B to 45% B in 120 min (A: 0.1% formic acid in H₂O, B: 0.1% formic acid in acetonitrile) at a flow rate of 300 nl/min. All tandem MS were produced following higher collision energy dissociation method.

Protein identification and function annotation

Protein identification was performed using a decoy database search with the false discovery rate < 1.0% and more than one identified peptide. Protein quantitation was analyzed using an iTRAQ 8-plex combined with Mann-Whitney Test. Differentially expressed proteins (DEPs) were selected with a fold-change (FC) value ≥1.2 or ≤0.82 (The ratio of T2 vs T1 or T1 vs T3) and *P*-value ≤0.05 [69]. Protein functions were annotated against the databases including Swiss-Prot and Gene Ontology (GO) [70-72].

RNA extraction and quantitative real-time PCR (qRT-PCR)

Western blotting was used to identify protein expression due to high resolution and sensitivity [73, 74], while the decrease of immune reactivity and nonspecific bands exist in plants [75]. qRT-PCR was alternatively selected to indirectly validate the protein expression [67, 76, 77]. In this study, the qRT-PCR was used to identify the expression level for genes encoding the candidate proteins. Briefly, RNA samples were extracted from roots using a plant RNA kit. Primer sequences (Table 2) were designed in primer-blast of NCBI. First-strand cDNA was synthesized using a FastKing RT Kit. PCR amplification was carried out using a SuperReal PreMix. *Actin* was used as an internal reference and the relative expression level (REL) was calculated using a $2^{-\Delta\Delta C_t}$ method [78].

Table 2

Sequences of primer used in qRT-PCR analysis.

Protein Name (Abbreviation)	Sequences (5' to 3')	Amplicon Size (bp)	Accession No.
Actin	Forward: CGCAGAGCACTTCTTTCCCA Reverse: ATAGTGATGCCGCCTGATCC	181	XM_010657947.2
Dehydrin (DHN1)	Forward: ACCCAGTCCATCAAACCGAG Reverse: GGATGAAGAGCTGCCGGATT	113	NM_001281292.1
Ferritin (FER)	Forward: GGAGCAGGACCAAGACCAAG Reverse: GGAGATGGTGGGAAGCTCTG	138	AM472371.2
Glutathione peroxidase (GPX)	Forward: CACCGTTAAGGATGCTGAGG Reverse: GGCCTTGATCTTTGTACTTCTCG	153	XM_002272900.4
Serine/threonine-protein phosphatase (S/TPP)	Forward: TCAACTGCCTTCCTGTAGCC Reverse: TGGTACATCAACAGGGCGAG	122	XM_002277780.3
Ubiquitin receptor RAD23 (RAD23)	Forward: CAATGGGTTTTGACCGTGCC Reverse: TGGTTCTAGGGGGATGGAGG	170	XM_002282316.3
Alpha-1,4 glucan phosphorylase (GluP)	Forward: GAGGCTTTGCGTGAAGTTGG Reverse: CAGAAAGCAGGAAGCAAGCC	105	XM_002279039.3
Pectinesterase (PE)	Forward: TGCTGATGTTGGTGGGAGAC Reverse: CACTGCTTGGTGATTGCTCG	173	XM_002271629.4
Methyltransferase (MT)	Forward: TAGGCGTGAGATGTGTGTGG Reverse: GACCTGCCTGCTTCGGTAAG	197	AM447844.2
ADP/ATP carrier protein	Forward:	160	AM472940.2

(AAC)	CCCTTGGGGCTTTTTCCCAT		
	Reverse: GGGCAAAGCATGTCCACTAC		
NAD(P)-bd dom domain-containing protein (NADCP)	Forward: TGGTTGGGTCTATGGGAGGA	174	XM_010655958.2
	Reverse: GTAATTCCCGGATGCCACCT		
Phytoeyanin domain-containing protein (PCP)	Forward: GCCCAGACCATTACGGATAGG	182	AM480712.2
	Reverse: CCACATTGGTCGGCTTTGAG		
Ribosomal protein L14 (rpL14)	Forward: CCGCGACTTCGGTCTTTTTC	134	FN595512.1
	Reverse: GCCTTACGTCTGTCTGGAGG		
Peptidylprolyl isomerase (PPI)	Forward: TCGGGGGAAACTCACAGATG	141	XM_002271020.4
	Reverse: TTTCGCTTCTCACCCACACA		

Statistical Analysis

All the measurements were performed using three biological replicates. Statistical analysis was performed using SPSS 22.0 One-way analysis of variance, Duncan multiple comparison tests were used with $P \leq 0.05$ as the basis for significant differences.

Declarations

Author contributions

S.J.C: data curation, investigation and methodology; H.Y.S: data curation and formal analysis; H.X: writing—original draft preparation; D.L.Y and J.M: resources; P.S: project administration and supervision; M.F.L: conceptualization and writing—review and editing.

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Competing interests

The authors declare that they have no competing interests.

Data availability

The datasets are publicly available at ProteomeXchange with the Dataset Identifier PXD009435 and PXD009837.

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Figures

Figure 1

Differences in root activity in *Vitis riparia* × *Vitis labrusca* and Cabernet Sauvignon at different treatments. T1: *V. riparia* × *V. labrusca* at -4°C ; T2: *V. riparia* × *V. labrusca* at -15°C ; and T3: Cabernet Sauvignon at -4°C . Different lowercase letters represent a significant difference ($P\leq 0.05$) at the different treatments.

Figure 2

Volcano plot for roots of *V. riparia* × *Vitis labrusca* and Cabernet Sauvignon at different treatments. T1: *V. riparia* × *V. labrusca* at -4°C ; T2: *V. riparia* × *V. labrusca* at -15°C ; and T3: Cabernet Sauvignon at -4°C (UR, up-regulated; DR, down-regulated).

Figure 3

Cluster heat maps of the differentially expressed proteins (DEPs) at T1, T2 and T3 treatments.

Figure 4

Functional classification of DEPs based on GO database at different treatments. A: T2 vs T1, B: T1 vs T3 (CC, Cellular component; BP, Biological process; MF, Molecular function).

Figure 5

Distribution and classification of DEPs in roots of *V. riparia* × *V. labrusca* and Cabernet Sauvignon at different treatments.

Figure 6

The relative expression level of genes involved in stress response (A), bio-signaling (B), metabolism (C), energy (D), and translation (E) at different treatments, as determined by qRT-PCR.

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